

Pathogenic *Elizabethkingia miricola* Infection in Cultured Black-Spotted Frogs, China, 2016

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Multiregional outbreaks of meningitis-like disease caused by *Elizabethkingia miricola* were confirmed in black-spotted frog farms in China in 2016. Whole-genome sequencing revealed that this amphibian *E. miricola* strain is closely related to human clinical isolates. Our findings indicate that *E. miricola* can be epizootic and may pose a threat to humans.

Elizabethkingia is a genus of gram-negative, nonmotile, non-spore-forming bacilli occasionally associated with human clinical infections (1–6). Although *E. meningoseptica* is the most commonly identified nosocomial pathogen of the genus (2), many descriptions of this species are misidentifications of *E. anophelis* and *E. miricola* (3–5). *E. anophelis*, initially isolated from the midgut of mosquitoes, caused a large outbreak centered in Wisconsin during 2015–2016 (5). *E. miricola* was found in 2003 in condensation water at the Mir space station (7). The first reported case of *E. miricola* infection was in a hematology patient in the United States in 2008 (8). Subsequently, *E. miricola* has been increasingly documented as causing bacteremia and sepsis in immunocompromised and immunocompetent patients, mostly in European countries (6). Until now, pathogenic *E. miricola* has seldom been isolated from Asia, and whether *E. miricola* can be pathogenic to animals is unknown.

The black-spotted frog, *Pelophylax nigromaculatus*, is a typical amphibian species, largely endemic to east Asia. Owing to the success of rearing it on an artificial diet, this frog has been widely farmed under special government approval as an edible animal in south-central China in recent years. In 2016, epidemic meningitis-like disease outbreaks in cultured black-spotted frogs occurred in separate farms. We identified *E. miricola* as the predominant pathogen and used whole-genome sequencing (WGS) to further characterize this Asian epizootic isolate and phylogenetically compare it with the available typical *Elizabethkingia* genomes.

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The Study

Since May 2016, many black-spotted frogs in farms in Hunan Province in south-central China have experienced an emerging, contagious disease characterized mainly by severe neurologic dysfunction. The first clinical sign is intermittent swimming in circles. Thereafter, the frogs develop signs of torticollis (Figure 1, panel A), disorientation (Video, <https://wwwnc.cdc.gov/EID/article/23/12/17-0942-V1.htm>), and anepithymia or meteorism (Figure 1, panel E). These signs are followed by cataracts (Figure 1, panel C); proptosis or hyperemia (Figure 1, panels B, D); agitation or lethargy; and, ultimately, death. The frogs are farmed in artificial ecologic wetlands or ponds with running water and shelter (online Technical Appendix Figure 1, <https://wwwnc.cdc.gov/EID/article/23/12/17-0942-Techapp1.pdf>). Most ponds in 1 farm, which share a common water supply, were infected sequentially within a short time. More than 60% of the frogs in the infected farms had signs of varying appearance, and 60%–90% of the diseased frogs died in the next few days or weeks. The disease continued until hibernation and returned the following spring.

During July–October 2016, we collected 213 abnormal frogs from 7 separate farms in Hunan Province, China (online Technical Appendix Figure 2). Histopathologic examination showed severe meningitis with denatured, incassate meninges. We observed inflammatory infiltrates, moderate multifocal gliosis, and perivascular cuffing in the cerebellum (online Technical Appendix Figure 3). Results of the diagnostic tests for *Batrachochytrium dendrobatidis* and ranaviruses were negative (Table 1). Although we observed Myxosporidia protozoa in the gallbladder and some protists in the intestine, they were not identified as the etiologic agents, considering the proportion of infection (online Technical Appendix Figure 4).

We confirmed bacterial infections in 190 (89.2%) of the 213 frogs; 90% were *E. miricola* according to the 16S rRNA gene sequence, which shared 99.36%–99.86% similarity with *E. miricola* DSM14571 (online Technical Appendix). We selected bacterial strain FL160902, isolated from frog no. 160, as the representative isolate and conducted experimental pathogenicity testing by various infection routes, including intramuscular injection, immersion infection, and cohabitation with infected frogs. All animal handling was done in compliance with the National Institutes of Health protocols (online Technical Appendix). After 2 weeks of observations

