New Microbiologica, 40, 1, 151-154, 2017, ISN 1121-7138

CASE REPORT

Genotyping of *Toxoplasma gondii* strain directly from human CSF samples of congenital toxoplasmosis clinical case

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SUMMARY

This report describes a case of congenital toxoplasmosis in a newborn in Southern Italy. A pregnant mother had been admitted at the 20th week of her pregnancy on account of pharyngodynia and laterocervical lymphadenopathy. Although serological testing of the mother's serum documented a seroconversion with positive IgG and IgM anti-*Toxoplasma* antibodies during II trimester, the woman refused to perform prenatal diagnosis for congenital toxoplasmosis. Fetal ultrasound scan already showed mild asymmetrical triventricular hydrocephaly and cerebral calcifications. After birth, real-time PCR on cerebrospinal fluid and blood samples of the newborn showed a positive result for 529bp-repeat element DNA of *T. gondii*, In addition brain magnetic resonance imaging and computed tomography showed a characteristic diffuse brain tissue loss associated with hydrocephalus. For the first time molecular characterization of *T. gondii* isolate was performed directly from the newborn's CSF samples by using nested-PCR-RFLP of *sag-2* and *pk1* genes. The PCR-RLFP analysis revealed that the isolate belongs to the clonal type II, the predominant lineage causing human toxoplasmosis, as confirmed by DNA sequencing.

Received July 5, 2016 *Accepted* February 4, 2017

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects all warm-blooded vertebrates. Human infections are primarily caused by ingesting undercooked meat containing viable tissue cysts or by ingesting food or water contaminated with oocysts shed in the faeces from infected cats. Primary infection in adults is mostly asymptomatic but lymphadenopathy or ocular toxoplasmosis can occur in some patients (Su *et al.,* 2010). In immunocompromised patients, the reactivation of a latent infection can cause life-threatening encephalitis (Montoya *et al.,* 2004). Infection acquired during pregnancy can lead to maternal-foetal transmission. Connatal toxoplasmosis (CT) is associated with a wide spectrum of clinical signs and symptoms, such as retinochoroiditis, intracerebral calcification, hydrocephalus, and mental retardation, which may be present at birth or develop later in life (Kasper *et al.,* 2009). Clinical symptoms of *T. gondii* infection are non-specific and unreliable for diagnosis. The conventional diagnosis of *T. gondii* infection usually employs serological tests and recently the detection of parasite-specific DNA in biological samples using PCR-based molecular methods. In recent years, to achieve high sensitivity, molecular detection has been based on quantitative real-time PCR (qPCR) that targets the repetitive DNA sequence (Su *et al.,* 2009). Here we describe a case of severe congenital toxoplasmosis in an infant. For the first time, the *T. gondii* strain responsible for the infection was directly genetically characterized from CSF samples using RFLP nested-PCR.

Key words:
Congenital toxoplasmosis, Nested-PCR-RFLP, Genotyping, *Toxoplasma gondii*.

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CASE REPORT

An Italian woman, aged 27, was pregnant with her third baby. She was admitted at the 20th week of her pregnancy on account of pharyngodynia and laterocervical lymphadenopathy. At 15th week, *Toxoplasma* serological tests were found positive for immunoglobulin M (IgM) (28.4, reference positive range >0.65), afterwards, at 17th week both immunoglobulin G (IgG) and IgM were found to be

