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Neurological symptoms and mortality associated with *Streptococcus gallolyticus* subsp. *pasteurianus* in calves

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Highlights:

- Neonatal suppurative meningitis-meningoencephalitis occurs sporadically in calves
- *Streptococcus gallolyticus* subsp. *pasteurianus* (SGP) is a broad host-range pathogen
- A neonatal neurological syndrome associated with SGP is reported in calves
- Diagnosis was made by bacteriological, histopathological and biomolecular tests
- The zoonotic potential and pathogenicity of SGP in calves require further studies

Abstract: Suppurative meningitis-meningoencephalitis (M-ME) is a sporadic disease in neonatal ungulates and only a few studies have reported the involvement of *Streptococcus bovis*/*Streptococcus equinus* complex (SBSEC) members in bovine neonatal M-ME. The SBSEC taxonomy was recent revised and previous biotype II/2 was reclassified as *S. gallolyticus* subsp. *pasteurianus* (SGP). The aim of this study was to describe a case of fatal neonatal neurological syndrome associated with SGP in calves. Ten calves were monitored because of neurological hyperacute symptoms associate with bilateral hypopyon and death. They were not fed with maternal colostrum; two of them died and were subjected to bacteriological, histopathological and biomolecular analysis as well as antibiotic susceptibility test. Both animals presented lesions mostly concentrated to meninges and brain and had bilateral hypopyon. Nine strains isolated in purity from brain, ocular humors and colon were identified as *S. bovis* group by using the API Strep system and as *S. gallolyticus* by using the 16S rRNA sequence. Two of these strains where subjected to WGS analysis that confirmed the sub-species identification and the clonality of the two SGP strains. The strains were found resistant to OT, SXT, MTZ and EN and susceptible to AMP, AMC, KZ and CN. We hypothesized that the syndrome observed could be due to the lack of maternal colostrum feeding. A timely and precise diagnosis could have likely prevented the death of the calves and, since the zoonotic potential of SBSECs members is known, accurate and rapid identification is required.

Key words: calves; meningitis-meningoencephalitis; *Streptococcus bovis*/*Streptococcus equinus* complex; *Streptococcus gallolyticus sbsp. pasteurianus.*

1. Introduction

Suppurative meningitis-meningoencephalitis (M-ME) in neonatal ungulates is a sporadic inflammation of the meninges, choroid plexuses and ventricular wall with involvement of central nervous system (CNS) (Seymia et al., 1999). Indeed, neonatal M-ME due to streptococci has been reported in piglets (Pan et al., 2015) while in bovines, mastitis (Sasaki et al., 2004) and purulent lesions in various organs (Seimiya et al., 1992) are often caused by streptococci and only a few studies have reported their involvement in neonatal and pediatric M-ME (Seimiya et al., 1992; Aydin et al., 2018). The *Streptococcus bovis*/*Streptococcus equinus* complex (SBSEC) members were previously comprised in the Lancefield group D streptococci and are currently divided into several species (Ben-Chetrit et al., 2017). Previous phenotypic characterization (mainly mannitol fermentation) allowed the distinction of three biotypes of *S. bovis*: biotype I, biotype II/1 and biotype II/2. In view of their biochemical and genetic divergences, biotypes were recent reclassified as *Streptococcus gallolyticus* subsp. *gallolyticus* (SGG) (biotype I), *S. infantarius* subsp. *infantarius* (SII) (biotype II/1), S. *lutetiensis* (SL) (biotype II/1) and *S. gallolyticus* subsp. *pasteurianus* (SGP) (biotype II/2) (Schlegel et al., 2003; Dekker and Lau, 2016). Members of SBSEC were first discovered in cattle, subsequently were described as colonic commensals of healthy humans and other animals (Sasaki et al., 2004), but they are now also recognized as opportunistic pathogens (Schlegel et al., 2003). In humans, they have been associated with a variety of diseases such as bacteraemia, colonic malignant diseases (Corredoira-Sánchez et al., 2012), endocarditis (Aydin et al., 2018), as well as adult and pediatric meningitis (Beneteau et al., 2015). Certain diseases are associated with specific sub-species; endocarditis, bacteraemia and colorectal cancer are usually associated with SGG while human meningitis, biliary and urinary tract infection are associated with SGP and SL (Pompilio et al., 2019). Shared strain lineages between food products, animals and humans suggest a zoonotic potential and

possible transmission by food contaminated through the oro-fecal route (Jans et al., 2016). In this study, a case of neonatal neurological syndrome associated with *Streptococcus gallolyticus sbsp. pasteurianus* is reported in calves.

