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# Genetic diversity among sea otter isolates of *Toxoplasma gondii*

N. Sundar [a], R.A. Cole [b], N.J. Thomas [b], D. Majumdar [c], J.P. Dubey [a,*], C. Su [c]

[a] *United States Department of Agriculture, Agricultural Research Service, Animal Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, MD 20705-2350, USA*
[b] *Department of Interior, United States Geological Survey, National Wildlife Health Center, 6006 Schroeder Road, Madison, WI 5371, USA*
[c] *Department of Microbiology, The University of Tennessee, Knoxville, TN 37996-0845, USA*

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**Abstract**

Sea otters (*Enhydra lutris*) have been reported to become infected with *Toxoplasma gondii* and at times succumb to clinical disease. Here, we determined genotypes of 39 *T. gondii* isolates from 37 sea otters in two geographically distant locations (25 from California and 12 from Washington). Six genotypes were identified using 10 PCR-RFLP genetic markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico, and by DNA sequencing of loci SAG1 and GRA6 in 13 isolates. Of these 39 isolates, 13 (33%) were clonal Type II which can be further divided into two groups at the locus Apico. Two of the 39 isolates had Type II alleles at all loci except a Type I allele at locus L358. One isolate had Type II alleles at all loci except the Type I alleles at loci L358 and Apico. One isolate had Type III alleles at all loci except Type II alleles at SAG2 and Apico. Two sea otter isolates had a mixed infection. Twenty-one (54%) isolates had an unique allele at SAG1 locus. Further genotyping or DNA sequence analysis for 18 of these 21 isolates at loci SAG1 and GRA6 revealed that there were two different genotypes, including the previously identified Type X (four isolates) and a new genotype named Type A (14 isolates). The results from this study suggest that the sea otter isolates are genetically diverse.
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*Keywords:* *Toxoplasma gondii*; Sea otters (*Enhydra lutris*); Genotyping; DNA sequencing; Washington State; California

## 1. Introduction

*Toxoplasma gondii* infections are widely prevalent in human beings and other 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 or other animals develop clinical signs of disease. It is unknown whether the severity of toxoplasmosis in immunocompetent hosts is due to the parasite strain, host variability, or to other factors. Recently, attention has been focused on the genetic variability among *T. gondii* isolates from apparently healthy and sick hosts.

Most *T. gondii* isolates from human and animal sources have been grouped into one of three clonal lineages by multi-locus enzyme electrophoresis, PCR-RFLP and microsatellite typing (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002a,b). We have recently found that the isolates of *T. gondii* from Brazil are biologically and genetically different from those in North America and Europe (Dubey et al., 2002, 2007a,b,c; Lehmann et al., 2006). *T. gondii* isolates

* Corresponding author. Tel.: +1 301 504 8128; fax: +1 301 504 9222.
*E-mail address:* jitender.dubey@ars.usda.gov (J.P. Dubey).

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Table 1
Summary of California and Washington state sea otter materials

