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Genotyping of viable *Toxoplasma gondii* from the first national survey of feral swine revealed evidence for sylvatic transmission cycle, and presence of highly virulent parasite genotypes

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Abstract

Feral swine are known reservoirs of various pathogens, including *Toxoplasma gondii*. Here, we report the first national survey of viable *T. gondii* in feral swine in the USA. We paired serological surveys with parasite isolation and bioassay to evaluate the prevalence and genetic diversity of these parasites. From 2012–2017, sera and tissues from 1517 feral swine across the USA were collected for the isolation of viable *T. gondii*. Serum samples were initially screened for antibodies to *T. gondii*, and then the tissues of seropositive feral swine were bioassayed in mice. Antibodies were detected in 27.7% of feral swine tested by the modified agglutination test (1:25 or higher). Antibody positive rates increased significantly with age, with 10.1% of juveniles, 16.0% of sub-adults and 38.4% of adults testing seropositive. Myocardium (50 g) from 232 seropositive feral swine was digested in pepsin and bioassayed in mice. Viable *T. gondii* was isolated from 78 feral swine from 21 states. Twelve of the 78 isolates were pathogenic to outbred Swiss Webster mice and 76 of the 78 isolates could be propagated further in cell culture and were genotyped. For genotyping, deoxyribonucleic acid extracted from cell culture-derived tachyzoites was characterized by polymerase chain reaction restriction fragment length polymorphism using the genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico. Genotyping revealed 15 ToxoDB genotypes, including 43 isolates for genotype #5 (haplogroup 12), 11 isolates for #24, four isolates for #2 (haplogroup 3), two isolates for each of genotypes #3 (haplogroup 2), #4 (haplogroup 12), #216, #221, #289 and #297 and one isolate for each of genotypes #1 (haplogroup 2), #39, #66, #260, #261 and #299. Genotype #5 was the most frequently isolated, accounted for 57% (43/76) of the isolates, followed by #24, accounted for 14% (11/76). Genotypes #260, #289, #297 and #299 are new types. Genotype #289 was highly virulent to mice and originated from feral swine collected in Louisiana on the same day at the same location. Genotype #216 was previously demonstrated to be highly virulent to mice. Our results indicate moderate genetic diversity of *T. gondii* in feral swine in the USA, with the genotype #5 (haplogroup 12) dominant in the continental USA, whereas genotype #24 (10/14) was dominant in Hawaii, suggesting different population structures of the parasites among the two distinct geographical locations.

Introduction

The protozoan *Toxoplasma gondii* infects virtually all warm-blooded animals, including birds, humans, livestock and marine mammals (Dubey, 2010). Domestic pigs are considered important in the epidemiology of toxoplasmosis in the USA (Dubey, 2010), but little is known of the role of feral swine.

Feral swine (*Sus scrofa*) populations in the USA are estimated to exceed five million and their geographic range continues to expand. Feral swine pose a threat to non-biosecure domestic pig facilities by serving as reservoirs for pathogens which may be transmitted to domestic pigs. In a national survey, antibodies to *T. gondii* were detected in ~20% of feral swine (Hill *et al.*, 2014). The presence of *T. gondii* in feral swine is considered a good indicator of contamination in the environment because they are omnivores with a generalist diet, and can become infected by ingesting oocysts while rooting and eating tissues of infected animals. Transmission of *T. gondii* has been documented in free-ranging domestic pigs through cannibalism (Dubey *et al.*, 1986; Hill *et al.*, 2010). The objective of the present investigation was to isolate and characterize *T. gondii* from feral swine across the USA.

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Table 1. Serological prevalence of *T. gondii* in feral swine collected across the USA from 2012–2017

Year	Samples received	Male	Female	Juvenile	Sub-adult	Adult	MAT positive (%; 95% CI)	Samples bioassayed	*T. gondii* isolates
2012	235	102	133	23	51	161	27.6 (22.3–33.7)	46	15
2013	848	407	439	44	170	634	28.5 (25.6–31.7)	97	29
2014	79	48	31	2	16	61	26.6 (18.1–37.2)	10	7
2015	132	65	66	27	31	72	23.5 (17.1–31.4)	27	9
2016	162	85	77	30	37	95	23.5 (17.6–30.1)	34	12
2017	61	27	34	3	13	45	39.3 (28.1–51.9)	18	6
Total	1517	734	780	129	318	1068[a]	27.7 (27.4–32.1)	232	78

[a]Age not recorded for two swine. Sex not recorded for three swine.

Materials and methods

Animals and sampled areas

The United States Department of Agriculture's (USDA) Wildlife Services has a task to control feral swine for wildlife damage management purposes and routinely collects sera from a subset for pathogen surveillance. For this study, sera and hearts were collected from 1517 feral swine between September 2012 and October 2017 from 30 states (Table 1). Sex, age (juvenile, sub-adult or adult), date of collection and location information were recorded for each feral swine (Hill *et al.*, 2014). Samples were submitted for *T. gondii* testing to the USDA's Animal Parasitic Diseases Laboratory in Beltsville, Maryland as described previously (Hill *et al.*, 2014).

Serology

Sera were tested for antibodies to *T. gondii* by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). Sera were screened at 1:25, 1:50, 1:100 and 1:200 dilutions or higher.

