In the United States, negligible rates of zoonotic sarcocystosis occur in feral swine that, by contrast, frequently harbour infections with *Sarcocystis miescheriana*, a related parasite contracted from canids

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SUMMARY

Transmission of pathogens between domestic and wild life animals plays an important role in epidemiology. Feral pig populations are increasing and expanding in the USA, and may constitute a risk to non-biosecure domestic pig facilities by serving as reservoirs for pathogens. We surveyed, for *Sarcocystis* infection, the myocardium of 1006 feral pigs (*Sus scrofa*) trapped or hunted in 29 states during the Comprehensive Feral Swine Disease Surveillance Program of the USDA's Animal and Plant Health Inspection Service, Wildlife Services unit during 2012–2014. Sarcocysts were detected in histological sections of 25% (251/1006) of myocardium with an average parasitic load/intensity of infection of 3·03 sarcocysts/section (1·5 × 0·7 cm), and higher prevalence of myocarditis in severe infections. Microscopic examination of pepsin digests of 147 hearts revealed a higher prevalence of *Sarcocystis* bradyzoites (49%, 72/147) than when diagnosed by histology. A fragment of *Sarcocystis* 18S rRNA was amplified and digested with a restriction endonuclease, revealing a pattern consistent with *Sarcocystis miescheriana* in all 44 selected samples. Sequencing 31 of these 44 isolates confirmed their correspondence to *S. miescheriana*. Thus, *S. miescheriana* infection, but not the zoonotic parasite *Sarcocystis suihominis*, appears to be prevalent and widespread in feral pigs in the USA.

Key words: *Sarcocystis*, feral pigs, *Sus scrofa*, PCR-RFLP, parasitic load, food safety, USA.

INTRODUCTION

Species of *Sarcocystis* have obligatory two-host life cycles. Definitive hosts are infected by eating parasitized tissues from intermediate hosts, and these become infected by ingesting sporocysts excreted by the definitive host into the environment (Dubey *et al*. 1989). Pigs are intermediate hosts of at least two species of *Sarcocystis, Sarcocystis miescheriana* and *Sarcocystis suihominis*. Dog (*Canis familiaris*), raccoon (*Procyon lotor*), wolf (*Canis lupus*), red fox (*Vulpes vulpes*) and jackal (*Canis aureus*) are definitive hosts for *S. miescheriana* (Dubey *et al*. 1989).

Clinical fatal sarcocystosis attributed to *S. miescheriana* has been reported once in a naturally infected pig (Caspari *et al*. 2011). The affected pig was a 2-year-old boar that had fever, anorexia and died after a short period of illness. Histopathological examination revealed severe myocarditis associated with *S. miescheriana*-like schizonts; sarcocysts were not seen. Molecular characterization of DNA extracted from paraffin-embedded myocardium indicated *S. miescheriana* infection (Caspari *et al*. 2011). In experimental infections, *S. miescheriana* is pathogenic for pigs, with clinical signs including weight loss, anorexia, dyspnea, purpura of the skin, muscle tremors, weakness, thrombocytopenia, fever, abortion and death (Barrows *et al*. 1982*b*; Reiner *et al*. 2002).

Unlike *S. miescheriana, S. suihominis* is zoonotic. Humans and nonhuman primates (*Macaca mulatta, Macaca irus, Pan troglodytes* and *Papio cynocephalus*) are definitive hosts for *S. suihominis* (Dubey *et al*. 1989). Humans who ingested raw pork containing *S. suihominis* sarcocysts became ill, some with severe symptoms (diarrhoea, nausea, anorexia, fever, headache, dizziness and rapid pulse) (Piekarski *et al*. 1978; Li *et al*. 2007). It is also pathogenic for pigs and may cause similar clinical signs to those caused by *S. miescheriana* (Heydorn, 1977).

