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Comparison of phenotypic traits and genetic relatedness of *Salmonella enterica* subspecies *arizonae* isolates from a colony of ridgenose rattlesnakes with osteomyelitis

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Abstract. Reptiles are well-known sources of human *Salmonella* infections; however, little is known about the ability of *Salmonella* to cause disease in reptiles. Thirty-seven isolates of *Salmonella enterica* subspecies *arizonae* (*S. arizonae*) were obtained from retrospective and prospective studies of a closed colony of ridgenose rattlesnakes (*Crotalus willardi*) with osteomyelitis. All isolates ($N = 7$) from bone lesions were of a single serotype, 56:z4,z23, and this serotype was found on only 1 occasion among 8 other serotypes isolated from 21 cloacal and intestinal samples. The remainder ($N = 7$) of serotype 56:z4,z23 isolates were from other extraintestinal sites, including liver, ovary, blood, and testis. *S. arizonae* isolates were susceptible to most antimicrobials, and plasmid profiles did not correlate with serotype or antimicrobial resistance. Isolates of the 56:z4,z23 serotype ($N = 14$) formed a tight cluster with 95% similarity by *Xba*I macrorestriction analysis. Individual isolates of serotypes, 56:z4,z23, 38:(k)-z35, and 48:i-z invaded HeLa cells but an isolate of serotype 50:r-z did not. The same individual isolates of serotype 56:z4,z23 and 48:i-z also invaded viper heart cells. The *Salmonella Inv*A gene was detected by polymerase chain reaction (PCR) in all *S. arizonae* serotypes tested, including 5 serotype 56:z4,z23 isolates and individual isolates of serotypes 48:i-z and 50:r-z. A source or possible explanation for increased virulence of *S. arizonae* serotype 56:z4,z23 in this unique host has not been found.

Contact with reptiles is a well-known risk factor for *Salmonella* infections especially in children and immunosuppressed individuals.[6,23] More than 1,000 of the nearly 2,500 serotypes of *Salmonella* are isolated primarily from cold-blooded animals and the environment. *Salmonella* isolates from reptiles belong to subgroups II, IIIA, IIIB, IV, and V (*Salmonella enterica* subspecies *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *Salmonella bongori*, respectively).[5]

Although reptiles are known sources of *Salmonella*, very little is known about the ability of *Salmonella* to cause disease in reptiles. *Salmonella* has been isolated from diseased reptiles and is regarded as an opportunistic pathogen.[2,10,19,20] Whether some *Salmonella* isolates have a greater degree of adaptation to, or virulence for, particular species of cold-blooded animals is not known. Among reptiles, there have been a disproportionately greater number of *S. enterica* subspecies *arizonae* (henceforth referred to as *S. arizonae*) isolations reported from snakes when compared with terrapins or lizards.[11]

The association of a single serotype (56:z4,z23) of *S. arizonae* with osteomyelitis in a colony of ridgenose rattlesnakes was previously reported.[21] The snakes were individually housed in a single collection with little opportunity for direct contact. Some had been caught in the wild years earlier and some were born on site. The source of infection was not known. It was questioned whether this serotype was of increased virulence for ridgenose rattlesnakes and whether it came from a point source of contamination or represented exposure to genotypically diverse strains from multiple sources. The purpose of the present study was to characterize the *S. arizonae* isolates from these snakes with in vitro tests to further differentiate extraintestinal from intestinal isolates and assess virulence and genetic relatedness.

Salmonella arizonae isolates used in this study are listed in Table 1. Serotyping was performed at the National Veterinary Services Laboratory, Ames, IA. All but 1 isolate of serotype 56:z4,z23 were from extraintestinal sites. All but 1 extraintestinal isolate were serotype 56:z4,z23, and all *S. arizonae* isolates from bone lesions were serotype 56:z4,z23.

Antimicrobial susceptibility tests were performed using a fully automated microbroth dilution system[a] according to the manufacturer's instructions and interpreted according to the National Committee for Clinical Laboratory Standards guidelines for broth microdilution methods.[18]

Plasmid DNA was isolated using the lysis in solution method as previously described.[7] Plasmids were separated in 0.8% agarose by electrophoresis at 10 V/cm for 30 minutes and stained with ethidium bromide for visualization. Molecular weights were determined by comparison with a standard of supercoiled DNA size markers (plasmids <15 kb) and plasmids of known sizes isolated from *Escherichia coli* V517 (plasmids >15 kb).[17]

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Table 1. *Salmonella enterica* subspecies *arizonae* isolates collected from a colony of ridgenose rattlesnakes with osteomyelitis.