| Serial no. (case no.) | Date collected (month/year) | Location, county in CA[a] or WA state[b] | Cause of mortality | *Toxoplasma gondii* MAT titer | Isolation method | Reference for *T. gondii* isolation | DNA extracted from | Isolate designation |
|---|---|---|---|---|---|---|---|---|
| 1 (15822) | September/1998 | SCR | *S. neurona* encephalitis and *T. gondii* infection[c] | 50 | Cell culture | This study | Cell culture | TgSoUs1 |
| 2 (14293) | May/1996 | MON | *S. neurona* encephalitis and *T. gondii* infection[c] | ≥500 | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs2 |
| 3 (16061) | November/1998 | SCR | Bacterial infection (vegetative endocarditis) | nd[c] | Cell culture | This study | Cell culture | TgSoUs3 |
| 4 (13790) | July/1995 | SLO | Pneumonia | 50 | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs4 |
| 5 (13761) | July/1995 | MON | Undetermined | nd | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs5 |
| 6 (15687) | February/1998 | MON | Emaciation | ≥500 | Cell culture | This study | Cell culture | TgSoUs6 |
| 7 (14623) | December/1996 | SLO | Bacterial infection (septicemia) | ≥500 | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs7 |
| 8 (14211) | April/1996 | SLO | Bacterial infection (enteritis) | nd | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs8 |
| 9 (14337) | June/1996 | MON | Parasite infection (acanthocephalan peritonitis) | nd | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs9 |
| 10 (14651) | January/1997 | MON | Intestinal perforation, peritonitis | nd | Cell culture | This study | Cell culture | TgSoUs10 |
| 11 (14335) | June/1996 | SLO | Undetermined (neonatal drowning) | 50 | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs11 |
| 12 (14374) | July/1996 | SLO | Myocarditis | nd | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs12 |
| 13 (14379) | July/1996 | SLO | Undetermined | nd | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs13 |
| 14 (13729) | July/1995 | SLO | *S. neurona* encephalitis and *T. gondii* infection[c] | nd | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs14 |
| 15 (13791) | July/1995 | SLO | Pneumonia | ≥500 | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs15 |
| 16 (14382) | July/1996 | SLO | Bacterial infection (peritoneal abscess) | ≥500 | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs16 |
| 17 (14385) | July/1996 | SLO | Bacterial infection (abscess) | ≥500 | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs17 |
| 18 (14513) | October/1996 | SCR | Pneumonia | 50 | Cell culture | Cole et al. (2000) | Cell culture | TgSoUs18 |
| 19 (14226) | April/1996 | SLO | Myocarditis | ≥500 | Cell culture | This study | Cell culture | TgSoUs19 |
| 20 (13758) | July/1995 | MON | *S. neurona* encephalitis, *T. gondii* infection, skull fracture[c] | ≥500 | – | This study | Brain of otter | TgSoUs20 |
| 21 (12749) | April/1994 | SCR | *S. neurona* encephalitis and *T. gondii* infection[c] | nd | – | This study | Brain of otter | TgSoUs21 |
| 22 (17482) | December/2001 | SLO | Intestinal volvulus, *T. gondii* encephalitis[c] | nd | – | This study | Brain of otter | TgSoUs22 |
| 23 (17200) | April/2001 | SMA | Shark attack, *T. gondii* infection[c] | ≥3200 | – | This study | Brain of otter | TgSoUs23 |
| 24 (12806) | May/1993 | SLO | Skull fracture, *T. gondii* encephalitis[c] | Not done | – | This study | Brain of otter | TgSoUs24 |
| 25 (14498) | September/1996 | MON | Encephalitis, *T. gondii* infection[c] | Not done | – | This study | Brain of otter | TgSoUs25 |
| 26 (15713) | March/1998 | WA | *S. neurona* encephalitis[c] | Not done | – | This study | Brain of otter | TgSoUs26 |
| 27 (16961) | July/2000 | WA | *T. gondii* encephalitis and morbillivirus infection[c] | Not done | – | This study | Brain of otter | TgSoUs27 |

[a] County in California = MON = Monterey; SCR = Santa Cruz; SLO = San Luis Obispo; SMA = San Mateo.
[b] WA = Washington state.
[c] Thomas et al. (2007).

from asymptomatic chickens from Brazil were more pathogenic to mice than isolates from Europe or North America, irrespective of the genotype (Dubey et al., 2006a). Additionally, most isolates from chickens from Brazil were not clonal, and Type II was absent (Dubey et al., 2007a).

Recent studies on the mortality associated with toxoplasmosis in sea otters contributed new information on the host parasite relationship linking clinical outcome and *T. gondii* genotype and the description of a new genotype X (Miller et al., 2004; Conrad et al., 2005). In the present study, we compared *T. gondii* genotypes of isolates derived from distinct and non-overlapping populations of sea otters from two geographically distant locations, California and the state of Washington.

## 2. Materials and methods

### 2.1. Isolates from California sea otters

In total, DNA from 25 *T. gondii* isolates from 25 California sea otters (*E. l. nereis*) were used in the present study. Fifteen of these isolates were those previously reported by Cole et al. (2000). Four additional isolates were obtained by bioassay of sea otter tissues in cell culture and were maintained in cell culture at the Department of Interior, United States Geological Survey, National Wildlife Health Center (NWHC), WI (Table 1). Cell cultures containing tachyzoites of these 19 isolates and six additional frozen brain samples of sea otters from California had been stored at −20 °C for 8–10 years at NWHC. Material from these 25 nonviable *T. gondii* samples were sent frozen by overnight courier to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, MD for genetic characterization.