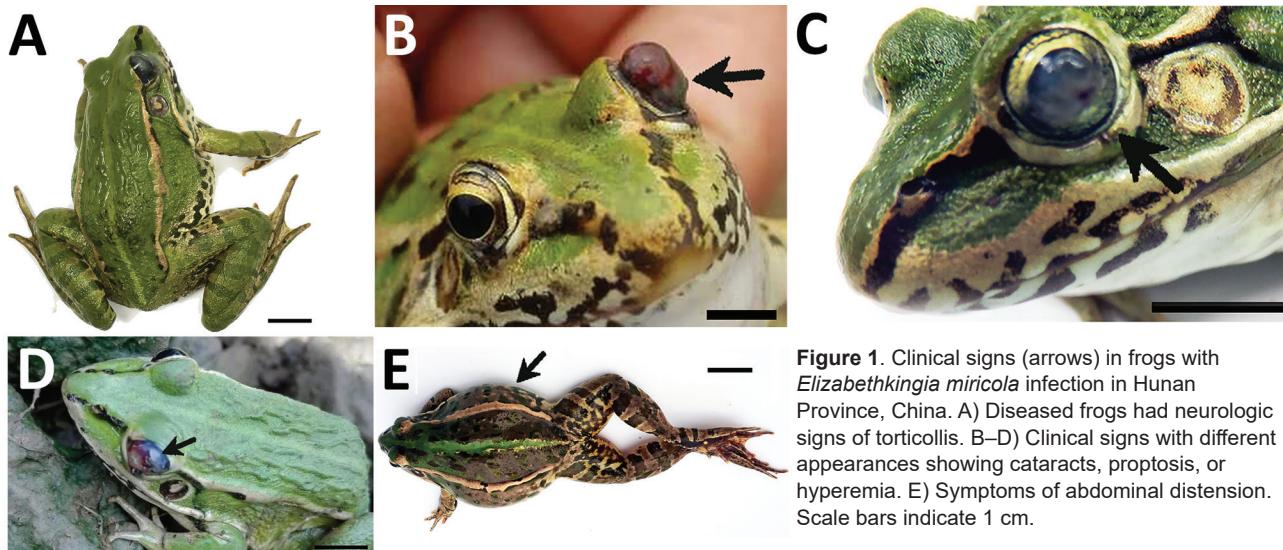


Figure 1. Clinical signs (arrows) in frogs with *Elizabethkingia miricola* infection in Hunan Province, China. A) Diseased frogs had neurologic signs of torticollis. B–D) Clinical signs with different appearances showing cataracts, proptosis, or hyperemia. E) Symptoms of abdominal distension. Scale bars indicate 1 cm.

(Table 2), we found that the cumulative mortality (10%–70%) increased with dose in the injection trial and that 100% of frogs exposed to *E. miricola* by immersion died. In the cohabitation studies, 30% mortality was recorded, indicating cross-infection. Koch's postulates were satisfied by identification of isolates from dead frogs as *E. miricola*, identical to FL160902.

To characterize *E. miricola* FL160902, we conducted WGS with the Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA), producing 2 × 150-bp paired-end reads. We assembled the trimmed reads using SOAPdenovo (<http://soap.genomics.org.cn/soapdenovo>).

We constructed a phylogenetic tree (Figure 2) of orthologous genes using RAxML (9) with 100 bootstrap replicates to examine the evolutionary relatedness between *E. miricola* FL160902 (GenBank accession no. NHPR000000000) and other *Elizabethkingia* genomes. The results showed that FL160902 was most closely related to CSID_3000517120, a clinical isolate of *E. miricola* from the United States sequenced by the Centers for Disease Control and Prevention (CDC) (10), revealing the potential of *E. miricola* FL160902 for pathogenicity in humans.

Before WGS was commonly used, *E. meningosep-*

Table 1. Results from etiologic detection in 213 frogs collected in Hunan, China, July–October 2016*

Pathogen	Tested organ									No. positive
	Skin	Liver	Spleen	Kidney	Brain	Intestine	Muscle	Gallbladder	Heart	
Bacteria	NT	+	+	+	+	NT	NT	NT	NT	190†
Parasite‡	–	–	–	–	–	–	–	+	–	9
Fungus§	–	NT	NT	NT	NT	NT	NT	NT	NT	0
Ranaviruses	NT	NT	–	–	NT	NT	–	NT	NT	0

* NT, not tested; +, positive; –, negative.

†Predominant bacterial infection. The results were considered positive if any one of the tested organs was positive.

‡Class Myxosporea.

§*Batrachochytrium dendrobatidis*.

Table 2. Results of the experimental exposure of frogs to *Elizabethkingia miricola* isolate FL160902, China, 2016*

Route of infection	Concentration, CFU/mL	No. frogs per trial	Cumulative no. deaths, by days after exposure†							Mortality, %
			2	4	6	8	10	12	14	
Intramuscular injection‡	10 ⁵	10	0	1	1	1	1	1	1	10
	10 ⁶	10	0	0	1	1	5	5	5	50
	10 ⁷	10	1	3	6	7	7	7	7	70
	SPSS§	10	0	0	0	0	0	0	0	0
Immersion inoculation¶	10 ⁵	10	3	7	10	10	10	10	10	100
Cohabitation inoculation#	NA	10	0	0	1	3	3	3	3	30
Control	NA	10	0	0	0	0	0	0	0	0

*NA, not applicable.

†Deaths after 14 d were not included.

‡Injection volume 200 µL.

§An equivalent volume injection of 0.70% stroke-physiologic saline solution.

¶Immersed for 30 min in *E. miricola* suspension.

#Frogs in this trial cohabited with frogs previously infected with *E. miricola*.

tica (previously *Flavobacterium meningosepticum*) was found to be separated into 2 main hybridization groups, UBI and UBII, that were ≈40%–55% interrelated; UBII could be further divided into 4 subgroups (11,12). However, because the isolates from different groups are phenotypically very similar, these genomic groups remain assigned at this time to *E. meningoseptica* (13). In our

phylogenetic tree, UBI group *E. meningoseptica* isolates did not group with the other *Elizabethkingia* spp. and were distantly related to UBII. Considering the low DNA–DNA relatedness (<70%) between the 2 groups and phylogenomic analysis based on WGS (3,11,12), we propose that UBII are not *E. meningoseptica*. The UBII subgroups branching separately supports the view

