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Imaging and genetic investigations of neural tube defect in a calf: case report and review of the literature

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Running head: Split cord malformation in a calf

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Abstract

A 15-day-old female crossbreed calf was referred because of paraplegia since birth. Clinical examination revealed a skin defect covered by hair on the dorsal midline in the thoracic area of the spine. Thoracolumbar neuroanatomical localization was determined based on the neurological examination. Computed tomography (CT) of the thoracolumbar spine revealed incomplete fusion of the vertebral arches from T6 to T9 and duplication of the vertebral arch of T7. At this level, duplication of the spinal cord with two segments completely separated by a septum of hyperdense, probably cartilaginous, tissue was noted. The spinal segments showed different degrees of duplication at histopathology. Three central canals where detected in one point. Genetic investigation for the presence of methylenetetrahydrofolate reductase (MTHFR) polymorphism, based on a study carried out by Song et al. in 2011 on Holstein cattle, was carried out and resulted negative in both the calf and the mother.

Key words: Anomaly of nervous system, Diplomyelia, Dyastematomyelia, Cattle

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A 15-day-old female crossbreed calf was referred to the Mobile Clinic Service, Veterinary Teaching Hospital (VTH), Department of Veterinary Science of Turin (Italy) because of inability to stand on her hind limbs since birth. Owing to podalic presentation, she was delivered by Cesarean section performed without complications by the referring veterinarian. The dam had given birth to other normal calves previously, but pedigree information about the sire was unavailable.

Clinical examination, performed in the field, revealed a skin defect covered by hair on the dorsal midline in the thoracic area of the spine. After cleaning and shaving of the area, the lesion was seen to be about 10 cm long surrounded by severely inflamed tissues. Other malformations included mandibular deviation and mild evidence of arthrogryposis affecting both forelimbs. Thorough neurological examination performed by a board-certified neurologist (ADA) revealed a moderately obtunded mental status. The calf was able to stand on forelimbs if supported. Gait observation showed paraplegia. Cranial nerve examination was normal, except for a bilaterally absent menace response due to the animal's young age. Patellar, tibialis cranialis, withdrawal and perineal reflexes were all normal on both hind limbs. Extensor carpi and withdrawal reflexes on both forelimbs were difficult to evaluate because of tendon contracture but were considered normal. No pain at palpation of the spine was noted. Thoracolumbar neuroanatomical localization was determined based on the neurological examination; a further possible secondary intracranial involvement was suspected on the basis of mental status because of the presence of an infected lesion at the thoracic area of the spine. A blood sample for complete blood count (CBC) and biochemistry profile was collected. A computed tomography (CT) scan was scheduled. CBC showed a mild neutrophilia (7.64 x 10^9 cells/L, reference range 1.1 – 3.6 x 10^9 cells/L) probably due to inflammation at the site of the defect. The biochemistry profile was otherwise unremarkable.

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CT (GE Highspeed Fx/i CT, GE Healthcare, General Electrics Company) revealed incomplete fusion of the vertebral arches from T6 to T9, associated with duplication of the vertebral arch of T7. The spinous processes appeared completely merged together at the level of T11. The vertebral canal had an elliptical shape at the level of T7, where the transversal diameter was increased from 2.2 cm to 2.57 cm and the spinal cord duplicated, with two segments completely separated by a septum of hyperdense (+111HU), probably cartilaginous, tissue. Figure 1 shows a CT dorsal reconstruction of the thoracic spine. Iodinated nonionic contrast medium (Iomeron ® 400, Bracco S.p.A., Milan, Italy) was administered by lumbosacral puncture. The post-contrast phase showed homogeneous distribution of the contrast media until T8. From this point to 3.2 cm further cranially, the contrast media surrounded the two spinal cord segments, enhancing two different subarachnoid spaces, and flowed dorsally from the right spinal cord segment to the skin, creating the appearance of a dermoid sinus.

A cerebrospinal fluid (CSF) sample was collected by lumbosacral puncture just before iodinated nonionic contrast medium administration. The sample appeared slightly yellow but clear at gross physical evaluation. CSF analysis showed increased total microprotein concentration (5.38 g/L, reference range < 0.4 g/L), increased total nucleated cell count (0.11 x 10^9 cells/L, reference range 0 - 0.01 x 10^9 cells/L), and marked blood contamination with an increased total erythrocyte count (0.5 x 10^9 cells/L, reference range 0 cells/L). Differential leukocyte count revealed mixed mononuclear pleocytosis with numerous activated vacuolated macrophages (61%) and occasional signs of leukophagocytosis. The remaining part consisted of activated lymphoid cells (30%) and neutrophils (9%).