2. Materials and Methods

2.1 Animals

An Apulian herd of 350 Holstein Friesian and Angler cows (with 150 of them in production) was monitored from June 2018 to January 2019 because of neurological hyperacute symptoms observed in newborn and young calves. A total of ten calves presenting a hyperacute syndrome were included in the study: three calves were 3 days-old and seven calves were 15 up to 50 days-old. The syndrome started with lethargy and lack of suckling reflex in all calves but in the newborns it was followed by incoordination, hyperesthesia and coma, ultimately leading to death (within 8-10 hours). The syndrome regressed in 15-50 days-old calves. None of the calves suffered from respiratory symptoms or had fever and none of them received antibiotics. Newborns presented bilateral cloudiness of the ocular aqueous humor which was concomitant with the onset of severe neurological symptoms and worsened before death. The anamnesis revealed that calves were bred in single cages and were fed with artificial colostrum for 24 hours post partum and subsequently were fed with milk powder reconstituted with potable water. Immediately after birth the umbilical region was routinely sanitized with iodine solution and no infections were reported. In January 2019, two out of the three newborn calves died (calf 1-calf 2) due to neurological disorders and were examined at the Infectious Disease Laboratory of the Department of Veterinary Medicine, University of Bari.

2.2 Gross findings and Histopathology

Brain, cerebellum, spleen, liver, lung and kidneys were collected from both calves and were fixed with 10% neutral formalin, embedded in paraffin, sectioned at 5 micron and then stained with haematoxylin and eosin (HE). The slides were observed under optical microscope (Leica Microsystems Nussloch GmbH, Leica DM 4000, Germany), at 10X and 40X magnification.

2.3 Bacteriological analysis

Using separate sterile instruments for each region, three different portions of the CNS (dorsal, anterior region of the brain and ventricular grooves indicated as sites 1, 2 and 3, respectively) were sampled and the ocular aqueous humor of both animals was collected for microbiological investigations. Spleen, liver, kidneys, lungs, and colon were also analyzed in both calves. In addition, two blood samples (collected immediately before death), artificial milk and colostrum were tested. All the samples (n=22) were plated onto Columbia blood agar (CBA), McConkey agar (MCK), Mannitol salt agar (MSA), (Liofilchem, Teramo, Italy) and incubated at 37°C for 48 h with anaerobic, aerobic and microaerophilic conditions. Nine strains, grown on CBA, were selected and tested with the Gram staining. The API Strep System (bioMerieux, France) was employed for biochemical identification. Antimicrobial susceptibility testing was performed on isolated bacteria using 5% sheep blood Muller Hinton (Bauer et al., 1966). Metronidazole (MET, 30 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), gentamicin (CN, 10 μg), ampicillin (AMP, 10 μg), amoxicillin-clavulanic acid (AMC, 30 μg), cephazolin (KZ, 30 μg), enrofloxacin (ENR, 5 μg) and oxitetracyclin (OT, 5 μg) (Liofilchem, Teramo, Italy) were tested. Results from disk diffusion tests were interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, 2013).

2.4 Diagnostic PCR analyses

A Real-time PCR for the presence of *Toxoplasma gondii* (Lin et al., 2002), a multiplex real-time RT-PCR for the presence of bovine viral diarrhea virus 1 and 2 (BVDV) (Mari et al., 2016) and 2 PCRs for *Listeria monocytogenes* (Le Monnier et al., 2011) and bovine herpesvirus-1 (BHV-1) (Vilček, 1993) were performed on all samples, based on anamnesis and pathological findings of the calves. DNA was extracted from tissues and pure colonies (isolated on CBA), by using the kit Cador (QIAampCador Pathogen® Mini Kit), following the manufacturer's instructions.

