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Case report

Diagnosis of *Yersinia enterocolitica* serovar O:9 in a commercial 2400-sow farm with false-positive *Brucella suis* serology using western blot, competitive ELISA, bacterial isolation, and whole genome sequencing

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Summary

Despite eradication of swine brucellosis from US commercial swine, *Brucella suis* still exists in feral swine. Therefore, brucellosis surveillance occurs to detect and eliminate any disease introduction from feral swine to domestic swine. As serology for swine brucellosis has imperfect specificity, false-positive serological reactions (FPSRs) occur and true brucellosis infection must be ruled out. In this case report, we detail a process to rule out *B suis* infection in a commercial sow herd using additional diagnostics including bacterial culture, whole genome sequencing, western blot, and competitive enzyme-linked immunosorbent assay. It was determined *Yersinia enterocolitica* serovar O:9 caused the FPSRs.

Keywords: swine, *Brucella suis*, false-positive, *Yersinia enterocolitica*, serology

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Resumen – Diagnóstico de *Yersinia enterocolitica* serovar O:9 en una granja comercial de 2400 cerdas con un falso-positivo de serología de *Brucella suis* usando Western blot, ELISA competitiva, aislamiento bacteriano, y secuenciación del genoma completo

A pesar de la erradicación de la brucelosis porcina de los cerdos comerciales de EE UU, *Brucella suis* todavía existe en los cerdos salvajes. Por lo tanto, la vigilancia de la brucelosis se usa para detectar y eliminar cualquier introducción de enfermedad de cerdos salvajes a cerdos domésticos. Como la serología para la brucelosis porcina tiene una sensibilidad imperfecta, se producen reacciones serológicas falsas-positivas (FPSRs), y se debe descartar una verdadera infección por brucelosis. En este reporte de caso, detallamos un proceso para descartar la infección por *B suis* en una piara de cerdas comerciales utilizando diagnósticos adicionales que incluyen cultivo bacteriano, secuenciación del genoma completo, western blot y ensayo competitivo de inmunoadsorción ligado a enzimas. Se determinó que *Yersinia enterocolitica* serovar O:9 causó las FPSRs.

Résumé – Détection de *Yersinia enterocolitica* serovar O:9 dans une ferme commerciale de 2400 truies présentant des résultats faux-positifs à *Brucella suis* par sérologie en utilisant l'immunobuvardage, un ELISA compétitif, l'isolement bactérien, et le séquençage du génome entier

Malgré l'éradication de la brucellose porcine chez les porcs américains commerciaux, *Brucella suis* est présent chez les porcs sauvages. Ainsi, la surveillance pour la brucellose porcine existe afin de détecter et d'éliminer toute transmission de la maladie des porcs sauvages aux porcs domestiques. Étant donné que le test sérologique pour la brucellose a une sensibilité imparfaite, des réactions faussement-positives (FPSRs) se produisent, et une véritable infection brucellique doit être exclue. Dans le présent rapport de cas, nous détaillons un processus pour exclure l'infection à *B suis* dans un troupeau commercial de truies en utilisant des méthodes diagnostiques additionnelles incluant la culture bactérienne, le séquençage du génome complet, l'immunobuvardage, et une épreuve ELISA compétitive. Il fut déterminé que *Yersinia enterocolitica* O:9 était responsable des FPSRs.

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Swine brucellosis was eradicated in the US commercial swine herd in 2011 when Texas was added as the final validated brucellosis-free state.[1] In spite of this eradication success, *Brucella suis* continues to exist in a wildlife carrier, feral swine.[2,3] *Brucella suis* presents a risk of disease reintroduction to domestic swine via contact with feral swine and presents an ongoing risk of zoonotic disease to people who have contact with blood or other body fluids from infected swine.[4] Therefore, swine brucellosis disease surveillance programs exist at US slaughter plants to allow prompt detection and removal of infected domestic swine and to provide assurance to international trading partners that US commercial swine herds are brucellosis-free.

False-positive serological reactions (FPSRs) are common when testing for swine brucellosis, and *Yersinia enterocolitica* serovar O:9 appears to be the most common cause of these false-positive tests due to the similar lipopolysaccharide (LPS) O-antigens in both organisms.[5,6] Additionally, *Y enterocolitica* serovar O:9 has also been shown to cause FPSRs in cattle that are serologically tested for *Brucella abortus* for the same reason.[7] Many researchers have sought to create serologic tests[7-9] that cancel out cross-reactivity and either prevent or rule out these FPSRs.

In spite of these efforts, there is still no dependable serological test for the diagnosis of swine brucellosis in an individual animal.[10] Hence, ruling out a true swine brucellosis infection in a seropositive animal or herd comes at a considerable cost to the swine producer due to time spent under quarantine and to the state or federal government due to required additional testing to ensure a herd is not infected with *B suis*.[11,12] In the absence of an alternative method, a serologic surveillance program with specificity less than 100% will continue to be used and FPSRs will need to be investigated. This case report details a diagnostic work-up to rule out swine brucellosis in a herd with FPSRs, and *Y enterocolitica* serovar O:9 was isolated and deemed to be the cause of the FPSRs.

Case description

Initial herd investigation

In February 2017, the National Veterinary Services Laboratories (NVSL) notified the North Carolina Veterinary Services office of a swine brucellosis reactor animal found by slaughter surveillance. Serology results from the cull sow collected at slaughter revealed fluorescent polarization assay (FPA) values of 85/80 Delta millipolarization units (mP; each sample was analyzed twice for comparison and reported as two values, eg, 85/80; negative reference range: < 10 Delta mP, suspect reference range: 10-20 Delta mP, and positive reference range: > 20 Delta mP) and complement fixation (CF) value of 2+ at a 1:10 dilution (negative reference range: no complement fixation occurs at a 1:10 dilution). This animal was traced to a 2400-sow farm in North Carolina. The source herd did not have clinical signs suggesting swine brucellosis infection. The herd was kept in closed buildings and potential exposure to feral swine was considered negligible. Pigs that were weaned from the source farm were destined for market production only after shipment to a nursery and then to a finishing unit. No females weaned from this farm were kept as replacement gilts. Serological testing of 160 breeding females was conducted within the source herd. The herd was placed under quarantine due to the positive herd test serology and replacement females could not enter and cull sows could not leave during the investigation period. The finishing units that ultimately received pigs from the sow farm flow could move pigs to slaughter under permit during the investigation period.

