

## AN OUTBREAK OF BESNOITIOSIS IN MINIATURE DONKEYS

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**ABSTRACT:** Fourteen miniature donkeys (*Equus asinus*) in a mid-Michigan herd of 38 animals presented with clinical signs of besnoitiosis, including the presence of typical tissue cysts in the ocular sclera, the buccal and nasal mucosa, together with characteristic dermatitis in specific areas of the body. The common histopathological change seen was the presence of many 100–200- $\mu$ m diameter, thick walled, typical *Besnoitia* sp. tissue cysts together with a chronic cellular response associated with degenerating cysts. Microscopy of isolated scleral cysts and skin biopsies showed the presence of protozoal organisms consistent in morphology with that of *Besnoitia bennetti* bradyzoites. Molecular analysis of these parasites indicates that they differ from previously described coccidia, including *Besnoitia* sp., from rabbits and opossums. Isolated cases of infection with this agent have been reported infrequently in equids; however, this is the first report of an outbreak in a herd of donkeys in the United States.

*Besnoitia* sp. is a tissue-cyst-forming coccidian parasite belonging to Sarcocystidae, Toxoplasmatinae. The disease it causes, besnoitiosis, is recorded worldwide in different animal species and has considerable economic importance in subtropical regions of Africa and Asia. It is very much less common in nontropical regions of Asia, Europe, and the Americas, including the United States (Mayhew and Greiner, 1986; Bigalke and Prozesky, 1994; Yager and Scott, 1994; Dubey et al., 2002). The definitive host for many of these parasites is presumed to be a member of the cat family and the intermediate hosts vary with each of the parasite species. Cattle (Njagi et al., 1998), sheep, and goats (Oryan and Sadeghi, 1997) are all reported as important intermediate hosts, with clinically less significant infections described as occurring in wildebeest, impala, zebra, horses, donkeys, reindeer, mules, and opossums (Stabler and Welch, 1961; Bigalke, 1968, 1994; Bigalke and Prozesky, 1994; Ayroud et al., 1995; Dubey et al., 2002).

Besnoitiosis in donkeys was first described in Sudan early last century (Bennett, 1927, 1933) and has been noted infrequently in the United States (Smith et al., 1966; Terrell and Stookey, 1973; Wendell et al., 1997). It has also been described in horses in Africa (Schultz and Thornburn, 1955; Bigalke, 1970; Van Heerden et al., 1993). The present report describes a cluster of miniature donkeys in Michigan that are naturally infected with this parasite and exhibited obvious clinical disease.

### MATERIALS AND METHODS

#### Animals

The animals in the herd (N = 38) studied were all kept on a small peri-urban farm in mid-Michigan. Most donkeys were from 1 to 8 yr

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of age. Only a few animals were over 8 yr of age, with the oldest a 27 yr old. Twenty of the herd had been born on the farm in question and the remainder came originally from herds either in Michigan or surrounding states. Most of the affected animals were actually born on the farm in question, with only 2 coming originally from other farms; all were females.

The donkeys were kept in relatively high-density housing in 4 fenced areas (a total of 1.2 ha), each with an open small barn provided for their protection from inclement weather. The animals were fed a standard donkey food regimen predominantly of hay, supplemented with 12% protein-containing grain. Previous medical treatments included routine vaccinations and prophylactic deworming. Other species that had ready access to the farm and the paddock buildings included feral cats, rabbits, opossums, raccoons, squirrels, humans, chickens, Canada geese, and other birds.

Two other herds of donkeys, totaling 65 animals, were also examined for clinical signs of besnoitiosis. Both these herds had had some interaction with the affected herd in the past few years and are both located in mid-Michigan.

#### Physical examination

Initially, the condition was detected in a single animal undergoing a soundness examination for sale. Following detection of characteristic lesions in this animal, all animals from the originating herd were carefully examined for signs of *Besnoitia* sp., with particular attention being paid to the external ocular tissues, the buccal mucosa, the external and inner pinnae, externally accessible mucosal sites, as well as the skin. The general condition and the history of each animal were recorded and all obvious medical conditions identified. Five of the animals (including the animal undergoing treatment) were examined on a number of occasions for any changes in clinical presentation. Loose hair from the affected areas was collected and examined microscopically for the presence of lice and heavy fungal loads. Skin areas with obvious clinical change were also scraped and the samples examined microscopically; larger pieces were fixed and processed for histopathology. Punch biopsies (7 mm) were taken from 4 animals. Scleral nodules were removed from 1 animal for identification of their content. Changes in general medical condition were recorded. The examinations took place in late summer/early fall.

