Isolation of *Toxoplasma gondii* from the brain of a dog in Australia and its biological and molecular characterisation

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ABSTRACT

Toxoplasma gondii was isolated from the brain of a young dog for the first time in Australia. The identity of the parasite was confirmed by PCR, Western blotting, electron microscopy and cat bioassay. Genotyping of the isolate (TgDgAu1) was determined by PCR-RFLP markers that showed it to be a Type II strain. Serology demonstrated the presence of IgM antibodies to *T. gondii* suggesting the bitch was probably infected during pregnancy and the *T. gondii* was transmitted to the pups congenitally. We believe this represents the first description of a natural case of congenital transmission of *T. gondii* in the dog.

Key words: *Toxoplasma*; Dog; Isolation; Genotyping; Australia; Congenital transmission.

1. Introduction

Toxoplasma gondii is a common parasite of most warm blooded animals and birds. The majority of *T. gondii* strains from different human and animal sources have been divided into three clonal but closely related lineages: I, II and III based on restriction fragment length polymorphism (PCR-RFLP), isoenzyme analysis and microsatellite analyses (Howe and Sibley, 1995; Dardé, 1996; Howe et al., 1997; Lehmann et al., 2000; Ajzenberg et al., 200**2**). Most recently, high resolution genetic markers were developed and revealed an even greater level of polymorphism within this species than previously thought. In addition "atypical strains" were identified, which are different from the clonal type I, II and III lineages (Su et al., 2006; Dubey et al., 2007e). These atypical lineages could be either recombinants from type I, II and III lineages, or unique diverged lineages. Speculations about the global evolution of *T. gondii* have led to a world-wide effort to study genetic diversity within this species (Lehmann et al., 2006).

Little is known about the type of *T. gondii* found in Australia. Before the discovery of a related parasite, *Neospora caninum* in 1988 (Dubey et al., 1989), there were many reports of fatal toxoplasmosis in dogs worldwide, including Australia (Dubey and Beattie, 1988). The main aim of this study was to isolate *N. caninum* from the brains of pups born to a bitch known to be infected with this parasite. However *T. gondii* was unexpectedly isolated. Consequently we report here the isolation and genetic characterization of *T. gondii* from a young pup in Australia. Serology showed the bitch was probably infected during pregnancy and the *T. gondii* was transmitted to the pups congenitally. We believe this represents the first description of a natural infection of *T. gondii* occurring via congenital transmission in the dog.

2. Material and methods

2.1. Host history

A female cattle dog, aged 18 months, was previously determined to have a titre of 1:200 in the immunofluorescent antibody test (IFAT, VMRD Inc, USA) to *N. caninum* (Hall et al., 2005). In February 2007, the bitch gave birth to six pups and sera from her and all pups were tested in the IFAT and the commercially available latex agglutination test (LAT) (Eiken, Japan) for *N. caninum* and *T. gondii* antibodies ,respectively. All sera were negative to *T. gondii* in the LAT (<1:8 serum dilution), but the female cattle dog had an IFAT titre of >1:2800 to *N. caninum*. All pups were weakly positive at 1/50 in the IFAT to *N. caninum*. Serial bleeds were subsequently obtained from the bitch in two-weekly intervals in July and August of 2007 and demonstrated a persistent IFAT titre of 1/1000 for *N. caninum* yet remained negative for *T. gondii* using LAT.

2.2. Preparation of brain and cultivation of the parasite

All animal research was performed with approval from the University of Technology Sydney and Australian National University Animal Care & Ethics Committees. The six pups were killed at 14 days of age using barbiturate anaesthesia. Whole brains were removed for attempted protozoal isolation by bioassays in cell culture and mice. Brains were homogenized aseptically in saline (0.95 % NaCl), trypsinized and overlayed on to African Green Monkey kidney (VERO) cells seeded in 75 cm^2 vented flasks. Cells were grown in RPMI-1640 medium (Sigma) supplemented with 50 U/ml penicillin G, 50 μg/ml streptomycin (Sigma) and 2% heat-inactivated horse serum (Sigma) at 37 ˚C in a 5% CO_2 humidified incubator. Each flask was sub-cultured once after three weeks of inoculation and divided into two. For PCR, a small portion of pup brains were stored in the -80 ˚C freezer.

2.3. Bioassay in mice

The brain homogenates were also inoculated subcutaneously into female in-bred BALB/c mice (Gore Hill Research Laboratories, Sydney) maintained on drinking water supplemented with dexamethasone (Sigma,1 mg/L, Nicoll et al., 1997) beginning at 2 weeks prior to inoculation until the end of experiment.

