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IDENTIFICATION OF *BRUCELLA SUIS* FROM FERAL SWINE IN SELECTED STATES IN THE USA

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ABSTRACT: Serologic tests currently available for brucellosis diagnosis detect antibodies to *Brucella* but do not distinguish between species of *Brucella*. Although *Brucella suis* is known to circulate within various feral swine (*Sus scrofa*) populations, our objective was to determine the primary species of *Brucella* circulating in feral swine populations in areas of the US with high brucellosis prevalence. We cultured lymph nodes from 183 feral swine. We identified 22 isolates from 21 animals, and all isolates were genotyped as *B. suis*. Most isolates were *B. suis* biovar 1, with the exception of two genetically distinct isolates from one feral swine in Hawaii, which were identified as *B. suis* biovar 3. Serum from each feral swine was also tested by the fluorescence polarization assay when possible, but only 52% (95% CL=29.8–74.3) of culture-positive animals were antibody positive. Our results indicate that brucellosis infections in feral swine within the US are typically caused by *B. suis*. However, improved serologic tests are needed to more accurately determine exposure to *Brucella* spp. and to monitor disease trends in feral swine populations.

Key words: *Brucella suis*, brucellosis, culture, feral swine, genotype, isolate, *Sus scrofa*, VNTR.

INTRODUCTION

Feral swine (*Sus scrofa*) populations have spread across the US through natural expansion, intentional translocation, and accidental release. The current US feral swine population is estimated to exceed 5 million individuals (Pimentel 2007) in at least 38 states (Wyckoff et al. 2009). The increasing number of feral swine is problematic because they damage public and private property and also pose disease threats to native ecosystems, livestock, and humans. Transmission of *Brucella* spp. between feral swine and domestic livestock is particularly important in the US because of the economic importance of the commercial swine (*Sus scrofa domesticus*) and cattle (*Bos primigenius*) industries (Thorne 2001; USDA 2010) and the threat it poses to their brucellosis-free status. However, humans with occupational or recreational activities that overlap with infected swine, such as wildlife biologists, abattoir workers, and hunters, are at increased risk for transmission (CDC 1994, 2009; Wu et al. 2012).

Feral swine have been documented to carry more than 30 diseases and 37 parasites that can be transmitted to humans, livestock, and wildlife (Seward et al. 2004). One of those diseases—brucellosis—is of particular interest because it can infect domestic animals, such as swine, cattle, and dogs (*Canis lupus familiaris*), in addition to humans. Although each of the six classical species of *Brucella* has a primary mammalian host or hosts, four are known to occasionally cause disease in other animals: *B. suis* (pigs), *B. abortus* (cattle), *B. melitensis* (sheep [*Ovis aries*] and goats [*Capra aegagrus hircus*]),

and *B. canis* (dogs). In the US, the sources of *Brucella* spp. include free-ranging bison (*Bison bison*) and elk (*Cervus elaphus*), which are reservoirs of *B. abortus*, and *B. suis*, which is endemic in feral swine (Olsen, 2010).

The role of feral swine in the transmission and maintenance of *B. suis*, and possibly *B. abortus*, are of concern to the domestic livestock industry for many reasons. Commercial swine are considered brucellosis-free in the US, and reintroduction of brucellosis could jeopardize the economic livelihood of the $34 billion industry (Witmer et al. 2003). In addition to causing disease in swine, *B. suis* has also been detected in cattle, and although it does not result in abortion or decreased production, economic losses can be incurred as a result of the amount of time necessary for trace-back investigations, removal of infected animals, and subsequent whole-herd testing because the serologic titers of cattle infected with *B. suis* cannot be distinguished from those infected with *B. abortus* (Ewalt et al. 1997). Bovine brucellosis, typically caused by *B. abortus*, has significant animal health, public health, and national and international trade consequences. *Brucella abortus* has previously been detected in feral swine surrounding an infected cattle herd (Higgins et al. 2012), validating the possibility that feral swine infected with *B. abortus* could pose a source of infection to cattle. The US has made significant efforts to obtain disease freedom in both the commercial cattle and swine industries (Olsen 2010).