Isolation by bioassay in mice

A total of 1100 Swiss Webster (SW) mice and 275 INF-γ gene knock-out (KO) mice were used for bioassay and propagation of *T. gondii*. Myocardium samples (50 g) were homogenized in saline, digested in acidic pepsin, centrifuged and aliquots of homogenates were inoculated subcutaneously into 3–5 outbred albino SW mice, and/or one or two KO mice, which are especially susceptible to toxoplasmosis (Dubey, 2010). Inoculated mice that showed symptoms of toxoplasmosis were terminated and their lungs and brain imprints were examined for *T. gondii* tachyzoites or tissue cysts, respectively (Dubey, 2010). Survivors were bled 45 days post-inoculation (p.i.) and a 1:25 dilution of serum was tested for *T. gondii* antibodies by MAT. Mice were euthanized 46 days p.i. and brains of all mice were examined for tissue cysts as described previously (Dubey, 2010). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in their tissues.

Pathogenicity of oocysts of T. gondii strains in mice

To determine mouse pathogenicity of the parasite isolates, four *T. gondii* isolates showing different virulence levels based on initial observation on bioassay in SW mice were selected. For this, four 3–4 months old *T. gondii*-free cats (Dubey, 1995) were fed tissues of infected mice. Oocysts collected from the faeces of cats (Dubey, 2010) were sporulated in 2% sulphuric acid for a week on a shaker at room temperature, washed, counted and diluted 10-fold from 10^{-1} to 10^{-7} to reach an endpoint of ≅1 oocyst. Aliquots from each dilution of oocysts were fed to each of five SW mice and the recipient mice were examined for *T. gondii* infection. Mice were examined daily for illness for 2 months, and ill mice were euthanized. Survivors were bled and their sera were tested for *T. gondii* antibodies and their brains were examined for tissue cysts (Dubey, 2010).

Ethical considerations

All experimental procedures were approved by the Beltsville Area Animal Care and Use Committee (Protocol # 15-017, and 15-018), United States Department of Agriculture. Outbred SW and KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) incompliance with the Institutional Animal Ethics Committee guidelines.

The feral swine were euthanized in the field, often in remote locations, and tissues were transported by the collector to the office, and then shipped by overnight mail. Samples were shipped with ice packs. By the time tissues were received at the USDA laboratory, they often were contaminated with bacteria and not suited for cell culture to isolate *T. gondii*. A previous study with tissues of naturally infected domestic sows from Iowa indicated that the probability of isolation of *T. gondii* is very low unless large numbers of mice are used. In this case, of 109 *T. gondii* isolates obtained from 1000 naturally exposed sows, in most instances only 1 of 10 mice inoculated with sow heart tissue was positive for *T. gondii* (Dubey *et al.*, 1995). To increase the probability of isolating parasites and minimizing the number of mice, we decided to use five mice for the bioassay of each feral swine in the current study.

All mice and cats used in the present study were treated humanely and examined twice daily for any signs of illness and were supervised by a veterinarian assigned exclusively to the toxoplasmosis project. Any sick mice were euthanized because our objective was isolation of *T. gondii* and not testing for mortality. We wanted to collect mouse tissues aseptically for cultivation in cell culture or subpassage to other mice. Cats usually do not become ill within 10 days of ingesting *T. gondii* infected tissues, even though they can excrete many oocysts (Dubey, 2010). In the present study, cats were euthanized 2–3 days after they started excreting *T. gondii* oocysts.

In vitro cultivation

Infected mouse tissues were seeded onto CV1 cell culture flasks and tachyzoites were harvested from the medium as previously described (Dubey, 2010).