Isolate	Animal ID	Body site	Serotype	Isolate	Animal ID	Body site	Serotype
95-2589	311	ovary	56:z4,z23	00-4244	1414	cloaca	38:(k)-z35
95-2685	311	rib	56:z4,z23	00-4245	1463	cloaca	56:z4,z23
96-1129	1147	cloaca	48:i-z	00-4246	1350	cloaca	50:r-z
96-1130	1007	cloaca	50:r-z	00-4247	495	cloaca	61:i-z35
96-1131	368	cloaca	48:i-z	00-4248	498	cloaca	50:r-z
96-1132	1413	cloaca	38:(k)-z35	00-4274	906	cloaca	Rough O:k-z35
96-1133	1005	cloaca	38:(k)-z35	00-4275	911	cloaca	65:l,v-z
96-1134	1414	cloaca	38:(k)-z35	00-4276	910	cloaca	48:i-z
96-1135	911	cloaca	48:i-z	00-4277	908	cloaca	50:k-z
96-1136	1350	cloaca	50:r-z	00-4370	911	vertebra	56:z4,z23
00-1118	912	liver	56:z4,z23	00-4371	911	rib	56:z4,z23
00-1119	912	testes	48:i-z	00-4372	911	gonad	56:z4,z23
00-1120	912	rib	56:z4,z23	00-4373	911	rib	56:z4,z23
00-1121	912	cloaca	48:i-z	01-3416	1350	blood	56:z4,z23
00-1121†	912	cloaca	48:i-z	01-3423	1350	rib	56:z4,z23
00-4241	1413	cloaca	6,14:z10-z	01-3424	1350	liver	56:z4,z23
00-4241†	1413	cloaca	38:(k)-z35	01-3425	1350	testes	56:z4,z23
00-4242	1462	cloaca	38:(k)-z35	01-3426	1350	rib	56:z4,z23
00-4243	369	cloaca	38:(k)-z35				

* Knoxville Zoological Park, Knoxville, TN.

† Separate colony type from same specimen.

Cell invasion assays were performed using HeLa cells as previously described.[1] Briefly, the cells were exposed to bacteria at a multiplicity of infection of 100:1, incubated for 1 hour, washed 3 times, treated with gentamicin for 1 hour, washed 3 times and lysed. On the basis of preliminary experiments, a countable range of viable bacteria were best obtained by plating 0.01-ml samples from a 1:10 dilution of cell lysate. Four cell lysate replicates and 4 dilution sample replicates were used in each experiment. Two intestinal and 2 extraintestinal isolates representing different *S. arizonae* serotypes were tested. Field strains of *Salmonella* Typhimurium and *Salmonella* Choleraesuis were used as positive controls, and *E. coli* HB 101 was used as a negative control. The procedure was also performed using a viper heart cell line[b] with the modification that all incubations were performed at ambient room temperature.

The distribution of colony forming units (CFUs) was tested for normality by visualization and use of the Shapiro–Wilk statistic. Non-normally distributed data were converted to ranks and the total CFU for each experiment, cell lysate replicate, and plate count replicate within a bacterial isolate were compared using a mixed model procedure. In the model, CFU was the dependent variable, and bacterial isolate, interaction between experiment and bacterial isolate and replicate nested within experiment and bacterial isolate as the independent variables. Experiment was considered a random factor in the model. All statistical analyses were done using commercial software. A P value of 0.05 was used to identify statistical significance.

Macrorestriction profiles (MRPs) of each isolate were generated by pulsed-field gel electrophoresis (PFGE) as previously described.[9] One millimeter slices from each sample plug were digested with 10 U *Xba*I.

