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Original Research

Isolation and Characterization of a Novel Alpha-Hemolytic *Streptococcus* spp. from the Oral Cavity and Blood of Septicemic Periparturient Immunodeficient Mice

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MISTRG is an immunodeficient mouse strain that expresses multiple human cytokines that support hematopoietic stem cell maintenance and myelopoiesis. While establishing a breeding colony of MISTRG mice in a dedicated barrier room, 6 cases of death or disease occurred in pregnant or postpartum mice. Clinically, this manifested as hunched posture, dyspnea, and 1 case of emaciation with ataxia. Pathologic analysis of 7 mice revealed multisystemic necrosuppurative inflammation variably affecting the uterus and placenta, joints, meninges, inner and middle ears, kidneys, and small intestine. Bacteria cultured from the blood of septic mice were identified with 89% probability by the Vitek 2 identification system as *Streptococcus sanguinus* with atypical biochemical parameters; the API 20E/NE system fully differentiated the isolates as a novel *Streptococcus* species. MALDI Biotyper-based mass spectrometry also indicated that the phenotype represented a novel *Streptococcus* spp. Sequencing revealed that the full-length 16S rRNA gene identity was below 97% with known *Streptococcus* species, including the 2 closest species *Streptococcus acidominimus* and *Streptococcus azizii*. We propose the name *Streptococcus murisepticum* spp. nov to our novel isolates. All male mice in this colony remained healthy despite their association with diseased female mice. Overall, 19% of the colony carried the novel *Streptococcus* in their oral cavity, but it could not be detected in feces. The organism was sensitive to amoxicillin, which was administered via drinking water throughout pregnancy and weaning to establish a colony of pathogen-negative future breeders. The colony remained disease-free and culture-negative for *Streptococcus murisepticum* spp. nov after treatment with amoxicillin. We suspect that oral colonization of MISTRG mice with the novel *Streptococcus* species and its associated unique pathology in periparturient mice is potentially the principal cause of loss of this strain at several institutions. Therefore, screening the oral cavity for α-hemolytic streptococci followed by targeted antibiotic treatment may be necessary when establishing MISTRG and allied immunodeficient mouse strains.

Abbreviations and Acronyms: MISTRG, M-CSF$^{h/h}$ IL-3/GM-CSF$^{h/h}$ SIRPa$^{h/h}$ TPO$^{h/h}$ RAG2$^{-/-}$ IL2Rg$^{-/-}$; MISTRG-EPO, M-CSF$^{h/h}$ IL-3/GM-CSF$^{h/h}$ SIRPa$^{h/h}$ TPO$^{h/h}$ RAG2$^{-/-}$ IL2Rg$^{-/-}$ EPO; TMS, Trimethoprim Sulfamethoxazole; VGS, Viridans Group Streptococci

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Introduction

Several immunodeficient mouse strains, including NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ), have low engraftment and differentiation potential for human myeloid cell lineages.[33] However, the MISTRG (M-CSF$^{h/h}$ IL-3/GM-CSF$^{h/h}$ SIRPa$^{h/h}$ TPO$^{h/h}$ RAG2$^{-/-}$ IL2Rg$^{-/-}$) mouse strain is highly permissible for engraftment of functional human myeloid cells (monocytes, basophils, eosinophils, dendritic cells, and natural killer cells) in the bone marrow and in lymphoid and nonlymphoid tissues, enabling engraftment of certain acute myeloid leukemia and myelofibrosis xenografts.[19,28] The MISTRG strain is characterized by the expression of human cytokines (macrophage colony-stimulating factor, interleukin-3, granulocyte-macrophage colony-stimulating factor, and thrombopoietin) that replace endogenous mouse cytokines through gene knock-in and that drive human myeloid development and differentiation in the mice.[12,33] Complementarily, the expression of the human signal regulatory protein α transgene, which blocks phagocytosis of human cells by mouse macrophages on a highly immunodeficient background (RAG2 and IL2Rg mutation), makes such xenotransplantation highly efficient and reproducible.[26,33,40] As such, MISTRG and the allied MISTRG-EPO strain, which harbors human erythropoietin (EPO) as the fifth knock-in cytokine needed to support the development of human erythroid lineage, are excellent patient-derived xenograft (PDX) models for studying leukemia and myelodysplastic syndromes. Recombination activating gene 2 (RAG2) and interleukin-2 receptor subunit gamma (IL2Rg) mutations impair adaptive

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and innate immunity in MISTRG/MISTRG-EPO strains[19]. Furthermore, the lack of murine cytokines may functionally compromise both neutrophils and macrophages in these strains. As such, these mice may be highly susceptible to infection, including with opportunistic agents like α-hemolytic *Streptococcus* spp., and noncoagulase-positive *Staphylococcus* spp., that may be viewed as nonpathogenic commensals in routinely used immunodeficient mouse strains.

Alpha-hemolytic streptococci, also known as viridans group streptococci (VGS), are gram-positive bacteria found in the oro-nasopharyngeal, gastrointestinal, and genitourinary tracts of animals, including humans.[6] Except for *Streptococcus pneumoniae*, the best-known and most well-studied member of this group, most of the species in this group are either nonpathogenic or of low pathogenicity.[30] Despite their low pathogen potential, α-hemolytic streptococci can cause septicemia and invasive infections like meningoencephalitis in children,[6] weanling mice,[2] and patients with chemotherapy-induced oral mucositis.[29] Patients with neutropenic cancer are susceptible to VGS bacteremia-induced streptococcal toxic shock syndrome, which can have mortality rates as high as 40% to 100%, with *Streptococcus mitis* as the most frequent isolate.[30]

Except for a study reporting on a well-characterized α-hemolytic *Streptococcus azizii* infection in weanling mice[2] and a report on oral colonization in Sprague–Dawley rats by a novel *Streptococcus orisratti* spp.,[42] rare natural *Streptococcus* infections in rats and mice are due to β-hemolytic streptococci.[32,34] Also, α-hemolytic streptococci are not included in the standard microbial test panels for research rodents, possibly because of the ubiquitous nature of this bacterial group. Even if identified in culture, the phenotypic similarities between VGS make species-level identification difficult when testing relies on standard biochemical and mass spectrometry-based methods; marker gene-based molecular methods are more reliable for species determination.[5,11,30] As such, novel α-hemolytic streptococci that colonize or cause disease in research mice are either not identified at the species level or are misidentified as a known VGS.

In this article, we use phenotypic testing and whole genome sequencing to characterize a novel α-hemolytic *Streptococcus* spp. that colonized the oropharynx of a recently procured colony of male and female MISTRG mice. Cultures of mouse oral cavities and feces can reveal the presence of most VGS species (*Streptococcus sanguinus* and *Streptococcus mitis*); however, we were unable to grow this novel *Streptococcus* spp. from the feces of our mice. This novel *Streptococcal* spp. manifested predominantly as septicemia and multisystemic necrosuppurative inflammation and occurred only in pregnant and lactating mice. We eliminated the bacteria from this colony through the careful use of antibiotics. Antibiotic treatment may be necessary for the successful establishment of MISTRG and related immunodeficient strains colonized with this novel α-hemolytic *Streptococcus* species.

