Comparative Medicine
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Vol 57, No 1
February 2007
Pages 97-104

Newly Identified *Mycobacterium* Species in a *Xenopus laevis* Colony

Denice Godfrey,[1,*] Heather Williamson,[2] Jerald Silverman,[1] and Pamela LC Small[2,*]

The University of Massachusetts Medical School maintains 3 separate research colonies of *Xenopus laevis*, with each colony located in a separate building on campus. After a 5-wk in-house quarantine period, 34 wild-caught *X. laevis* were transferred into one of the existing colonies. As a result, this colony grew from 51 to 85 frogs. All animals were housed in a recirculating frog housing system. During the first 2 mo, 6 frogs died suddenly, and health reports were generated for another 10 frogs in this colony. The majority of health reports were written in response to acute coelomic distention. These patterns continued until, after 1 y, only 25 of the original 85 animals remained. Necropsies performed showed large accumulations of serosanguinous fluid in the subcutaneous space or body cavity. Granulomatous inflammatory lesions with acid-fast bacilli were generally present in the liver, lung, or spleen. Culture of affected tissues grew *Mycobacterium* sp. within 40 d. Polymerase chain reaction analysis confirmed the isolated organism to be the same species of *Mycobacterium* (provisionally named *M. liflandii*) recently reported by 2 other groups. However, previous clinical publications suggested that this bacterium originated only from *X. tropicalis*. The cases we present highlight the rapidly lethal effects of *M. liflandii* in a colony of wild-caught *X. laevis* and illustrate the need to dedicate further attention to this emerging *Xenopus* disease.

Abbreviations: PCR, polymerase chain reaction; ppm, parts per million; UMMS, University of Massachusetts Medical School

Xenopus laevis, the African clawed frog, is an aquatic species native to South Africa. *X. laevis* is commonly used as a research model for developmental biology, toxicology, and cellular and molecular biology. It has become one of the most commonly used amphibians in research today.[11]

Mycobacteria are aerobic, nonmotile, acid-fast organisms that are commonly found in aquatic environments.[6] They can be isolated not only from water but also from soil, dust, and vegetation.[6] An estimated 50 species of *Mycobacterium* are thought to be agents of human disease.[18] Several species are considered important human pathogens, including the *M. tuberculosis* complex, *M. leprae*, and *M. ulcerans*. However, disease in humans also has resulted from many other species of *Mycobacterium*, including *M. cheonae, M. xenopi, M. marinum,* and *M. fortuitum*, all of which have been isolated from amphibians.[4,11,17,18] Species of *Mycobacterium* isolated from *X. laevis* include *M. marinum, M. chelonei,* and *M. xenopi.*[1,7,8,15] *M. ulcerans* has been shown to be capable of growth in *X. laevis* cell lines at low temperatures.[3]

Recently, a new species of *Mycobacterium* was isolated from a colony of *X. tropicalis*. This new species is most closely related genetically to *M. ulcerans* and *M. marinum* and has provisionally been named *M. liflandii*.[10,16] *M. liflandii* originally was reported to be an apparent natural pathogen of only *X. tropicalis*, with spread to *X. laevis* possible in captivity.[10,16] However, as noted by Mve-Obiang and colleagues,[10] the University of Massachusetts Medical School (UMMS) colony of wild-caught *X. laevis* experienced great morbidity and mortality in a facility devoid of any other *Xenopus* species. In light of the published clinical presentation and histopathologic findings in *X. tropicalis*,[10,16] we hypothesized that the UMMS colony of wild-caught *X. laevis* was infected with this new species of *Mycobacterium* despite having no contact with *X. tropicalis*. In this report, we document the details of the clinical presentation and subsequent diagnosis of disease caused by *M. liflandii* in the *X. laevis* colonies maintained at the UMMS.

Received: 30 Mar 2006. Revision requested: 24 Jul 2006. Accepted: 1 Aug 2006.
[1]Department of Animal Medicine, University of Massachusetts Medical School, Worcester, MA; [2]Department of Microbiology, University of Tennessee, Knoxville, TN.
[*]Corresponding authors. Email: denice.godfrey@umassmed.edu; psmall@utk.edu

Materials and Methods

Housing and husbandry. The UMMS has 3 rooms of adult female *X. laevis*, with each room in a separate building (Buildings A, B, and C). One of the 3 rooms (Building A) houses animals used by 3 different laboratories. The other 2 rooms are single-user rooms. Building B houses the only wild-caught frogs on campus. The other areas house only purpose-bred *X. laevis*. No other species of frog are housed anywhere in the school. Husbandry in each area is performed by different animal-care technicians, thus ensuring that crossover of personnel does not occur. Colony animals are housed in either static tanks (Buildings A and C) or a recirculating frog housing system (X-Mod, Marine Biotech, Beverly, MA, Buildings A and B). Static tanks are supplied with reverse osmosis water and 12.5 ml/gal of a salt solution (1740 g NaCl, 49.5 g $CaCl_2H_2O$, 25.3 g KCl, 10.5 g $NaHCO_3$, 2 l distilled water) and are changed a minimum of twice weekly. The recirculating system was maintained at a pH of 6.6 to 7.0, conductivity of 1550 to 1650 µS, ammonia levels of 0 to 0.8 parts per million (ppm), nitrites of 0 to 0.75 ppm, nitrates 0 to 20 ppm, and a flow rate of 4.5 to 5.5 gal/min. Room temperatures were maintained at approximately 20 °C, with humidity at 30% to 70%. Room lights were on a 12:12-h light:dark cycle. The frogs each were fed 3 to 4 pellets of Frog Brittle (Nasco, Ft Atkinson, WI) twice weekly. They were housed at densities of 1 frog/2 l in the static system and 1 frog/3.8 l in the recirculating system.

At the time of this report, all new frogs were routinely quarantined upon arrival, during which time they were housed in static tanks in an isolated room in Building A. Quarantine procedures consisted of general observation and a series of 3 ivermectin (Ivercide, Pheonix Pharmaceutical, St Joseph, MO) injections (200 µg/kg subcutaneously), with a 2-wk interval between treatments. The total length of quarantine was 5 wk, after which all healthy animals could be moved into the designated colony room.