Table 2. Median and range of colony forming units (CFU) obtained in cell invasion assays for each bacterium over all experiments, and replicates.*

Bacterial isolate†	HeLa cell line	Viper heart cell line
Escherichia coli HB 101	0 (0-0)/16‡[a]	1.5 (0-12)/48[a]
Salmonella arizonae 38:(k)-z35	11 (0-34)/64[b]	not determined
Salmonella arizonae 48:i-z	16 (0-51)/64[c]	359 (219-518)/48[b]
Salmonella arizonae 50:r-z	0 (0-5)/64[a]	not determined
Salmonella arizonae 56:z4,z23	32 (3-255)/64[d]	143 (21-334)/48[c]
Salmonella Choleraesuis	8 (2-16)/12[be]	51 (43-70)/16[d]
Salmonella Typhimurium	5 (1-10)/16[e]	8 (5-26)/32[e]

* Number of CFU with different letter in the same column are significantly different ($P < 0.05$).

† A single bacterial strain was used in all experiments. *Salmonella arizonae* O groups 38, 48, 50 and 56 were represented by isolates 00-4243, 00-1119, 00-1136, and 00-1118, respectively.

‡ Median (range)/number of observations (total number of observations from all plate count, cell lysate and experiment replications).

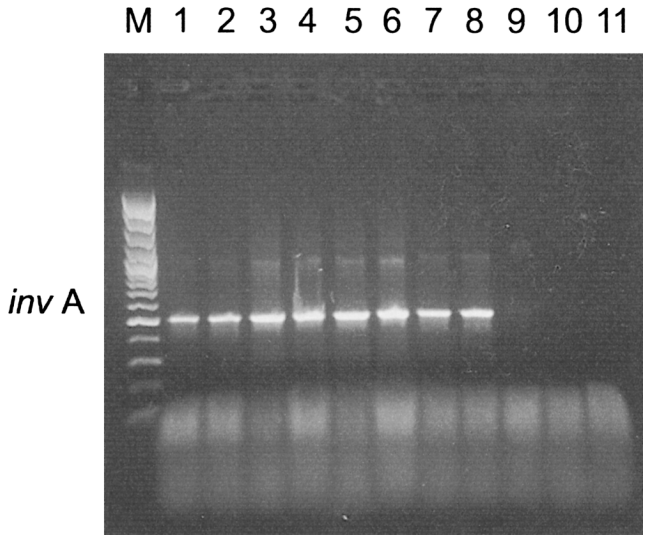

Figure 1. Amplification of a 521-bp *inv*A sequence in *Salmonella arizonae* isolates. Polymerase chain reaction products separated in 1.5% agarose and stained with ethidium bromide for visualization. Lane M = 100-bp ladder; lane 1 = 00-1118; lane 2 = 00-1119; lane 3 = 95-2589; lane 4 = 95-2685; lane 5 = 00-4245; lane 6 = 00-4373; lane 7 = 96-1130; lane 8 = *Salmonella* Typhimurium DT104 (positive control); lane 9 = *Escherichia coli* pcd[3] (negative control); lane 10 = *E. coli* pcd5 (negative control); lane 11 = *E. coli* pcd2 (negative control). Location of the *inv*A DNA fragment on the gel is indicated with an arrow.

Fragments were separated in 1.0% agarose[c] using an electrophoresis apparatus[d] with a linearly ramped pulse time of 2.16–35.07 seconds (6.0 V/cm gradient), at an angle of 120°, with a total run time of 14 hours at 14 C. A standard reference strain of *Salmonella* Newport (strain AM01144, kindly provided by Barbara Gillespie, University of Tennessee) was included in multiple locations on each gel. Gels were stained with ethidium bromide for visualization. Relatedness of MRP band patterns was determined by generating unweighted pair group method with arithmetic averages clusters based on the Dice coefficients of each band pattern.[d]

Detection of a 521-bp *Salmonella inv*A gene sequence in selected *S. arizonae* isolates was performed by polymerase chain reaction (PCR). DNA was prepared by boiling cultures diluted 1:1 with 0.2% Triton X-100 for 5 minutes.[3] Reactions were conducted in a 50 μl total volume containing 50 ρmol of each primer[12] (f = ttgttacggctattttgacca; r = ctgactgctaccttgctgatg), 10 μl reaction buffer (12.5 mM $MgCl_2$), 5 μl deoxynucleoside triphosphate (dNTP) solution (2.5 mM each dNTP), 32 μl H_2O, and 1 μl DNA solution. Polymerase chain reaction products along with standard weight markers were separated in 1.5 agarose by electrophoresis at 17 V/cm for 1 hour and stained with ethidium bromide for visualization.

