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Outbreak of *Aeromonas hydrophila* bacterial dermatosepticemia in wild leopard frogs (*Rana pipiens* complex) in Mexico

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Abstract

This report presents a case of dermatosepticemia in ten wild leopard frogs (*Rana pipiens* complex) captured in Mexico, where the presence of *Aeromonas hydrophila* was confirmed. This polyphasic study included macroscopic, microbiological, histological, and molecular analyses. Externally, the specimens exhibited erythema, hemorrhage, and erosions on various body parts. Internal observations revealed hemorrhaging of the oral cavity and stomach, as well as sero-sanguineous ascites. Parasites were also identified, including *Ochoterenella* sp. in the mesentery, *Glypthelmins* sp. in the liver, *Clinostomum* sp. in the thigh muscles, and *Opalina* sp. and *Glypthelmins* sp. in the intestine. Histopathological analyses showed skin hemorrhaging, erosion and ulceration, myositis, coagulative myonecrosis with the presence of bacteria, and damage to internal organs with an abundant presence of bacteria. *Aeromonas hydrophila* was isolated in pure culture from the skin, muscle, mouth, heart, liver, and kidney of all ten frogs. The Mexican isolate (cos-ciesa 01) had high biochemical-enzymatic similarity to the strain *A. hydrophila* subsp. *hydrophila* CECT 4330. Additionally, the Mexican strain's 1 414 bp nucleotide sequence of 16S ribosomal RNA gene (GenBank Accession MN795652) showed high identity with *A. hydrophila* strain ATCC 7966. *Ranavirus* was not recovered in the cell cultures and PCR did not detect its DNA from paraffin-embedded tissues. These results suggest *A. hydrophila* as the causative agent of the dermatosepticemia disease outbreak.

Keywords: Dermatosepticemia; *Aeromonas hydrophila*; Rana virus; Frog; Bacteria; Disease.

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Study contribution

Bacterial dermatosepticemia is a systemic disease affecting both wild and captive amphibians. Traditionally, the disease is associated with *Aeromonas hydrophila* but pathogens such as bacteria, *Ranavirus* and *Batrachochytrium dendrobatidis* have also been implicated, therefore exhaustive analyzes are required to obtain an accurate diagnosis. Mexico has high frog biodiversity; however there are few studies on diseases affecting frogs, and no reports on dermatosepticemia exist. This work presents a case of dermatosepticemia in wild leopard frogs (*Rana pipiens* complex). The study included macroscopic, microbiological, histological, and molecular analyses, confirming *A. hydrophila* as the causative agent of the outbreak. The bacteria isolated from the skin and internal organs from frogs showed high biochemical-enzymatic similarity to the *A. hydrophila* strain subsp. *hydrophila* CECT 4330. In this study, *Ranavirus* was not recovered from cell cultures, and viral DNA was not detected by endpoint PCR from paraffin-embedded tissues.

Introduction

Bacterial dermatosepticemia is a systemic infectious disease that results in high morbidity and mortality rates among wild and captive amphibians worldwide.[1] This condition is considered to have multifactorial etiology associated with opportunistic bacterial agents that inhabit the skin and digestive tract of healthy amphibians, often becoming pathogenic when animals undergo stressful conditions.[2–4] These stressors may arise due to deforestation and habitat destruction, pollution, climate change, the presence of invasive species, and other infectious diseases.

Aeromonas, especially *Aeromonas hydrophila*, has been reported as the main etiological agent of dermatosepticemia,[1,5,6] but *Pseudomonas* spp., *Chryseobacterium indologenes*, *Chryseobacterium meningosepticum*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia liquefaciens*, and *Flavobacterium* spp. have also been associated with this disease.[3] Unlike other bacteria involved in cases of bacterial dermatosepticemia, *A. hydrophila* can synthesize proteolytic enzymes that affect skin integrity and the innate defense activity of antimicrobial peptides produced in the granular glands of amphibians,[7] allowing tissue colonization and the establishment of an infection.

Affected frogs may suffer a septicemic process, showing skin ulcerations as external clinical signs and sometimes even manifesting symptoms associated with a syndrome.[5] Dermatosepticemia in frogs begins with anorexia, inactivity, apathy, skin reddening, or cutaneous erythema in the ventral region and inner thighs. Other manifestations include local or generalized edema, erosion, epidermal ulcerations, necrosis, and coelomic effusions.[8, 9]

Some cases clinically similar to bacterial dermatosepticemia have been confirmed as infections by the viral pathogen *Ranavirus*. Ranavirosis is an emerging disease that affects at least 60 species of frogs, toads, and salamanders (Anura and Caudata orders) in North America,[10] including Mexico.[11] Retroactive investigations with samples from cases occurring in the USA between 1996 and 2001 ruled out *A. hydrophila* as a cause of disease and death in frogs,[10] instead postulating that *A. hydrophila* is an opportunistic or secondary invader that takes advantage of morbid amphibians infected with other pathogens. However, outbreaks caused

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by *A. hydrophila* are frequently reported in frog populations.[5] Cases of co-occurrence of bacterial and viral infections in frogs cannot be ruled out, making it difficult to identify the agent responsible for the death.

