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ORIGINAL ARTICLE

Neonatal calf meningitis associated with *Streptococcus gallolyticus* subsp. *gallolyticus*

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Abstract
Here, we report a case of neonatal calf meningitis due to *Streptococcus gallolyticus* subsp. *gallolyticus* (SGG). Clinical, pathological and microbiological findings were evaluated. API Strep, 16S rRNA gene sequencing, *rpoB* gene sequencing and *sodA* gene sequencing were used for the complete identification of SGG. This is the first documented report of neonatal calf meningitis due to SGG in veterinary medicine.

Introduction

Until 2003, *Streptococcus bovis* (*S. bovis*) was divided into three different biotypes (biotype I, biotype II/1 and biotype II/2). Thereafter, in view of their phenotypic and genotypic characteristics, *S. bovis* biotypes were reclassified as *Streptococcus gallolyticus* subsp. *gallolyticus* (SGG) (biotype I), *S. infantarius* subsp. *infantarius* (biotype II/1), *S. lutetiensis* (biotype II/1) and *S. gallolyticus* subsp. *pasteurianus* (biotype II/2). Also, *S. gallolyticus* subsp. *macedonicus* was placed as a new subspecies of *S. gallolyticus.* (Beck et al. 2008; Osawa et al. 1995; Poyart et al. 2002; Schlegel et al. 2003; Jans et al. 2015; Dekker and Lau 2016). SGG is a Gram-positive inhabitant bacterium usually found in the gastrointestinal tract of healthy humans (Dumke et al. 2017) and animals (Sasaki et al. 2004; Schulz et al. 2015). Also known to be an opportunistic pathogen (Dumke et al. 2017; Kambarev et al. 2017), this bacterium has been isolated from cases of meningitis (Beneteau et al. 2015), colonic malignant diseases (Boleij and Tjalsma 2013; Butt et al. 2016), septic arthritis and osteomyelitis (García-País et al. 2016), bacteraemia (Corredoira-Sánchez et al. 2012) endocarditis (Vollmer et al. 2010) and biliary tract infections (Corredoira et al. 2014) in humans. Furthermore, this microorganism has also been reported to be isolated from animals in cases of endocarditis in chickens (Sekizaki et al. 2008), mastitis in cows (Sasaki et al. 2004), mortality in turkey poults (Droual et al. 1997), infection in ducklings (Hogg and Pearson 2009), purulent lesions in various organs in calves (Seimiya et al. 1992) and haemorrhages in the pulmonary artery and aortic valve in a roe deer (Velarde et al. 2009). Recent research has shown that SGG is also of zoonotic nature (Dumke et al. 2014; Dumke et al. 2015).

In this study, it was aimed to report the isolation of SGG from a neonatal calf with meningitis. In addition, advanced molecular analysis of the isolated bacterium was performed and the results were evaluated.

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Materials and methods

Patient's description and clinical data

A 5-day-old male Simmental calf, weighing 40 kg with central nervous system (CNS) signs, was admitted to the Internal Medicine Clinic of the Education, Research and Practice Hospital of Faculty of Veterinary Medicine, Erciyes University. The patient's anamnesis revealed that the calf, which was born to a dam with dystocia, had

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received neither enough colostrum nor the required care of the navel region, and had been administered antibacterial, vitamin and mineral treatment after being examined by a veterinarian. But we could not get more information from the owner about this treatment including dose and name of drugs used. At clinical examination, the animal showed a general deterioration of the body condition, lethargy, stiffness of the neck muscles, painful paraspinal muscle spasm, hyperaesthesia, anorexia, dyspnoea, opisthotonus, ataxia, tension of the extremities, slight dehydration, mild cough, increased vesicular sounds on the auscultation of the thorax, exophthalmos and excitation. Measurements demonstrated that while the body temperature of the animal was 38.4 °C, its pulsation and respiration rate were 140/min and 35/min, respectively. Five-millilitre blood samples were collected into EDTA-coated tubes for haematological analyses, and 9-ml blood samples were collected into dry tubes for biochemical analyses. The patient was hospitalised so as to be monitored, and was regularly checked and treated in the following days. The calf underwent regular daily examinations and was sampled for blood. Furthermore, in view of the CNS signs that the animal showed, 4-ml sterile cerebrospinal fluid (CSF) samples were taken from the lumbosacral region into EDTA-coated and dry tubes using a spinal cannula (Spinocan® 0.90 × 88 mm/20 G × 3.5″). It was observed that the CSF was yellowish grey in colour, opaque and cloudy. Treatment was started with the parenteral administration of enrofloxacin (Baytril® 10%, 5 mg/kg, intravenous, twice daily (IV BID), metronidazole (Flagyl®, ampoule 0.5% inj. 100 ml solution, 20 mg/kg IV BID), sulfamethoxazole/trimethoprim (Animar®, 5 mg/kg IV BID), vitamins B1 and B6 (Nervit®, 10 ml IV, once daily) and dexamethasone (Deksavet®, 0.1 ml/kg intramuscular (IM) once daily). Also, balanced electrolyte solutions (0.9% NaCl Polifleks®, Polifarma, Turkey) and lactated Ringer's solution (Medifleks®, Eczacıbaşı, Turkey) were given intravenously via the auricular vein. Furthermore, 5% O_2 therapy was administered by nasal route.