The calf was euthanized due to poor prognosis and a post-mortem examination was carried out. The central nervous system was removed and fixed in 10% neutral buffered formalin, embedded in paraffin-wax, sectioned and stained with hematoxylin and eosin for histology. The

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vertebral column and spinal cord malformations identified on the CT scans were confirmed on autopsy. At gross examination, the spinal cord was progressively enlarged at the level of T6, with a complete split of the central part and a hole in the sagittal plane, and thickening of the meninges closely adherent to the bone (Fig. 2). The spinal cord was transversally sectioned at different levels to inspect it for macroscopic and microscopic lesions. The spinal segments showed different degrees of duplication at histopathology. Complete fusion of two spinal cords with central shrinkage was noted in the rostral section (at the level of T6) where two histologically normally organized hemicords, each with a central canal, were detected (section a – Fig. 3A). Spinal cord duplication was more evident caudally: two hemicord smaller and regularly organized (section b - Fig. 3B). Moving caudally, histological duplication of the spinal cord was complete where it was separated into two well-organized but atrophic sections (section c - Fig. 3C). Severe disorganization of the neuroparenchyma was noted on transverse section after resolution of the split into two segments, with three central canals (one of which ramified) and disseminated nonsuppurative perivascular cuffs with multifocal foci of neovascularization and gliosis (section d - Fig. 3D). The meninges all along this segment of the spinal cord were severely inflamed and showed severe suppurative-necrotizing meningitis. Multifocal meningeal fibrosis was also observed, particularly in the section caudal to cord duplication. Moderate, diffuse chronic meningitis associated with multifocal nonsuppurative cuffings was detected in the white and gray matter of the cerebellum and brainstem, particularly in the submeningeal and subventricular areas. No lesions were detected in the other parts of the central nervous system.

A CSF sample was submitted for the detection of *Toxoplasma gondii* [by means of a nested real-time polymerase chain reaction (rtPCR)] and of *Neospora caninum* (by means of simplex rtPCR). A spleen sample of the calf taken during autopsy was submitted for the detection of Schmallenberg virus and Bovine Viral Diharrea Virus by RT-rtPCR. All these investigations

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were carried out at the Istituto Zooprofilattico Sperimentale del Piemonte e della Valle d'Aosta and had negative result.

In order to exclude, as reported in human medicine[1,4,10], a correlation of the malformations identified with a deficiency of methylenetetrahydrofolate reductase (MTHFR), and subsequently of folate, a further genetic investigation was carried out, based on a previous study[12].

A muscle sample of the calf was taken during autopsy and stored at -80°C; a muscle sample of the mother was also collected at slaughtering. Genomic desoxyribonucleic acid (DNA) was obtained from muscle using the NucleoSpin ® Tissue kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol. DNA purity was evaluated by absorbance readings using the UV Spectrophotometer NanoDrop™ 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The primer set designed by Song and colleagues was used to amplify a fragment of MTHFR exon 4[12]. The primers for exon 7, designed on the bovine genomic sequence AC_000173.1, were: TGGAGGCCATTGTCTGGAGTAT (forward), CGAGAGGTAGTGGGCAAAGA (reverse). rtPCR reactions were performed in 25 µL volumes consisting of 0.03 U/µL of a HotStarTaq ® DNA Polymerase (Qiagen, Hilden, Germany), 0.2 mM each of deoxyribonucleotide triphosphate, 0.5 µM of each primer, and 50–100 ng of DNA template. The rtPCR profile consisted of an initial activation step at 95 °C for 15 min, followed by 35 denaturation cycles at 95 °C for 60 s, annealing at 54 °C for 60 s, and extension at 72 °C for 60 s. A final extension step of 72 °C for 7 min was added to all reactions. Amplifications were carried out using a GeneAmp® PCR system 2720 Thermal Cycler (Thermo Fisher Scientific, Life Technologies Italia, Monza, Italy). Amplicons were resolved on 2.0% agarose gel. Amplified fragments were cycle sequenced on an ABI Prism® 310 Genetic Analyser (ThermoFisher Scientific) using the ABI Prism BigDye ™ Terminator version 1.1 terminator cycle sequencing ready reaction kit (ThermoFisher Scientific) by the dideoxy chain termination