Materials and Methods

Case presentation. During the summer of 2020, we imported 10 male and 20 female MISTRG (strain number 017712) and MISTRG-EPO strains of mice from Regeneron Pharmaceuticals (Tarrytown, NY) as part of a material transfer agreement. The mice were placed on Institutional Animal Care and Use Committee protocol approved by the St Jude Children's Research Hospital Animal Care and Use Committee for breeding, genotyping, sample collection, and investigation of hematopoietic neoplasms in mice. The mice were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*. On arrival, animals underwent PCR testing and were negative for viral, bacterial, and parasitic agents that are typically excluded in immunodeficient mice colonies, including lymphocytic choriomeningitis, mouse adenovirus 1 and 2, mouse hepatitis virus, murine norovirus, mousepox, mouse parvovirus/minute virus of mice, mammalian orthoreovirus, murine chapparvovirus, pneumonia virus of mice, reovirus, Sendai virus, Theiler's murine encephalomyelitis virus/GDVII subgroup, β-hemolytic streptococci Groups A, B, C, and G, *Bordatella bronchiseptica*, *Bordatella pseudohinzii*, *Corynebacterium bovis*, cilia-associated respiratory bacillus, *Corynebacterium kutscheri*, *Citrobacter rodentium*, *Clostridium piliforme*, *Klebsiella oxytoca, Klebsiella pneumoniae*, *Helicobacter* genus, *Mycoplasma pulmonis, Rodentibacter heylii, Rodentibacter pneumotropicus, Pseudomonas aeruginosa*, *Salmonella* genus, *Staphylococcus aureus, Streptobacillus moniliformis, S. pneumoniae*, *Cryptosporidium*, Demodex, *Entamoeba*, *Giardia*, mites, pinworms, *Pneumocystis*, *Proteus mirabilis*, *Spironucleus muris*, and *Tritrichomonas* genus. The mice were also negative by bacteriological culture for the opportunistic bacterial agents *Klebsiella* spp., *Rodentibacter* spp., *Proteus* spp., and *Staphylococcus aureus*.

A dedicated room was used for the breeding and expansion of these mice and was maintained under strict biosecurity to avoid fomite, murine, and human pathogen contamination. Mice were housed in breeding pairs or in groups of 5 after weaning in autoclaved, individually ventilated cages (Allentown Jag cages, Allentown, NJ) with quarter-inch corncobs (The Andersons, Maumee, OH)as bedding. They received reverse osmosis, ultraviolet-light–irradiated, and chlorinated (4 ppm) water through an automatic watering system (Avidity Science, Waterford, WI) and a rodent diet that contained trimethoprim-sulfamethoxazole (TMS; 5TK4, modified LabDiet 5058, 0.025% trimethoprim and 0.124% of sulfamethoxazole). Cages were changed every 2 wk or were spot-changed as needed. Sentinel screening was based on multiplex PCR screening of exhaust air dust (Charles River Laboratories Inc, Wilmington, MA). The agents screened for were the same as those tested at arrival.

Over the next 3 to 4 mo, we noticed sporadic deaths of breeding females and pups, initially attributed to the advanced age of breeding females (> 6 mo) and the possible fastidious nutritional and husbandry needs of these mouse strains. The remaining cohort of pregnant females delivered small litters that were successfully raised and were used as next-generation breeders. One of these female mice was noticeably thin and ataxic at 5 wk of age and subsequently was euthanized for humane reasons. Over the next 5 mo, 5 breeding females presented with lethargy, hunched posture, and respiratory distress, and one was found dead. The signalment and clinical signs from these cases are presented in Figure 1.

Necropsy, clinical pathology, and histopathology. Oral swabs and feces were collected for bacteriology and mice were then euthanized with carbon dioxide. Blood was then collected for bacteriology and a complete blood count (CBC), followed by necropsy and tissue collection for histopathologic examination. For CBC, blood was collected into microfuge tubes containing a 10% volume of disodium enthylenediaminetetraacetic acid anticoagulant (10% w/v concentration). CBCs were performed on a ForCyte Hematology Analyzer (Oxford Science, Inc, Oxford, CT). For each mouse, a full set of tissues was routinely fixed in 10% neutral-buffered formalin for a period ranging from 48 h to 2 wk. Bone specimens were decalcified in 10% formic acid. Tissues were embedded in paraffin and sectioned

Case No.	Signalment (Strain; Age; Parity)	Clinical Sign(s)	Pathology findings
1	28 weeks; MISTRG; Post-partum lactating	Dyspnea	Necrosuppurative placentitis with intralesional bacteria (Fig 1A); placental detachment with prolapse into vagina; necrosuppurative aortic endocarditis with intralesional bacteria
2	5 weeks; MISTRG-EPO; Nulliparous	Thin, hunched, and ataxic with tachypnea	Vaginitis with necrosis and rupture of the wall (Fig 1B); polysynovitis (temporomandibular, stifle [Fig 1F] and vertebral facet joints); otitis interna and media; aortitis
3	19 weeks; MISTRG; Post-partum lactating	Hunched, lethargic with tachypnea	Necrosuppurative aortic endocarditis (Fig 1C) and papillary nephritis with intralesional bacteria; polysynovitis (temporomandibular and knee joints); otitis interna
4	11 weeks; MISTRG; Post-partum lactating	Dyspnea	Meningitis (Fig 1D inset upper right) and otitis interna (Fig 1D inset lower right); lumbar radiculoneuritis; pancreatitis
5	20 weeks; MISTRG; Post-partum lactating	Lethargic, ataxic	Necrotizing placentitis with fetal resorption; vaginal ulceration with submucosal vaginitis and intralesional bacteria; otitis media; aortic endocarditis; papillary nephritis (Fig 1E)
6	20 weeks; MISTRG; Post-partum lactating	Scruffy, hunched with tachypnea	Neutrophilic meningitis of brain and spinal cord; optic neuritis; neutrophilic periostitis of cranium; serositis; mastitis with focal area of cellulitis
7	20 weeks; MISTRG-EPO; Post-partum lactating	Found dead	Necrosuppurative placentitis with intralesional bacteria; placental detachment; necrotizing Ileo-jejunitis

Figure 1. Case findings from breeding females from different cages that had positive blood cultures for the novel α-hemolytic *Streptococcus* spp.

at 4-µm thickness. Sections were mounted on slides and either stained with routine hematoxylin and eosin (HE) or used for immunohistochemistry (IHC) analysis. All assay steps for CD68 IHC analysis were performed on the Bond Max with Bond wash buffer (#AR9590; Leica Biosystems, Deer Park, IL), with rinses between steps. Heat-induced epitope retrieval was performed with Bond Epitope Retrieval Solution 2 (ER2), and the slide was incubated with the primary antibody (Cell Signaling, #97778) at 1:900 for 15 min and then with Bond Polymer Refine Detection (DS9800). Labeling was visualized with streptavidin conjugated to horse radish peroxidase (ThermoShandon, #TS-125-HR, 10 min) and substrate containing the chromagen DAB (ThermoShandon, #TA-125-HDX, 5 min).