Table 2. *Toxoplasma gondii* isolates from feral swine in the USA from 2012–2017

Feral pigs					Bioassays in mice		
Isolate number	State	County	Collection date	MAT	SW (no. of infected/no. of inoculated)	KO (no. infected/no. inoculated)	Genotype
(1) TgFpAL1	AL	Pike	4/15/2013	400	1/3	ND	#5
(2) TgFpAL2	AL	Montgomery	5/17/2013	200	3/3	1/1	#5
(3) TgFpAR1	AR	Desha	10/23/2012	50	3/4	1/1	#5
(4) TgFpAR2	AR	Desha	11/27/2012	100	2/4	1/1	#5
(5) TgFpAR3	AR	Desha	11/27/2012	200	4/4	1/1	#5
(6) TgFpAR4	AR	Desha	11/27/2012	200	4/4	1/1	#5
(7) TgFpAR5	AR	Union	2/19/2013	200	3/3	ND	#221
(8) TgFpAR6	AR	Union	2/19/2013	400	1/3	ND	#5
(9) TgFpAR7	AR	Union	2/19/2013	200	1/3	ND	#5
(10) TgFpAR8	AR	Chicot	3/14/2013	200	1/3	ND	#5
(11) TgFpAR9	AR	Chicot	3/18/2013	200	3/3	ND	#5
(12) TgFpAR10	AR	Chicot	3/18/2013	200	1/3	ND	#5
(13) TgFpAR11	AR	Phillips	5/8/2013	200	1/3	ND	#5
(14) TgFpAZ1	AZ	Mohave	4/7/2013	200	3/3	ND	#5
(15) TgFpCA1	CA	Nevada	2/17/2017	200	2/4	1/1	#66
(16) TgFpFL1	FL	Pasco	2/12/2013	400	1/3	1/1	#221
(17) TgFpHI1	HI	Honolulu	10/6/2012	200	4/4	1/1	#24
(18) TgFpHI2	HI	Honolulu	10/15/2012	400	4/4	1/1	#24
(19) TgFpHI3	HI	Honolulu	11/7/2012	100	3/4	1/1	#24
(20) TgFpHI4	HI	Honolulu	11/12/2012	50	1/4	0/1	#3, Type II
(21) TgFpHI5	HI	Honolulu	2/9/2013	100	3/3	0/1	#24
(22) TgFpHI6	HI	Honolulu	4/11/2017	100	5/5	ND	#24
(23) TgFpHI7	HI	Kalawao	2/28/2017	>200	1/4	1/1	#297 (New)
(24) TgFpHI8	HI	Honolulu	12/19/2013	200	4/4	1/1	#24
(25) TgFpHI9	HI	Honolulu	1/7/2014	400	3/4	1/1	#260 (New)
(26) TgFpHI10	HI	Honolulu	1/7/2014	100	2/4	0/1	#24
(27) TgFpHI11	HI	Honolulu	1/14/2014	>200	3/4	1/1	#24
(28) TgFpHI12	HI	Honolulu	1/30/2014	>200	1/4	1/1	#261
(29) TgFpHI13	HI	Honolulu	2/2/2014	>200	4/4	1/1	#24
(30) TgFpHI14	HI	Honolulu	2/11/2014	1600	2/5	ND	#24
(31) TgFpIL1	IL	Du Page	10/20/2016	200	0/4	1/1	#1, Type II
(32) TgFpIL2	IL	Fulton	5/23/2013	200	2/3	2/2	#3, Type II
(33) TgFpIN1	IN	Lawrence	12/23/2012	100	0/4	1/1	#39
(34) TgFpIN2	IN	Lawrence	10/15/2015	>200	1/4	1/1	#2 likely
(35) TgFpIN3	IN	Washington	11/3/2015	200	1/4	1/1	#216 likely
(36) TgFpIN4	IN	Lawrence	4/12/2017	25	0/4	1/1	#4
(37) TgFpKS1	KS	Bourbon	3/27/2013	200	2/3	ND	#5
(38) TgFpLA1	LA	Orleans	11/6/2012	400	4/4	1/1	#289, New
(39) TgFpLA2	LA	Orleans	11/6/2012	3200	2/4	1/1	#289, New
(40) TgFpLA3	LA	East Feliciana	3/6/2013	200	1/3	ND	ND
(41) TgFpLA4	LA	West Feliciana	3/7/2013	1600	1/3	ND	#5
(42) TgFpMI1	MI	Bay	10/29/2015	200	4/4	1/1	#5 likely
(43) TgFpMI2	MI	Marquette	11/12/2015	>200	4/4	1/1	#5
(44) TgFpMI3	MI	Midland	2/2/2016	50	4/4	1/1	#299 (New)
(45) TgFpMO1	MO	Reynolds	9/19/2012	100	3/4	1/1	#5

(*Continued*)

Table 2. (*Continued.*)

Feral pigs					Bioassays in mice		
Isolate number	State	County	Collection date	MAT	SW (no. of infected/no. of inoculated)	KO (no. infected/no. inoculated)	Genotype
(46) TgFpMS1	MS	Yazoo	10/1/2012	200	4/4	1/1	#5
(47) TgFpMS2	MS	Sharkey	1/3/2013	100	4/4	1/1	#5
(48) TgFpMS3	MS	Yazoo	3/22/2013	400	3/3	ND	#5
(49) TgFpMS4	MS	Yazoo	3/26/2013	800	3/3	ND	#5
(50) TgFpMS5	MS	Yazoo	3/26/2013	200	3/3	ND	#5
(51) TgFpMS6	MS	Yazoo	5/28/2013	200	1/3	1/2	#5
(52) TgFpNC1	NC	Bladen	10/16/2012	400	0/4	1/1	ND
(53) TgFpNC2	NC	Bladen	7/28/2015	200	4/4	1/1	#5 likely
(54) TgFpNC3	NC	Columbus	8/3/2015	>200	3/4	0/1	#5 likely
(55) TgFpNC4	NC	Duplin	4/13/2016	50	4/4	1/1	#5 likely
(56) TgFpNY1	NY	Clinton	8/21/2013	800	2/3	1/1	#5
(57) TgFpOH1	OH	Lorain	11/21/2013	400	1/4	0/1	#4
(58) TgFpOH2	OH	Jackson	5/25/2016	100	4/4	1/1	#24
(59) TgFpOH3	OH	Gallia	8/10/2016	200	1/4	1/1	#2
(60) TgFpOH4	OH	Gallia	8/10/2016	100	2/4	0/1	#297 (New)
(61) TgFpOH5	OH	Gallia	10/20/2016	200	1/4	1/1	#5
(62) TgFpOH6	OH	Gallia	10/20/2016	200	4/4	1/1	#2 likely
(63) TgFpOH7	OH	Gallia	10/20/2016	400	0/4	1/1	#5 likely
(64) TgFpOH8	OH	Gallia	10/20/2016	200	3/4	1/1	#5
(65) TgFpOK1	OK	Tillman	1/14/2013	100	3/4	1/1	#5
(66) TgFpOK2	OK	Tillman	1/14/2013	400	2/4	1/1	#5
(67) TgFpOK3	OK	Choctaw	3/8/2013	800	1/3	ND	#5
(68) TgFpPA1	PA	Fulton	3/12/2016	25	3/4	0/1	#5 likely
(69) TgFpPA2	PA	Bedford	4/27/2016	800	4/4	1/1	#5 likely
(70) TgFpSC1	SC	Georgetown	3/5/2014	>200	2/5	ND	#5 likely
(71) TgFpSC2	SC	Georgetown	1/25/2013	400	1/3	1/1	#5
(72) TgFpSC3	SC	Georgetown	3/6/2013	400	1/3	ND	#5
(73) TgFpTX1	TX	Hemphill	12/3/2012	100	1/4	1/1	#5
(74) TgFpVA1	VA	Lee	6/14/2015	>200	1/4	0/1	#5 likely
(75) TgFpVA2	VA	Culpeper	10/1/2015	25	4/4	1/1	#2 likely
(76) TgFpVA3	VA	Lee	12/18/2015	100	1/4	0/1	#216
(77) TgFpVA4	VA	Chesapeake City	4/10/2017	>200	3/4	1/1	#5
(78) TgFpVA5	VA	Chesapeake City	4/10/2017	50	2/4	1/1	#5