#### 2.1.1. Revival of cryopreserved isolates from California sea otters

After completion of the genetic typing in the present study, an attempt was made to revive 15 *T. gondii* isolates from California sea otters reported by Cole et al. (2000). In March 1997, cell-culture derived tachyzoites of these isolates were inoculated subcutaneously into Swiss Webster (SW) mice to obtain tissue cysts to feed cats for oocyst production at APDL. Two to three months later, mouse brains containing tissue cysts were digested in acid pepsin to release bradyzoites (Dubey and Beattie, 1988). After neutralization, free bradyzoites were stored in liquid nitrogen at APDL. For the present study, cyropreserved bradyzoites were thawed and immediately inoculated into 2 Swiss Webster (SW) mice obtained from Taconic Farms, Germantown, NY. Mice that became sick were euthanized and lung smears were examined microscopically for tachyzoites. Lung tissues with *T. gondii* tachyzoites were homogenized and inoculated subcutaneously into 2 SW mice each to observe the mortality pattern. The surviving mice were bled on day 39 p.i. and serology was conducted as described in Section 2.2.1.

Table 2
Isolation from Washington state sea otters by mouse bioassay

| Otter number | Date collected (month/year) | Cause of mortality | MAT titer | Isolation in SW mice (unless stated otherwise) inoculated with sea otter | | | | Isolate designation |
|---|---|---|---|---|---|---|---|---|
| | | | | Muscle | Heart | Tongue | Brain | |
| 19247 | October/2004 | *S. neurona* encephalitis, morbillivirus | >1600 | Not done | Not done | 4/4[a] | 1/4 | TgSoUs28 |
| 19481 | July/2005 | *S. neurona* encephalitis | >3200 | 4/5 | 5/5 | 5/5 | 5/5 | TgSoUs29 |
| 19482 | July/2005 | *S. neurona* encephalitis, *morbillivirus* | >3200 | 0/5 | 0/5 | 0/5 | 5/5 | TgSoUs30 |
| 19493 | July/2005 | *S. neurona* encephalitis | >3200 | 2/5 | 0/5 | 1/5 | 4/5 | TgSoUs31 |
| 19689 | February/2006 | *S. neurona* encephalitis, *T. gondii* encephalitis | >1280 | 4/5 | 5/5 | 5/5 | 5/5 | TgSoUs32 |
| 19956 | September/2006 | Morbillivirus | >1280 | 0/4 | 0/4 | 0/4 | 0/4 | |
| | | | | 2/2KO | 0/2KO | 0/2KO | 0/2KO | TgSoUs33 |
| 20040 | October/2006 | Trauma | >3200 | 0/5 | 0/5 | 0/5 | 4/4 | TgSoUs34 |
| | | | | 0/2KO | 2/2KO | 0/2KO | 2/2KO | TgSoUs35 |
| 20288 | May/2007 | Morbillivirus | >800 | 3/4 | 0/4 | – | 3/4 | TgSoUs36, TgSoUs37 |
| 20295 | May/2007 | *S. neurona* encephalitis | >800 | 0/4 | 0/4 | – | 4/4 | TgSoUs38 |
| 20334 | July/2007 | Morbillivirus | >1600 | 0/4 | 3/4 | 0/4 | 0/4 | TgSoUs39 |

[a] Number of mice *T. gondii* positive of the number of mice inoculated with sea otter tissues.

### 2.2. Isolates from Washington state sea otters

Brain, muscle, heart, tongue, and sera of 10 sea otters from Washington state (*E. l. kenyoni*) (Table 2) were shipped refrigerated from NWHC to the APDL and used in this study. Additionally, DNA extracted from frozen brains of two Washington State sea otters (see Table 1) were also included in the study.