In order to differentiate an FPSR situation from a truly infected swine brucellosis herd, the North Carolina Department of Agriculture, the US Department of Agriculture, and the herd veterinarian agreed that 4 of the sows with high titers should be humanely euthanized by the herd veterinarian and necropsied at the North Carolina Veterinary Diagnostic Laboratory System (NCVDL). Because the herd had no clinical signs of swine brucellosis, more sows were not sacrificed for tissue collection, thus preventing unnecessary loss to the producer. Tissues sampled from each euthanized sow were submitted to the NVSL for culture. Tissue samples included mandibular lymph nodes, retropharyngeal lymph nodes, hepatic lymph nodes, internal iliac lymph nodes, superficial inguinal lymph nodes, mesenteric lymph nodes, kidney, and tonsil. These tissues were examined in order to maximize the likelihood of isolating *B suis* if it was present in the animals. Three of the 4 euthanized sows were pregnant and fetal lung, amniotic fluid, and placenta samples were submitted for culture (Table 1).

If *B suis* was isolated from the collected tissues, whole-herd depopulation and further tissue collection would be the likely outcome. More samples for culture (placenta and milk) would become available as sows farrowed, which would provide further evidence of a negative herd status and further prevent the need for sacrificing additional animals. Resampling of the remaining seropositive sows in the source herd was accomplished 39 days after initial samples were taken and titers were compared (Table 2). One-time milk and placenta samples were collected from 8 sows with titers when they farrowed and were submitted for isolation of *B suis* at the NCVDL (Table 3). A third set of serological testing was completed on 8 of the seropositive sows on the source farm between 88 to 104 days after the initial herd test (Table 2).

Serological sampling of breeding females in the source herd and from swine in epidemiologically linked herds was conducted to approximate a 95% confidence level of finding an infected animal assuming a 2% herd prevalence and 90% diagnostic test sensitivity. The brucellosis card test (NVSL SOP-SERO-0020) was used for sample screening and FPA (NVSL SOP-SERO-0021) and CF (NVSL SOP-SERO-0015) were used as confirmatory tests. In addition, for selected secondary sow samples, a competitive enzyme-linked immunosorbent assay (cELISA; NVSL SOP-SERO-0023; Boehringer Ingelheim Svanova) was performed as a potential highly specific differential test. All serological testing was conducted using standard operating procedures administered by the NVSL which are controlled documents and available through the NVSL Quality Assurance program section (**nvsl.mastercontrol@usda.gov**).

Testing of the 160 breeding females at the source herd identified 35 animals as card positive, and these positive serum samples were sent to the NVSL for confirmatory testing. Of these 35 card-positive animals, 23 animals were positive in both the FPA and the CF, 3 animals were suspect in the FPA and positive in CF (Table 2). Four animals were positive in the FPA and negative in the CF, and the remaining 5 animals were negative in both the FPA and the CF. Of the 26 sows resampled from the source farm, 21 had a decrease in the mean FPA value and 20 had a decrease or no change in the CF value (Table 2). Half of the 26 animals were negative in the cELISA (Table 2).

Serologic investigation of epidemiologically linked herds

The source herd received replacement gilts from a single 2400-sow multiplier herd. Serological testing was conducted on 164 animals from the multiplier herd. Gilts from the multiplier were sent to a nursery and then a finisher before arriving at the source herd for breeding. The multiplier finisher (7920-head farm) that supplied gilts to the source farm also had serum collected from 167 gilts. The source herd had no boars, but had received semen from 2 boar studs in the previous 12 months. The 2 boar studs, which housed 430 and 532 boars, had 143 and 150 animals sampled, respectively.

During quarantine, farms within 2.4 km of the quarantined herd were identified by the North Carolina Department of Agriculture. Five farms in this radius were commercial finishing units, and 2 were backyard swine producers. The backyard swine producers each had 1 breeding female on their respective farms.

All boars tested from the first boar stud were negative in the card test. Two boars from the second boar stud were positive in the card test; these samples were shipped to the NVSL for confirmatory serological testing and both samples were negative in the FPA and the CF test.

Serological testing of 164 animals from the multiplier herd indicated 32 of the 164 breeding females were positive using the card test. These positive samples were sent to the NVSL for confirmatory serological testing, and all 32 samples were negative using the FPA and the CF tests. Serum samples collected from the 167 gilts at the finisher farm resulted in 2 samples being positive using the card test. These samples were sent to the NVSL for confirmatory serological testing and were both negative using the FPA and the CF test. The 2 breeding females from the backyard swine producers were found to be serologically negative for brucellosis. All epidemiologically linked herds were considered negative for swine brucellosis.