The US Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Wildlife Services, National Wildlife Disease Program (NWDP) conducts routine monitoring for brucellosis in feral swine. The primary goal is to identify geographic areas of potential risk to commercial swine, assess the disease status of newly established populations of feral swine, monitor epidemiologic trends, and inform public health officials. Annual submissions of feral swine serum samples nationwide indicate antibodies to *Brucella* spp. are widespread; however, currently available serologic diagnostic tests, which were originally designed to detect *B. abortus* in cattle (Nielsen 2002), do not distinguish infections caused by the various species of *Brucella* (Ewalt et al. 1997; Olsen 2010). Lymph nodes can be cultured to identify the species of *Brucella*, and subsequent genotyping elucidates the relationships among isolates based on similarities in the variable number tandem repeat (VNTR) profile. That information can be used to examine geographic and evolutionary differences in the DNA of specific species of *Brucella* and to learn more about the source of infection. Our study targeted lymph node collection from feral swine in US states with known high *Brucella* antibody prevalence (Pedersen et al. 2012) to increase the probability of culturing and genotyping *Brucella* to identify the primary species of *Brucella* circulating in feral swine populations in these areas and to provide insight into the potential risk posed by transmission to domestic livestock and humans.

MATERIALS AND METHODS

Sample collection

From 1 October 2010 through 30 September 2012, we collected lymph nodes from 183 feral swine in eight states across the US and also collected a serum sample from each animal when possible. Specific counties within those eight states were selected based on the following criteria: 1) the detection of *Brucella* antibodies in feral swine over multiple years, and/or 2) *Brucella* antibody detection in at least 1 yr (Pedersen et al. 2012) in a county listed as one of the top five domestic swine producing counties in that state. Wildlife disease biologists from the NWDP were asked to collect serum and lymph nodes from 10 animals per target county whenever possible (Table 1). Samples were collected opportunistically from adult (≥1 yr) and subadult (2 mo–1 yr) (Matschke 1967) feral swine. A standardized data sheet was provided to record site- and animal-specific information, including latitude and longitude (WGS 84), county, state, date of sample collection, sex and age

TABLE 1. *Brucella* culture and fluorescence polarization assay (FPA) results from feral swine (*Sus scrofa*) collected from eight states in the USA, from 1 October 2010 to 30 September 2012.

State	Counties	Animals sampled for culture (No.)	Culture-positive animals (No.)	FPA-positive animals (No.)	Culture and FPA-positive animals (No.)[a]
Alabama	Clarke	10	2 (*B. suis* bv 1)	7	2
Arkansas	Desha	12	3 (*B. suis* bv 1)	7	2
Florida	Highlands	1	0	0	N/A
	Hillsborough[b]	33	4 (*B. suis* bv 1)	11	2
	Marion[c]	5	1 (*B. suis* bv 1)	0	0
	Palm Beach[d]	15	1 (*B. suis* bv 1)	4	1
	Polk[e]	18	3 (*B. suis* bv 1)	4	1
Hawaii	Honolulu	20	1 (*B. suis* bv 3)	8	0
Louisiana	Cameron	1	0	0	N/A
	Evangeline	9	2 (*B. suis* bv 1)	1	1
Mississippi	Bolivar	6	1 (*B. suis* bv 1)	0	0
	Yazoo[f]	30	0	0	N/A
South Carolina	Calhoun	3	0	0	N/A
	Georgetown	5	0	0	N/A
	Richland	12	3 (*B. suis* bv 1)	3	2
Texas	Freestone	3	0	1	N/A

[a] N/A = not applicable.

[b] 1 animal with no serum and 1 too hemolyzed to test.

[c] 1 serum sample too hemolyzed to test.

[d] 3 serum samples too hemolyzed to test.

[e] 1 animal with no serum and 9 too hemolyzed to test.

[f] 1 animal with no serum.

class of the animal, and a unique barcode number that was assigned to all samples from the same animal.