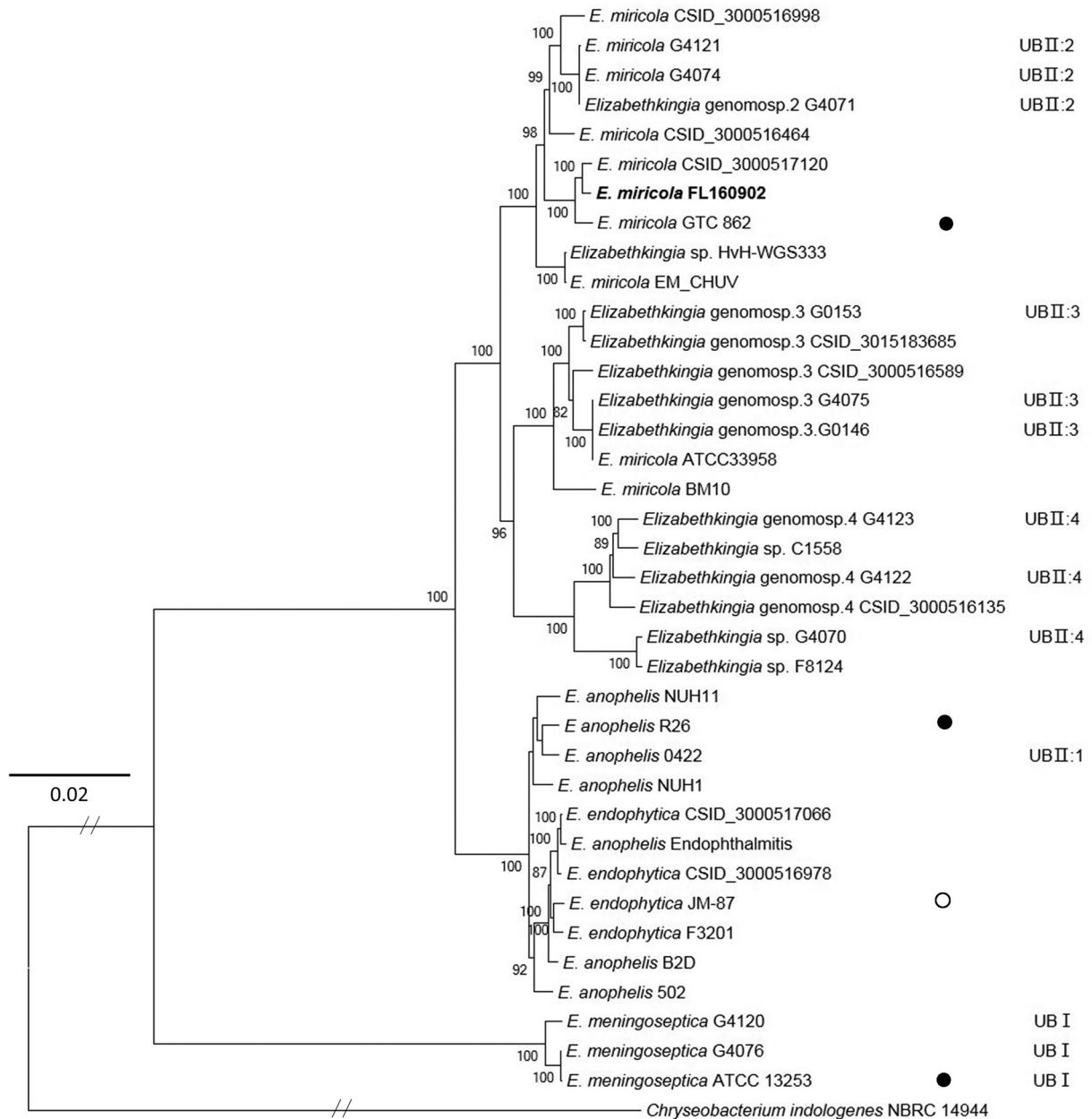


Figure 2. Maximum-likelihood phylogenetic tree of *Elizabethkingia miricola* FL160902 from an infected frog in Hunan Province, China, and reference genomes. The tree was constructed by using the single-copy orthologous genes of all the 38 genomes with 100 bootstrap replicates. Species identifications strictly followed the National Center for Biotechnology Information submitted names. Isolates assigned into UB groups and subgroups are according to Holmes et al. (12) and Bruun and Ursing (13). Solid circles indicate type strains; open circle indicates a former type strain. Bold indicates strain isolated in this study. Scale bar indicates nucleotide substitutions per site.

that they are different *Elizabethkingia* species (3). The UBII:1 group species *E. anophelis* and *E. endophytica* formed a clade with strong support of 100%, favoring the suggestion that *E. endophytica* is a later subjective synonym of *E. anophelis* (14). Our FL160902 isolate grouped with *E. miricola*, which is thought to be closely related to UBII:2 (3,10). The taxonomic status of *E. miricola* ATCC 33958 and BM10 should be reconsidered because they clustered with UBII:3 and not with UBII:2 *E. miricola* species. Our results agree with Eriksen's conclusion about the genetic diversity in *Elizabethkingia*; a more comprehensive taxonomic system is needed to clarify the *Elizabethkingia* genus (3).

Conclusions

In this natural outbreak of meningitis-like disease in cultured frogs in Hunan Province, China, in 2016, *E. miricola* was the most predominant pathogen. The neurologic signs and pathologic brain lesions suggested that *E. miricola* could break through the blood–brain barrier and damage the nervous system. The etiologic analyses combined with the results of experimental challenge support the conclusion that the *E. miricola* strain represented by isolate FL160902 is highly contagious for frogs, especially by immersion infection. We suspect that contaminated water is the primary vehicle of transmission, considering the infection assay and the epidemiology in 1 farm with different ponds. However, diverse transmission routes might be involved because there is no obvious interconnectivity among independent farms, which needs to be investigated further. Close attention should be paid to whether this disease affects the wild population of amphibians. Our results indicated the gradual expansion of its host and suggest that amphibians may serve as a reservoir for infection in humans. Black-spotted frog farming is a major aquaculture industry in south-central China; thus, animals and humans that have close contact with infected frogs should be continually monitored for emerging *E. miricola* infections, even though no human *E. miricola* infection cases were reported related to frog consumption or farming in Hunan in 2016. Our results demonstrate a contagious disease in frogs caused by *E. miricola* that poses a potential zoonotic threat to humans, generating a need for consideration of the role of *Elizabethkingia* bacteria in public health.