Although *Sarcocystis* infections in pigs have been reported in several countries such as Uruguay, India, Japan and China (Saleque and Bhatia, 1991; Freyre *et al*. 1992; Saito *et al*. 1998; Yan *et al*. 2013), we are only aware of few old reports from the USA. In USA, *Sarcocystis* bradyzoites were found in trypsin digests

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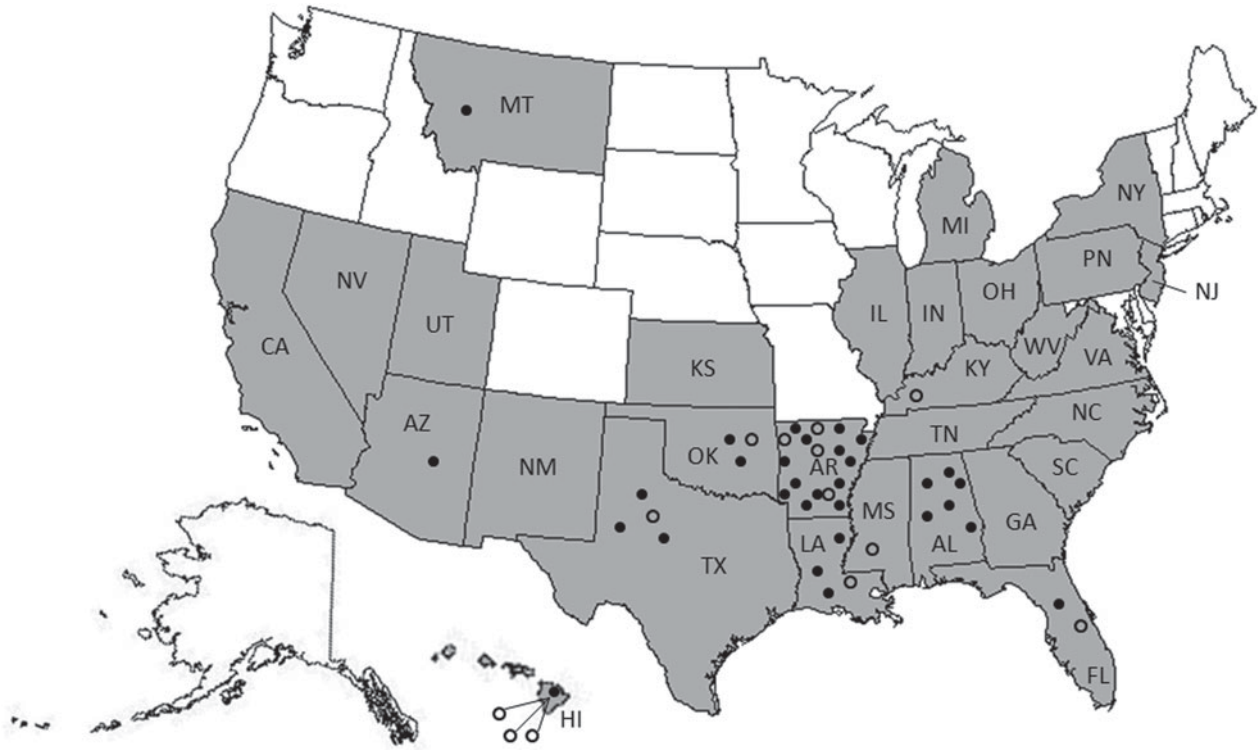

Fig. 1. Sampling areas in the USA. Shaded areas corresponding with those US states in which feral swine were collected: Alabama (AL), Arkansas (AK), Arizona (AZ), California (CA), Florida (FL), Georgia (GA), Hawaii (HI), Illinois (IL), Indiana (IN), Kansas (KS), Kentucky (KY), Louisiana (LO), Michigan (MI), Montana (MT), Mississippi (MS), North Carolina (NC), New Jersey (NJ), New Mexico (NM), Nevada (NV), New York (NY), Ohio (OH), Oklahoma (OK), Pennsylvania (PN), South Carolina (SC), Tennessee (TN), Texas (TX), Utah (UT), Virginia (VA), West Virginia (WV). Spots are referred to *Sarcocystis* spp. PCR positive animals; black dots correspond to samples from which sequencing was successful.

of 8 (3·4%) of 235 domestic sows from Ohio (Dubey, 1979), in pepsin digests of 28 (16·5%) of 168 domestic sows from Georgia (Prestwood *et al*. 1980), and in 163 (18·2%) of 893 adult sows from Iowa (Dubey and Powell, 1994); all of these pigs were destined for human consumption. In addition, Coombs and Springer (1974) detected sarcocysts in five feral swine (feral pig crossed with European wild boar) from Texas; and Barrows *et al*. (1981) found *Sarcocystis* bradyzoites in pepsin digests of 62 (32%) of 192 feral swine from 11 southern US states. Most of the above citations diagnosed the infections as *S. miescheriana* by bioassay in dogs (Prestwood *et al*. 1980; Barrows *et al*. 1981) or histological examination (Dubey and Powell, 1994).