Microbiology. Blood, oral, and fecal samples were cultured on Trypticase Soy Agar (TSA). Morphologically distinct colonies were biochemically phenotyped by using the Vitek 2 Compact System (bioMérieux, Durham, NC) and the GP identification card. Colonies were then cryopreserved as glycerol stocks. To recover organisms, glycerol stocks were streaked on to Columbia Agar (CA) with 5% sheep blood under 5% CO_2 microaerophilic conditions. Individual colonies from CA were used for whole- genome sequencing, and biocheminal phenotypes were revalidated by inoculation onto API20 Strep strips in duplicate (bioMérieux, Marcy- I'Etoilel'Étoile, France). At the same time, antibiotic sensitivity testing was performed using MIC and breakpoint standards listed for VGS using the Clinical and Laboratory Standards Institute guidelines (version 11.0). We also analyzed antibiotic sensitivity by using the Kirby-Bauer method based on inoculation of a TSA plate with the McFarland Standard of a test organism to provide a uniform lawn of growth. A disc stamper was used to apply specific antibiotics indicated for *Streptococcus* spp. (clindamycin, chloramphenicol, enrofloxacin, erythromycin, amoxicillin/clavulanic acid, ampicillin, amikacin, tetracycline, cephalothin, vancomycin, and penicillin) to the CA plates. For MALDI-TOF mass spectrometry, samples were sent to IDEXX BioAnalytics (Columbia, MO).

Whole-genome characterization and phylogenetic analysis. The whole genomes of the novel *Streptococcal* spp. strains (isolates B01 from blood and O1 from the oral cavity of the ataxic and thin mouse, Figure 1, Case 2) were sequenced at the St. Jude Children's Research Hospital Hartwell Center for Biotechnology with MiSeq (Illumina, Inc, San Diego, CA) and at St. Jude Children's Pathology Laboratory with MinION (Oxford Nanopore Technologies, Littlemore, Oxford, United Kingdom). DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corp, Madison, WI). For short-read sequencing, libraries and paired-end reads (2 × 150 bp) were generated using Nextera XT and MiSeq reagent kits and instruments

(all Illumina). For long-read sequencing, MinION was used to prepare libraries and generate reads. Illumina paired-end reads were trimmed by fastp (version 0.20.1; parameters: -detect_adapter_for_pe, -cut_front, -cut_tail, -cut_window_size 4, -qualified_quality_phred 25, -length_required 20)[3] and the nanopore reads were trimmed by porechop (version 0.2.4) with default parameters. Filtered nanopore reads were then assembled by Minimap2 (version 2.17)[18] and miniasm (version 0.3_r179)[17] with default parameters. The resulting miniasm assembly was polished by Racon (version 1.4.13, parameters: -m 8 -x -6 -g -8 -w 500)[37] and Medaka (version 1.2.0, with r941_min_fast_g303 model). This corrected nanopore long-read assembly was further corrected and extended with the Illumina paired-end data using POLCA (built-in MaSuRCA 3.4.2).[43] CheckM (version 1.1.3, taxonomic genus *Streptococcus*-specific workflow)[24] was used to assess the complete assembly of all scaffolds, including the largest scaffold. The final assembly was 100% complete and, as a result, was designated a complete draft genome. The draft genome was annotated using the National Center for Biotechnology Information's (NCBI) Prokaryotic Genome Annotation Pipeline (PGAP, version 6.1)[35] and further characterized with the curated virulence factor database, MEGARes, and PHASTER to identify virulence-associated genes, antibiotic resistance mechanism genes, and prophages, respectively. Digital DNA-DNA hybridization estimates were generated with the Genome-to-Genome Distance Calculator 2.1 (https://ggdc.dsmz.de/ggdc.php/) using Equation 2 (identities/HSP length). In addition, OrthoANI (Orthologous Average Nucleotide Identity)[16] values were calculated for both complete and draft genome assemblies by the OrthoANIu tool (version 1.2; https://www.ezbiocloud.net/tools/orthoaniu). Average amino acid identity was calculated from the following web server: http://enve-omics.ce.gatech.edu/aai/.

For phylogenetic analysis, 16S rRNA gene sequences of the closely related taxa of the genus *Streptococcus* were obtained by performing a BLAST search against the nucleotide database. The 16S rRNA gene sequences of strain B01 and closely related members of the genus *Streptococcus* were aligned using MAFFT (version 7.471).[13] The 16s rRNA gene phylogenetic tree was calculated in Mega X[15] using the maximum likelihood method and the general time-reversible model with a discrete γ distribution and rapid bootstrap (1000 replicates) analysis. Pairwise sequence similarity was calculated by the needle program in the EMBOSS Software Suite (version 6.6.0).[27]

The core phylogeny of 22 *Streptococcus* genomes (B01 and O1 isolates and 20 representative strains of species of the genus *Streptococcus* from the NCBI GenBank) and the *Enterococcus faecalis* V583 was constructed using 433 core genes determined by the roary[23] pan genome pipeline (version 3.13.0, -i 70 -cd 90). Nucleotide sequences of these 433 core genes in each genome were concatenated. Four additional marker genes (*rpoB*, *sodA*, *recN*, and *gyrB*) were extracted and concatenated. Multiple sequence alignments were performed by MAFFT (version 7.471),[13] and the maximum-likelihood trees were constructed by FastTree (version 2.1.10).[25]

Data analysis. CBC parameters of healthy and affected mice were analyzed using Excel (Microsoft 350 ProPlus). Welch's *t* test was used due to an unequal variance in parameters of the infected and healthy mice; differences were considered statistically significant at a *P* value less than 0.05.

Data availability. The GenBank/ENA/DDBJ accession numbers for the Whole Genome Shotgun projects of *Streptococcus* isolate B01 and *Streptococcus* isolate O1 are JALJAW000000000 and JALJAX000000000, respectively.

The GenBank/ENA/DDBJ accession numbers for the 16S rRNA of isolate B01 is OP501805.1 and for isolate O1 is OP501806.1.

Results

Clinical and histopathologic findings. Compared with healthy controls of the same strain, the 7 affected breeding females, of which 6 were periparturient, had statistically significant neutrophilic leukocytosis, lower red blood cell count, hematocrit, hemoglobin, and platelets, and an absence of reticulocytes (Table 1). Histopathologic examination of the affected female mice revealed multisystemic necrosuppurative inflammation from bacterial septicemia (Figure 2). The most affected system was the reproductive tract, with 3 of the mice having placentitis and one with infection and rupture of the vaginal wall. Septicemia manifested in a combination of tissues: 4 mice with otitis, 3 with aortic valve endocarditis, 2 each with renal papillary nephritis, meningitis, serositis, and arthritis, and one with jejunitis (Figure 2). Varying degrees of alveolar proteinosis and histiocytosis (Figure 3), splenic white pulp hypoplasia, and thymic hypocellularity were seen in the affected mice and were considered to be strain-related background findings because identical changes were also present in healthy MISTRG/MISTRG EPO mice.