This study provides the first report of clinical and histological pathology observed in wild leopard frogs (*Rana pipiens* complex) captured in Mexico. The clinical signs, including macroscopic and histological injuries, are consistent with a bacterial dermatosepticemia, and *A. hydrophila* was isolated and identified as the ultimate cause of death. In addition, *Ranavirus* did not grow in cell lines, and *Ranavirus* DNA was not detected by PCR.

Materials and methods

Ethical statement

The analysis of the animals in the present work does not have bioethical approval from the Comité Institucional de Bioética of the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Autónoma del Estado de Mexico, since the leopard frog specimens were sent by the farmers themselves for diagnostic purposes and to identify the cause of death of the frogs. The specimens were moribund and were transported by an official of the Comité de Sanidad Acuicola of Querétaro (Mexico), so the protocol for handling animals for scientific research does not apply. However, in our laboratory, the sanitary diagnostic procedures are carried out according to the Manual de diagnóstico del área de Sanidad Acuicola from the Centro de Investigación y Estudios Avanzados en Salud Animal, Facultad de Medicina Veterinaria y Zootecnia (FMVZ), which is accredited and authorized by the Mexican Accreditation Entity under the ISO 17025-2005 Standard.

Sick frogs and maintenance conditions

As part of a health-monitoring program for aquatic organisms, in October 2016, ten leopard frogs were sent to the Laboratorio de Sanidad Acuícola at the Universidad Autónoma del Estado de Mexico. The sampled frogs (83.1 ± 20 g and 24.4 ± 2.1 cm, average weight, and length, respectively) exhibited a septicemic disease process characterized by a poor body condition, erythema in the ventral area and thighs, and ulcerations on the toes, mouth, and thighs.

The sampled specimens were part of a 1 000-animal batch of wild frogs captured in the warm, sub-humid region of Sinaloa State, Northwest Mexico in the autumn season in 2016, where the average minimum temperature is 28 °C. After capture, the frogs were transported to a collection center in central Mexico, where they were kept in concrete pools in a greenhouse at an average temperature of 24 °C. The frogs were routinely maintained under these conditions for 30 d (quarantine) before being shipped to the market for sale. During this time, they were fed live and artificial feed commonly used for rainbow trout (*Oncorhynchus mykiss*). The animals began to show signs of disease and mortality shortly after arriving at the collection center.

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Macroscopic and parasitological analyses

Upon arrival at the laboratory, the frogs were euthanized using 240 mg/L tricaine methane sulfonate 222 (Sigma) for 30 min to ensure their death. Immediately, fresh samples of damaged skin were aseptically collected from each specimen and viewed at 10× and 40× magnifications under a Primo Star Zeiss® contrast microscope to evaluate the presence of parasites and fungi. Furthermore, a postmortem examination was conducted on all remitted frogs. Alterations in the appearance and color of the internal organs and the contents of the coelomic cavity were recorded. The specimens were thoroughly examined for parasites in the lungs and in the contents of the stomach and intestine.

The parasites from the coelomic cavity, mesentery, and intestine were removed with fine brushes, cleaned, and placed in Petri dishes with saline solution (0.85 % NaCl). Trematodes were fixed with hot 4 % formalin and preserved in 70 % alcohol until staining. The trematodes were stained using Mayer's paracarmine technique, rinsed with methyl salicylate, and mounted in permanent preparations in Canadian balsam.[12] Once the preparations were dry, they were analyzed under an optical microscope to observe morphological structures (e.g., position of the oral and ventral suction cups, the presence of the pharynx, esophagus, and length of the intestinal cecum, etc.)

These observations allowed for identification of specimens up to the genus level with the help of the taxonomic codes reported by Yamaguti[13] and Gibson et al.[14] Similarly, nematodes were fixed with hot 4 % saline formalin, preserved in 70 % alcohol, and rinsed with gradually increasing glycerin concentrations, and finally mounted in temporary preparations to determine the taxonomic classification following the criteria of Anderson et al.[15] and Caspeta-Mandujano.[16]

Histological analysis

For histological analyses, skin, liver, kidney, heart, spleen, and lung samples were taken from each frog, fixed in vials containing 10 % buffered formalin, dehydrated, and embedded in paraffin wax following standard procedures.[4, 11] Each tissue was sectioned at 5 µm and stained with hematoxylin and eosin to describe histopathological alterations, according to conventional methods. Sections were observed at various magnifications under an Olympus BH-2 light microscope.

Virus isolation and Ranavirus diagnosis by PCR

For virus isolation, two pools of kidney and spleen tissues, each containing approximately 1 mL of tissue from three randomly chosen frogs, were placed in 15 mL Falcon tubes containing 9 mL of Leibowitz's L-15 Medium (Gibco BRL). The samples were macerated and centrifuged at 4 000 ×g for 15 min at 4 °C. The obtained supernatant was recovered and filtered at 0.22 µm. Each sample was diluted to 1:10 and 1:100 and inoculated, in duplicate, into 12-well cell culture plates containing cell monolayers at ≥ 90 % confluence of (i) Chinook salmon embryo (CHSE-214) incubated at 18 °C, (ii) Bluegill fry (BF) incubated at 20 °C, and (iii) Epithelioma papulosum cyprini (EPC) incubated at 25 °C.