Blood was collected via cardiac puncture as soon as possible after death. Blood was transferred to sterile, glass collection tubes and allowed to clot for 15–20 min before being placed into a cooler. Once clotting occurred, the collection tubes were refrigerated for no longer than 12 hr before extracting serum. Serum was transferred to 2-mL cryogenic vials using a pipette and was labeled with a unique barcode. Serum was shipped to the laboratory within 3 days of collection. Samples that could not be shipped within 3 days were frozen at −20 C and shipped no later than 2 wk after collection. Samples were shipped via overnight carrier with ice packs or dry ice to the NWDP Feral Swine Tissue Archive (Fort Collins, Colorado, USA), where they were stored in a −80 C ultracold freezer until they were batch-shipped to the testing laboratory every other week.

One to three of the following lymph nodes were submitted for testing: submandibular, gastrohepatic, internal iliac, medial retropharyngeal, and superficial inguinal. Upon collection, lymph nodes were immediately transferred to sterile, resealable, plastic bags and were grouped by body region. Lymph nodes were frozen at −20 C and shipped overnight no later than 3 days after collection to the NWDP Feral Swine Tissue Archive, where they were stored in a −80 C ultracold freezer and batch-shipped monthly to the USDA-APHIS, National Veterinary Services Laboratories (NVSL; Ames, Iowa, USA).

Testing procedures

Serology: Serum samples were submitted to the Kansas State-Federal Brucellosis Laboratory in Topeka, Kansas or the Kentucky Eastern Regional Federal Brucellosis Laboratory (Frankfort, Kentucky, USA) for evidence of *Brucella* antibodies. Standardized testing was conducted using the fluorescence polarization assay (FPA), as described by Nielsen et al. (1999). Samples with a result of 20 millipolarization units or greater than the mean, negative control were considered positive.

Isolation and identification: Isolation of *Brucella* bacteria from lymph nodes and subsequent biochemical identification of isolates were performed by conventional methods at NVSL (Alton et al. 1988). Briefly, each node was

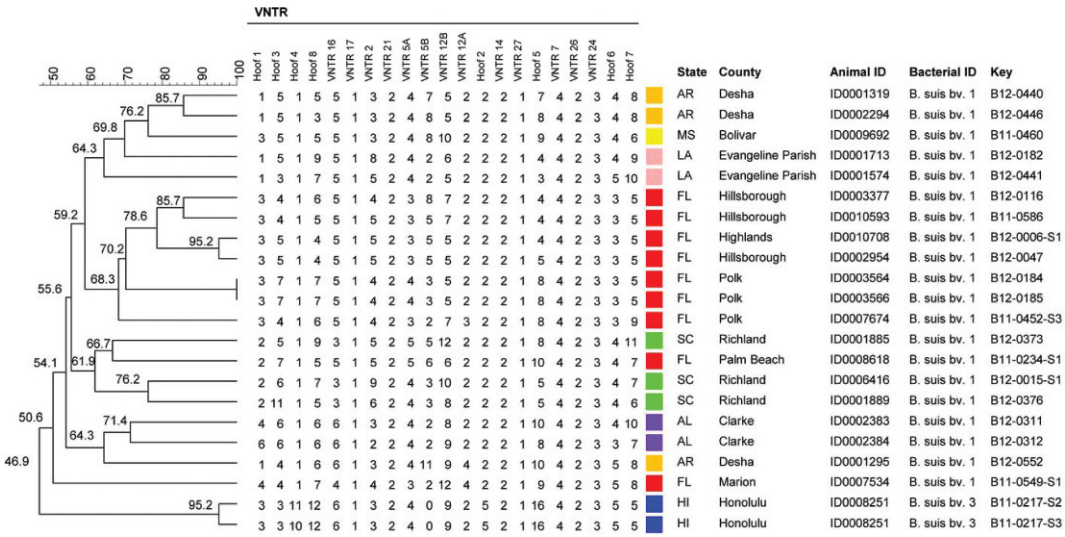

FIGURE 1. Unweighted pair group method with arithmetic mean analysis of the variable number tandem repeat (VNTR) profiles for feral swine (*Sus scrofa*) collected in selected states in the USA ($n=22$). State of feral swine origin is indicated by color coding: Hawaii (HI) = blue, Florida (FL) = red, Mississippi (MS) = yellow, South Carolina (SC) = green, Louisiana (LA) = pink, Alabama (AL) = purple, and Arkansas (AR) = orange.