#### 2.2.1. Serology

Sera from animals were tested for antibodies to *T. gondii* by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

#### 2.2.2. Bioassay in mice

Tissues of 10 sea otters from Washington state were bioassayed in mice for *T. gondii* infection. Tissues were homogenized, digested in acidic pepsin, washed, and aliquots of homogenates were inoculated subcutaneously into four out bred SW mice (Dubey, 1998) and two gamma interferon knock out (KO) mice from Jackson Laboratories (Dubey and Lindsay, 1998). Tissue imprints of lungs and brains of inoculated mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 38 p.i. and a 1:25 dilution of serum was tested for *T. gondii* antibodies by MAT. Mice were killed 43 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.

### 2.3. Genotyping of T. gondii by PCR-RFLP

DNA was extracted from infected tissues or cell cultures using a Qiagen DNeasy tissue kit (Qiagen). The strain typing was performed using genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, L358, PK1, c22-8, c29-2 and Apico (Su et al., 2006; Dubey et al., 2006b).

### 2.4. Sequencing

To sequence representative isolates at loci SAG1 and GRA6, the target sequences were amplified by PCR. For

Table 3
Summary of genotyping

| Genotype | Genetic markers | | | | | | | | | | | Isolate ID |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SAG1[a] | 5′ + 3′ SAG2[b] | SAG2[c] | SAG3 | BTUB | GRA6 | c22-8 | c29-2 | L358 | PK1 | Apico | |
| Reference | I | I | I | I | I | I | I | I | I | I | I | RH88 |
| Reference | II or III | II | II | II | II | II | II | II | II | II | II | PTG |
| Reference | II or III | III | III | III | III | III | III | III | III | III | III | CTG |
| Reference | I | II | II | III | II | II | II | u-1 | I | u-2 | I | COUGAR |
| Reference | u-1 | I | II | III | III | III | u-1 | I | I | III | I | MAS |
| Reference | I | III | III | III | III | III | I | I | I | u-1 | I | TgCatBr5 |
| #1 | II or III | II | II | II | II | II | II | II | II | II | II | TgSoUs5, 20, 27, 36[d] |
| #2 | II or III | II | II | II | II | II | II | II | II | II | I | TgSoUs3, 4, 18, 19, 21, 26, 29, 30, 37[d] |
| #3 | II or III | II | II | II | II | II | II | II | I | II | II | TgSoUs1, 2 |
| #4 | II or III | II | II | II | II | II | II | II | I | II | I | TgSoUs35 |
| #5 | u-1 | II | II | II | II | II | II | II | I | II | I | Type A: TgSoUs6, 7, 9, 10, 12, 13, 14, 22, 23, 24, 25, 32, 33, 34. Type X: TgSoUs8, 11, 15, 31. Not done: TgSoUs16, 17, 38 |
| #6 | II or III | II | II | III | III | III | III | III | III | III | II | TgSoUs39 |
| Mixed | II or III | II and III? | II and III? | III | II | II and III? | II and III? | II | I | II | I | TgSoUs28 |

[a] At SAG1 locus, Type II and III are indistinguishable.
[b] The SAG2 marker based on 5′- and 3′-end DNA sequence polymorphisms of SAG2 gene (Howe et al., 1997).
[c] The SAG2 marker developed recently based on 5′-end DNA sequence of SAG2 gene is able to identify additional alleles often seen in atypical *T. gondii* strains (Su et al., 2006).
[d] Two different genotypes from muscle, tongue and brain, respectively.

SAG1, primers SAG1-S2, 5′-CAATGTGCACCTGTAGGAAGC-3′, and SAG1-Rext, 5′-GTGGTTCTCCGTCGGTGTGAG-3′ were used. For GRA6, primers GRA6-For, 5′-GTAGCGTGCTTGTTGGCGAC-3′, and GRA6-Rev, 5′-TACAAGACATAGAGTGCCCC-3′ were used (Fazaeli et al., 2000). DNA products were sequenced using the sequencing primers. For SAG1, sequencing primer SAG1-AS2, 5′-TTATCTGGGCAGGTGACAAC-3′ was used. For GRA6, two sequencing primers GRA6-SqF, 5′-GTGGACAGCAAGAAGCAGTG-3′ and GRA6-SqR, 5′-GCACCTTCGCTTGTGGTT-3′ were used to generate sequences from both directions. Sequences were processed using BioEdit (available free at http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and compared to previously published sequences of a variety of *T. gondii* isolates (Fazaeli et al., 2000; Miller et al., 2004; Conrad et al., 2005).

