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Investigation of the spread of *Brucella canis* via the U.S. interstate dog trade

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Summary
Objectives: The aim of this study was to illustrate and help address a growing need for regulatory or molecular tools to track and control the spread of canine brucellosis. Our study objectives were to first characterize *Brucella canis* outbreaks in Wisconsin kennels in the context of the dog trade in the USA, and then to identify a molecular technique that may be useful for strain differentiation of *B. canis* isolates.
Methods: Wisconsin Veterinary Diagnostic Laboratory (WVDL) *B. canis* serology data from 1995 to 2005 were reviewed, three canine brucellosis outbreaks in Wisconsin dog kennels were investigated, and eight *B. canis* isolates recovered from Wisconsin outbreaks and kennels in Missouri and Arkansas and four isolates received from outside sources were subjected to ribotyping, pulsed-field gel electrophoresis (PFGE), outer membrane protein analysis (OMPA), and cellular fatty acid profiling (CFAP).
Results: WVDL has received increasing numbers of *B. canis* positive samples from Wisconsin kennels, and Wisconsin outbreaks are associated with the interstate dog trade. All of the *B. canis* isolates we examined were genetically homogenous and as such could not be differentiated by ribotyping, PFGE and OMPA. However, dendrogram analysis of CFAP divided the isolates into two groups, indicating that CFAP methyl ester analysis has discriminatory power.
Conclusions: CFAP methyl ester analysis has promise as a tool for epidemiological tracing of *B. canis* outbreaks and will be useful in comparison studies as isolation of *B. canis* continues to expand globally.

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Introduction

Brucella canis is a bacterial disease of dogs that is orally and sexually transmitted. The disease causes epididymitis and orchitis in male dogs, endometritis, placentitis and abortions in females, and often presents as infertility in both sexes. Non-reproductive lesions are less common and include inflammation in eyes and axial and appendicular skeleton, lymphadenopathy and splenomegaly.[1–5]

Since it was first isolated in 1966,[1] *B. canis* has commonly been reported in Central and South America, has been identified in breeding kennels in China and Japan, and has become a major source of economic loss in large and small dog breeding facilities in the USA. A single outbreak occurring in a large breeding facility can lead to the euthanasia of hundreds of dogs. Clear identification of outbreak sources is rarely achieved because of poorly documented sales and breeding practices, absence of regulated testing for *B. canis*, and a delay in clinical evidence of infection.

Human infection with *B. canis* is generally considered uncommon and mild,[2,4,5] but reports have suggested that inappropriate serologic testing for the disease in humans has led to misconceptions concerning prevalence.[6–8] Human cases have occurred through exposure to infected dogs, most often aborting bitches,[3] accidental laboratory infections,[9] and by unknown sources, and have been described with a wide range of clinical presentations and disease severity.[7,8,10]

The annual transport and exchange of hundreds of thousands of dogs between states without regulated testing for *B. canis* has resulted in significant losses to the dog industry and has increased human exposure to this potentially zoonotic pathogen. Our purpose is to identify a molecular method for use in backward source tracing, which may encourage regulation and accountability, thus helping the dog industry to curb the spread of canine brucellosis.

Materials and methods

Epidemiologic data and pathology

Canine brucellosis outbreaks in three Wisconsin dog kennels (A, B and C) were examined. Owners and staff, veterinarians, and animal health and welfare regulatory personnel were interviewed for information regarding breeding facilities, canine purchase, breeding and sales practices, and *B. canis* control efforts in Wisconsin. Diagnoses of dogs from A and B kennel outbreaks were made by necropsy, tissue culture and histopathology of aborted fetuses. Necropsy with bacterial culture from tissues was used to confirm *B. canis* in adult dogs in kennel C.

Serologic and bacteriological analysis

Serum samples from the three outbreaks, as well as serologic data referenced from Wisconsin Veterinary Diagnostic Laboratory (WVDL) archives from 1995 to 2005 were reviewed. Serum samples were screened for *B. canis* using a rapid slide agglutination test (RSAT). Positive samples were then subjected to a 2-mercaptoethanol RSAT, provided with the same test kit (canine brucellosis antibody test kit; Synbiotics Corporation, San Diego, CA, USA). Samples that remained positive after this modified procedure were considered positive for *B. canis*.

