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NOTE

Pathology

Suppurative meningoencephalitis and perineuritis caused by *Streptococcus gallolyticus* in a Japanese Black calf

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ABSTRACT. A 179-day-old calf, which was weak and stunted, showed neurological signs and was euthanized. Postmortem examination revealed extensive and severe cloudy area in the meninges, and pleural pneumonia. Gram-positive cocci were isolated from systemic organs. Biochemical and 16S rRNA gene sequence analyses identified the isolate as *Streptococcus gallolyticus*, and its subspecies was suggested to be *gallolyticus* (SGG). The isolate was classified as a novel sequence type (ST115) by the multilocus sequence typing scheme for SGG and showed susceptibility to penicillin, ampicillin, amoxicillin, florfenicol, sulfamethoxazole-trimethoprim, and chloramphenicol. Histopathologically, suppurative meningoencephalitis and perineuritis were detected. As SGG has been isolated solely from a cow with mastitis in Japan, this is the first SGG infection in a calf with suppurative meningoencephalitis and perineuritis in this country.

KEY WORDS: calf, Multilocus sequence typing, perineuritis, *Streptococcus gallolyticus* subsp. *gallolyticus*, suppurative meningoencephalitis

The *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) members originally comprised of Lancefield group D streptococci [4]. Thereafter, *S. bovis* was classified into three different biotypes (biotypes I, II/1, and II/2) based on their phenotypic (mainly mannitol fermentation) and genotypic characteristics. On the basis of their biochemical and genetic divergences, *S. bovis* biotypes were reclassified as *Streptococcus gallolyticus* subsp. *gallolyticus* (SGG) (biotype I), *Streptococcus infantarius* subsp. *infantarius* (biotype II/1), *Streptococcus lutetiensis* (biotype II/1) and *S. gallolyticus* subsp. *pasteurianus* (SGP) (biotype II/2) [7, 34]. *S. gallolyticus* subsp. *macedonicus* (SGM) was further added as a new subspecies of *S. gallolyticus* [3, 7, 14, 24, 30, 34].

S. gallolyticus can be identified by a PCR assay targeting the manganese-dependent superoxide dismutase gene (*sodA*) [30, 33]. Among the three subspecies of *S. gallolyticus*, SGG strains were reported to specifically hydrolyze tannic acid and decarboxylate gallic acid, while SGP strains only showed gallate decarboxylase activity, and SGM strains did not have either activity [25]. In addition to molecular techniques such as 16S rRNA gene sequence analysis, differences in these enzymatic activities have been used to identify the subspecies of *S. gallolyticus* [35].

SGG has been described as a normal inhabitant of the rumen of herbivores and digestive tract of birds [28]. Although not frequently, it can be detected in the intestinal tract of healthy humans (2.5–15%) [28]. Recently, SGG has been considered a potential zoonotic pathogen [10, 11]. This bacterium is known to be an opportunistic pathogen in humans [12, 15] and has been isolated from cases of endocarditis [22]. In addition, SGG has long been known to have a strong association with colorectal cancer

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in humans [18]. SGG is also known to pathogenic to turkey poults [9], chickens [35], ducklings [13], and roe deer [38]. In cattle, SGG has been isolated from a Japanese Black cow with gangrenous mastitis [20] and a neonatal calf with meningitis [2]. However, information on the antibiotic susceptibility and genetic and immunohistochemical characteristics of SGG associated with diseases in ruminants is still limited, which hampers establishment of effective strategies for control of SGG infections in animals.

Here, we present a case of suppurative meningoencephalitis and perineuritis due to suspected SGG infection in a calf wherein we genetically and phenotypically characterized the causative strain.

In October 2017, a 179-day-old castrated male calf at a breeding farm with 61 Japanese Black cattle in Fukushima prefecture, Japan, showed astasia, respiratory distress, nystagmus, and tremor. The calf was weak, stunted, and was affected with intermittent pneumonia from birth. The calf had no history of vaccination. When approximately 5-months old, the calf was treated with florfenicol (FFC) for pneumonia, and its left displaced abomasum was corrected by rolling. Due to the poor prognosis, the calf was moved to Fukushima Prefectural Chuo Livestock Hygiene Service Center from the farm and was euthanized. The mother cow showed no clinical signs, and no gross placental lesions were observed.