AL, Alabama; AR, Arkansas; AZ, Arizona; CA, California; FL, Florida; HI, Hawaii; IL, Illinois; IN, Indiana; KS, Kansas; LA, Louisiana; MI, Michigan; MO, Missouri; MS, Mississippi; NC, North Carolina; NY, New York; OH, Ohio; OK, Oklahoma; PA, Pennsylvania; SC, South Carolina; TX, Texas; VA, Virginia; SW, Swiss Webster albino mice; KO, Interferon-γ Knockout mice; ND, Not done.

Genotyping of DNA samples

For successful genotyping of *T. gondii* strains from asymptomatic naturally infected animals, it is necessary to obtain good quality parasite deoxyribonucleic acid (DNA) with minimal contamination of host tissue. Therefore, parasite isolates from mouse tissues were expanded in cell culture. Genotyping of DNA samples by multilocus polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) markers were carried out following previously reported protocols (Su *et al.*, 2010). Samples with missing data for one to three of the 10 PCR-RFLP markers, but otherwise matching with previously reported genotypes were designated as 'likely' of that genotype.

Results

Antibodies to *T. gondii* were detected in 27.7% (421 of 1517) of feral swine (Table 1). The prevalence of *T. gondii* antibodies varied only slightly (23.5% to 28.5%) by year from 2012 to 2016. However, seroprevalence was much higher (39.3%) in samples collected in 2017. Among the 734 males and 780 female swine tested, seroprevalence did not differ significantly (26.3% *vs* 29.2%, $\chi^2 = 1.62$ $P > 0.20$). Seroprevalence increased significantly with age, with 10.1% of juveniles ($n = 129$), 16.0% of sub-adults ($n = 318$) and 38.4% of adults ($n = 1068$) testing positive ($\chi^2 = 78.73$, $P = 0$) (Table 1).

Viable *T. gondii* was isolated from 78 feral swine from 21 states (Table 2, Fig. 1). The isolation rate increased with MAT titer;

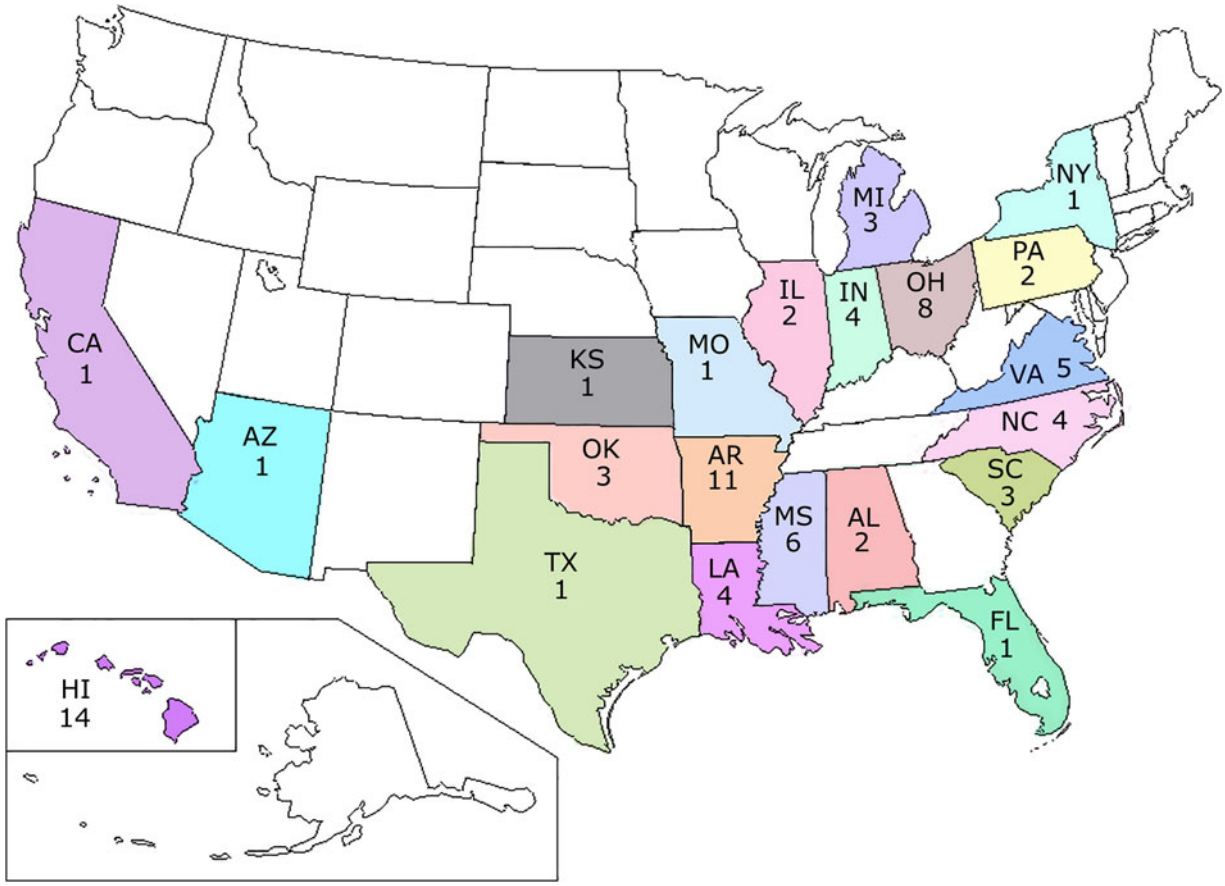

Fig. 1. Map of USA showing the *T. gondii* isolates from feral swine.