Current molecular methodologies allow us to detect and type the specific species in meats, meat products (Moré *et al*. 2014) and animal samples (Yang *et al*. 2002); moreover, PCR methods open avenues of knowledge on parasite–host relationship, epidemiology and phylogenetic studies (Kia *et al*. 2011; Yan *et al*. 2013).

The aim of this study was to determine the prevalence of *Sarcocystis* species in feral pigs from the USA, study possible pathological changes associated to infections, and provide molecular characterization and phylogenetic analyses of the isolates collected. Because feral swine may be hunted as a food source, or may serve as a reservoir for infection for domesticated herds, we were especially interested to determine whether feral swine, might be infected with the form of porcine sarcocystosis established as a zoonotic agent, *S. suihominis*.

MATERIALS AND METHODS

Samples collection

During an epidemiological investigation of *Toxoplasma gondii* infection in feral pigs (*Sus scrofa*), hearts from 1006 individuals sampled from throughout the country (29 states; Fig. 1) were collected and submitted to the Animal Parasitic Diseases Laboratory (APDL), United States Department of Agriculture, Beltsville, Maryland (Hill *et al*. 2014). A piece (5 × 2 cm) of each heart was fixed in 10% buffered neutral formalin for subsequent examination. Information concerning gender, and age class of the animals based on lower jaw tooth eruption criteria (incisor #2 absent = juveniles, less than 2 month old; incisor #2 erupted, deciduous canine = sub-adults, between 2 months and 1 year old; permanent canine = adults, over 1 year old; Matschke, 1967) were recorded for each collected sample.

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Histopathological examination

Tissue samples were cut into sections (1·5 × 0·7 cm), embedded in paraffin and sectioned 5 μm thick; the sections were stained with haematoxylin and eosin (H and E) and observed under the microscope. Intensity of infection was estimated as number of sarcocysts per tissue section.

Pepsin digestion examination

Samples of 147 myocardium (50 g) were homogenized and digested in acidic pepsin (Dubey, 2010). To test for the presence of bradyzoites of *Sarcocystis*, 50 μL of digested tissue were placed on a slide, covered with a coverslip and screened under the microscope at 400× magnification. Approximately 300 μL were stored in microtubes, labelled and maintained at −70 °C until the molecular processing.

Molecular characterization

DNA was extracted from each of the above digests using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, California, USA) according to manufacturer's instructions. DNA quantification and quality were determined using a NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). PCR was performed using the *Sarcocystis* spp. specific primers amplifying a 915 bp portion of the 18S ribosomal RNA gene, 2L and 3H (2L forward 5′-GGATAAACCGTGGTAATTC-TATG-3′ and 3H reverse 5′-GGCAAATGCTTT-CGCAGTAG-3′) as described previously (Holmdahl et al., 1994; Yang *et al*. 2001). Briefly: initial denaturation at 95 °C for 5 min; 40 cycles of amplification (94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min) and final extension at 72 °C for 10 min.

The PCR products were subjected to restriction endonuclease digestion using *Ssp*I to reveal the polymorphism between *S. miescheriana* and *S. suihominis* as described by Yang *et al*. (2002). After *Ssp*I digestion, the 915 bp PCR product of *S. miescheriana* generates two fragments (650 and 265 bp), while with the *S. suihominis* amplicon no digestion occurs. Digestion was performed in a 20 μL reaction mixture containing 6 μL of 18S PCR product and *Ssp*I enzyme (5U) at 37 °C for 12 h. The amplified and digested PCR products were run on 2·5% (w/v) agarose gel with ethidium bromide stain and visualized using Gel Logic 212 Imaging Systems (Eastman Kodak Company, Rochester, New York, USA).

PCR products of 31 samples, derived from various geographic locales, were selected and sequenced directly using the same primers by Macrogen Corporation (Rockville, Maryland, USA). Sequencing was performed in both directions. These sequences were trimmed and edited in Geneious prior to being compared to each other, and to related sequences identified through BLAST from the non-redundant GenBank database.