At necropsy, the cerebrum, cerebellum, spinal cord, superficial cervical lymph node, subiliac lymph node, bronchial lymph node, liver, spleen, kidneys, heart, lungs, stomach, and intestinal tissue samples were fixed in 20% neutral-buffered formalin. Fixed tissues were embedded in paraffin, sectioned (~3 µm), and stained with hematoxylin and eosin (H&E) for histological examination. Gram staining was also performed on sections from the cerebrum, cerebellum, spinal cord, spleen, and kidney.

For immunohistochemical examination, formalin-fixed, paraffin-embedded sections of the cerebrum were deparaffinized and incubated with 3% hydrogen peroxide in methanol solution to suppress endogenous peroxidase activity. Antigen was retrieved using 0.1% actinase E solution in phosphate-buffered saline at 37°C for 20 min. After adding 10% normal goat serum to block non-specific reactions, sections were incubated with anti-group D *Streptococcus* (Article number 85207, Statens Serum Institut, Copenhagen, Denmark), anti-*S. bovis* (*equinus*) (kindly provided by Dr. Yasushi Kataoka) and anti-*Streptococcus ruminantium* capsular polysaccharide synthesis gene (*cps*)-type IA (corresponding to *Streptococcus suis cps*-type 33) (article number 22430, Statens Serum Institut, Copenhagen, Denmark) and IB (kindly provided by Dr. Fumiko Suzuta and Dr. Kengo Shimojo, Nagasaki Prefecture, Japan) for 30 min at room temperature.

Sections were incubated with the secondary antibody (Histofine Simple Stain MAX-PO Multi; Nichirei Bioscience Inc., Tokyo, Japan) for 30 min at room temperature, and then treated with aminoethyl carbazole (AEC) substrate solution (Histofine Simple Stain AEC solution; Nichirei Bioscience Inc.) at room temperature. Finally, the sections were counterstained with hematoxylin. Sections of organs injected with bacterial solutions were used as controls. The bacteria used were SGG (strains DTK270 and DTK422), *S. lutetiensis* (strain DTK428), *S. equinus* (strain DAT99), *Streptococcus alactolyticus* (strain DTK211) and *S. ruminantium* (*cps-type* IA, IB, IIA, IIB, and III strains).

At clinical examination before necropsy, the calf presented with weak heartbeat, muddy diarrhea, astasia, respiratory distress, nystagmus, and tremor during clinical examination. The body temperature was 39.0°C, and the body size was very small and thin, similar to a 120-day-old calf (Fig. 1a). At necropsy, extensive and severe cloudy areas in the meninges were detected (Fig. 1b), with a small increase in the amount of CSF. The right anterior pulmonary pleura was fibrously thickened (Fig. 1c) and adhered to the parietal pleura. In addition, the omasum and abomasum adhered to the peritoneum and intra-abdominal organs with diffuse fibrinous masses on the serosal surface. Thymus size was normal. No other gross lesions were observed.

The histopathological finding in the central nervous system was severe suppurative meningoencephalitis and perineuritis with numerous gram-positive cocci. In the cerebral parenchyma, perivascular cuffs of neutrophils and macrophages were scattered (Fig. 1d). The lesions were severe and diffused to the cerebrum grooves, meninges (Fig. 1e), and cerebellum and extended around the spinal cord (Fig. 1f). Fibrous pleural pneumonia was observed in the right anterior lung lobe. Numerous gram-positive cocci embolisms were present in the renal glomerulus, with sporadic micro-abscesses of neutrophils and macrophage infiltration. Gram-positive cocci were also found in the interstitium of renal tubules. Atrophy of white pulp and gram-positive cocci was detected in the spleen. Neutrophil infiltration and fibrosis were observed in the area where the omasum and abomasum adhered. Atrophy of lymphoid follicles was detected in the superficial cervical, subiliac, and tracheobronchial lymph nodes.

The anti-group D *Streptococcus* serum used in this study reacted with all the *Streptococcus* strains in positive control sections, including SGG strains (DTK270 and DTK422). The same serum showed positive reactions to the antigens in gram-positive cocci and infiltrated macrophage cytoplasm in the lesions (Fig. 1g, 1h), while no reaction was observed in lesions with the other antisera tested.