Haematological analyses

Blood with EDTA samples were analysed by the haemocytometer (Mindray BC-2800Vet, China) during hospitalisation. In addition, CSF taken from first day of the hospitalisation was evaluated in the same way.

Biochemical analyses of serum and CSF

Biochemical analyses of serum and CSF samples obtained only on the first day of the hospitalization were performed by using an autoanalyzer (BT-3000 Plus; Italy).

Glutaraldehyde coagulation test

We performed glutaraldehyde coagulation test in the detection of failure of passive transfer according to Turgut et al. (1998) and Weaver et al. (2000).

Cytological analysis

Cytospin smears from CSF were prepared for morphological evaluation by cytocentrifugation (Cytospin3, ThermoShandon, Pittsburgh, PA, USA) for 5 min at 1000 rpm using disposable plastic chambers (Cytofunnel, ThermoShandon) and glass slides (Cytoslide, ThermoShandon) (Scott and Penny 1993). Then, cytology smears were stained with Diff-Quik (Richard Allan Scientific, Thermo Electron Corporation, 9990700, USA).

Bacteriological analysis

Gram staining was used for slides prepared from the CSF. The CSF was inoculated onto 7% sheep blood agar (blood agar base no. 2, CM0271, Oxoid), McConkey (105,465, Merck, Germany) and eosin methylene blue (EMB, CM0069B, Oxoid, UK) agar; the plates were incubated at 37 °C under aerobic, anaerobic (Anaerocult A, Merck) and microaerobic (Anaerocult C, Merck) conditions, and were examined daily. Three colonies were selected at random to obtain pure cultures of the bacteria. Gram staining, and the oxidase, catalase, oxidation fermentation and motility tests were used for the phenotypic identification of the colonies grown (Quinn et al. 2011). API 20 Strep system (bioMerieux SA, Marcy-l'Etoile, France) was performed for the identification at the species and subspecies levels of the bacteria. In addition, antimicrobial susceptibility testing was done for the bacteria isolated (Bauer et al. 1966). Metronidazole (MET, 5 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), gentamicin (CN, 10 μg), ampicillin (AM, 10 μg), amoxicillin-clavulanic acid (AMC, 30 μg), cephazolin (KZ, 30 μg) and enrofloxacin (ENR, 5 μg) discs were used in the test. The disc diffusion test results were interpreted using the criteria published by Clinical and Laboratory Standards Institute (CLSI 2008a). After the necropsy, samples (lungs, liver, spleen, kidneys and brain) taken from calf were used for microbiological examinations. The isolation and identification procedure followed for the CSF was also used for the organ samples.

DNA extraction

DNA extraction for the molecular analyses was performed by using UltraClean® Microbial DNA Isolation Kit (12224-50, Mo Bio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions.

