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Review

Feral swine brucellosis in the United States and prospective genomic techniques for disease epidemiology



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ABSTRACT

Brucellosis is a common infection of feral swine throughout the United States. With the recent expansion of feral swine populations across the country, this disease poses an increasing threat to agriculture and hunters. The standard approach to *Brucella* surveillance in feral swine has been serological testing, which gives an indication of past exposure and is a rapid method of determining populations where *Brucella* is present. More in-depth analyses require bacterial isolation to determine the *Brucella* species and biovar involved. Ultimately, for a comprehensive understanding of *Brucella* epizootiology in feral swine, incorporation of genotyping assays has become essential. Fortunately, the past decade has given rise to an array of genetic tools for assessing *Brucella* transmission and dispersal. This review aims to synthesize what is known about brucellosis in feral swine and will cover prospective genomic techniques that may be utilized to develop more complete understanding of the disease and its transmission history.

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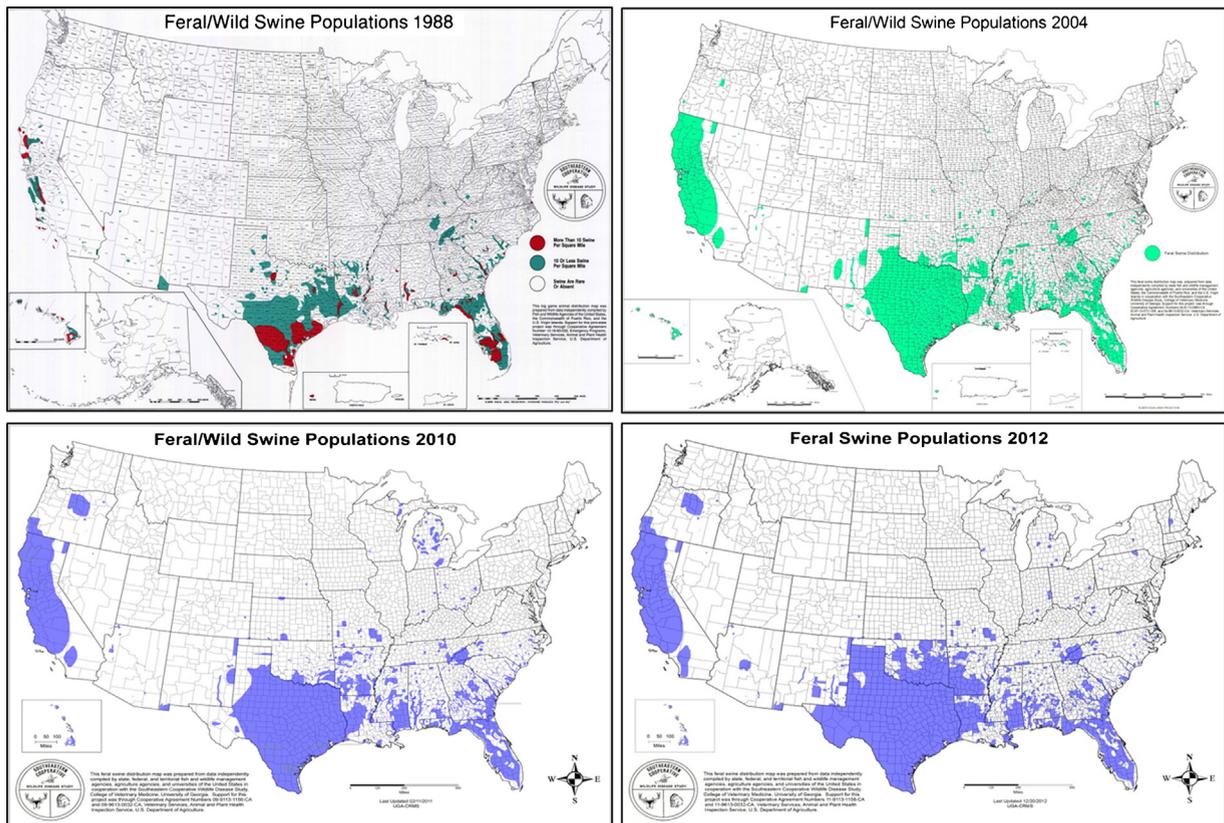