Brucella culture testing

At the NVSL, culture for *B suis* was performed as previously described,[13] with a modification for the use of a blender to homogenize tissues. At the NCVDL, tissues were aseptically placed in a sterile plastic bag with trypticase-soy broth and macerated for up to 10 minutes. A sterile swab was used to inoculate the following media: 1) *Brucella* serum tryptose agar plate (made in-house) composed of horse serum (5 mL/500 mL of prepared media), polymixin B (1.5 mL/500 mL of prepared media), cyclohexamide (2.5 mL/500 mL of prepared media), and bacitracin (1 mL/500 mL of prepared media); 2) *Brucella* crystal violet tryptose agar plate (made in-house) composed of 1% crystal violet solution (0.7 mL/500 mL of prepared media), polymixin B (1.5 mL/500 mL of prepared media), cycloheximide (2.5 mL/500 mL of prepared media), and bacitracin (1 mL/500 mL of prepared media); and 3) *Brucella* selective tryptose agar plate composed of heat inactivated horse serum (25 mL/500 mL of prepared media) and *Brucella* Selective Supplement (Oxoid *Brucella* Selective Supplement, ThermoFisher Scientific; 10 mL/500 mL of prepared media) containing 2500 IU of polymyxin B, 12,500 IU of bacitracin, 50 mg of cycloheximide, 2.5 mg of nalidixic acid, 50,000 IU of nystatin, and 10 mg of vancomycin.

The plates were incubated at 37°C in 5% to 7% CO_2. Plates were examined daily for 14 days. Any colonies with a morphology consistent with *Brucella* species would have been subcultured to a blood agar plate and examined by Gram stain. Isolates exhibiting the typical *Brucella* Gram stain (gram-negative coccobacilli, or short rods) would have been further tested by performing a Koster's stain, an oxidase test, and inoculating a triple sugar iron (TSI) slant and a urea slant. If presumptive tests were positive for *Brucella* species, the isolate would have been forwarded to the NVSL.

Yersinia culture testing

Bacterial culturing for *Yersinia* at the NVSL was conducted by cutting tissues into 1 to 2 mm pieces with sterile scissors or sterile scalpels and put into peptone sorbitol bile broth (PSBB; made in-house) in a 1:10 ratio and thoroughly vortexed. The PSBB consisted of 8.23 g sodium phosphate dibasic anhydrous (Sigma-Aldrich), 1.2 g sodium phosphate monobasic monohydrate (Avantor), 1.5 g bile salts mixture (Becton, Dickinson and Co), 5 g sodium chloride (Fisher Scientific), 10 g D-sorbitol (Sigma-Aldrich), 5 g Bacto peptone (Becton, Dickinson and Co), and was brought to 1000 mL with sterile water.[14] The PSBB was incubated at 10°C for 10 to 12 days.

After incubation was complete, the PSBB was thoroughly vortexed. A swab was used to sample the PSBB and then plated directly onto MacConkey (MAC; Remel) and *Yersinia* Selective agar (cefsulodin-irgasan-novobiocin; CIN; Remel) and streaked for isolation. Also, 0.1 mL PSBB was transferred to 0.9 mL of 5% potassium hydroxide (Sigma-Aldrich) in normal saline and vortexed. This

Table 1: Serologic titers and culture results from four sows that were euthanized and tissues collected to determine swine brucellosis status

Sow ID (Parity)	Card test	FPA, Delta mP	CF value (dilution)	MLN	RLN	Tonsil	ALN	Kidney*	AF*	PL*	FL*
1979 (5)	Pos	98/92	2+ (1:60)	YE	NI	YE	NI	NI	NI	NI	NI
5218 (0)	Pos	94/96	4+ (1:80)	YE	YE	YE	YE	NI	NI	NI	NI
2870 (4)	Pos	52/47	3+ (1:10)	NI	NI	YE	NI	NI	NS	NS	NS
2672 (5)	Pos	91/100	2+ (1:10)	NI	NI	YE	NI	NI	NI	NI	NI

* *Brucella* isolation attempt only, no *Yersinia* isolation attempt.
ID = identification; FPA = fluorescent polarization assay; mP = millipolarization units; CF = complement fixation (cold) test; MLN = mandibular lymph node; RLN = retropharyngeal lymph node; ALN = additional lymph nodes; AF = amniotic fluid; PL = placenta; FL = fetal lung; Pos = positive; YE = *Yersinia enterocolitica*; NI = no isolation of *Brucella suis* or *Y enterocolitica*; NS = not submitted (sow not pregnant).

Table 2: Chronological decrease of titers in sows seropositive for swine brucellosis

Sow ID	Initial FPA, Delta mP*	Follow-up FPA, Delta mP*	Final FPA, Delta mP*	Initial CF value (dilution)	Follow-up CF value (dilution)	Final CF value (dilution)	cELISA, %I†	Follow-up cELISA, %I†
5128	21/20	25/26	NS	2+ (1:80)	2+ (1:40)	NS	31.5	NS
4613	14/15	34/31	NS	3+ (1:10)	1+ (1:40)	NS	40.1	NS
5053	117/113	72/74	36/31	2+ (1:160)	1+ (1:80)	2+ (1:10)	76.0	28.8
4505	21/19	9/10	NS	2+ (1:10)	Neg (1:10)	NS	20.2	NS
5097	16/16	10/9	7/6	3+ (1:10)	Neg (1:10)	Neg (1:10)	12.0	-4.9
4029	42/40	19/20	NS	Neg (1:10)	Neg (1:10)	NS	28.6	NS
3647	34/35	22/30	NS	1+ (1:10)	1+ (1:10)	NS	29.8	NS
4177	32/25	20/19	NS	1+ (1:40)	1+ (1:10)	NS	28.7	NS
2623	29/28	17/16	8/7	Neg (1:10)	Neg (1:10)	Neg (1:10)	25.9	4.65
4146	86/89	39/51	NS	4+ (1:40)	1+ (1:20)	NS	54.1	NS
3284	41/46	27/23	NS	3+ (1:10)	1+ (1:10)	NS	32.3	NS
2169	24/27	13/13	-1/-2	4+ (1:20)	1+ (1:10)	Neg (1:10)	23.6	10.8
4026	34/29	32/28	NS	2+ (1:10)	2+ (1:10)	NS	52.0	NS
5004	70/66	54/53	NS	4+ (1:40)	1+ (1:20)	NS	40.6	NS
4259	54/52	25/22	NS	3+ (1:20)	1+ (1:10)	NS	26.1	NS
5142	50/50	29/26	10/11	3+ (1:20)	Neg (1:10)	Neg (1:10)	21.7	-20.7
4539	25/21	15/13	5/5	4+ (1:40)	1+ (1:10)	Neg (1:10)	29.0	1.6
5191	117/115	79/70	NS	2+ (1:160)	1+ (1:40)	NS	84.1	NS
4530	61/61	41/37	NS	3+ (1:40)	3+ (1:10)	NS	81.2	NS
4167	18/18	42/41	NS	2+ (1:10)	3+ (1:20)	NS	61.8	NS
3160	49/42	24/26	NS	2+ (1:10)	3+ (1:10)	NS	25.6	NS
3163	50/47	55/52	NS	3+ (1:10)	2+ (1:80)	NS	64.5	NS
3153	73/74	69/63	NS	Neg (1:10)	1+ (1:10)	NS	48.8	NS
4216	47/50	22/19	NS	4+ (1:20)	2+ (1:10)	NS	35.7	NS
4702	23/19	27/28	5/4	Neg (1:10)	2+ (1:10)	Neg (1:10)	20.1	10.0
5217	19/23	19/18	6/6	2+ (1:10)	2+ (1:10)	Neg (1:10)	20.0	-1.9

