

Short communication

Genetic characterization of *Toxoplasma gondii* isolates in dogs from Vietnam suggests their South American origin

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Abstract

Dogs are considered a potential risk for transmission of *Toxoplasma gondii* to humans because they can mechanically transmit oocysts to people and in certain parts of the world dog meat is consumed by humans. The prevalence of *T. gondii* in 42 dogs from rural Vietnam was determined. Antibodies to *T. gondii* were assayed by the modified agglutination test, and found in 21 (50%) of 42 dogs with titers of 1:20 in six, 1:40 in seven, 1:80 in two, 1:160 in two, 1:320 in two, 1:640 in one, and 1:1280 or higher in one. Hearts, tongues and brains of 21 seropositive dogs were bioassayed in cats, mice or both. Tissues from eight seropositive dogs were fed to eight *T. gondii*-free cats. Feces of cats were examined for oocysts. *T. gondii* was isolated from eight dogs by bioassay in cats. Genotyping of these eight *T. gondii* isolates using polymorphisms at 10 nuclear markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and a new SAG2, and an apicoplast marker Apico revealed two genotypes. Both genotypes were previously identified from the dog isolates in Colombia, suggesting their South America origin. However, they are different from the predominant Type I, II and III lineages that are widely spread in North America and Europe. This is the first report of isolation of viable *T. gondii* from any host in Vietnam.

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1. Introduction

Toxoplasma gondii infections are widely prevalent in human beings and animals worldwide (Dubey and Beattie, 1988). Humans become infected post-natally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or by accidentally ingesting oocysts from the environment. However, only a small percentage of exposed adult

humans develop clinical signs. It is unknown whether the severity of toxoplasmosis in immunocompetent persons is due to the parasite strain, host variability, or to other factors.

T. gondii isolates have been classified into three genetic Types (I, II, III) based on restriction fragment length polymorphism (RFLP) (Howe and Sibley, 1995; Howe et al., 1997) and until recently, *T. gondii* was considered to be clonal with very little genetic variability. Based on newer markers for genetic characterization and using recently isolated strains from many countries, a higher genetic variability has been revealed than previously reported (Lehmann et al., 2006). We have initiated a study of worldwide genetic

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diversity of *T. gondii* (Lehmann et al., 2006). This is the report of isolation and characterization of viable *T. gondii* from any host in Vietnam.

2. Materials and methods

2.1. Naturally infected dogs

The 42 dogs surveyed were of local breeds and came from 7 provinces (Binh Phuoc 6 dogs, Binh Duong 6 dogs, Dong Nai 13 dogs, Tay Ninh 5 dogs—these provinces are east of Ho Chi Minh City, and Tien Giang 4 dogs, Ben Tre 3 dogs, Long An 5 dogs—these provinces are west of Ho Chi Minh City, Fig. 1). Each dog was from a different home and the houses were at least 2 km apart. They were 1–4-year-old, and there were 21 males and 21 females. In rural Vietnam most households raise several dogs. They keep three to five dogs for guarding their homes and the excess are sold for human consumption; these dogs are not considered pets. For the present study, these dogs were purchased from individual homes and euthanized by a veterinarian with an overdose of sodium thiopental (30–40 mg/kg given intravenously). Samples of serum, brain, heart, and tongue of these 42 dogs were received in two batches (20 in batch 1 and 22 in batch 2) in March and June 2006. At necropsy, brain, heart, and blood samples were collected and sent by air to Beltsville, MD. Samples were stored at 4 °C for 2–3 days after euthanasia and then shipped by air to USA. Two to 4 days elapsed between killing of dogs and receipt of samples at Beltsville. Tissues had autolysed during transit.



Fig. 1. Map of Vietnam showing the location of *T. gondii* positive dogs.