Fig. 2. Increase in the range of feral swine in the United States. Data compiled from multiple independent sources through 2012 identify established populations of feral swine in 36 states. Data from 1988 are reported as fewer (teal) or greater (red) than ten feral swine per square mile. Data from 2004, 2010, and 2012 are reported as established populations. Maps provided by the Southeastern Cooperative Wildlife Disease Study, University of Georgia.

continue to be discovered: an unnamed species present in Australian rodents (Tiller et al., 2010), *Brucella microti* in the common vole (Scholz et al., 2008a) and red fox (Scholz et al., 2009), a novel species from a captive baboon (Schlabritz-Loutsevitch et al., 2009), and the novel *Brucella inopinata* isolated from a human breast implant infection (Scholz et al., 2010). Formal taxonomic classification remains unresolved in many of these groups and the genus will likely expand to include new species. It is also likely that additional hosts will be described as more animal species are tested. Although *Brucella* spp. are largely host specific, the barriers to cross-species infection are poorly understood. For instance, *B. melitensis* was recently found in Nile catfish (El-Tras et al., 2010). Additionally, *B. melitensis* and *B. suis* are known to infect cattle (Corbel, 1997; Ewalt et al., 1997), and reports of *Brucella* infection in African camels (Gwida et al., 2011) highlight the fact that *Brucella* is a genus of bacteria with a wide array of vertebrate hosts.

1.1. Brucellosis in feral swine and wild boar

Brucella species have traditionally been split into different biovars based on distinct biochemical properties (Alton et al., 1988). Infections in domestic and feral swine are predominantly from *B. suis* biovars 1, 2, and 3.

Brucellosis has been eliminated from domestic swine herds in the United States (9 CFR 78:43 2011b; Federal Register §76:97 2011a), so the main reservoir for *B. suis* biovar 1 in the U.S. is feral swine; the same is true in Australia. In contrast, the main European reservoir of *B. suis* biovar 2 consists of wild boar (Wood et al., 1976; Pavlov and Edwards, 1995; Godfroid and Kasbohrer, 2002; Ruiz-Fons et al., 2006; Olsen, 2010). Feral swine in the U.S. are likely descendants of domestic swine, European wild boar, and hybrids of the two (Mayer and Brisbin, 1991). Originally derived from escaped domestic swine brought to the Americas by European settlers, in some areas feral swine descended from domestic swine later interbred with wild boar introduced from Europe. Feral swine herds have since spread to large portions of the southeastern U.S., Texas and California (SCWDS, 1982, 1988; van der Leek et al., 1993; Gresham et al., 2002; SCWDS, 2004, 2010; Stoffregen et al., 2007). Feral swine have been introduced, often illegally, into many states for hunting purposes, further expanding the range of these animals and thus increasing the areas of contact with humans and livestock. Established populations of feral swine are now reported in 36 of 50 states (SCWDS, 2012).

Feral swine are a significant problem in other parts of the world. Recent cases of human brucellosis in Australia and the U.S. have been attributed to contact with *B. suis*

when dressing, transporting, and/or cooking the infected animals, demonstrating the importance of these animals from a public health standpoint (Starnes et al., 2004; CDC, 2009; Irwin et al., 2010). In Europe, *B. suis* biovar 2 has historically been isolated from European wild boar, although European hares (*Lepus europaeus*) are also infected (Sterba, 1983; Gyuranecz et al., 2011). As is the case with feral swine in the U.S., the range of wild boar in Europe has expanded due to stocking for hunting (Acevedo et al., 2006). As these populations expand into areas containing free range, backyard, traditional and commercial domestic swine production, the possibility of spreading *B. suis* has increased. In fact, European outbreaks of *B. suis* biovar 2 that occurred from 1999 to 2000 among outdoor domestic pig herds were strongly suspected to have originated in wild boar (Godfroid and Kasbohrer, 2002). Interestingly, wild boar in Europe are most often infected with *B. suis* biovar 2, which is not commonly associated with human infections in the U.S. (Godfroid et al., 1994), likely due to the lack of any introduction event from Europe into the United States. In contrast, feral swine in the U.S. are most commonly infected with *B. suis* biovars 1 and 3 (Zygmunt et al., 1982; van der Leek et al., 1993), which in turn are the most commonly isolated biovars in humans (Fretin et al., 2008). The increased potential for human infections by strains from biovars 1 and 3 (but less so by biovar 2) warrants future research.