* FPA reference ranges: < 10 Delta mP = negative; 10-20 Delta mP = suspect; > 20 Delta mP = positive.
† cELISA reference ranges: ≥ 30% inhibition = positive; < 30% inhibition = negative.
ID = identification; FPA = fluorescence polarization assay; mP = millipolarization units; CF = complement fixation (cold) test; cELISA = competitive enzyme-linked immunosorbent assay; I = inhibition; NS = not submitted; Neg = negative.

was plated onto MAC and CIN agar using a swab and streaked for isolation. Another 0.1 mL PSBB was transferred to 0.9 mL normal saline and swabbed on MAC and CIN agar and streaked for isolation. All plates were incubated at 30°C for 1 to 2 days.

After incubation the plates were read and suspect colonies were streaked on trypticase soy agar with 5% sheep blood agar plates (Remel) which were incubated at 30°C for 1 to 2 days. Isolated colonies were identified by Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) using Bruker Biotyper software (Bruker Daltonics) on a Bruker Autoflex MALDI-TOF (Bruker Daltonics).

For bacterial culturing for *Yersinia* at the NCVDL, tissues were aseptically placed in a sterile plastic bag with trypticase-soy broth and macerated using a stomacher for up to 10 minutes. A sterile swab was used to inoculate a MAC agar plate and a CIN agar plate. The plates were incubated at 30°C in ambient air for 48 hours. The swab was also used to inoculate a sterile tube containing phosphate buffered saline (PBS; pH 7.4). This tube was stored at 2°C to 8°C for up to 21 days with weekly subcultures to MAC and CIN agar plates which were also incubated at 30°C for 48 hours.

Original plates and plates from weekly subcultures were observed for colonies exhibiting morphologies consistent with *Yersinia* species. Suspicious colonies, if they had been found, would have been further tested by inoculating biochemicals including a TSI slant, a urea slant, and two sulfide, indole, motility tubes (one at 30°C and one at 37°C). Oxidase and indole tests would also

Table 3: Initial serologic titers and culture results from sows immediately post-partum

Sow ID	Initial FPA, Delta mP	Initial CF value (dilution)	Milk culture	Placenta culture
2623	29/28	Neg (1:10)	NI	NI
5053	117/113	2+ (1:160)	NS	NI
4539	25/21	4+ (1:40)	NI	NI
2169	24/27	4+ (1:20)	NI	NI
5217	19/23	2+ (1:10)	NI*	NS
5142	50/50	3+ (1:20)	NI*	NS
5097	16/16	3+ (1:10)	NI*	NS
4702	23/19	Neg (1:10)	NI*	NI

* Isolation of *Yersinia enterocolitica* attempted in addition to *Brucella suis* isolation attempt.
ID = identification; FPA = fluorescent polarization assay; mP = millipolarization units; CF = complement fixation (cold) test; NI = no isolation of *Brucella suis*; NS = not submitted.

have been performed. If presumptive tests were consistent with *Yersinia*, an Analytical Profile Index 20E (bioMérieux, Inc) would have been set up.

Yersinia isolate sequencing and serovar determination

One isolate of *Y enterocolitica* from sow 1979 and sow 5218 were streaked on blood agar plates and incubated at 37°C for 18 to 24 hours. Genomic DNA was extracted from each isolate using the Promega Maxwell RSC 48 instrument with the Maxwell RSC whole blood DNA kit (Promega). Isolates were sequenced on an Illumina MiSeq (Illumina) using 2 × 250 paired end chemistry and the NexteraXT (Illumina) library preparation kit. Each isolate was aligned using the Burrows-Wheeler Aligner-MEM algorithm to reference genomes for *Y enterocolitica* serovar O:3 strain Y11 (GenBank accession NC_017564), *Y enterocolitica* serovar O:8 strain 8081 (GenBank accession NC_008800), and *Y enterocolitica* serovar O:9 strain 105.5R(r) (GenBank accession CP002246). Alignments and annotation were viewed using Integrative Genomics Viewer version 2.3.97. Samtools was used to output depth of coverage at each position, which was used to determine percent coverage of the O-antigen clusters. In addition, the Genome Annotation Toolkit's Unified Genotyper was used to call single-nucleotide polymorphisms for determining percent identity of the O-antigen clusters.