After alignment using the MUSCLE algorithm, these sequences were subjected to phylogenetic analysis under the criterion of maximum likelihood using PHYML as implemented by Geneious assuming the HKY substitution model and allowing estimation of the transition/transversion ratio and the gamma distribution parameter.

Table 1. Prevalence of *Sarcocystis* sarcocysts and bradyzoites detected in the feral pigs surveyed in the USA

	Presence of sarcocysts (H and E)	Presence of bradyzoites (pepsin digestion)
Number of positive	251 (24·9%)	73 (49·3%)
Number of negative	755 (75·1%)	75 (50·7%)
Total examined	1006	148

Statistical analyses

Statistical analyses were performed with SPSS software, version 15·0 (SPSS Inc., Chicago, Illinois, USA). According to data, appropriate parametric or non parametric tests were employed. A P value of $\leqslant 0{\cdot}05$ was considered significant.

RESULTS

Sarcocysts were detected in 24·95% (251/1006) of myocardial sections examined histologically; bradyzoites were found in 49·0% (72/147) of digested tissues (Table 1). Infection intensity varied by over two orders of magnitude (range 1–184 sarcocysts/section). The average parasitic load was 3·03 ± 12·10 sarcocysts/section (n = 251); a maximum of 35 foci of myocarditis in the same section were detected; degenerated sarcocysts were found in only one section, seven sarcocysts were found in the centre of a focus of necrosis with mononuclear cell infiltration (Fig. 2A–E); higher prevalence of myocarditis was found in severe infections (r=0·974) (Table 2).

Statistically significant differences were detected among prevalence of infection detected in feral pigs from particular US states (Fig. 1; Table 3); the highest prevalence was observed in Texas (54·29%; 38/70) and the lowest prevalence in Mississippi (13·64%; 12/88). Slightly lower prevalence was observed in male swine (23·59%) than in female swine (26·54%); prevalence increased with age (juvenile: 6·52%; sub-adults: 15·38%; adults: 28·92%) (Table 4); finally, higher parasitic loads were detected in adult individuals (3·23 ± 13·02 sarcocysts/section) and in females (4·02 ± 16·20 sarcocysts/section) (Table 4).

A region of the *Sarcocystis* 18S rRNA gene was amplified in 44 samples from 11 US states (AL, AR,

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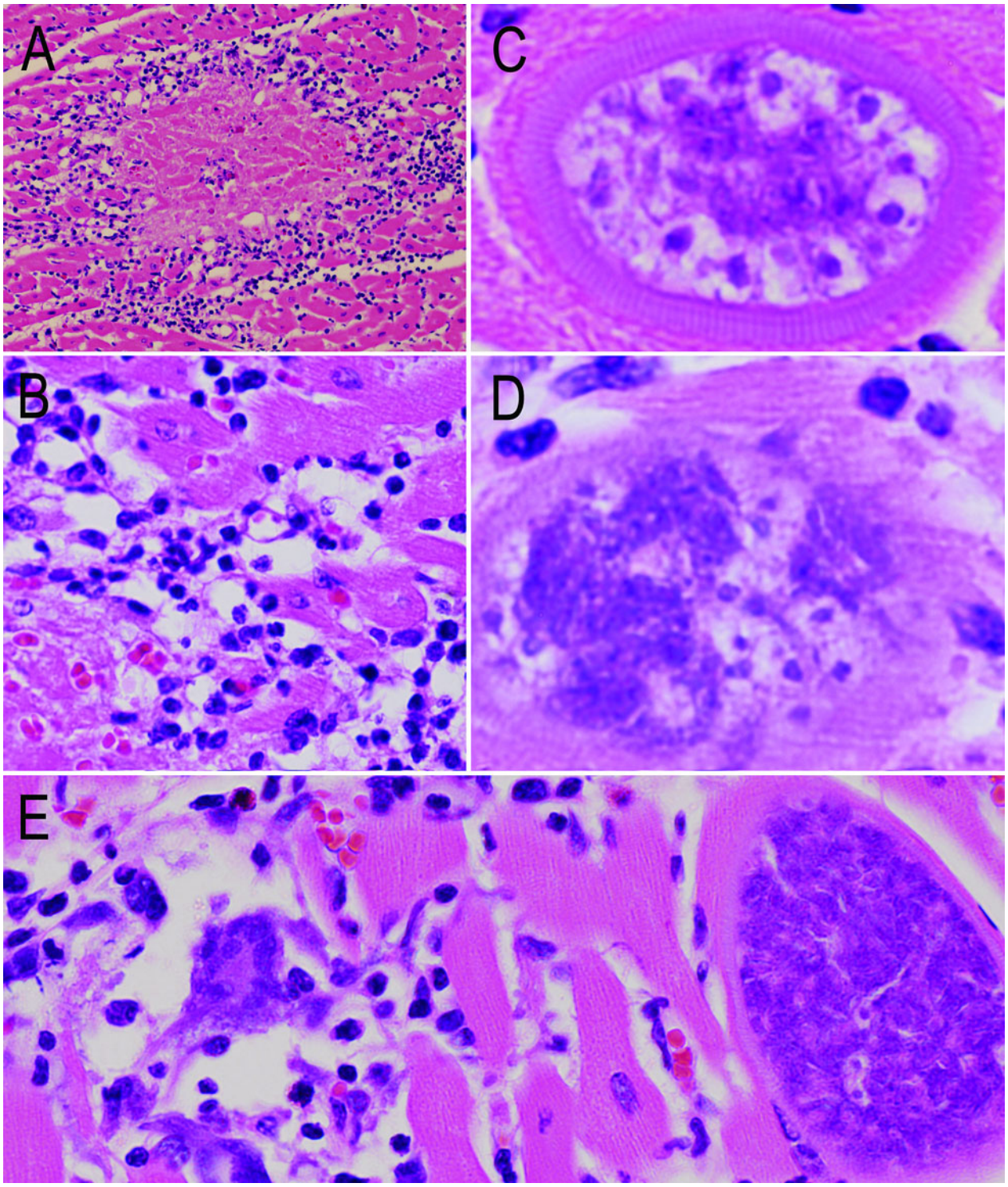