1.2. Transmission of *B. suis* among feral swine

The exact mechanisms of *B. suis* transmission in feral swine are poorly understood, due to the reclusive nature of the animals and difficulty observing their behavior in the wild. Transmission mechanisms in feral swine are likely similar to those in other animals including domestic swine and humans. *B. suis* enters the host through damaged skin or through damaged or intact mucosal membranes, such as those found in the respiratory, reproductive, and gastrointestinal tracts (Buchanan et al., 1974). Brucellosis is a sexually transmitted disease in domestic pigs, and can be readily transmitted during breeding and artificial insemination when using semen from an infected boar (Alton, 1990; Whatmore et al., 2006; Maes et al., 2008).

B. suis has not been shown to survive as a free-living organism in the environment (but see Scholz et al., 2008b for example in *B. microti*), thus most animals become infected during mating, through direct contact with contaminated placenta and aborted fetuses, or by inhalation of aerosols during or after septic abortions (Alton, 1990). Swine are opportunistic feeders (Taylor, 1999; Schley and Roper, 2003; Ditchkoff and Mayer, 2009; Jolley et al., 2010), and will likely consume abortion products if available. Feral swine and wild boar are known to be reservoirs for many diseases, including brucellosis (Meng et al., 2009), and several studies have pointed to encroachment by feral swine to the areas surrounding domestic swine facilities as a potential source of *B. suis* transmission back into domestic swine herds (Corn et al., 1986, 2009; Frolich et al., 2002).

1.3. *B. suis* seroprevalence in feral swine

Brucella exposure is commonly detected in feral swine (see below), although current serological tests are not able to distinguish down to the species level and can give false positive results in animals infected with *Yersinia enterocolitica* O:9 (Weynants et al., 1996). Select *B. suis* cultures isolated from feral swine (USDA unpublished data) support widespread serological data suggesting that *B. suis* is endemic in feral swine across large portions of their current geographic distribution (Table 1). *Brucella* exposure has been detected in feral swine by serology in at least 16 states (Table 1). The percentage of seropositive animals in feral swine populations varies from state to state, as well as over time, ranging from 0.3% to 52.6%; variation that may be due to true prevalence differences or methodological issues (Table 1). Therefore, *Brucella* serologic data must be interpreted with caution, and improved standardized sampling is necessary to assess the broad scale implications of these findings. Nonetheless, serologic data indicate that feral swine in populations sampled across the U.S. are infected with, or at the very least exposed to *B. suis*. Worldwide, the rates of *Brucella* seroprevalence in feral swine and wild boar vary in a similar manner to those found in the U.S. (Table 2). Interestingly, the collared peccary, a distant relative of *Sus scrofa* native to the American Southwest (also known as javelinas; *Tayassu tajacu*) has consistently been *Brucella* seronegative (Randhawa et al., 1977; Corn et al., 1987; Gruver and Guthrie, 1996). Javelinas belong to the same taxonomic order (*Artiodactyla*), but are members of a different family (*Tayassuidae*) than pigs (*Suidae*). The ranges of javelinas and feral swine do overlap in some areas (Gruver and Guthrie, 1996), and it is therefore unclear why U.S. populations of javelinas have remained seronegative, considering the fact that feral swine can transmit *Brucella* to other animals such as domestic cattle and wild deer (Cooper et al., 2010). Furthermore, peccaries in Venezuela have been shown to be seropositive for *B. suis* at a rate of 87.8%, with many animals containing viable and culturable organisms – most likely due to their close proximity to infected domestic swine herds (Lord and Lord, 1991).

