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# CLINICOPATHOLOGIC FEATURES OF INFECTION WITH NOVEL *BRUCELLA* ORGANISMS IN CAPTIVE WAXY TREE FROGS (*PHYLLOMEDUSA SAUVAGII*) AND COLORADO RIVER TOADS (*INCILIUS ALVARIUS*)

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*Abstract:* Two novel and distinct *Brucella* strains were recovered from 5 of 10 adult, sex undetermined, captive waxy tree frogs (*Phyllomedusa sauvagii*) and two of five adult, sex undetermined, captive Colorado river toads (*Incilius alvarius*) held in a zoologic collection with clinical and pathologic findings of bacterial disease. These amphibians originated from three separate private breeding facilities over several years and exhibited disease 9–49 mo following release from quarantine. Common presenting signs were vague but included focal abscessation, weight loss, change in coloration, anorexia, and decreased perching. Two waxy tree frogs and one Colorado river toad recovered with supportive care and antimicrobial treatment based on susceptibility testing. Microgranulomatosis, subcutaneous and renal abscessation, femoral osteomyelitis, and multicentric infection were the most common histologic findings. The organisms were identified antemortem in samples from subcutaneous abscesses, cloaca, and skin and from a variety of organ systems postmortem, and demonstrated a consistent susceptibility pattern. Initial isolates were misidentified as *Ochrobactrum anthropi*. Polymerase chain reaction and sequencing of the 16S rRNA gene identified the two organisms as novel *Brucella* strains similar to *Brucella inopinata*–like sp. and other novel organisms within the emerging "BO clade." *Brucella* strain oaks (isolated from waxy tree frogs) and *Brucella* strain leathers (isolated from Colorado river toads) differed from each other by 16 of 571 base pairs in a region of chromosome 2, and did not closely match any previous GenBank entries. This report describes the clinicopathologic features of infection by these bacteria in two amphibian species and expands the range of novel *Brucella* organisms from amphibian reservoirs.

*Key words:* Amphibians, *Brucella*, Colorado river toad, *Incilius alvarius, Phyllomedusa sauvagii,* waxy tree frog.

## INTRODUCTION

Brucellosis is a major disease concern in humans and domesticated animals worldwide. Animal reservoirs are the source of human infection, which is most often associated with *Brucella melitensis*, *Brucella abortus*, and *Brucella suis*.[3] With the isolation of marine mammal *Brucella* species from clinically affected humans,[12,19] new wildlife reservoirs are emerging as a potential source for human brucellosis. *Brucella*-like organisms have been isolated from a variety of amphibians,[5,7,13,18,20,25] expanding the wildlife reservoir while raising questions regarding potential zoonotic risks and population health concerns that could affect the captive breeding and management of taxa of which some species are undergoing a global decline.

All *Brucella* species have a specific insertion sequence, IS711, and core species share identical 16S rRNA sequences.[13,16,22,25] All core species are from mammals and include *B. melitensis*, *B. suis*, *B. abortus*, *Brucella canis*, *Brucella neotamae*, *Brucella ovis*, *Brucella pinnepedialis*, *Brucella ceti*, and *Brucella papionis*. Atypical *Brucella* exhibit slight differences in 16S rRNA sequence, biochemical features, growth rate, and motility. Atypical *Brucella* spp. include *Brucella inopinata* and *B. inopinata*–like (human),[4,16,22] *Brucella microti* (voles, *Microtus arvalis*),[11,14,15] *Brucella vulpes* (red fox, *Vulpes vulpes*),[17] and numerous isolates collected from a diversity of amphibians, including African bullfrog (*Pyxicephalus edulis*),[5] big-eyed tree frog (*Leptopelis vermiculatus*),[7] cane toad (*Rhinella marina*, formerly *Chaunus* [*Bufo*] *marinus*),[18] Argentine horned (Pac-Man) frog (*Ceratophrys ornata*),[20] and White's tree frog (*Litoria caerulea*),[24] as well as red-eyed tree frog (*Agalychnis callidryas*), Amazonian

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milk frog (*Trachycephalus resinifictrix*), tomato frog (*Dyscophus antongilii*), and Cranwell's horned frog (*Ceratophrys cranwelli*).[13] These atypical *Brucella* organisms, distinct from currently described *Brucella* sp. and genetic relatives, pose an undetermined risk for human, domesticated animal, and wildlife populations.