2.2. Serological examination

Sera of dogs were tested for *T. gondii* antibodies using two-fold serum dilutions from 1:20 to 1:1280 with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

2.3. Bioassay of canine tissues for *T. gondii* infection

Tissues of all 21 dogs with titers of 1:20 or higher were bioassayed for *T. gondii* infection in mice (13 dogs), cats (8 dogs), 1–3 days after results of serologic examination were available. In batch 1, brains, hearts, and tongue of each seropositive dog were pooled and bioassayed in cats (eight dogs, Table 1) and mice (five dogs). In batch 2, brains, tongues, and hearts of 10 dogs were each bioassayed individually only in female Swiss

Table 1
Toxoplasma gondii from tissues of dogs from Vietnam

Dog no.	Source ^a	Age (years)	Sex	MAT	Cat no., oocysts shed	Bioassay in mice		Genotype			
						Oocysts fed	Tachyzoites inoculated	Strain designation	SAG1, SAG2, SAG3, BTUB, GRA6	c22-8, c29-2, L358, PK1, new SAG2, Apico	
3	Dong Xaoi, BH	2.5	Female	1280	294-yes	11 ^b , 13	15, 15	TgDgVi1	I, I, III, I, III	II, I, III, III, I, I,	
4	Ben Cat, BD	3	Female	160	261-yes	13, 13	14, 14	TgDgVi2	I, I, III, I, III	II, I, III, III, I, I	
5	Thuan An, BD	3	Male	640	278-yes	13, 13	13, 14	TgDgVi3	I, I, III, I, III	II, I, III, III, I, I	
12	Bien Hoa, DN	3	Female	40	277-yes	10, 10	15, 15	TgDgVi4	I, I, III, I, III	II, I, III, III, I, I	
15	Long Thanh, DN	1.5	Female	80	257-yes	4, 4	22, 23	TgDgVi5	u-1, II, III, III, II	II, III, II, II, II, I	
16	Tan An, LA	3	Female	320	269-yes	5, 5	49 ^c , 49 ^c	TgDgVi6	u-1, II, III, III, II	II, III, II, II, II, I	
18	Tan An, LA	2	Male	40	274B-yes	4, 4	11, 23	TgDgVi7	u-1, II, III, III, II	II, III, II, II, II, I	
20	Moc Hoa, LA	2	Female	80	275-yes	5, 5	49 ^c , 49 ^c	TgDgVi8	u-1, II, III, III, II	II, III, II, II, II, I	

^a Town and Provinces (BH = Binh Phuoc, BD = Binh Duong, Dn = Dong Nai, LA = Long An). Distances (km) of these towns for dogs 3, 4, 5, 12, 15, 16, 18, and 20 from Ho Chi Minh City were 80,60, 35, 30, 75, 47, 50, and 65 km, respectively.

^b Day mice died or euthanized.

^c Day mice were killed.

Webster mice obtained from Taconic Farms, Germantown, New York, as described by Dubey et al. (2002). Each tissue (20–25 g) was homogenized individually, digested in acidic pepsin, neutralized, and washed; the homogenate inoculated subcutaneously into three to five mice (Dubey, 1998). Brains, tongues and hearts from eight dogs were pooled and fed separately to eight *T. gondii*-free cats. Feces of cats were examined for shedding of *T. gondii* oocysts 3–14 days post-ingesting canine tissues. Fecal floats were incubated in 2% sulfuric acid for 1 week at room temperature on a shaker to allow sporulation of oocysts and were bioassayed orally in mice (Dubey and Beattie, 1988). Four to 7 days after feeding oocysts, mesenteric lymph nodes of mice that died or killed were removed and after ascertaining the presence of tachyzoites, homogenates of lymph nodes were inoculated into new mice to exclude *Hammondia hammondi* infection (Dubey and Beattie, 1988). Tissue imprints of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on days 40–42 post-inoculation (p.i.) and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed 6 weeks post-inoculation (p.i.) and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

2.4. Genetic characterization

T. gondii DNA was extracted from the tissues of infected mice and strain typing was initially performed using PCR-RFLP genetic markers SAG1, SAG2, SAG3, BTUB and GRA6 (Dubey et al., 2006). In brief, the target DNA sequences were amplified by multiplex PCR using external primers for all five markers. Multiplex PCR amplified products were then used for nested PCR with internal primers for each marker separately. Nested PCR products were treated with restriction enzymes and resolved in agarose gel by electrophoresis to reveal the RFLP patterns of the isolates. These five markers allow us to quickly characterize all samples and to identify potential mixed infection in dogs. One or two representative DNA extracts from mice infected with the same dog sample were genotyped with six additional genetic markers including c22-8, c29-2, L358, PK1, a new SAG2 and Apico to further identify isolates with high resolution (Dubey et al., 2007a; Su et al., 2006) by the same method described above. Allele types for all isolates were determined based on the RFLP patterns of six

reference strains including RH88, PTG, CTG, COUGAR, MAS and TgCatBr5 (Su et al., 2006). These reference strains allow us to capture all known alleles for each marker and to identify potential unique alleles in new samples.