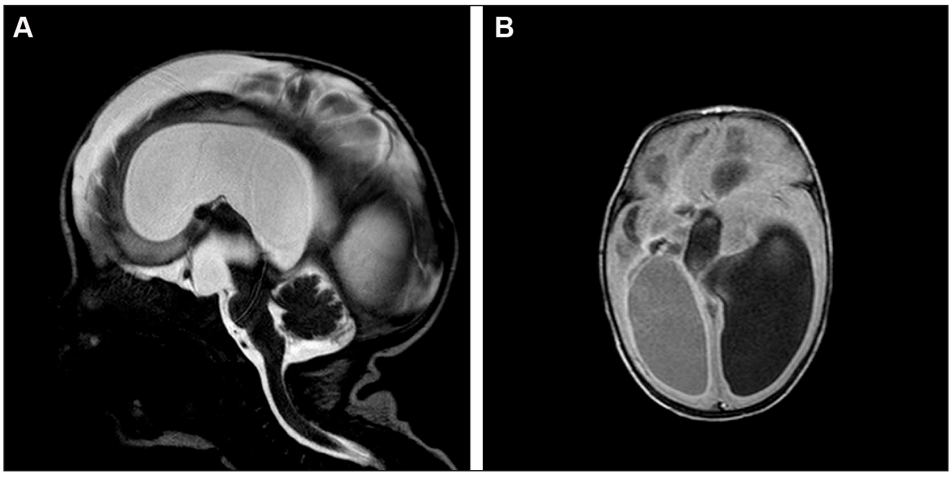

Figure 1 - *MRI sagittal CSF drive scan and axial FLAIR scan. MRI sagittal CSF drive scan (A) and axial FLAIR (B) scan showing slight aqueductal obstruction associated to large left ventricular system that compress controlateral ventricle and the third ventricle, with marked brain tissue loss, abnormal gyri and fluid flap at convexity.*

strongly positive (IgG 148 IU/ml, reference positive range >8 IU/ml, VIDAS Toxo IgG II, and IgM 24.6, reference positive range >0.65 D.O., VIDAS Toxo IgM), and at 19th week the degree of avidity was low (9%) (VIDAS Toxo Avidity). Clinicians discussed with the patient about the risk of seroconversion in the II trimester of pregnancy but the woman decided not to perform prenatal diagnosis for congenital toxoplasmosis. At the 29th week of pregnancy, repeated foetal ultrasound scan showed mild asymmetrical (right more than left) triventricular hydrocephaly and cerebral calcifications (data not show). She gave birth to a boy at 34 weeks of gestation in August 2014. The newborn's brain magnetic resonance imaging (MRI) and computed tomography (CAT) showed calcifications and hydrocephalus pattern with mixed aqueductal and Monro foraminal obstruction. The analysis showed direct communication between the lateral ventricle and adjacent cerebral surface, corpus callosum hypoplasia, poroencephaly, encephalomalacia, intracerebral haemorrhage, and multiple calcifications, so ventriculo-peritoneal shunt placement was performed (*Figure 1*). Real-time PCR on the CSF and blood samples showed a positive result for *T. gondii* 529 bp-repeat element DNA (ELITech group, Molecular Diagnostics). Serologic tests showed positive results for *T. gondii*-specific IgG, but negative results for *T. gondii*-specific IgM antibody.

MOLECULAR CHARACTERIZATION

To better understand the epidemiology of the parasite, genetic characterization of *T. gondii* isolate was performed directly on CSF samples of the newborn. DNA was extracted from CSF samples 5, 16, 41 and 73 days after birth. The extraction was done with Extrablood kit (ELITech group, Molecular Diagnostics) according to manufacturer's instructions. For the genetic typing of strain a nested-PCR-restriction fragment length polymorphism (RFLP) of *sag2* and *pk1* genes, encoding for surface tachyzoite protein p22 and serine/threonine kinase protein, respectively, was performed from CSF samples. The selected markers belong to a panel of genes for molecular epidemiology studies and population genetics of *T. gondii*, developed by Su and coworkers (Dubey *et al.,* 2007). The choice of using *sag2* gene as genetic marker relies on its high discriminatory power between lineages I and II (Alghamdi *et al.,* 2016; Rico-Torres *et al.,* 2016). To confirm the result obtained from *sag2* analysis, *pk1* gene marker was also associated.