Native to the semiarid Chaco Plain of Argentina, Bolivia, Brazil, and Paraguay, waxy tree frogs (*Phyllomedusa sauvagii*) are an arboreal amphibian species threatened by the international pet trade and habitat alteration through agriculture and wood harvesting.[1] The Colorado river toad (*Incilius alvarius*) is a common terrestrial amphibian native to the arid lowlands, grasslands, and mountain canyons of southern California, Arizona, and New Mexico in the United States, extending south into Mexico.[9] This report describes the clinicopathologic features of two novel *Brucella* strains from two species of captive amphibians, *Brucella* strain oaks, isolated from South American waxy tree frogs (*P. sauvagii*), and *Brucella* strain leathers, isolated from Colorado river toads (*I. alvarius*), from a zoologic collection in the United States.

## CASE REPORT

Two groups (group 1, group 2) of waxy tree frogs were obtained from private facilities approximately 1 yr apart. Group 1 consisted of six adult frogs of undetermined age and sex from a private breeder located in California, USA, captive bred from wild-caught frogs originating from Paraguay. Group 2 consisted of four adult frogs, captive bred, of undetermined age and sex from a private facility located in Nevada, USA, but may have originated from the same breeder from which group 1 frogs were obtained. Health history and preshipment testing were not available for either group. Three of six frogs from group 1 died or were euthanized during the 110-day quarantine period. Cause of death in two frogs was attributed to respiratory disease with histologic findings of mild diffuse interstitial pneumonia, focal bronchopneumonia, and marked multifocal unilateral granulomatous pneumonia, but no culture testing was performed. Cause of death for the third frog was attributed to intestinal nematodiasis and inanition. Culture testing for chytrid fungus (skin) and polymerase chain reaction (PCR) testing for ranavirus (kidney, liver, and skin) were negative for deceased frogs, and surviving frogs tested negative for chytrid fungus from skin swab samples. Surviving frogs were treated for intestinal nematodes with fenbendazole (Panacur, 100 mg/ml, Intervet Inc., Millsboro, Delaware 19966, USA) 100 mg/kg po q 14 days for five doses. All frogs from group 2 survived the 49-day quarantine period and tested negative for chytrid fungus from skin swab samples.

Two groups (group 3, group 4) of Colorado river toads were obtained from the same private facility located in Washington, USA, approximately 1 yr apart. Group 3 consisted of two adult toads of undetermined age and sex, listed as "undetermined place of hatch." Group 4 consisted of three adult toads, captive bred, of undetermined age and sex. Health history and preshipment testing were not available for either group. All toads survived the quarantine period (81 days for group 3 and 31 days for group 4) and tested negative for chytrid fungus on skin swab samples. During quarantine all toads were treated for intestinal nematodes with fenbendazole (Panacur, 100 mg/ml) 50 mg/kg po sid for 3–10 days. Group 3 toads were successfully treated for presumptive ivermectin toxicosis following ivermectin (Vetrimec 1%, Norbrook Laboratories Limited, Newry, Co. Down, Northern Ireland) administered 0.2 mg/kg po once for intestinal nematodiasis. Symptoms of bloating, lethargy, and increased sternal recumbency with a "spread-out" appearance developed in both toads approximately 72 hr following ivermectin treatment, and both toads responded to supportive care. Group 4 toads were not treated with ivermectin during quarantine. Group 3 toads underwent quarantine during the same time period as group 1 frogs but were housed in a separate enclosed room with separate air flow.

Case 1 is a waxy tree frog that originated from group 1. Approximately 14 mo following release from quarantine, the frog presented with right stifle swelling and radiographic evidence of osteomyelitis. Cytologic evaluation of Wright-Giemsa–stained aspirate smears from the stifle revealed numerous degenerative neutrophils and macrophages, necrotic cell debris, and numerous approximately 2.0 × 3.0-μm intracellular and extracellular coccobacillary structures. Culture and API® 20NE test strips (bioMerieux, Inc., Durham, North Carolina 27712, USA) performed at a commercial laboratory misidentified the bacteria as *Ochrobactrum anthropi*. Based on sensitivity testing of the bacterial isolate, the right stifle was infused with enrofloxacin (Baytril, 22.7 mg/ml, Bayer Corp., Shawnee Mission, Kansas 66201, USA) 20 mg/kg as a single dose and systemic enrofloxacin administered at 10 mg/kg im sid. The frog died 12 days following initial