In order to investigate virulence of the tachyzoites isolated into culture, untreated mice were inoculated subcutaneously with1, 100 or 1000 culture-derived tachyzoites. Mice were observed for 2 months.

2.4. Bioassay in cats

To exclude the possibility of mixed infection with *N. caninum*, mice were infected with 10,000 cultured tachyzoites of TgDgAu1 and subsequently their brains were fed to five *T. gondii*-free cats as described (Dubey, 1995); *T. gondii* oocysts are produced by cats and *N. caninum* oocysts are produced in dogs. Faeces of cats were examined for shedding of *T. gondii* oocysts 3-21 days post-ingesting mouse tissues.

2.5. DNA extraction and PCR

DNA was extracted from the brains of pups, mice and *in vitro* grown tachyzoites. Tissues were snap frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. Tissues powdered in this way and tachyzoites were lysed in 2ml of lysis buffer containing 10mM Tris-Cl (pH 7.6), 100mM EDTA, 1% SDS containing 40 units of proteinase K [50mg/ml] (Sigma) at 65ºC for 4 hours. DNA was purified by extraction with phenol-chloroform followed by ethanol precipitation. The DNA obtained was quantified using a Nanodrop spectrophotometer and stored at -20ºC.

Various primers were used to confirm the parasite identity and to detect *T. gondii* and *N. caninum* in cultures and dog and mouse tissues. The primer pairs were Tim3/Tim 11 (Payne and Ellis, 1996); T1/T4 for *T. gondii* (Burg et al. 1989; Chabbert et al. 2004); and the nested PCR described by Barratt et al. (2008) for *N. caninum*. All PCR reactions were performed on a PTC-100 Programmable Thermal Controller (MJ Research Inc.). DNA from the isolates Me49 of *T. gondii* (sourced from ATCC) and NC-Liverpool of *N. caninum* were used as controls.

The PCR product produced from cultured tachyzoites isolated from the pup using Tim3 and Tim11 primer sets was purified applying a QIAquick gel extraction kit (QIAGEN), according to the protocol described by the manufacturer and sequencing was performed by the service provider SUPAMAC (Sydney University). Blast searches were conducted using GenBank.

2.6. PCR-RFLP genotyping

Strain typing of *T. gondii* was performed using ten PCR-RLFP genetic markers including *SAG1, SAG2, SAG3,* β*TUB, GRA6, c22-8, c29-2, L358, PK1* and *Apico* using nested PCR and following the published protocol (Su et al., 2006; Dubey et al., 2007e).

2.7. Immunoblot analysis

Tachyzoites of Me49, NC-Nowra (*N. caninum*) and TgDgAu1 were purified from cultures by filtration through 3 µm filters (Isopore membrane filter, Millipore) and then centrifuged at 3000 rpm for 10 min, after which the pellet was snap-frozen in liquid nitrogen. The tachyzoites were then resuspended in 0.5 ml PBS and frozen/thawed three times. The mixture was centrifuged at 10,000 *g* for 10 min and the supernatant containing the soluble fraction was removed and stored at -80 ˚C. Western blotting experiments was performed as follows using tachyzoite extracts and either rat anti *T. gondii* sera or sera from the female dog as primary antibodies.

Tachzyoites were solubilised in NuPage LDS buffer (Invitrogen) containing 5% β-mercaptoethanol and electrophoresed on a NuPage 4-12% Bis-Tris gel (Invitrogen) using 1x XT MES SDS running buffer (Bio-Rad) for 1hr at 180 V in a Bio-Rad minigel apparatus. A SeeBlue plus2 pre-stained standard (Invitrogen) was used as a marker. Proteins were transferred to a 0.45 µm PVDF membrane (immobilon-P, Millipore) using a Novex transfer system (100 V for 1 hr, which was cooled with ice) and blotting buffer (0.192 M Glycine, 0.025 M Tris and 20% Methanol). The membrane was stained with Ponceau S to visualise the proteins. The membrane was incubated overnight in 5% skim milk at 4 ˚C to block non-specific antibody binding and then washed three times with cold PBS containing 0.02% Tween 20 (PBST). All subsequent washing of the membrane was also done using PBST.

Serum from a Wister rat infected with the Me49 strain of *T. gondii* (bled at 8 weeks post infection) was used as primary antibody (1:1000 dilution in 5% skim milk) and anti-rat IgG (whole molecule)-alkaline phosphatase conjugated antibody (rabbit; Sigma) was used as secondary antibody (1:1000 dilution in 5% skim milk). Antibody binding was visualised using the Sigma Fast BCIP/NBT alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium;

Sigma) dissolved in water. The membrane was then washed with distilled water and allowed to dry.