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References

1. Kim KK, Kim MK, Lim JH, Park HY, Lee ST. Transfer of *Chryseobacterium meningosepticum* and *Chryseobacterium miricola* to *Elizabethkingia* gen. nov. as *Elizabethkingia meningoseptica* comb. nov. and *Elizabethkingia miricola* comb. nov. *Int J Syst Evol Microbiol*. 2005;55:1287–93. <http://dx.doi.org/10.1099/ijs.0.63541-0>
2. Moore LSP, Owens DS, Jepson A, Turton JF, Ashworth S, Donaldson H, et al. Waterborne *Elizabethkingia meningoseptica* in adult critical care. *Emerg Infect Dis*. 2016;22:9–17. <http://dx.doi.org/10.3201/eid2201.150139>
3. Eriksen HB, Gumpert H, Faurholt CH, Westh H. Determination of *Elizabethkingia* diversity by MALDI-TOF mass spectrometry and whole-genome sequencing. *Emerg Infect Dis*. 2017;23:320–3. <http://dx.doi.org/10.3201/eid2302.161321>
4. Lau SK, Chow WN, Foo CH, Curreen SO, Lo GC, Teng JL, et al. *Elizabethkingia anophelis* bacteremia is associated with clinically significant infections and high mortality. *Sci Rep*. 2016;6:26045. <http://dx.doi.org/10.1038/srep26045>
5. Perrin A, Larssonneur E, Nicholson AC, Edwards DJ, Gundlach KM, Whitney AM, et al. Evolutionary dynamics and genomic features of the *Elizabethkingia anophelis* 2015 to 2016 Wisconsin outbreak strain. *Nat Commun*. 2017;8:15483. <http://dx.doi.org/10.1038/ncomms15483>
6. Opota O, Diene SM, Bertelli C, Prod'homme G, Eckert P, Greub G. Genome of the carbapenemase-producing clinical isolate *Elizabethkingia miricola* EM_CHUV and comparative genomics with *Elizabethkingia meningoseptica* and *Elizabethkingia anophelis*: evidence for intrinsic multidrug resistance trait of emerging pathogens. *Int J Antimicrob Agents*. 2017;49:93–7. <http://dx.doi.org/10.1016/j.ijantimicag.2016.09.031>
7. Li Y, Kawamura Y, Fujiwara N, Naka T, Liu H, Huang X, et al. *Chryseobacterium miricola* sp. nov., a novel species isolated from condensation water of space station Mir. *Syst Appl Microbiol*. 2003;26:523–8. <http://dx.doi.org/10.1078/072320203770865828>
8. Green O, Murray P, Gea-Banacloche JC. Sepsis caused by *Elizabethkingia miricola* successfully treated with tigecycline and levofloxacin. *Diagn Microbiol Infect Dis*. 2008;62:430–2. <http://dx.doi.org/10.1016/j.diagmicrobio.2008.07.015>
9. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*. 2014;30:1312–3. <http://dx.doi.org/10.1093/bioinformatics/btu033>
10. Nicholson AC, Humrighouse BW, Graziano JC, Emery B, McQuiston JR. Draft genome sequences of strains representing each of the *Elizabethkingia* genomospecies previously determined by DNA–DNA hybridization. *Genome Announc*. 2016;4:e00045-16. <http://dx.doi.org/10.1128/genomeA.00045-16>
11. Ursing J, Bruun B. Genetic heterogeneity of *Flavobacterium meningosepticum* demonstrated by DNA–DNA hybridization. *Acta Pathol Microbiol Immunol Scand B*. 1987;95:33–9.
12. Holmes B, Steigerwalt AG, Nicholson AC. DNA–DNA hybridization study of strains of *Chryseobacterium*, *Elizabethkingia* and *Empedobacter* and of other usually

- indole-producing non-fermenters of CDC groups IIc, IIe, IIh and III, mostly from human clinical sources, and proposals of *Chryseobacterium bernardetti* sp. nov., *Chryseobacterium carnis* sp. nov., *Chryseobacterium lactis* sp. nov., *Chryseobacterium nakagawai* sp. nov. and *Chryseobacterium taklimakanense* comb. nov. *Int J Syst Evol Microbiol.* 2013;63:4639–62. <http://dx.doi.org/10.1099/ijss.0.054353-0>
13. Bruun B, Ursing J. Phenotypic characterization of *Flavobacterium meningosepticum* strains identified by DNA-DNA hybridization. *Acta Pathol Microbiol Immunol Scand B.* 1987;95:41–7.
14. Doijad S, Ghosh H, Glaeser S, Kämpfer P, Chakraborty T. Taxonomic reassessment of the genus *Elizabethkingia* using whole-genome sequencing: *Elizabethkingia endophytica* Kämpfer et al. 2015 is a later subjective synonym of *Elizabethkingia anophelis* Kämpfer et al. 2011. *Int J Syst Evol Microbiol.* 2016;66:4555–9. <http://dx.doi.org/10.1099/ijsem.0.001390>

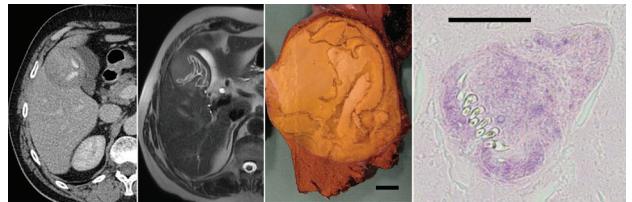
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- Variably Protease-Sensitive Prionopathy, a Unique Prion Variant with Inefficient Transmission Properties