exam. Histologic examination identified abscess formation in the stifle region with extension of inflammation into the lumen of the stifle joint and distal femur, associated bone resorption, and minimal reactive bone formation. Histiocytes and neutrophils predominated, with fewer lymphocytes and plasma cells present. Inflammation was accompanied by edema, cell necrosis, and rhabdomyolysis where inflammation extended into adjacent muscle (Fig. 1). Visceral lesions included microgranulomatosis in the lamina propria of the colon and interstitium of the kidney and intestinal nematodiasis. Cellular phagocytosis in melanomacrophage centers of the liver and spleen and mild granulocytic interstitial pneumonia were also noted. Fites acid-fast, tissue Gram (Brown and Brenn), and Gimenez stains did not identify organisms in the lesions.

Molecular testing to further characterize this isolate classified the bacteria as *Brucella* species rather than *Ochrobactrum*. DNA was purified and a real-time PCR assay determined the presence of the *Brucella*-specific insertion sequence 711 (IS711).[10] Sequencing of the 16S rRNA gene provided further confirmation that the isolated bacterium was a *Brucella* species. The isolate was designated as *Brucella* strain oaks.

Case 2 is a waxy tree frog that originated from group 1. Approximately 15 mo following release from quarantine this frog was examined for lethargy, anorexia, decreased perching, weight loss, and diminished coloration. Culture of a firm subcutaneous mass located along the ventral pectoral girdle was initially reported as *O. anthropi* but molecular testing as performed in case 1 identified *Brucella* strain oaks, with a similar drug sensitivity pattern (Table 1). Treatment with enrofloxacin 10 mg/kg im sid q 30 days was initiated, and the frog was also treated by soaking in a shallow pan containing amphibian Ringer solution placed in a small chamber under oxygenation (5 L/min) for 15 min q 48 hr (AR-O2 treatment). The patient responded to treatment, the subcutaneous mass resolved, and no further signs or health concerns were noted.

Case 3 is a waxy tree frog that originated from group 2. Approximately 9 mo following release from quarantine this frog was examined for lethargy, anorexia, decreased perching, weight loss, and diminished coloration. Physical exam findings were unremarkable, but cytology and culture of a bloody cloacal discharge identified *Brucella* strain oaks with a similar drug sensitivity pattern (Table 1). The patient responded to enrofloxacin and AR-O2 treatments as prescribed for case 2 and symptoms resolved within the 30-day treatment period. The patient was unexpectedly found dead approximately 6 wk following case resolution. Postmortem exam findings included cachexia, coelomic fluid accumulation, and multiple renal nodules. Histologic examination identified renal bacterial abscesses with associated sepsis, articular gout, and marked atrophy of fat. Fites acid-fast, tissue Gram (Brown and Brenn), and Gimenez stains did not identify organisms in the lesions. Renal abscessation was the presumptive cause of the bloody cloacal discharge. Culture of the liver, kidney, and coelomic fluid identified *Brucella* strain oaks. Immunohistochemical staining was performed on kidney sections at the National Veterinary Services Laboratories, Ames, Iowa, USA as previously described.[2] *Brucella* antigen was detected using two rabbit polyclonal antibodies to *B. abortus* and *B. ovis* (provided by Dr. Steven Olsen, National Animal Disease Center, Ames, Iowa, USA). Both antibodies labeled numerous intracellular organisms in renal abscesses (Fig. 1d, inset). However, the *B. ovis* antibody labeled the antigen at a higher dilution, suggesting a stronger affinity for the organism. *B. ovis* lacks the oligopolysaccharide side chain common to field strains of *B. abortus*, *B. mellitensis*, and *B. suis*.