All isolates were susceptible to the antimicrobials tested except for 3 serotype 48:i-z isolates that had intermediate susceptibility to enrofloxacin (MIC = 1 μg/ml). These isolates (2 from an antemortem cloacal swab and 1 from testicular tissue taken at necropsy) were from a single snake. Estimated MIC_{90} values for the *S. arizonae* isolates were amikacin, ≤2; amoxacillin/clavulanic acid, ≤8; ampicillin, ≤0.25; carbenicillin, ≤2; ceftazidime, ≤8; ceftiofur, ≤1; cephalothin, ≤2; chloramphenicol, ≤4; ciprofloxacin, ≤0.5; enrofloxacin, ≤0.25; gentamicin, ≤0.5; nitrofurantoin, ≤32; piperacillin, ≤8; tetracycline, ≤1; ticarcillin, ≤16; tobramycin, ≤0.5; trimethoprim/sulfamethoxazole, ≤10. Slight variation was observed in MIC values for chloramphenicol. Two isolates (14%) of serotype 56:Z4-Z23 and 11 isolates (50%) of other serotypes had MIC values equal to or greater than the MIC_{90} for chloramphenicol. Host-specific standards for performance and interpretation of antimicrobial susceptibility tests on *S. arizonae* isolates from rattlesnakes are lacking; therefore, results of these tests may not be suitable for making therapeutic choices. Human *Salmonella* infections often fail to respond clinically to treatment with first- or second-generation cephalosporins or aminoglycosides, despite the isolates appearing susceptible in vitro.[18]

Plasmids were detected in 21 of 32 isolates examined. Twelve different plasmid profiles were recognized, with plasmid sizes ranging from 2 to 77 kb. Up to 5 plasmids were seen in a single isolate. There was no apparent correlation between possession of plasmids, serotype, and antimicrobial resistance profiles.

Three of 4 *S. arizonae* isolates, including serotype, 56:z4,z23, invaded HeLa cells as well or better than a wild-type *S.* Typhimurium isolate (Table 2). One intestinal isolate of serotype 50:r-z and the *E. coli* strain used as a negative control did not invade HeLa cells. Although it is possible that some of the cell-associated bacteria observed in this assay were externally attached, isolates had similar growth rates and were equally susceptible to gentamicin, as determined in preliminary experiments, suggesting that internalization had occurred.

It is not known if HeLa cell invasion is related to extraintestinal spread of *S. arizonae* in the snake species *Crotalus willardi.* Live animals and fresh tissues from this rare venomous species are not readily available for study. There are few established cell lines from snakes, none from this species, and none from tissues (e.g., intestinal epithelial or macrophage derived) that are suitable to study extraintestinal bacterial spread. The extraintestinal *S. arizonae* isolate of serotype 56:z4,z23 and an intestinal isolate of serotype 48:i-z also invaded viper heart cells. Although it may be an artifact of the assay procedure, both isolates and the positive *S.* Typhimurium control, had up to 5 times