- Geographic Divergence of Bovine and Human Shiga Toxin–Producing *Escherichia coli* O157:H7 Genotypes, New Zealand
- Bacterial Pathogens Associated with Hidradenitis Suppurativa, France
- Replication and Shedding of MERS-CoV in Upper Respiratory Tract of Inoculated Dromedary Camels
- Transmission Characteristics of Variably Protease-Sensitive Prionopathy
- Seroconversion for Infectious Pathogens among UK Military Personnel Deployed to Afghanistan, 2008–2011
- Circulation of Reassortant Influenza A(H7N9) Viruses in Poultry and Humans, Guangdong
- Molecular Evolution of Peste des Petits Ruminants Virus Province, China, 2013
- Effects of Knowledge, Attitudes, and Practices of Primary Care Providers on Antibiotic Selection, United States
- Accuracy of Herdsmen Reporting versus Serologic Testing for Estimating Foot-and-Mouth Disease Prevalence
- Residual Infestation and Recolonization during Urban *Triatoma infestans* Bug Control Campaign, Peru
- Two *Anaplasma phagocytophilum* Strains in *Ixodes scapularis* Ticks, Canada
- *Francisella tularensis* Bacteria Associated with Feline Tularemia in the United States
- Avian Bornavirus in Free-Ranging Psittacine Birds, Brazil
- Gouleako and Herbert Viruses in Pigs, Republic of Korea, 2013
- Human Infection with Influenza Virus A(H10N8) from Live Poultry Markets, China, 2014
- Molecular Epidemiology of Influenza A(H1N1)pdm09 Virus among Humans and Swine, Sri Lanka
- Novel Amdoparvovirus Infecting Farmed Raccoon Dogs and Arctic Foxes
- Novel Porcine Epidemic Diarrhea Virus Variant with Large Genomic Deletion, South Korea
- MERS Coronavirus Neutralizing Antibodies in Camels, Eastern Africa, 1983–1997
- Equine Influenza A(H3N8) Virus Infection in Cats
- *Echinococcus ortleppi* Infections in Humans and Cattle, France
- Human Hantavirus Infections in the Netherlands
- *Mycobacterium* Species Related to *M. leprae* and *M. lepromatosis* from Cows with Bovine Nodular Thelitis
- Human Metapneumovirus Infection in Chimpanzees, United States
- Putative New West Nile Virus Lineage in *Uranotaenia unguiculata* Mosquitoes, Austria, 2013
- Novel Bluetongue Virus in Goats, Corsica, France, 2014



Pathogenic *Elizabethkingia miricola* Infection in Cultured Black-Spotted Frogs, China, 2016

Technical Appendix

Methods

Outbreak Investigations

During July–October 2016, we investigated the outbreaks of emerging, contagious disease, characterized mainly by severe neurologic dysfunction, in black-spotted frog farms in south-central China (Hunan province). Clinical observations and epidemiologic information were recorded and analyzed to determine the cause of these outbreaks. A total of 213 abnormal frogs were collected from 7 separate farms in Hunan. When the frogs were captured, potassium permanganate solution was used to disinfect equipment to avoid cross-contaminating possible pathogens between ponds.

Etiologic Examination

To figure out the etiology of this disease, we conducted bacteria isolation, microscopic parasite examination, and PCR tests for fungi and viruses in the 213 field-collected frogs. The frogs were euthanized by immersion in a 0.4% aqueous solution of MS222 followed by pithing. We then performed a routine necropsy and fixed representative tissues with 4% paraformaldehyde for histopathology. Samples taken from brain, liver, spleen, and kidney were directly streaked onto brain–heart infusion agar at 28°C for bacteria isolation. Although 190 frogs were bacteria positive, most bacterial colonies showed the same phenotype (size, color, shape). We purified 70 bacterial colonies isolated from different frogs and farms by streaking and restreaking on fresh medium, and then the 70 purified isolates were identified by Eztaxon (1) based on their 16S rRNA gene sequence. Among all the identified bacteria, 63 (90%) were *E.miricola*. We selected the bacteria strain FL160902, isolated from the liver of frog no. 160, as the representative strain. Its 16S rRNA gene sequence (KY461715) and *gyrB* sequence

(KY461716) were submitted to GenBank. In our etiologic examination, *Batrachochytrium dendrobatidis* (*Bd*) and ranaviruses were selected as the targeted pathogens, considering that they both contribute to global population declines in amphibians (2). Specifically, we tested skin samples to detect *Bd* by PCR using specific primers Bd1a and Bd2a, as described by Annis et al. (3). For virus detection, we extracted DNA of spleen, kidney, and muscle using a DNA extraction kit (ComWin Biotech Co. Ltd, Beijing, China). A pair of ranavirus-specific primers (4) and universal primers AdenoF, HVF, and ConsR (5) were used successively to detect ranaviruses, large DNA viruses, and adenoviruses. The primers used in our study are shown in Technical Appendix Table 1. Parenchymal tissues were checked for parasitic infections by microscopy. The results were considered positive if any of the tested organ samples was positive.