Organism identification. Gross necropsies were performed in house, samples were prepared with hematoxylin and eosin and acid-fast stains, and histopathology slides were read by a board-certified contracted veterinary pathologist (Charles River Laboratories, Worcester, MA).

Tissue samples taken at the time of necropsy were submitted to IDEXX Preclinical Research Services (West Sacramento, CA) or to the State Tuberculosis Laboratory (Jamaica Plain, MA) for culture. IDEXX performed routine bacterial (not *Mycobacteria*-specific) and fungal cultures on submitted samples. The State Tuberculosis Laboratory performed *Mycobacteria* cultures by incubating specimens at 36 °C on 7H11 and LG slants and in MGIT liquid media.

Polymerase chain reaction (PCR) identification and characterization of *M. liflandii* in infected tissues was performed by the Department of Microbiology (University of Tennessee, Knoxville). The following regions were amplified with the described PCR primers: conserved regions of the 16S rRNA gene[16] (16S A, B, and C) with primers 16Sa1 (5' CGA ACG GGT GAG TAA CAC G 3'), 16Sa2 (5' CAC CTA CGA GCT CTT TAC G 3'), 16Sb1 (5' CGT TGT CCG GAA TTA CTG 3'), 16Sb2 (5' TGC ACA CAG GCC ACA AGG GA 3'), 16Sc1 (5' CGA TGC AAC GCG AAG AAC CTT 3'), and 16Sc2 (5' CGA TCC CAC CTT CGA CAG CT 3'); the enoyl reductase domain (ER) in *M. ulcerans mlsA* with primers ER1 (5' GAG ATC GGT CCC GAC GTC TAC 3') and ER2 (5' GGC TTG ACT CAT GTC ACG TAA G 3'); and *esxA* and *esxB* (encoding Esat6 and Cfp10 in the RD1 motif) from *M. marinum* with primers CFP101 (5' TGA CGG ATG TTC GTC GAA ATC 3'), CFP102 (5' TTT TGA AGA ACG ATG CCG CTA 3'), ESAT61 (5' CTT CTG CTG CAC ACC CTG GTA 3'), and ESAT62 (5' GAC AGA ACA GCA GTG GAA TTT CG 3'). The following primers were used for 2426 PCR[13] for genotype analysis: MU4 (5' ATC GCC GAA GCC TGG CGG AT 3') and MU9 (5' TCT TCG TCG TTT TGT GAT GCC 3'). The 2426 PCR system is based on the identification of 2 high-copy-number insertion sequences, IS2404 and IS2606, which show restriction fragment length polymorphism between *Mycobacterium* strains. Each PCR reaction had a final volume of 50 µl. Each 0.5 ml tube contained 1.0 µl *Taq* DNA polymerase (Promega, Madison, WI), 5.0 µl 10× PCR buffer (Promega), 1.0 µl 10 mM dNTPs (BioSeq, Woburn, MA), 1.0 µl each of the appropriate forward and reverse primers, 5.0 µl template DNA (~200 ng DNA per 50 µl PCR reaction), and 36 µl double-distilled H_2O. Amplification conditions were denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 1 min, annealing for 1 min at the temperature appropriate for the primer pair used, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. The annealing temperatures were 55 °C for the 16S RNA primers, 58 °C for enoyl reductase, 62 °C for CFP10, and 60 °C for ESAT 6 and 2426 PCR. Cloning and transformation of the PCR products were performed using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The resulting plasmid DNA was purified using Wizard Plus Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. Purified plasmid DNA was sequenced at the University of Tennessee Molecular Biology Research Facility (Knoxville, TN).

In addition to PCR, bacterial culture was performed at the University of Tennessee. Tissue samples were decontaminated using the modified Petroff method[8] and were inoculated onto Lowenstein-Jensen media. The media were allowed to incubate at 32 °C and 5% CO_2. After primary culture, strains were maintained on Middlebrook media with 10% oleic acid–dextrase–albumin supplement.

To isolate mycolactones, lipids were extracted from the cell wall of mycobacterial colonies by use of chloroform:methanol (2:1, vol:vol) followed by precipitation with ice-cold acetone to enrich for mycolactones.[5] The resulting acetone-soluble lipids were analyzed by silica thin-layer chromatography using a solvent system of chloroform:methanol:H_2O (90:10:1, vol:vol:vol) and visualized with cerric sulfate and ammonium molybdate in 2 M H_2SO_4.[10]

Cytopathicity assays were conducted on bacterial cultures or partially purified mycolactones by their addition to a semiconfluent monolayer of L929 murine fibroblasts in a 96-well plate. Cytopathicity was detected by cell rounding in 24 h followed by monolayer detachment by 48 h, as described.[2]

Case Reports

On 16 Mar 2004, 40 adult (110 to 170 g), female wild-caught (from South Africa) *X. laevis* frogs arrived at UMMS from a commercial vendor and were immediately placed in the isolated quarantine room. On 30 Mar 2004, 2 animals died acutely while in quarantine, and 1 animal was diagnosed with a minor growth of *Heteropolaria* sp. on the tips of the toes. Because of pronounced tissue autolysis, no useful information was gained from necropsy of the 2 animals that died suddenly. However, the stress of shipment was considered to have contributed to the deaths. On 20 Apr 2004, 3 more frogs were noted to have *Heteropolaria* growth on the tips of their toes. Diagnosis was made by microscopic examination of the organisms and was confirmed by histopathologic examination of biopsied toe samples. Fungal and routine bacterial cultures performed on toe skin samples from these animals revealed moderate growth of *Citrobacter freundii* complex, *Klebsiella oxytoca*, and *Aeromonas hydrophila*. No fungal organisms were noted.