Molecular analysis

16S rRNA gene sequencing, *rpoB* gene sequencing and *sodA* gene sequencing were used for the molecular analysis of the recovered isolate. The universal primers 27F and 1492R were used to amplify the 16S rRNA gene (Lane 1991). The primers 31F and 830R were used to amplify the *rpoB* gene, which encodes the beta subunit of RNA polymerase (Drancourt et al. 2004). In addition, *sodA* partial sequencing has been performed by using the degenerated primers d1 (5-CCI TAY ICI TAY GAY G CI YTI GAR CC-3) and d2 (5-ARR TAR TAI GCR TGY TCC CAI ACR TC-3) reported by the Poyart et al. (1998). The amplified products were purified using the QIA-quick PCR Purification Kit (Qiagen, USA), and sequence analysis was performed using the Big Dye Direct Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions. After cycle sequencing, the amplicons were purified with Sephadex G-50 (Sigma-Aldrich, USA) by using spin columns and sequenced on the Applied Biosystems 3500 Genetic Analyser (Applied Biosystems, USA). All sequences were analysed with the CLC Main Workbench 6 and compared with reference sequences available on the website of the National Centre for Biotechnology Information using the Basic Local Alignment Search Tool for Nucleotides (BLASTn) programme according to the criteria of the CLSI, MM18-A guideline (CLSI, 2008b).

The genotyping of the isolates obtained from the CSF and the organ samples was performed with Enterobacterial repetitive intergenic consensus (ERIC)-PCR (Aydin et al. 2007).

Histopathological analysis

After the animal died (on fifth day after the administered to the clinic), a necropsy was performed. Samples were taken from several organs (lungs, liver, spleen, kidneys, small intestine and brain) for histopathological examinations. Tissue samples were fixed in 10% formalin, routinely processed and stained with haematoxylin and eosin for histopathological evaluation. Tissue sections were also stained with Brown-Brenn Gram's stain to investigate the presence of bacteria.

Results

Clinical data

On day 3, treatment with metronidazole and sulfamethoxazole/trimethoprim, to which the bacteria were found to be resistant, was ceased and therapy was continued with enrofloxacin and amoxicillin-clavulanic acid. Treatment was continued until day 5, when the animal died.

Haematological analyses

Haematological analysis results pointed out to leucocytosis (18.9×10^6/μl), monocytosis (1.1×10^6/μl), granulocytosis (13.4×10^6/μl) and low-packed cell volume (PCV) level (24.0%), and no significant alteration was observed in these values until day 4 (Table 1). CSF analysis revealed the presence of leucocytosis (0.1×10^6/μl). While the platelet level was within the normal range on day 1 (572×10^6/μl), thrombocytosis was detected in the measurements on days 2 (2524×10^6/μl), 3 (2993×10^6/μl) and 4 (1814×10^6/μl) (Table 1). The other haematological parameters measured were within the normal range (Klinkon and Ježek 2012).

Biochemical analyses

Biochemical analyses of serum and CSF

BUN (blood urea nitrogen) (80.5 mg/dl) and GGT (gamma-glutamyl transferase) (46 U/L) levels were higher than those of references values reported previously for bovine (10–25 mg/dl and 6–17.4 U/L respectively) (Table 2). Results of serum biochemistry were evaluated according to Russell and Roussel (2007) and Kaneko et al. (2008). While levels of LDH (lactate dehydrogenase), CPK (creatine phosphokinase), K^+, BUN, Na^+ and Cl^- were found to be high, albumin and glucose levels were low in CSF of the calf (Table 2). Biochemistry results of CSF were evaluated according to publications conducted by various researchers (St Jean et al. 1997; Welles et al. 1992; Al-Sagair et al. 2005).

Glutaraldehyde coagulation test

Negative result was detected in the glutaraldehyde coagulation test, so it was confirmed to the failure of colostrum uptake of the calf.

Cytological analyses

Cytology smears of the CSF stained with Diff-Quik showed abundant neutrophils and a few aggregates of degenerated neutrophils and numerous Gram-positive cocci (Fig. 1A–C).

Bacteriological analyses

The Gram staining of CSF and organs revealed the presence of Gram-positive cocci. Following 24 h of incubation, bacterial growth was not observed in the EMB and McConkey plates, whereas in the blood agar plates incubated under all three atmospheric conditions, grey-white coloured non-haemolytic smooth colonies of a diameter of 2–3 mm were observed. On the basis of the results of phenotypic tests, the bacteria were identified as *Streptococcus* spp. Results of the Api 20 Strep

Table 1 Haematological values of the calf during hospitalisation

Hematologic parameters	1st day	2nd day	3th day	4th day	References
WBC (10^3 cells/μl)	18.9	27.1	20.4	14.5	4–12
Monocyte (10^3 cells/μl)	1.1	1.3	0.9	0.7	0–0.9
Granulocyte (10^3 cells/μl)	13.4	23.1	16.5	10.8	
PLT (10^3 cells/μl)	572	2524	2993	1814	100–800

PLT platelet, *WBC* white blood cells

test were analysed by the API 20 Strep V7.0 software available at http://210.242.211.31/servlet/Identify?action=prepareNew&stripId=6. Three isolates recovered from CSF were identified as *S. bovis* I (*S. gallolyticus* subsp. *gallolyticus,* Api Strep profile: 5240573, ID%, 98.9) by using Api 20 Strep.