3. Results

Antibodies to *T. gondii* were found in 21 (50%) of 42 dogs with titers of 1:20 in six, 1:40 in seven, 1:80 in two, 1:160 in two, 1:320 in two, 1:640 in one, and 1:1280 or higher in one.

In batch 1, antibody titers were: 1:20 in three, 1:40 in four, 1:80 in two, 1:160 in one, 1:320 in one, 1:640 in one, and 1:1280 in one and *T. gondii* was isolated from two of four dogs with titers of 1:40 and from all six dogs with titers of 1:80 or higher. *T. gondii* was not isolated in mice inoculated with tissues of four dogs with titers of 1:20 in two and 1:40 in two; the mice inoculated with tissues of the fifth dog with titer of 1:20 died of bacterial infection 2–3 days p.i. and these mice were discarded without further evaluation.

The eight cats fed tissues of dogs in batch 1 shed *T. gondii*-like oocysts; in six of these oocysts were detected by microscopic examination of fecal floats and in two cats only after bioassay. The mice fed sporulated oocysts of these eight isolates became sick or had to be euthanized between 3 and 12 days p.i. (Table 1). All mice inoculated with tissue homogenates of mesenteric lymph nodes of mice fed oocysts became infected with *T. gondii*; tachyzoites were seen in lungs of mice that became sick or died after inoculation of lymph node homogenate of six isolates and tissue cysts were found in the brains of mice that were killed 49 days p.i. Thus, tachyzoites of six of the eight isolates from dogs were pathogenic for mice (Table 1).

The eight isolates were from dogs that came from houses that were at least 2 km apart and thus the isolates were likely to be independent.

T. gondii was not isolated from tissues of any of the six dogs from batch 2 that were bioassayed directly in mice. All mice inoculated with heart tissue of all six dogs (and additionally brain of one) died within 3 days p.i., presumably of bacterial infection and were not evaluated further.

4. Discussion

In the present study six of the eight canine isolates from Vietnam were pathogenic for mice. *T. gondii* isolates differ markedly in their virulence to outbred mice. Isolates of *T. gondii* were considered virulent

when mice inoculated with tachyzoites or bradyzoites died within 28 days p.i. Virulence to mice depends on several factors including the stage of the parasite, route, dose, types of mice used, host, and the strain of the parasite. Among the three infectious stages of *T. gondii*, oocysts are more virulent than tachyzoites or bradyzoites for non-feline hosts (Dubey and Beattie, 1988). However, oocysts are not pathogenic for cats (Dubey, 2006). Results of our previous studies indicated that mouse virulent strains are present in apparently clinically normal dogs from Colombia (Dubey et al., 2007b) and Brazil (Dubey et al., 2007c). Results of the present study indicated that *T. gondii* isolates virulent for mice were present in apparently healthy dogs from Vietnam. Therefore, *T. gondii* virulence phenotype in mouse is unique and it does not necessarily reflect the parasite's virulence in other animals.

Genetic analysis of the eight *T. gondii* isolates in dogs from Vietnam revealed only two different genotypes with each genotype having four isolates. Recently, we have identified 12 genotypes from 19 dog isolates in Brazil (Dubey et al., 2007c), and 10 genotypes from 20 dog isolates in Colombia (Dubey et al., 2007b). Comparing these dog isolates showed that only one genotype overlaps between Brazil and Colombia dog isolates at the 11 markers used here. Interestingly, the two genotypes identified from the dogs in Vietnam were also found in dogs from Colombia. In addition, the exact genotypes were identified from eight cat isolates from southeast China (Dubey et al., 2007d). These findings suggest that *T. gondii* genetic diversity in the southeast Asia is limited. A recent population genetic study on 275 isolates from free-ranging chickens worldwide demonstrated high diversity in South American populations but much less so in other regions of the world, and it suggested that *T. gondii* originated from South America and then spread to Eurasia by human migration and trading in recent history (Lehmann et al., 2006). All these results are in agreement with a more general scheme of evolution, in which most groups of organisms have a pronounced latitudinal decrease in biodiversity from the tropics to the poles (Marshall, 2006). The state of such a latitudinal gradient is maintained as the majority of genera that originated in the tropics later expanded into extratropics, while it is very rare to see the reverse scenario in that extratropical originated genera expanded into the tropics (Jablonski et al., 2006). Taken together, it is likely that the isolates in Vietnam were originated from South America. However, to better understand *T. gondii* population in Asia, more parasite samples are needed. Nevertheless, the results of

this study show that the genotypes identified in Vietnam are different from the predominant Type I, II and III lineages that are widely spread in North America and Europe, further supporting the idea that *T. gondii* has a very diverse global population structure.