Serum from the bitch was also used as primary antibody (1:125 dilution in 5% skim milk) and anti-dog IgG (whole molecule) alkaline phosphatase conjugated antibody (rabbit, Sigma; 1:500 dilution in 5% skim milk) and anti-dog IgM alkaline phosphatase conjugated antibody (goat, Bethyl Laboratories Inc.; 1:500 dilution) were used as secondary antibodies. Antibody binding was visualised using the Sigma Fast BCIP/NBT alkaline phosphatase substrate.

2.8. Electron microscopy

Tachyzoites of TgDgAu1 were washed in three changes of 0.1 M PBS (pH 7.2), and fixed in 3% glutaraldehyde at 4 ˚C then washed three times with 0.1 M PBS. The tachyzoites were post-fixed in 1% osmium tetroxide in the same buffer for 2 hours than washed in PBS and enbloc stained with 2 % aqueous uranyl acetate for 20 minutes. Dehydration in ethanol was used, and the tachzyoites infiltrated and embedded in LR White resin. Blocks were polymerised overnight at 65 ˚C. Semi-thin (1µm) sections were stained with 1% methylene blue. Ultrathin (70nm) sections were cut using a diamond knife (Drukker) and an ultramicrotome (Ultracut S, Leica). Sections were mounted on 300 mesh copper grids and stained with 2% aqueous uranyl acetate for 20 min and lead citrate (Reynold's) for 4 min. All samples were viewed and imaged in a Philips CM10 TEM.

3. Results

3.1. In vitro isolation of the parasite

PCR of DNA derived from the female dog sera showed that she contained DNA from both *T. gondii* and *N. caninum*. Homogenates from the brains of the six pups were individually injected into mice or inoculated into tissue culture. Tachyzoites were observed in all tissue culture flasks after four weeks post inoculation, but flasks inoculated with tissues of five of the six pups were discarded without characterization of the parasites.. After 60 days, tachyzoites started dividing only in the culture derived from pup3 and the present isolate (called TgDgAu1) is derived from this culture.

3.2. Mouse infection

All mice (except one) that were injected with brain tissue from the pups and TgDgAu1 tachyzoites remained healthy. One mouse injected with brain tissue from pup4 showed clinical signs manifest as kyphosis from 55 days post-infection and died one week later. This mouse was later shown by PCR to be infected with *N. caninum* but no parasite was recovered into culture.

T. gondii-like tissue cysts of TgDgAu1 were observed in impression smears from brains of mice infected with 10000 tachyzoites after 25 days post infection.

3.3. PCR analysis

PCR of DNA derived from sera of the bitch showed it contained DNA from both *T. gondii* and *N. caninum*. PCR of DNA from pup brains showed that all brain tissues contained *T. gondii* but only pup3 and pup4 brains were positive to *N. caninum*. PCR of tissue cultures that were inoculated with brain tissue were positive to *T. gondii* in all flasks, while all were negative by PCR to *N. caninum*.

Analyses of the DNA sequences of PCR products (derived using Tim3 and Tim11 primers or T1 and T4 primers) from TgDgAu1 showed them to be identical to *T. gondii* sequences (e.g. GenBank accession no. L49390 or AF179871) thereby confirming the identity of the new isolate as *T. gondii*.

3.4. Cat infection

All cats fed infected mouse tissues shed *T. gondii*-like oocysts. DNA isolated from them generated a PCR product using *T. gondii*-specific primers. Bioassay of oocysts in mice was not performed.

3.5. Genotyping

Genotyping results at all ten loci are summarised in Table 1. TgDgAu1 displayed the features of a Type II strain at nine loci, while at the SAG1 locus alleles II and III are identical.

3.6. Western blotting

Immunoblotting of proteins derived from Me49, NC-Nowra and TgDgAu1were probed with antisera from a rat infected with Me49. The antigen profile (Fig. 1a) showed that TgDgAu1 is similar, but not identical to that obtained from Me49, thereby providing additional evidence that the isolate is *T. gondii*. Western blotting using sera from the bitch obtained on the day of the euthanasia of its pups, and sera from different bleeds taken over six months, showed the bitch had IgG and IgM antibodies to *T. gondii* (Fig1b, c).

3.7. Electron microscopy

Electron microscopy of the tachyzoites of TgDgAu1 showed that they had the characteristic morphology of *T. gondii* with few electron-lucent rhoptries.