Microbiologic characterization. Bacterial cultures of the blood and oral cavity of affected females revealed an α-hemolytic pattern on trypticase soy broth, identified by Vitek 2 automated system using GP cards to be *S. sanguinis* with an 89% probability but with contraindicating biopatterns of salicin and d-mannose fermentation and β-galactosidase expression. API20 Strep strips revealed positive β-glucuronidase activity and sorbitol fermentation, negative α-glucuronidase activity, and a lack of fermentation of raffinose and pullulane sugars, allowing differentiation from *S. azizii* 12.5202, *Streptococcus acidominus* CCUG 27296, and *Streptococcus cuniculi* CCUG 65085 (Table 2). MALDI-TOF mass spectra of the blood and oral cavity isolates against the MaldiBiotyperCompass-BDAL-8.0 database provided logarithmic identification scores of 1.7 and 1.8, respectively; scores of ≥ 1.7 and 2.0 indicate reliable genus- and species-level identification, respectively. Based on the above phenotypic characteristics, the isolates were deemed to be a novel species of *Streptococcus*.

Genomic characterization. Genomic features of the blood and oral isolates of the novel *Streptococcus* spp. involved 2119 to 2447 predicted genes and 2048 to 2376 coding sequences, including 4almost identical 16S rRNA and 55 tRNA sequences (Table 3). These 4almost identical 16S rRNA sequences from the blood isolate were phylogenically closely related to a cluster of undescribed *Streptococcus* spp., including 99.6% identity with 2 of the sequences (KM609123.1 (*Streptococcus* spp. AHSI00047 and AB969442.1, an uncultured bacterium, clone: DE1) in the NCBI nucleotide database (Figure 4). Matches to established *Streptococcus* spp. were less than 97%, which is below the

Table 1. Statistically and clinically significant blood parameters (Mean ± SEM) from clinically healthy and septic MISTRG mice

Blood Parameter	Healthy mice	Septic mice
WBC (10^3/uL)	1.8 ± 0.3	4.2 ± 0.8
Neutrophils × 103/uL	1.3 ± 0.2	3.8 ± 0.7
RBC x 106/uL	9.1 ± 0.1	7.5 ± 0.6
Hematocrit %	52 ± 1	40 ± 3
Hemoglobin g/dL	13.3 ± 0.2	10.8 ± 0.6
Platelets × 103/uL	536 ± 417	320 ± 21
Reticulocytes / uL	343 ± 32	0

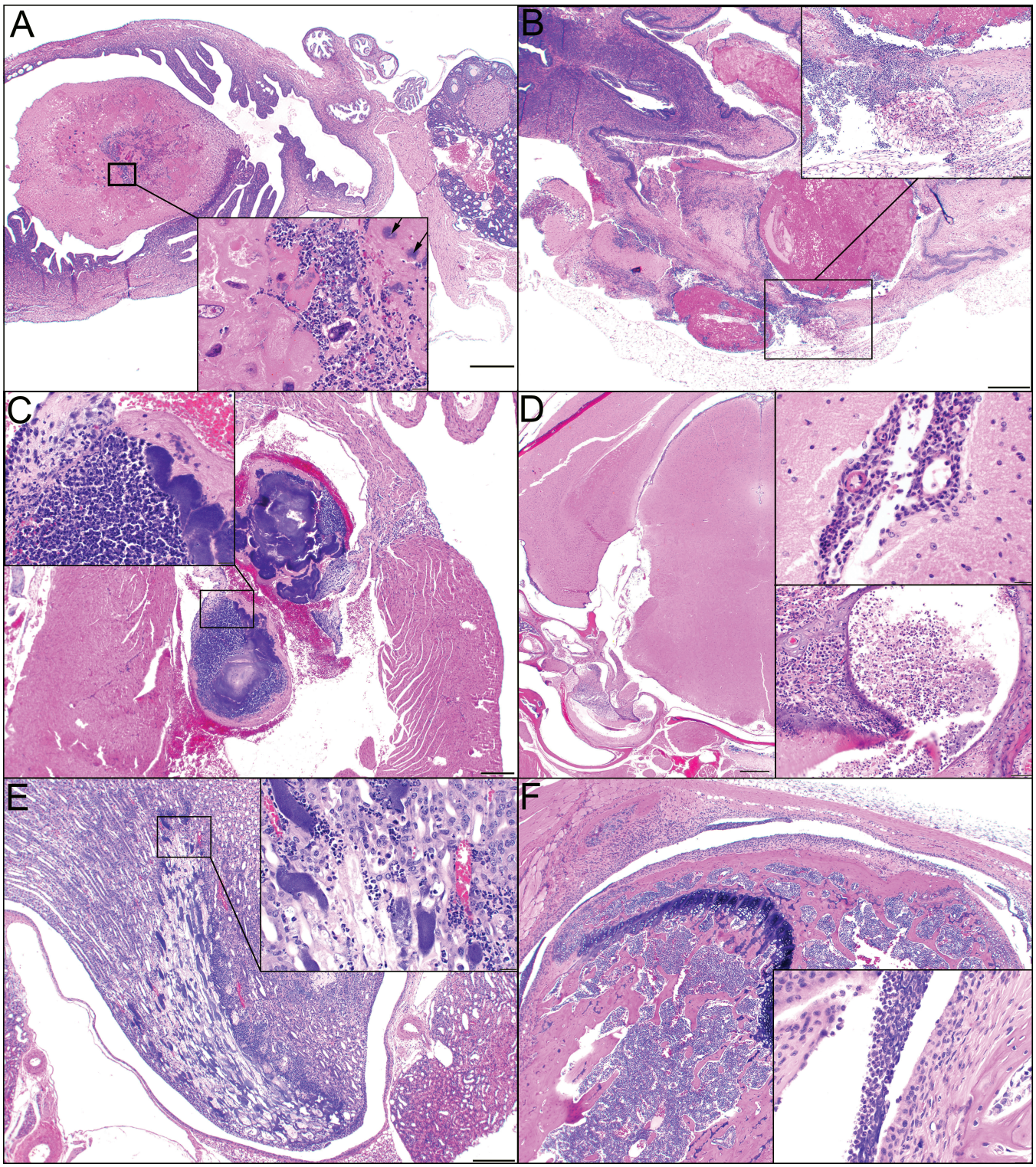

Figure 2. Histopathology of lesions associated with novel *Streptococcus* spp. infection. (A) Case 1. Mouse uterus with necrosuppurative placentitis and bacteria (inset, arrows indicate bacterial colonies within the necrotic placental trophoblasts, accompanied by degenerate neutrophils). Scale bar in large image is 500 µm, in inset is 20 µm. (B) Case 2. Rupture of vaginal wall with necrosis, fibrinosuppurative inflammation, and bacteria. The vaginal lumen contains fibrin, necrotic debris, and degenerate neutrophils (scale bar 500 µm). Inset shows site of rupture (scale bar 20 µm). (C) Case 3. Heart, aortic valve endocarditis with large mass of bacteria surrounded by fibrinosuppurative inflammation (scale bar 200 µm). Inset shows valve overlain with degenerate neutrophils, bacteria, and fibrin (scale bar 20 µm). (D) Case 4. Cross section of whole head (scale bar 200 µm) with suppurative meningitis (inset, upper right, scale bar 20 µm) and otitis interna (inset, lower right, scale bar 50 µm). (E) Case 5. Necrosuppurative papillary nephritis with bacteria (scale bar 100 µm). A large portion of the renal papilla is necrotic and infiltrated by degenerate neutrophils and large colonies of bacteria (inset, scale bar 20 µm). (F) Case 1. Cross section of stifle joint with suppurative arthritis (scale bar 200 µm). Inset shows degenerate neutrophils in joint space (scale bar 20 µm).