2.5 Biomolecular analysis

The universal primers BV5 and AV6 were used to amplify the 16S rRNA gene (Stecher et al., 2010). The primers were: BV5 5'-ATTAGATACCCYGGTAGTCC-3' (tm 55° C) and AV6 5'-ACGAGTGACGACARCCATG-3' (tm 69.8° C). Cycling conditions were as follows: 95°C, 10 min;

35 cycles of (94°C, 30 s; 57°C, 30 s; 72°C, 30 s); 72°C, 8 min. Reaction conditions (50 µl) were as follows: 50 ng template DNA; 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM Mg^{2+}, 0.2 mM dNTPs; 40 pmol of each primer, 5U of Taq DNA polymerase (AmpliTaq Gold™, Thermofisher, Italy). PCR amplified products (300 bp) were purified by using enzymes QIA-quick PCR Purification Kit (Qiagen, USA), and sequence analysis was performed using the MiSeq NGS (Illumina, San Diego, California) technology. All sequences were analysed with the Geneious 9.1 Software and compared with reference sequences available on the BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Purified genomic DNA were subjected to WGS using the Nextera XT library preparation workflow (Illumina, San Diego, CA) and 2 × 250-nucleotide paired-end reads were generated on an Illumina MiSeq instrument. *De novo* genome assembly was performed using SPAdes v.3.12 (Nurk et al., 2013).

3. Results

3.1 Pathology and histopathology

Both animals presented lesions mostly concentrated to eyes, meningeal surfaces and brain and had bilateral hypopyon. Aqueous humors were cloudy and there was a fibrinous exudate adhering to the surface of the iris and lens (Fig.1, 2). The cerebro-spinal fluid was cloudy and increased in amount. There were congestion, petechiae and cloudiness of the meningeal surface and diffuse petechiae on the brain surface. No omphalitis or other macroscopically visible lesions were found in others organs. In both calves, the major histopathological finding in the CNS was a marked suppurative-meningitis with infiltration of neutrophils, lymphocytes and macrophages, forming diffuse micro-abscesses. The lesions were more diffused to the meninges, choroid plexuses and ventricular grooves and extended to the ependymal zone and cerebellum (Fig. 3, 4). Ectasia of the middle vessels was observed, moreover, capillars were destroyed by the perivascular neutrophilic infiltration (Fig. 5, 6). Diffuse micro-abscesses were found within brain cortices. No significant lesions were observed in the remaining organs studied.

3.2 Bacteriological Analysis

Numerous grey-white non-haemolytic smooth colonies with a diameter of 2-3 mm (grown on CBA plates following 24 h incubation in aerobic conditions) were observed in: the three different sites of the CNS, the ocular aqueous humor of both animals the colon of Calf 1. No bacterial growth was observed in the remaining samples even after 48h of incubation, in all the media used. Bacteria from the nine positive samples were Gram stained and pure colonies of Gram positive cocci were found in all cases. All the nine isolates were then identified as *S. bovis* group by using the API Strep system and they were found resistant to OT, SXT, MTZ and EN and susceptible to AMP, AMC, KZ and CN (Table 1).

3.3 Diagnostic PCR analysis

All the samples tested negative for *Toxoplasma gondii, Listeria monocytogenes,* BVDV and BHV-1.