Pathogenicity Testing of *E. miricola* FL160902 to Frogs

Experimental exposure of frogs to *E. miricola* FL160902 was conducted to determine its pathogenicity. We conducted infection trials with batches of 10 healthy frogs (20.5 ± 6.7 g) using various infection routes, including intramuscular injection, immersion infection, and cohabitation with infected frogs. We performed all assays in 50-L tanks supplied with wet cotton wool at 30°C. Three groups received 200 μ L *E. miricola* suspensions (10^5 , 10^6 , 10^7 cfu/mL) by intramuscular injection, with sterile 0.70% stroke-physiologic saline solution (SPSS) injection in the fourth group as a parallel control. We performed the immersion infection by bathing frogs in 1500-mL *E. miricola* suspensions (10^6 cfu/mL) for 30 minutes. Frogs in the sixth group cohabited with 4 diseased frogs previously infected with *E. miricola* by IM injection (10^7 cfu/mL). Ten frogs in the seventh group were used as a negative control. Deaths of frogs in each of the 7 groups were recorded every day over a period of 2 weeks. Deaths after 14 days were not included in this recording. Samples from brain, liver, and kidney were tested to confirm the cause of death. *E. miricola* FL160902 was reisolated from the dying frogs, indicating that Koch's postulates had been satisfied in the challenge studies. All animal challenges in this study were carried out following the National Institutes of Health protocols and the International Society for Development Psychobiology standards (6).

Whole-Genome Sequencing and Phylogenetic Analysis of *E. miricola* FL160902

We extracted genomic DNA of *E. miricola* FL160902 using the SDS method (7). The harvested DNA was detected by agarose gel electrophoresis and quantified by Qubit (Thermo

Fisher, Waltham, MA, USA). Whole-genome sequencing was performed on the Illumina HiSeq 2500-PE150 platform with MPS (massively parallel sequencing) Illumina technology. A-tailed, ligated to paired-end adaptors and PCR amplified with a 500 bp insert and a mate-pair library with an insert size of 5 kb were used for the library construction at the Beijing Novogene Bioinformatics Technology Co., Ltd. Illumina PCR adaptor reads and low-quality reads from the paired-end and mate-pair library were filtered in the quality control step using our own compiling pipeline. All good-quality paired reads were assembled using SOAPdenovo (<http://soap.genomics.org.cn/soapdenovo.html>) into several scaffolds. The 15 scaffolds ranged in size from 1215 bp to 1302244 bp with a mean coverage of 160-fold. The genome of *E. miricola* FL160902 contained 4219019 bp with a GC content of 35.65%. This whole-genome project has been deposited at GenBank (BioProject PRJNA387126, BioSample SAMN07139315). The filter reads were handled by the next step of the gap closing, followed by genome components and gene function prediction.

Genomic alignments between *E. miricola* FL160902 and 37 other reference genomes were performed using the MUMmer (8) and LASTZ (9) tools. Under the analysis of ProtTest 3.2 (10), the best model according to BIC was JTT+I+G+F, with a confidence interval of 100.0. The phylogenetic tree of orthologous genes was constructed using the RAxML with 100 bootstrapping replicates.

References

1. Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, et al. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol.* 2012;62:716–21. <http://dx.doi.org/10.1099/ijs.0.038075-0>
2. Latney LV, Klaphake E. Selected emerging diseases of amphibia. *Vet Clin North Am Exot Anim Pract.* 2013;16:283–301. <http://dx.doi.org/10.1016/j.cvex.2013.01.005>
3. Annis SL, Dastoor FP, Ziel H, Daszak P, Longcore JE. A DNA-based assay identifies *Batrachochytrium dendrobatidis* in amphibians. *J Wildl Dis.* 2004;40:420–8. <http://dx.doi.org/10.7589/0090-3558-40.3.420>
4. Mao J, Hedrick RP, Chinchar VG. Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. *Virology.* 1997;229:212–20. <http://dx.doi.org/10.1006/viro.1996.8435>

5. Hanson LA, Rudis MR, Vasquez-Lee M, Montgomery RD. A broadly applicable method to characterize large DNA viruses and adenoviruses based on the DNA polymerase gene. *Virology*. 2006;3:28. <http://dx.doi.org/10.1186/1743-422X-3-28>
6. NIH National Institutes of Health. Guide for the Care and Use of Laboratory Animals. Washington, DC: National Academies Press; 1985.
7. Chen WP, Kuo TT. A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. *Nucleic Acids Res.* 1993;21:2260. <http://dx.doi.org/10.1093/nar/21.9.2260>
8. Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, et al. Versatile and open software for comparing large genomes. *Genome Biol.* 2004;5:R12. <http://dx.doi.org/10.1186/gb-2004-5-2-r12>
9. Harris RS. Improved pairwise alignment of genomic DNA. State College, PA: Pennsylvania State University; 2007.
10. Darriba D, Taboada GL, Doallo R, Posada D. ProtTest 3: fast selection of best-fit models of protein evolution. *Bioinformatics.* 2011;27:1164–5. <http://dx.doi.org/10.1093/bioinformatics/btr088>

Technical Appendix Table 1. Primers used for virus and fungi detection

Targeted pathogen	Primer name	Primer sequence	Size	Reference
Large DNA viruses and adenoviruses	AdenoF	5-gggaattctaGAYATHTGYGGNATGTAYGC-3	1200 bp	Hanson LA et al. (5)
	HVF	5-cggaattctaGAYTTYGCNWSNYTNTAYCC-3	400–700 bp	
Ranaviruses (Iridoviridae; Ranavirus)	ConsR	5-cccgaattcagatcTCNGTRTCNCCRTA-3		
	RGV-P1	5'-GACTTGGCCACTTATGAC3'	531 bp	Mao J et al. (4)
<i>Batrachochytrium dendrobatidis</i>	RGV-P2	5'-GTCTCTGGAGAAGAAGAA-3'		
	Bd1a	5'-CAGTGTGCCATATGTCACG-3'	300 bp	Annis SL et al. (3)
	Bd2a	5'-CATGGTTCATATCTGTCCAG-3'		

Technical Appendix Table 2. Detailed results of the etiologic detection of frogs collected in 7 farms in Hunan province, China, July–October 2016