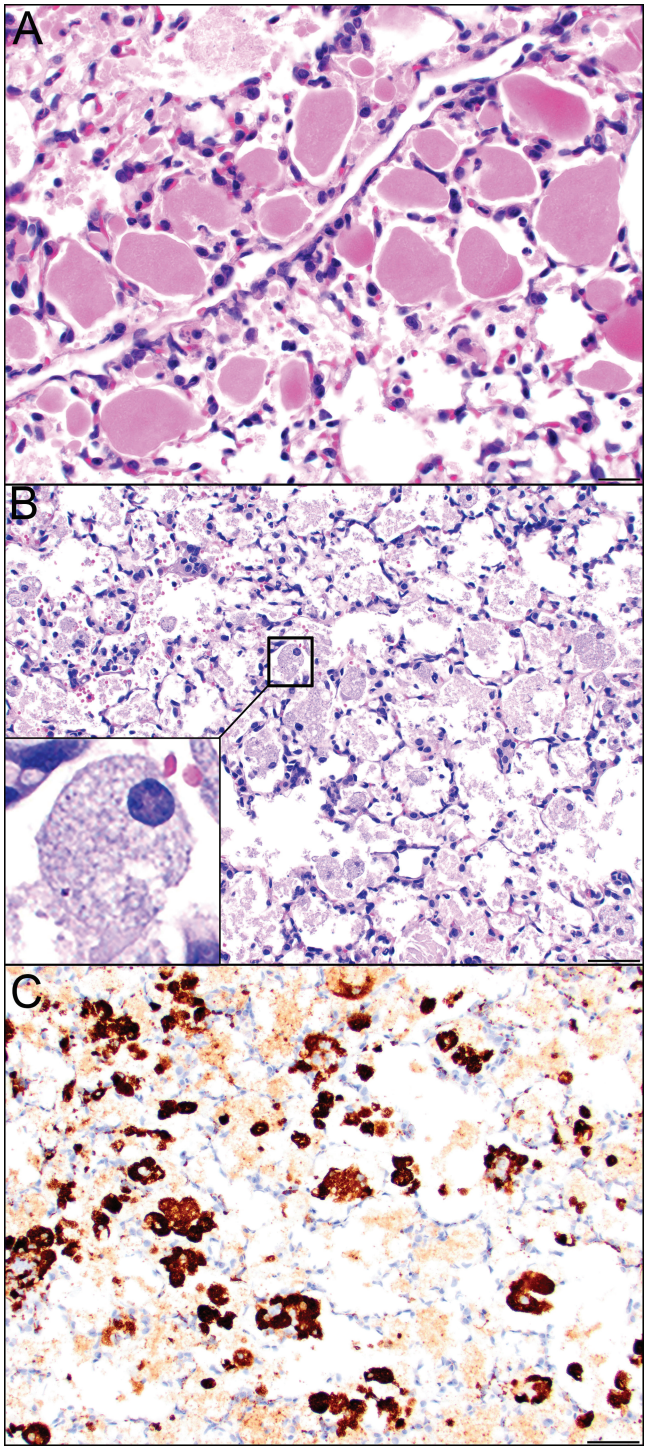

Figure 3. Background histopathology of proteinosis and histiocytosis in MISTRG mice. (A) Mouse lung alveoli filled with eosinophilic protein (scale bar 20 µm). (B) Mouse lung alveoli containing both protein and histiocytes with engulfed cytoplasmic protein (scale bar 50 µm). (C) Histiocytes are positive for mouse CD68 (scale bar 50 µm).

threshold of 98.7% for species-level matching.[4] The isolate B01 16S rRNA sequences shared 94.4% to 96.4% similarity and average nucleotide identity (ANI) and amino acid identity values of < 85% and < 87%, respectively, with closely related species *S. acidominimus* strain CCUG 27296, *S. azizii* strain 12-5202, *S. cuniculi* strain CCUG 65085, and *Streptococcus respiraculi* strain HTS25 (Table 4). These values were well below the ANI species delineation boundary of 95% to 96%.[10,14] Pairwise digital DNA-DNA hybridization (dDDH) estimates of the previously mentioned, closely related *Streptococcus* strains were well below the species boundary of 70%,[22] ranging from 27.2 to 29.6 (Table 4). Further, core genome phylogenetic analysis, based on 433 core genes of the *Streptococcus* B01 and O1 isolates against closely related 20 *Streptococcus* spp., confirmed their unique identity (Figure 5). In this analysis, *S. acidominimus* strain CCUG 27296 and *S. azizii* strain 12 to 5202 were the next 2 closely related species. Finally, phylogenetic analysis of 4 marker genes (*rpoB*, *sodA*, *recN*, and *gyrB*), which are more discriminative for the genus *Streptococcus*,[8,9] confirmed the distinctive placement of these isolates from other *Streptococcus* species (Figure 6). Overall, genomic analysis established the unique species-level identity of the isolates from septicemic MISTRG and MISTRG-EPO mouse strains.

Colony screening and treatment. Using bacterial culture, a colony-wide screening of 42 mice, comprising breeding pairs and offspring, revealed a 19% prevalence of the novel *Streptococcus* species in the oral cavity; however, the organism was not found in fecal cultures. The organism was sensitive to amoxicillin/clavulanic acid, ampicillin, benzylpenicillin, cephalothin, chloramphenicol, clindamycin, enrofloxacin, and erythromycin, and was resistant to trimethoprim-sulfamethoxazole.

Breeding pairs continuously received drinking water that contained amoxicillin doses of 0.57 mg/mL; pups had access to amoxicillin only before weaning. Weaned mice did not receive amoxicillin. The entire colony, including the breeders, was screened for the presence of the novel *Streptococcus* species in the oral cavity every 2 wk for 22 wk by using bacterial culture. Weaned mice that tested positive were culled or placed on amoxicillin if selected for breeding. The entire colony was negative by week 16 of this regimen of treatment and screening. No cases of septicemia or death occurred in the breeders or weanlings during this period. This colony remained free of septicemia and colonization with the novel *Streptococcus* spp. 15 mo after cessation of antibiotic treatment.