3.4 Molecular analysis

The nine bacterial strains which had been biochemically identified, were selected for 16S rRNA PCR. In particular, three strains isolated from the CNS (Site 1, 2, 3), one strain from acqueous humor and one strain from colon were tested in Calf 1 while three strains isolated from the CNS (Site 1, 2, 3) and one strain from acqueous humor were tested in Calf 2. Analysis of the 16S rRNA sequence revealed that all strains were *S. gallolyticus,* with an identity score of 99 %. Two of these strains both from site 1 of SNC of the two calves (strain 1 and strain 2) where subjected to WGS analysis. *De novo* genome assembly of selected isolates resulted in a final N50 of 113 kb and 32 contigs for strain 1 and an N50 of 38 kb and 100 contigs for strain 2. The first draft genome sequences were approximately of 2.217 (strain 1) and 2.203 (strain 2) Mbp in length (approx. 60×coverage) respectively. Strain 1 and 2 had a G+C content of 37.24 mol % and 37.21 mol %, respectively. To confirm the taxonomical identification, the Average Nucleotide Identity based on Blast (ANIb) analysis was performed as previously described (Richter and Rossello-Mora, 2009), using the genomes of strains 1 and 2 as a dataset and a publicly-available genome of *Streptococcus pasteurianus* type strain (ATCC 43144-GCA_000270165.1). Computation of ANIb values was performed using the Pairwise genome comparisons on-line tool

(http://jspecies.ribohost.com/jspeciesws/#home). Both *S. pasteurianus* strains showed ANIb values with *S. pasteurianus* ATCC 43144, of 99.78 % (strain 1) and 99.83% (strain 2) confirming the sub-species identification of *Streptococcus gallolyticus* sbsp. *pasteurianus* (Richter and Rossello-Mora, 2009). The analysis confirmed also the clonality of the two strains. The results are summarized in Table 1.

Table 1.

Animal	Samples	Api Strep	Resistance-pattern	16S rRNA	Whole Genome Sequencing
Calf 1	Site 1 SNC (dorsal region)	*S. bovis*[1]	OT-SXT-MTZ- EN[2]	*S.gallolyticus*[3]	*S. gallolyticus* subsp.*pasteurianus*
	Site 2 SNC (anterior region)	*S. bovis*	OT-SXT-MTZ- EN	*S.gallolyticus*	n.d. [4]
	Site 3 SNC (ventricular groove)	*S. bovis*	OT-SXT-MTZ- EN	*S.gallolyticus*	n.d.
	Acqueous humor	*S. bovis*	OT-SXT-MTZ- EN	*S.gallolyticus*	n.d.
	Colon	*S. bovis*	OT-SXT-MTZ- EN	*S.gallolyticus*	n.d.
	Blood	/	/	/	/
	Spleen	/	/	/	/
	Liver	/	/	/	/
	Kidney	/	/	/	/
	Lung	/	/	/	/
Calf 2	Site 1 SNC (dorsal region)	*S. bovis*	OT-SXT-MTZ- EN	*S.gallolyticus*	*S. gallolyticus subsp. pasteurianus*
	Site 2 SNC (anterior region)	*S. bovis*	OT-SXT-MTZ- EN	*S.gallolyticus*	n.d.
	Site 3 SNC (ventricular groove)	*S. bovis*	OT-SXT-MTZ- EN	*S.gallolyticus*	n.d.
	Acqueous humor	*S. bovis*	OT-SXT-MTZ- EN	*S.gallolyticus*	n.d.
	Colon	/	/	/	/
	Blood	/	/	/	/
	Spleen	/	/	/	/

	Liver	/	/	/	/
	Kidney	/	/	/	/
	Lung	/	/	/	/

[1] Identity percentage: 98.9%
[2] OT: Oxitetracyclin; SXT: trimethoprim-sulfametoxasole; MTZ: metronidazole; EN: Enrofloxacin
[3] *S. gallolyticus* subps. *gallolyticus*, Identity percentage: 99%.
[4] n.d.: Not done