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method with fluorescence dye terminators. Sequencing on both strands was performed using the PCR primer. The resulting sequences were compared and aligned with the human messenger-ribonucleic acid (mRNA) sequence U09806.2 using the sequences nucleotide database NCBI blastn suite-2sequences software (https://blast.ncbi.nml.nih.gov); 198 bp and 205 bp fragments were generated from exons 4 and 7 of MTHFR, respectively. The amplicon sequences were compared and aligned with corresponding human sequences to identify the position corresponding to C677T and A1298C (Figs. 4 and 5). Both calf and mother were homozygous for the two single nucleotide polymorphisms (SNPs) showing the genotypes CC and AA for the normal alleles. Our results for exon 4 are in agreement with previous investigations.[12] Regarding the mutation in exon 7, Song et al. detected a C/T polymorphism in Chinese Holstein cows in position 1484 (NM_001011685.1) corresponding to position 1308 in the human sequence, while no polymorphism was found at nucletide 1474 (NM_001011685.1), corresponding to 1298 in the human sequence.

In this calf, a condition of spina bifida occulta and meningocele was associated with different degrees of duplication of the spinal cord. In veterinary medicine this condition is defined as diastematomyelia (from the Greek *diastema* = cleft) when the duplication is complete; usually the two histologically well-organized spinal cords are separated by a bony partition and contained in their own meningeal sheaths.[13] When the two spinal cords are merged together and covered by the same meninges, and histological disorganization of the white and gray matter is present, this condition is referred to as dyplomyelia (*diplouz* = double).[17] Tripartition of the spinal cord, also known as trifid cord, was noted at one point of the thoracic spine in this patient; it has been histologically reported only once previously in veterinary medicine by Zani et al. in 2010.[19] Few cases have been identified in human medicine.[7] Spinal cord malformations are an uncommon finding in large animals. [3,13] Usually occurring in the thoracolumbar segments, they

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are often associated with vertebral column malformations.[2,3] In fact, a close correlation exists between the embryological development of these two structures: the mesenchyme that gives rise to the axial skeleton is derived from the sclerotomal portion of the somites. These bilateral segmental structures originate from paraxial mesoderm and are located next to the neural tube and notochord. They begin to develop during the third week of gestation. Over the following two weeks, differentiation of these somites is influenced by the adjacent structures: the notochord and the neural tube stimulate the secretion of epimorphin, which induces sclerotome cells to move close to the notochord and the neural tube and promote the differentiation into vertebral cartilage and bone.[6]

In human medicine, there is a lack of consensus on the classification and terminology used to describe these malformations. Dyplomyelia was classically defined as spinal cord duplication while diastematomyelia referred to spinal cord splitting.[15] In 1992 Pang et al. proposed replacing these terms with the general term "split cord malformations", which are further classified in type I and II based on easily identifiable imaging hallmarks. Type I is characterized by the presence of a rigid bony or cartilaginous septum that gives rise to two different dural tubes containing two completely separated hemicords, whereas type II lacks a rigid septum (eventually only fibrous or fibrovascular) and the two spinal cords are contained in a single dural tube.[8] On the basis of the human classification, the present case could be defined as split cord malformation type I.

The embryogenesis of spinal cord tripartition is not well understood. The presence of more than one accessory neuroenteric canal is thought to be involved. Moreover, it is not known whether predisposing factors for the development of dyplomyelia exist. Several have been associated with the onset of spina bifida and other neural tube defects in human medicine.[4] Various environmental and genetic factors have been studied, including geography, maternal age, maternal diet, maternal diabetes and obesity, and exposure to antiepileptic drugs. The most

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significant finding to date was the protective effect of folic acid consumption during pregnancy, with a reduction in the incidence of neural tube defects by as much as 60 to 70%.[1]

An essential nutrient for mammalian cell growth, folic acid, is involved in the conversion of homocysteine in methionine and in the synthesis of purine and pyrimidine, essential components of fetal development. A deficiency of folate leads to elevated homocysteine levels in the blood, causing a delay in neural tube closure. The conversion of homocysteine in methionine requires the reduction of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, which represents the primary form of serum folate. This reaction is promoted by the 5,10-methylenetetrahydrofolate reductase enzyme.[1-3] Genetic studies have identified two different alleles and different genetic mutations for the MTHFR enzyme that can cause a deficiency of this enzyme.[16]