The inoculated cells were examined daily under an inverted Axiovert 40 C/40 CFL ZEISS microscope to detect the presence of cytopathic effects. After seven days post-inoculation, the supernatant from plates with a negative cytopathic effect

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were recovered, reinoculated in the same cell lines, and incubated as previously described to confirm the negative result. Negative controls consisted of duplicate sets of wells containing monolayers of cell lines inoculated with 0.1 mL of filter-sterilized (0.45 µm pore diameter) Hank's balanced salt solution.

For molecular diagnosis of *Ranavirus*, total DNA was extracted from formalin-fixed paraffin-embedded tissues according to Pikor et al.[17] except that the process of paraffin removal with 1 mL xylene was extended, being repeated three times over two days to release the tissues from the paraffin. The resulting DNA was resuspended in 70 µL of ultrapure water and stored at -80 °C. A volume of 1 µL of each sample was used in the amplification reaction.

PCR assays were performed on a DLAB TG-1000 Thermocycler using the PCR method based on the major capsid protein (MCP) gene, as recommended by the World Organization for Animal Health[18] for infection with *Ranavirus*. The primer pair M153 (5'-ATG-ACC-GTC-GCC-CTC-ATC-AC-3') and M154 (5'-CCA-TCG-AGC-CGT-TCA-TGA-TG-3') were used for amplification of the target MCP sequence, with an expected amplification product of 625 bp. The PCR products were separated on a 1 % (w/v) agarose gel for 60 min at 90 V in 1× TAE buffer 1× GelRed® nucleic acid gel stain (Biotium). Positive results showed amplification bands around the expected size using a 1 Kb DNA ladder (Bioline, USA). A positive control for *Ranavirus* was included using DNA from *Lithobates catesbeianus* infected with *Ranavirus*.

Bacterial isolation and phenotypic characterizations

For bacterial analysis, samples were aseptically taken from the ulcerated areas of the skin and muscle, the oral cavity, liver, heart, and kidney of each specimen. These samples were streaked onto brain heart infusion agar (BHI, Oxoid) and Columbia blood agar (Oxoid). All plates were aerobically incubated at 28 °C and examined every 24 h for 7 d. Samples were also seeded on Cytophaga agar[19] and incubated at 18 °C for 10 d. If no colony growth was observed after a week, the bacterial cultures were considered negative.

Seven frogs tested positive for bacterial isolation, with the same colony morphotype detected on microbiological plates in pure culture, resulting in 30 isolates. However, only one isolate was kept as a representative. This isolate, coded as cos-ciesa 01, was selected from a kidney BHI plate, streaked onto a new BHI plate to obtain pure cultures, and stored at -80 °C in Criobille tubes (AES Laboratories). For phenotypic characterizations, the initial growth of the isolate was tested on different culture media, including marine agar (BBL Difco), nutrient agar (Oxoid), BHI (Oxoid), blood agar with 5 % defibrillated sheep blood (BioMérieux), MacConkey agar (Oxoid), thiosulphate citrate bile salts sucrose agar (Oxoid), and tryptone soy agar (BBL Difco) with and without the supplementation of 1 % NaCl.

The hydrolysis of starch (0.4 % w/v), gelatin (1 % w/v), tween 80 (1 % v/v), and DNase (Liofilchem) was tested using tryptone soy agar as the basal medium, except for DNAse, which was prepared according to the manufacturer's instructions. Further tests included the oxidation and fermentation of glucose, arginine, lysine, and ornithine decarboxylase, as well as the Simmons citrate reaction.[20] All tubes and plates were incubated at 25 °C and observed daily for 14 d for any growth or reaction.

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Growth was also assessed on tryptone soy agar plates at different temperatures (i.e., 4, 15, 18, 20, 25, 28, 37, and 42 °C) and in tryptone soy broth to test salt tolerance (0-10 % NaCl [w/v], with a 1 % range) and pH ranges (pH 4-10, adjusted using 1 M NaOH and 1 M HCl). The enzyme activity of the Mexican isolate was determined using the API ZYM, API 50CH, API 20E, and API 20NE (BioMérieux) systems, following the manufacturer's instructions, except that temperature was set at 25 °C and the incubation periods were established as 24, 48, and, for only API 20E and API 20NE, 72 h. Since the obtained results presumably identified the Mexican isolate as a mobile *Aeromonas*, the reference strain *A. hydrophila* subsp. *hydrophila* CECT 4330 was included in all tests for comparison purposes. The strain was acquired from the Spanish Type Culture Collection (CECT).

16S rRNA amplification and nucleic acid sequencing

To identify the Mexican isolate cos-ciesa 01, the 16S rRNA sequence was amplified; total bacterial DNA was extracted using the InstaGene™ DNA Purification Matrix (Bio Rad) according to the manufacturer's instructions. For PCR-amplification of the 16S rRNA gene, the isolate was sequenced using the universal bacterial 16S ribosomal RNA sequence targeting the primers 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′).[21] The expected 1 500 bp amplicon was sequenced by Macrogen in Seoul, Korea, and the sequence was edited using the Geneious Prime v2020.03 software.[22]

Phylogenetic relationships were inferred between the obtained sequence and 32 nucleotide sequences of *Aeromonas* type strains published in GenBank (see accession numbers in Figure 1). Inferences were determined using the neighbor-joining method,[23] implemented in the MEGA X software,[24] and the Metropolis-Hastings coupled Markov chain Monte Carlo for Bayesian phylogenetic inference, implemented in the MrBayes v3.0B4 software.[25] The nucleotide sequence of *Serratia marcescens* (KU724077) was incorporated as an outgroup. A multiple sequence alignment was performed in Geneious Prime (https://www.geneious.com) and manually ver ified in GeneDoc.[26] The Bayesian information criterion, implemented in MEGA X, was used to determine the nucleotide substitution model that best described the evolutionary process of the multiple sequence alignment for the 16S rRNA ribosomal gene.[27] The selected best-fit model of nucleotide substitution was the Kimura-2 parameter model with a discrete gamma distribution (shape parameter = 4) and invariant sites (K2 + G + I).