## 3. Results

### 3.1. Antibody titers of the sea otters

#### 3.1.1. California sea otters

Serology was performed on sera from 13 of 25 sea otters and all had detectable antibodies to *T. gondii*. Of 13 sea otters, four had antibody titers of 1:50, eight had 1:500, and one had 1:3200 (Table 1). The isolates from California sea otters were designated as TgSoUs1–25 (Table 1).

#### 3.1.2. Washington state sea otters

All 10 sea otters from Washington had antibodies to *T. gondii* with the titers of >800 in two, >1:1280 in two, >1:1600 in two, and >1:3200 in the remaining four.

Viable *T. gondii* was isolated from various tissues from nine of 10 sea otters by bioassay in SW mice (Table 2). The SW mice inoculated with these nine isolates remained asymptomatic. The tenth isolate was from KO mice inoculated with muscle digest from sea otter 19956 (Table 2). Mixed infection with two genotypes (see Section 3.2) was identified in sea otter 20040 (from brain by bioassay in SW mice and from heart by bioassay in KO mice) and sea otter 20288 (from muscle, tongue, and brain in SW mice (Table 2). These mouse-derived *T. gondii* isolates as well as DNA extracted from two frozen brain samples from otters from Washington state were designated as TgSoUs26–39 (Table 2).

### 3.2. Genotyping

#### 3.2.1. PCR-RFLP

Seven genotypes were found among the isolates from the sea otters (Table 3). Genotypes #1 and #2 differ at the locus Apico and have a total of 13 isolates that

(A) DNA Sequence analysis at GRA6

| | 41 | 71 | 95 | 106 | 146 | 162 | 171 | 291 | 292 | 293 | 296 | 304 | 336 | 418 | 561 | 576 | 614 | 635 | 637 | 640 | 641 | 642 | 643 | 644 | 645 | 646 | 647 | 648 | 649 | 650 | 651 | 652 | 653 | 654 | 655 | 656 | 657 | 660 | 677 | 690 | 692 | 702 | 711 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Type I (RH, AF239283) | C | G | G | C | G | G | A | - | - | - | A | A | T | C | A | A | G | T | C | A | G | G | C | A | G | A | G | G | T | G | A | A | G | G | A | G | G | C | C | A | C | T | A |
| Type II (BEV, AF239284) | T | T | . | . | . | . | G | - | - | - | . | T | C | . | . | G | . | G | A | -[a] | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | G | . | G | A | A | T |
| Type III (NED, AF239286) | . | T | . | T | . | A | . | - | - | - | . | T | . | . | C | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | G | . | . | . | . |
| Type II* (sea otter isolates) | T | T | . | . | . | . | G | - | - | - | . | T | C | . | . | G | . | G | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | G | . | G | A | A | T |
| Type A** | T | T | A | . | T | . | G | A | T | G | G | T | C | T | . | G | . | G | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | . | . | . | G | . | . | . | A | . |
| Type X# | T | T | A | . | T | . | G | A | T | G | G | T | C | T | . | G | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | A | . |

a deletion.

(B) DNA Sequence analysis at SAG1

| | 193 | 211 | 246 | 263 | 356 | 444 |
|---|---|---|---|---|---|---|
| Type I (RH) | A | C | T | C | A | T |
| Type II (Me49) | G | T | C | G | G | C |
| Type III (CEP) | G | T | C | G | G | C |
| Type II* (sea otter isolates) | G | T | C | G | G | C |
| Type A** | G | . | C | G | G | C |
| Type X# | G | . | C | G | G | C |

Fig. 1. (A) DNA sequence analysis at GRA6. [a]Deletion. (B) DNA sequence analysis at SAG1. *Type II include Washington isolate TgSoUs35 and California isolates TgSoUs1, 2, 5, 21, 26. **Type A include Washington isolate TgSoUs32 (GenBank EU180624) and California isolate TgSoUs6, 7, 9, 10, 14 (GenBank EU180619 to EU180623). #Type X include California isolate TgSoUs11 and previously published California isolate 3160 (Conrad et al., 2005).