Fig. 2. *Sarcocystis* infection in myocardium of feral pigs. H and E stain. (A) Mononuclear cell infiltration surrounding a focus of necrosis in an adult female from Louisiana. (B) Higher magnification of the periphery of lesion in Fig. 1A. (C) Cross-section of an immature sarcocyst with a radially striated sarcocyst wall and metrocytes, detected in an adult male from Kansas. (D) Degenerating immature sarcocyst with metrocytes, the sarcocyst wall is no longer visible, detected in an adult male from Florida. (E) Focal inflammatory focus with mononuclear and giant cell on left and an intact intramuscular sarcocyst on the right, detected in an adult female from Texas.

AZ, FL, HI, KY, LA, MT, MS, OK and TX) which yielded approximate 915 bp PCR product (Fig. 3A). Digestion of 18S PCR products with restriction enzyme *Ssp*I generated two distinct fragments of 650 and 265 bp, belonging to *S. miescheriana*, in all positive samples studied (e.g., Fig. 3B). No product failed to be digested, which would have suggested the presence of *S. suihominis*. PCR-RFLP analysis of all *Sarcocystis* spp. positive samples revealed only *S. miescheriana* infection in feral pigs from the USA.

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Table 2. Intensity of infection and frequency of myositis associated to *Sarcocystis* sarcocysts detected in myocardium of feral pigs from the USA

Number of sarcocysts/ section (1·5 × 0·7 cm)	Pigs parasitized	Presence of myositis (%)
1	153	27 (17·6)
2–5	82	23 (28·0)
6–9	8	4 (50·0)
>10	8	6 (75·0)

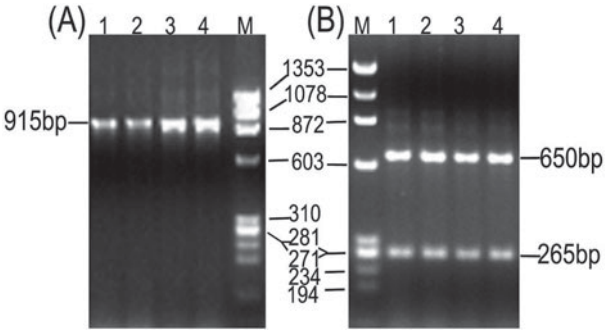

Fig. 3. Restriction pattern of *Sarcocystis miescheriana* revealed for the feral pigs sampled in the USA. PCR amplification of *Sarcocystis* 18S rRNA gene and digestion of 18S PCR product with restriction enzyme *Ssp*I. Visualized on 2% agarose gel with ethidium bromide stain. (A) Lanes 1, 2, 3 and 4: 18S PCR products of four different feral pigs, Lane M: DNA Ladder, size 915 bp band corresponding to 18S rRNA gene of *Sarcocystis* spp. (B) Digestion of 18S PCR product. Lane M: DNA Ladder; Lanes 1, 2, 3 and 4: digested products of four feral pigs. The two bands with size 650 bp and 265 bp of digested 18S PCR products represented *S. miescheriana*.