#### Tissue examination

Areas of skin containing small, superficial nodules, including the inner ear pinnae, were scraped, and the samples stained with Giemsa for microscopical examination. Isolated tissue cysts were ruptured and the contents suspended in normal saline and examined under an inverted microscope for form and motility. Seven-millimeter punch biopsies were taken from the affected skin and fixed in 10% buffered formalin solution, processed routinely for paraffin-embedded, and the staining of 8- $\mu$ m sections with hematoxylin and eosin (H&E). The dimensions of the tissue cysts were measured on histological sections using calibrated ocular micrometers, and those of bradyzoites obtained from calibrated electron microscopic images.

Tissues were also prepared for transmission electron microscopy

TABLE I. Characteristics of the infected animals in the herd.

Age group	No. infected	Total	Severity of condition
1–2	2 (66.6%)	3*	Severe cases
2–3	9 (83.4%)	11*	The most severe cases
3–4	1 (100%)	1*	Lightly affected
4–5	1 (100%)	1*	Lightly affected
5–6	1 (33.3%)	3	Lightly affected
>6	0 (0%)	19	Free of disease

\* Born on the current farm.

(TEM) by postfixing in 1% osmium in 0.1 M phosphate buffer for 3 hr, dehydrated through graded alcohols, rinsed in propylene oxide, then transferred to resin : propylene oxide mixtures of 50, 75, and then 100% resin (approximately 12 hr for each incubation). Thick sections (1  $\mu\text{m}$ ) were made with glass knives, and thin sections (40 nm) made with a diamond knife. Thick sections were stained with a mixture of 1% toluidine blue and 0.1% basic fuchsin. Thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips 301 TEM.

#### Molecular systematics

Bradyzoites from a fresh skin biopsy were subjected to proteinase K digestion and DNA extraction using DNAeasy columns following the manufacturer's recommendations (Qiagen, Valencia, California). A portion of the beta subunit of RNA polymerase B, encoded in the extranuclear plastid genome of apicomplexans, was then amplified using primers designed to portions of the gene that are well conserved among the coccidia, as previously described (Dubey et al., 2002). In addition to the target-specific 3' terminus, each of these primers also incorporates a GC-rich 5' domain intended to increase the affinity of the primers in later rounds of PCR to templates synthesized in earlier rounds. The resulting PCR product was then directly sequenced in each direction using an ABI 3100 instrument. Sequence chromatograms were aligned and edited using Sequencher (Genecodes Corp., Ann Arbor, Michigan) and aligned to homologues from other coccidian parasites, including to homologues previously characterized from *Besnoitia darlingi* and *Besnoitia oryctofelisi*. (sequenced twice with consistent results). This alignment and 1,000 bootstrap replicates, were then used in phylogenetic reconstructions using the neighbor joining method and under the minimum evolution and maximum parsimony criteria as implemented by MEGA v. 2.1.

## RESULTS

### Clinical presentation

The animals were generally in good condition, with only 3 animals showing clearly overt signs of poor health that included a minor degree of ventral edema, excessive hair loss, and a generally ragged appearance; these animals appeared to be depressed and had reduced appetite. Vital signs of all animals were within normal ranges. The major changes seen were in skin condition and in the external eye; 14 of the 38 animals in the herd showed some clinical manifestation that was consistent with besnoitiosis (Table I).

The most common dermal change was a nodular, focal alopecia, giving a spotted appearance to the muzzle and other light-colored areas of the coat, and was associated with thickening and roughening of the skin in the specific areas; these skin changes appeared not to be pruritic. The focal areas of rough lichenified skin were present on the head, the base of the ears, the shoulders, and in the inner aspects of the hind limbs, as well as the perineal and perivulvar regions. The most common sites of skin change in affected animals were the inner surfaces of the hind legs (85.7% of the animals) and the muzzle skin of the head (57.1%). The most severely affected animal showed, in addition, considerable general alopecia, with tufts of hair easily pulled out. Three animals had apparently unrelated medical conditions, i.e., bacterial dermatitis of the mammary gland, severe dental problems, and traumatic lesions in the corner of the mouth.