On 21 April 2004, 34 of the 40 frogs were transferred out of quarantine to an investigator's existing frog colony (all wild-caught) housed in Building B. The 2 deaths and 4 cases of *Heteropolaria* growth represent the 6 animals not transferred into the colony. All animals housed in Building B belonged to 1 researcher, and these were the only wild-caught frogs housed on campus. The 34 new animals were added to tanks in a recirculating frog system (Marine Biotech, X-mod, Beverly, MA) that already held a total of 51 colony animals, thus bringing the colony total to 85 frogs. The investigator randomly placed the new frogs into the tanks of the X-Mod housing system. Most of the new frogs were placed into unoccupied tanks, but some went into tanks with pre-existing colony animals. No more than 6 frogs were housed in each 23-l tank.

During the following month, 3 frogs died acutely, with no obvious gross signs of disease. In addition, 5 health reports were generated for animals with various clinical presentations. The first of these animals had a bloated appearance. Two animals had localized areas of skin petechication or ulceration but no other signs. The fourth animal had a bloated appearance and an area of localized skin ulceration. The fifth animal had sustained trauma adjacent to the right eye. Skin samples were submitted for routine bacterial culture and fungal culture, which showed moderate growth of *Aeromonas* spp, *Enterobacter* spp, and *Klebsiella oxytoca*.

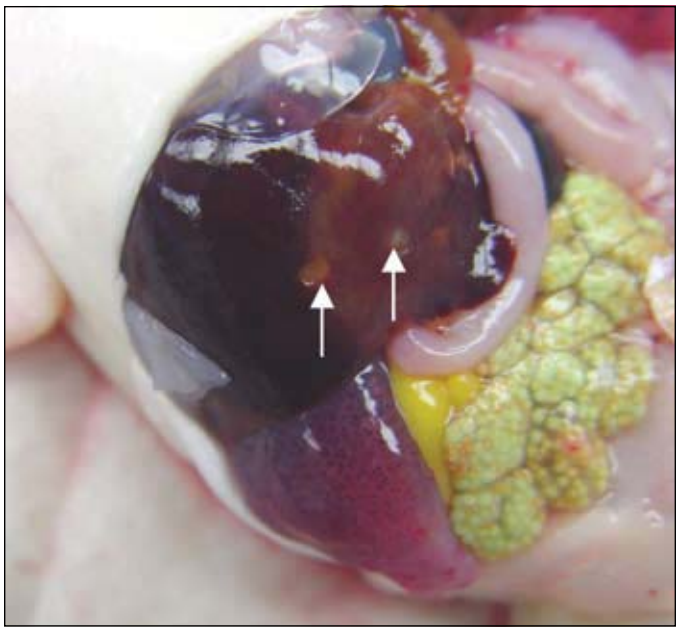

Figure 1. Liver of frog from case 1, showing grossly visible focal areas of palor (white arrows).

Treatment was initiated for the 3 animals with skin lesions and consisted of trimethoprim–sulfamethoxazole (Sulfamethoxazole and trimethoprim oral suspension, 200 mg and 40 mg per 5 ml; Hi-Tech Pharmacal, Amityville, NY) every other day at 15 ml per 2 gal water in a static tank.[19] The skin lesions on 2 of the frogs cleared and never returned after treatment; however, they both eventually died during the 6 mo that followed. The frog with both skin lesions and a bloated appearance became moribund within 24 h and was euthanized by injection of sodium pentobarbital (200 mg/kg) into the dorsal lymph sac. The frog with the eye wound was also euthanized. A necropsy was performed on both animals in addition to 1 of the frogs that died acutely. In addition, samples were submitted for routine bacterial and fungal cultures, which yielded *Aeromonas* spp, *Acinetobacter* spp, *Citrobacter* spp, *Enterobacter* spp, and *Klebsiella* spp were reported. Necropsy findings were as follows:

Case 1 (acute death). Gross observations included coelomic distention; serosanguinous coelomic fluid; mottled, irregular liver with multiple pale foci 1 to 3 mm in diameter (Figure 1); and congested lungs. Histopathologic findings included moderate, multifocal granulomatous inflammation and moderate, diffuse congestion in lungs; diffuse, marked nodular granulomatous inflammation in liver; and diffuse splenic congestion.

Case 2 (euthanized frog). Gross observations included deep ulcerative lesion adjacent to right eye, congested lungs, and hepatomegaly. Histopathologic findings were moderate necrohemorrhagic subcutaneous inflammation, with intralesional rod-shaped bacteria extending into underlying musculature; moderate, multifocal alveolar hemorrhage in lungs; and mild, multifocal granulomatous inflammation and mild extramedullary hematopoiesis in the liver.

Case 3 (euthanized frog). Gross findings were coelomic distention; localized facial petechiation and ulceration of the right maxillary region; diffuse, serosanguinous subcutaneous and coelomic fluid; and hepatomegaly. Histopathology revealed diffuse, subcutaneous necrohemorrhagic inflammation, with intralesional rod-shaped bacteria; markedly congested spleen; and moderate to marked diffuse granulomatous inflammation of the liver.

Figure 2. Frog from case 4, with accumulation of coelomic and subcutaneous fluid.

In light of the granulomatous inflammation in the necropsied animals, liver samples were resubmitted for acid-fast staining. Furthermore, the timely publication of an article documenting *M. ulcerans*-like infections in a colony of *X. tropicalis*[16] prompted us to investigate possible PCR testing for the bacteria. In the interim, during June 2004, health reports were generated for 5 additional animals with acute coelomic distention; 1 of these animals also had a localized area of skin irritation and swelling. There were also 3 acute deaths in June, and 3 animals with acute bloat were euthanized and underwent necropsy. The following findings were noted:

Case 4. Gross observations comprised coelomic distention (Figure 2), serosanguinous fluid in the body cavity and subcutaneous space, mild lung congestion, mottled liver and spleen, enlarged kidneys, and hemorrhagic feces in intestine. Histopathologic findings included minimal, diffuse, histiocytic pneumonia, with infrequent intralesional acid-fast bacilli; mild, diffuse lung congestion; hepatic necrosis with intralesional acid-fast bacilli; severe, diffuse splenic congestion with necrosis and abundant acid-fast bacilli; histiocytic, multifocal nephritis, with moderate, diffuse hemorrhage; and mild, diffuse congestion of the gastrointestinal tract.