Cerebrum, cerebrospinal fluid (CSF), liver, spleen, kidneys, heart, lungs, and ascites were sampled and used for the isolation of bacteria. All the samples were stamped or inoculated onto 5% sheep blood-supplemented blood agar base (BD BBL™) (SBA), 5% sheep blood-supplemented modified GAM agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) (MGAM) and MacConkey agar (MCK) (BD Difco™) and incubated at 37°C for 72 hr in air plus 5% CO_2 (SBA), anaerobic (MGAM), and aerobic conditions (SBA and MCK). The cultures were maintained in anaerobic and 5% CO_2 conditions using the Anaero Pack system (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). The agar plates were examined daily, and three colonies growing on SBA were randomly selected and subcultured for purification. Pure-cultured bacterial isolates were Gram stained, and species of *Streptococcus*-suspected isolates were determined using API Strep20 and API 20 Strep V7.0 (bioMerieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. Tannase and gallate decarboxylase activities of a representative isolate were determined by the previously described methods [26, 27], except that brain heart infusion broth (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) was used to prepare the substrate medium in the gallate decarboxylation test.

Bacterial species of the selected gram-positive cocci were identified by molecular methods. Genomic DNA was extracted using InstaGene Matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, and 16S rRNA gene

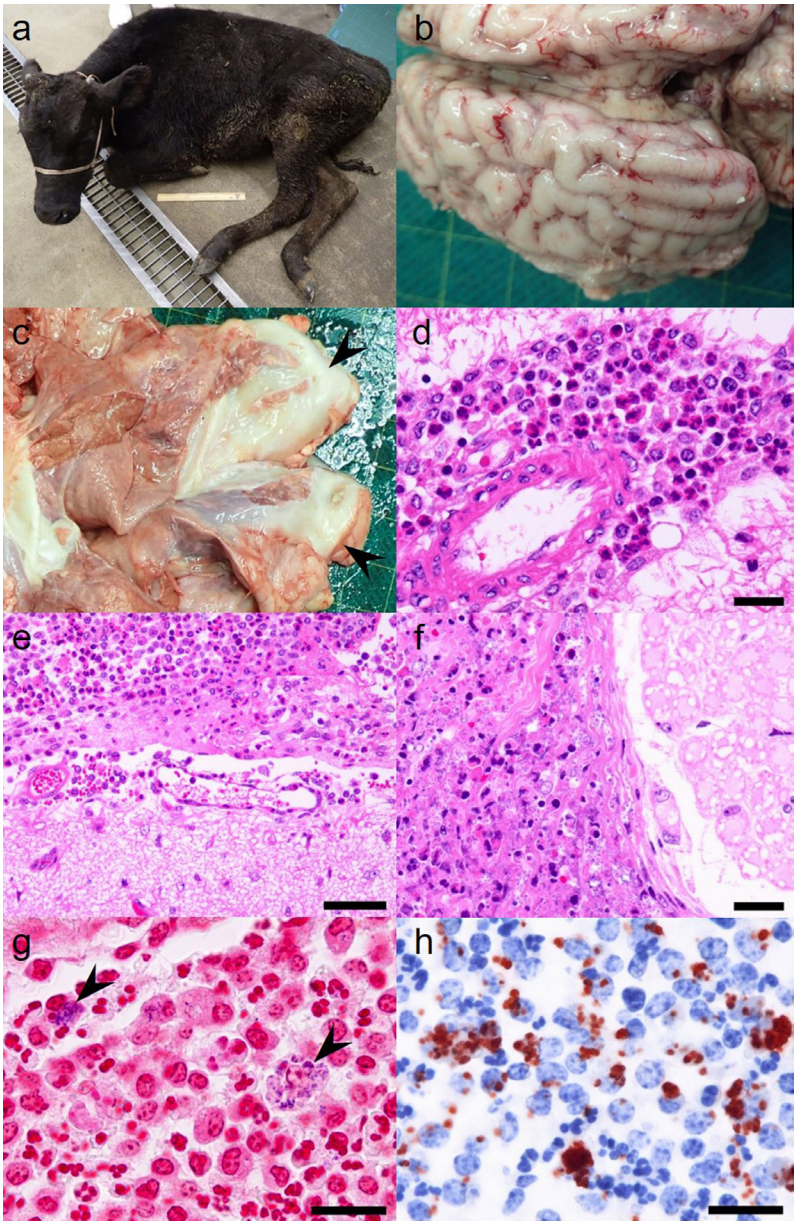

Fig. 1. **a**. At necropsy, a 179-day-old calf was very small and thin similar to 120-day-old calf. The buttock and limbs were covered with watery feces. **b**. Extensive and severe cloudy areas in the meninges were detected on the brain surface. **c**. Fibrously thickened pulmonary pleura in the right anterior lung lobe (arrowheads). **d**. Perivascular cuffing of neutrophils and macrophages in the cerebral parenchyma. Hematoxylin and eosin (H&E) staining. Bar=20 µm. **e**. Infiltration of neutrophils and macrophages in the temporal lobe meninges of the cerebrum. H&E staining. Bar=50 µm. **f**. Infiltration of neutrophils and macrophages around the spinal cord. H&E staining. Bar=20 µm. **g**. Gram-positive cocci phagocytosed by macrophages in lesions of cerebrum (arrowheads). Gram staining. Bar=20 µm. **h**. Positive reactions of Gram-positive cocci to anti *Streptococcus* group D serum antibody were found in lesions of the cerebrum. Immunohistochemical staining. Bar=20 µm.