The results of the antibacterial susceptibility testing showed that all of the isolates were resistant to MET, SXT and CN, susceptible to AM, AMC, KZ and moderately susceptible to ENR. Results of bacteriological analyses performed from organs were similar to CSF results. No growth was observed on the McConkey and EMB plates. Growth was observed in all of the blood agar plates incubated under aerobic, microaerobic and anaerobic conditions. Similar to the method used for the CSF, with the use of the Api 20 Strep, all isolates (15) were identified as *S. gallolyticus* subsp. *gallolyticus*. In the genotyping, it was determined that all of the 18 isolates (3 from CSF and 15 from organs) had the same band patterns (data not shown); thus, a single representative isolate was used in the further stages of the study and this isolate was named as Erugall17.

Molecular analysis

According to result of the 16S rRNA BLASTn, Erugall17 was found to be *S. gallolyticus* with the identity score of 99%. On the other hand, *rpoB* gene sequence and *sodA* gene sequence revealed that Erugall17 had the identity scores of 99 and 100% for SGG respectively.

The 16S rRNA gene sequence, *rpoB* gene sequence and *sodA* gene sequence of Erugall17 were deposited in GenBank under accession numbers KY952169, KY952170 and MH817021, respectively.

Table 2 Biochemical values of the serum and CSF samples of the calf

	Samples analysed	
Biochemical parameters	Serum (reference values)	CSF (reference values)
LDH (U/L)	ND	850 (13.94 ± 1.32)
CPK (U/L)	ND	144 (11.44 ± 3.43)
K^+ (mmol/L)	ND	15.30(2.96 ± 0.03)
ALT (U/L)	26.6 (25–74)	ND
Total protein (g/dl)	6.9 (6.7–7.5)	1.2 (39.16 ± 3.39)
BUN (mg/dl)	80.5 (10–25)	30 (3.15 ± 0.09) for camel
Albumin (g/dl)	3 (2.5–3.8)	0.6 (15.75 ± 1.53)
Creatinine (mg/dl	0.06 (0.9–1.7)	ND
Glucose (mg/dl)	53 (40–100)	2 (42.88 ± 0.99)
ALP U/L	81 (30–145)	ND
GGT U/L	46(6–17.4)	ND
AST U/L	52.8 (60–125)	ND
Na^+ (mmol/L)	ND	164 (140 ± 0.78)
Cl^- (mmol/L)	ND	160 (134 ± 6.5) for lama
Specific gravity	ND	1030 (1.005–1.006)

ND not done, *LDH* lactate dehydrogenase, *CPK* creatine phosphokinase, *ALT* alanine aminotransferase, *BUN* blood urea nitrogen, *ALP* alkaline phosphatase, *GGT* gamma-glutamyl transferase, *AST* aspartate aminotransferase, K^+ potassium, Na^+ sodium, Cl^- chlorine

Biochemistry of CSF was evaluated according to St Jean et al. 1997, Welles et al. 1992, Al Sagair et al. 2005

Biochemistry of serum was evaluated according to Russell and Roussel 2007 and Kaneko et al. 2008