The most common sign of besnoitiosis, however, was the presence of scleral nodules (13/14 animals). The scleral lesions were small, round, white to yellow, glistening papules, 1 mm or less in diameter, usually close to the limbus and were more common toward the medial canthus (Fig. 1A). Two of the most severely affected animals also presented with obvious scleral injection and swelling of the palpebral conjunctiva.

Five of the animals were examined in detail over a period of 4 mo. Two of these (1–2 yr of age) developed further scleral pearls over the period and their general condition deteriorated

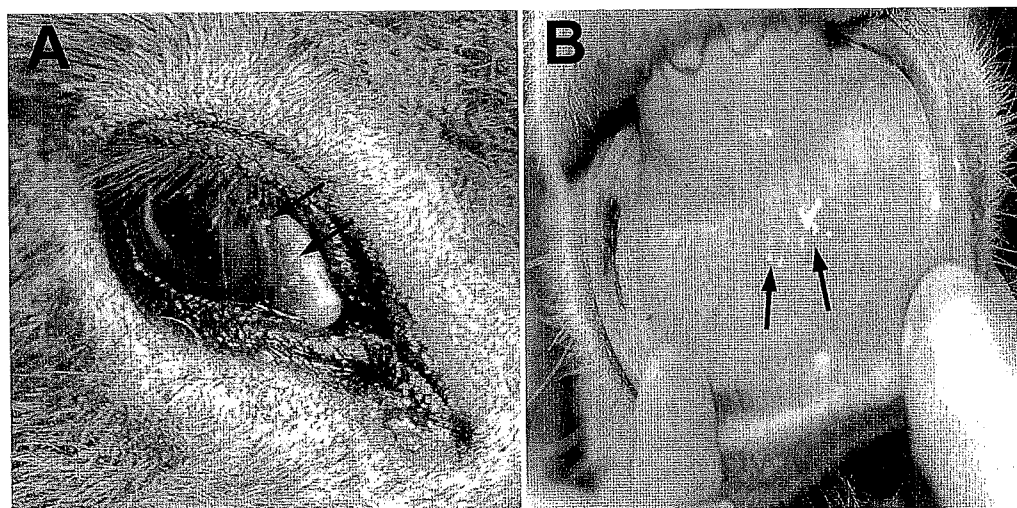


FIGURE 1. Clinical presentations. Cystic lesions (arrows) are present (A) at the limbal border of the sclera (scleral pearls) and (B) in the mucosa of the lower lip.

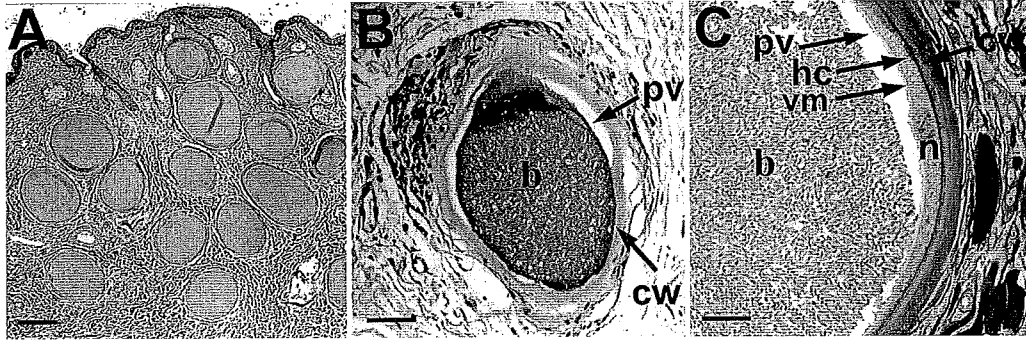


FIGURE 2. Subcutaneous biopsy specimens from donkey naturally infected with *Besnoitia bennetti* infection. (A) *Besnoitia bennetti* cysts are present with a minor mononuclear cell and eosinophil infiltrate beneath the basal layer of epidermis. Many melanocytes are present in the epidermis. (H&E stain). (B) A dermal *B. bennetti* cyst filled with bradyzoites (b); the parasitophorous vacuole (pv) and cyst wall (cw) are evident (H&E stain). (C) Close-up of the cyst, showing the cyst wall (cw), host cell cytoplasm (hc), the host cell nucleus (n), and a vacuolar membrane (vm) enclosing myriads of bradyzoites (b). Basic fuchsin & toluidine blue stain. Scale bars in micrometers: A = 100; B = 50; C = 10.

with apparent weight loss and their coat hair becoming dry and standing upright.