Case 5. Gross findings included coelomic distention; serosanguinous subcutaneous fluid; 2-cm area of darkening of skin and underlying muscle ventral to the mandible, with central area of muscle necrosis; and bright green fecal material in gastrointestinal tract. Histopathology revealed mild, diffuse, subcutaneous inflammation with intralesional bacteria and ulceration of overlying epidermis; marked, diffuse, necrotizing myositis with intralesional bacteria (acid-fast stain not performed); moderate, diffuse, histiocytic hepatitis, with occasional intralesional acid-fast bacilli; splenic inflammation, with infrequent intralesional acid-fast bacilli; and mild, multifocal, histiocytic nephritis, with moderate, diffuse hemorrhage.

Case 6. Gross observations included coelomic distention; serosanguinous fluid in subcutaneous space and in body cavity; thickened pericardium with multinodular appearance and pericardial adhesions; congested lung; general mottling of and 3-mm raised, pale nodule on liver; and mottled kidneys. Histopathologic findings comprised epicardial and pericardial adhesions with granulomatous inflammation and intralesional bacteria (acid-fast stain not performed); mild to moderate, diffuse, histiocytic pneumonia;

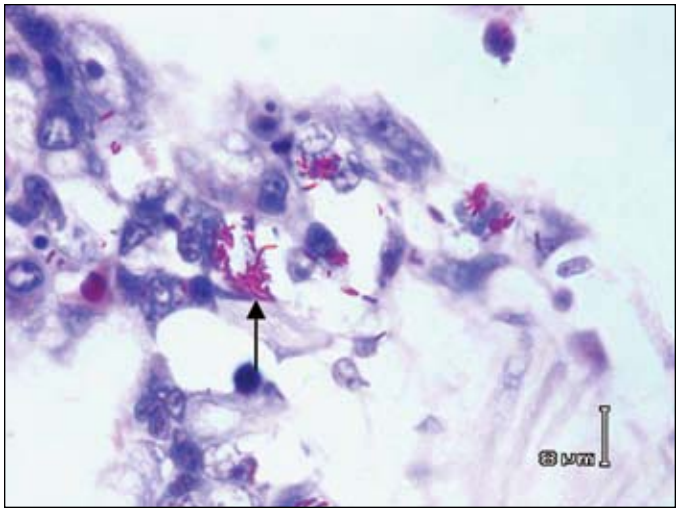

Figure 3. Acid-fast stain of liver from case 1, showing lavender-stained bacteria (dark arrow).

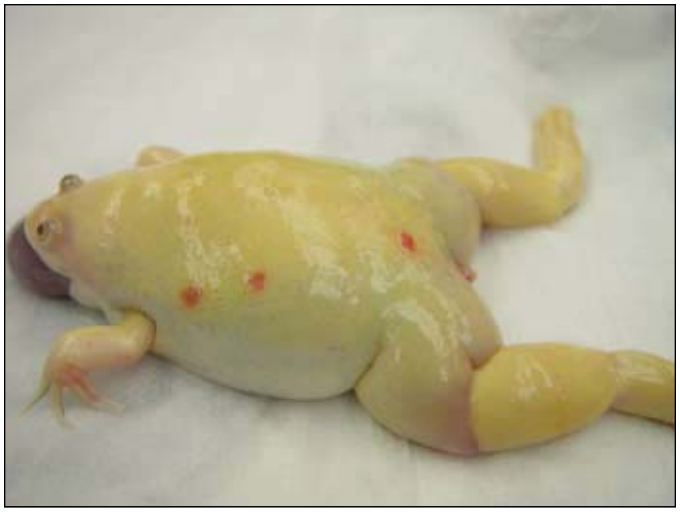

Figure 4. Affected frog with multiple raised, red foci on skin.

moderate to marked nodular, necrotizing, histiocytic hepatitis, with occasional intralesional acid-fast bacilli; and mild, diffuse, histiocytic nephritis with moderate, diffuse hemorrhage.

The liver samples from the first 3 necropsies also demonstrated acid-fast bacilli in histiocytes of all 3 frogs (Figure 3). At the time these necropsy reports arrived, another frog with a bloated appearance was found. This animal was the first and only animal noted also to have cutaneous raised, red foci on the skin (Figure 4). The animal was euthanized, and gross examination revealed hepatomegaly and lung congestion. Because tissue samples from other colony animals revealed findings consistent with mycobacterial infection, we sent liver, lung, and skin samples from this latest frog to the Massachusetts State Tuberculosis Laboratory for culture. By 40 d, acid-fast staining organisms from all tissues were identified as *Mycobacterium sp*. Additional liver, lung, and skin samples had been submitted to the Department of Microbiology (University of Tennessee, Knoxville) for PCR analysis; this lab was involved with the PCR identification of the previously identified *M. ulcerans*-like organism.[16] PCR analysis using probes to 16S RNA, mycolactone genes (*enoyl redutase*, *polyketide synthase*), RD1-associated genes encoding Esat6 and Cfp10 (*esxA* and *esxB*), and the intergenic regions between IS2404 and IS2606[14] were performed on the samples. As described later in the Results section, these analyses confirmed the identity of the same species of *Mycobacterium* described in previous publications,[10,15] which has now provisionally been named *M. liflandii*.

After receipt of the PCR results, investigator use of the affected frog colony ceased, and the room was quarantined. The investigator immediately ordered new animals from the same vendor, but this time brought in purpose-bred, rather than wild-caught, frogs. These new animals were housed in the same building (Building B) and were maintained under the primary investigator's original study protocol, but they were held in static cages in a separate room from the affected colony. The affected colony remained quarantined in the facility and was maintained under a new institutionally approved protocol, to gather additional information on this new species of *Mycobacterium*. Additional personal protective equipment—comprising plastic booties, gloves with full arm sleeves, a nonpermeable gown, face mask with shield, and a hair-covering—was required for entry into the room containing the affected colony. This room was also last in the room-entry order.