4. Discussion

Bacterial neonatal M-ME is a severe and frequently fatal condition in calves and SBSEC complex members are sporadically the aetiological agents of M-ME (Aydin et al., 2018; Seimiya et al., 1992). In the present note, M-ME due to SGP and associated with ocular symptoms is reported in calves. The neurological symptoms and histopathological findings reported in this study are similar to those described by others (Seimiya et al., 1992; Aydin et al., 2018), although the SGG-mediated meningitis reported by Aydin et al. (2018) was not associated with ocular symptoms and the meningo-ventriculitis described by Semiya et al. (1992) was not ascribed to a specific biotype. The ocular lesions described here were diagnosed as septic hypopyon resulting from the spreading of bacteria to CNS. The bacterial strains isolated from brains, aqueous humor and colon (only from Calf 1) were successfully identified at the genus level using both the API Strep System and 16S rRNA gene sequence analyses but the sub-species identification required WGS, which was also necessary to confirm the clonality (Pompilio et al., 2019). In humans, SGG- and SGP-mediated meningitis has been described in neonates (Fikar and Levy, 1979; Beneteau et al., 2015) and in the last two decades invasive infections have been reported in infants and in adults (Alex et al., 2013; Corredoira et al., 2014). Geographic differences in prevalence have also been reported with SGG being the most frequent species causing infective endocarditis in Europe whereas SGP is responsible for the majority of infections in Asia (Pompilio et al., 2019). In ruminants, these bacteria are commensals of rumen and intestine (playing a major role in digestion) but they are recognized also as opportunistic pathogens. A recent review indicated that SBSEC members are capable to adapt to different ecological niches and that no reliable differentiation between human and animal SBSEC strains is

always possible (Jans et al., 2016). Invasive SGP infection presumably occurs either via an ascending route or via rectal and vaginal delivery or, post-natally, via maternal milk and contact; in fact, in humans, the maternal vaginal carriage is responsible for infections in neonates (Fikar and Levy, 1979). The association between lack of passively acquired immunoglobulins and neonatal septicemia is well established in bovines because in neonatal calves neutrophil function is less effective than adults and colostral leukocytes enhance humoral immunity (Fecteau et al., 2009). Indeed, the neonatal meningitis observed in this study could be due to the lack of maternal colostrum feeding. It could be also hypothesized that the source of infection in newborn calves was the maternal vaginal or rectal mucosa because calves were immediately separated from cows after birth and therefore did not receive maternal care and colostrum/milk. Although bacteria can spread hematogenously to the leptomeninges, blood coltures give high rates of false negative results and the definitive diagnosis relies on pathological findings on CNS and bacterial isolation (Biolatti et al., 2012). In addition, little is known about the pathogenicity of SBSECs, as virulence factors are limited to a very few adhesion and pro-infiammatory molecules (Jans et al., 2016). Streptococci are one of the most abundant genus both in human and ruminant gut microbiota and they carry antibiotic resistance genes; SBSEC isolates represent the major species displaying high antibiotic-resistance rate and this could be due to extensive horizontal gene transfer between streptococci and other gut microbiota bacteria (Jans et al., 2016; Pompilio et al., 2019). Recently, the highest resistance rates were reported in SGP for tetracyclin and macrolides, with extremely varying resistance rates for SXT (Pompilio et al., 2019). Most SBSEC members are susceptible to β-lactamases even though reduced susceptibility to penicillin has been reported in humans for SGP strains causing neonatal meningitis (Pompilio et al., 2019). However, the strains described here were susceptible to several classes of antibiotics indicating that a timely and precise diagnosis could have likely prevented the death of the calves.

5. Conclusion

Accurate and rapid identification of SBSECs is required in neonatal M-ME although the lack of reliable molecular tools and marker genes hampers accurate differentiation of SBSEC subspecies and

definitive taxonomic identification. The evidence of horizontal gene transfer between streptococci, the rapidly changing taxonomy and a broad host-range suggest that SBSEC members can be considered as emerging pathobionts that require continuous monitoring (Pompilio et al., 2019). Furthermore, since little is known about the zoonotic potential of several SBSEC strains, further characterization studies are warranted.

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Figure 1 (calf 1) and figure 2 (calf 2): Bilateral Hypopyon: storing of exudative-fibrinous materials in the anterior chamber of the eye which appeared macroscopically filled with yellowish materials