The optimal neighbor-joining tree had a summed branch length of 0.21562926. Shown next to the branches is the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates). The presented tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura-2 parameter model and are presented as the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution. All ambiguous positions were removed for each sequence pair (i.e., pairwise deletion option).

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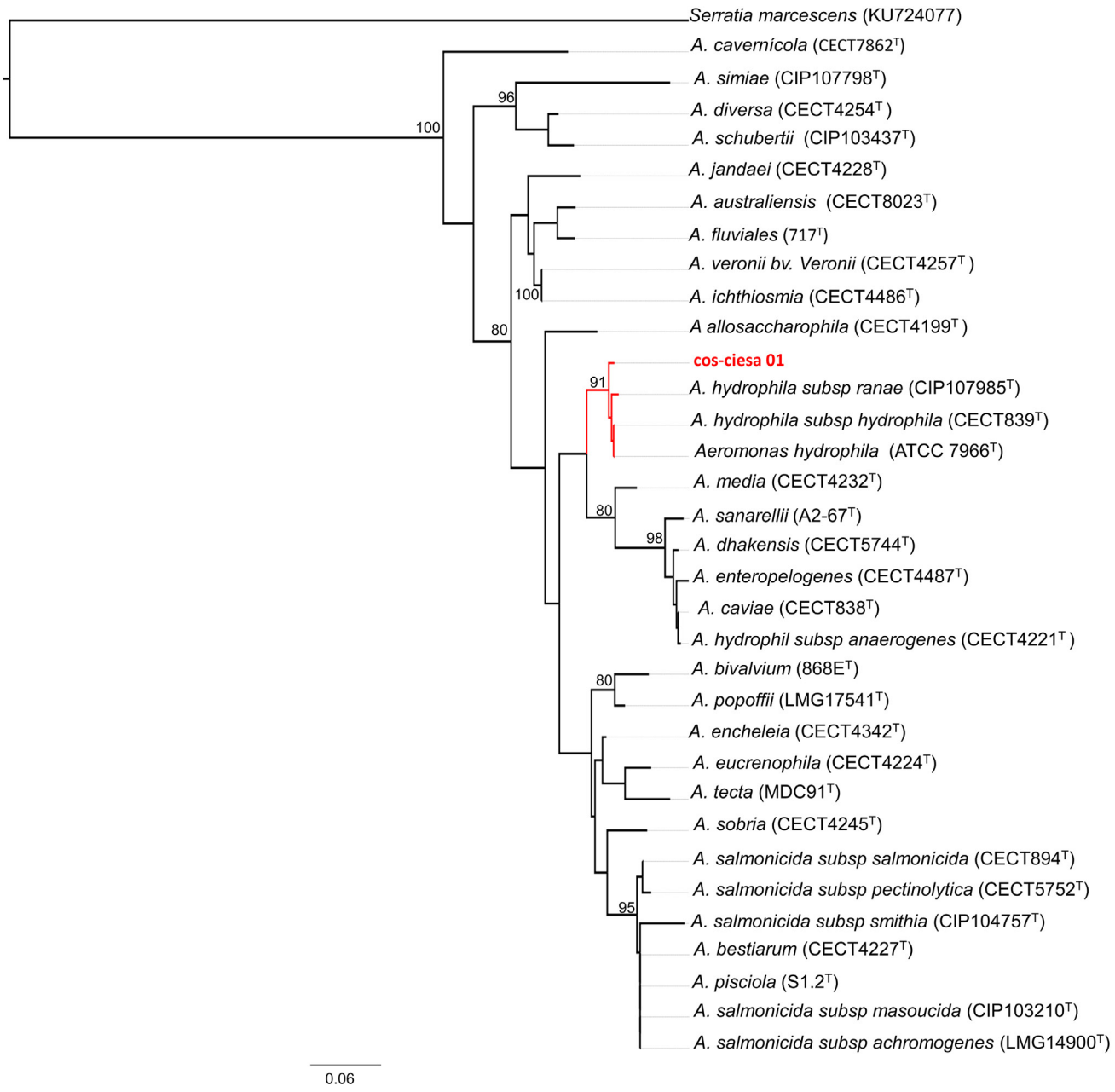

Figure 1. Phylogenetic tree of publicly available 16S rRNA sequences of *Aeromonas* species. The tree was constructed using the neighbor-joining algorithm. Well-supported nodes with bootstrap support ≥ 80 % are indicated.

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Figure 2. Leopard frog (*Rana pipiens* complex) affected by dermatosepticemia, presenting (A) mouth erythema; (B) ulcerations on the ventral region and palmar areas; (C) ulcerations on the thigh; (D) plantar ulcerations; (E) ulcerations (thin black arrows) and bone exposure (thick white arrow).