In veterinary medicine, based on a study carried out by Song et al. in 2011 on Holstein cattle, it is believed that a MTHFR polymorphism exists and that one of these genotypes is associated with a higher risk of abortion and higher homocysteine plasma concentration during pregnancy.[12] Further studies are needed to better understand the role of this mutation and resulting hyperhomocisteinemia in the development of neural tube defects. Genetic investigation for the presence of MTHFR polymorphism in both the calf and the mother resulted negative.

To our knowledge, no information is available about an appropriate CT imaging technique to diagnose dyplomyelia. Testoni et al. in 2010 reported on the use of ultrasound examination in the case of a 40-day-old crossbreed female calf diagnosed with dyplomyelia in the lumbosacral region of the spinal cord.[14] The authors remarked that because the vertebral spinous processes in human neonates are not yet ossified, ultrasound evaluation of the spinal cord without acoustic shadowing can be performed. Differently, the only acoustic window in calves is at the lumbosacral junction, which allows for the evaluation of just 1 cm of the spinal cord.[14] In the

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case of spilt cord malformation reported by Zani et al. in 2010, the dyplomyelia was diagnosed by magnetic resonance imaging (MRI).[19] As compared to MRI, CT takes considerably less time for image acquisition, with a shorter time required for general anesthesia and better evaluation of bony structures. The images allowed the diagnosis of both spina bifida and dyplomyelia, confirmed on post mortem examination in the present case.

Like those described by Vitellozzi et al. in 1983, Gülbahar et al. in 2005, Zani et al. in 2010, and Testoni et al. in 2010, [5,14,18,19] the animal presented in this case report was female. Large case series and retrospective studies on this type of malformations in human medicine have highlighted its higher prevalence among females, with a female to male ratio between 1.6:1 and 3:1.[9,11]

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Declaration of conflicting interests

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289 **Figure legends**

290 **Figure 1.** CT dorsal reconstruction of the thoracic spine

291

292 **Figure 2.** Gross pathology. Spinal cord with a complete split of the central part and a hole in the
293 sagittal plane at the T7 level.
294 Legend. a,b,c,d: levels of inclusion corresponding to histological sections of Figure 3.

295

296 **Figure 3.** Histopathology. Spinal cord. A. Rostral section corresponding to the level a of figure 2.
297 Two fused hemicords with central shrinkage, histologically normally organized, each with a
298 central canal. Suppurative-necrotizing meningitis. B. Section caudal to A, corresponding to
299 the level b of figure 2. Spinal cord complete duplication: one hemicord smaller and regularly
300 organized. Suppurative-necrotizing meningitis. C. Section caudal to B, corresponding to the level
301 c of figure 2. Spinal cord complete duplication: one well-organized but atrophic hemicord. D.
302 Section caudal to C, corresponding to the level d of figure 2. Two fused hemicords after the
303 resolution of the split. Disorganization of the neuroparenchyma, presence of three central canals
304 (arrows) and disseminated nonsuppurative perivascular cuffs with multifocal foci of
305 neovascularization and gliosis. Suppurative-necrotizing meningitis. Hematoxilin and eosin. Bar =
306 5mm.

307

308 **Figure 4.** Fragment of MTHFR exon 4 aligned with the human reference mRNA sequence. The
309 boxes correspond to the mutation site.

310

311 **Figure 5.** Fragment of MTHFR exon 7 aligned with the human reference mRNA sequence. The
312 boxes correspond to the mutation site**.**