sequence of the isolate was determined as described previously [1]. Briefly, approximately 1.5-kb region of 16S rRNA gene was amplified from the genomic DNA of the isolate using TaKaRa *Ex Taq* (Takara Bio Inc., Kusatsu, Japan) and primers F1 (5′-GAGTTTGATCCTGGCTCAG-3′) and R13 (5′-AGAAAGGAGGTGATCCAGCC-3′) [8]. The amplified fragments were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and sequenced using the BigDye Terminator v3.1 cycle sequencing kit and 3130*xl* Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Tokyo, Japan). SEQUENCHER Ver. 5.4.1 (Gene Codes Corp., Ann Arbor, MI, USA) was used for sequence assembly. Isolated species was identified by analyzing the 16S rRNA gene sequence using the EzBioCloud server (https://www.ezbiocloud.net) [41]. The determined sequence has been deposited in the DDBJ/EMBL/GenBank database under the accession number LC644657.

Antibiotic susceptibility test of the isolate was performed using disk diffusion methods, according to the standard methods of the Clinical and Laboratory Standards Institute (VET01S) [6]. Antibiotic disks, including the BD BBL Sensi-Discs (Becton, Dickinson and Co.) for amoxicillin (AMPC), oxytetracycline (OTC), KB disks (Eiken Chemical Co., Ltd., Tokyo, Japan) for penicillin

(PCG), ampicillin (ABPC), erythromycin (EM), josamycin (JM), FFC, cephazolin (CEZ), sulfamethoxazole trimethoprim (SXT), chloramphenicol (CP), and VKB disks (Eiken Chemical Co.) for enrofloxacin (ERFX) were used in the tests.

Multilocus sequence typing (MLST) analysis of the isolated bacterium was performed by sequencing seven genes, as described previously [10]. The allelic numbers of the sequenced genes were determined by comparing their sequences with those in the SGG MLST database (https://pubmlst.org/organisms/streptococcus-gallolyticus). Novel alleles and sequence types (STs) were assigned by submission of data to the database. The novel allele sequences of *aroE* and *nifS* have also been deposited in the DDBJ/EMBL/GenBank database under accession numbers LC645157 and LC645158, respectively.

To detect bovine viral diarrhea virus (BVDV), reverse transcriptase (RT)-PCR assay was performed with cerebrum, spleen, and urine samples as described previously [39].

Numerous grey-white colored, non-hemolytic, smooth colonies with a diameter of 2–3 mm were detected on SBA plates from the cerebrum, CSF, liver, spleen, kidneys, lungs, and heart after 24-hr incubation under aerobic and air plus 5% CO_2 conditions. The isolated bacteria were gram-positive cocci and biochemically identified as SGG (API Strep profile: 5040533, ID%, 99.8). The 16S rRNA gene sequence of this isolate (accession no. LC644657) showed highest identity to that of the type strain of SGG (ATCC 700065; accession no. FOLZ01000015; 99.86%), followed by the type strains of SGP (CIP 107122; accession no. DQ232528; 99.73%), and SGM (NCTC 13767; accession no. UHFM01000006; 98.98%), supporting the biochemical identification results. However, tannase and gallate decarboxylase activities of the isolate were negative and positive, respectively, and the results matched the characteristics of SGP strains but not those of SGG strains (i.e., tannase, positive and gallate decarboxylase, positive) [25]. The isolate was assigned to a new ST (ST115; allele profile, *aroE*/*glgB*/*nifS*/*p20*/*tkt*/*trpD*/*uvrA*=29/13/34/4/3/1/1) using the MLST scheme designed for SGG [10].