Sampling location	Sampling time	No. bacteria positive/total	No. Myxosporea positive/total	No. Bd* positive/total	No. virus† positive/total
Yiyang	Aug 9	36/42	7/42	0/42	0/42
Ningxiang	Aug 14	30/30	2/30	0/30	0/30
Changsha	Aug 14	33/35	0/35	0/35	0/35
Zhuzhou	Oct 3	17/26	0/26	0/26	0/26
Hengyang	Sept 5	21/22	0/22	0/22	0/22
Shaoshan	Jul 20	26/31	0/31	0/31	0/31
Wugang	Jul 21	27/27	0/27	0/27	0/27

**Batrachochytrium dendrobatidis*.

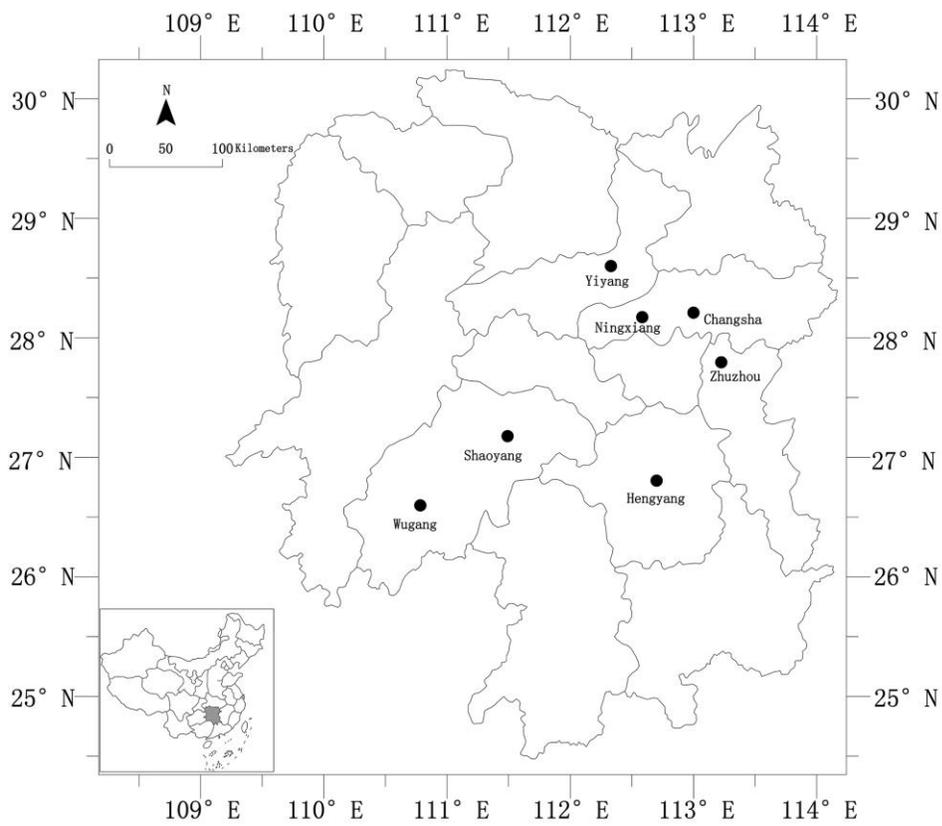
†Ranaviruses or large DNA viruses.

Technical Appendix Table 3. Results of the identified bacteria isolated from different organs

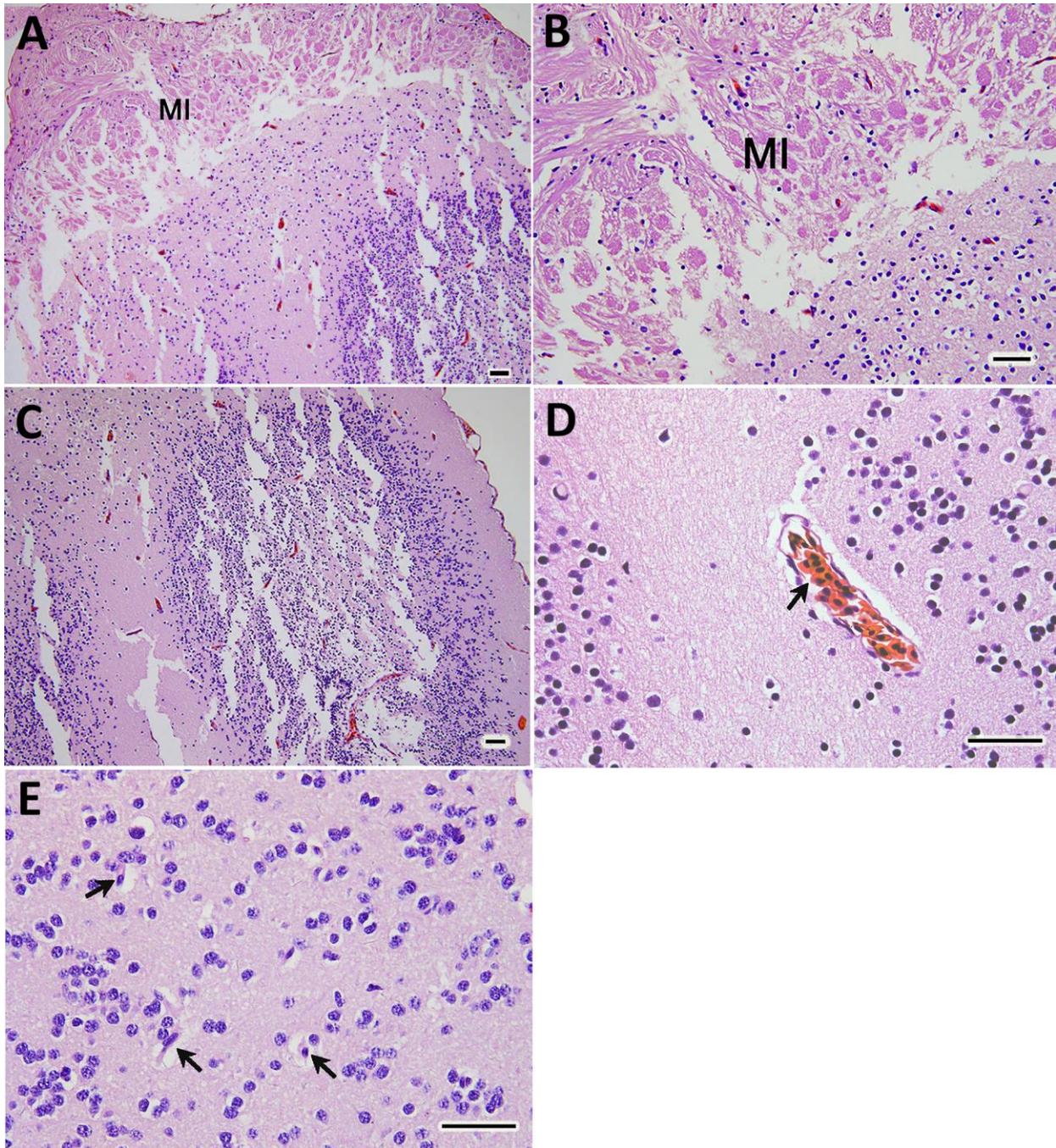
Organ	Identified isolates	<i>Elizabethkingia miricola</i>	Proportion
Brain	23	20	86.9%
Liver	16	14	87.5%
Spleen	13	12	92.3%
Kidney	18	17	94.4%
Total	70	63	90%



