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# Biologic and genetic characteristics of *Toxoplasma gondii* isolates in free-range chickens from Costa Rica, Central America

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**Abstract**

The prevalence of *Toxoplasma gondii* in free-ranging chickens is a good indicator of the prevalence of *T. gondii* oocysts in the soil because chickens feed from the ground. The prevalence of *T. gondii* in 144 free-range chickens (*Gallus domesticus*) from Costa Rica was determined. Antibodies to *T. gondii* were assayed by the modified agglutination test (MAT), and found in 60 (40.1%) of 144 chickens with titers of 1:5 in 16, 1:10 in 5, 1:20 in 2, 1:40 in 3, 1:80 in 5, and 1:160 or higher in 29. Tissues of all chickens were bioassayed for *T. gondii* in mice or cats. Hearts and brains of 52 chickens with titers of 1:5 or higher and 16 chickens with doubtful titers were pooled and bioassayed in mice. Tissues from 76 chickens with MAT titers of 1:10 or less were pooled and fed to three *T. gondii*-free cats. Fecal floats of cats were bioassayed orally in mice but were negative for *T. gondii* oocysts. *T. gondii* was isolated by bioassay in mice from 32 chickens with MAT titers of 1:10 or higher. All infected mice from 4 of the 32 isolates died of toxoplasmosis. Genotyping of these 32 isolates using polymorphisms at the loci SAG1, SAG2, SAG3, BTUB and GRA6 revealed five genotypes. Five isolates had type I alleles and one isolate had type III alleles at all loci. The rest 26 isolates contained the combination of type I and II or I and III alleles and were divided into three genotypes. None was found to have genotype II alleles at all five loci. This is the first report of genetic characterization of *T. gondii* isolates from Costa Rica, Central America.
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*Keywords:* *Toxoplasma gondii*; Chickens; *Gallus domesticus*; Free-range; Costa Rica; Central America; Genotype

## 1. Introduction

*Toxoplasma gondii* infections are widely prevalent in human beings and animals worldwide (Dubey and Beattie, 1988). Humans become infected post-natally

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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; Mondragon et al., 1998; Owen and Trees, 1999; Fuentes et al., 2001; Grigg et al., 2001; Ajzenberg et al., 2002a,b; Boothroyd and Grigg, 2002; Jungersen et al., 2002; Aspinall et al., 2003; Ajzenberg et al., 2004; Dubey et al., 2004a,d; da Silva et al., 2005; Khan et al., 2005; Ferreira et al., 2004, 2006). The parasite was previously considered clonal with very low genetic variability. However, most of the information was derived from isolates from Europe and North America. Using newer markers for genetic characterization and using recently isolated strains from Brazil and French Guiana, higher genetic variability was revealed than previously reported (Ajzenberg et al., 2004; Lehmann et al., 2004).

We have initiated a worldwide study of *T. gondii* population structure. For this we have chosen the free-range chicken as the indicator host for soil contamination with *T. gondii* oocysts because they feed from the ground. Thus far, we have characterized strains from South America (Brazil [Dubey et al., 2002,

Table 1
Isolation of *Toxoplasma gondii* from batch A free-range chickens from Costa Rica