Case 4 is a waxy tree frog that originated from group 2. Approximately 14 mo following release from quarantine, this frog was examined for lethargy, anorexia, decreased perching, weight loss, and diminished coloration. No other abnormalities were identified and treatment with enrofloxacin and AR-O2 treatments as prescribed for cases 2 and 3 was initiated, but the frog died within 72 hr of presentation. Coelomic fluid accumulation and cachexia were noted at necropsy. Histologic examination identified severe nephrosclerosis with chronic interstitial nephritis, tubular protein casts and oxalate-like crystals, lymphoid depletion in the spleen and thymus, mild interstitial pneumonia, cholelithiais, and marked atrophy of fat. Culture of the heart, liver, and coelomic fluid identified *Brucella* strain oaks.

Case 5 is a waxy tree frog that originated from group 2. Approximately 22 mo following release from quarantine, this frog was evaluated for lethargy, decreased perching, and darkening skin coloration. A single swab of the skin and cloaca identified *Brucella* strain oaks. Antibiotic susceptibility pattern of this isolate showed resistance to enrofloxacin (Table 1), so treatment with amikacin (Amiglyde-V, 250 mg/ml, Ft. Dodge, Ft. Dodge, Iowa 50501, USA) 8 mg/kg sc q 48 hr × 5 doses