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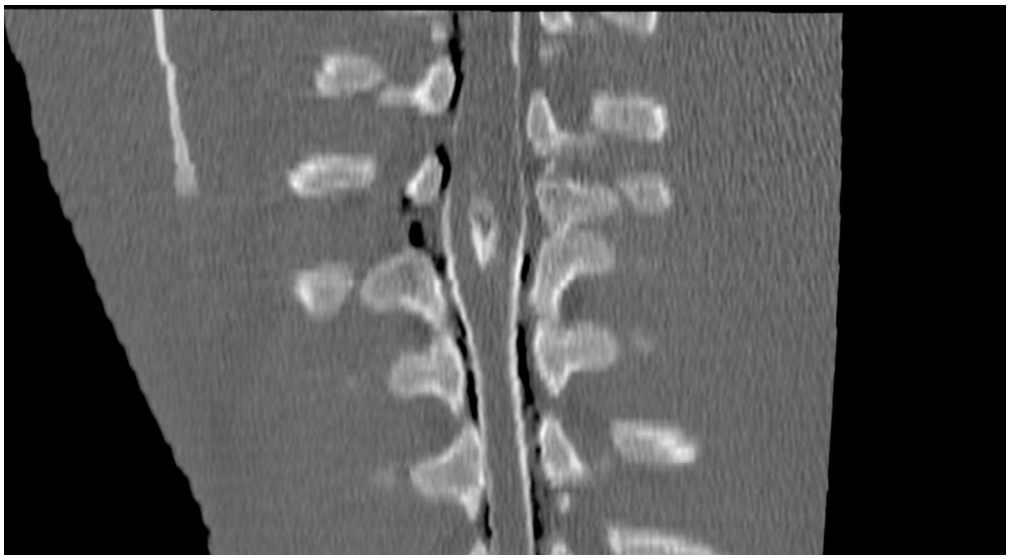

Figure 1. CT dorsal reconstruction of the thoracic spine

46x25mm (600 x 600 DPI)

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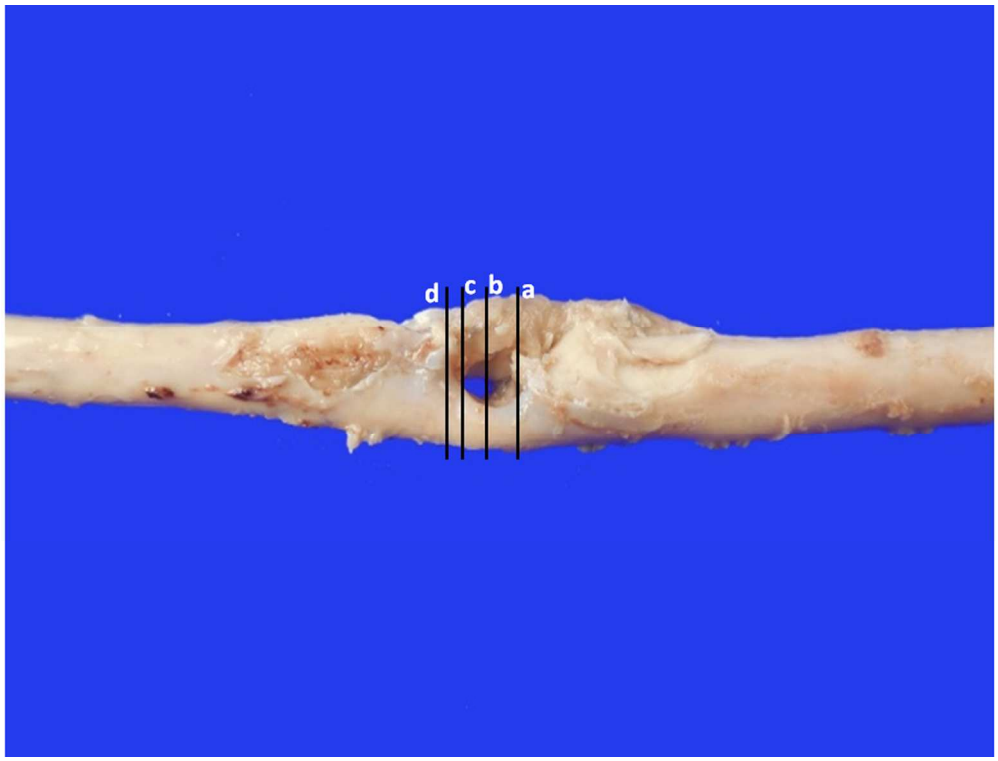

Figure 2. Gross pathology. Spinal cord with a complete split of the central part and a hole in the sagittal plane at the T7 level.
Legend. a,b,c,d: levels of inclusion corresponding to histological sections of Figure 3.