None of the clinical signs defined above was detected in any of the 65 animals in the 2 other local donkey groups involved in this study.

### Histopathology

The characteristic component of the skin biopsies was the presence of numerous medium-sized, thick-walled, *Besnoitia* sp. tissue cysts, mostly present in the middle to deep dermis within alopecic areas of skin (Fig. 2A); some tissue cysts, however, were closely apposed to the base of the epidermis. Those cysts within the deep dermis commonly were present in clusters and were either surrounded by inflammatory reactions that included lymphocytes, macrophages, and multinucleated giant cells or were without any cellular evidence of host response. Cysts were fewer in number and usually were free of inflammatory cells in the superficial dermis and at the dermal-epidermal junction. The overlying epidermis had varying degrees of orthokeratotic hyperkeratosis, parakeratotic hyperkeratosis, acanthosis, and pseudoepitheliomatous hyperplasia.

Cysts were approximately 100–200  $\mu\text{m}$  in diameter and were circular in cross-section. The cyst wall varied from 6.3 to 6.9  $\mu\text{m}$  in thickness, and variation occurred within individual cysts. The parasitized host cell was severely enlarged and its nucleus was hypertrophied. Numerous banana-shaped bradyzoites (Fig. 2B, C) were prominent and filled out the entire cell cavity, compressing the cell cytoplasm and nucleus into a thin rim at the periphery to form an inner coat to the cyst. There was a condensed, hyalinized, and laminated layer of collagen fibers outside the cyst. Histological sections of *Besnoitia* sp. cysts were deposited at the United States National Parasite Collection (USNPC), Beltsville, Maryland; USNPC accession number 90685.

### Parasitology and cyst ultrastructure

Samples of extirpated nodules produced motile parasites of short, curved, banana shapes. Giemsa staining of these samples demonstrated the protozoa typical of *Besnoitia* spp. (Jack et al., 1989; Paperna and Lainson, 2001; Dubey et al., 2002). The ultrastructure of the tissue cysts and bradyzoites was compatible

with that previously reported for *B. bennetti* (Terrell and Stookey, 1973; Van Heerden et al., 1993; Wendell et al., 1997).

Ultrastructurally, the external capsular wall of *B. bennetti* cysts comprised a dense outer layer of circumferentially arranged collagen fibers and granular, electron-dense material. The extracellular layer had a loosely woven pattern that blended into a more condensed linear banding pattern. The inner portion adjacent to the host-cell plasma membrane was composed of loosely arranged fibrillar material. The infected host cell had a thin cytoplasmic host-cell layer that was in direct contact with the external fibrous cyst wall-layer. The densely granulated cytoplasmic layer contained several grossly visible hypertrophic nuclei (Fig. 3A).

The parasitophorous vacuole contained numerous bradyzoites (Fig. 3). Bradyzoites were crescentic or piriform and measured 6.7–8.1 ( $7.2 \pm 0.54$ )  $\mu\text{m}$  by 1.7–2.1 ( $1.9 \pm 0.2$ )  $\mu\text{m}$ . They had a typical coccidia 2-layered pellicle. A polar ring (electron-dense anterior collar) was found around the opening of the inner membranous layer of the pellicle at the anterior end. A cone-shaped conoid with 6–7 annular elements with a helical arrangement was located within the polar ring and extended posteriorly. The nucleus was located toward the middle to posterior third of the bradyzoite. Two to 4 rhoptries were occasionally observed interspersed with numerous, randomly arranged micronemes. Other organelles were observed, such as amylopectin and lipid granules (Fig. 3B). Micropores were occasionally noted in bradyzoites.