parasites were isolated from 11.7% of 17 feral swine with titers of 1:25, from 25% of 20 with titers of 1:50, from 22.6% of 53 with titers of 1:100 and from 39.8% of 143 with titers of ⩾1:200 (Supplementary Table 1).

The SW mice inoculated with tissue digest of hearts from 12 of the 78 infected feral swine showed symptoms of *T. gondii* infection and a few died or were euthanized between 11 and 27 days p.i. (Table 3).

The four cats fed infected mice excreted *T. gondii* oocysts but remained clinically healthy and were euthanized in good health 7–10 days after feeding infected mouse tissues. Oocysts of two isolates (TgFpLA1 and TgFpLA2, both are genotype #289) were very pathogenic to SW mice; all mice fed their oocysts died/or euthanized of acute toxoplasmosis enteritis or pneumonia and tachyzoites were found in lungs of all infected mice (Table 4). The isolate TgFpHI1 (genotype #24) was mildly pathogenic; mice fed 100 oocysts had signs of acute toxoplasmosis whereas mice fed fewer than 100 oocysts survived and remained asymptomatic. For isolate TgFpMS1 (genotype #5), only a few oocysts were present and low doses (10 and 1 oocysts) were used to challenge mice, all infected mice survived (Table 4).

Seventy-six of the 78 isolates were genotyped (Table 5); typing results for individual isolates are shown in Supplementary Table 2. The results revealed 15 ToxoDB genotypes, including 43 isolates for genotype #5 (haplogroup 12), 11 isolates for #24, four isolates for genotype #2 (haplogroup 3), two isolates for each of genotypes #3 (haplogroup 2), #4 (haplogroup 12), #216, #221, #289 and #297 and one isolate for each of genotypes #1 (haplogroup 2), #39, #66 (haplogroup 11), #260, #261 and #299. Genotype #5 was the most frequently isolated, accounted for 57.5% (43/76) of the isolates, followed by #24, accounting for 14% (11/76). Genotypes #260, #289, #297 and #299 have not been previously reported. Genotype #289 was mouse-virulent, and originated from each of two feral swine collected concurrently from a location in Louisiana; no other information was available regarding these pigs.

Discussion

The primary objective of the present study was to isolate and genetically characterize *T. gondii* occurring in feral swine in the USA. We have previously reported seroprevalence of *T. gondii* in feral swine samples collected between 2006–2013, which was 17.7% by ELISA and 28.4% by MAT (Hill *et al.*, 2014). The results of this study (27.7% seroprevalence) supplement previously reported data and indicate that *T. gondii* infection remains high in feral swine in the USA. This prevalence of *T. gondii* antibodies was like the 23% (4759 of 16 788) seroprevalence detected in wild pigs worldwide (Rostami *et al.*, 2017).

Most isolates from feral swine in the present study were identified as ToxoDB genotype #5, which is the dominant type in North American wildlife (Dubey *et al.*, 2011; Khan *et al.*, 2011; Jiang *et al.*, 2018). This contrasts with what has been most frequently derived from domestic pigs in USA, in which the dominant *T. gondii* genotypes are #1 and #3 (collectively known as type II, haplogroup 2) and #3 (type III, haplogroup 3) (Velmurugan *et al.*, 2009; Jiang *et al.*, 2018). Importantly, these data substantiate a distinction between transmission among feral and farmed pigs in North America (Jiang *et al.*, 2018). In Europe, the type II *T. gondii* lineage is dominant in human population, and genotyping data showed that it is also true in wildlife including wild hogs (Richomme *et al.*, 2009; Aubert *et al.*, 2010), suggesting *T. gondii* population is largely homogeneous and no partition of parasite genotypes in the region. Several studies in China indicated dominance of ToxoDB genotype #9 in domestic pigs (Zhou *et al.*, 2010; Jiang

Table 3. Isolates of pathogenic *T. gondii* identified in feral swine collected across the USA from 2012–2017

								Bioassayed in SW mice[a]	
Isolate number	Feral pig ID	Collection date	State	County	Age class	Sex	MAT	No. of mice infected with *T. gondii*	No. of mice died/euthanized (day)	Genotype
15 TgFpCA1	ID0034613	2/17/2017	CA	Nevada	Sub-Adult	Male	200	2	1 (26)	#66
17 TgFpHI1	ID0016782	10/6/2012	HI	Honolulu	Sub-Adult	Female	200	4[b]	1 (11)	#24
26 TgFpHI10	ID0019838	1/7/2014	HI	Honolulu	Adult	Male	100	2	1 (19)	#24
27 TgFpHI11	ID0019841	1/14/2014	HI	Honolulu	Adult	Male	>200	3	1 (23)	#24
35 TgFpIN3	ID0030648	11/3/2015	IN	Washington	Adult	Female	200	1[c]	1 (14)	#216 Likely
38 TgFpLA1	ID0017224	11/6/2012	LA	Orleans	Adult	Female	400	4[b]	4 (15, 20, 20, 22)	#289, New
39 TgFpLA2	ID0017225	11/6/2012	LA	Orleans	Adult	Female	3200	2	2 (20, 20)	#289, New
40 TgFpLA3	ID0017282	3/6/2013	LA	East Feliciana	Adult	Male	200	1	1 (12)	Not done
46 TgFpMS1	ID0017303	10/1/2012	MS	Yazoo	Adult	Female	200	1[b]	1 (14)	#5
54 TgFpNC3	ID0026419	8/3/2015	NC	Columbus	Adult	Male	>200	3	1 (27)	#5 Likely
75 TgFpVA3	ID0031871	12/18/2015	VA	Lee	Adult	Male	100	1	1 (15)	#216
76 TgFpVA4	ID0031573	4/10/2017	VA	Chesapeake City	Adult	Female	>200	3	1 (19)	#5