63x47mm (600 x 600 DPI)

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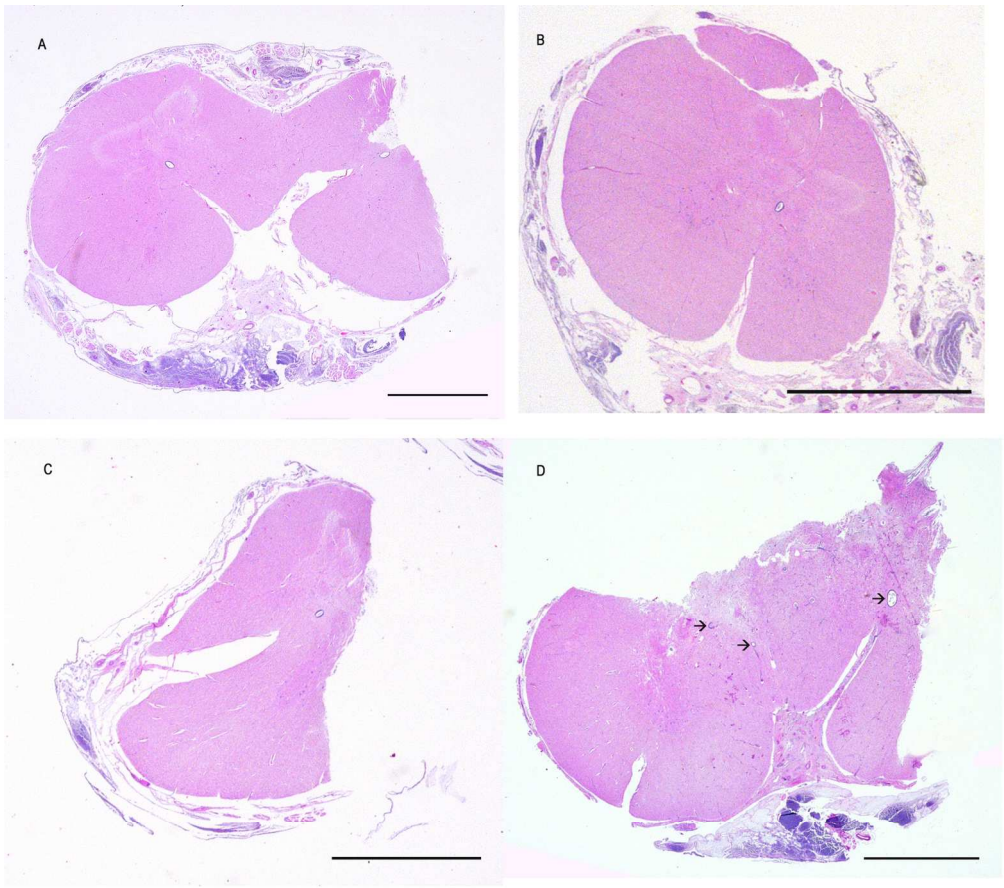

Figure 3. Histopathology. Spinal cord. A. Rostral section corresponding to the level a of figure 2. Two fused hemicords with central shrinkage, histologically normally organized, each with a central canal. Suppurative-necrotizing meningitis. B. Section caudal to A, corresponding to the level b of figure 2. Spinal cord complete duplication: one hemicord smaller and regularly organized. Suppurative-necrotizing meningitis. C. Section caudal to B, corresponding to the level c of figure 2. Spinal cord complete duplication: one well-organized but atrophic hemicord. D. Section caudal to C, corresponding to the level d of figure 2. Two fused hemicords after the resolution of the split. Disorganization of the neuroparenchyma, presence of three central canals (arrows) and disseminated nonsuppurative perivascular cuffs with multifocal foci of neovascularization and gliosis. Suppurative-necrotizing meningitis. Hematoxilin and eosin. Bar = 5mm.

77x71mm (600 x 600 DPI)