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References

- Dubey, J.P., 1998. Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. *Vet. Parasitol.* 74, 75–77.
- Dubey, J.P., 2006. Comparative infectivity of oocysts and bradyzoites of *Toxoplasma gondii* for intermediate (mice) and definitive (cats) hosts. *Vet. Parasitol.* 140, 69–75.
- Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. *Equine Vet. J.* 19, 337–339.
- Dubey, J.P., Beattie, C.P., 1988. *Toxoplasmosis of Animals and Man*. CRC Press, Boca Raton, FL, pp. 1–220.
- Dubey, J.P., Graham, D.H., Blackston, C.R., Lehmann, T., Gennari, S.M., Ragozo, A.M.A., Nishi, S.M., Shen, S.K., Kwok, O.C.H., Hill, D.E., Thulliez, P., 2002. Biological and genetic characterization of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*) from São Paulo, Brazil: unexpected findings. *Int. J. Parasitol.* 32, 99–105.
- Dubey, J.P., Patitucci, A.N., Su, C., Sundar, N., Kwok, O.C.H., Shen, S.K., 2006. Characterization of *Toxoplasma gondii* isolates in free-range chickens from Chile, South America. *Vet. Parasitol.* 140, 76–82.
- Dubey, J.P., Sundar, N., Gennari, S.M., Minervino, A.H.H., Farias, N.A.R., Ruas, J.L., dos Santos, T.R.B., Cavalcante, G.T., Kwok, O.C.H., Su, C., 2007a. Biologic and genetic comparison of *Toxoplasma gondii* isolates in free-range chickens from the northern Pará state and the southern state Rio Grande do Sul, Brazil revealed highly diverse and distinct parasite populations. *Vet. Parasitol.* 143, 182–188.
- Dubey, J.P., Cortés Vecino, J.A., Vargas-Duarte, J.J., Sundar, N., Velmurugan, G.V., Bandini, L.M., Zambrano, L., Mora, L.E., Kwok, O.C.H., Smith, T., Su, C., 2007b. Prevalence of *Toxoplasma gondii* in dogs from Colombia, South America and genetic characterization of *T. gondii* isolates. *Vet. Parasitol.* 145, 45–50.
- Dubey, J.P., Gennari, S.M., Sundar, N., Vianna, M.C.B., Bandini, L.M., Yai, L.E.O., Kwok, O.C.H., Su, C., 2007c. Diverse and atypical genotypes identified in *Toxoplasma gondii* from dogs in São Paulo, Brazil. *J. Parasitol.* 93, 60–64.
- Dubey, J.P., Zhu, X.Q., Sundar, N., Zhang, H., Kwok, O.C.H., Su, C., 2007d. Genetic and biologic characterization of *Toxoplasma gondii* isolates of cats from China. *Vet. Parasitol.* 145, 352–356.
- Howe, D.K., Sibley, L.D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J. Infect. Dis.* 172, 1561–1566.
- Howe, D.K., Honoré, S., Derouin, F., Sibley, L.D., 1997. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. *J. Clin. Microbiol.* 35, 1411–1414.

- Jablonski, D., Roy, K., Valentine, J.W., 2006. Out of the tropics: evolutionary dynamics of the latitudinal diversity gradient. *Science* 314, 102–106.
- Lehmann, T., Marcet, P.L., Graham, D.H., Dahl, E.R., Dubey, J.P., 2006. Globalization and the population structure of *Toxoplasma gondii*. *Proc. Natl. Acad. Sci.* 103, 11423–11428.
- Marshall, C.R., 2006. Fossil record reveals tropics as cradle and museum. *Science* 314, 66–67.
- Su, C., Zhang, X., Dubey, J.P., 2006. Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. *Int. J. Parasitol.* 36, 841–848.