trimmed of excess fat, dipped in 95% ethanol, and flamed to remove surface contaminants. The node was trimmed into small (~1 cm^2) pieces and homogenized with an equal volume of phosphate-buffered saline in a stomacher blender for ≥2 min. The resulting homogenate was then swabbed (transferring ~100–200 μL) onto nonselective and selective agars, including trypticase soy agar with 5% serum, antibiotics and ethyl-violet, Ewalt's medium, and Farrell's medium with 5% serum (Nielsen and Duncan 1990). Agar plates were incubated at 37 C and 10% CO_2 for ≥10 days, with observations typically at 5 and 10 days. Bacterial growth that appeared typical of *Brucella* was tested for catalase production, and if positive, at least one colony was chosen for further biochemical identification. This included the ability to grow in air and on various dyes and antibiotics, production of hydrogen sulfide and urease, lysis by Tbilisi phage at the routine test dilution (RTD) and at an RTD×10^4, and agglutination with monospecific antisera. In an attempt to detect a potential mixed infection, up to 30 colonies per animal were tested by modified *Brucella* AMOS PCR (AMOS is an acronym for the *Brucella* species identified: *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis*; Bricker and Halling 1994, 1995) with visualization of the PCR products by gel electrophoresis.

Genotyping

A VNTR was conducted for at least one *B. suis* isolate from each animal. The VNTR analysis targeted 21 loci (VNTR-21) within the *B. suis* genome (see Fig. 1 for names of specific loci), consisting of the seven loci associated with the "HOOF-Prints" assay developed by Bricker et al. (2003), as well as 13 additional loci and the redesigned HOOF-Prints 7 primers determined by Whatmore et al. (2006). The PCR assays were performed using dye-labeled primers in 16 reactions, with some assays being multiplexed. Each 10 μL reaction contained 1× PCR buffer, 5% dimethyl sulfoxide, 0.2 mM each deoxyribonucleotide triphosphates, 400–600 nM of each primer, 0.25 U FastStart High-Fidelity *Taq* polymerase (Roche, Indianapolis, Indiana, USA), and ~10 ng of *B. suis* DNA. Thermal cycling conditions were 5 min at 95 C, then 35 cycles of 30 sec at 95 C, 1 min at 55 C, 1.5 min at 75 C, and a final hold of 5 min at 75 C. All PCR reactions included *B. suis* biovar 1, strain 1330 (NVSL ID 9-1-2010) as a positive control and no-template controls. The PCR products were stored at 4 C in light-safe containers no more than 48 hr and subjected to electrophoresis on a BI 3500XL Genetic Analyzer using the GeneScan® 600 LIZ® size standard (Applied Biosystems, Carlsbad, California, USA). Fragment data were analyzed using

GeneMapper® 4.1 software package (Applied Biosystems). Isolates that generated unambiguous, well-resolved peaks in GeneMapper electropherograms were included in the analysis. To ensure accuracy, electropherograms indicative of zero repeats, or those that generated ambiguous peaks for a given locus, were reanalyzed, and a confirmatory, singleton PCR assay for the locus was performed. Genetic distances among the sampled *B. suis* genotypes were visualized through a cluster analysis of categorical data with the unweighted-pair group method with arithmetic mean (UPGMA) analysis using BioNumerics 6.3 software (Applied Maths, Austin, Texas, USA; Fig. 1). To further investigate clustering we used a factorial correspondence analysis (FCA) implemented in GENETIX v4.05.2 (Belkhir et al., 2004) to compare our feral swine VNTR profiles to *B. suis* genotypes from previous work (Whatmore et al. 2006).