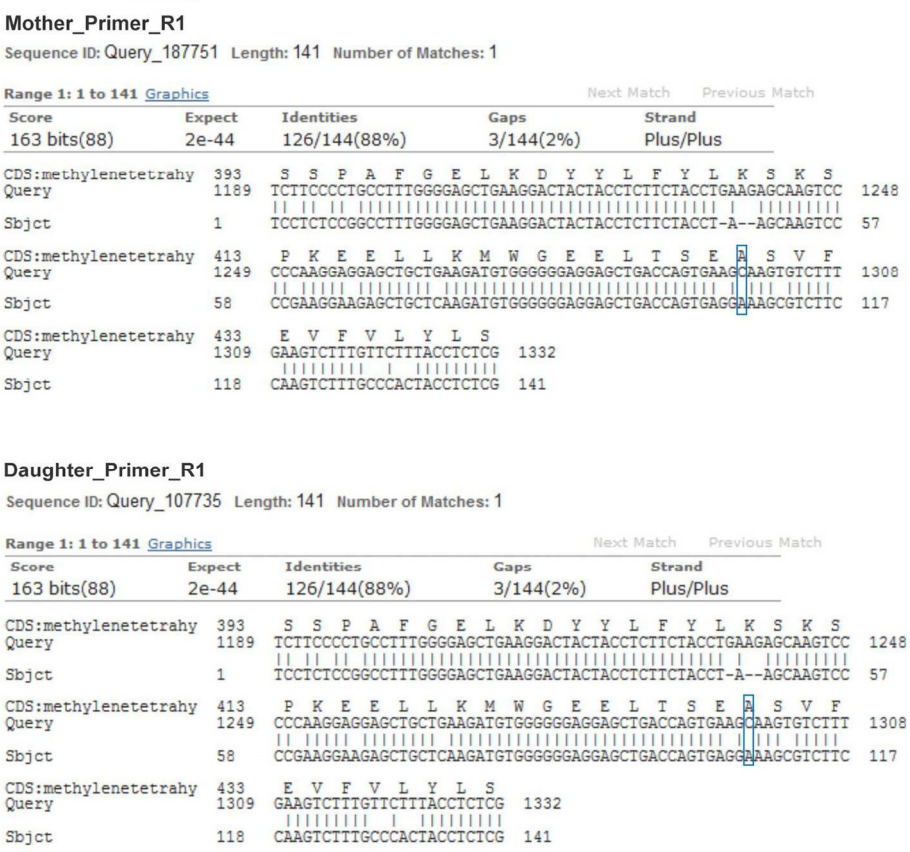

Figure 4. Fragment of MTHFR exon 4 aligned with the human reference mRNA sequence. The boxes correspond to the mutation site.

75x67mm (600 x 600 DPI)

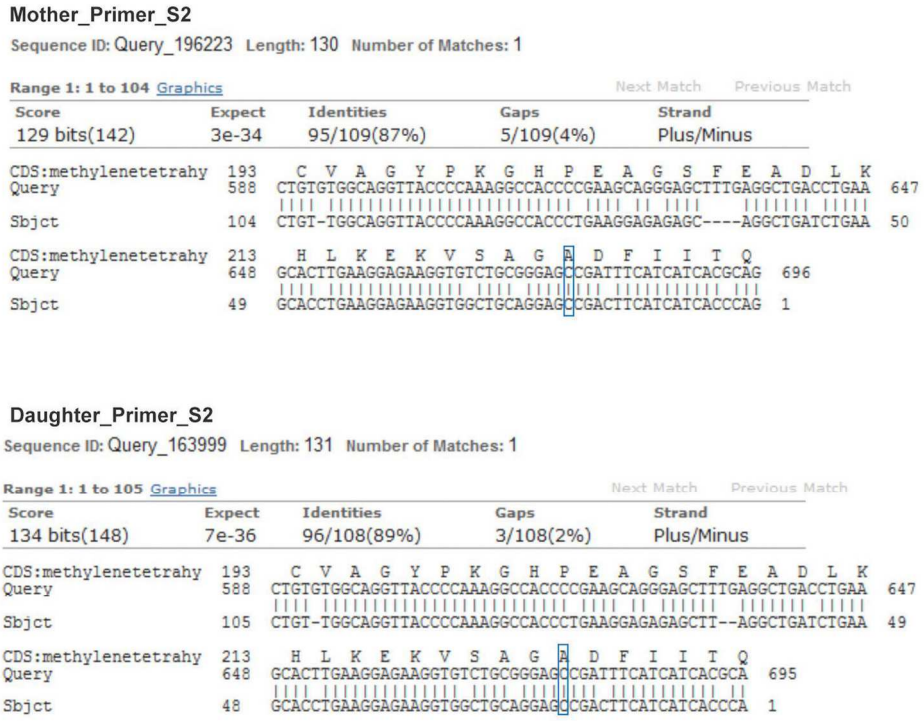

Figure 5. Fragment of MTHFR exon 7 aligned with the human reference mRNA sequence. The boxes correspond to the mutation site.

70x58mm (600 x 600 DPI)