| Chicken no. | Location, city, house number | Chicken MAT titer | Isolation in mice from chicken tissues | | | Isolate designation | Genotype[e] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | No. infected[a] | No. died | Day of death | | SAG1 | SAG2 | SAG3 | BTUB | GRA6 |
| 3 | La Cruz[b], house 1 | 10 | 3 | 2 | 16, 19 | TgCkCr1 | I (3) | I (3) | I (3) | I (3) | I (3) |
| 6 | La Cruz, house 1 | 80 | 3 | 0 | Not applicable | TgCkCr2 | I (3) | I (3) | I (3) | III (3) | I (3) |
| 14 | La Cruz, house 2 | 80 | 1 | 1 | 24 | TgCkCr3 | I (1) | I (1) | I (1) | I (1) | I (1) |
| 16 | La Cruz, house 2 | 320 | 5 | 5 | 14, 14, 15, 16, 16 | TgCkCr4 | I (5) | I (5) | I (5) | I (5) | I (5) |
| 18 | La Cruz, house 2 | 320 | 5 | 5 | 13, 14, 14, 15, 15 | TgCkCr5 | I (5) | I (5) | I (5) | I (5) | I (5) |
| 23 | La Cruz, house 3 | 640 | 5 | 5 | 17, 17, 18, 18, 21 | TgCkCr6 | I (4) | I (4) | I (4) | I (4) | I (4) |
| 55 | Los Chiles[c], house 2 | 320 | 5 | 3 | 10, 18, 18 | TgCkCr7 | I (4) | I (4) | I (4) | II (4) | I (4) |
| 62 | Los Chiles, house 3 | 320 | 3 | 0 | Not applicable | TgCkCr8 | I (3) | III (3) | III (3) | III (3) | III (3) |
| 72 | Los Chiles, house 4 | 20 | 2 | 1 | 30 | TgCkCr9 | I (2) | I (2) | I (2) | II (2) | I (2) |
| 73 | Los Chiles, house 4 | 80 | 5 | 4 | 14, 15, 15, 16 | TgCkCr10 | I (5) | I (5) | I (5) | II (5) | I (5) |
| 79 | Alajuela[d], house 1 | 640 | 4 | 0 | Not applicable | TgCkCr11 | II or III (5) | III (4) | III (4) | III (4) | III (4) |
| 81 | Alajuela, house 2 | 640 | 5 | 1 | 45 | TgCkCr12 | I (5) | I (5) | I (5) | III (5) | I (5) |

[a] Five mice were inoculated with tissues of each chicken.
[b] Latitude 11°05′00″N, longitude 085°39′00″W.
[c] Latitude 11°04′00″N, longitude 084°44′00″W.
[d] Latitude 10°00′00″N, longitude 084°12′00″W.
[e] Genotyping based on DNA from the stated no. of mice in parenthesis.

2003a,d, 2006a], Peru [Dubey et al., 2004b], Venezuela [Dubey et al., 2005h], Argentina [Dubey et al., 2003e, 2005f]), Colombia [Dubey et al., 2005d], Central America and the Caribbean (Guatemala [Dubey et al., 2005e], Grenada, West Indies [Dubey et al., 2005b]), North America (USA [Dubey et al., 2003c; Lehmann et al., 2003], Mexico [Dubey et al., 2004c]), Africa and Middle East (Egypt [Dubey et al., 2003b], Israel [Dubey et al., 2004e], Mali, Kenya, Burkina Faso, and Democratic Republic of Congo [Dubey et al., 2005a]), Asia (Sri Lanka [Dubey et al., 2005g], India [Sreekumar et al., 2003]), and Europe (Austria [Dubey et al., 2005c], and Portugal [Dubey et al., 2006b]). These studies are still not complete, nevertheless, a pattern is emerging that isolates from Brazil are genetically distinct (Lehmann et al., 2004).

In the present paper, we attempted to isolate and genotype *T. gondii* from chickens from Costa Rica, Central America.

## 2. Materials and methods

### 2.1. Naturally-infected chickens

Chickens ($n = 144$) were obtained in two batches (A, B) from free-range chickens in rural farms from eight different houses that were at least 500 m apart (Tables 1 and 2). Adult chickens were purchased, identified, and killed on two farms. Samples of brain, whole heart, and blood were collected from each chicken, and kept at 4 °C until sent with cold packs by air to Beltsville, MD. Batch A of 94 chickens was obtained in August 2005 and batch

Table 2
Isolation of *Toxoplasma gondii* from batch B free-range chickens from Costa Rica