4. Discussion

The main aim of this study was to isolate *N. caninum* from the brains of pups born to a bitch known to be infected with this parasite. However *T. gondii* was unexpectedly isolated giving rise to a tachyzoite population (called TgDgAu1) that was stably maintained in culture.

A serological test (a commercial LAT) showed the bitch was seronegative to *T. gondii* whilst seropositive to *N. caninum*. Similar results were obtained from sera obtained from the bitch up to six months after the birth of its six pups. However Western blotting and PCR of sera from the dog provided evidence that she was co-infected with both *T. gondii* and *N. caninum*. Co-infection with these two parasites has also been reported by others (Dubey et al., 2008). The pups were also seronegative to *T. gondii* by LAT, although PCR on DNA extracted from their brain tissue showed that all six pups contained *T. gondii* DNA while only pup3 and pup4 were positive to *N. caninum* (representing a mixed infection). Western blotting using anti-IgG and IgM demonstrated that the cattle dog was probably infected with *T. gondii* during pregnancy (Fig 2b, c). Although transplacental transmission of *T. gondii* in naturally infected dogs has not previously been demonstrated, there is evidence from experimentally-infected dogs that congenital infection does occur (Bresciani *et al*., 1999).

There is little information on the genotyping of Australian isolates of *T. gondii*. Only the TG96 strain isolated from a non AIDS patient (see Sibley and Boothroyd, 1992) is reported and was genotyped using PCR-RFLP, allozyme analysis and microsatellite (Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Dardé, 1996; Ajzenberg et al., 2002). These studies showed that TG96 stain was avirulent and a Type II (= zymodeme 4 and microsatellite group 2) strain. The relatedness of Australian *T. gondii* to the rest of the world remains unclear.

Genotyping of *T. gondii* from Asia has been reported in more detail. Studies on dogs in Sri Lanka revealed four genotypes; one of the Type III lineage plus three other atypical genotypes (Dubey et al., 2007d). Two atypical genotypes were detected in dogs from Vietnam (Dubey et al., 2007c). The Type III lineage from Sri Lanka is identical to a genotype isolated from a dog in Brazil (Dubey et al., 2007b). An atypical lineage 4 from Sri Lanka and an isolate from Vietnam (from a dog) are

identical to a genotype isolated from dog in Colombia (Dubey et al., 2007a), and also to genotypes isolated from chicken and cat in Brazil and China irrespectively (Dubey et al., 2007e, f) suggesting a South American origin of *T. gondii* currently found in South East Asia (Lehmman et al., 2006; Dubey et al., 2007c).

In conclusion, this study is the first isolation of *T. gondii* from the brain of a young dog in Australia. Serology showed the bitch was probably infected during pregnancy and *T. gondii* was transmitted to the pups congenitally. The genotyping of TgDgAu1 confirmed it to be of the Type II linage. Further studies are needed for a better understanding of the diversity of *T. gondii* from this region.

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Figure and Table legends

Fig. 1. Western blotting using rat anti- *T. gondii* (A) or dog serum (B,C) and tachyzoite extracts. Arrows point to differences between the two isolates (Dog3 is TgDgAu1). B and C were developed with anti-IgG (B) or anti-IgM (C).

Table 1. Summary of genotyping data for the TgDgAu1 isolate of *T. gondii*.

Table

Genotype	*SAG*1	5'+3' *SAG*2[a]	*SAG*2[b]	*SAG*3	β*TUB*	*GRA*6	*c22-8*	*c29-2*	*L358*	*PK1*	*Apico*	Isolate IDs
Type I	I	I	I	I	I	I	I	I	I	I	I	RH88
Type II	II or III	II	II	II	II	II	II	II	II	II	II	PTG
Type III	II or III	III	III	III	III	III	III	III	III	III	III	CTG
Atypical	I	II	II	III	II	II	II	u-1	I	u-2	I	TgCgCa1
Atypical	u-1	I	II	III	III	III	u-1	I	I	III	I	MAS
Atypical	I	III	III	III	III	III	I	I	I	u-1	I	TgCatBr5
Type II	II or III	II	II	II	II	II	II	II	II	II	II	TgDgAu1

[a] The *SAG*2 marker based on 5'- and 3'-end DNA sequence polymorphisms of SAG2 gene (Howe et al., 1997).

[b] The *SAG*2 marker developed recently based on 5'-end DNA sequence of *SAG*2 gene is able to identify additional alleles often seen in atypical *T. gondii* strains (Su et al., 2006).

Figure