Western blot testing

Twelve serum samples from seropositive sows were subjected to western blot testing to differentiate between *Yersinia* and *Brucella* antibodies. Antigens were prepared from *B abortus* strain 2308 and strain RB51, and from *Y enterocolitica* serovar O:8 (*Y enterocolitica* subspecies *enterocolitica* ATCC 51871) and serovar O:9 (*Y enterocolitica* subspecies *enterocolitica* ATCC 55075), using a cell lysis extraction kit (CellLytic B cell lysis solution, Sigma-Aldrich) according to the manufacturer's directions followed by centrifugation at 5018*g*. The supernatant was retained with subsequent filtration using a 0.2 µm syringe filter. The antigen preparations were a crude extract containing outer membrane and cytoplasmic proteins. Resulting suspensions were tested for inactivation. Precast 4% to 12% Novex Bis-Tris gels (12 well, 1 mm thickness, ThermoFisher Scientific) were used for SDS-PAGE separation of proteins. Respective protein suspensions were prepared by the addition of 60 µL of sample buffer (4x NuPage LDS Sample Buffer, ThermoFisher Scientific) to 180 µL of antigen. Preparations were heated at 70°C for 10 minutes prior to loading 15 µL into pre-assigned gel lanes. The approximate protein concentrations for each respective antigen well was *B abortus* 2308 = 9 mg; *B abortus* RB51 = 8 mg; *Y enterocolitica* serovar O:8 = 40 mg; *Y enterocolitica* serovar O:9 = 25 mg.

Electrophoresis was conducted in an Invitrogen XCell SureLock Mini-Cell system (ThermoFisher Scientific) at a constant current of 125 mA for 35 minutes. A control gel to be used as a western blot comparative standard was prepared by including Invitrogen SeeBlue Plus2 prestained molecular standard (ThermoFisher Scientific) to serve as a marker for molecular weight determination in one lane of the respective gel.

Electrophoretic transfer of proteins onto nitrocellulose was performed using the Invitrogen XCell II Blot Module (ThermoFisher Scientific) and Invitrogen NuPAGE transfer buffer (ThermoFisher Scientific) at 160 mA for 1 hour. After transfer, membranes were blocked with PBS (pH 7.0) with 0.5% Tween 20 plus 2% bovine serum albumin (PBST+BSA) at room temperature for 1 to 2 hours with rocking. Membranes were washed 3 times with PBS plus 0.5% Tween 20 (PBST). Nitrocellulose sheets were then cut into 3 sections, with each section containing duplicate antigen lanes, for incubation with swine sera. Swine sera were diluted at either 1:50 or 1:200 in PBST+BSA and incubated with the membranes at room temperature on a rocker platform for approximately 60 minutes. Membranes were washed 3 times with PBST.

Membranes were incubated for approximately 3 minutes at room temperature on a rocker with Pierce peroxidase conjugated Protein A (ThermoFisher Scientific) diluted 1:20,000 in PBST+BSA. Membranes were then washed 3 times with PBST. Membranes were developed in Sigma TMB Substrate (Sigma-Aldrich) according to the manufacturer's directions.

Bacterial isolation results

Three of the 4 sows at the source farm that were euthanized after the herd quarantine were pregnant, and none of the 4 sows had gross lesions on necropsy. *Brucella suis* was

not isolated from any tissue sample from the 4 euthanized sows. *Yersinia enterocolitica* was isolated from all 4 sows, with tonsil being the most common tissue of successful isolation (4 of 4 animals). One animal also yielded *Y enterocolitica* from the mandibular lymph node. *Yersinia enterocolitica* was isolated from multiple lymph nodes of the fourth animal including mandibular, suprapharyngeal, internal iliac, and superficial inguinal nodes (Table 1).

Alignment to the O-antigen cluster of *Y enterocolitica* serovar O:3 had 18% coverage with 99.6% identity, *Y enterocolitica* serovar O:8 had 58% coverage with 97.8% identity, and *Y enterocolitica* serovar O:9 had 100% coverage with 99.97% identity. Alignments of both isolates with the O-antigen cluster are consistent with an identification as *Y enterocolitica* serovar O:9 as previously described.[15] The regions of *Y enterocolitica* serovar O:3 and *Y enterocolitica* serovar O:8 O-antigen clusters with sequence coverage correspond directly with genes that are homologous to genes present in the *Y enterocolitica* serovar O:9 O-antigen cluster. Unique regions of the O-antigen clusters showed no sequence coverage, consistent with absence of the O:3 and O:8 O-antigen clusters.

Western blot evaluation

The 1:50 serum dilution resulted in an overload of antibody preventing clear interpretation of the blot results. There was excessive smearing observed at the bottom of the *Yersinia* antigen lanes and across other lanes on the blot. Multiple protein band reactivity against *B abortus* strain RB51 antigen was observed with the 1:50 serum dilutions and is normally not observed. This was attributed to non-specific binding due to the overload of antibody. A 1:200 serum dilution improved the ability to decipher banding patterns and reduce smearing and nonspecific binding (Figure 1). However, due to very high antibody levels to *Yersinia* the incubation time was kept to a minimum, with the reactivity resulting in heavy staining with moderate smearing between the 38 and 14 kDa molecular weight ranges in both the *Y enterocolitica* serovar O:8 and *Y enterocolitica* serovar O:9 antigen lanes. Immunoreactivity was observed against multiple protein bands in both the *Y enterocolitica* serovar O:8 and *Y enterocolitica* serovar O:9 antigen lanes with strong reactivity noted at bands of approximately 35, 28, 20, 12, and 5 kDa molecular weight. Moderate to strong immunoreactivity was also observed in both the *Y enterocolitica* serovar O:8 and *Y enterocolitica* serovar O:9 antigen lanes corresponding to molecular weights of approximately 98, 62, 60, 58, and 50 kDa. Moderate staining intensity accompanied by smearing was observed against multiple proteins of the *B abortus* strain 2308 antigen in ranges between 28 and 90 kDa, but of less intensity than observed against both the *Yersinia* antigen proteins.