| Chicken no. | Location, city, house number | Chicken MAT titer | Isolation in mice[a] from chicken tissues | | Genotype[f] | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | No. infected[a] | Isolate designation | SAG1 | SAG2 | SAG3 | BTUB | GRA6 |
| 6 | San José (Colón), house 1[b] | 200 | 2 | TgCkCr13 | I (2) | III (2) | III (2) | III (2) | III (2) |
| 7 | | 1600 | 5 | TgCkCr14 | I (5) | III (5) | III (5) | III (5) | III (5) |
| 12 | | 1600 | 5 | TgCkCr15 | I (5) | III (5) | III (5) | III (5) | III (5) |
| 15 | | 400 | 5[c] | TgCkCr16 | I (5) | III (5) | III (5) | III (5) | III (5) |
| 16 | San José (Colón), house 2[d] | 800 | 5 | TgCkCr17 | I (5) | III (5) | III (5) | III (5) | III (5) |
| 17 | | 200 | 5 | TgCkCr18 | I (5) | III (5) | III (5) | III (5) | III (5) |
| 19 | | 800 | 4 | TgCkCr19 | I (4) | III (4) | III (4) | III (4) | III (4) |
| 20 | | 200 | 5 | TgCkCr20 | I (5) | III (5) | III (5) | III (5) | III (5) |
| 21 | | 200 | 3 | TgCkCr21 | I (3) | III (3) | III (3) | III (3) | III (3) |
| 22 | | 200 | 5 | TgCkCr22 | I (5) | III (5) | III (5) | III (5) | III (5) |
| 24 | | 800 | 1 | TgCkCr23 | I (1) | III (1) | III (1) | III (1) | III (1) |
| 25 | | 800 | 5 | TgCkCr24 | I (5) | III (5) | III (5) | III (5) | III (5) |
| 28 | | 80 | 2 | TgCkCr25 | I (2) | III (2) | III (2) | III (2) | III (2) |
| 29 | | 40 | 4 | TgCkCr26 | I (3) | III (3) | III (3) | III (3) | III (3) |
| 30 | | 200 | 5 | TgCkCr27 | I (5) | III (5) | III (5) | III (5) | III (5) |
| 31 | Heredia (San Rafael), house 3[e] | 200 | 4 | TgCkCr28 | I (5) | I (5) | I (5) | III (5) | I (5) |
| 33 | | 800 | 5 | TgCkCr29 | I (4) | I (4) | I (4) | III (4) | I (4) |
| 37 | | 200 | 5 | TgCkCr30 | I (4) | I (4) | I (4) | III (4) | I (5) |
| 40 | | 800 | 5 | TgCkCr31 | I (5) | I (5) | I (5) | III (5) | I (5) |
| 48 | | 1600 | 5 | TgCkCr32 | I (5) | I (5) | I (5) | III (5) | I (5) |

[a] Five mice were inoculated with tissues of each chicken.
[b] Latitude 094°00′00″N, longitude 084°00′00″W.
[c] One mouse died on day 37.
[d] Latitude 094°00′00″N, longitude 084°00′00″W.
[e] Latitude 10°00′00″N, longitude 084°08′00″W.
[f] Genotyping based on DNA from the stated no. of mice in parenthesis.

B of 50 chickens was obtained in December 2005. Four (batch B) to eight days (batch A) elapsed between killing of chickens and receipt of samples at Beltsville. Samples were received in excellent condition.

### 2.2. Serological examination

Sera of chickens were tested for *T. gondii* antibodies using eight dilutions, from 1:5 to 1:640 with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

### 2.3. Bioassay of chickens for T. gondii infection

Tissues of all chickens were bioassayed for *T. gondii* infection. From batch A brains and hearts of 46 chickens (30 positive at 1:5 or higher and 16 questionable at 1:5) were bioassayed individually in outbred female Swiss Webster (SW) mice obtained from Taconic Farms, Germantown, New York, as described (Dubey et al., 2002). Tissues were homogenized, digested in acidic pepsin, washed, and homogenate inoculated subcutaneously into five mice (Dubey, 1998). Brains and hearts from 48 chickens with MAT titers of <1:5 were pooled and fed to one *T. gondii*-free cat (Dubey et al., 2002).