2. Traditional methods of detecting and typing *brucellae*

Detection of exposure to *Brucella* spp. in feral swine and other wild animals is commonly performed serologically (see above), screening sera for *Brucella*-specific antibodies. However, this approach is complicated by several factors. First, the available serum agglutination tests suffer from a lack of sensitivity and specificity (Kassahun et al., 2006; Gomez et al., 2008; Mukhtar and Kokab, 2008; Vancelik et al., 2008; Swai and Schoonman, 2009). Second, a seropositive animal may not contain culturable organisms, which is problematic since the gold standard for diagnosis of brucellosis remains the isolation and culturing of viable cells. Finally, successful isolation of *Brucella* becomes more and more challenging as an infection progresses, largely due to the fact that the organisms grow slowly and intracellularly (Espinoza et al., 2009).

Table 1
Positive serologic test results for antibodies against *Brucella* spp. in United States feral swine.^a

State	Seropositive	Total tested	Prevalence	Study
Alabama	11	102	10.8%	Pedersen et al. (2012)
Arkansas, Florida, Georgia, Louisiana, S. Carolina	21	352	6.0%	Zygmunt et al. (1982) ^b
Arkansas	11	350	3.1%	Pedersen et al. (2012)
California	21	136	15.4%	Clark et al. (1983)
California	23	611	3.8%	Drew et al. (1992)
Florida	50	95	52.6%	Becker et al. (1978)
Florida	238	1015	23.4%	van der Leek et al. (1993)
Florida	29	464	6.3%	Pedersen et al. (2012)
Georgia	5	296	1.7%	Pedersen et al. (2012), Hanson and Karstad (1959)
Hawaii	33	229	14.4%	Pedersen et al. (2012), Griffin (1972)
Kansas	1	142	0.7%	Pedersen et al. (2012)
Louisiana	3	136	2.2%	Pedersen et al. (2012)
Mississippi	9	238	3.8%	Pedersen et al. (2012)
Missouri	1	321	0.3%	Martin et al. (2007)
Missouri	2	201	1.0%	Pedersen et al. (2012)
N. Carolina	6	157	3.8%	Pedersen et al. (2012)
Oklahoma	18	181	9.9%	Pedersen et al. (2012)
S. Carolina	46	255	18.0%	Wood et al. (1976)
S. Carolina	NA	NA	44.0%	Gresham et al. (2002) ^c
S. Carolina	39	80	48.8%	Stoffregen et al. (2007)
S. Carolina	7	50	14.0%	Corn et al. (2009)
S. Carolina	20	173	11.6%	Pedersen et al. (2012), Wood and Brenneman (1977), Wood et al. (1992)
Texas	1	1	100.0%	Randhawa et al. (1977)
Texas	4	124	3.2%	Corn et al. (1986)
Texas	24	102	23.5%	Wyckoff et al. (2005)
Texas	41	368	11.1%	Wyckoff et al. (2009)
Texas	11	884	1.2%	Pedersen et al. (2012)

NA: data not available.

^a SCWDS (1995). Wild swine databases on swine brucellosis and pseudorabies. Southeastern Cooperative Wildlife Disease Study, University of Georgia, Athens, Georgia. 26pp. This report lists published and unpublished data collected 1959–1995, including a total of 1465/16268 feral swine in 9 states seropositive for brucellosis.

^b Study did not break out results by state; percentage reflects mean prevalence across the five states.

^c Study listed only prevalence data.

Traditional microbiological methods have provided the foundation for the typing of *Brucella*. The classical strains have historically been differentiated on the basis of differences in LPS molecules, differential bacteriophage sensitivity, sensitivity to and/or uptake of various dyes, production of H₂S, the requirement for CO₂ during growth, and fermentation of various sugars (Alton et al., 1988).

These techniques, however, suffer from a lack of resolution beyond strain and biovar level, as well as presenting a significant danger of exposure to the organism in laboratory personnel. Although *Brucella* spp. require biosafety level 3 facilities, brucellosis is the most commonly reported laboratory-acquired infection (Weinstein and Singh, 2009) and route of exposure is often

Table 2
Reported serologic test results for antibodies against *Brucella* spp. worldwide in feral swine and European wild boar.