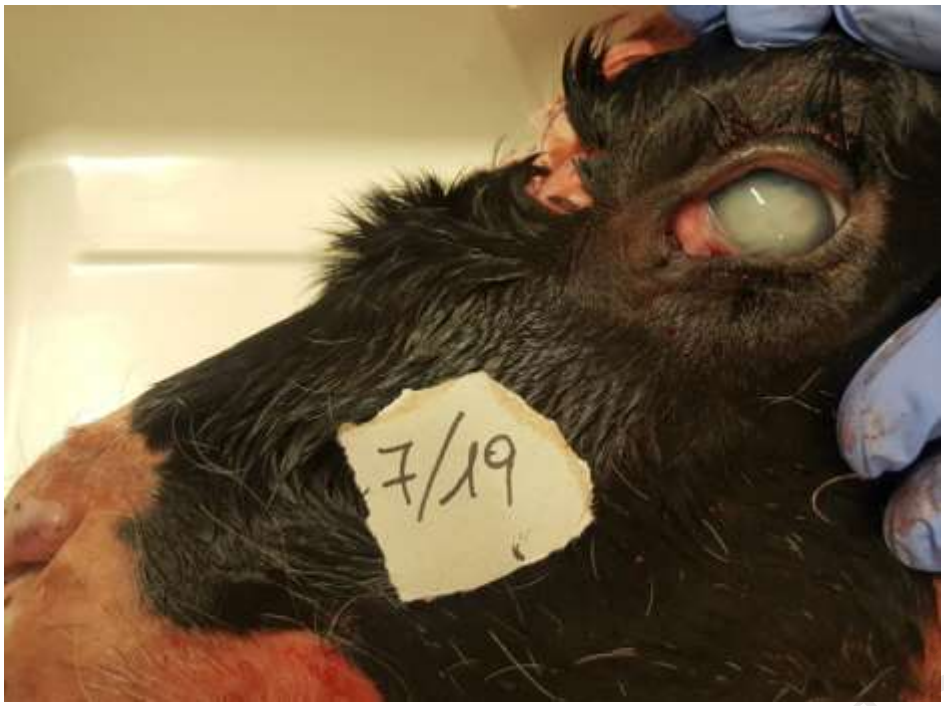


Figure 2. Calf 2

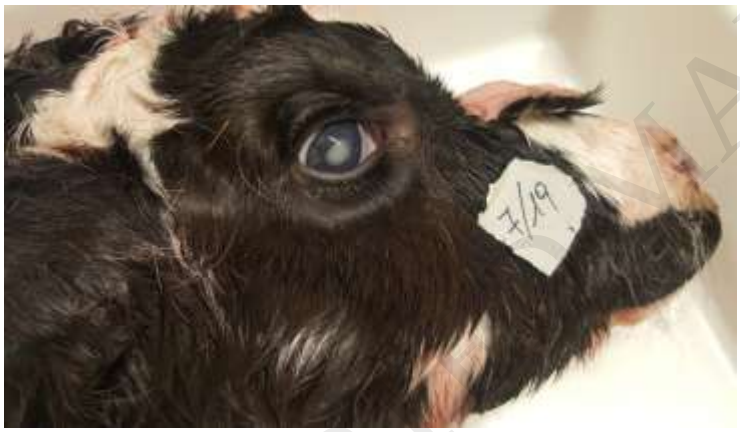


Figure 3. Calf 1**:** Massive and diffusive meningitis-meningoencephalitis involving mainly the choroid plexus and ventricular grooves, characterized by massive neutrophilic, lymphocytic and macrophage infiltration, with widespread abscessual areas; EE, 10X.