The results of antibiotic susceptibility testing are shown in Table 1. The isolated bacteria showed resistance to EM and intermediate susceptibility to OTC, JM, and ERFX and were susceptible to AMPC, PCG, ABPC, FFC, SXT, and CP.

The result of RT-PCR test was negative for BVDV.

In this study, suppurative meningoencephalitis was detected in a calf in Japan, and *S. gallolyticus* was isolated from the lesions as the putative causative agent. The *S. gallolyticus* isolate was identified as SGG, based on the results of a commercially available identification kit and 16S rRNA gene sequencing analysis (99.86% identical to that of the type strain of SGG). However, the 16S rRNA gene sequence was very similar to SGP (99.73%), and tannase and gallate decarboxylase activities of the isolate matched the reported characteristics of SGP strains; therefore, there is some possibility that the isolate is SGP.

SGG and SGP are known to be opportunistic pathogens [12, 15]. In birds, SGG is known to cause splenic multifocal necrosis and Kupffer cell hypertrophy with necrosis of macrophages [9], endocarditis [35], and meningitis [13]. In humans, SGG and SGP are known to cause endocarditis [22, 40], sepsis, and meningitis [5, 37]. Recently, SGG was isolated from a neonatal calf with meningitis in Turkey [2], and the calf also exhibited neurological signs. In this case, several factors, including poor intake of colostrum (failure of passive transfer) and insufficient care of the navel region were suspected to be the cause of infection [2]. SGP is also reported to cause suppurative meningitis-meningoencephalitis with neurological symptoms and mortality in calves in Italy [36]. In Japan, SGG has been isolated only from a Japanese Black cow with gangrenous mastitis [20]. Therefore, the present case is the first report of SGG infection in a calf with suppurative meningoencephalitis and perineuritis in East Asia. Since the calf was weak and stunted from birth and had intermittent pneumonia, the case was considered as an opportunistic infection. Moreover, the initial disease was pleural pneumonia and the bacteria spread from lung lesions to various organs via septic condition.

Although immunohistochemical staining of *S. gallolyticus* was performed in pigeons [16], to the best of our knowledge, this has not been reported in cattle. As SGG strains may have Lancefield group D antigen, anti-group D *Streptococcus* serum was

Table 1. Results of antimicrobial susceptibility testing

Antibiotic disk	Disk content	Zone diameter (mm)			
		Inhibition circle	Resistant	Intermediated	Susceptible
Amoxicillin (AMPC)[4)]	25 µg	30	≤14	15–20	≥21
Oxytetracycline (OTC)[4)]	30 µg	18	≤14	15–18	≥19
Penicillin (PCG)[1)]	10 U	32	-	-	≥24
Ampicillin (ABPC)[1)]	10 µg	26	-	-	≥24
Erythromycin (EM)[2)]	15 µg	15	≤15	16–20	≥21
Josamycin (JM)[3)]	30 µg	12	≤11	12–15	≥16
Florfenicol (FFC)[1)]	30 µg	25	≤18	19–21	≥22
Cephazolin (CEZ)[5)]	30 µg	29	-	-	-
Sulfamethoxazole trimethoprim (SXT)[3)]	23.75/1.25 µg	20	≤15	16–18	≥19
Chloramphenicol (CP)[1)]	30 µg	23	≤17	18–20	≥21
Enrofloxacin (ERFX)[1)]	5 µg	18	≤16	17–22	≥23

To determine the antibiotic susceptibility of *Streptococcus gallolyticus* isolate, disk diffusion method was performed according to the Clinical and Laboratory Standard Institution (CLSI) Guidelines [6], and the results of antibiotic susceptibility were interpreted according to the zone-interpretative chart provided by [1)] CLSI (VET01) [6] for *Streptococcus* spp. and [2)] for *Streptococcus* spp. viridans group, [3)] handling instructions [https://www.info.pmda.go.jp/downfiles/ivd/PDF/170005_09A2X10001000010_A_01_07.pdf], [4)] handling instructions [https://www.bdj.co.jp/micro/products/1f3pro00000qho5o-att/54-sd-hantei-other.pdf] and [5)] as a reference value because no target bacterial species was available.