Country	Number seropositive	Total tested	Prevalence	Study
Australia	NA	NA	1.0%	Pavlov and Edwards (1995) ^a
Belgium	NA	NA	39.0%	Godfroid et al. (1994) ^a
Croatia	NA	NA	26.0%	Cvetnic et al. (2003) ^{a,b}
Croatia	NA	NA	11.3%	Cvetnic et al. (2004) ^{a,c}
Croatia	NA	NA	27.6%	Cvetnic et al. (2009) ^a
Czech Republic	18	204	8.8%	Hubalek et al. (2002)
Germany	168	763	22.0%	Al Dahouk et al. (2005)
Germany	NA	NA	12.1%	Melzer et al. (2007) ^a
Italy	29	562	5.2%	Ebani et al. (2003)
Italy	448	2267	19.8%	Bergagna et al. (2009)
Spain	NA	NA	29.7%	Ruiz-Fons et al. (2006) ^a
Spain	NA	NA	29.5%	Muñoz et al. (2010) ^{a,b}
Switzerland	NA	NA	13.5%	Köppel et al. (2007) ^a

NA: data not available.

^a Study listed only prevalence data.

^b Prevalence varied by region; average seroprevalence listed.

^c Prevalence varied by assay used; average seroprevalence listed.

undetermined. Additionally, these methods are often subjective in nature, resulting in the potential for conflicting results reported by different laboratories.

Because of the danger posed during routine laboratory culturing, various molecular approaches have been utilized to distinguish different members of *Brucella*. The first assays to differentiate members of the genus were PCR based, such as examination of unique banding patterns obtained during arbitrarily primed PCR (AP-PCR; Fekete et al., 1992), PCR followed by reverse hybridization (Rijpens et al., 1996), and restriction fragment length polymorphism (RFLP; Cloeckert et al., 1995). PCR-based assays often target polymorphisms in outer membrane proteins (OMPs; Leal-Klevezas et al., 1995; Bardenstein et al., 2002; Imaoka et al., 2007), or in the 16S ribosomal RNA sequence (Romero et al., 1995). These assays, while useful for the rapid detection of *Brucella* DNA, experience limited resolution, sometimes even at the species level. To combat this weakness, multiplex PCR assays were developed, in which multiple oligonucleotide primers amplify differential PCR products, depending on which *Brucella* species is present. The first such assay, called AMOS (abortus-melitensis-ovis-suis) was developed around polymorphisms in the chromosomal IS711 element (Bricker and Halling, 1994, 1995). It was refined by Lopez-Goni et al. (2008) to differentiate among the six classical *Brucella* species, *B. ceti* and *B. pinnipedialis*, and the vaccine strains Rev1, RB51 and S19.

While these types of assays provide relatively rapid diagnostic resolution (when considered against classical microbiological approaches), they have the limitation of only giving binary data; that is, the tests tell only whether a member of *Brucella* is present or absent. They do not describe genetic relatedness among *Brucella* isolates; such information would be useful during investigation of illegal feral swine translocations, for example.

3. Contemporary molecular genetic approaches to *Brucella* detection

Although serological and microbiological studies are important for maintaining healthy herds of food animals, there are significant limitations in classical strategies for the detection of *B. suis* in swine from an epidemiological standpoint. Fine-scale genetics-based approaches improve epidemiological investigations by allowing for increased resolution of transmission routes, more effective tracking of disease spread, and allow for targeted implementation of disease control and/or prevention strategies. Traditional molecular approaches to subtyping *B. suis* isolated from swine have been hindered by the genetically monomorphic nature of the genus (Gandara et al., 2001). However, developments following the completion of the *B. suis* genome sequence (Paulsen et al., 2002) have opened the door for high-resolution genotyping studies. Currently, the two primary PCR-based techniques available to establish genetic evolutionary relationships among *B. suis* isolates are multilocus sequence typing (MLST) and multiple-locus [variable number tandem repeat (VNTR)] analysis (MLVA).