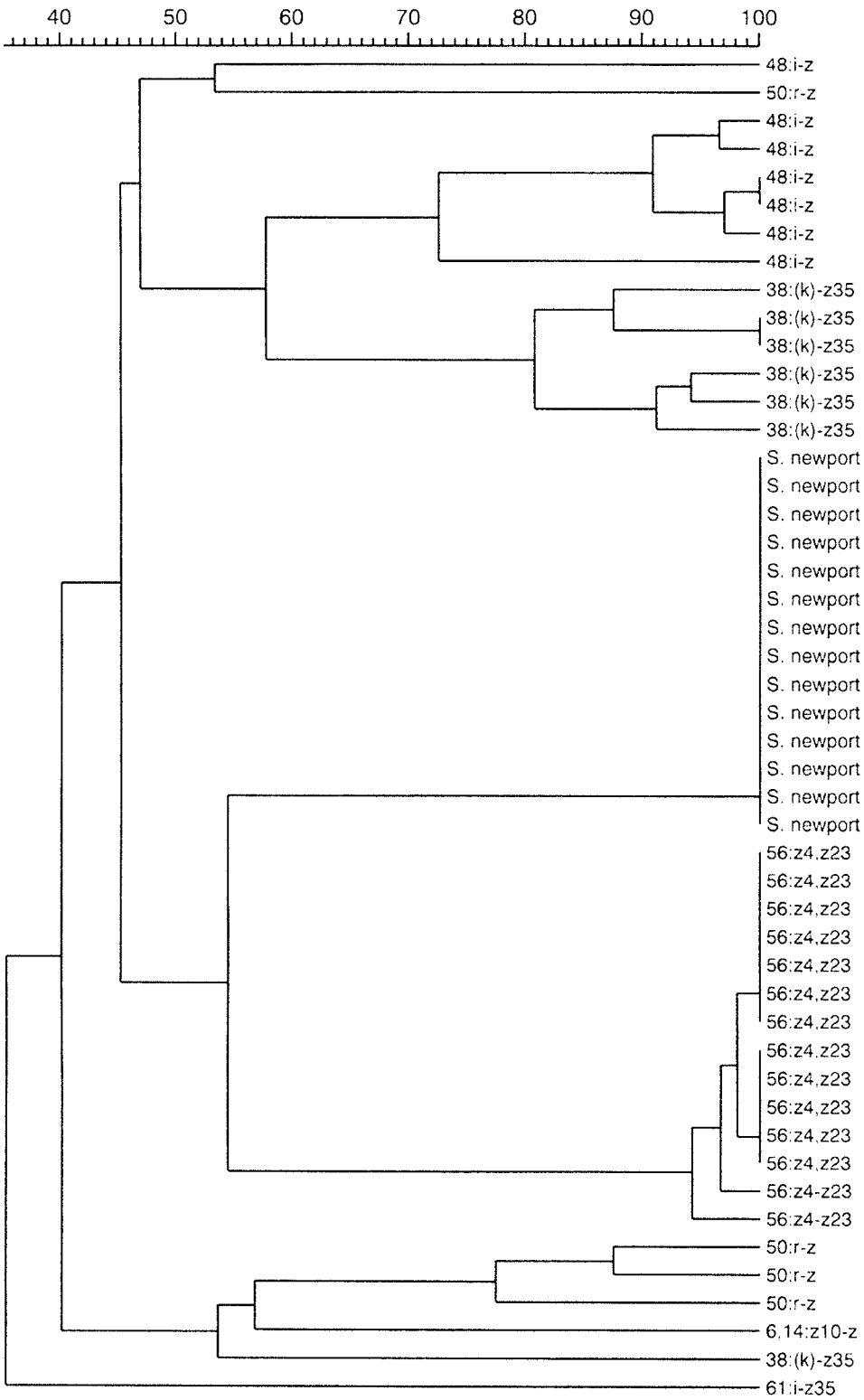

Figure 2. Relatedness of pulsed-field gel electrophoresis profiles of *S. arizonae* isolates from ridgenose rattlesnakes. Similarity scale (percent) is indicated at the top of the dendrogram. *Salmonella arizonae* serotype and *S.* Newport control strain are designated in the column to the right.

more bacteria associated with viper heart cells than had been observed with HeLa cells.

As has been reported for a few other isolates in the literature,[4,8] the *Salmonella inv*A gene was present in all *S. arizonae* isolates examined (Fig. 1). A search for other genetic markers that may be associated with invasion and intracellular survival in serotype 56:z4,z23 has not been performed.

Relatedness of 34 *S. arizonae* isolates from 16 snakes, based on PFGE MRPs, is illustrated by the dendrogram in Fig. 2. Isolates of serotype 56:z4,z23 formed a cluster at 95% similarity that was distinctly different from all other *Salmonella*. *Salmonella arizonae* serotypes 38:(k)-z35 and 48:i-z also showed a tendency to cluster but had 1 or more isolates with dissimilar MRPs. Three isolates (00-4274, 00-4275, and 00-4277) repeatedly failed to yield macrorestriction fragments presumably due to ineffective DNA extraction or degradation during the procedure. Recent technical improvements in the PFGE method may overcome the problem with these isolates.[13,16] Correlations between PFGE macrorestriction patterns and serotypes have been reported for several *Salmonella* spp. implicated in human salmonellosis.[14] However, PFGE has also been useful for distinguishing strains within a variety of different *Salmonella* serotypes.[15,22] Although 3 of the snakes that these isolates were obtained from were wild-caught, they had been in the present collection for more than 5 years and had limited opportunity for contact with each other or with other snakes in the collection. The native habitat and breeding range (mountainous desert regions of southwestern United States and northern Mexico) of this snake species is also quite limited. It is tempting to speculate that limited diversity among isolates of the 56:z4,z23 serotype could reflect a special host–parasite relationship. Additional isolates and analytical procedures must be performed before conclusions can be drawn regarding diversity within the *S. arizonae* 56:z4,z23 serotype.