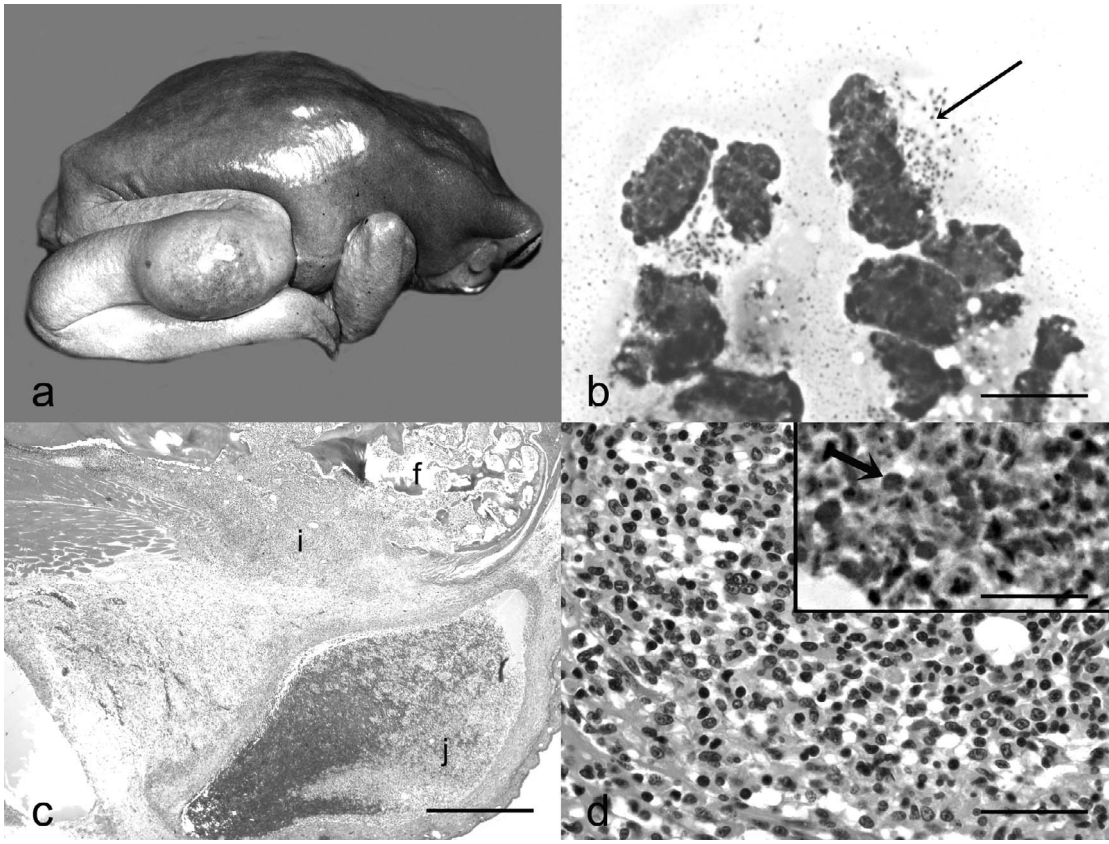


**Figure 1.** Brucella strain oaks infection in captive waxy tree frogs (*P. sauvagii*). **a.** Waxy tree frog (case 1) with abscess involving the stifle region. **b.** Smear of stifle aspirate from case 1 showing degenerative neutrophils containing intracytoplasmic bacteria. Wright-Giemsa, bar = 18 μm. **c.** longitudinal section of stifle (case 1). An inflammatory infiltrate (i) is present within the joint space (j) and extends into the distal femur (f), with associated bone lysis. Hematoxylin and eosin (H&E), bar = 350 μm. **d.** Higher magnification of the stifle inflammatory infiltrate composed of histiocytes, neutrophils, and rare multinucleate giant cells. H&E, bar = 80 μm. Inset: inflammatory cells stain positive for intracytoplasmic *Brucella* antigen using antibodies to *B. ovis* (arrow). Rabbit *Brucella* polyclonal antibody, hematoxylin counterstain, bar = 80 μm.

and AR-O2 treatments q 48 hr × 5 doses was effective in returning this frog to normal appearance and health. Approximately 23 mo following case resolution, this frog was found dead following a 2-day illness consisting of lethargy, discoloration, and lack of perching activity. A skin swab collected the day prior to death identified *Brucella* strain oaks with a similar resistance pattern to the previous isolate obtained from this frog (Table 1). Histology identified moderate to severe, chronic nephritis with interstitial fibrosis and oxalate nephrosis as the cause of death. Postmortem liver culture identified *Brucella* strain oaks. However, there was no histologic evidence of bacterial infection, and pathogenic infection was not considered as a contributing factor in the death of this frog.

Case 6 is a Colorado river toad that originated from group 4. Approximately 33 mo following release from quarantine, this toad was examined for a focal, circular shallow wound with inflammation on the lateral right rear limb. Radiographs were unremarkable and topical treatment with a commercial spray combining gentamicin sulfate with betamethasone valerate (GenOne Gentocin Spray, Vetone, Boise, Idaho 83705, USA) topically bid for 10 days was initiated. Treatment was changed to enrofloxacin 10 mg/kg im sid q 10 days, continuing as enrofloxacin (Baytril, 22.7-mg tablets) 10 mg/kg po bid q 21 days based on sensitivity testing (Table 1) of a second novel *Brucella* strain, designated Brucella strain leathers, cultured from the wound. The wound resolved and no further concerns or symptoms

**Table 1.** Antibiotic susceptibility pattern for isolates of *Brucella* strain oaks isolated from captive waxy tree frogs (*P. sauvagii*; cases 1–5) and *Brucella* strain leathers isolated from Colorado River toads (*I. alvarius*; case 6).[a]

| | Case 1 | Case 2 | Case 3 | Case 5 | | Case 6 |
|---|---|---|---|---|---|---|
| Antibiotic | Right stifle swelling | Ventral mass | Cloacal | Combined swab of oral cavity and skin | Skin | Right rear limb mass |
| Amikacin | S | S | S | S | S | S |
| Amoxicillin/clavulanic acid | S | S | S | S | S | S |
| Ampicillin | R | S | S | R | R | I |
| Cefazolin | R | R | I | R | R | R |
| Cefoxitin | R | I | I | R | R | R |
| Ceftiofur | R | R | R | R | R | R |
| Chloramphenicol | S | S | S | S | S | S |
| Doxycycline | S | S | S | S | S | S |
| Enrofloxacin | S | S | S | I | R | S |
| Gentamicin | S | S | S | S | R | S |
| Imipenem | S | S | S | S | S | S |
| Ticarcillin/clavulanic acid | I | I | I | I | I | I |
| Ticarcillin | I | I | I | I | I | I |
| Trimethoprim/sulfadiazine | S | S | S | S | S | S |

[a] S = sensitive; R = resistant; I = intermediate susceptibility.

developed. Like *Brucella* strain oaks, isolated from the waxy tree frogs (cases 1–5), *Brucella* strain leathers was originally misidentified by the API 20NE strip as *O. anthropi*, but although *Brucella* strain oaks was mannosidase positive, *Brucella* strain leathers was mannosidase negative. The 16S rRNA sequence of *Brucella* strain leathers was identical to that of *Brucella* strain oaks.