Blood and tissue bacterial culture was used for confirmation of seropositive kennels and individual dogs. The bacteria were cultured aerobically and the identity confirmed by standard biochemical tests.

Molecular methods

Ribotyping using the restriction endonucleases PvuII, EcoRI, SspI, and PstI and outer membrane protein analysis (OMPA) were performed using previously published protocols.[11,12] Pulsed-field gel electrophoresis (PFGE) using the restriction endonucleases PvuII and XbaI was performed according to the following protocol. Bacteria were grown aerobically in 5 ml of tryptic soy broth (BBL) at 37 °C. Three hundred microliters of each sample was transferred into a sterile microfuge tube and the cells were harvested by centrifugation at 11 000 rpm for 2 min. Cells were then resuspended in 200 μl of TE buffer (10 mM Tris-HCl, 10 mM EDTA, pH 7.4) followed by the addition of 200 μl of 1.2% low melting temperature agarose. The suspension was immediately transferred into disposable plug mold and allowed to solidify. Plugs were incubated in TE buffer containing 5 mg/ml lysozyme for 3 h at 37 °C. Cells were then treated overnight at 50 °C with the same volume of solution containing 100 μg proteinase K/ml and washed three times with 1 ml of cold TE buffer per wash. Restriction endonucleases were used according to the manufacturer's instructions (Promega). The fragments were resolved by PFGE within electrophoresis grade agarose (1.1%) by using a CHEF-DR III system (Bio-Rad). The following parameters were used: running time, 18.5 h; gradient, 63 milliamps; included angle, 120 °C; initial switch time, 1.0 s; final switch time, 12.0 s; final shape, non-linear; ramp v/cm, 6. The gels were stained with ethidium bromide, destained in distilled water, and photographed under UV light. A lambda marker (Promega G3011) was used as the molecular weight standard.

Gas chromatographic and dendrogram analyses on whole cell fatty acid methyl esters (cellular fatty acid profiling, CFAP) using Sherlock MIS Software were performed at the MIDI facility (Microbial ID, Inc., Newark, DE, USA) using previously published protocols.[13]

Results

Interviews revealed a regular, brokered trade route for the purchase and sale of breeding dogs between Wisconsin and Missouri (WI–MO route) in which health certificates are obtained and *B. canis* testing is not performed. All three of the Wisconsin outbreaks followed purchase of new breeding dogs through this route.

From 1995 to 1996 a total of 510 serum samples were tested for *B. canis* at WVDL and 10 (1.96%) were identified as positive. The number of requests for *B. canis* serology at the WVDL dropped sharply from 1996 to 1997, but has steadily risen since then. From 2003 to 2004 a total of 174 samples were tested and eight (4.60%) were positive. The outbreak at kennel C resulted in a large increase in submissions to 317 in the first three months of 2005, with 85 (26.80%) positive samples.

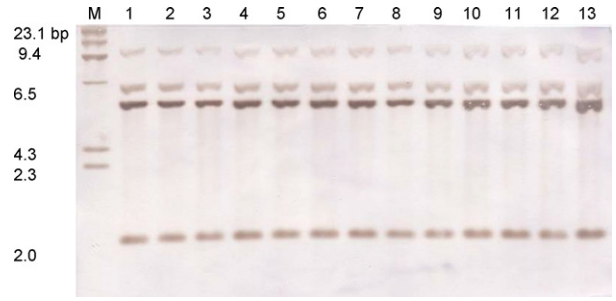

Figure 1 Example of *Brucella canis* isolate ribotyping using SspI. See details in Table 1.

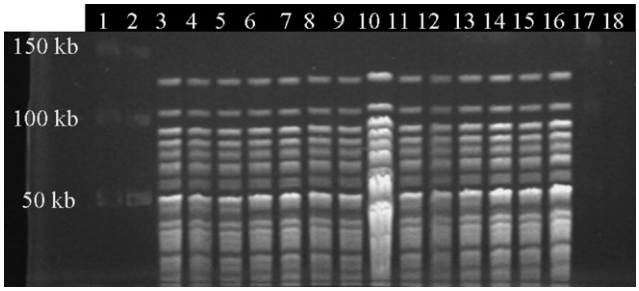

Figure 2 Example of *Brucella canis* isolate PFGE using PvuII. See details in Table 1.