used in this study. The serum successfully detected group D *Streptococcus* in the positive-control sections including SGG strains and antigens in the lesions from which SGG was isolated, whereas no cross-reaction was observed by the other antisera tested, including anti-*S. bovis* (*equinus*) serum. These results suggest the presence of SGG in the lesions and usefulness of anti-group D *Streptococcus* serum for detection of SGG in histopathological analyses. However, as the serum may react with streptococci other than SGG, such as *S. lutetiensis*, *S. equinus*, *S. alactolyticus*, and *S. ruminantium*, as shown in this study, the development of serum specific to SGG will be needed in future for more specific detection of SGG and determination of the subspecies on tissue sections.

Using the MLST scheme designed for SGG [10], the isolate in this study was assigned to a novel ST, ST115. Among the 115 STs of SGG isolates, ST12, ST31, and ST115 are the only three STs that have been found in the isolates from diseased cattle (https://pubmlst.org/organisms/streptococcus-gallolyticus), and ST31, the only double locus variant of ST115, is the most closely related genotype of ST115. ST31 was found in an isolate from a bovine with mastitis in the United Kingdom, and both ST31 and ST115 have not been detected in other sources, including humans and healthy animals. Therefore, the SGG strains that are genetically closely related to these two types are more likely to cause opportunistic infections in cattle, although further MLST analysis using both healthy and diseased cattle isolates is required to verify this hypothesis.

SGG is generally susceptible to frequently-used antimicrobial compounds, but previous studies have reported relatively high resistance rates of *S. gallolyticus* to macrolides (45–59%) and tetracyclines (56–78%) [17, 19, 23, 32]. The isolate in the present study also showed resistance or reduced susceptibility to these drugs (i.e., EM, OTC, and JM). Although further studies using SGG isolates from various origins are necessary, trends in antimicrobial resistance in Japanese SGG strains might be similar to previous reports. In addition to macrolides and tetracyclines, the present SGG isolate also showed reduced susceptibility to ERFX. Notably, macrolides and new quinolones have often been used in the farm in this case, which might have affected the antimicrobial resistance profile of the SGG isolate. In contrast, in the *in vitro* analysis, the isolate was susceptible to FFC that was used for treatment of this case. In addition, the present isolate was also susceptible to SXT and all the penicillins tested (AMPC, PCG, and ABPC). Penicillins are the most important drugs for the treatment of streptococcal infections. Although reduced susceptibility to penicillin has been detected in SBSEC strains, it has rarely been observed [29, 31]. As FFC was insufficient for this case, the use of penicillins may be worth considering as an option for treatment if veterinarians encounter similar cases in this farm in future. In contrast to the present isolate, SGG from a neonatal calf [2] and SGP from calves [36] were resistant to SXT. Although extremely varying resistance rates have been reported for SXT in SGP [21, 32], information on susceptibility to SXT is limited in SGG; therefore, further studies are needed to evaluate the usefulness of SXT in the treatment of SGG infections.

In conclusion, we report a case of histopathological diagnosis of calf suppurative meningoencephalitis and perineuritis caused by *S. gallolyticus*. The phenotypic and genetic characteristics of the causative strain suggest that the strain was SGG. Our results also suggest the usefulness of anti-Group D *Streptococcus* serum for immunohistochemical analysis of *S. gallolyticus* infections and provide important insights into the characteristics of *S. gallolyticus*, which may cause diseases in cattle. As *S. gallolyticus* is considered a potential zoonotic pathogen [10, 11] and has long been known to have a strong association with colorectal cancer in humans [18], accurate diagnosis including the subsp. identification of *S. gallolyticus* is important for both animal and public health. However, the anti-group D *Streptococcus* serum may detect other group D streptococci. In addition, it is still difficult to unambiguously identify *S. gallolyticus* subsp. Therefore, the development of novel diagnostic tools, including SGG- and SGP-specific antisera, and easy and reliable subsp. identification methods such as PCR will help in diagnosing SGG and SGP infections and accumulate case data to understand this important zoonotic pathogen.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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