Case 7 is a Colorado river toad that originated from group 3. Approximately 49 mo following release from quarantine this toad was examined for lethargy, bloating, and heat-seeking behavior, but died shortly following examination. Histologic findings included foci of histiocytic inflammation and discrete granuloma formation present throughout the lung, liver, skin, coelomic surfaces, mesentery, urinary bladder, ovary, ureter, kidney, and nasal mucosa. Histiocytic cells contained large numbers of minute granular structures in the cytoplasm that stained lightly magenta with Gimenez stain and blue with Giemsa stain. Culture, PCR, and histologic examination confirmed disseminated granulomatosis from *Brucella* strain leathers.

The remaining waxy tree frog from group 1 was euthanized 3 mo following release from quarantine after failure to respond to enrofloxacin and AR-O2 treatments initiated for head tilt and anisocoria. Histologic examination identified marked subacute meningitis and mild interstitial pneumonia, but culture failed to grow any organisms. The remaining waxy tree frog from group 2 died 5 mo following release from quarantine without premonitory signs, with histologic findings of marked atrophy of fat, intestinal nematodiasis, hepatic microgranulomas, and mild interstitial pneumonia. Culture of the liver and intestines identified several gram-negative and anaerobic pathogens, but did not identify any *Brucella* organisms.

The remaining Colorado river toad from group 3 was found dead approximately 9 mo following release from quarantine. Significant postmortem exam findings included significant generalized swelling along the left brachium and antebrachium with generalized erythema and skin abrasions and less prominent erythema and skin abrasions on the left rear limb, with ventral erythema of the distal ventral abdomen and medial thighs. The carcass was in an advanced state of autolysis with liquefaction of the coelomic organs that precluded further gross examination, histology, or other diagnostic sample collection. The two remaining Colorado river toads from group 4 have remained clinically healthy.

*Brucella* strain oaks and *Brucella* strain leathers are small gram-negative cocci or coccobacilli that exhibit a rapid urease reaction (<30 min), characteristic of *Brucella*. Unlike established *Brucella* species, both strains are motile and exhibited motility readily after 24 hr culture in agar tube motility (motility nitrate) media (Hardy Diagnostics, Santa Maria, California 93455, USA) which differs from other emergent species in this clade[19] that report motility demonstrable only by swarming on semisolid agar media. Both strains were

identified by API 20NE as *O. anthropi*, with a probability of >99%. Both *Brucella* strain oaks and *Brucella* strain leathers differed from strain B13-0095 in the following API 20NE tests: positive reduction of nitrates, positive hydrolysis of gelatin, positive potassium gluconate assimilation, positive trisodium citrate assimilation, negative capric acid assimilation, and negative mannose assimilation. They differed from each other in arabinose and mannitol assimilations. Using the Hinić series of seven real-time *Brucella* typing assays,[10] *Brucella* strain oaks isolates were positive for only the *Brucella*-specific target IS711 and BMEII0466. *Brucella* strain leathers isolates were positive for IS711, BMEII0466, and one additional target, BMEII0635. Both *Brucella* strain oaks and *Brucella* strain leathers generated four fragments each (272, 450, 587, and 794 bp) by Bruce ladder PCR, a multiplex PCR that can differentiate all established species of *Brucella*.[8] This Bruce ladder pattern is different from that of previously described *Brucella* species, including amphibian strains. Sequencing of the hypothetical protein gene in chromosome 2 region target from Bruce ladder PCR[6] showed that *Brucella* strain oaks and *Brucella* strain leathers differed from each other by 16 of 571 base pairs (bp), and did not closely match any previous GenBank entries.

The virtually complete (1,471-bp) 16S rRNA sequences for both strains were determined and found to be identical. Sequences from *Brucella* strain oaks and *Brucella* strain leathers show only 95.8% (1,400 of 1,471 bp) identity with the nine core *Brucella* species and 96% (1,356 of 1,412 bp) identity with *B. inopinata*, the first *Brucella* species shown to have a divergent 16S rRNA sequence.[16] A 46-bp 16S rRNA insertion sequence similar to that identified in *Ochrobactrum intermedium*[21] was found in all *Brucella* strain oaks and *Brucella* strain leathers isolates. A BLASTN search found that this insertion is also present in the big-eyed tree frog isolate *Brucella* species 152,[7] the motile *Brucella* isolate HE603360 from African bullfrogs,[5] *Brucella* sp. 141012304 (GenBank LT 605586) isolated from the bluespotted ribbontail ray (*Taeniura lymma*),[6] *Ochrobactrum tritici*, and *B. ceti*. The 46–bp insertion sequence identified in *O. intermedium*, folded into a stem–loop structure, took place in and prolonged helix H184 of the 16S rRNA molecule.[21] Helix H184 had been described as conserved in length at 5 bp among all known bacteria until identification of the 46-bp insertion in *O. intermedium*.