From batch B brains and hearts of 22 chickens with MAT titers of 1:20 or higher were bioassayed individually in mice. Tissues of 20 chickens with MAT titers of <1:5 were pooled and fed to a cat and tissues of eight chickens with titers of 1:5 and 1:10 were pooled and fed to another cat. Feces of cats were examined for shedding of *T. gondii* oocysts 3–14 days post-ingesting chicken tissues as previously described (Dubey, 1995). 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). Tissue imprints of lungs and brains of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 41 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 47 or 48 days 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 for T. gondii

*T. gondii* DNA was extracted from the tissues of all infected mice from each group (Tables 1 and 2) and strain typing was performed using genetic markers SAG1, SAG2, SAG3, BTUB and GRA6 as described with modification (Grigg et al., 2001; Howe et al., 1997; Khan et al., 2005). In brief, the target DNA sequences were first amplified by multiplex PCR using external primers for all five markers. The reaction was carried out in 25 μl of volume containing 1× PCR buffer, 2 mM $MgCl_2$, 200 μM each of the dNTPs, 0.15 μM each of the forward and reverse primers, 0.5 units of FastStart DNA polymerase and 1.5 μl of DNA extract. The reaction mixture was treated at 95 °C for 4 min, followed by 25 cycles of 94 °C for 30 min, 55 °C for 1 min and 72 °C for 2 min. Multiplex PCR amplified products (1.5 μl) were then used for second round amplification (35 cycles) with internal primers for each marker separately, using an annealing temperature of 60 °C in 25 μl volume reaction mixture. To reveal the RFLP pattern of each reference strain, 3 μl of PCR products were mixed with 17 μl of digestion reaction containing 1× NEB buffer, 0.1 mg/ml BSA and one unit of restriction enzyme. The reaction was carried out by incubating at the proper temperature for each restriction enzyme by the manufacturer's instruction (New England BioLab, Beverly, MA). The digested PCR products were resolved in a 2.5–3% agarose gel by electrophoresis in the presence of 0.3 μg/ml ethidium bromide and visualized under UV light.

### 2.5. Oocyst shedding by cats fed mice infected with type I strains of T. gondii

Until recently there was concern that if there are type I strains that circulate in nature in the absence of oocyst shedding because the mice infected with these strains can die acutely before tissue cysts are formed (see Dubey et al., 2002). For this two strains (TgCkCr4 from chicken no. 16, and TgCkCr5 from chicken no. 18, Table 1) that had type I allele at all loci were used to obtain oocysts. Both of these strains were lethal for mice (Table 1). Homogenates of lung tissue of mice that had died on day 13 (TgCkCr5) or day 14 after inoculation with tissues of naturally infected chickens were cryopreserved in liquid nitrogen. After 2 months frozen strains were thawed and inoculated

s.c. into two mice each. The recipient mice were killed on day 16 p.i. because they were ill and their tissues were fed to two cats (cat no. 210 fed strain TgCkCr4 and cat no. 218 fed strain TgCkCr5).

## 3. Results

### 3.1. Batch A chickens

Antibodies to *T. gondii* were found in 30 of 94 (31.9%) chickens with titers of 1:5 in four, 1:10 in three, 1:20 in one 1:40 in one, 1:80 in eight, 1:160 in eight, 1:320 in three, and 1:640 or higher in four. Sixteen chickens had questionable titers at 1:5 dilution and were considered seronegative. *T. gondii* was isolated from tissues of 12 chickens (Table 1); from one of three with a titer of 1:10, one of one with a titer of 1:20, from three of eight with a titer of 1:80, and seven of eight with titers of 1:320 or higher (Table 1). All infected mice from 4 of the 12 isolates died of toxoplasmosis. Overall, 46 (76.6%) of 60 mice became infected after inoculation with chicken tissues and 27 (58.7)% of infected mice died of toxoplasmosis. Most of the infected mice died of toxoplasmic pneumonia during the second and third week p.i. Eight of the 12 isolates were from different locations. The cat fed tissues from seronegative chickens did not shed oocysts as ascertained with bioassay of fecal floats in mice.

The *T. gondii* isolates obtained by bioassay in mice were designated TgCkCr1–12 (Table 1). Genotyping of these 12 isolates using polymorphisms at the SAG1, SAG2, SAG3, BTUB, and GRA6 loci revealed that five isolates (TgCkCr1, 3, 4, 5, 6) had type I alleles and one isolate (TgCkCr11) had type III alleles at all five loci. Six isolates contained the combination of type I and II or I and III alleles and were divided into three genotypes. None was found to have genotype II alleles at all five loci. All infected mice from each group had identical genotype; mixed infections were not found (Table 1). The two cats fed TgCkCr4 and TgCkCr5 strains of *T. gondii* shed 150 and 25 million oocysts, respectively.