Reactivity to a single protein band (approximately 38 kDa) within the *B abortus* strain RB51 antigen was consistently observed for all sow samples. Stronger immunoreactivity against both the *Y enterocolitica* serovar O:8 and *Y enterocolitica* serovar O:9 low and mid-molecular weight antigens in comparison to lower reactivity observed within the two *Brucella* antigen lanes were indicative of positive *Yersinia* antibody reactivity. In addition, comparing results obtained with a control blot using brucellosis positive bovine field samples, bovine positive control serum, and *Y enterocolitica* serovar O:8 and *Y enterocolitica* serovar O:9 control serum, there was a lack of strong reactivity of the sow serum to low to medium molecular weight proteins (3 to 28 kDa) against the *B abortus* strain 2308 antigen (Figure 2). The sow serum also resulted in a greater number of protein bands staining within both *Yersinia* antigen lanes as compared to results observed with the brucellosis control sera to the *Yersinia* antigen preparation. The reactivity of the sow sera to a single protein band in the *B abortus* strain RB51 antigen at approximately the 38 kDa molecular weight range was also consistent with that observed in the control blot using the *Y enterocolitica* serovar O:8 and *Y enterocolitica* serovar O:9 control serum.

As was noted in the control blot the *Brucella* control and field serum samples react with a higher molecular weight RB51 antigen at approximately 49 kDa. This higher molecular weight RB51 protein band was not visible from the swine sera tested on the immunoblot procedure. Strong reactivity to both *Yersinia* antigens, the lack of similar reactivity to the *B abortus* strain 2308 antigen, and specific reactivity to the *B abortus* strain RB51 antigen 38 kDa protein band indicated the sow sera contained high levels of *Yersinia* antibody.

After culture results became available on the euthanized sows, coupled with the western blot results and declining serologic titers, the herd received a partial quarantine release that enabled the herd to move cull sows direct to slaughter but not to buying stations and to receive replacement gilts. *Brucella* was not isolated from any milk or placental samples taken from farrowing sows between 88 and 104 days from the initial herd test (Table 3). *Yersinia* was not isolated from any of these samples (Table 3). Once culture results became available on milk and placenta samples, the herd received a full quarantine release.

Discussion

This report describes the difficulties associated with FPSRs for swine brucellosis. These FPSRs cause significant economic costs to both the producer and the state government due to time spent under quarantine, labor for follow-up testing, and costs associated with confirmatory diagnostic tests including serology and culture. This case reveals potential methods for dealing with this situation in the future. Serologic titers in this case report did decline over time and can be used as evidence for FPSRs as has previously been discussed.[5] However, this is not ideal as the herd must remain under quarantine during the waiting period between serial sampling. *Yersinia enterocolitica* was readily cultured from the tissues of sows with swine brucellosis titers, but this requires the sacrifice of productive females from the herd.

As *Y enterocolitica* has been isolated from bovine raw milk samples,[16,17] attempts were made to isolate the organism from some of the post-partum milk samples. Swabbing the tonsils of swine has been shown to be a possible method of isolating *Y enterocolitica* from carrier swine.[18,19] Reports of similar cases in species other than swine have cultured *Y enterocolitica* from the feces of infected animals.[20,21] However, these methods would not rule out the potential for dual infection with *Yersinia* and *Brucella,* and therefore would not be a suitable test for ruling out FPSRs. It should be noted that newer cell-mediated assays[11,12,22] have shown promise when used to rule out FPSRs, however, they were not utilized for this investigation.

The use of a developmental western blot assay in this investigation added to the evidence that the herd was not infected with *B suis.* However, interpretation is somewhat subjective and does not provide ample evidence by itself for a diagnosis of FPSR and subsequent quarantine release. Western

Figure 1: Western blot of 2 sow samples (sows 4539 and 4529) tested using 2 *Brucella* and 2 *Yersinia* antigen preparations. Lanes 1and 5: *Brucella abortus* strain 2308 antigen; Lanes 2 and 6: *B abortus* strain RB51 antigen; Lanes 3 and 7: *Yersinia enterocolitica* serotype O:8 antigen; Lanes 4 and 8: *Y enterocolitica* serotype O:9 antigen. Sow serum was diluted 1:200 in blot blocker. Note strong reactivity in *Yersinia* antigen lanes corresponding to approximate 35, 28, 20, 12, and 5 kD molecular weights. Moderate reactivity in *Yersinia* antigen lanes corresponding to approximate molecular weights ranging between 48 and 62 kD. A strong band of reactivity is noted in the *B abortus* RB51 antigen lane corresponding to an approximately 38 kD protein band.