For each marker, the target DNA sequence was amplified by nested PCR using Hot Taq DNA polymerase (Microtech). The reaction with external primers was carried out in 50 µl of volume containing 1X PCR buffer, 2 mM $MgCl_2$, 200 µM of the dNTPs, 0.15 µM each of the forward and reverse primers, pK1-F ex/pK1-R ex and Sag2-F ex/Sag2-R ex, for *pK1* and *sag2* genes respectively (*Table 1*), 3 Units of Hot Taq DNA polymerase and 20 µl of DNA sample. The reaction mixture was treated at 95°C for 15 s, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 2 min. The nested PCR reaction with internal primers was carried out in a volume of 50 µl containing 1X PCR buffer, 2 mM $MgCl_2$, 200 µM of the dNTPs, 0.30 µM each of internal forward and reverse primers pK1-F/pK1-R and Sag2-F/Sag2-R, for *pK1* and *sag2* genes respectively (*Table 1*), 3 Units Hot Taq DNA polymerase and diluted (1:1) PCR

Table 1 - *Primers used for the Toxoplasma gondii identification.*

Name	*Sequence 5-3*	*Reference*
pK1-F	CGCAAAGGGAGACAATCAGT	Khan *et al.*, 2005; Su *et al.*, 2006
pK1-R	TCATCGCTGAATCTCATTGC	Khan *et al.*, 2005; Su *et al.*, 2006
pK1-F ex	GAAAGCTGTCCACCCTGAAA	Khan *et al.*, 2005; Su *et al.*, 2006
pK1-R ex	AGAAAGCTCCGTGCAGTGAT	Khan *et al.*, 2005; Su *et al.*, 2006
Sag2-F	ACCCATCTGCGAAGAAAACG	Lehmann *et al.*, 2000; Su *et al.*, 2006
Sag2-R	ATTTCGACCAGCGGGAGCAC	Lehmann *et al.*, 2000; Su *et al.*, 2006
Sag2-F ex	GGAACGCGAACAATGAGTTT	Khan *et al.*, 2005; Su *et al.*, 2006
Sag2-R ex	GCACTGTTGTCCAGGGTTTT	Khan *et al.*, 2005; Su *et al.*, 2006

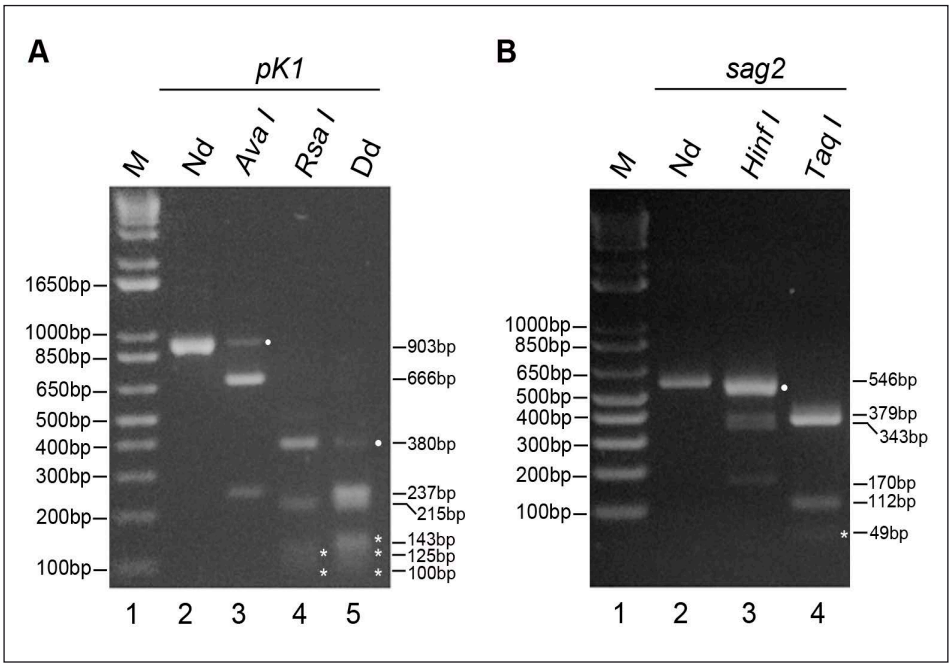

Figure 2 - *Nested PCR-RFLP gel image from CSF sample based on* pk1 *and* sag2 *markers. (A) Nested PCR product of* pk1 *(lane 2) was digested with 2 restriction enzymes* Ava I, Rsa I *(Biolabs) in single digestion (lane 3 and 4, respectively) and in a double digestion (lane 5). (B) Nested PCR product of* sag2 *(lane 2) was digested with 2 restriction enzymes* Hinf I, Taq I *(Roche) in single digestion (lane 3 and 4, respectively). The digest products were resolved in 2.5% agarose gel by electrophoresis and visualized under UV light. M: molecular weight DNA marker (lane 1 panel A and B); Nd: no digested amplicon; and Dd: double digested. Asterisks indicate bands present in the gel, but limited visible in the figure. Circles indicate partially digested fragments.*