Sequences of the 16S rRNA from *Brucella* strain oaks and *Brucella* strain leathers were compared to available sequences from other amphibian isolates within the "BO clade," with 96.8% (1,375 of 1,421 bp) sequence identity to the White's tree frog isolate,[24] 99.3% identity to the big-eyed tree frog isolate 152,[7] and 99.6% (1,202 of 1,212 bp) to the African bullfrog isolates.[5] GenBank accession numbers for the 16S rRNA gene, rpoB gene, and a portion of chromosome 2 for *Brucella* strain oaks and *Brucella* strain leathers are MF120465, MF120466, MF120467, MF320271, MF325034, and MF 325035.

## DISCUSSION

Novel *Brucella* infection in the amphibians from this report was associated with a wide range of pathologic and clinical findings, most notably osteomyelitis, subcutaneous and renal abscessation, and sepsis. Infection with novel *Brucella* species was suspected but not confirmed in a waxy tree frog with meningitis and a Colorado River toad with limb swelling and generalized erythema. No single organ system was involved in all cases and the reproductive system was not affected in any of the examined frogs. With the exception of subcutaneous abscessation, clinical presentation in both amphibian species was vague and consisted of coloration changes, lethargy, anorexia, and weight loss. Decreased perching behavior was also a common finding in the arboreal waxy tree frogs. Localized lesions or systemic infection is consistent with the variable pathogenicity reported in other amphibians with *Brucella* infections.[5,7,13,18,24] A review of institutional records did not identify any prior cases of *Ochrobactrum* sp. or *Brucella* sp. in any taxa from this collection.

The *Brucella* strain oaks and *Brucella* strain leathers isolates obtained in this report shared a similar multidrug sensitivity pattern (Table 1) comparable to isolates from other amphibians.[7] Antimicrobial sensitivity was determined by a commercial veterinary laboratory using an automated broth microdilution system. As for many amphibian pathogens, break-point values are not available for these novel *Brucella* strains and sensitivity data reflect an amalgam of break-point values for gram-negative veterinary pathogens from the CLSI and EUCAST databases. Aminoglycoside and fluoroquinolone antibiotics are the most attractive options for amphibians based on available pharmacokinetic data. Systemic enrofloxacin combined with surgical excision of localized lesions and chloramphenicol baths was successfully used to treat amphibians in other reports.[7,13,24] The addition of AR-O2 treatments to systemic antibiotic therapy appeared to shorten

time to resolution of clinical symptoms in cases 2, 3, and 5 from this report. Lowered survivability in case 3 was attributed to renal abscesses potentially present at the time of treatment.

Cytologic evaluation of the abscesses was needed to identify the *Brucella* organism in lesions. These bacteria were very small, but visible within and around inflammatory cells in cytologic preparations. Care should be taken, however, to avoid misdiagnosis with morphologically similar organisms, such as *Coxiella burnetii*, *Chlamydophila*, or *Chlamydia*.[3] The organism was not reliably identified using conventional histochemical stains, and was evident in only one Gimenez-stained specimen, whereas the other Gimenez-stained sections from affected frogs did not illustrate the intracellular bacteria. This is likely due to the small size of the organism and the suboptimal sensitivity of tissue Gram stains for identifying some gram-negative bacteria in histologic section.[26] Length of time in formalin prior to paraffin embedding, laboratory variability in staining technique, and degree of autolysis may have contributed to the sensitivity of the cytochemical stains. Importantly, gross and histologic lesions closely resembled those of mycobacteriosis, a common disease process in amphibians. Negative acid-fast stain results should prompt investigators to consider brucellosis as a differential for the lesion.