3.1. MLST

MLST analyses involve sequencing of 400–500 bp fragments of housekeeping genes and utilize single nucleotide polymorphisms (SNPs) and other mutations in these genes to assess variation among isolates (Maiden et al., 1998). A significant advantage of MLST over traditional typing methods, for example, subjecting cultures to the tests described in Bergey's manual (Holt et al., 1994), is the fact that they prevent the need for isolation of organisms; loci can be amplified by PCR directly from clinical samples such as blood. In the case of *B. suis* and other select agents, this lessens the opportunity for infection of lab personnel and/or accidental release of viable organisms. MLST is most effective when analyzing variation in species with high genetic polymorphism, and is a poor choice for species with too few polymorphic loci (Foster et al., 2009). However, MLST analysis has been used to reveal taxonomically informative variation among various *Brucella* isolates (Whatmore et al., 2007), and is a useful tool for further analysis of *B. suis* isolates. It should be kept in mind that MLST analysis is costly in *Brucella* because the limited genetic variation necessitates using far more than the seven standard loci, and even then differentiation of closely related isolates within a species is not always possible.

3.2. MLVA

MLVA exploits repeat regions (i.e. VNTRs) in the chromosome of the target organism that are polymorphic when tested across a set of samples. The procedure is based on the fact that during replication DNA polymerase occasionally adds or removes individual repeats through a process known as slipped-strand mispairing (Torres-Cruz and van der Woude, 2003). MLVA has been used successfully to type multiple bacteria species possessing low overall genetic diversity, such as *Bacillus anthracis*, *Yersinia pestis* and *Francisella tularensis* (Keim et al., 2000; Klevytska et al., 2001; Johansson et al., 2004; Van Belkum, 2007). This technique has recently been applied for *Brucella* genotyping, and is excellent for discriminating among and within members of this genetically monomorphic genus. Bricker et al. (2003) developed the first VNTR assays, which target highly variable and rapidly evolving loci most suitable for monitoring outbreak situations. Subsequent MLVA assays have been developed, with three schemes most commonly used (Le Fleche et al., 2006; Whatmore et al., 2006; Huynh et al., 2008). With any of these schemes it is now possible to genotype and differentiate *B. suis* isolates and assign relationships for epizootiological or epidemiological analysis. For example, recent outbreaks of brucellosis in feral swine populations could conceivably be traced back to their source, although no such studies have yet been performed. In human cases, however, MLVA has been shown to be a useful tool to examine outbreaks of infection by *Brucella* spp. worldwide (for examples, see Tiller et al., 2009; Her et al., 2009; Valdezate et al., 2010; Kilic et al., 2011; Jiang et al., 2011). A further advantage to this approach is that, like MLST, MLVA can be performed on samples initially collected from an

infected individual such as sera or tissue, avoiding the need to culture the bacteria themselves. Analyses like this will be critical in establishing relationships of *B. suis* transmitted among and between feral swine populations and, potentially, domestic swine herds.

3.3. Whole genome sequencing

Despite the discriminatory power of MLST and MLVA, whole genome sequencing is becoming the preferred method for assigning phylogenetic relationships among organisms (Mardis, 2008b,a; Pearson et al., 2009). With the advent of next-generation sequencing methods, entire bacterial genomes can now be accurately sequenced in less than two weeks, at a rapidly diminishing cost per sequenced base. There are currently three predominant systems for sequencing whole genomes: Roche/454 FLX Pyrosequencer, Illumina Genome Analyzer II/HiSeq and Applied Biosystems SOLiD Sequencer (reviewed in Shendure and Ji, 2008; Mardis, 2008a; Duan et al., 2010). Even though each individual DNA fragment sequenced is small relative to the size of the entire genome (~50–500 bp), enormous multiplexing capacities allow the instruments to achieve large depth and breadth of sequencing coverage. Two additional sequencing platforms, the Ion Torrent Personal Genome Machine (PGM; Life Technologies) and the MiSeq (Illumina) have been recently introduced, and numerous others will undoubtedly be brought to market in the near future. These latest sequencing platforms boast incredible speed (~2 h sequencing runs) and high accuracy, although they suffer from a lack of coverage relative to their larger cousins when used to sequence large genomes. Still, for diagnostic and/or epidemiological studies, high sequence coverage is not always essential when typing isolates, and the low setup cost combined with high speed of the PGM and MiSeq platforms will likely prove very useful.