### Molecular systematics

A portion of the Beta subunit of the plastid-encoded RNA polymerase was amplified from an isolate of parasites from 1 of the animals, sequenced, and compared with homologues available from 2 *Besnoitia* parasites and from other coccidian genera. Both the sequence (Fig. 4) and the alignment are available (GenBank Accession AY665399). Phylogenetic reconstructions using the neighbor-joining, minimum-evolution, and maximum-parsimony criteria yielded congruent topologies and confirmed that *B. bennetti* is distinct from previously characterized parasite species and that it comprises, with its congeners, a monophyletic group. Representative analyses, based on

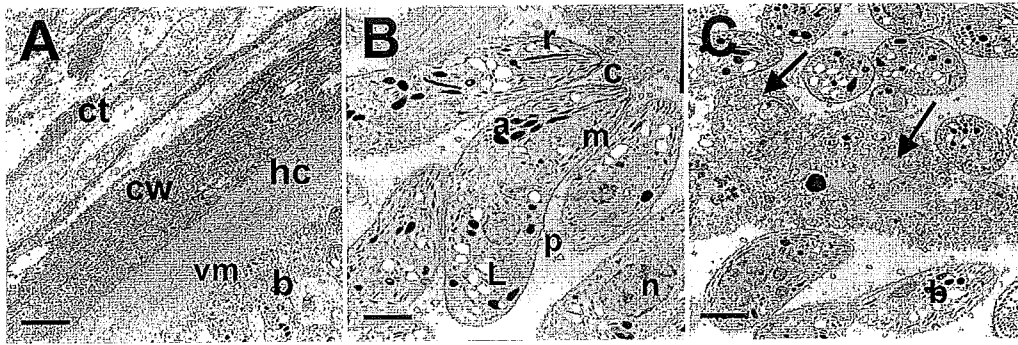


FIGURE 3. Transmission electron micrographs (TEM) of *Besnoitia bennetti* in the skin of a naturally infected donkey. Lead acetate stain. (A) A thick host connective tissue (ct) overlies the cyst wall (cw), the host cell cytoplasm (hc), and the vacuolar membrane (vm); bradyzoites (b) are in close apposition to the cyst wall. (B) Longitudinal and tangential sections of crescent-shaped bradyzoites showing pellicle (p), numerous randomly distributed micronemes (m) located mostly anterior to the nucleus (n) and extending up to the posterior end, rhoptries (r), conoid (c), amylopectin (a), and lipid granules (L). (C) Cross- and tangential sections of bradyzoites. Degenerating bradyzoites (arrows) are present. Scale bars in micrometers: A = 2; B and C = 1.

all variable positions and on transversional substitutions only, are illustrated in Figure 5.

DISCUSSION

*Besnoitia* sp. has been reported in sporadic individual cases in equids in the United States but has never been described as

the cause of an outbreak of clinical importance, as presented in this article. These animals showed similar clinical presentations in the skin, conjunctiva, and elsewhere to that previously described to be associated with *Besnoitia* sp. cysts in donkey and burros (Terrell and Stookey, 1973; Wendell et al., 1997).

Two animals were seen to have actively changing lesions, both developing scleral nodules within 3 mo of each other, suggesting that they had active, and possibly recent, infections. The older infected animals had relatively minor clinical presentations and were probably either lightly infected or were recovering from more severe infections. It is possible that *Besnoitia* sp. is more common and more severe in younger animals; the previous reports of this infection are in young donkeys (Terrell and Stookey, 1973; Wendell et al., 1997).

The severe, more generalized clinical disease described previously in a horse (Van Heerden et al., 1993) was seen in 3 of the present group of donkeys. Why the disease in these animals was more severe is unclear. The factors that determine whether infection with *Besnoitia* spp. will induce clinical disease remain unclear (Lewis, 1992), but it was noted that the animals in this present article were either living in a stressful housing situation and/or had been subjected to travel; thus, stress may have contributed to the worsening of their condition.

The parasites found in these animals are, in all likelihood, *B. bennetti*. This is supported by the histological structure of the bradyzoite-containing cysts in these cases. Other genera within the Sarcocystidae that form large intracellular cysts containing bradyzoites include *Sarcocystis* spp., *Toxoplasma gondii*, *Neospora caninum*, *Hammondia* spp. and *Frenkelia* spp., but these lack the thick connective tissue capsule, with a thick hyaline wall with eccentrically placed host-cell nuclei, found around *Besnoitia* spp. cysts (Gardiner et al., 1988); these were seen in the present cases.