Table 4
Details of revival of cryopreserved *T. gondii* isolates from California sea otters

| Otter number | Isolate number | Number of mice dead[a] (day of death) of 2 mice inoculated | Number of mice *T. gondii* infected of 2 inoculated | Subpassage, number of mice dead[a] (day of death) of 2 mice inoculated |
|---|---|---|---|---|
| 14293 | TgSoUs2 | 0 | 2 | 0/2 |
| 13790 | TgSoUs4 | 0 | 0 | nd |
| 13761 | TgSoUs5 | 0 | 1 | 0/2 |
| 14623 | TgSoUs7 | 1 (16) | 1 | 2/2 (14) |
| 14211 | TgSoUs8 | 1 (15) | 1 | 1/2 (15) |
| 14337 | TgSoUs9 | 1 (16) | 1 | 0/2 |
| 14651 | TgSoUs10 | 0 | 2 | 0/2 |
| 14335 | TgSoUs11 | 0 | 2 | 0/2 |
| 14374 | TgSoUs12 | 0 | 2 | 0/2 |
| 14379 | TgSoUs13 | 0 | 2 | 0/2 |
| 13729 | TgSoUs14 | 2 (15) | nd | 0/2 |
| 13791 | TgSoUs15 | 2 (15) | nd | 2/2 (11) |
| 14382 | TgSoUs16 | 0 | 2 | 0/2 |
| 14385 | TgSoUs17 | 0 | 0 | nd |
| 14513 | TgSoUs18 | 0 | 0 | nd |
| 14226 | TgSoUs19 | 1 (30) | 1 | nd |

nd = not done.

belong to the typical Type II lineage. Genotype #3 has two isolates (TgSoUs1, 2) that have Type II alleles at all loci except a Type I allele at locus L358. Genotype #4 has one isolate (TgSoUs35) that has Type II alleles at all loci except the Type I alleles at loci L358 and Apico. Genotype #5 has 21 isolates with a unique allele reported previously in California sea otters as Type X isolates (Miller et al., 2004; Conrad et al., 2005). It is interesting that two otters from Washington state had two different isolates (TgSoUs34, 35 and 36, 37) from heart and brain from one and from muscle, tongue, and brain, respectively one of the other. The genotype #6 had Type III alleles at all loci except Type II alleles at SAG2 and Apico.

#### 3.2.2. Sequencing

DNA sequence analysis for seven of the 21 isolates belonging to genotype #5 was carried out for markers SAG1 and GRA6. Within about 280 bp region in the 5′ end of SAG1, all seven isolates were identical to the previously reported Type X (Miller et al., 2004; Conrad et al., 2005). However, DNA sequences of GRA6 revealed two different subtypes, including the previously identified Type X and a new type with 15-bp deletion, here designated as Type A (Fig. 1A and B). The restriction enzyme MaeIII which recognizes sequence within the 15-bp deletion was used to genotype isolates in group #5 to distinguish Type X from Type A. Four Type X (TgSoUs8, 11, 15, 31) and 14 Type A isolates were identified (Tables 2 and 3).

#### 3.2.3. Geographic distribution of T. gondii isolates

Seventy percent of the isolates in the present study were from California sea otters. The geographic locations of isolates within California were fairly evenly divided between the northern (12 of 25 from San Mateo, Santa Cruz, and Monterey Counties) and southern (13 of 25 from San Luis Obispo County) halves of sea otter range. Within California 64% of the isolates were Type A or X. In the northern half of California sea otter range only 5 of 12 isolates (42%) were Type A or X, while in the southern half of the range 11 of 13 (85%) of the isolates were Type A or X. In Washington state 5 of 13 isolates (38.4%) were Type A or X.

### 3.3. Revival of cryopreserved isolates from California sea otters

Thirteen of 15 isolates could be revived after 10-year storage in liquid nitrogen (Table 4). Two of these 13 isolates were pathogenic for mice (Table 4). These isolates have now been cryopreserved at APDL for future studies.