[a]Four mice were inoculated with pig hearts.
[b]One SW mouse from the group fed to cat.
[c]Three out of four mice died on day 2 – it was no due to toxoplasmosis.

Table 4. Pathogenicity of oocysts of four *T. gondii* isolates derived from feral swine

	Isolate number (ToxoDB genotype)			
Dose[a]	TgFpMS1 (#5)	TgFpHI1 (#24)	TgFpLA1 (#289)	TgFpLA2 (#289)
100	Not done	5 (10, 10, 10, 12, 15)	5 (9, 9, 9, 9, 9)	5 (8, 8, 9, 9, 9)
10	5 (S, S, S, S, S)[b]	4 (S, S, S, S)	3 (10, 10, 13)	5 (8, 9, 9, 9, 12)
1	5 (S, S, S, S, S)	2 (S, 16)	1 (13)	5 (9, 12, 12, 12, 12)
<1	0	Not done	0	0

S, Survived, infected with *T. gondii*.
Five mice per group. Oocysts were inoculated orally.
[a]Based-on estimation that the last infective dilution has one infective organism.
[b]No. of mice dead, and day of death in parenthesis.

Table 5. *Toxoplasma gondii* isolates and genotype by State of feral swine collected from 2012–2017

State	Samples bioassayed	No. of isolates	Toxo DB genotype
AL	10	2	#5
AR	26	11	#5; #221
AZ	1	1	#5
CA	3	1	#66
FL	7	1	#221
HI	28	14	#24; #3 Type II; #260 (New); #261; #297 (New)
IL	2	2	#1 Type II; #3 Type II
IN	9	4	#39; #2 likely; #4; #216 likely
KS	18	1	#5
LA	10	4	#289 (New); #5
MI	10	3	#5 likely; #1 or #3 Type II; #299 (New)
MO	14	1	#5
MS	14	6	#5
NC	14	4	#5 likely
NY	1	1	#5
OH	16	8	#4; #24; #2; #2 likely; #5 likely; #5; 297 (New)
OK	3	3	#5
PA	9	2	#5 likely
SC	5	3	#5 likely; #5
TX	6	1	#5
VA	13	5	#5 likely; #2 likely; #5; #216
GA	2	0	
Guan	3	0	
KY	1	0	
NV	1	0	
TN	4	0	
UT	2	0	
Total	232	78	#1 ($n = 1$), #2 ($n = 4$), #3 ($n = 2$), #4 ($n = 2$), #5 ($n = 43$), #24 ($n = 11$), #39 ($n = 1$), #66 ($n = 1$), #216 ($n = 2$), #221 ($n = 2$), #260 ($n = 1$), #261 ($n = 1$), #289 ($n = 2$), #297 ($n = 2$), #299 ($n = 1$)

Two of the 78 isolates were not typed.

et al., 2013; Wang *et al.*, 2016), however, there is limited information regarding genotypes in wildlife for a comparison. Recent data from Brazil indicated high genetic diversity of *T. gondii* in domestic pigs (Feitosa *et al.*, 2017). But information is still limited to compare the parasites from domestic animal *vs* wildlife. To better understand the partition of transmission, studies of *T. gondii* genotypes in domestic animals and wildlife in other regions such as Asia, Africa, Australia and South America are needed.

Pathogenicity of oocysts of four *T. gondii* strains in mice suggested that the newly identified genotype #289 (isolates TgFpLA1 and TgFpLA2) is highly virulent. However, genotype #24 (isolate TgFpHI1), common in Hawaii, is mildly pathogenic. The genotype #5 (isolate TgFpMS1), prevalent in wildlife in North America, is also mildly pathogenic to mice. This result indicates that, even though most *T. gondii* strains in the U.S. are not highly virulent, there is a low frequency of highly virulent parasites circulating in wildlife.

Genotype #24 was the second-most frequently isolated type in this study. Ten of the 14 isolates identified in Hawaii belong to #24, which accounted for 71% (10/14) of those isolates (Table 2). Genotype #24 has previously been identified in chickens from Costa Rica and Brazil (Dubey *et al.*, 2006; Ferreira *et al.*, 2018), and in bobcats from Mississippi, USA (Verma *et al.*, 2017), suggesting it is widely distributed in the USA. Bioassays in mice indicate #24 strains are not highly virulent to mice (Tables 3 and 4). Among the four new genotypes identified in this study, two (#260 and #297) were from Hawaii. In addition, genotype #261 was also first identified in Hawaiian geese (Work *et al.*, 2016). These results indicate that the *T. gondii* population in Hawaii differs from those in the continental USA.