This is an infrequently encountered *Salmonella* serotype. Review of a database[e] representing more than 200,000 *Salmonella* isolates from animals revealed a history of only 3 other isolates of serotype 56:z4,z23.[21] Several snakes remaining in the present collection have bone lesions that have yet to be cultured. Additional isolates from bone lesions and of this serotype are being sought for further comparisons. This study confirms that isolates of *S. arizonae* serotype 56:z4,z23 have the potential to invade cells and supports further study to determine if this serotype has other extraintestinal survival advantages in *C. willardi.*

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Sources and manufacturers

a. Vitek AMS with GNS-VB susceptibility card, bioMerieux, St. Louis, MO.
b. VH2, ATCC no. CCL-140, American Type Culture Collection, Rockville, MD.
c. SeaKem Gold, FMC BioProducts, Rockland, ME.
d. CHEF Mapper System and Molecular Analyst Software, Bio-Rad Laboratories, Hercules, CA.
e. National Veterinary Services Laboratory, Ames, IA.

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Clinical coccidiosis in a boar stud

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Abstract. This report describes an outbreak of coccidiosis in a boar stud. A live, untreated, adult boar with a history of diarrhea was submitted to the Iowa State University Veterinary Diagnostic Laboratory, Ames, IA. For a 3-month period, approximately 40% of the boars in this stud had developed gray to brown diarrhea that lasted 1–3 days. Affected boars did not lose condition, and antibiotic therapy did not appear to affect the clinical course of the disease. At necropsy, the distal ileum was palpably thickened and covered by a thick, yellow-green, fibrinous exudate. Microscopic changes in the ileum consisted of an erosive enteritis associated with the presence of numerous coccidia within mid to superficial villus enterocytes. The mucosa was covered by a fibrinous exudate admixed with numerous nonsporulated coccidian oocysts. A light growth of *Salmonella enterica* serovar Derby was isolated from the small intestine of this animal, but laboratory tests were negative for *Lawsonia* and *Brachyspira* spp. Individual or paired fecal samples were obtained from 6 additional boars experiencing similar clinical signs. Numerous *Eimeria spinosa* oocysts were identified in these samples. Neither *Salmonella* nor *Brachyspira* spp. were cultured from submitted fecal samples. Necropsy of a live boar and examination of feces from 6 additional animals confirmed that the mild, sporadic, transient diarrhea in this boar stud was due to coccidiosis.

The principal agents identified in adult swine with diarrhea include *Lawsonia intracellularis, Salmonella* spp., *Brachyspira* spp., and transmissible gastroenteritis.[5] Coccidiosis is primarily a disease of suckling and recently weaned pigs.[5,6] Reports of clinical coccidiosis in grow/finish and adult swine are rare.[2] This report describes an outbreak of clinical coccidiosis in a boar stud due to *Elimeria spinosa.*

A live, untreated, adult boar was submitted to the Iowa State University Veterinary Diagnostic Laboratory, Ames, IA (ISU VDL), from a boar stud experiencing an ongoing problem with diarrhea. Clinical signs were initially observed in 3% of the boars. For a 3-month period, approximately 40% of the boars developed gray to brown diarrhea that lasted 1–3 days. Affected boars did not lose condition. Treatment with 400 g/ton chlortetracycline or 100 g/ton of tylosin in the feed did not appear to affect the clinical course of disease. This boar was reported to be exhibiting clinical signs typical of the herd problem and was available, in part, because of poor semen quality.

The animal was from a 250-boar stud. Boars were housed in large gestation crates, arranged in 6 rows in a single barn. Crates were on slatted floors with a single common water trough per row located at the end of each crate. The lip of the trough was even with the surface of the slatted flooring and walkway. The boars had access to a clean collection pen 1–2 times per week. The entire barn was washed once per week with water and the aisles were scraped and swept daily. New boars were introduced every 60 to 90 days.

At necropsy the boar was in good flesh. The wall of approximately 70–80 centimeters of the distal ileum

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