Thirty one isolates from nine US states (AL, AR, AZ, FL, HI, LA, MT, OK and TX) were subjected to sequencing (Fig. 1) and phylogenetic analyses, a neighbour joining tree (Fig. 4) illustrates the correspondence of feral swine isolates from USA with either of two sequences previously reported for *S. miescheriana* (GenBank accession GU395554, JN256123). Each of these was distinct from a sequence representing *S. suihominis* (AF176936) (Fig. 4).

DISCUSSION

This represents the first comprehensive *Sarcocystis* survey of feral swine from the USA. Prevalent infection was also documented previously (60·0%, Coombs and Springer, 1974 and 32·0%, Barrows *et al*. 1981). Prevalence estimated are strongly influenced by the methodology used; our prevalence estimate doubled (from 24·9 to 49%) when using pepsin digestion rather than microscopical examination of histological sections. Our study surveyed a wide area and documented infection in 16 of 29 US states. A previous survey developed by Barrows *et al*. (1981) over an area of 11 US states showed animals infected in most of them, but failed to document infection in Florida and rarely found infection in the lower coastal plains of Georgia, Mississippi and South Carolina. The current situation continues to reflect that distribution. We detected no infection in Georgia, and lower rates in Florida and Mississippi. On the other hand, we estimated higher prevalence in the Southern states of Alabama, Arkansas, Arizona, Louisiana, Oklahoma and Texas, consistent with the findings over 30 years ago (Barrows *et al*. 1981).

We also confirmed the earlier finding that older animals are more likely to be infected and more likely, if infected, to harbour greater parasitic loads. This has been reported for feral swine (Barrows *et al*. 1981) and domestic pigs (Hinaidy and Supperer, 1979; Ohino *et al*. 1993; Avapal *et al*. 2003).

The average parasitic load detected in 251 feral pigs in our study was 3·03 sarcocysts/section, with maximum parasitization of 184 (in an adult female from TX); this far exceeded the maximum of four sarcocysts/section observed in heart from Iowa sows (Dubey and Powell, 1994). The markedly greater intensity of infection identified, in certain feral swine, may indicate that feral pigs have greater contact with sporocysts contaminating environment; alternatively, this may reflect a greater accumulation of infection in longer-lived feral swine. Notably, parasitic load was 2·2 times higher in females than in male feral swine; perhaps periodic gestational stress may depress immunity, thereby accounting for this difference. High intensity of infection in female (ratio: 0·54) was also observed in wild boars from the Slovak Republic (Hvizdošová and Goldová, 2009). According to Caspari *et al*. (2011), naturally occurring *Sarcocystis* infections in pigs are usually unapparent, presumably due to natural immunization by repeated, low-dose infections, more common in free-ranging pigs. Even so, cases in wild swine may have more varied clinical outcomes.

According to Avapal *et al*. (2004), little information is available concerning pathological changes in natural infections of swine with *Sarcocystis*. Recently, Kia *et al*. (2011) and Yan *et al*. (2013) did not found inflammatory reactions around sarcocysts of *S. miescheriana* in tissues from wild boar or domestic pigs, respectively. But in previous studies in experimentally infected pigs, Barrows *et al*. (1982*a*, *b*) detected various stages of cystic development between 35 and 92 days post-infection, and also, dissolution and reabsorption. Later, Yan *et al*. (2013) reported the occurrence of atrophy, degeneration and necrosis of the myosites with sarcocysts present. Our findings agree with these two reports in the sense that sarcocysts undergoing destruction were accompanied by focal areas of mononuclear cell infiltrates and eosinophils. In several individuals, foci of myositis

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Table 3. Geographical distribution of *Sarcocystis* infection detected in feral pigs from the USA