Other genotypes, including #1, #2, #3, #4, #39 #66, #216 and #221, have previously been identified in animals in the USA, with the first four being common (Jiang *et al.*, 2018). Among these genotypes, #216 is highly virulent to mice (Dubey *et al.*, 2013*a*, 2013*b*, 2015).

Recent evidence indicates that wildlife *T. gondii* strains can also cause clinical disease in humans (Jokelainen *et al.*, 2018; Pomares *et al.*, 2018) and domestic cats (Dubey and Prowell, 2013; Crouch *et al.*, 2019). It is suggested that partition of *T. gondii* genotypes among domestic animals and wildlife is mainly due to distinct sylvatic and domestic transmission cycles, though both cycles overlap to a certain degree (Shwab *et al.*, 2018).

Our results revealed moderate genetic diversity of *T. gondii* in feral swine in the USA, with genotype #5 (haplogroup 12) dominant in continental USA, whereas genotype #24 (10/14) was dominant in Hawaii, suggesting different population structures of the parasites among the two distinct genographical locations. The *T. gondii* isolates detected in feral swine generally resembled those found in other wildlife species and were distinct from those that are typically identified in domestic pigs, and include novel genotypes including ones that are highly virulent to mice. The

contribution of feral swine as a reservoir of infection deserves additional scrutiny, as well as their potential in disseminating parasites to humans.

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References

Aubert D, Ajzenberg D, Richomme C, Gilot-Fromont E, Terrier ME, de Gevigney C, Game Y, Maillard D, Gibert P, Dardé ML and Villena I (2010) Molecular and biological characteristics of *Toxoplasma gondii* isolates from wildlife in France. *Veterinary Parasitology* **171**, 346–349.

Crouch EEV, Mittel LD, Southard TL, Cerqueira-Cézar CK, Murata FHA, Kwok OCH, Su C and Dubey JP (2019) Littermate cats rescued from a shelter succumbed to acute, primary toxoplasmosis associated with TOXO DB genotype #4, generally circulating in wildlife. *Parasitology International* **72**, article 101942.

Dubey JP (1995) Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. *Journal of Parasitology* **81**, 410–415.

Dubey JP (2010) Toxoplasmosis of Animals and Humans, 2nd Edn. Boca Raton, Florida, USA: CRC Press, pp. 1–313.

Dubey JP and Desmonts G (1987) Serological responses of equids fed *Toxoplasma gondii* oocysts. *Equine Veterinary Journal* **19**, 337–339.

Dubey JP and Prowell M (2013) Ante-mortem diagnosis, diarrhea, oocyst shedding, treatment, isolation and genetic typing of *Toxoplasma gondii* associated with clinical toxoplasmosis in a naturally-infected cat. *Journal of Parasitology* **99**, 158–160.

Dubey JP, Murrell KD, Hanbury RD, Anderson WR, Doby PB and Miller HO (1986) Epidemiologic findings on a swine farm with enzootic toxoplasmosis. *Journal of the American Veterinary Medical Association* **189**, 55–56.

Dubey JP, Thulliez P and Powell EC (1995) *Toxoplasma gondii* in Iowa sows: comparison of antibody titers to isolation of *T. gondii* by bioassays in mice and cats. *Journal of Parasitology* **81**, 48–53.

Dubey JP, Su C, Oliveira J, Morales JA, Bolaños RV, Sundar N, Kwok OCH and Shen SK (2006) Biologic and genetic characteristics of *Toxoplasma gondii* isolates in free-range chickens from Costa Rica, Central America. *Veterinary Parasitology* **139**, 29–36.

Dubey JP, Velmurugan GV, Rajendran C, Yabsley MJ, Thomas NJ, Beckmen KB, Sinnett D, Ruid D, Hart J, Fair PA, McFee WE, Shearn-Bochsler V, Kwok OCH, Ferreira LR, Choudhary S, Faria EB, Zhou H, Felix TA and Su C (2011) Genetic characterisation of *Toxoplasma gondii* in wildlife from North America revealed widespread and high prevalence of the fourth clonal type. *International Journal for Parasitology* **41**, 1139–1147.

Dubey JP, Choudhary S, Kwok OCH, Ferreira LR, Oliveira S, Verma SK, Marks DR, Pedersen K, Mickley RM, Randall AR, Arsnoe D and Su C (2013a) Isolation and genetic characterization of *Toxoplasma gondii* from mute swan (*Cygnus olor*) from the USA. *Veterinary Parasitology* **195**, 42–46.

Dubey JP, Hill D, Zarlenga D, Choudhary S, Ferreira LR, Oliveira S, Verma SK, Kwok OCH, Driscoll CP, Spiker H and Su C (2013b) Isolation and characterization of new genetic types of *Toxoplasma gondii* and prevalence of *Trichinella murrelli* from black bear (*Ursus americanus*). *Veterinary Parasitology* **196**, 24–30.

Dubey JP, Verma SK, Calero-Bernal R, Cassinelli AB, Kwok OCH, van Why K, Su C and Humphreys JG (2015) Isolation and genetic characterization of *Toxoplasma gondii* from black bears (*Ursus americanus*), bobcats (*Lynx rufus*), and feral cats (*Felis catus*) from Pennsylvania. *The Journal of Eukaryotic Microbiology* **62**, 410–415.

Feitosa TF, Vilela VLR, de Almeida-Neto JL, dos Santos A, de Morais DF, Alves BF, Nakashima F, Gennari SM, Athayde ACR and Pena HFJ (2017) High genetic diversity in *Toxoplasma gondii* isolates from pigs at slaughterhouses in Paraíba state, northeastern Brazil: circulation of new genotypes and Brazilian clonal lineages. *Veterinary Parasitology* **224**, 76–80.