In July 2004, 2 additional frogs in the quarantined room in Building B developed acute coelomic distention and were euthanized; another 2 frogs died acutely. Tissue samples (including liver, lung, and spleen) were submitted from all 4 of these animals for PCR evaluation. All samples were positive for *M. liflandii*.

In August 2004, 2 animals in the quarantined colony developed coelomic distention, and 1 additional animal died acutely. A little over a month after their arrival at UMMS, the investigator's new room of replacement frogs also experienced 4 instances of sudden death. Although no animals had been transferred between the 2 rooms, the purpose-bred replacement animals had been brought to and treated in the same laboratory that had been used for work with the original, and now quarantined, group. The lab benches had all been cleaned with bleach prior to the arrival of the replacement colony, and it was not immediately clear whether the deaths in this new colony were related to the *Mycobacterium* findings in the now-quarantined room. To help determine the cause of death, samples from 1 of the frogs were submitted for PCR evaluation; tissues from the other 3 animals were in too poor a condition to evaluate. Results came back positive for *M. liflandii*. It remained unclear, however, whether the new animals arrived at the facility already infected or they had contracted the bacteria from the quarantined colony.

In September 2004, 3 more animals in the quarantined room were found with coelomic distention and were euthanized. In addition, 3 animals in this room died acutely. Only 56 of the original 85 animals remained at this time.

The sporadic cases of coelomic distention and sudden death in the quarantined colony continued over the next several months. At 1 y after the wild-caught animals were introduced, only 25 of the original 85 remained. All tissues evaluated from animals in this group revealed acid-fast organisms, primarily in the spleen, liver, or lungs. Further, all PCR analyses performed on tissues submitted from animals in this room came back positive for *M. liflandii*. During this year, several sporadic deaths also occurred in animals in the replacement frog room (Building B) as well as in a separate colony housed in Building A. Animals located in Building A were purpose-bred animals purchased from a different vendor than those housed in Building B. They were housed in both static and recirculating systems and cared for by a separate group of animal-care technicians. A few of these animals in

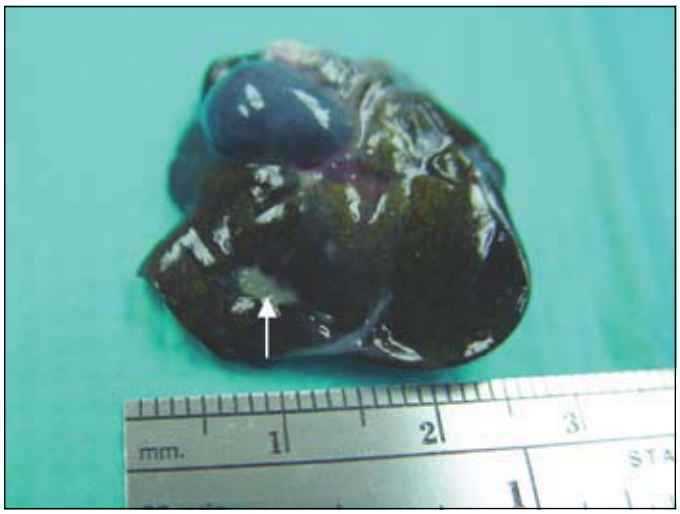

Figure 5. Liver from affected frog, with grossly visible 3-mm pale lesion (white arrow).

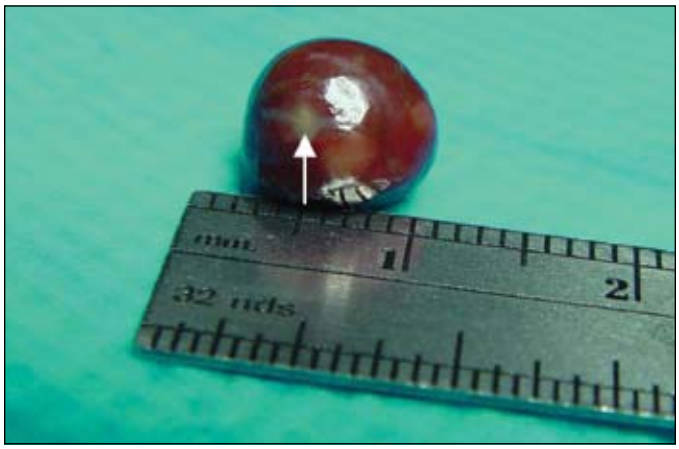

Figure 6. Spleen of affected frog, showing grossly visible 1- to 2-mm pale lesion (white arrow).

Building A showed gross evidence of granulomatous lesions in the liver, spleen, or lung (Figures 5 to 7) as well as acid-fast staining organisms on histopathologic examination and positive PCR findings consistent with *M. liflandii*. These cases from areas beyond the original quarantined room in Building B were sporadic and few. Whether and how the organism might have spread from the quarantined room to the other colonies, particularly those in a separate building, remains uncertain. One animal (which had been ordered from a purpose-bred colony of a different vendor) died while in postarrival quarantine, where it had contacted no other animals and had not been used for any studies. Postmortem analysis revealed positive PCR findings for *M. liflandii*.

Results

M. liflandii is characterized by a unique 16S RNA sequence as well as by the presence of *mlsA*, which encodes the lactone core of the mycolactone toxin. *M. liflandii* is further distinguished from *M. ulcerans* by the presence of *esxA* and *esxB,* which are lacking in most isolates of *M. ulcerans.* Multiple organs from each of the first 4 frogs submitted for analysis were PCR positive for *mlsA, esxA*, as well as for *esxB* (Figure 8). DNA sequence obtained after cloning the 16S RNA gene was 100% identical to that of *M. liflandii*

Figure 7. Lung from affected frog, with grossly visible 2- to 3-mm brown-gray foci (white arrows).

(GenBank accession number AY845224).