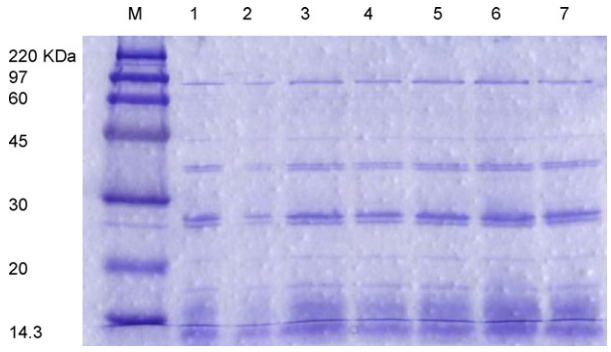

Figure 3 Example of *Brucella canis* isolate outer membrane protein profile. See details in Table 1.

Ribotyping results using SspI (Figure 1), EcoRI, PvuII and PstI (results not shown) as restriction endonucleases revealed that all of the isolates detailed in Table 1 shared genetic identity. Results from PFGE (Figure 2) and OMPA (Figure 3) showed the same homologous results between the isolates.

Only when CFAP was employed were variations between the isolates identified. Dendrogram analysis using Sherlock software showed two distinct strains, identified as Northern and Southern (Figure 4). All of the Missouri and Arkansas isolates were Southern strain. The isolates from the Splitter

Table 1 *Brucella canis* isolates and their sources

Isolate ID	Lane	Number of samples and origin	Location
1. 918270	1—3	3; Abdominal swabs	Kennel B — WI
2. 866568	4	1; Fetal lung and intestine	Kennel A — WI
3. M05-39757	5—8	4; Uterus and placenta	Kennel C — WI
4. M05-46984	9—11	3; Blood	MO — 1
5. M05-49529	12	1; Blood	AK
6. M05-55455	13	1; Blood	MO-2
7. 870018	14	1; Mixed fetal tissues	WI
8. 57	15	1; Unknown	Splitter lab — CDC
9. 3192	16	1; Unknown	Splitter lab — isolate — 1
10. H966	17	1; Unknown	Splitter lab — isolate — 2
11. Hog	18	1; Unknown	Splitter lab — isolate — 3

WI, Wisconsin; MO, Missouri; AK, Arkansas; CDC, Centers for Disease Control and Prevention.

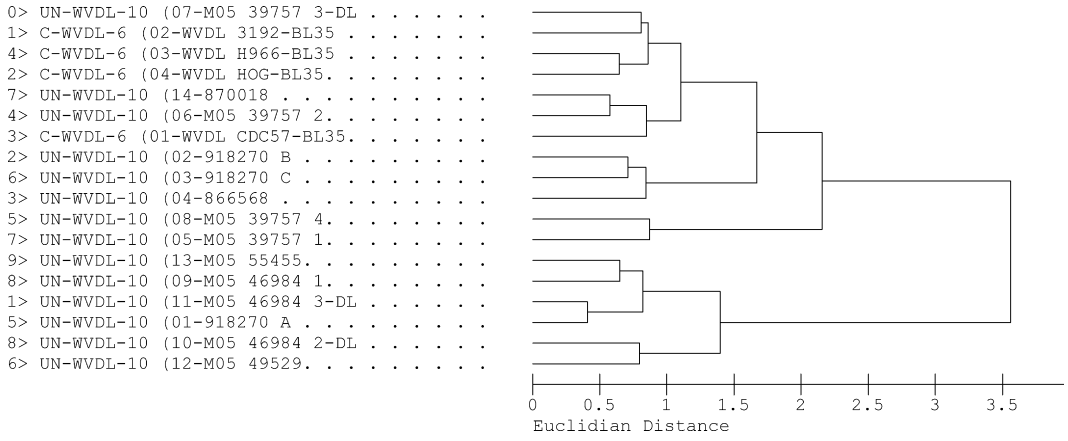

Figure 4 Dendrogram analysis of fatty acid profiles for *Brucella canis* isolates 1—11. See details in Table 1.

laboratory collection, Wisconsin kennels A and C, and the additional Wisconsin case (870018), were Northern strain. Both the Northern and Southern strains were identified in the same brucellosis outbreak at Wisconsin kennel B.