RESULTS

Culture and serology

Lymph nodes and serum samples were collected from apparently healthy feral swine in 16 counties in Alabama, Arkansas, Florida, Hawaii, Louisiana, Mississippi, South Carolina, and Texas (Table 1). Of the 183 samples collected, 92% were collected from adults and 8% were collected from subadults; one sample was collected from an animal of unknown age class. Samples were collected from 91 females and 92 males.

Twenty-one animals had culture-positive results; all colonies tested by PCR indicated *B. suis*. All isolates recovered were identified as *B. suis* biovar 1, except for those from an animal in Hawaii, which were identified as *B. suis* biovar 3. All culture-positive animals were adults; nine were female and 12 were male. At least one *B. suis* culture-positive animal was obtained from all states included in this survey, except Texas.

Serum was submitted from 180 of the 183 animals sampled, but 14 samples were too hemolyzed to allow reliable testing. Based on the 166 serum samples that were suitable for testing, the apparent prevalence was 28% (Table 1). Eleven of 21 culture-positive animals (52.3%; 95% CL=29.8–74.3) were also antibody positive (Table 1). Ten antibody-negative animals had positive culture results (48%), and 35 antibody-positive animals had negative culture results (24%).

Genotyping

Genotyping results indicated that all isolates from a single individual shared the same VNTR-21 profile, with the exception of an individual from Hawaii, in which two distinct profiles were identified (Fig. 1). That difference occurred at Hoof 4, with a difference of one repeat. Two individuals had isolates with identical VNTR-21 profiles; they were collected from an adult male and an adult female feral swine from the same location on the same day. The UPGMA tree revealed that genetic distances among genotypes were not associated with geographic proximity (Fig. 1). In the FCA, the *B. suis* collected from feral swine appeared to cluster in a relatively tight group, as opposed to other species (Figs. 2, 3).

DISCUSSION

Our results indicate that feral swine are carriers of *B. suis* and the FPA serologic diagnostic test is likely detecting *B. suis*. However, serologic results did not appear to be a good indicator of infection because only 52% of sera from culture-positive animals were antibody positive. One explanation for that discrepancy was that the feral swine identified as culture positive in this study may have been recently infected and had not yet seroconverted. Additionally, serologic tests for brucellosis are known to have widely varying sensitivity and specificity (Nielsen 2002), so it is not surprising that serologic results were not always consistent with the positive culture results. There are also potential confounding factors associated with using a test validated for domestic swine (Nielsen et al. 1999) for pathogen exposure in feral swine. Disease progression in feral swine can differ markedly