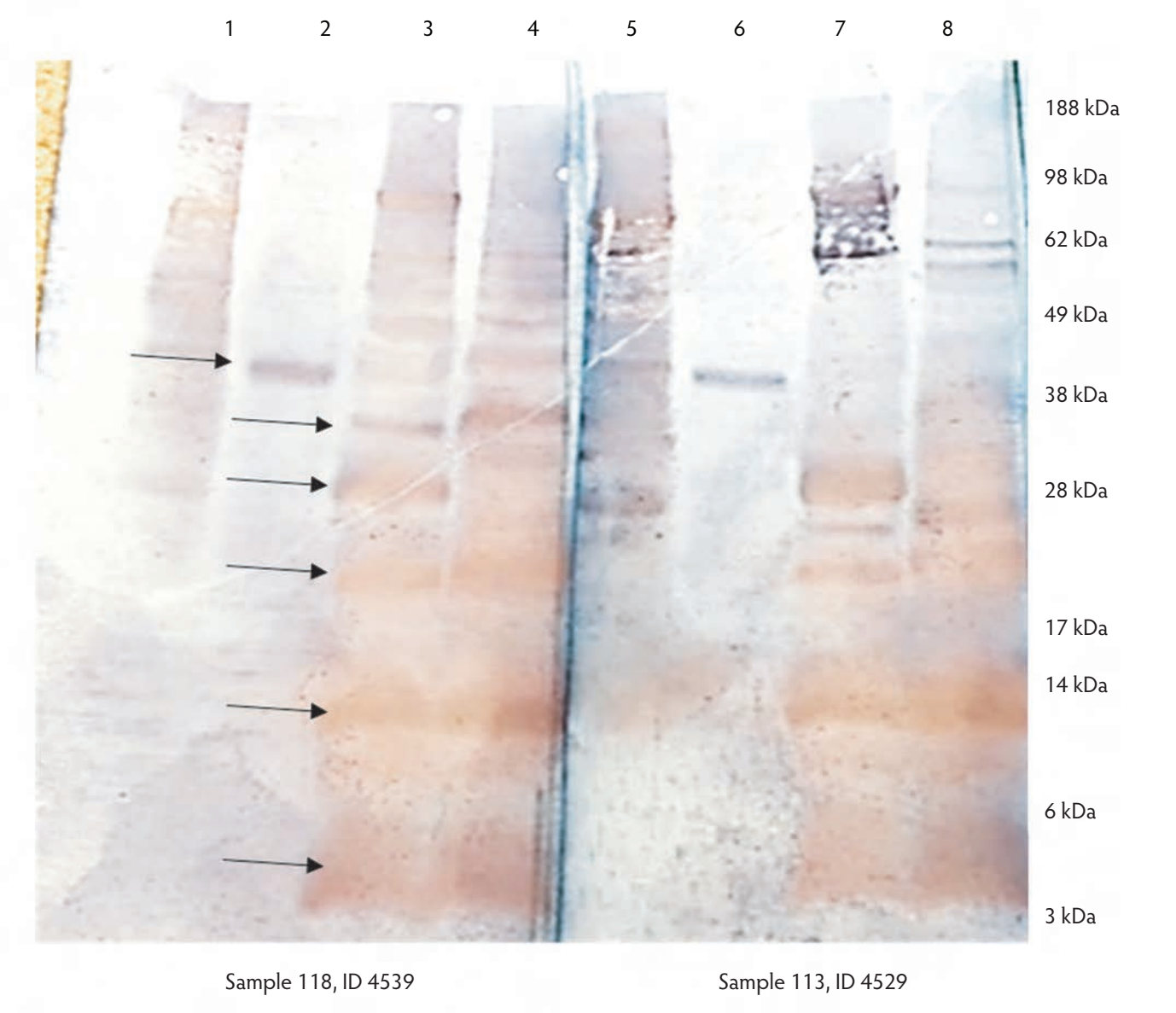
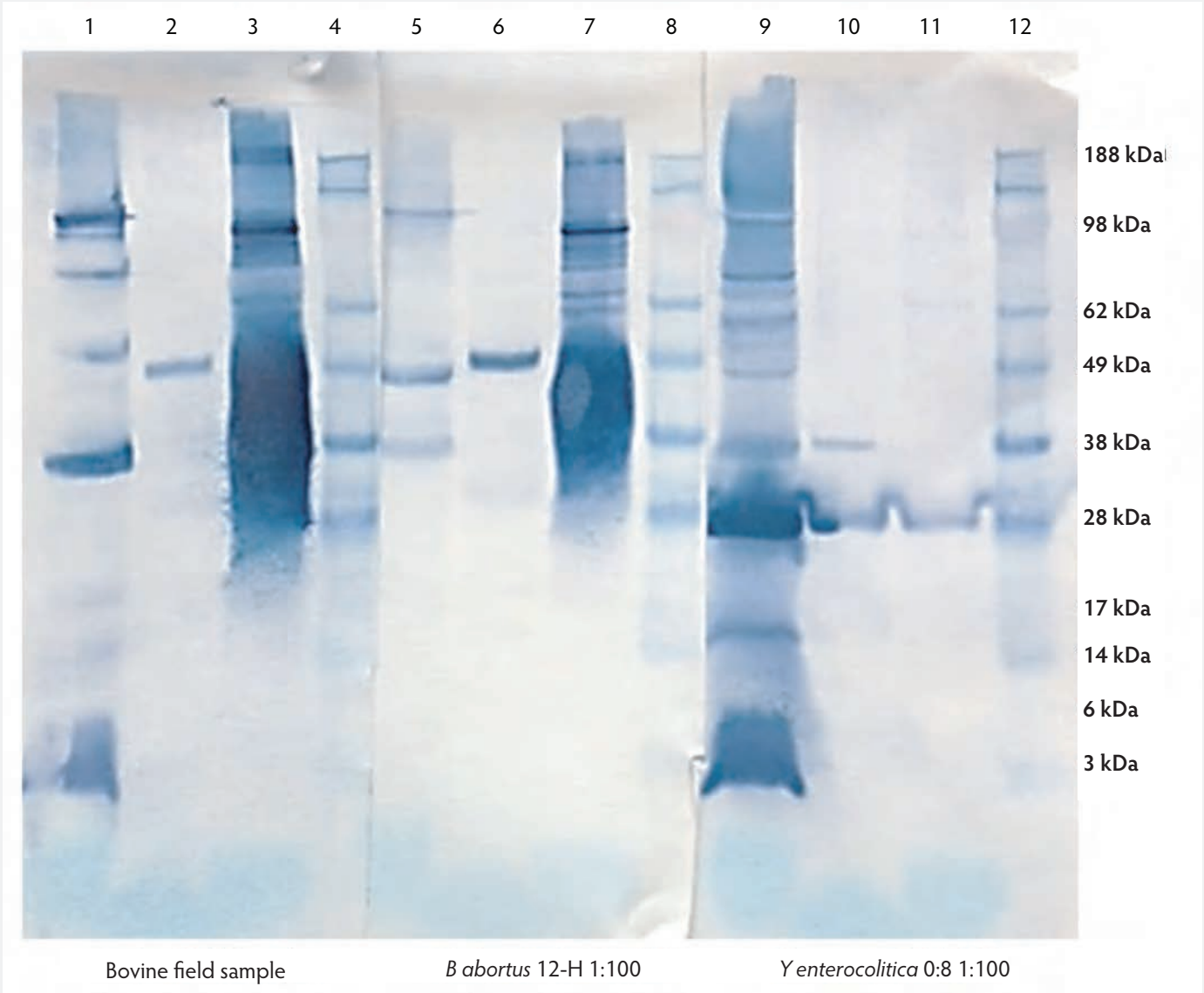