State	*N*	Positive	%	95% CI	Intensity of infection (mean ± SD)
Alabama (AL)	51	17	33·33	21·97–47·03	2·35 ± 1·33
Arkansas (AK)	137	43	31·39	24·22–39·58	1·69 ± 1·92
Arizona (AZ)	31	11	35·48	21·11–53·05	1·18 ± 0·40
California (CA)	20	10	50·00	29·93–70·07	3·03 ± 2·68
Florida (FL)	93	15	16·13	10·03–24·92	1·36 ± 0·65
Georgia (GA)	49	0	0·00	–	–
Hawaii (HI)	50	14	28·00	17·47–41·67	3·33 ± 4·30
Illinois (IL)	27	7	25·93	13·17–44·68	1·36 ± 0·48
Indiana (IN)	22	0	0·00	–	–
Kansas (KS)	41	10	24·39	12·91–40·64	1·30 ± 0·54
Kentucky (KY)	7	0	0·00	–	–
Louisiana (LO)	94	35	37·23	28·14–47·32	1·81 ± 1·12
Michigan (MI)	7	0	0·00	–	–
Montana (MT)	20	2	10·00	2·79–3·01	2·00 ± 1·41
Mississippi (MS)	88	12	13·64	7·55–23·00	1·43 ± 0·67
North Carolina (NC)	38	0	0·00	–	–
New Jersey (NJ)	2	0	0·00	–	–
New Mexico (NM)	12	0	0·00	–	–
Nevada (NV)	5	0	0·00	–	–
New York (NY)	6	2	33·33	9·68–70·00	1·25 ± 0·35
Ohio (OH)	13	0	0·00	–	–
Oklahoma (OK)	64	24	37·50	26·67–49·75	1·92 ± 1·23
Pennsylvania (PN)	1	0	0·00	–	–
South Carolina (SC)	30	7	23·33	11·79–40·92	1·64 ± 0·85
Tennessee (TN)	17	4	23·53	9·56–47·26	1·00 ± 0·00
Texas (TX)	70	38	54·29	42·70–65·43	9·64 ± 30·31
Utah (UT)	5	0	0·00	–	–
Virginia (VA)	5	0	0·00	–	–
West Virginia (WV)	1	0	0·00	–	–
Total	1006	251	24·95	22·37–27·72	3·03 ± 12·10

CI, confidence interval; SD, standard deviation.

Table 4. Study of variables on the prevalence and intensity of infection of *Sarcocystis* infection in feral pigs from the USA

Variable	*N*	Positive	%	95% CI	Intensity of infection (mean ± SD)
Gender					
Male	479	111	23·59	20·01–27·59	1·81 ± 1·77
Female	520	134	26·54	22·93–30·51	4·02 ± 16·20
Age					
Juvenile	46	3	6·52	2·24–17·5	2·33 ± 1·53
Sub-adult	208	32	15·38	11·11–20·91	1·75 ± 1·50
Adult	747	216	28·92	25·78–32·27	3·23 ± 13·02

CI, confidence interval; SD, standard deviation.

without apparent presence of parasites were detected, in those cases it is no longer possible to know with exactitude the aetiology of such lesions, owing to likely reabsorption of *Sarcocystis* sarcocysts. Also, in several cases, oedema, fibrinous exudates and hyaline degeneration was observed according to Avapal *et al*. (2004) in pigs from India. No signs of acute infections, as reported by Caspari *et al*. (2011), were detected.

Sarcocystis species can be identified based on the morphology of their sarcocysts wall, but specific morphological diagnosis often requires transmission electron microscopy (Dubey *et al*. 1989). The sarcocysts of *S. miescheriana* have walls 3–6 μm thick that appear radially striated; the villar protrusions on the sarcocysts walls are up to 5 μm long and 1·3 μm wide. While for *S. suihominis*, the wall is 4–9 μm thick and appears hirsute, with villar protrusions up to 13 μm long (Dubey *et al*. 1989). Also, bioassay can be useful in their differential diagnosis. Saito *et al*. (1998) detected sarcocysts morphologically consistent with *S. suihominis* in pigs from Japan; confirmation was done by bioassay in dogs and cats, which did not shed sporocysts.