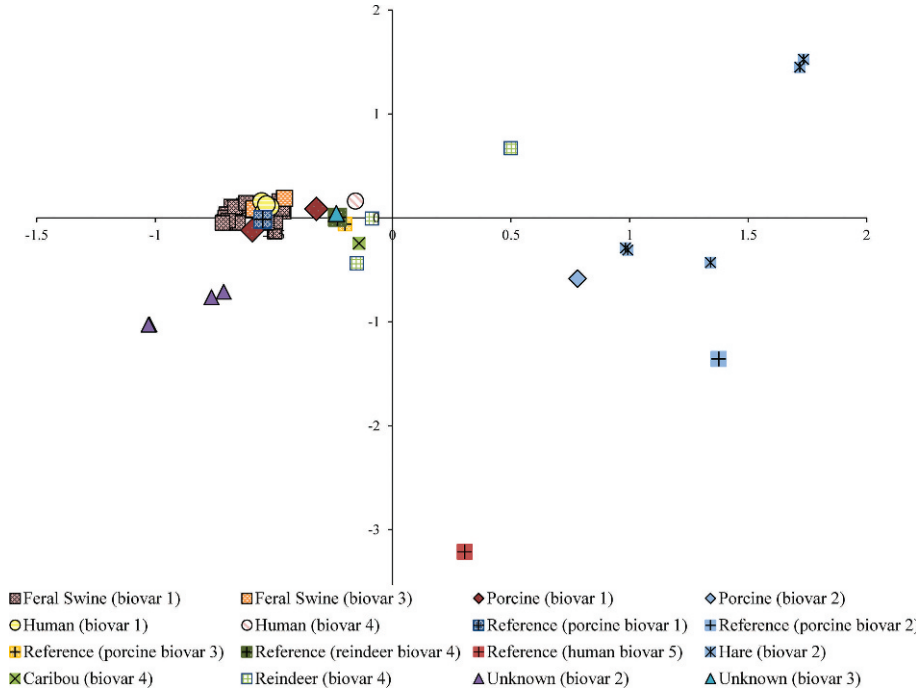

FIGURE 2. Genetic clustering of *Brucella suis* by host species (feral swine = *Sus scrofa*; "porcine" = *Sus scrofa*; caribou = *Rangifer tarandus* ssp.; reindeer = *Rangifer tarandus* ssp.; hare = *Lepus* sp).

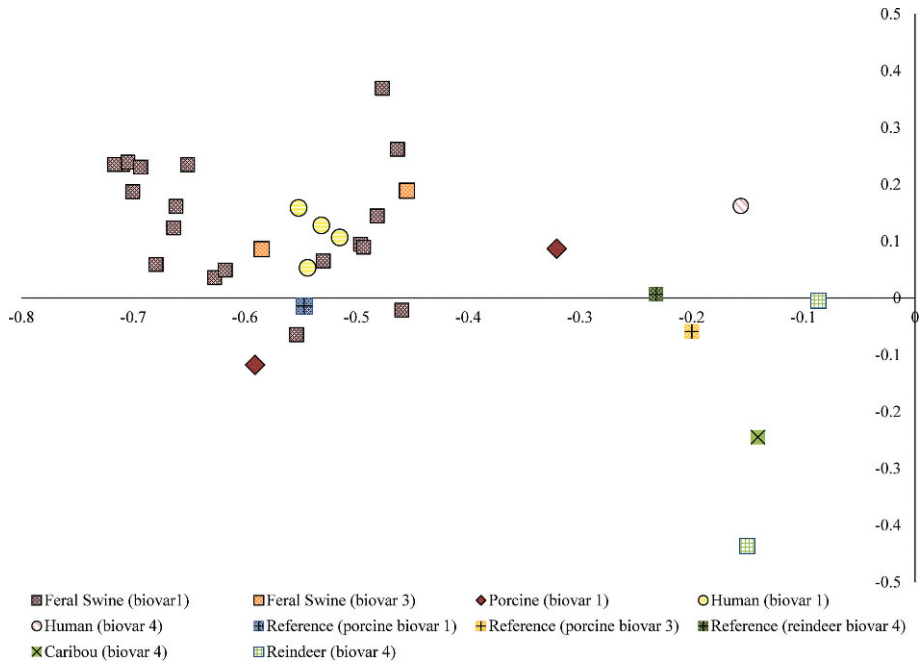

FIGURE 3. Genetic clusters of *Brucella suis* most closely related to feral swine (*Sus scrofa*) genotypes (feral swine = *Sus scrofa*; "porcine" = *Sus scrofa*; caribou = *Rangifer tarandus* ssp.; reindeer = *Rangifer tarandus* ssp.).

from what is seen in domestic swine (Mohamed et al. 2011), and differences in test performance can be attributed to serologic response variations between species (Roffe et al. 1999). Because our data suggest that the sensitivity of the FPA may be less than the 80% previously calculated in swine (Paulo et al. 2000), we recommend additional studies be conducted with larger sample sizes to allow for a robust sensitivity calculation. The nature of our results prevented a similar specificity calculation.

Another explanation for the apparently low congruency between the serology and culture results may be because *Brucella* infections tend to localize in specific tissues. From our relatively small data set, it appears that *B. suis* was most frequently isolated from head lymph nodes and the gastrohepatic lymph node. However, it was common for *B. suis* to only be isolated from one of several lymph nodes from an animal suggesting that some culture-negative animals may not have been truly negative and that *Brucella* spp. was not localized in the specific subset of lymph nodes tested. Yet another explanation may be that, because we froze the samples before culture, there may have been some associated bacterial mortality that resulted in decreased detection.