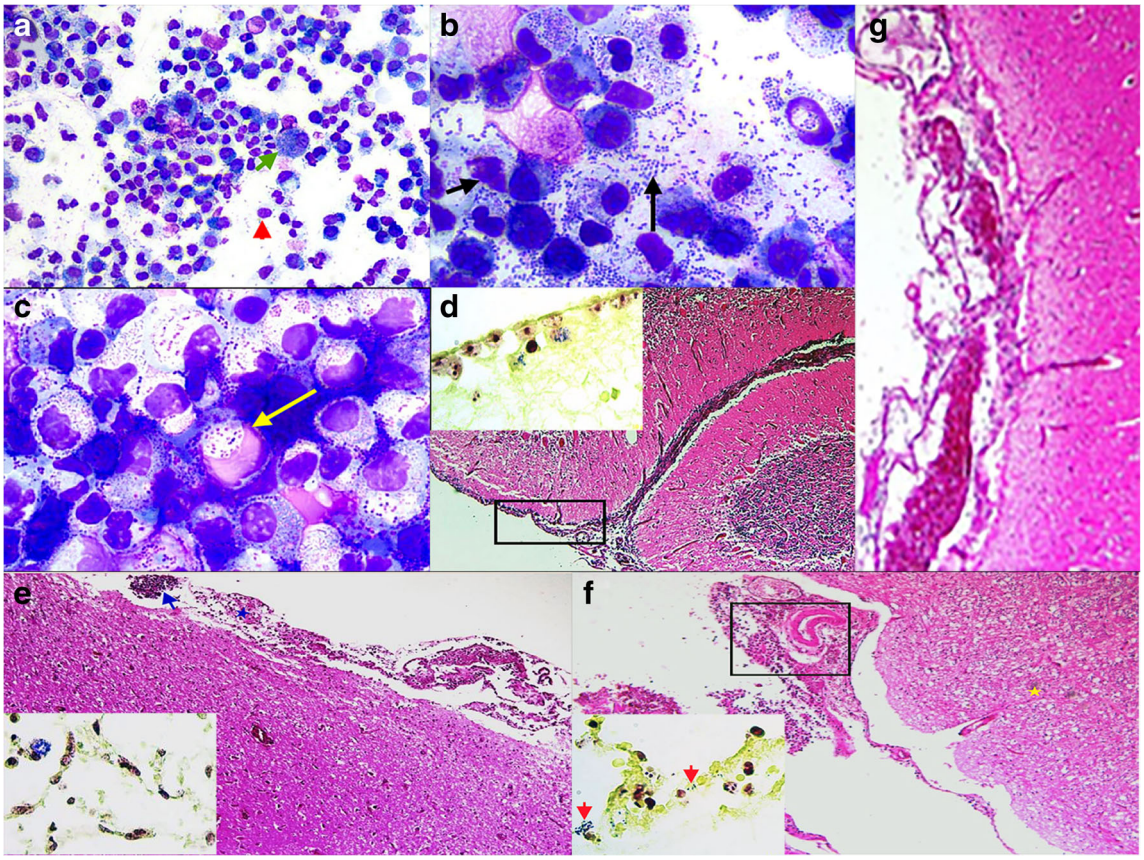


Fig. 1 **a**–**c** Cytological analysis of the CSF of a calf. **a** Numerous small foci of infiltrating cells, mainly of mononuclear cells; lymphocytic (red arrowhead) and monocytic lineage (green arrow, one shown with an arrow). **b**, **c** Intra/extracellular, bacteria (black arrow) in the cytoplasm of lymphocytes and macrophages and karyolytic neutrophils. Mixed inflammatory cell population including many degenerate neutrophils (black arrowhead). Giant cell (yellow arrowhead) with two large kidney-shaped nuclei and several cocci within the cytoplasm. Diff-Quick. Bars, 20 μm (**a**); 10 μm (**b**, **c**). **d**–**g** Major histopathological findings in cerebellum. **d** Neutrophils in the subarachnoid space. Focal area of necrosis and neutrophilic encephalitis in the molecular layer (boxed area). Inset: Higher magnification of boxed area (arrows) Gram-positive cocci. **e** Cerebral cortex. Necrosis and neutrophilic meningitis (star); focal mononuclear cell infiltration (blue arrow). Inset demonstrates Gram-positive small cocci in the lesion. **f** Lymphohistiocytic infiltration (boxed area) in the leptomeninges of the midbrain (star). Inset: Gram-positive small cocci (arrows) in the lesion shown in the boxed area. **g** Cerebral cortex, large amount of fibrinopurulent exudate in the leptomeninges and prominently dilated vessels. Haematoxylin x Eosin (**d**–**g**); Brown-Brenn Gram (inset of Fig. 1**d**–**g**). Bars, 10 μm (inset of Fig. 1**d**–**g**); 200 μm (**d**–**g**)