As in this report, *Brucella* isolates from amphibians,[5,7,18] humans,[16,22] and mammals[11,14] have been misidentified as *O. anthropi* by commercial systems that utilize biochemical analysis to make a rapid identification. A close genetic relative of *Brucella* sp., *O. anthropi* has historically been considered an opportunistic human pathogen of immunocompromised patients, with recent evidence of more severe pathogenicity and *Brucella*-like disease.[23] Because brucellosis serology testing[3] as utilized in humans and domestic animals is usually impractical or unavailable for many zoologic species, particularly amphibians, real-time PCR and 16S rRNA sequencing should be utilized to provide correct identification of *Brucella* infections, especially where initial testing suggests *Ochrobactrum* sp.

This report describes two additional novel amphibian *Brucella* strains belonging to the emerging novel "BO clade" of atypical *Brucella*. *Brucella* strain oaks is the first reported *Brucella* strain infecting waxy tree frogs, and *Brucella* strain leathers is the first reported *Brucella* strain infecting Colorado river toads. A BLASTN search found that only 4 other *Brucella* species (*Brucella* species 251, HE603360, LT605586, and *B. ceti*) contain the 46-bp 16S rRNA insertion sequence found in *Brucella* strain oaks and *Brucella* strain leathers, similar to the 46-bp insertion found in *Ochrobactrum*.[21] *Brucella* strain oaks and *Brucella* strain leathers, like others in this clade, were initially misidentified as *Ochrobactrum* species based on positive motility and API 20NE results. Whole-genome sequencing and intracellular replication assays on these isolates will be performed and published separately, providing valuable information regarding these new strains. Laboratories identifying *Ochrobactrum* species by conventional testing should consider using real-time or conventional PCR targeting the IS711 insertion sequence common to all *Brucella* species, or 16S rRNA sequencing, in order to exclude the possibility of atypical *Brucella*. Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry (MS) is becoming available for bacterial identification in many clinical laboratories. Construction of a MALDI-TOF MS *Brucella* database that includes emergent amphibian *Brucella* species is underway and will be an invaluable tool.

The pathogenicity of brucellosis in amphibians is not well understood and may represent a commensal organism or facultative pathogen, rather than direct pathogenicity. In the amphibians from this and other reports,[13] *Brucella* could be isolated from the skin or cloaca, suggesting possible sampling sites for clinically normal individuals. The incidence, distribution, and host specificity of brucellosis in amphibians are also unclear. In addition to the two strains from this report and spanning a 10-yr period (2006–2016), numerous atypical brucellae isolates were obtained from nine amphibian species native to Australia, South America, and Africa, and obtained as wild caught or from a zoologic collection, private breeder, or pet store.[5,7,18,20,24] The amphibians from this report were captive bred and obtained from three separate private sources that, like the zoologic collection in this report, house multiple amphibian and reptile species. The possibility that commensal organisms of one species were introduced as novel pathogens to another species cannot be ruled out. The implication of novel brucellae to captive amphibian breeding programs or impact on wild populations is unknown. Although amphibian brucellae may demonstrate a close genetic relationship to *B. inopinata*, the natural reservoir or host range of this human isolate is currently unknown. To date,

brucellosis in humans has not been linked to contact with amphibians, and no human cases were associated with the amphibians in this report. Although the zoonotic potential of amphibian brucellae remains unclear, human brucellosis remains a significant disease concern.[3]

Nondomestic animal reservoirs are emerging as a source for novel brucellae with potential human and animal health risks. It is unclear if amphibians are a natural host for *Brucella* organisms or if breeding or holding facilities, changes in biosecurity or quarantine protocols, contact with other species, pet trade, importation of wild-caught animals, or environmental pressures or other population health concerns create opportunities for cross-species infection, transmission, or pathogenicity. Exotic animal veterinarians should be aware that amphibians may harbor novel *Brucella* organisms that can induce morbidity and mortality in these taxa. Culture findings of *Ochrobactrum* sp. should be followed by PCR testing for novel *Brucella* organisms, especially when clinical or pathology findings support health concerns. Surgical, antibiotic, and supportive treatments may be rewarding for critical species. The potential zoonotic risk for amphibian *Brucella* isolates is undetermined, are is the host species, geographic range, and range of species affected.

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