products obtained with external primers. The reaction mixture was treated at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 1.5 min. For the genetic typing of *T. gondii* strain, RFLP pattern was evaluated. Each nested PCR product was treated with the restriction enzymes *Ava I* and *Rsa I* for *pK1* amplicon, and *Hinf I* and *Taq I* for *sag2* amplicon, as indicated by Dubey and coworkers (Su *et al.,* 2006) in a volume of 100 µl and the digested samples were resolved in 2.5% agarose gel to reveal DNA banding patterns (Su *et al.,* 2006). PCR-RFLP results of *T. gondii* markers were represented in *Figure 2*. Both *loci* analyzed showed a restriction pattern corresponding to clonal type II according to the multilocus genotyping results of Dubey and coworkers (Su *et al.,* 2006). For *pK1 locus*, the digestion of type II strain of *T. gondii* produces a unique banding patterns with 5 bands as compared with PCR-RFLP of reference type I, II and III strains published by Dubey and coworkers (Su *et al.,* 2006) (*Figure 2A*). For *sag2 locus* analysis, *Taq I* recognizes only allele II sequence, whereas *Hinf I* cuts allele I and II sequences (Su *et al.,* 2006) (*Figure 2B*).

To further confirm the results obtained, nested PCR products were subjected to sequencing analysis, performed by Ceinge Advanced Biotechnologies, Naples (*Figure 3*). Purified nested PCR products were sequenced, using the same primers described for the amplification process (Table 1). BLAST analysis was used to compare the sequences with those of available *T. gondii* sequences in the GenBank (KT881317.1 for *sag2* gene and KT881375.1 for *pk1* gene). The homology sequence analysis demonstrated that a 546bp nested PCR product corresponding to *sag2* gene has 98% identity with the available sequence of *T. gondii* isolate TgCatAu_7 and a 903bp fragment corresponding to *pk1* gene has 99% identity with those of available sequences for *T. gondii* isolate TgCatAu_6, both belonging to genotype *T. gondii* type II in Genbank (Brennan *et al.,* 2016).

Nested PCR *pk1*

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5'- CTCGANTAGG TGCATTAGTG GGTTTCCCTG CGAGAGTGTT ACTGCGATGC GAGTGGCAAC
    GTGTTCGGAG CGCAATACCT TATGTGTAGG ACAGTTAAGA TGCAGAATTT GTATGGGTGT
    CGTTCTTCCC GCATTTCGCT GAATAATTAA CTGCGCAACA TAATCCCAAC AGTGTCACAT
    GCTGACCCGA GTATCAGGGG CTTTTTTGTT GCCCATATGC TTTTCANAAG CAGTAGGGCA
    ATGGAAGACG ATTTTGTCCA GACTGTTTCC GCACCTGAAT TTGCCACGTG CAGGTCATGC
    ACAATTTTCT CTAGTGTAAA CCAAAGTACA CCTGTATTAG NTCTGAAAGA GAGAGTTTTG
    CTAGTTGAGA TCTATAGCCA ACTTGTATAA GGCGATCCAC ATTGAACTGC GTTAAGGTGC
    AATCTGTACG GAATACAGGG ATCGAATGTT TGGTGGCCTG GTACCAGTNT CGCGTTCGTT
    GNGCACACGT TTTCGCCGTG ATGCAAAACC ATGGGTTTCC TTAAAACTCT CTCTTNTTGT
    GTCATCGACA ACAAAGTGGC GAAATGCGTG CACTTTGTCA CCACCAGGAG AGATCGTTGC
    AATCAAGCTT ATAAACAANA GATCATTCAA CGAGATCACC GATGCTGACC GCGTTTTCGT
    CGAGATCCAG GTAACGTACN GACCACTCTA CGAGACAGCT TGTTCTGGAG AACGGTTCTT
    CACGTACTGA TGAACATACG TCACATGCAC TCGTTATTCG CCATTTGGCA GCAGATGATT
    AGAGTCGCTG CCGACACANT CCAAAC-3'
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Nested PCR *sag2*

