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Reproductive loss attributed to *Lactococcus petauri* infection in a black-and-white ruffed lemur

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Abstract. Lactococci have been associated with fetal and neonatal infections in humans and cattle. Here we describe a case of reproductive loss attributed to *Lactococcus petauri* in a lemur. A full-term black-and-white ruffed lemur (*Varecia variegata*) was found dead in the indoor area of a zoologic exhibit. Classification as a late-term abortion or stillbirth was unclear as the precise gestational time was unknown. A medical checkup of the dam revealed fever and neutrophilic leukocytosis; recovery followed treatment with enrofloxacin. The main histologic findings were placental edema and hemorrhage, hepatic necrosis, desquamated amniotic epithelial cells in alveoli, and subendocardial and myocardial hemorrhages. Tissue Gram stain revealed abundant gram-positive cocci arranged in short chains in the placenta and liver. *Toxoplasma gondii* was not detected by immunohistochemistry. Bacterial isolates from the placenta and fetal liver were identified as *Lactococcus garvieae* by MALDI-TOF MS. However, the isolates were found to be *L. petauri* by determining their in-silico DNA–DNA hybridization and average nucleotide identity values using pairwise comparisons of their whole-genome sequences and the genomes of the type strains. The antimicrobial susceptibility of isolates by the disk diffusion method revealed resistance to tylosin, gentamicin, apramycin, neomycin, amikacin, ampicillin, and florfenicol. We attributed the reproductive loss in this lemur to placental and fetal infection by *L. petauri*.

Keywords: abortion; *Lactococcus petauri*; lemurs; placentitis; stillbirth.

Lemurs comprise a diverse group of primates that includes 107 different species. They evolved separately for tens of millions of years in Madagascar and the nearby Comoros Islands.[12] Lemurs are of significant interest to scientists studying primate evolution, behavior, and ecology. They play a crucial ecologic role in their forest ecosystems by feeding on fruits and nectar and dispersing seeds, which promotes plant diversity in their habitats.[12]

The black-and-white ruffed lemur (*Varecia variegata*) is a critically endangered species of lemur native to the lush rainforests and tropical forests of eastern Madagascar. They are also frequently found in zoologic collections. The International Union for Conservation of Nature (IUCN) has classified them as critically endangered due to habitat loss caused by deforestation and fragmentation (https://www.iucnredlist.org/species/22918/115574178).

Conservation efforts for lemurs typically focus on protecting their habitat and understanding their reproductive biology in zoologic collections to support wild lemur populations.[12] Breeding in black-and-white ruffed lemurs is highly seasonal, occurring during the austral spring (October–December). Females give birth to 1 or 2 offspring after a gestation of 102–120 d. Infant lemurs are carried by their mothers during the early stages of life.[3]

A full-term black-and-white ruffed lemur fetus was found dead in the indoor exhibit of a zoologic collection. The mother had expelled the placenta. A medical checkup revealed fever and neutrophilic leukocytosis. The mother was subsequently treated with enrofloxacin (15 mg/kg BW PO q24h) for 10 d. Both the placenta and fetus were exam-

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ined at the zoo. The gross findings included placental edema, collapsed lungs, and mild diffuse liver pallor. The stomach was empty. Samples of the placenta, liver, and lungs were collected for bacteriologic culture. Samples of the placenta and fetal organs were fixed in 10% neutral-buffered formalin and processed for histologic examination.

After 48 h of incubation at 37°C on blood agar plates, pure cultures of hemolytic catalase-negative cocci were obtained from the placenta and liver. Bacterial isolates were subjected to a protein–peptide extraction protocol based on formic acid–acetonitrile (Bruker) to obtain matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker) profiles and tentatively identified as *Lactococcus garvieae* (MALDI-TOF MS score = 2.294).