Discussion

In the USA there is a large and growing dog industry that exists with minimal regulation. Profitability is based on high volume breeding and the subsequent sale of puppies, stud dogs, and breeding bitches. Canine brucellosis is an infectious, venereal transmitted bacterial disease perfectly adapted to the production goals of the dog industry. The common practices of using outside dogs for breeding, and frequently buying, selling and trading breeding dogs without testing for *B. canis* have propagated this disease. The interstate purchase of dogs through the WI—MO route was temporally linked to the three Wisconsin kennel outbreaks that we investigated, and WVDL submissions suggest that *B. canis* is on the rise in Wisconsin dog kennels. Similar reports of increasing serology submissions and positive cases in other states indicate the disease is pervasive in dog kennels in the USA.[14,15]

Although canine brucellosis is a reportable disease, accurate state-by-state serologic data are not available. This is due in part to serologic testing being conducted in out-of-state diagnostic laboratories or in-house by practitioners who may not report positive cases to state agencies.

Though federal dog kennel licensure is controlled by the United States Department of Agriculture (USDA), the dog industry is dissociated from agriculture, where an effective *Brucella abortus* eradication program for cattle has been in place in the USA since 1954.[16] Because of the absence of regulated *B. canis* testing, the highly infectious nature of *B. canis*, and an industry that is willing to tolerate infection, we do not anticipate that this disease will diminish in the canine population without intervention similar to that employed in the cattle industry. Regulation would be dependent on state-to-state enforcement of kennel licensing to designate dog breeding facility locations. Once facilities are identified, required testing for *B. canis* prior to the sale and interstate transport of dogs could be implemented.

The propagation of *B. canis* not only impacts the health and welfare of dogs, but is also a public heath concern.[3,6—8,10,17] Common clinical symptoms in humans infected with *B. canis* include fever and headaches, often accompanied by lymphadenopathy.[3,8] Accurate serologic data on human exposure to *B. canis* is lacking because serologic screening in humans is generally directed against *B. abortus*, which does not cross-react with *B. canis*.[6—8,17] Further, it is unknown how many subclinical and mild human infections resolve without diagnostic investigation.

Published serologic surveys of *B. canis* in the USA have looked at pound dogs, animal shelter workers, veterinarians,[18] and military recruits,[19] and there are reports that include serologic data from newborn infants, blood donors, hospital employees and hospitalized patients.[6] These studies suggest a relatively low prevalence when compared to reports from Mexico and Argentina,[6,7,20] but they have not included the populations most at risk in the USA, which are those closely tied to the dog industry. These populations include breeding kennel owners and employees, and those dogs specifically used for breeding. This select group is repeatedly exposed to highly infectious materials when *B. canis* outbreaks occur.

Difficulties in species differentiation due to limited genetic diversity in the genus *Brucella* are well documented.[21,22] It is therefore not surprising that the commonly used molecular-based methods for bacterial strain differentiation, including ribotyping, PFGE and OMPA[21,23—26], failed to discriminate *B. canis* isolates. Using CFAP we were able to discriminate our isolates into Northern and Southern strains, and as we expand our database with wider geographic sampling we expect we will find additional regional isolate types. Both the Northern and Southern strains were identified in the brucellosis outbreak at Wisconsin kennel B following purchase of infected dogs from Missouri. This finding suggests that the Missouri dogs were the source of the Southern strain in this kennel. However, additional data necessary to document the introduction of the Southern strain, such as analysis of isolates from kennel B prior to the Missouri dog purchases, were not available. In the absence of regulated *B. canis* testing, subjecting isolates to CFAP with dendrogram analysis when outbreaks occur, and maintaining kennel isolate records may prove to be valuable tools in canine brucellosis outbreak investigations.

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Conflict of interest: No conflict of interest to declare.

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