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5'- CGGGTTGCNG TTCTAGGAAC TGAGTTGTGA TTGTGCACAA TTGCGGTGTG ACACCTTCTG
    TCTCGTTCCA ATCTTTGTCT TGNCGGAACT ATGAGTTTCT CAAAGACCAC GAGCCTAGCG
    TCGCTAGCGC TCACGGGCTT GNTTGTTGTG TTCAAGTTCG CTCTTGNGTC CACCACCGAG
    ACGCCAGCGC CCATTGAGTG NACTGNCGGC GCAACGAAGA CTGTNGAGGC ACCCTCCAGT
    GGTTCCGTTG TCTTCCAATG NGGGGATAAA CTAACCATCA GTCCCAGTGG CGAAGGTGAT
    GTCTTTTATG GCAAGGAATG CACAGACTCG AGGAAGTTGA CGACTGNCCT TCCAGGTGCG
    GTCTTGAAAG CTAAGGTCGA GCAGCCCCCG AAAGGTCCTG CTACCTACAC ACTGTCTTAC
    GACGGTACTC CCGAGAAACC TCAGGTTCTC TGTTACAAGT GCGTTGCCGA AGCAG-3'
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Figure 3 - *Genomic sequence of nested PCR product of* pk1 *and* sag2 *genes.*

DISCUSSION

To the best of our knowledge, the present study is the first in Italy to carry out the molecular characterization of a clinical isolate of *T. gondii* directly from CSF samples.

The PCR-RFLP analysis showed that the *T. gondii* isolate, responsible for the CT case report, belongs to type II. The majority of human infections studied in North America and Europe are caused by type II strains (Sibley *et al.,* 2009). In Europe, this genotype is related to acquired and also congenital toxoplasmosis (Ajzenberg *et al.,* 2002; Rico-Torres *et al.,* 2016). The role of parasite type on congenital toxoplasmosis is still controversial (Ajzenberg, 2012; Ajzenberg, 2015). Although type II parasites are very effective in activating an early immune response which destroys tachyzoites and induces cyst formation, neonatal cases infected by type II strains during the first half of pregnancy presented severe clinical problems, suggesting a crucial role of the immature status of the immune re-

sponse of the foetus in disease susceptibility (Rico-Torres *et al.,* 2016).

Indeed, congenital infections with type II strain are frequently associated with hydrocephalus characteristic patterns (Hutson *et al.,* 2015). In our clinical case, brain MRI and CAT analysis showed diffuse brain tissue loss associated with hydrocephalus with bilateral obstruction of the foramina of Monro with asymmetric dilatation of the lateral ventricles.

Although a substantial decrease in *T. gondii* seroprevalence, from 40 to 20-30% in the adult population was observed in the last 20 years and no national register of congenital infections is available (Tomasoni *et al.,* 2014), the CT incidence rate in Italy was 0.06% per 10347 births, as reported in a recent study (Capretti *et al.,* 2014). The incidence observed was higher than previously reported in other European countries (Signorell *et al.,* 2006; Villena *et al.,* 2010; Schmidt *et al.,* 2006) probably due to recent advances in diagnostic methods (Capretti *et al.,* 2014). Prenatal treatment substantially reduced the proportion of infected fetuses who developed severe neurological sequelae or death (Cortina-Borja *et al.,* 2010). The psychosocial burden of CT is also high for families: parents of children with serious neurological damage report psychological distress. The application of an integrated approach, combining molecular detection and high resolution genetic characterization, enhance our understanding of the molecular epidemiology, population genetics and phylogeny of *T. gondii* and could help clinicians control parasite transmission and reduction of toxoplasmosis in humans and animals.

Acknowledgments

This research was funded in part by PRIN 2012 [grant number 2012WJSX8K]: "Host-microbe interaction models in mucosal infections: development of novel therapeutic strategies".

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