Three parallel Bayesian Markov chain Monte Carlo analyses were run simultaneously, with each run consisting of 20 × 106 generations with four Markov chains (one cold and three heated). Output parameters were visualized with Tracer v1.6[28] to assess the stationary status of the runs and whether the duplicate runs had converged on the same mean likelihood. Runs appeared stationary before 106 generations. The first 2×10^6 generations of each run were conservatively excluded as burn-ins. All post-burn-in estimates (sampled every 1 000 generations) were combined, and phylogeny and parameter estimate from this combined posterior distribution were summarized. Nodes were considered well-supported if posterior probabilities > 0.95. The maximum clade credibility tree was visualized with FigTree v1.4.3 (http://beast.bio.ed.ac.uk/figtree).

Results

The originating frog population suffered a 20 % mortality rate during the outbreak, with specimens showing anorexia and lethargy prior to death. The observed external changes among the collected specimens included erythema and hemorrhages on the rostral area in 4 out of 10 frogs (Figure 2A) and epidermal erosions on the ventral region (Figure 2B) and inner side of the hind limbs at the thigh muscles in 7 out of 10 frogs (Figure 2C). The same lesions were recorded on the plantar and palmar surfaces, as well as the toes of both extremities, in 7 out of 10 frogs (Figures 2B, 2D, 2E). In some cases, crusts, deep ulcers, and even bone exposure were observed in three animals (Figure 2E). No parasites or fungi were detected through analyses of fresh lesion samples.

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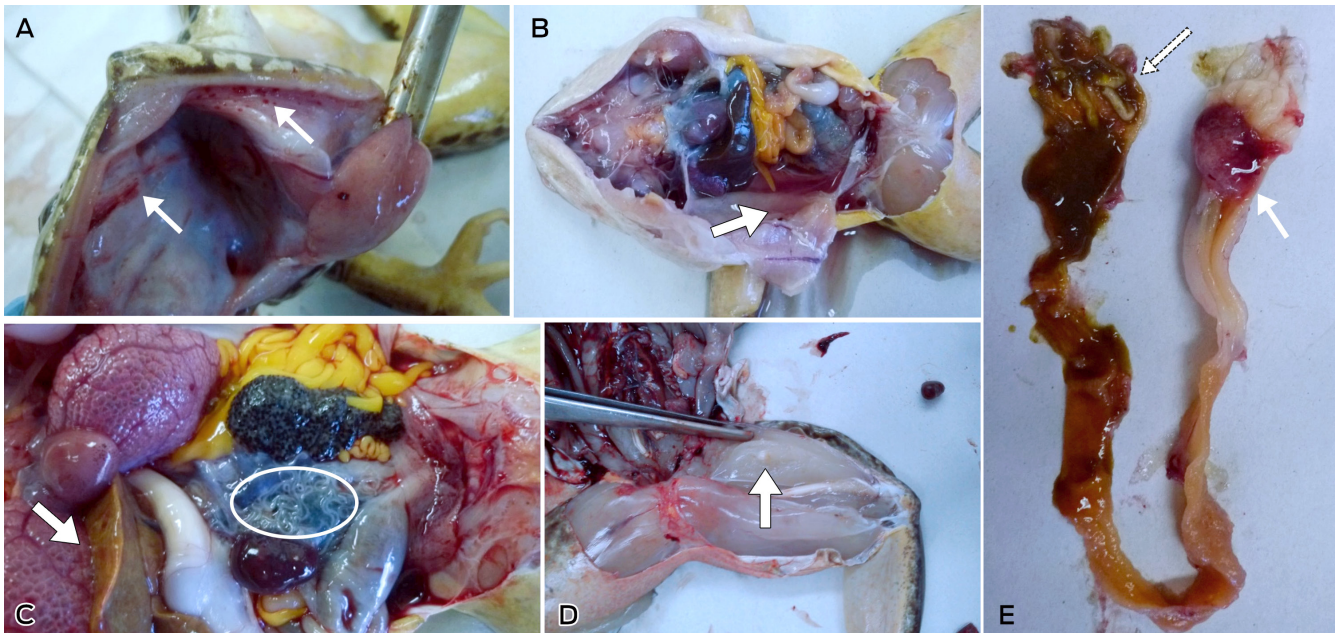

Figure 3. Leopard frog (*Rana pipiens complex*) affected by dermatosepticemia. Shown are (A) hemorrhages in the oral cavity (arrows), (B) serosanguinous ascites (arrow), (C) pale yellowish liver discoloration (arrow) and the presence of *Ochoterenella* sp. (circle) in the coelomic cavity, (D) *Clinostomum* sp. cysts in the muscle (arrow), and (E) hemorrhages in the stomach mucosa (arrow) and the presence of *Glypthelmins* sp. in the intestine (dotted arrow).

Internally, three of the ten evaluated specimens presented hemorrhages in the oral cavity at the base of the tongue (Figure 3A). The coelomic cavity displayed serosanguineous ascites in three frogs (Figure 3B) and an abundant presence of the parasite *Ochoterenella* sp. in the mesentery (Figure 3C). Other lesions included a pale liver with an irregular surface and whitish nodules associated with the presence of digenean *Glypthelmins* sp. in four frogs (data not shown), splenomegaly, a pale kidney, and the existence of cysts with metacercariae of *Clinostomum* sp. in the muscle at the level of the femur of two frogs (Figure 3D). The gastric mucosa of four frogs showed petechiae and ecchymosis (Figure 3E); in the intestinal lumen, there was an abundance of the protozoan *Opalina* sp. (data not shown) and of digenean *Glypthelmins* sp. (Figure 3E).