In a genetically monomorphic genus such as *Brucella*, whole genome sequencing is the most accurate method of determining population-level relationships as well as directionality of pathogen movement, as recently demonstrated in *Vibrio cholera* (Mutreja et al., 2011), but not yet well explored in wildlife or livestock pathogens. However, whole genome sequencing has been carried out to differentiate between epidemiologically related strains of the human pathogens *Cryptococcus gattii* (Gillece et al., 2011), *Escherichia coli* (Rasko et al., 2011), methicillin-resistant *Staphylococcus aureus* (Harris et al., 2010), *Klebsiella pneumoniae* (Snitkin et al., 2012), as well as to determine molecular signatures of laboratory growth in *F. tularensis* (Sjodin et al., 2010). In the case of endemic *B. suis* infection, such as is found in feral swine herds, whole genome sequencing will be the definitive method by which the history of nationwide and worldwide dispersal can be evaluated. Whole genome sequencing has already been carried out in a limited fashion to phylogenetically assign and determine the divergence of species within *B. suis* (Chain et al., 2005; Foster et al., 2009). In these studies, isolates of *B. suis* representing the recognized biovars readily separated into different clades despite possessing genome-level differences. Thus, *B. suis* contains sufficient

genomic polymorphisms for population genetic analyses, such as the distribution of genotypes, or for tracing domestic swine outbreaks back to potential feral or domestic swine sources. The rapid drop in costs per genome will soon make this approach feasible with large numbers of samples, as is evidenced by recent efforts at the Broad Institute of MIT and Harvard (Cambridge, MA) to sequence nearly 500 *Brucella* strains, which include many strains isolated from swine in the U.S. and Europe.

4. Conclusions

The rapid spread of feral swine can be attributed to commercial and private hunting creating a market for illegal translocation, accidental escapes from transitional swine facilities, as well as natural range expansion and habitat alteration (Hutton et al., 2006). Once established, a population of feral swine can be very difficult (if not impossible), expensive, and time consuming to eradicate (Seward et al., 2004). This range expansion is often accompanied by the spread of diseases endemic to U.S. feral swine such as pseudorabies and swine brucellosis. Feral swine pose a continual challenge for wildlife management agencies, the domestic swine industry, and the general public due to their destructive nature and propensity for carrying diseases of importance to livestock and human health. Facing the inevitability of a long term feral swine problem within the United States, it becomes increasingly important for wildlife disease epidemiologists to fully understand disease dynamics so that they may be effectively managed.

The high numbers of domestic swine, coupled with the continued range expansion of feral swine, suggest an increasing risk of transmission of *B. suis* into the commercial swine industry (Corn et al., 2009; Wyckoff et al., 2009; Cooper et al., 2010). The potential for brucellosis transmission from feral to domestic swine exists in nearly every state that contains feral swine although greatest transmission potential would be expected in states with relatively high populations of feral swine in close proximity to low biosecurity domestic production situations. States like North Carolina, with \$792 million in 2010 pork sales, would face large economic losses if brucellosis were to enter the domestic swine industry there from infected feral swine in the region.

Recent advances in genomic techniques should allow wildlife epidemiologists to further describe the ecology of *B. suis* within feral swine populations. A handful of laboratories are currently engaged in the process of constructing genetic relationships among *Brucella* isolates (Al Dahouk et al., 2007; Garcia-Yoldi et al., 2007; Foster et al., 2009), and this work should provide a framework for finer scale molecular epidemiological investigations of brucellosis in domestic and feral swine. With the advent of next-generation sequencing methods, disease transmission routes can now be clearly described and appropriate measures taken to protect existing herds of domestic pigs. Highly accurate phylogenetic analysis allows for subsequent reconstruction of disease transmission history and, ultimately, introduction of measures designed to prevent further infection of domestic and feral swine.

Conflict of interest statement

The authors declare no conflict of interest with this manuscript.

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