Figure 2: Western blot using control sera for evaluation of expected results for analysis. Lanes 1, 5, and 9: *Yersinia enterocolitica* serotype O:8 antigen; Lanes 2, 6, and 10: *Brucella abortus* strain RB51 antigen; Lanes 3, 7, and 11: *B abortus* strain 2308 antigen; Lanes 4, 8, and 12: pre-stained molecular weight marker. Control sera used were: Lanes 1-4: *B abortus* bovine field sample; Lanes 5-8: *B abortus* 12-H (high positive control serum); Lanes 9-12: *Yersinia enterocolitica* O:8 positive rabbit control serum. Serum was diluted 1:100 in blot blocker. Strong homologous reactivity was evident in lane 9 of the *Yersinia* control serum at the 38, 28, and 3-5 kD range. Specific reactivity of the *Yersinia* control serum was noted at the 38 kDa protein band to the *B abortus* RB51 antigen and moderate reaction at the 28 kDa range for both the *B abortus* RB51 and Strain 2308 antigens. Of significant interest was the distinction noted of the *B abortus* field sample and control serum reacting to the 49 kDa protein of the RB51 antigen. Multiple bands of reactivity to high molecular weight proteins visible against the homologous *Yersinia* control serum evident in Lane 9 that is not as prevalent in Lanes 1 and 5 with the *Brucella* control serum. Strong contrast of reactivity is noted in the 30-50 kDa range between Lanes 3, 7, and 11 with the varying control serum, indicating strong reactions of the *Brucella* positive serum and lack of reaction of the *Yersinia* control serum.



blot results from our study were consistent with previous studies [23,24] using *Brucella* positive bovine control serum resulting in intense protein band staining between 29 and 68 kDa against a smooth *Brucella* antigen preparation. In addition, during development of the western blot assay *Brucella* positive bovine field samples and *Brucella* positive bovine control serum were evaluated against *Y enterocolitica* serovar O:8 antigen. The brucellosis positive bovine samples had limited reactivity detected at approximately 38, 48, and 98 kDa against the *Y enterocolitica* O:8 antigen. These results are similar to a previous study[25] indicating little to no reaction of *B abortus* positive serum against *Y enterocolitica* O:8 LPS and proteins, whereas there was greater cross-reactivity against *Y enterocolitica* O:9 LPS and proteins. The use of *Y enterocolitica* O:8 control serum against the *Y enterocolitica* O:8 antigen during assay development consistently resulted in blot staining detected at locations corresponding to approximately 12 to 15, 20, 28, 35, 48, 62, and 98 kDa molecular weight proteins. These developmental test results indicated that positive brucellosis bovine serum samples would have limited reaction to the *Y enterocolitica* O:8 antigen. In contrast, cross-reactions of either *Yersinia* O:8 or O:9 antibody would be expected against both *Yersinia* antigens. Inclusion of the O:8 antigen in our study allowed us an additional component to decipher the level and characteristics of possible cross reactions if *Brucella* antibody was present. The high level of reactivity observed against both *Yersinia* antigens supported the presence of high level of *Yersinia* antibody. Additional supporting information for the lack of *Brucella* antibody was indicated by the lack of strong reactivity in the mid-molecular weight range (28-49 kDa) against the *B abortus* strain 2308 antigen as is observed with the use of positive control serum. In our study, an additional higher molecular weight band (approximately 98 kDa) was observed with the NVSL *B abortus* strain 2308 antigen not previously reported with immunoblot procedures.

Use of the *B abortus* strain RB51 antigen in this study provided information related to possible antibody reactions against core *Brucella* proteins. Results indicated specific differences between reactions of *Yersinia* antibody reacting at approximately 38 kDa versus *Brucella* antibody which indicated reactivity with a protein band at approximately 49 kDa. This variance may provide additional support in the future for differentiating *Yersinia* from *Brucella* immunological reactions in these situations.

One difficulty associated with use of western blot is unknown antibody titers that may be present in field samples. During antigen standardization trials this variable antibody titer of field samples continued to result in difficulties establishing antigen concentrations that would provide clear blot results and yet ensure adequate sensitivity. Decreasing protein concentrations of the *Brucella* antigens allowed better delineation of banding patterns from *Yersinia*-positive samples, but still results in variable smearing. Initially, higher concentrations of the *Yersinia* antigens proved useful for low titer brucellosis serum samples but does present continued difficulties when encountering *Yersinia* field samples containing high antibody titers. This may result in having to repeat immunoblot testing if serum samples were over- or under-diluted during initial testing and may add time onto the testing period. As further work proceeds with immunoblot procedures it may be possible to determine an initial serum dilution based upon a correlation with brucellosis serological results.

The amount of additional diagnostics performed in this investigation was extensive since the implications for the company and the state pork industry would have been immeasurable if the herd would have truly been infected with swine brucellosis. Therefore, the efforts were necessary to rule out swine brucellosis infection and to prevent unnecessary depopulation of the herd.

Implications

- Due to imperfect specificity, other diagnostics were used to rule out *B suis* infection.
- A joint effort was needed to determine herd status and relieve the burden of quarantine.
- Several diagnostic tools helped confirm FPSR for *B suis* and remove the herd quarantine.

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Conflict of interest

None reported.

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