Histopathological analysis

Necropsy revealed hyperaemia and cloudy areas in the meninges. No gross abnormalities were noted in the brain of the calf. The animal had gross anatomic lesions, including marked diffuse chronic passive hyperaemia (nutmeg liver). The lungs were dark red, uncollapsed, heavy and firm. A clear frothy fluid was observed on the cut section of the lung. Microscopically, the primary pathological finding was purulent meningitis associated with the presence of Gram-positive cocci. Acute mild diffuse interstitial pneumonia was evident in the lungs. The renal glomeruli contained numerous fibrinous thrombi. The CNS lesions were prominent and limited to the meninges, where a fibrinous exudate and infiltrations of neutrophils, macrophages, and lymphocytes were observed (Fig. 1D–G). Small clusters of Gram-positive cocci were evident within the foci in the kidneys, lungs, small intestine, brain and liver.

Discussion

Bacterial meningitis is severe and mostly a fatal condition. Several factors, including the calf not receiving enough colostrum and the hygiene of the navel region not being attended to, are so effective in the occurrence of neonatal calf meningitis. Meningitis cases due to SGG have been reported in humans (Beneteau et al. 2015; van Samkar et al. 2015), but have not been reported in neonatal calves before. Nonetheless, Seimiya et al. (1992) reported to have observed fibrinopurulent meningoventriculitis, endophthalmitis and purulent lesions in several organs and tissues in three newborn calves, and isolated *S. bovis* from the lesions. Unlike this study, in this case, we did not observe or detect any symptoms or signs related to eyes and joints of the calf. However, these researchers did not indicate the biotype of *S. bovis*. The CNS signs and histopathological findings observed in this study

show similarity to the findings previously reported by Seimiya et al. (1992). However, in the present study, the Gram-positive cocci isolated from the CSF and organs were identified as SGG on the basis of the Api 20 Strep, *rpoB* gene and *sodA* gene sequencing results. To the best of our knowledge, the present study is the first veterinary report on the isolation of SGG from a neonatal calf with meningitis, which also provides a detailed assessment of the clinical, cytological (Fig. 1A–C), histopathological (Fig. 1D–G) and molecular findings.

Calves showing failure of passive transfer and suffering from poor hygiene conditions are particularly prone to septicaemia and meningitis. Negative glutaraldehyde coagulation test result revealed that the calf had failure of passive transfer, in our case. Similarly, failure of passive transfer in calves was reported in a study conducted by Turgut et al. 1998.

Serum biochemical values were between normal references except for GGT and BUN (Table 2). The high levels of this two parameters might be associated with failure of renal functions or dehydration (Russell and Roussel 2007; Kaneko et al. 2008.) This biochemical changes were supported by histopathological findings.

When the results of CSF biochemistry were compared with references values indicated for bovine, camel and lama, levels of LDH, CPK, K, BUN, Na and Cl were detected as high in contrast albumin and glucose levels were found as low (Table 2) (Welles et al. 1992; Al-Sagair et al. 2005). In particular, high levels of K, LDH and CK observed for CSF biochemistry were found compatible with the study of Nazifi et al. (1997).

Bacterial meningitis cases are characterised by high-mortality rates even if appropriately treated (Smith 2015; Fecteau et al. 2009). Therefore, in order to protect newborn calves from neonatal septicaemia and bacterial meningitis, neonatal stress should be reduced, an adequate amount of quality colostrum should be provided to the animals, navel care should be attended to and favourable hygiene conditions should be established.

In the present study, the isolate obtained was able to be successfully identified at subspecies level using the Api 20 Strep, *rpoB* gene sequencing (CLSI 2008b) and *sodA* gene sequencing methods. On the other hand, 16S rRNA gene sequence analysis was found to be sufficient for identification at genus level, but did not suffice for the identification of subspecies. Therefore, it is suggested that while Api 20 Strep can be used as a rapid, accurate, sensitive and reproducible method for the routine laboratory identification of *S. gallolyticus* isolates at subtype level, *rpoB* gene and *sodA* gene sequence analysis can be used for molecular and phylogenetic analyses. Resistance of the agent to antibiotics used in the treatment, organ failure (especially kidneys) and failure of passive transfer could be considered as important factors in the death of the calf. We believe that the identification of the subspecies or biotyping of *S. gallolyticus* is not only important for researches but also for the epidemiologic relationship of the isolates and the association with several diseases observed in humans and animals.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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