### 3.2. Batch B chickens

In the second batch, antibodies to *T. gondii* were found 30 (60%) of 50 chickens with titers of 1:5 in six, 1:10 in two, 1:20 in one 1:40 in two, 1:80 in one, 1:200 in eight, 1:400 in one, and 1:800 in six, and 1:1600 or higher in three. *T. gondii* was isolated from tissues of 20 of 22 chickens bioassayed in mice and are designated as TgCkCr13 to TgCkCr32 (Table 2). Contrary to results from batch A chickens, only one of the 82 *T. gondii* infected mice died (Table 2). The two cats fed tissues of the 28 chickens with titers of 1:10 or less did not shed oocysts. Genotyping of these 20 isolates using the five genetic markers revealed only two genotypes, with 15 isolates (TgCkCr13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27) had type I, III, III, III, III alleles and five isolates (TgCkCr28, 29, 30, 31, 32) had type I, I, I, III, I alleles at loci SAG1, SAG2, SAG3, BTUB and GRA6, respectively. Both genotypes were observed in the isolates from the first batch isolates (Table 2). The two cats fed tissues of the 28 chickens with titers of 1:10 or less did not shed oocysts.

## 4. Discussion

In the present study *T. gondii* was isolated by bioassay in mice from 32 chickens with titers of 1:10 or higher and not from 46 chickens with titers of 1:5. The cats fed tissues from 68 seronegative chickens did not shed oocysts. Data from this and other studies with chickens (see Dubey et al., 2005f) are being accumulated for the validity of MAT for the detection of *T. gondii* in chickens.

The success of isolation also depends on the number of mice inoculated, the amount of tissue bioassayed, and the concentration of the parasite in tissues sampled. In the present study, entire brains and hearts were used to isolate *T. gondii* and most of the tissue digest was inoculated into five mice. Ruiz and Frenkel (1980) isolated *T. gondii* from brains and skeletal muscles of 27 of 50 chickens from farmhouses on the outskirts of San Jose (Desamparados, Santa Ana), Costa Rica. Ruiz and Frenkel (1980) did not mention the pathogenicity of the chicken isolates to mice. In the present study the success of isolation and pathogenicity in two batches varied. In the first batch only 12 of 94 (12%) chickens had viable *T. gondii* whereas in the second batch viable *T. gondii* was found in 20 of 50 (40%) chickens. In the first batch 58% of infected mice died compared with 1% in the second

batch; identical techniques were used to process tissues of both batches.

Although *T. gondii* can be transmitted by many modes of infections, cats are considered essential in its life cycle in nature (Dubey and Beattie, 1988). Virtually all cats that ingest tissues infected with *T. gondii* shed oocysts, irrespective of the genotype. The results of the present study confirm our earlier observations of shedding of oocysts by cats fed tissues of mice infected with type I strains (Dubey et al., 2002, 2003a).

Before the recognition of three genotypes of *T. gondii* (Howe and Sibley, 1995), *T. gondii* isolates were phenotypically classified as mouse virulent or avirulent. Type I strains were considered mouse virulent whereas type II and type III strains were avirulent or mildly virulent for mice (Howe and Sibley, 1995); type I strains killed all mice within 2 weeks p.i., irrespective of the dose. However, these data are based on isolates that have been maintained in mice for an unknown time (Howe and Sibley, 1995). There are very few data on mouse mortality based on primary isolations. We have started to accumulate such data based on isolates from chickens using a specified protocol (subcutaneous inoculation of tissue digest into five SW mice). In the present study, 4 of the 12 isolates in the first batch were lethal for mice, whereas strains from North America did not kill inoculated mice. Genotyping result of the 12 isolates showed that the four lethal isolates had type I alleles at all five loci while seven remaining isolates had type III or combined alleles (I and II or I and III), which in general supporting the notion that type I is virulent in mice. However, the type I isolate TgCkCr1 was not lethal, indicating there was variation. It is of interest that 13 mice infected with six type I isolates did not die. Genotyping with additional markers may be necessary to reveal if TgCkCr1 is a strict type I strain.