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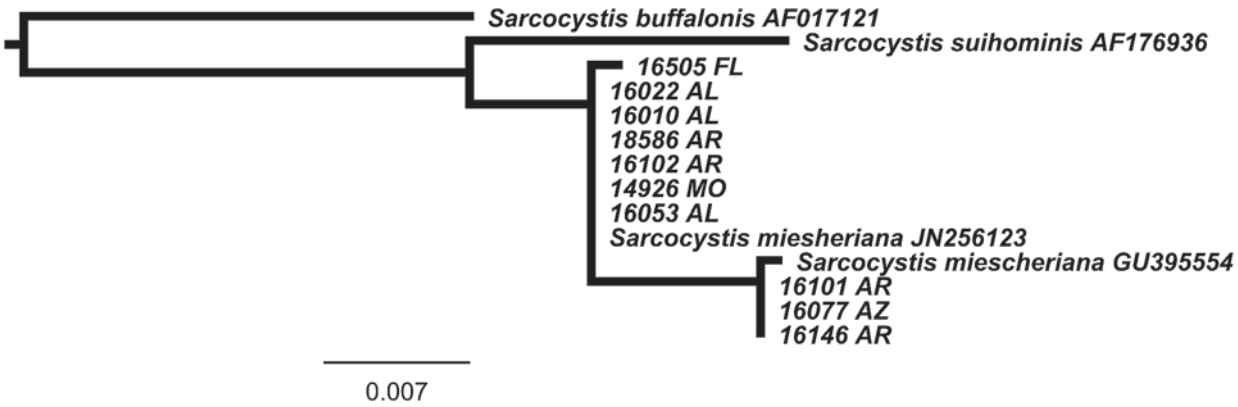

Fig. 4. Maximum likelihood 18S rRNA sequence phylogenetic tree for the 31 isolates of *Sarcocystis* spp. studied in feral pigs from the USA.

Molecular methods are useful to identify *Sarcocystis* species; 18S rRNA sequences are of special interest because they contain conserved regions that are easy to target via PCR, interspersed by more variable regions that differ among related species (Yang *et al*. 2002; Dahlgren and Gjerde, 2007). Our results confirm the presence of *S. miescheriana* in the 44 isolates studied from 11 US states. *S. miescheriana* is a ubiquitous parasite whose presence has been confirmed molecularly in different countries as China (Yan *et al*. 2013), Iran (Kia *et al*. 2011) and Switzerland (Caspari *et al*. 2011). The absence of zoonotic *S. suihominis* from any animal in our sample provides some reassurance that this zoonotic parasite continues to occur at low or negligible frequencies in feral swine in the United States. Assuming our sample of 44 is representative of the US population of feral swine, we can conclude with 95% of probability that the true prevalence of *S. suihominis* is less than 1·4%. Further studies should be focused in organic pigs that are raised in free or semi-free conditions, and would need to enrol thousands of individuals in order to have statistical power to evaluate, with precision, the true prevalence of this infection here.

Sequence analysis and phylogenetic results showed that genetic variability of *S. miescheriana* is very low; our sequences were compared with available sequences in GenBank, and found to correspond almost perfectly to either GU395554 reported by Kia *et al*. (2011) in Iran or JN26523 reported from Lithuania. Gene sequence analysis is also very useful to define the identity of morphologically similar species (Yang *et al*. 2001).

Recently, zoonotic *Sarcocystis* species have become a major concern for food safety in many countries; e.g., European Food Safety Authority stimulates efforts on the development of harmonized schemes for the monitoring and reporting of *Sarcocystis* in animals and foodstuffs in the European Union (Taylor *et al*. 2010). At present, there is no report if *S. suihominis* occurs in the United States. It constitutes a risk for the safety of pork, and a risk assessment should be developed, especially for swine raised in organic or non-biosecure pig farms. *S. suihominis* is a parasite that thrives in poor hygienic conditions; humans act as definitive hosts, supporting development of the parasite in the intestines; current systems of water purification, and control of human feces, allow the cycle of transmission to be broken. Also, according to Dubey and Powell (1994), the management of pigs and cultural habits of humans affect the prevalence of *Sarcocystis*. For example, the highest prevalence of *S. suihominis* has been reported in pigs from Germany and Austria, where people commonly eat undercooked or raw pork (Hinaidy and Supperer, 1979; Boch *et al*. 1980); absence of this habit could be a cause of the apparent absence of this species in the USA.

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