In addition, at the University of Tennessee, mycobacteria were isolated in pure culture from liver from 1 frog and kidney and spleen from a second frog after 30 d of incubation at 32 °C in 5% CO_2. All samples grew identical pure cultures of mycobacteria. DNA sequence of the 16S RNA gene obtained from 1 isolate, *M. liflandii* 1136, confirmed its identity as *M. liflandii.* Further strain characterization was obtained by genotyping using IS2426 PCR[14]. Although both *M. ulcerans* and *M. liflandii* contain the intervening sequence elements IS2404 and IS2606,[8] *M. liflandii* contains many fewer copies. IS2426 PCR analysis of *M. liflandii* 1136, the isolate obtained in this study, produced a pattern identical to that of the reference strain, *M. liflandii* XL5 (Figure 9).

Thin-layer chromatography of partially purified mycolactones from *M. liflandii* 1136 showed the presence of mycolactone E as a major lipid species, with a refractive index of 0.37 (Figure 10). The addition of purified mycolactone E from *M. liflandii* 1136 produced typical mycolactone-mediated cytopathicity in cultures of L929 cells, characterized by cell rounding in 24 h followed by monolayer detachment (data not shown).

In summary, a total of 23 necropsies were performed on frogs from the UMMS wild-caught colony. Of those 23 animals, 5 presented with gross coelomic distention; 5 presented with visible mottling of the liver, lung, or spleen; 7 presented with coelomic distention and visible mottling of the liver, lung or spleen; 1 presented with coelomic distention, raised skin lesions, and visible mottling of the liver, lung, or spleen; and 5 showed no gross lesions. Samples from 12 of these 23 animals were submitted to the veterinary pathologist for examination, and all 12 frogs had acid-fast organisms. Samples from 3 of the 23 animals were sent to the Massachusetts Tuberculosis Laboratory for culture. All were positive for *Mycobacterium*. All samples of the 9 frogs from this colony that were analyzed at the University of Tennessee showed PCR and mycolactone properties consistent with *M. liflandii*.

Discussion

Mycobacterium spp can cause devastating disease in humans and animals. Recently, increasing attention in human medicine has been placed on nontuberculous *Mycobacterium* due to increasing prevalence of AIDS and use of immunosuppressive therapies, which lead to increased risk of infection.[18] One organism that has gained much recognition is *M. ulcerans*, the causative

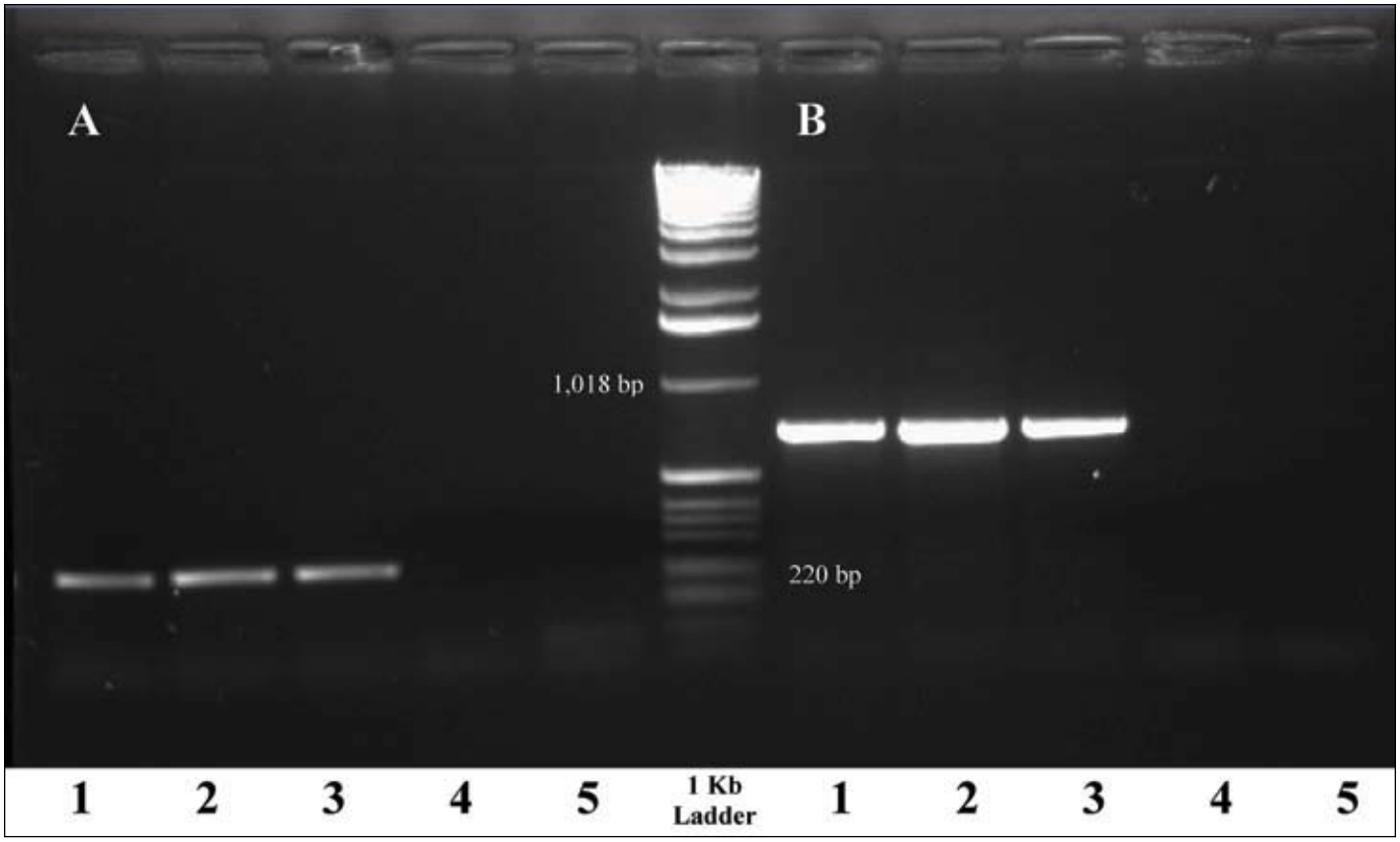

Figure 8. (A) PCR detection of *esxB* (226 bp). Lanes: 1, *M. liflandii* 1136; 2, *M. liflandii* 128; 3, *M. marinum* 1218; 4, *M. ulcerans* agy99; 5, no-DNA negative control. (B) PCR detection of enoyl reductase (*mlsA*, 720 bp). Lanes: 1, *M. liflandii* 1136; 2, *M. liflandii* 128; 3, *M. ulcerans* agy99; 4, *M. marinum* 1218; 5, no-DNA negative control.