Phenotypically and genetically, *T. gondii* isolates from chickens from Costa Rica were different from the isolates from North America and Grenada, West Indies but similar to those from Brazil and Colombia. Most isolates from chickens from Brazil and Colombia were lethal for mice whereas isolates from North America and the Caribbean did not kill inoculated mice. Genetically, none of *T. gondii* isolates from Colombia and Brazil was SAG2 type II, whereas most isolates from chickens from North America and Grenada were type II (Dubey et al., 2003c; Lehmann et al., 2003). The absence of type II strain at all loci from chickens from Costa Rica (Central America), Brazil and Colombia (South America) is remarkable. This is the first report of genetic characterization of *T. gondii* isolates from Costa Rica.

## References


Ajzenberg, D., Cogné, N., Paris, L., Bessières, M.H., Thulliez, P., Filisetti, D., Pelloux, H., Marty, P., Dardé, M.L., 2002a. Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. J. Infect. Dis. 186, 684–689.

Ajzenberg, D., Bañuls, A.L., Tibayrenc, M., Dardé, M.L., 2002b. Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. Int. J. Parasitol. 32, 27–38.

Ajzenberg, D., Bañuls, A.L., Su, C., Dumètre, A., Demar, M., Carme, B., Dardé, M.L., 2004. Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. Int. J. Parasitol. 34, 1185–1196.

Aspinall, T.V., Guy, E.C., Roberts, K.E., Joynson, D.H.M., Hyde, J.E., Sims, P.F.G., 2003. Molecular evidence for multiple *Toxoplasma gondii* infections in individual patients in England and Wales: public health implications. Int. J. Parasitol. 33, 97–103.

Boothroyd, J.C., Grigg, M.E., 2002. Population biology of *Toxoplasma gondii* and its relevance to human infection: do different strains cause different disease? Curr. Opin. Microbiol. 5, 438–442.

da Silva, A.V., Pezerico, S.B., de Lima, V.Y., d'Arc Moretti, L., Pinheiro, J.P., Tanaka, E.M., Ribeiro, M.G., Langoni, H., 2005. Genotyping of *Toxoplasma gondii* strains isolated from dogs with neurological signs. Vet. Parasitol. 127, 23–27.

Dubey, J.P., 1995. Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. J. Parasitol. 81, 410–415.

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., Beattie, C.P., 1988. Toxoplasmosis of Animals and Man. CRC Press, Boca Raton, FL, pp. 1–220.

Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet. J. 19, 337–339.

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., Graham, D.H., Silva, D.S., Lehmann, T., Bahia-Oliveira, L.M.G., 2003a. *Toxoplasma gondii* isolates of free-ranging chickens from Rio de Janeiro, Brazil: mouse mortality, genotype, and oocyst shedding by cats. J. Parasitol. 89, 851–853.

Dubey, J.P., Graham, D.H., Dahl, E., Hilali, M., El-Ghaysh, A., Sreekumar, C., Kwok, O.C.H., Shen, S.K., Lehmann, T., 2003b. Isolation and molecular characterization of *Toxoplasma gondii* from chickens and ducks from Egypt. Vet. Parasitol. 114, 89–95.

Dubey, J.P., Graham, D.H., Dahl, E., Sreekumar, C., Lehmann, T., Davis, M.F., Morishita, T.Y., 2003c. *Toxoplasma gondii* isolates from free-ranging chickens from the United States. J. Parasitol. 89, 1060–1062.

Dubey, J.P., Navarro, I.T., Graham, D.H., Dahl, E., Freire, R.L., Prudencio, L.B., Sreekumar, C., Vianna, M.C., Lehmann, T., 2003d. Characterization of *Toxoplasma gondii* isolates from free-range chickens from Paraná, Brazil. Vet. Parasitol. 117, 229–234.

Dubey, J.P., Venturini, M.C., Venturini, L., Piscopo, M., Graham, D.H., Dahl, E., Sreekumar, C., Vianna, M.C., Lehmann, T., 2003e. Isolation and genotyping of *Toxoplasma gondii* from free-ranging chickens from Argentina. J. Parasitol. 89, 1063–1064.