Discussion

In the current study, we identified a novel α-hemolytic *Streptococcus* spp. that was present in the oral cavity and blood of septicemic MISTRG/MISTRG-EPO female mice that were either pregnant or lactating. Clinical signs of disease or death from bacterial septicemia were not evident in weaned male or female mice or in breeding males cohoused with the females exhibiting clinical signs of sepsis. Affected periparturient mice

Table 2. Biochemical characteristics useful in differentiating novel *Streptococcus* spp. from the closest related species of the genus *Streptococcus*

Characteristics	*Streptococcus* isolate B01	*S. azizii* 12-5202	*S. acidominimus* CCUG 27296	*S. cuniculi* CCUG 65085
β-glucuronidase	+	+	+	-
α-Galactosidase	-	-	-	+
Sorbitol	+	-	+	+
Raffinose	-	+	+	-
Pullulan	-	+	+	+

Table 3. Genome features of the novel *Streptococcus* isolates

Item	Description	
Genome Assembly Data	**B01**	**O1**
Assembly Method	minimap2 + miniasm + racon + Medaka + POLCA	
Sequencing Technology	Illumina + Nanopore MinION	
No. of contigs	2	5
Genome Coverage	523.0×	300.0×
Genome features		
Size (Mbp)	2.449	2.703
Max contig length (Mbp)	2.415	2.206
Number of scaffolds > 50 KB	1	4
GC content (%)	41.1	41
No. of total predicted genes	2119	2447
No. of CDSs (total)	2048	2376
No. of Genes (coding)	1976	2223
rRNA (23S, 16S, 5S)	12 (4, 4, 4)	12 (4, 4, 4)
tRNA	55	55
ncRNAs	4	4
Pseudo Genes (total)	72	153
Repeat_region	1	1

showed systemic necrotizing infection involving multiple joints, aortic and cardiac tissues, middle and inner ears, meninges, abdominal organs (kidney and pancreas), and the reproductive tract (vaginitis and placentitis).

We have no documented information regarding death or disease in these 2 related strains of mice at other institutions, but we have heard anecdotes about the loss of colonies of this strain with recommendations for continuous use of antibiotics (enrofloxacin or TMS) in water in order to minimize opportunistic systemic, pulmonary, and reproductive tract infections.[36] As such, these strains of mice received TMS-medicated feed while in our facility based on husbandry and veterinary care information from the source institution. Given the broad antibacterial and antipneumocystis effects of TMS, we continued to provide this medicated diet to the MISTRG colony to suppress opportunistic infections. However, the reported minimum inhibitory concentration 90 (MIC90) for TMS against VGS is 4 mg/L.[39] This, in conjunction with a report of poor plasma bioavailability of TMS when delivered in drinking water (≤ 7.1 ng/mL),[20] makes the medicated feed, even with 55% more drug, ineffective against *Streptococcus* spp. This ineffectiveness was evident in our study, given the systemic infections and resistance to TMS. Antibiotic sensitivity assessment, however, indicated a pattern of sensitivity to β-lactam antibiotics, which is expected for VGS. We chose amoxicillin because it is the antibiotic of choice for *Streptococcus*, and is easy to administer through drinking water. Subsequently, within a few weeks, we successfully bred and weaned mice from this colony. Within 16 wk, the colony mice were culture-negative for the novel isolate. Like TMS, amoxicillin has poor systemic bioavailability in mice after oral dosing.[20] We therefore do not know whether the clearance of oral bacterial colonization is due to a localized effect of amoxicillin in the oral cavity while drinking or to systemic exposure.

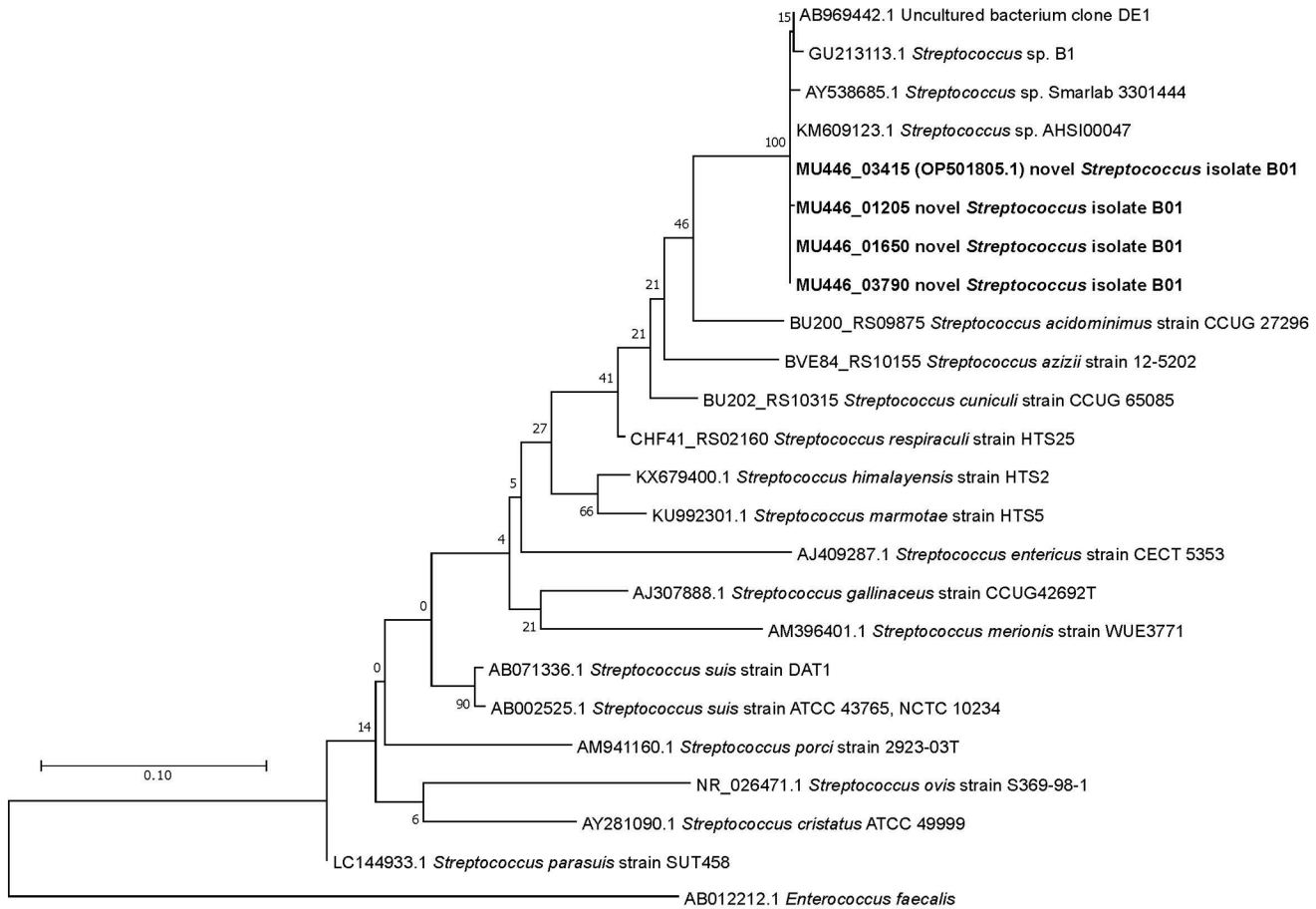

Figure 4. Phylogenetic tree inferred from comparison of 16S rRNA gene sequences of *Streptococcus* isolate B01 and closely related members of the genus *Streptococcus*. The 16S rRNA gene sequence of *Enterococcus faecalis* was used as an outgroup. Bar, 0.1 substitutions per site.