agent of Buruli ulcers. In sub-Saharan Africa, many countries are now deemed endemic for Buruli ulcer disease; in some regions prevalence rates are reportedly 150 to 280 per 100,000 people. Buruli ulcers can be devastating, not only because of the potentially painful and disfiguring ulcerative lesions present in advanced disease but also because of the social stigmatization that follows. In some regions, as many as half of those who are treated for Buruli ulcers are left with functional limitations.[9,17]

In *X. laevis*, disease resulting from infection with any of several species of *Mycobacterium* (for example, *M. marinum*, *M. fortuitum*, *M. xenopi*) can be severe. Clinical presentation can include lethargy, weight loss, cutaneous nodules or areas of ulceration, and single to multiple nodules in the subcapsular region of the liver, spleen, kidney, and other organs.[11,12,15] Histologically, granulomas are noted in the location of the gross nodules and generally are composed of macrophages surrounded by epitheliod cells and fibroblasts, with acid-fast bacilli.[12,15]

M. liflandii, an *M. ulcerans*-like bacterium, was discovered recently in colonies of *X. tropicalis* across the United States[10,16] and, as we report here, in wild-caught *X. laevis* at UMMS. In the UMMS colony 60 of 85 (70.6%) of wild-caught *X. laevis* housed in a recirculating frog housing system were lost in 1 y. In contrast to the disease reported in *X. tropicalis*, generalized cutaneous ulcers were not a primary component of disease in the *X. laevis* colony. The 2 animals with focal areas of skin ulcerations that responded favorably to treatment with trimethoprim–sulfamethoxazole had local infections due to organisms other than *Mycobacterium* sp. However, underlying pathology due to *M. liflandii* may have made the animals more susceptible to these cutaneous infections. The culture of *Aeromonas* spp, *Acinetobacter* spp, *Citrobacter* spp, and *Enterobacter* spp from these frogs may have been incidental findings,[12] but as noted previously, increased susceptibility to commensal organisms may occur in animals infected with *M. liflandii*. Only 1 of the 60 affected animals developed raised skin lesions that contained mycobacterial organisms; few subsequent animals showed any evidence of ulcerative lesions on the skin.

Consistent with the disease in *X. tropicalis* was the finding of acute coelomic effusion in *X. laevis*. Approximately half of the clinical cases in *X. laevis* had acute coelomic distention, and half died acutely with no prior clinical signs. In some cases, treatment was attempted with either trimethoprim–sulfamethoxazole or 25 g/L NaCl baths. However, treatment proved futile once animals were found with a bloated appearance, and death generally followed in 1 to 3 d. Therefore, animals routinely were euthanized and taken to necropsy as soon as evidence of coelomic distention appeared.

The hypothesis that the morbidity and mortality observed in the UMMS frogs was caused by the same species of *Mycobacterium*, *M. liflandii*, previously reported in *X. tropicalis* was supported not only by the clinical presentation (particularly the finding of coelomic distention) but also by histopathologic findings. The histopathologic findings of acid-fast organisms in granulomas (primarily within liver, spleen, and lung) in this colony of *X. laevis* were similar to those reported for *X. tropicalis*.[16] In addition, PCR analysis of tissue samples from affected organs were performed using probes to 16S RNA, mycolactone genes (*enoyl redutase*,

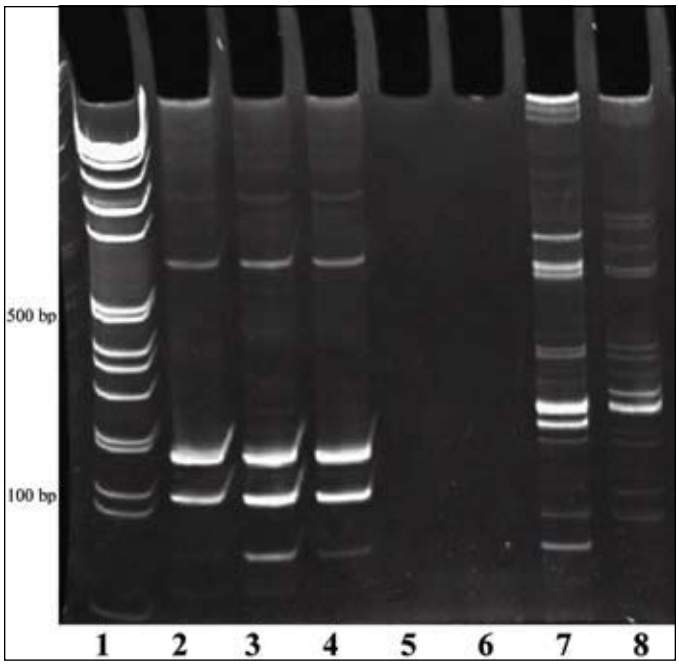

Figure 9. Genotype analysis of *M. liflandii* and *M. ulcerans* isolates by 2426 PCR. Lanes: 1, 100-base-pair ladder; 2 *M. liflandii* 1136; 3, *M. liflandii* 128; 4, *M. liflandii* XL5; 5, *M. marinum;* 6, no-DNA negative control; 7, *M. ulcerans* 1615; 8, *M. ulcerans* agy99.