Genome sequencing of the isolates was performed.[16] Identification was further confirmed by determining in-silico DNA–DNA hybridization (dDDH) and average nucleotide identity (ANI) values using pairwise comparisons of their whole-genome sequences and the genomes of the type strains of *L. garvieae* (CCUG 32208^T; GCA_008692985) and *L. petauri* (159469^T; GCA_002154895). *L. garvieae* and *L. petauri* were compared because of their genetic and phenotypic similarity, and due to the reported misclassification of *L. petauri* isolates as *L. garvieae*.[16]

Genome-to-Genome Distance Calculator 3.0 (GGDC; https://ggdc.dsmz.de/) and the ANI Calculator available in the EzBioCloud database (https://www.ezbiocloud.net/tools/ani) were used to determine the dDDH and ANI values, respectively, using threshold values of ≥70% for dDDH and 95–96% for ANI to delineate bacterial species. The average dDDH and ANI similarity values for *L. garvieae* CCUG 32208^T were 50.6%, and 93.0%, respectively; the average dDDH and ANI similarity values for *L. petauri* 159469^T were 81.5% and 98.3%, respectively. Based on these values, isolates were identified as *L. petauri*.

Antimicrobial susceptibility tests were performed as recommended by the Clinical and Laboratory Standards Institute, using the standard disk diffusion method. We tested the following commercial antimicrobials: amikacin (30 µg), amoxicillin (30 µg), ampicillin (10 µg), apramycin (30 µg), enrofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), gentamicin (100 µg), neomycin (30 µg), spectinomycin (25 µg), tylosin (30 µg), tetracycline (30 µg), and vancomycin (30 µg; Oxoid).

Inhibition zone diameters for the disk diffusion method are not available for *Lactococcus* spp.; hence, breakpoints were extrapolated from *Enterobacteriaceae* as follows: resistant <11 mm, intermediate 11–20 mm, or susceptible >20 mm.[19] Based on breakpoints, isolates were resistant to amikacin (8 mm), ampicillin (17 mm), apramycin (15 mm), florfenicol (24 mm), gentamicin (11 mm), neomycin (11 mm), and tylosin (18 mm); intermediate to enrofloxacin (18 mm), spectinomycin (12 mm), and tetracycline (23 mm); and susceptible to amoxicillin (20 mm), erythromycin (24 mm), and vancomycin (20 mm).

Histologically, placental villi were severely edematous, and the placenta was focally hemorrhagic (Fig. 1A). A tissue Gram stain revealed numerous gram-positive cocci arranged in short chains within and covering the surfaces of placental villi (Fig. 1B). The fetal liver had multifocal, predominantly periportal, areas of hepatocellular degeneration and necrosis (Fig. 1C), and rare gram-positive cocci were seen. Several pulmonary alveoli contained desquamated amniotic epithelial cells. The heart had multifocal subendocardial and myocardial hemorrhages.

For immunohistochemistry (IHC), paraffin sections of fetal organs were placed on charged slides. Deparaffination, rehydration, and heat-induced epitope retrieval were carried out using a PT module (Epredia). Then, samples were incubated in a hydrogen peroxide solution in methanol (Panreac Química) to quench endogenous peroxidase. Slides were then incubated in horse serum (Vector), followed by overnight incubation at 4°C with a mouse monoclonal anti–broad-spectrum cytokeratin (AE1/AE3; MAD-00001000QD, Vitro) antibody or a one-hour incubation with a rabbit polyclonal anti–*Toxoplasma gondii* (PA5-16921; Thermo Fisher) antibody. Commercial reagents were used for the secondary antibody (ImmPRESS-VR horse anti-rabbit IgG polymer kit; Vector) and chromogen (ImmPACT NovaRED peroxidase substrate; Vector). For negative controls, the primary antibody was replaced by a commercial universal negative control reagent. Finally, samples were counterstained with hematoxylin and coverslipped. IHC results for wide-spectrum cytokeratin confirmed desquamated amniotic epithelial cells in the fetal lung (Fig. 1D), and we found no evidence of *T. gondii* in fetal tissues.

L. petauri was first isolated from a facial abscess in a sugar glider (*Petaurus breviceps*),[7] and it has also been identified as an emerging pathogen in farmed fish.[13,16] Lactococci have been known to cause infections in immunocompromised individuals,[14] but there is only one report of a human urinary tract infection caused by *L. petauri*.[6] Our isolation of *L. petauri* from a black-and-white lemur placenta and liver associated with reproductive loss expands the range of susceptible mammals.