Table 4. Species delineation methods/gene comparisons for differentiating novel *Streptococcus* isolate B01 from related *Streptococcus* spp.

Assembly accession	Strains	16S rRNA	orthoANI	AAI	DDH	rpoB	soda	recN	gyrB
GCF_001921825.1	*S. acidominimus* strain CCUG 27296, **BU200**	96.2	84.4	86.12	29.6	90.1	89.1	85.7	88.9
GCF_001921845.1	*S. cuniculi* strain CCUG 65085, **BU202**	96.4	83.1	86.72	27.2	90.3	89.3	82.2	86.1
GCF_001984715.1	*S. azizii* strain 12-5202, **BVE**	94.4	82.4	84.55	27.2	88.2	86.5	84.4	86.4
GCF_003595525.1	*S. respiraculi* strain HTS25, **CHF**	96.4	83.1	85.47	27.8	90.3	87.5	82.6	88.1

The novel *Streptococcus* species characterized in this study is closely related to *S. acidominimus* strain CCUG 27296, *S. cuniculi* strain CCUG 65085, and *S. respiraculi* strain HTS25, with 96.2%, 96.4%, and 96.4%, respectively, of sequence similarity. This similarity is below the established species-level cutoff of 98.7% for prokaryotes.[4] The *Streptococcus* spp. Smarlab 3301444 is a partial 16S sequence nearly identical to our isolates and closely related to *S. azizii*.[2,31] However, partial 16S sequences are inadequate for establishing species-level identity. Evaluation of marker genes and core genome phylogeny of 433 genes established the unique species-level identity of the B01/O1 isolates and the close identity to the above-listed 4 known species of *Streptococcus*. B01/O1 isolates are identical species based on marker gene and core genome phylogenetic analysis. Based on clinical and pathologic findings, we name this organism *Streptococcus murisepticum* spp. nov. In addition, as elaborated in Table 4, the ANI of < 85%, with a species delineation boundary requirement of 95% to 96%, and pairwise dDDH estimates of 27.2 to 29.6, with species delineation cutoff at 70%, also confirmed the unique species-level identity of *Streptococcus murisepticum* spp. nov. We hope the biochemical characteristics of sugar alcohol sorbitol fermentation and lack of activity against raffinose and pullulane sugars will help to differentiate this novel isolate from *S. azizii*, a recently described organism that caused meningoencephalitis in weanling C57BL/6 mice.[2,31]

In this report, septicemia and associated pathology were restricted to pregnant and lactating mice, despite an oral prevalence of 19% for the novel *Streptococcus* spp. in a colony comprised of both sexes and weanlings. In humans, pregnancy and an associated elevation in hormones are associated with gingivitis and an alteration of the oral microenvironment that promotes bacterial growth and changes to the composition of microbial flora.[7,41] We have attempted to identify similar changes in the affected female mice. We saw a pronounced neutrophilic response to the infection at a systemic level in the affected

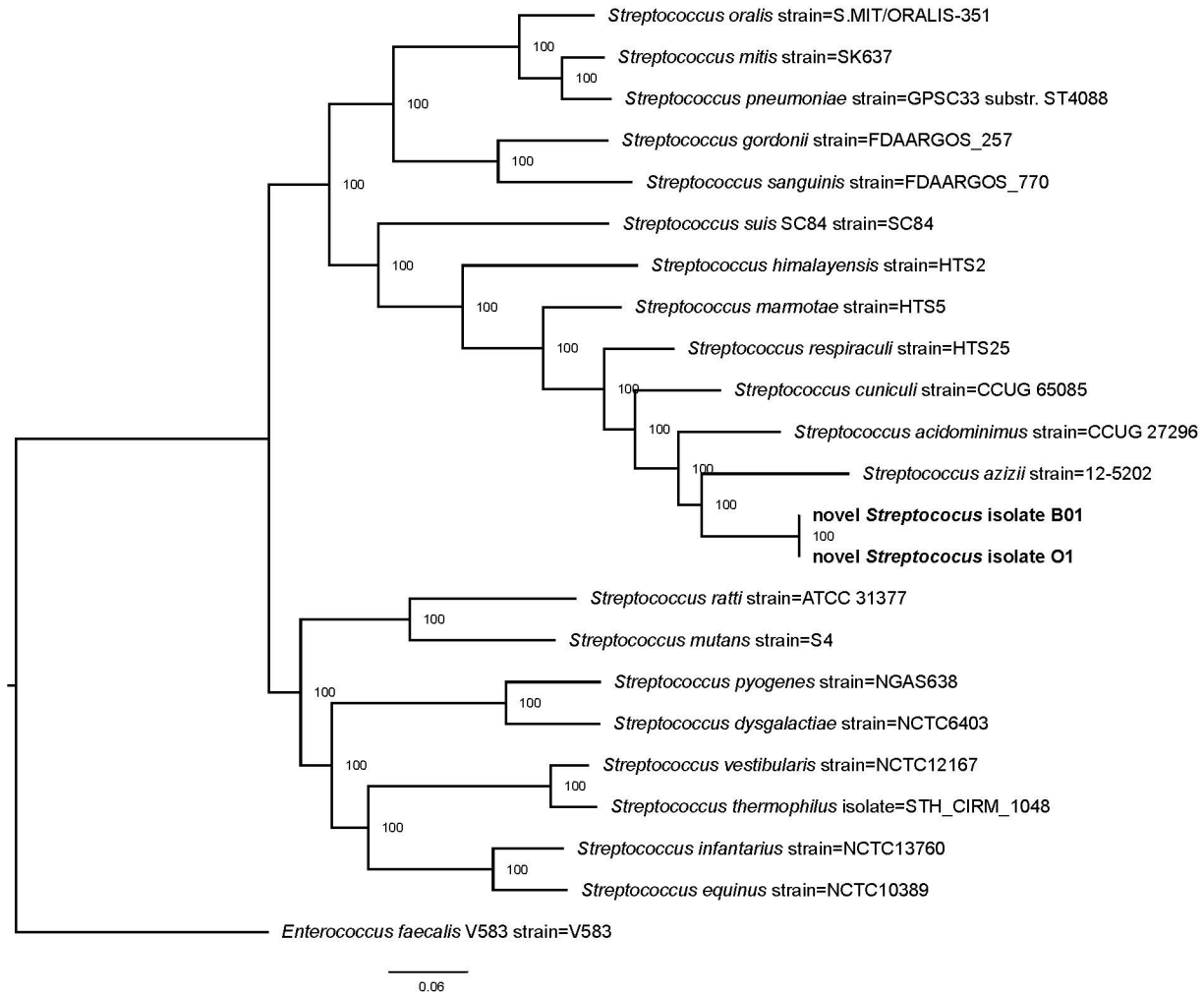

Figure 5. Core-genome phylogenetic tree based on 433 core genes from 22 representative genomes of members of the genus *Streptococcus*, including the 2 *Streptococcus* isolate genomes and 20 genomes from NCBI. The sequences of *Enterococcus faecalis* V583 were used as an outgroup.