The morphology of the parasite itself also defines this present organism as *B. bennetti* and distinguishes it from other species of *Besnoitia* and other coccidia. These donkey-derived bradyzoites had fewer micronemes (30–40) than most other species and the number of rhoptries was consistent with *B. bennetti*. Posteriorly located micropores, a consistently seen feature of other *B. bennetti* bradyzoite descriptions (Van Heerden et al., 1993), was only observed in a few of the current specimens.

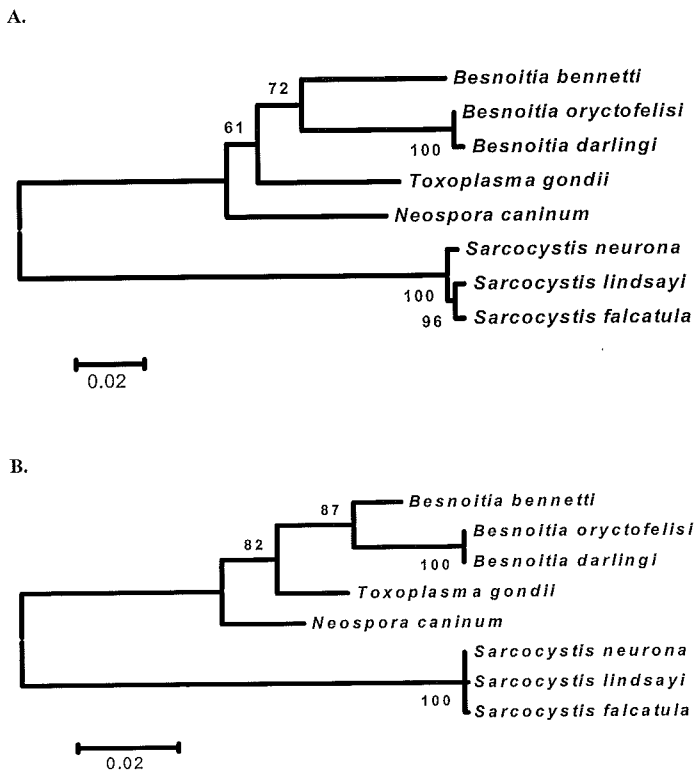


FIGURE 4. Phylogenetic reconstruction of midpoint rooted neighbor joining tree based on Kimura 2-parameter distances of a portion of the plastid-encoded beta subunit of RNA polymerase; 1,000 bootstrap replicates. (A) Distances calculated from all positions. (B) Distances calculated only from transversional substitutions. Comparison sequences: *B. bennetti* AY665399, *B. oryctofelisi* AY181999, *B. darlingi* AY181998, *T. gondii* AF095904, *S. neurona* AY165000, *S. lindsayi* AY164997, *S. falcatula* AY164999.

This might be due to fixation or other technical differences in the specimens. Enigmatic bodies are common features of other *Besnoitia* sp., such as *B. jellisoni* and *B. darlingi* but were not seen in any of the specimens examined in this present study.

Sequencing a portion of the plastid-encoded RNA polymerase corroborates both that *B. bennetti* may be readily distinguished from previously characterized congeners and that these represent an assemblage of specifically related parasite taxa. Evidence for the monophyly of the *Besnoitia* spp. was particularly strong when the analysis was limited to transversional substitutions, suggesting that this may be a particularly informative set of characters for assessing the membership of future parasite isolates presumed to also belong to the genus. Characterizing this sequence now provides an independent means for assessing how broad an intermediate host range this parasite species may occupy and may facilitate the confirmation of cats as a definitive host.

The origin of the infection in these donkeys cannot be determined from the current information, but the high number of infected animals being born on the primary farm suggests that active transmission may have taken place at this farm. The presence of infections in both a jenny and her offspring raises the possibility of transplacental transmission: in utero infections occur with others of this group of parasites.

A better understanding of the epidemiology of this infection in the donkey population as a whole is necessary. Whether this finding represents an unusual cluster of infections or reflects a wider distribution of a usually subclinical infection that has largely gone undetected to date requires further study. Donkeys are increasing in number, and now it is estimated that there are over 300,000 donkeys in the United States and countless numbers in Africa, the Middle East, and elsewhere. Wide-spread distribution of this infection would be of national concern and of importance to veterinary medicine in general.

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