L. petauri was initially misidentified as *L. garvieae* using MALDI-TOF MS, indicating that this method is not sufficiently sensitive to identify lactococci at a species level, as suggested previously.[7] MALDI-TOF MS offers the advantage of being rapid, cost-effective, and widely accessible but its accuracy in distinguishing closely related species can be limited in some cases due to overlap in protein spectra. In contrast, in-silico methods such as dDDH and ANI values provide higher resolution and accuracy for species-level identification, as they are based on genomic comparisons; however, these methods are more resource-intensive, requiring access to high-quality genome data and computational

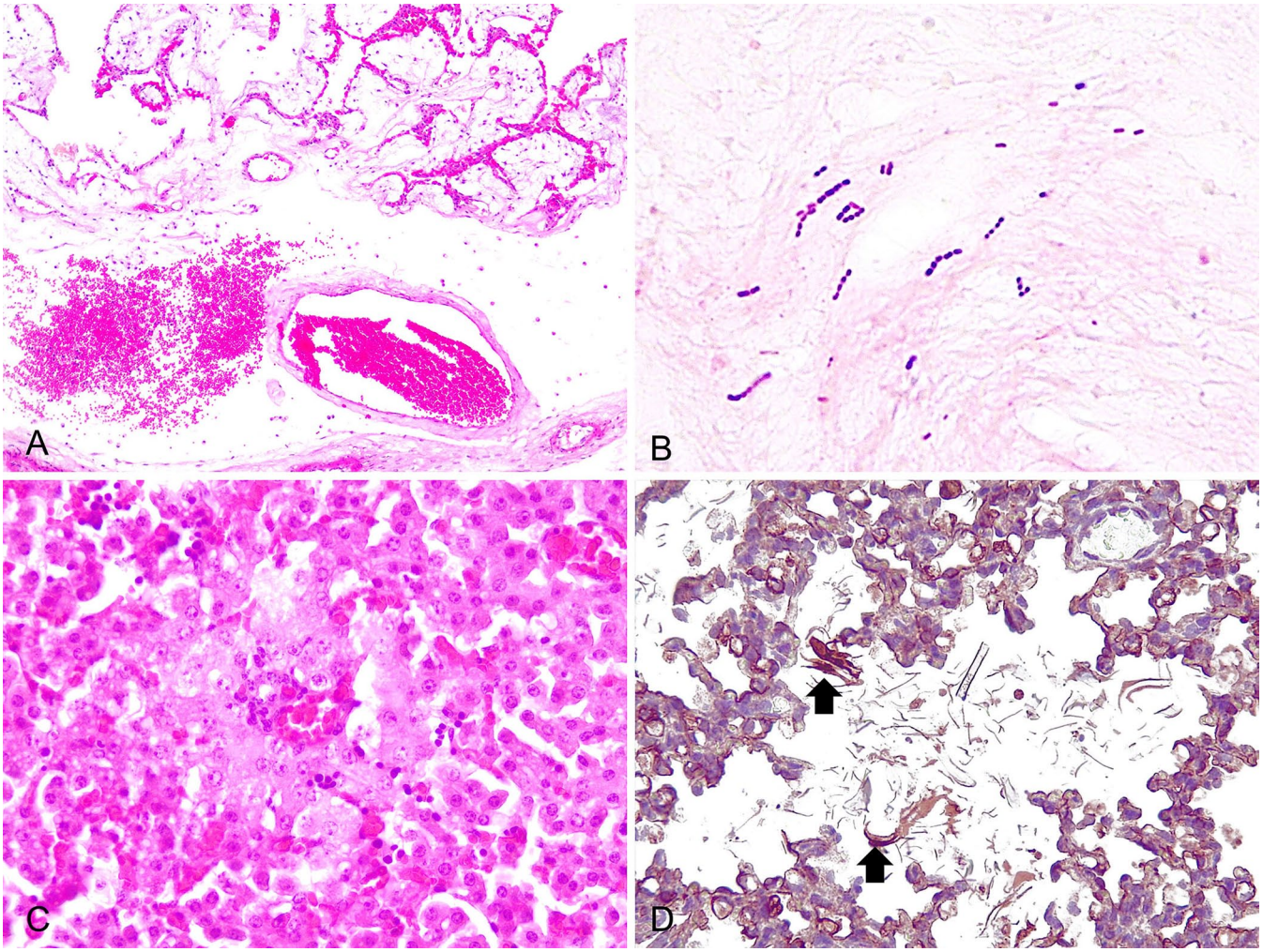

Figure 1. Reproductive loss in a black-and-white ruffed lemur attributed to *Lactococcus petauri* infection. **A.** Epitheliochorial placenta with villus edema, and an extensive area of hemorrhage. H&E. **B.** Numerous gram-positive cocci arranged in short chains in the edematous areas of the placenta. Gram stain. **C.** Fetal liver with periportal hepatocellular degeneration and necrosis. H&E. **D.** Desquamated amniotic epithelial cells that immunolabel for cytokeratin in the alveolar lumens (arrows). Mouse monoclonal anti–broad-spectrum cytokeratin (AE1/AE3) antibody.

tools. The identification of *Lactococcus* spp. isolates can also be performed through whole-genome sequencing (WGS), sequencing the *gyrB* gene or the 16S-23S ITS region, or PCR testing.[13,15,16]

Our *L. petauri* isolate was resistant to certain aminoglycosides (amikacin, apramycin, gentamicin, neomycin), macrolides (tylosin), beta-lactams (ampicillin), and florfenicol. However, a study of 78 *L. petauri* isolates from rainbow trout showed susceptibility to ampicillin, amoxicillin, erythromycin, florfenicol, oxacillin, oxytetracycline, and penicillin if an epidemiologic cutoff values-99 (ECOFFinder approach) is applied.[9] Although the *L. petauri* strains isolated in our study had moderate resistance to enrofloxacin, the signs of infection in the female lemur resolved after a 10-d enrofloxacin treatment. No further reproductive problems were registered one year after the *L. petauri*–associated reproductive loss.