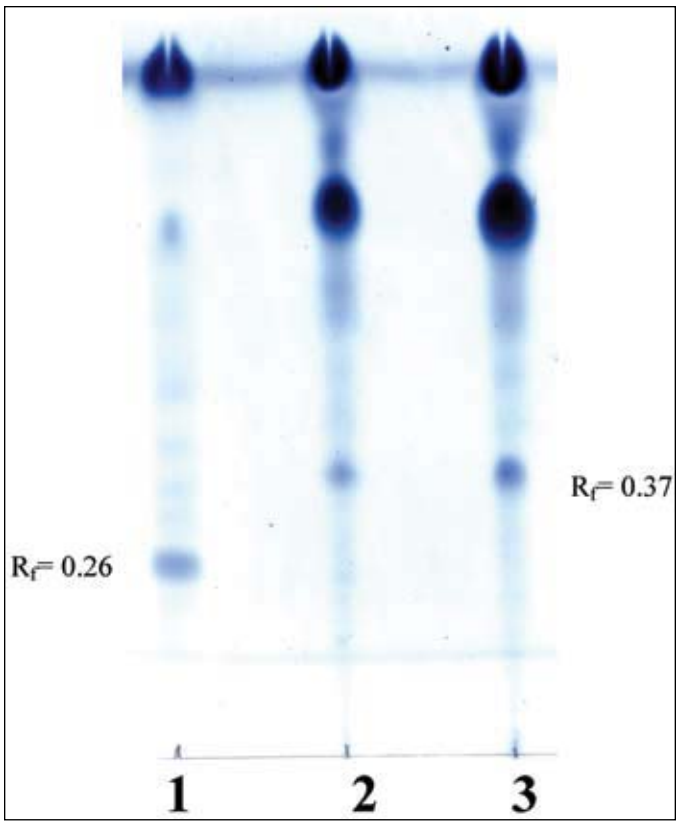

Figure 10. Identification of mycolactone E (refractive index, 0.37) in acetone-soluble lipid cell extracts by thin-layer chromatography. Lanes: 1, mycolactone A/B, *M. ulcerans* 1615; 2, mycolactone E, *M. liflandii* XL5; 3, mycolactone E, *M. liflandii* 1136.

polyketide synthase), the RD1-associated genes encoding Esat6 and Cfp10 (*esxA* and *esxB*), and the internal transcribed spacer region. These tests identified the presence of *M. liflandii.*

Several PCR probes have been used for the identification of *M. ulcerans*-like mycobacteria in aquatic samples. The recent demonstration of IS2404 in many aquatic species of mycobacteria[13] has decreased the usefulness of this probe. Although amplification of the internal transcribed spacer region is useful, the fact that it is present in single copy suggests that its diagnostic utility will be less than that of multicopy target regions. The advantages of a mycolactone-based probes, such as the *enoyl redutase* probe described here, are that they are present in 6 to 8 copies per bacterium and are based on a virulence determinant. The *enoyl redutase* probe also will detect *M. ulcerans* and thus is not species-specific.

Although 70.6% of the wild-caught animals died due to *M. liflandii* in 1 y, few cases of coelomic distention or sudden death occurred in the purpose-bred colonies. Remaining questions are whether the wild-caught animals carried a higher bacterial burden that led to increased morbidity, whether the 2 groups developed differing immune responses contributing to differences in morbidity, and whether the wild-caught animals were the source for the sporadic infections that occurred in other colonies. However, the 1 positive finding of *M. liflandii* from a newly acquired purpose-bred animal in quarantine does suggest that *M. liflandii* may exist at subclinical levels in at least 1 established, commercial purpose-bred colony. Clinical disease, as seen in the cases reported here, may be induced by factors such as shipment, change in housing or environmental conditions, increased handling, and other research-related manipulations or treatments. Cooperation from the vendor will be necessary to define the epidemiology. In addition, further investigation into the epidemiology of *M. liflandii* in wild *X. laevis* colonies would help to reveal the prevalence and origin of *M. liflandii*. Although *M. liflandii* is clearly an important frog pathogen, its potential as a human pathogen is unknown. To date, human infection has not been reported, and no UMMS employee has reported any clinical lesions or illness that could be attributed to the bacterium.

Since the initial outbreak of *M. liflandii* in the wild-caught colony, environmental samples from the housing system were collected and evaluated. Water and biofilm samples from a frog tank, the particulate filter, a supply line located after the particulate filter and immediately prior to the ultraviolet light source, the supply line immediately after the ultraviolet light source, and the main supply line were submitted for both general bacterial and mycobacterial culture. Cultures yielded positive results for *Aeromonas* spp, *Pseudomonas* spp, *Bacillus* spp, nonhemolytic *Staphylococcus*, and *Mycobacterium* from all locations except immediately after the ultraviolet light. The sample taken immediately after the ultraviolet light contained only nonhemolytic staphylococci and *Mycobacterium*. PCR and culture tests also were performed on swab samples from the tank and supply line, water and filtrate samples before and after exposure to ultraviolet light, biofilter beads, and aquarium water. Of the 7 samples, 5 were found positive for *M. liflandii* DNA by PCR using the previously described probes; the 2 negative samples were the biofilter beads and aquarium water.

Most of the environmental samples were heavily contaminated with fast-growing microbes, making isolation of slow-growing *M. liflandii* impossible. The only environmental sample that yielded a positive culture of *M. liflandii* was obtained from water exposed to ultraviolet light. This finding indicates that the standard ultraviolet-light source (sterilization rate of 225,000 µW-sec/cm^2, light

changed every 6 mo) in the recirculating system was not sufficient to remove *M. liflandii* from the water. Overgrowth of fast-growing organisms present in most other samples made it impossible to culture *M. liflandii*. Although mycobacteria are normally sensitive to ultraviolet light, this sensitivity is likely to be decreased if clumps of organisms are present. Ultraviolet light may have inhibited the growth of some of the rapid-growing organisms without killing *M. liflandii* present in clumps.

The affected UMMS wild-caught animals have since been depopulated and the recirculating housing system decontaminated by treating the system with bleach at 200 parts per million, followed by treatment with 1% Virkon (Antec Biosentry, Sudbury, Suffolk, UK) for 24 h. The system then was rinsed multiple times with reverse osmosis water and once with 70% denatured alcohol. However, more research is needed to explore appropriate means of preventing spread of the bacterium, particularly when recirculating housing systems are used.

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