Histological analysis

Extensive areas of skin erosion and deep ulceration were found in five frogs processed for histological analysis, with affected muscles presenting bacteria and showing septic coagulative necrotizing myositis (Figure 4A and 4B). The kidney showed interstitial congestion and bacteria (Figure 4C), granulomatous inflammation and bacteria, and septic thromboembolisms in two out of five frogs (Figure 4D). Observations in the liver included multifocal mononuclear hepatitis, marked congestion and distention of the sinusoids, hepatocyte degeneration, and multifocal hepatic necrosis (data not shown).

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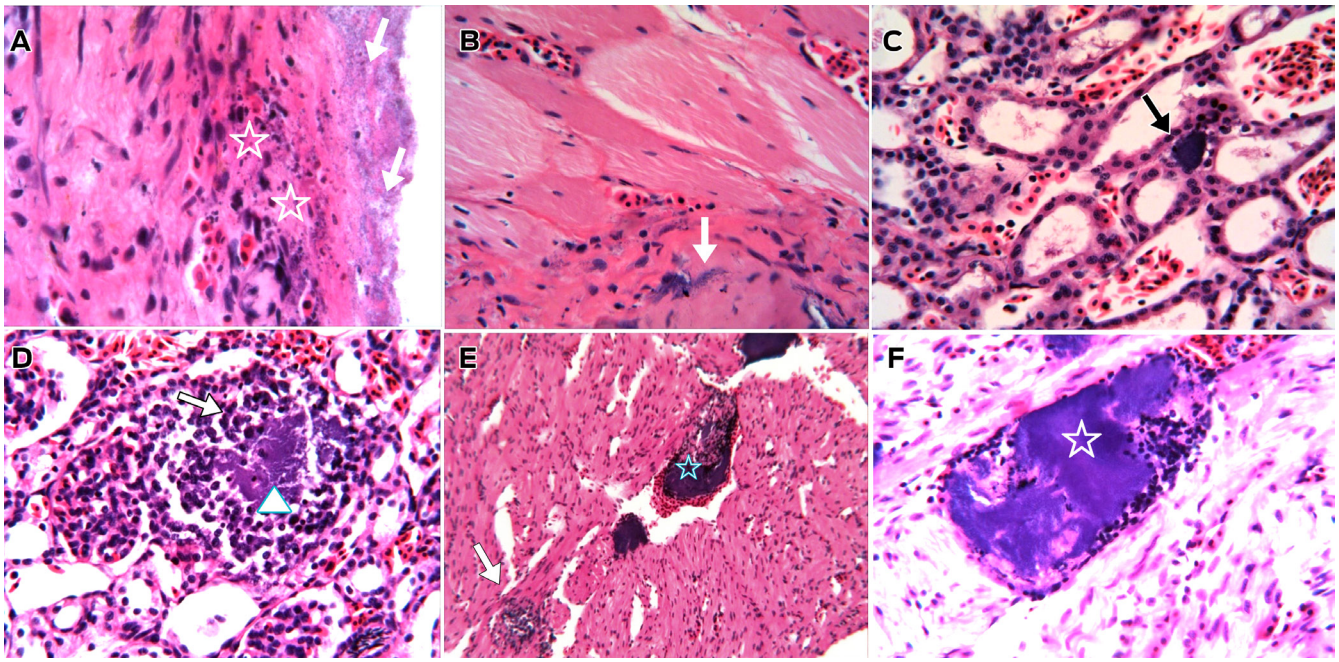

Figure 4. Leopard frog (*Rana pipiens* complex) affected by dermatosepticemia. Shown are (A) bacteria on ulcerated skin (arrow) and necrosis with cellular debris (stars); (B) bacteria on (arrow) and necrosis of the hamstring muscle; (C) a kidney with congestion and the presence of interstitial bacteria (arrow); (D) septic glomerulitis with thromboembolism (head arrow) and proliferative glomerulonephritis (arrow); (E and F) heart with bacterial thromboembolism (stars) and granulomatous cardiomyositis (arrow).

In turn, the spleen showed severe follicular depletion with a few small germinal centers (data not shown). Further recorded were bacterial thromboembolic cardiomyositis in two frogs (Figure 4E and 4F), valvular endocardiosis, granulomatous cardiomyositis (Figure 4E), congestion, and pulmonary microthrombosis (data not shown). None of the analyzed tissues presented intralesional basophilic intracytoplasmic inclusions, and skin samples showed no sporangia.

Viral isolation and Ranavirus diagnosis by PCR

The kidney and spleen samples inoculated in the CHSE-214, BF-2, and EPC cell cultures did not show any evidence of cytopathic effect or viral presence. Similarly, the PCR analyses against *Ranavirus* did not show products of the expected size with the DNA extracted from paraffin-embedded tissues. As expected, the positive DNA control amplified a band of ~625 bp (data not shown).