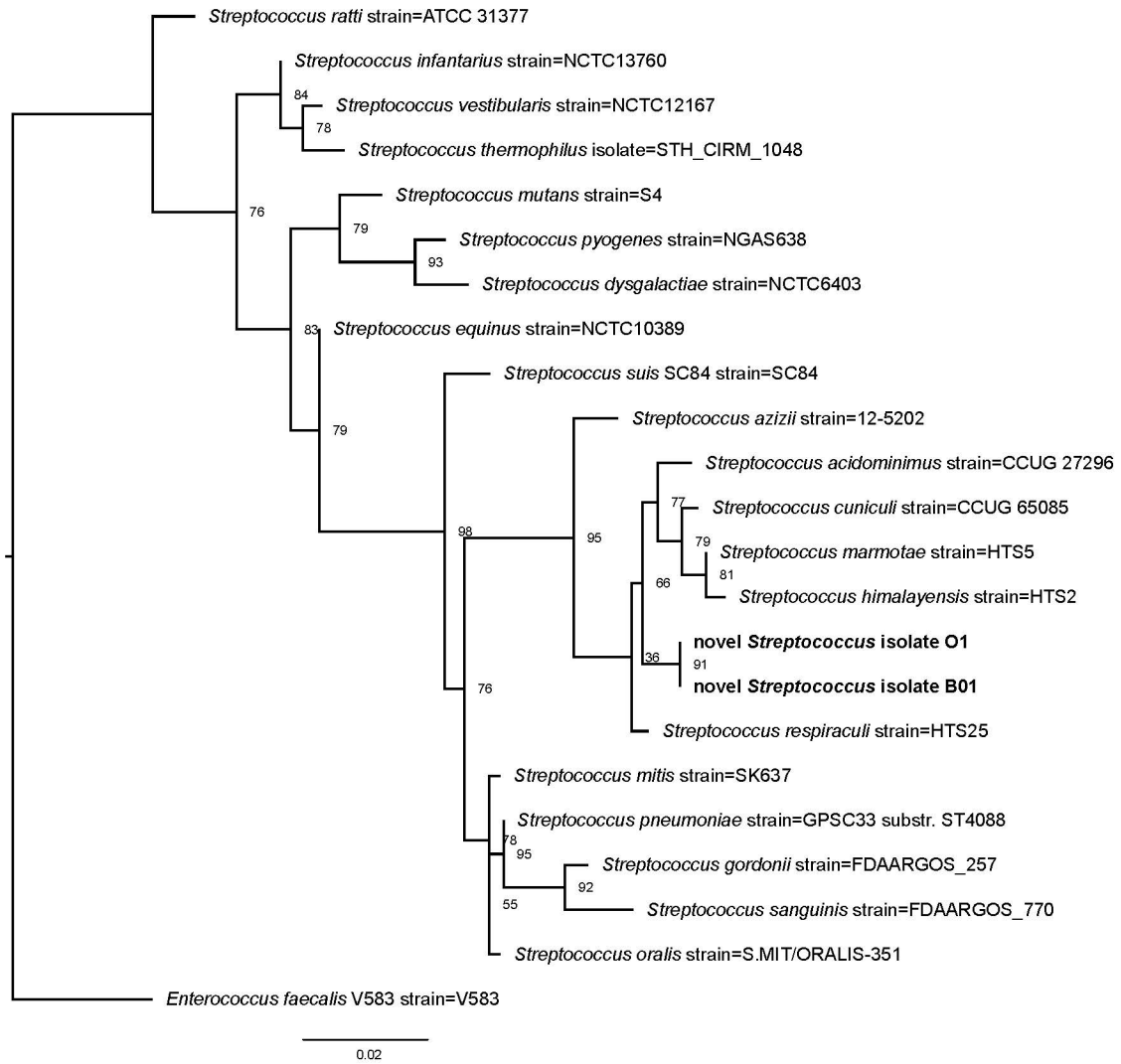

Figure 6. Phylogenetic tree for the novel *Streptococcus* isolates B01 and O1 based on 4 marker genes *gyrB*, *recN*, *rpoB*, and *sodA*. Genes in each genome were concatenated. The sequences of *Enterococcus faecalis* V583 were used as an outgroup.

periparturient females. This type of response is not surprising because the murine granulocyte colony-stimulating factor is not altered in MISTRG/MISTRG-EPO strains and potentially drives neutrophil growth, survival, differentiation, and terminal functions such as chemotaxis, respiratory burst, and degranulation.[21] The absence of other critical endogenous murine-specific cytokines in these strains (for example, granulocyte-macrophage colony-stimulating factor) may cause neutrophil dysfunction and ineffective clearance of bacteria. Similarly, mice that were deficient in granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor developed absolute neutrophilia in response to *Candida albicans* infection but failed to clear the organism from the body.[1] Alternatively, the knock-in human cytokines, especially granulocyte-macrophage colony-stimulating factor, may effectively support mouse neutrophil maturation and function, albeit secondary to endogenous mouse granulocyte colony-stimulating factor, and the vulnerability to septicemia in the MISTRG/MISTRG-EPO strain may stem from its dysfunctional monocyte/macrophage system. The lack of endogenous granulocyte-macrophage colony-stimulating factor in these strains of mice that can effectively drive monocyte/macrophage development and differentiation may be critical to clearance of bacteria from peripheral tissues, including joints, the reproductive tract, and the placenta. This impairment is indicated by the background pathology of pulmonary alveolar proteinosis and histiocytosis made up of CD68-positive macrophages in infected and uninfected MISTRG/MISTRG-EPO adult mice (Figure 3). Pulmonary alveolar proteinosis stems from failure of alveolar macrophages to terminally differentiate and effectively clear lung surfactant.[38] This macrophage defect may increase the susceptibility of these mice to bacterial infection.

Alpha-hemolytic *Streptococcu*s spp. are common commensal organisms in humans and animals, colonizing gastrointestinal and reproductive tracts and the oral mucosa. These species are considered to have low pathogenic potential in immunocompetent hosts. However, the VGS group of α-hemolytic *Streptococcus* spp. is gaining importance in humans as opportunistic agents capable of causing endocarditis, intraabdominal infection, and shock, especially after oral mucositis from chemotherapy and irradiation in cancer patients.[6,29] MISTRG strain mice colonized with the novel *Streptococcus* spp. may be a valid in vivo model for oral mucositis and systemic bacterial translocation. Currently, we have not compared the pathogenicity of this novel *Streptococcus* spp. to that of other immunodeficient mice, such as NSG mice. The novel *Streptococcus* sp was not identified when we tested other immunodeficient mice in our colony, and we assume that it was present only in the MISTRG colony. The current report

and the previously published study on *S. azizii*,[2,31] examined the 2 known α-hemolytic *Streptococcus* spp. with the potential to cause clinical disease in immature and immunocompetent mice.[2] However, given the challenge of identification and characterization of VGS, novel *Streptococcus* species may be more prevalent than expected in research mice, highlighting the need to carefully isolate and characterize infectious agents causing disease in animals, especially in newly characterized immunodeficient mice.

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