We cultured *B. suis* from feral swine in every state sampled, except Texas. Our culture success was most likely due to our study design, which targeted specific counties in states where previous serosurveys indicated high apparent prevalence of brucellosis (Pedersen et al. 2012). Although *B. suis* was the only species of *Brucella* we detected, *B. abortus* has been documented in feral swine (Stoffregen et al. 2007; Higgins et al. 2012). However, in one case (Stoffregen et al. 2007), positive samples were collected from feral swine on a property where *B. abortus* RB51 vaccine trials had been conducted, which may have led to exposure in feral swine. In another case (Higgins et al. 2012), *B. abortus* was isolated from a single feral swine sampled adjacent to a property with infected cattle, but the VNTR-21 analysis alone was insufficient to determine whether feral swine were the source or incidental hosts of the bacteria. Because we did not detect *B. abortus* in any of the lymph nodes and because it is widely accepted that *B. abortus* is self-limiting in swine, it is unlikely that feral swine act as a reservoir in the US under natural circumstances.

Similar to our nationwide serologic results (Pedersen et al. 2012), we found no significant difference in culture-positive results between males and females (57% and 43%); however, that is contrary to other studies where males were more likely to be antibody-positive than females were (Stoffregen et al. 2007).

Brucella suis biovars 1 and 3 have been isolated from infected swine in the US (NVSL unpubl. data; Meyer 1964; Cornell et al. 1989). We detected biovar 1 in the continental US and biovar 3 in Hawaii, which is noteworthy considering that those two biovars appear to be particularly virulent for humans (Young 1995). Also, *B. suis* is not only more virulent in humans than *B. abortus* is (Young 1995) but also greater numbers of *B. suis* organisms have been detected in the tissues of infected swine compared with cattle infected with *B. abortus* (Deyoe 1986). In addition, our analyses determined that some human and porcine genotypes (Whatmore et al. 2006) were clustered based on genotypic similarity (Figs. 2, 3). The human genotypes that originated from Finland, Tonga, and Holland clustered with our feral swine genotypes from the US. That pattern might be due to evolutionary relationships among *B. suis* genotypes attributed to recent transfer events among those taxa or to homoplasy. Further studies of *B. suis*, including a phylogenetic assessment, would be required to fully understand that pattern.

Contact between feral swine and domestic swine has been documented (Wyckoff et al. 2009), indicating that the

potential for pathogen transmission exists and appropriate biosecurity measures should be implemented to mitigate that risk. *Brucella suis* has not only been documented in swine (Wood et al. 1976) but also been documented in cattle (Cook and Noble 1984; Ewalt et al. 1997), demonstrating that cattle are susceptible to infection with *B. suis*. Feral swine have also been documented in proximity to cattle that have become infected with *B. suis* (Cook and Noble 1984), further emphasizing the risk of transmission if biosecurity does not prevent cross-species contact (e.g., grazing cattle on pastures occupied by infected feral swine [Norton and Thomas 1976; Cooper et al. 2010]).

Only two animals had isolates that shared identical profiles. Those two animals also shared an epidemiologic link in that both were captured at the same site on the same day. Two isolates from the same animal showed a difference of one repeat at Hoof 4, but it is not unusual to have variation in the VNTR profile of isolates from the same animal. It is not surprising that the remaining isolates showed a large degree of genotypic variation, given their widespread geographic distribution and collection dates. The UPGMA analysis (Fig. 1) indicates that isolates from the same state often cluster together, but not consistently.

As feral swine continue to inhabit new areas, the probability of pathogen transmission to livestock and humans is of greater concern. Wildlife biologists, hunters, and abattoir workers, in particular, have a higher risk than most people of coming into contact with infected feral swine, and consequently, should be aware of the risk and take appropriate cautions to prevent infection, not only from brucellosis but from other zoonotic pathogens feral swine may carry.

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