Ferreira TCR, Buery JC, Moreira NIB, Santos CB, Costa JGL, Pinto LV, Baraviera RCA, Vitor RWA and Fux B (2018) *Toxoplasma gondii*: isolation, biological and molecular characterisation of samples from free-range *Gallus gallus domesticus* from countryside Southeast Brazil. *Brazilian Journal of Veterinary Parasitology* **27**, 384–389.

Hill DE, Haley C, Wagner B, Gamble HR and Dubey JP (2010) Seroprevalence of and risk factors for *Toxoplasma gondii* in the US swine herd using sera collected during the National Animal Health Monitoring Survey (Swine 2006). *Zoonoses and Public Health* **57**, 53–59.

Hill DE, Dubey JP, Baroch JA, Swafford SR, Fournet VF, Hawkins-Cooper D, Pyburn DG, Schmit BS, Gamble HR, Pedersen K, Ferreira LR, Verma SK, Ying Y, Kwok OC, Feidas H and Theodoropoulos G (2014) Surveillance of feral swine for *Trichinella* spp. and *Toxoplasma gondii* in the USA and host-related factors associated with infection. *Veterinary Parasitology* **205**, 653–665.

Jiang HH, Huang SY, Zhou DH, Zhang XX, Su C, Deng SZ and Zhu XQ (2013) Genetic characterization of *Toxoplasma gondii* from pigs from different localities in China by PCR-RFLP. *Parasites & Vectors* **6**, 227.

Jiang T, Shwab EK, Martin RM, Gerhold RW, Rosenthal BM, Dubey JP and Su C (2018) A partition of *Toxoplasma gondii* genotypes across spatial gradients and among host species, and decreased parasite diversity towards areas of human settlement in North America. *International Journal for Parasitology* **48**, 611–619.

Jokelainen P, Murat JB and Nielsen HV (2018) Direct genetic characterization of *Toxoplasma gondii* from clinical samples from Denmark: not only genotypes II and III. *European Journal of Clinical Microbiology and Infectious Diseases* **37**, 579–586.

Khan A, Dubey JP, Su C, Ajioka JW, Rosenthal BM and Sibley LD (2011) Genetic analyses of atypical *Toxoplasma gondii* strains reveals a fourth clonal lineage in North America. *International Journal for Parasitology* **41**, 645–655.

Pomares C, Devillard S, Holmes TH, Olariu TR, Press CJ, Ramirez R, Talucod J, Estran R, Su C, Dubey JP, Ajzenberg D and Montoya JG (2018) Genetic characterization of *Toxoplasma gondii* DNA samples isolated from humans living in North America: an unexpected high prevalence of atypical genotypes. *Journal of Infectious Diseases* **218**, 1783–1791.

Richomme C, Aubert D, Gilot-Fromont E, Ajzenberg D, Mercier A, Ducrot C, Ferté H, Delorme D and Villena I (2009) Genetic characterization of *Toxoplasma gondii* from wild boar (*Sus scrofa*) in France. *Veterinary Parasitology* **164**, 296–300.

Rostami A, Riahi SM, Fakhri Y, Saber V, Hanifehpour H, Valizadeh S, Gholizadeh M, Pouya RH and Gamble HR (2017) The global seroprevalence of *Toxoplasma gondii* among wild boars: a systematic review and meta-analysis. *Veterinary Parasitology* **244**, 12–20.

Shwab EK, Saraf P, Zhu XQ, Zhou DH, McFerrin BM, Ajzenberg D, Schares G, Hammond-Aryee K, van Helden P, Higgins SA, Gerhold RW, Rosenthal BM, Zhao X, Dubey JP and Su C (2018) Human impact on the diversity and virulence of the ubiquitous zoonotic parasite *Toxoplasma gondii*. *Proceedings of the National Academy of Sciences of the United States of America* **115**, E6956–E6963.

Su C, Shwab EK, Zhou P, Zhu XQ and Dubey JP (2010) Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. *Parasitology* **137**, 1–11.

Velmurugan GV, Su C and Dubey JP (2009) Isolate designation and characterization of *Toxoplasma gondii* isolates from pigs in the United States. *Journal of Parasitology* **95**, 95–99.

Verma SK, Sweeny AR, Lovallo MJ, Calero-Bernal R, Kwok OC, Jiang T, Su C, Grigg ME and Dubey JP (2017) Seroprevalence, isolation, and co-infection of multiple *Toxoplasma gondii* strains in individual bobcats (*Lynx rufus*) from Mississippi, USA. *International Journal for Parasitology* **47**, 297–303.

Wang D, Liu Y, Jiang T, Zhang G, Yuan G, He J, Su C and Yang N (2016) Seroprevalence and genotypes of *Toxoplasma gondii* isolated from pigs intended for human consumption in Liaoning province, northeastern China. *Parasites & Vectors* **9**, 248.

Work TM, Verma SK, Su C, Medeiros J, Kaiakapu T, Kwok OC and Dubey JP (2016) *Toxoplasma gondii* antibody prevalence and two new genotypes of the parasite in endangered Hawaiian geese (nene: *Branta sandvicensis*). *Journal of Wildlife Diseases* **52**, 253–257.

Zhou P, Nie H, Zhang LX, Wang HY, Yin CC, Su C, Zhu XQ and Zhao JL (2010) Genetic characterization of *Toxoplasma gondii* isolates from pigs in China. *Journal of Parasitology* **96**, 1027–1029.