## 4. Discussion

Recently, Miller et al. (2004) identified two genotypes including Type II and a new type named Type X from 35 California sea otter isolates using five PCR-RFLP markers B1, SAG1, SAG2 and SAG3 and

sequencing data for three isolates at SAG1 and GRA6. In the current study, we genotyped 39 *T. gondii* isolates from 37 sea otters of California and Washington state using 10 multi-locus PCR-RFLP markers including SAG1, SAG2 (a.k.a SAG2A), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico, and sequenced 13 representative isolates at loci SAG1 and GRA6. From these sea otter isolates, six genotypes and one mixed genotypes were identified. Comparing our results with that of Miller et al. (2004), it is likely that genotypes #1, #2, #3 and #4 in our study would be classified as the Type II in their report even though we did not type B1 locus (Table 3). In our study, the genotype #5, which account for 54% of the 39 isolates, can be divided into two groups, Type A and Type X based on DNA sequencing at GRA6 (Table 3, Fig. 1). At DNA sequence level, Type A has a 15 bp deletion at GRA6, which eliminates the MaeIII restriction site, therefore, MaeIII cuts Type X sequence but not that of Type A. RFLP analysis of 18 of the 21 isolates in group #5 confirmed that 14 isolates were Type A, indicating its high prevalence in sea otters of both California and Washington. Since Miller et al. (2004) identified Type X by sequencing GRA6 for two isolates with the unique allele at the SAG1, it is not clear if the Type A is absent or missed from the 35 isolates reported in their study. Further analysis of these isolates at GRA6 by PCR-RFLP using restriction enzyme MaeIII would address the question. The presence of six genotypes from sea otters is an indication that the otters were infected with the parasites from different sources with possible genetic recombination.

Miller et al. (2004) observed localized clustering of Type X near Morro Bay and reported Type X *T. gondii* as the primary cause of meningoencephalitis in nine of 12 otters. In our study, based on the criteria defined by Thomas et al. (2007), *T. gondii* was considered a contributing cause of death in only three (Table 1) of 37 (25 California and 12 Washington) sea otters; these three otters also had other potentially fatal conditions. In the remaining 34 otters *T. gondii* infection was considered incidental. Isolates from the three significant *T. gondii* infections included one Type II from Washington state (TgSoUs27), and two Type A isolates (TgSoUs22, 25) from San Luis Obispo County and Monterey County California, in the southern half of California sea otter range.

Previously, nothing was known of the mouse pathogenicity of *T. gondii* isolates from sea otters. In the present study, none of the nine isolates from the sea otters from Washington state were pathogenic to SW mice. Of the 13 *T. gondii* isolates from California that could be revived, two were pathogenic to mice (TgSoUs7 and TgSoUs15), However, these isolates had been passaged in mice and cell culture for an unknown number of passages. The effect of these passages of *T. gondii* on mouse virulence remains unknown. *T. gondii* virulence can be altered by frequent passages in mice (Dubey and Beattie, 1988).

The MAT is considered highly specific for the detection of antibodies to *T. gondii* infection in animals but there are no data on its specificity in sea otters (Dubey et al., 2003). A dilution of 1:25 has been used in MAT to screen sera for antibodies to *T. gondii* in sea otters (Dubey et al., 2003). Miller et al. (2002) evaluated the diagnostic efficiency of indirect fluorescent antibody test (IFAT) for the diagnosis of *T. gondii* infection in 77 dead sea otters by comparing serology and demonstration of the parasite in tissues by immunohistochemistry and by isolation of the parasite in cell culture. *T. gondii* was demonstrable in 20 of 27 sea otters with IFAT titers of 1:320 or higher, in 1 of 5 animals with a titer of 1:160, and in none of the 24 sea otters with an IFAT titer of <1:80. Thus, they chose a diagnostic titer of 1:320 for IFAT. In the present study, four of the 13 sea otters from California proven positive for *T. gondii* by bioassay had a low MAT titer of 1:50. These differences in isolation efficiency and titer maybe related to the serologic technique and the quality of sera obtained from dead sea otters.

In conclusion, results of this study revealed high genetic diversity among sea otter *T. gondii* isolates and further studies are needed to relate genotype with *T. gondii*-associated mortality in sea otters.

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