Phenotypic and biochemical characterizations

Bacterial colonies were obtained from pure cultures of lesions affecting the skin (n = 7), muscle (n = 6), mouth (n = 5), heart (n = 4), liver (n = 4), and kidney (n = 4) of the ten frog specimens. The colonies of these cultures were macroscopically identical, measuring approximately 2 mm in diameter and being cream-colored, smooth, convex, circular with regular edges, and not producing brown pigmentation. Gram staining revealed rods with round-shaped edges.

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Cells and abundant diplo-bacilli were also observed. Given the macroscopic homogeneity, only one representative isolate taken from a kidney sample was used for further analyses of the outbreak.

The cos-ciesa 01 isolate was gram-negative, motile, bacilli, produced β-hemolysis on agar plates, and grew on all the different agar culture media tested. This included on thiosulphate citrate bile salts sucrose, with cos-ciesa 01 and the reference strain *A. hydrophila* subsp. *hydrophila* CECT 4330 producing yellow colonies. No brown pigments were produced on any of the tested culture media. The same results were obtained for *A. hydrophila* subsp. *hydrophila* CECT 4330. Both bacteria exhibited facultative anaerobic behavior and grew at all the tested incubation temperatures. Turbidity was observed at salinities of 0-4 % NaCl (w/v) and at pH 5—10, with results similar to those obtained for the strains *A. hydrophila* subsp. *hydrophila* CECT 4330 and *Aeromonas encheleia*. Data for the *A. encheleia* strain was retrieved from Esteve et al.[29]

When comparing reactions against those reported in the different API mini galleries, the Mexican isolate and *A. hydrophila* subsp. *hydrophila* CECT 4330 presented similar enzymatic profiles. A few reactions were negative for the isolate but positive for the reference strain, such as lipase (C14), β-galactosidase, and the Simmon´s citrate and assimilation of trisodium citrate. Results that were positive for the Mexican isolate but negative for the reference strain included esterase (C4), naphtol-AS-BI-phosphohydrolase, L-rhamnose, D-manitol, and methyl-αD-glucopyranoside.

When the results obtained for the Mexican isolate and *A. hydrophila* subsp. *hydrophila* CECT 4330 were compared to *A. encheleia*, the closest strain identified by 16S rRNA sequencing, both were citrate negative and D-mannitol and L-rhamnosa positive. More details are given in Table 1.

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Table 1. Phenotypical characteristics of the Mexican isolate cos-ciesa 01 and of the most closely related strains *A. hydrophila* CECT 4330 and *A. encheleia* CECT 4342

Characteristics	*Aeromonas*-frog	*A. encheleia* CECT 4342*	*A. hydrophila* CECT 4330
Organism	Frog	European eel	
Source	Kidney		Water
Country	Mexico	Spain	Spain
Gram	-	-	-
Motility	+	+	+
Catalase	+	+	+
Oxidase	+	+	+
Oxidation/fermentation	F	F	F
Arginine	-	-	-
Lysine	-	-	-
Ornithine	+	+	+
Citrate	+	-	+
Tween 80	+	+	+
Growth at			
Temperature (°C)	4–37	4–37	4–37
pH	7–10	8–9	8–10
% NaCl (w/v)	0–4	0–3	0–4
Blood agar	β-hemolysis	β-hemolysis	β-hemolysis
MacConkey	+, lactose -	+, lactose -	+, lactose -
TCBS	+	-	+
Fermentation			
Glycerol	+	-	+
Erythritol	-	-	-
L-arabinose	+	-	+
Glucose	+	+	+
L-sorbose	-	+	-
D-sorbitol	-	-	-
Arbutrin	+	+	+
Esculin	+	+	+
Salicin	+	+	+
D-celibiose	+	-	-
D-lactose	-	-	-
D-melibiose	-	-	-
D-raffinose	-	-	-
Hydrolysis of			
DNAse	+	+	+
Tween 80	+	+	+
Starch	-	+	-

*Information retrieved from Esteve et al.[29]
TCBS: thiosulphate citrate bile salts sucrose.
+: positive reaction; –: negative reaction.

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Amplification of the 16S rRNA gene

The nucleotide sequence obtained in this study for the cos-ciesa 01 strain consisted of 1 414 bp (GenBank accession MN795652). This sequence showed high identity with the *A. hydrophila* strain ATCC 7966 (accession number NR_074841, deposited in the NCBI 16S rRNA database) (Query cover: 100 %, E value: 0.0; Identity: 99.9 %). The cos-ciesa 01 isolate sequence and 32 other *Aeromonas* sequences were aligned to infer phylogenetic relationships, showing an identity percent range between 97.4 % and 99.9 % (Figure 1).

Phylogenetic analyses using both the neighbor-joining and Bayesian inference methods recovered a well-supported group formed by the query sequence (i.e., cos-ciesa 01), *A. hydrophila* ATCC 7966^T, *A. hydrophila* subsp. *hydrophila* CECT 839^T, and *A. hydrophila* subsp. *ranae* CIP 107985^T. This group presented a bootstrap support of 94 % for the neighbor-joining analysis (Figure 1) and a posterior probability of 0.98 for the Bayesian inference analysis. These results suggest that the cos-ciesa 01 query sequence can be confirmed as *A. hydrophila*.