Dubey, J.P., Graham, D.H., de Young, R.W., Dahl, E., Eberhard, M.L., Nace, E.K., Won, K., Bishop, H., Punkosdy, G., Sreekumar, C., Vianna, M.C.B., Shen, S.K., Kwok, O.C.H., Sumners, J.A., Demarais, S., Humphreys, J.G., Lehmann, T., 2004a. Molecular and biologic characteristics of *Toxoplasma gondii* isolates from wildlife in the United States. J. Parasitol. 90, 67–71.

Dubey, J.P., Levy, M., Sreekumar, C., Kwok, O.C.H., Shen, S.K., Dahl, E., Thulliez, P., Lehmann, T., 2004b. Tissue distribution and molecular characterization of chicken isolates of *Toxoplasma gondii* from Peru. J. Parasitol. 90, 1015–1018.

Dubey, J.P., Morales, E.S., Lehmann, T., 2004c. Isolation and genotyping of *Toxoplasma gondii* from free-ranging chickens from Mexico. J. Parasitol. 90, 411–413.

Dubey, J.P., Parnell, P.G., Sreekumar, C., Vianna, M.C.B., de Young, R.W., Dahl, E., Lehmann, T., 2004d. Biologic and molecular characteristics of *Toxoplasma gondii* isolates from striped skunk (*Mephitis mephitis*), Canada goose (*Branta canadensis*), blacked-winged lory (*Eos cyanogenia*), and cats (*Felis catus*). J. Parasitol. 90, 1171–1174.

Dubey, J.P., Salant, H., Sreekumar, C., Dahl, E., Vianna, M.C.B., Shen, S.K., Kwok, O.C.H., Spira, D., Hamburger, J., Lehmann, T., 2004e. High prevalence of *Toxoplasma gondii* in a commercial flock of chickens in Israel, and public health implications of free-range farming. Vet. Parasitol. 121, 317–322.

Dubey, J.P., Karhemere, S., Dahl, E., Sreekumar, C., Diabaté, A., Dabiré, K.R., Vianna, M.C.B., Kwok, O.C.H., Lehmann, T., 2005a. First biologic and genetic characterization of *Toxoplasma gondii* isolates from chickens from Africa (Democratic Republic of Congo, Mali, Burkina Faso, and Kenya). J. Parasitol. 91, 69–72.

Dubey, J.P., Bhaiyat, M.I., de Allie, C., Macpherson, C.N.L., Sharma, R.N., Sreekumar, C., Vianna, M.C.B., Shen, S.K., Kwok, O.C.H., Lehmann, T., 2005b. Isolation, tissue distribution, and molecular characterization of *Toxoplasma gondii* from chickens in Grenada. West Indies J. Parasitol. 91, 557–560.

Dubey, J.P., Edelhofer, R., Marcet, P., Vianna, M.C.B., Kwok, O.C.H., Lehmann, T., 2005c. Genetic and biologic characteristics of *Toxoplasma gondii* infections in free-range chickens from Austria. Vet. Parasitol. 133, 299–306.

Dubey, J.P., Gomez-Marin, J.E., Bedoya, A., Lora, F., Vianna, M.C.B., Hill, D., Kwok, O.C.H., Shen, S.K., Marcet, P.L., Lehmann, T., 2005d. Genetic and biologic characteristics of *Toxoplasma gondii* isolates in free-range chickens from Colombia, South America. Vet. Parasitol. 134, 67–72.

Dubey, J.P., Lopez, B., Alveraz, M., Mendoza, C., Lehmann, T., 2005e. Isolation, tissue distribution, and molecular characterization of *Toxoplasma gondii* from free-range chickens from Guatemala. J. Parasitol. 91, 955–957.

Dubey, J.P., Marcet, P.L., Lehmann, T., 2005f. Characterization of *Toxoplasma gondii* isolates from free-range chickens in Argentina. J. Parasitol. 91, 1335–1339.

Dubey, J.P., Rajapakse, R.P.V.J., Ekanayake, D.K., Sreekumar, C., Lehmann, T., 2005g. Isolation and molecular characterization of *Toxoplasma gondii* from chickens from Sri Lanka. J. Parasitol. 91, 1480–1482.