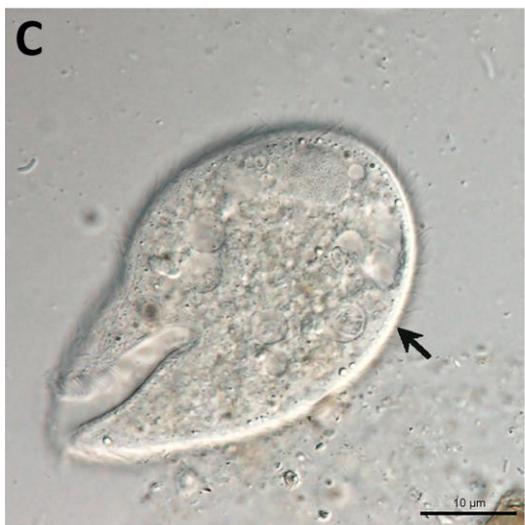
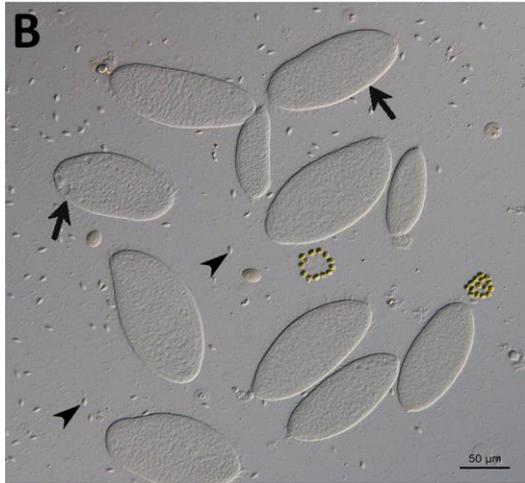
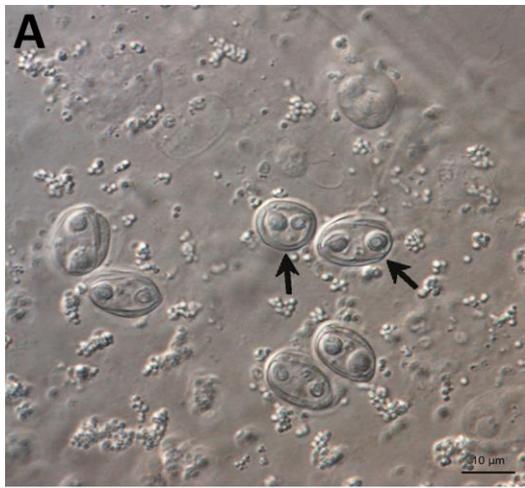
Technical Appendix Figure 1. Different frog farms in Hunan Province, China. A) Ponds with running water and shelter. B) Artificial ecologic wetlands with rice. C) Mass mortality because of disease.



Technical Appendix Figure 2. Map of sample locations of diseased frogs in Hunan Province, China.



Technical Appendix Figure 3. Pathologic brain lesions of diseased frogs in Hunan Province, China. A) Meningitis with meninges incrustation (MI). B) Denaturation in the incrustated meninges. C) Inflammation with lymphohistiocytic infiltration in cerebellum. D) Expanded perivascular space in brain parenchyma (single-arrow). E) Degenerated and necrotic nerve cell in ectocinerea (single arrow). All scale bars indicate 50 μm .



Technical Appendix Figure 4. Typical microorganisms observed in optical microscopy of diseased frogs in Hunan province, China. A) Parasitic myxospore observed in gallbladder; bar, 10 μm. B) Intestinal protist *Opalina* sp. (single arrow) and unknown flagellates (arrowhead); bar, 50 μm. C) Intestinal *Blantidium* sp.; bar, 10 μm.