Discussion

Dermatosepticemia, is the most important bacterial disease affecting captive/wild frogs and other amphibians, which has been primarily associated with *A. hydrophila*.[1, 3, 30] Prior to the present study, dermatosepticemia was unreported in amphibians in Mexico.[31] Other diseases known to affect frogs have been documented in Mexico; this includes *Batrachochytrium dendrobatidis*, reported in more than 50 amphibian species across the country,[32] and recently, a *Ranavirus* outbreak in American bullfrogs (*Lithobates catesbeianus*) in northeast Mexico.[11]

Cunningham et al.[5] described that dermatosepticemia can manifest as cutaneous ulcerations, as systemic hemorrhaging, or even, as a combination of both manifestations. A combined presentation was observed in the current study, with the recorded systemic signs including inflammatory or necrotic foci on the skin and internal organs, a conspicuous presence of bacteria, and thrombi. Together, these signs were indicative of an infection of bacterial origin.

Worth noting, hyperemia and other external injuries are not exclusive to bacterial dermatosepticemia and can occur with other infectious pathologies,[33] or in cases of toxicosis. In fact, injuries similar to those of dermatosepticemia are not just associated with saprophytic bacteria such as *A. hydrophila*, but also with viruses and fungi.[1, 11, 34] Despite *A. hydrophila* being widely implicated as an agent responsible for dermatosepticemia,[2, 3] studies have questioned diagnostic value when the bacterium is isolated from dead or moribund frogs, rather considering *A. hydrophila* to be a secondary agent to other primary factors[5] principal among which are *Batrachochytrium dendrobatidis* and *Ranavirus*.

Likewise, bacterial septicemia has been reported as a concomitant infection by *A. hydrophila* and *B. dendrobatidis*[1] or as one between *Ranavirus*, *B. dendrobatidis*, and *A. hydrophila*. In the present study, no clinical evidence was found for a concomitant infection among the analyzed frogs. Histological analyses did not show sporangia associated with chytridiomycosis,[35] nor were intralesional basophilic intracytoplasmic inclusions found, which are characteristic in cases of *Ranavirus* infection.[5, 10]

To rule out other possible causes for the injuries observed in the ten frogs, virus analyses were conducted by homogenizing kidney and spleen samples, with subsequent inoculation in CHSE-214, BF-2, and EPC cell lines. In addition, DNA samples were obtained from the same formalin-fixed paraffin-embedded tissues and processed for *Ranavirus* PCR diagnosis according to the World Organization for Animal Health manual. In all cases, negative findings were obtained for *Ranavirus*, supporting that only *A. hydrophila* could be the cause of the infection. Further research

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should aim to assess Koch's postulates in relation to the cos-ciesa 01 Mexican isolate. Another possible pathogen ruled out was *B. dendrobatidis*, which has been reported in Mexico, although without causing clinical disease.[36]

Nonetheless, the possible involvement of a viral etiology in the disease cannot be ruled out on the basis of a negative virus isolation or negative PCR result, especially considering that this frog batch originated in Sinaloa, a region where *Ranavirus* has been confirmed.[11] As such, it is possible that the affected frogs may have been in contact with a virus that went undetected. In this sense, the present study did not conduct follow-up or infection-kinetics analyses with the frogs since the research scope was diagnostic in nature and logistically limited to a single sample group collected at a particular time.

In the present study, microbiological analysis permitted the isolation of pure bacterial colonies consistent with dermatosepticemia from fresh external macroscopic lesions and internal organs of recently euthanized frogs. Furthermore, histological samples showed an abundant number of bacteria in infected tissues. Biochemical analyses determined isolate cos-ciesa 01 to be *A. hydrophila*, and confirmation through a molecular analysis of the 16S rRNA gene established this isolate to be the causative agent of death in the sampled frogs.

Aeromonas hydrophila is an opportunistic bacterium that, in association with various factors, can cause disease and death in frogs.[5, 37] Among captured and confined animals, it is additionally difficult to determine the moment at which skin injuries favoring bacterial invasion occur.[2, 8] However, stressful situations predispose frogs to infections by opportunistic agents, and malnourished amphibians that have been recently captured or are maintained under inappropriate conditions are particularly susceptible.[6] The combination of stressful conditions and the synergistic interactions of extracellular and intracellular products (i.e., exotoxin, endotoxin, and hemolysin)[36] in *A. hydrophila* may result in the development of bacterial dermosepticemia.

The resulting toxemia includes systemic necrotizing vasculitis, provoking congestion, and edema in affected tissues. Considering all the above, it is possible to speculate that the examined frogs were injured during capture and transport and were likely subjected to conditions of overcrowding, starvation, and drastic temperature variations. Furthermore, the analyzed frogs presented a severe mixed parasitic infestation that may have worsened the immune response. The detected parasites (i.e., *Ochoterenella* sp., *Clinostomum* sp., *Opalina* sp., and *Glypthelmins* sp.) have been widely reported affecting different amphibian species. Nevertheless, these species can also be found parasitizing animals not showing clinical signs.

Conclusions

This is the first report in Mexico of dermatosepticemia where *A. hydrophila* is proven to be the direct cause of death in wild leopard frogs. However, taking into account that the animals also had parasitic infestations, more research is needed to determine the interaction or concomitance of the bacterium *A. hydrophila* with other putative infectious agents. Further research to confirm Koch's postulates with the isolate cos-ciesa 01 is required.

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Data availability

All data that support the findings of this study are included within the manuscript. The corresponding author will be able to provide additional information upon request.

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Conflicts of interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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