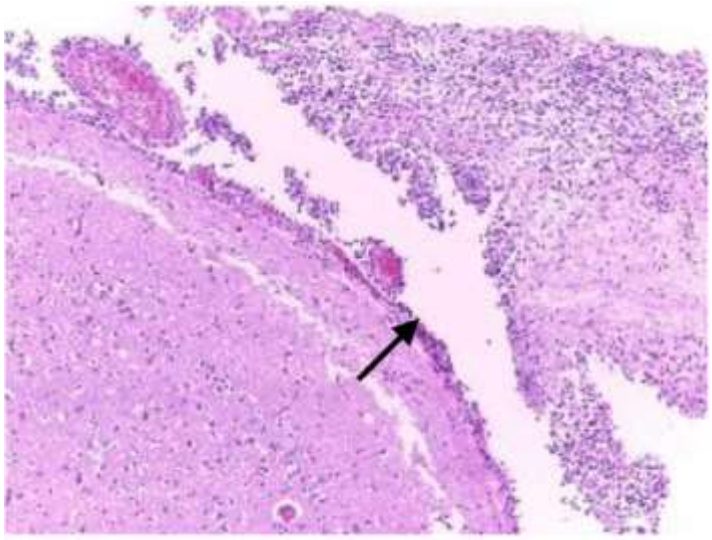
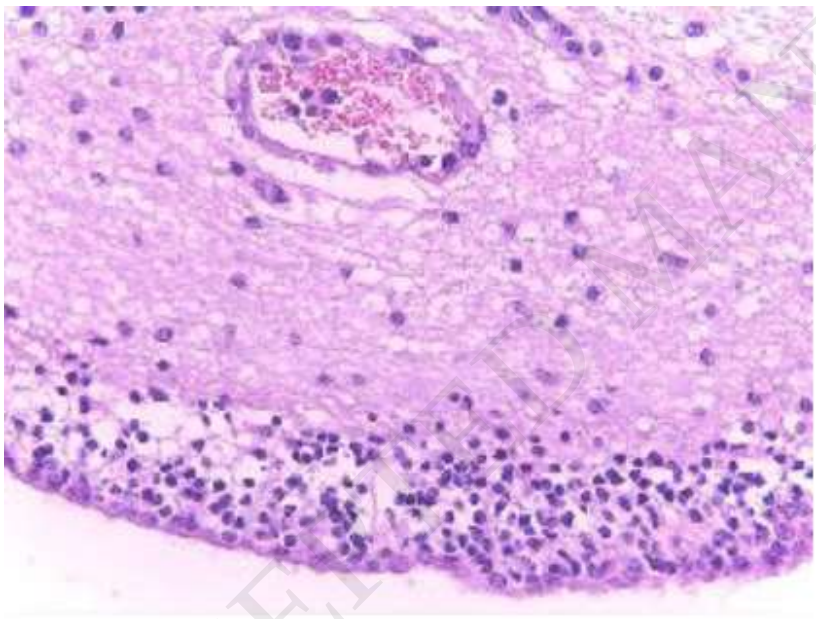
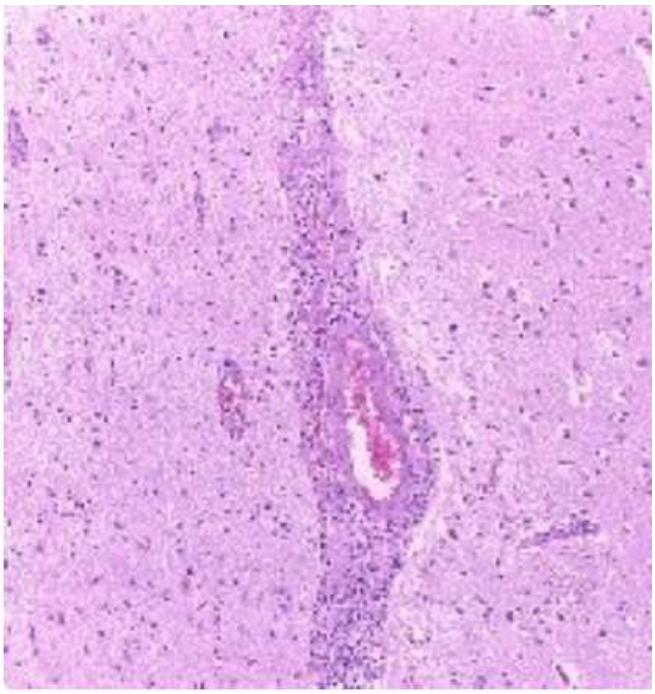


Figure 4. Calf 2: Granulocyte infiltration involving mainly choroid plexuses and ependyma; EE, 40X.



Figures 5. Calf 1: Vasculitis with blood congestion characterized by vascular lymphocytic infiltration of medium vessels. EE, 10X.



Figures 6. Calf 2: Perivascular neutrophilic accumulation in capillars with occlusion and destruction.

Respectively: EE, 40X.