Dubey, J.P., Lenhart, A., Castillo, C.E., Alvarez, L., Marcet, P., Sreekumar, C., Lehmann, T., 2005h. *Toxoplasma gondii* infections in chickens from Venezuela: isolation, tissue distribution, and molecular characterization. J. Parasitol. 91, 1332–1334.

Dubey, J.P., Gennari, S.M., Labruna, M.B., Camargo, L.M.A., Vianna, M.C.B., Marcet, P.L., Lehmann, T., 2006a. Characterization of *Toxoplasma gondii* isolates in free-range chickens from Amazon, Brazil. J. Parasitol. 92, 36–40.

Dubey, J.P., Vianna, M.C.B., Sousa, S., Canada, N., Meireles, C.S., Correia da Costa, J.M., Marcet, P.L., Lehmann, T., Dardé, M.L., Thulliez, F.D., 2006b. Characterization of *Toxoplasma gondii* isolates in free-range chickens from Portugal. J. Parasitol. 92, 184–186.

Ferreira, A.M., Vitor, R.W.A., Carneiro, A.C.A.V., Brandão, G.P., Melo, M.N., 2004. Genetic variability of Brazilian *Toxoplasma gondii* strains detected by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and simple sequence repeat anchored-PCR (SSR-PCR). Infect. Genet. Evol. 4, 131–142.

Ferreira, A.M., Vitor, R.W.A., Gazzinelli, R.T., Melo, M.N., 2006. Genetic analysis of natural recombinant Brazilian *Toxoplasma gondii* strains by multilocus PCR-RFLP. Infect. Genet. Evol. 6, 22–31.

Fuentes, I., Rubio, J.M., Ramírez, C., Alvar, J., 2001. Genotypic characterization of *Toxoplasma gondii* strains associated with human toxoplasmosis in Spain: direct analysis from clinical samples. J. Clin. Microbiol. 39, 1566–1570.

Grigg, M.E., Ganatra, J., Boothrooyd, J.C., Margolis, T.P., 2001. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. J. Infect. Dis. 184, 633–639.

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.

Jungersen, G., Jensen, L., Rask, M.R., Lind, P., 2002. Non-lethal infection parameters in mice separate sheep type II *Toxoplasma gondii* isolates by virulence. Comp. Immunol. Microbiol. Infect. Dis. 25, 187–195.

Khan, A., Su, C., German, M., Storch, G.A., Clifford, D.B., Sibley, L.D., 2005. Genotyping of *Toxoplasma gondii* strains from immunocompromised patients reveals high prevalence of type I strains. J. Clin. Microbiol. 43, 5881–5887.

Lehmann, T., Graham, D.H., Dahl, E., Sreekumar, C., Launer, F., Corn, J.L., Gamble, H.R., Dubey, J.P., 2003. Transmission dynamics of *Toxoplasma gondii* on a pig farm. Infect. Genet. Evol. 3, 135–141.

Lehmann, T., Graham, D.H., Dahl, E.R., Bahia-Oliveira, L.M.G., Gennari, S.M., Dubey, J.P., 2004. Variation in the structure of *Toxoplasma gondii* and the roles of selfing, drift, and epistatic selection in maintaining linkage disequilibria. Infect. Genet. Evol. 4, 107–114.

Mondragon, R., Howe, D.K., Dubey, J.P., Sibley, L.D., 1998. Genotypic analysis of *Toxoplasma gondii* isolates from pigs. J. Parasitol. 84, 639–641.

Owen, M.R., Trees, A.J., 1999. Genotyping of *Toxoplasma gondii* associated with abortion in sheep. J. Parasitol. 85, 382–384.

Ruiz, A., Frenkel, J.K., 1980. Intermediate and transport hosts of *Toxoplasma gondii* in Costa Rica. Am. J. Trop. Med. Hyg. 29, 1161–1166.

Sreekumar, C., Graham, D.H., Dahl, E., Lehmann, T., Raman, M., Bhalerao, D.P., Vianna, M.C.B., Dubey, J.P., 2003. Genotyping of *Toxoplasma gondii* isolates from chickens from India. Vet. Parasitol. 118, 187–194.