Lactococci are not part of the vaginal microbiome in the lemur species,[2,4,20] but belong to the gut microbiome of some others,[1] and *L. petauri* has been isolated from the urine of an elderly man.[6] Considering this, *L. petauri* infection could have been ascending (alimentary or urinary tracts), as reported in cases of *L. lactis cremoris* or *L. garvieae* infection in pregnant women.[11,14] Vertical transmission may have contributed to fetal infection, as occurs in humans (*L. lactis cremoris*)[11] and cattle (*L. lactis*), leading to sporadic abortions.[17]

L. petauri infected the lemur fetus through the placenta and umbilical cord, reaching the liver via the umbilical veins. Fetal distress may have led to amniotic fluid aspiration, as evidenced by the presence of amniotic epithelial cells in the fetal airways, which is a common, nonspecific finding in fetuses following intrauterine infections. However, based on the bacteriologic results (no growth from the lung) and

autopsy findings (absence of gastrointestinal contents), it seems that the infection was not extensive enough for *L. petauri* to be present in the amniotic fluid to be aspirated or swallowed. Determination of whether this is a late-term abortion or stillbirth was not possible as the precise gestational time was unknown.

L. garvieae and *L. lactis* have been identified as causative agents of sepsis, pneumonia, urinary tract infections, and meningitis in human neonates.[5,8,14] Additionally, *L. lactis* has been reported to cause sepsis and arthritis in neonatal calves, likely through milk consumption.[18] Hepatic lesions have not been reported in human neonates or calves. However, in our case, the most notable finding was hepatocellular degeneration and necrosis, probably due to local hypoxia caused by placental insufficiency rather than a direct effect of *L. petauri* in the tissue. In rainbow trout infected by *L. garvieae*, hepatic necrosis is only observed if bacteremia is established.[10]

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