Anencephaly, bifid tongue, and cleft palate in a Pomeranian dog: GFAP and NeuN immunoreactivities

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Abstract

Anencephaly is a congenital disease manifesting with the absence of the brain due to the failure of the cranial part of the neural tube to close during the embryonic stage. The disease may be accompanied by other anomalies and usually results in premature death. A stillborn puppy of a 2-year old female Pomeranian dog is examined in this case. The lack of brain tissue and accompanying abnormal skull formation was noted macroscopically. The eyes were protruding out of their normal position (protrusion), and a bifid tongue together with a secondary cleft palate was present. On serial sections stained with Haematoxylin-Eosin, only the medulla spinalis among the central nervous system structures could be inspected microscopically. Immunohistochemistry staining revealed GFAP immunoreactivity in the astrocytic glial cells. NeuN immunoreactivity was detected in the neurons in the medulla spinalis and spinal ganglions. Incomplete retinal layers were observed on the eye sections stained with Haematoxylin-Eosin and NeuN. The case was concluded to be coherent with skull and nervous system congenital malformations rarely observed in dogs. To the best of our knowledge, this represents the first description of a dog with anencephaly, bifid tongue and cleft palate.

Anencephaly is a type of neural tube defect (NTD) and affects the formation of brain and skull bones (8). Brain development occurs during embryonic development in animals (16). The leading cause of anencephaly is the failure of the neural tube to close properly during pregnancy (17). The neural tube originates from the neural plaque, which in the embryonic stage is fused with the neuroepithelium along the midline (16). A defect in the neural plaque may prevent the neural tube from closing completely, and the remaining gap in the neural tube can be observed as cranial, spinal, or both (8, 16). Defect in the cranial part of the neural tube leads to anencephaly (3, 8), and infectious agents, chemical substances, toxic plants and drugs, radiation, viruses, or nutritional disorders are the most important causes (16, 22). It is common in humans but rare in animals, and its etiology in dogs is not precisely known (8, 12, 16).

Bifid tongue and cleft palate are congenital defects rarely observed in humans and animals (9, 11, 13, 25). The bifid tongue is also defined as cleft tongue, accessory tongue, or double tongue (7, 9) and is reported to result from a fusion deficit in the embryonic stage. Persistent buccopharyngeal membrane, amniotic constriction bands in the region of the branchial archs, environmental factors, and large doses of vitamin A are described as some of the fusion deficit etiologies (18). The defect is believed to be caused by the failure of mesodermal migration into the midline structures of the mandibular portion of the first

branchial arch (18). Bifid tongue cases reported in mule foals, donkey foals, dogs, and a calf were often observed to have developed in conjunction with a cleft palate and a cleft mandible (5, 10, 19, 24, 25).

In this case, a stillborn puppy born to a 2-year-old female Pomeranian dog after her first labor is diagnosed with anencephaly, accompanied by a bifid tongue and a cleft palate, after macroscopic and microscopic examinations. The aim of this study is to show the presence of neurons and astrocytes by showing their immunoreactivity in the central nervous system.

The stillborn puppy with an anomaly born to a 2-year-old Pomeranian mother was brought to the Pathology Department of the Veterinary Faculty in Bursa Uludağ University for necropsy and histopathologic examination. Information obtained via anamnesis revealed that after 63 days of pregnancy, the mother gave birth to three puppies, of which two were normal, and one was stillborn. Incomplete skull formation of the stillborn puppy and a membrane cover over its skull was observed at necropsy (Figure 1). No cerebral hemispheres were found in the cavum cranii when this membrane was removed. Protrusion of the eyes out of their normal position (exophthalmos) was noted (Figure 2). It was observed in the examination of the mouth cavity that the tongue was split from the midline with an approximately 1.5 cm long cleft (bifid tongue), that there was also a 3 cm long cleft on the palate (palatoschisis) (Figure 3), and that the mandible protruded beyond the maxilla (brachygnathia superior). No pathological findings in the internal organs were found in the examination of the abdominal and thoracic cavities.

Specimens taken for microscopic examination were fixed in 10% neutral buffered formalin, subjected to routine procedures, and embedded into paraffin. 4-micron-thick sections were stained with routine H&E. Sections deparaffinized for immunohistochemical analysis were passed through the alcohol series. After washing in the buffers, it was incubated for 5 minutes in 3% H_2O_2 to suppress endogenous peroxidase activity after recovery of antigenicity for 30 minutes at pH 6.0 citrate buffer at 98°C. It was kept in Bloking Serum to prevent non-specific binding. As the primary antibody; were incubated with mouse anti-NeuN (1/500 dilution; Anti-NeuN Antibody, Clone A60, Sigma-Aldrich) and mouse anti-GFAP (1/200 dilution; Anti-GFAP Antibody, CloneG-A-S, Sigma-Aldrich) antibody for overnight at +4°C. The next day, incubation was performed with biotin-conjugated goat anti-mouse secondary antibody at a dilution of 1/300 for 2 hours at room temperature (Thermo Fisher Scientific). It was incubated with ABC enzyme for 1 hour. Sections were washed in buffers and then nuclear or cytoplasmic labeling was visualized with DAB. Negative control staining was performed for each staining. Microscopic examinations were made with Olympus BX50 microscope.

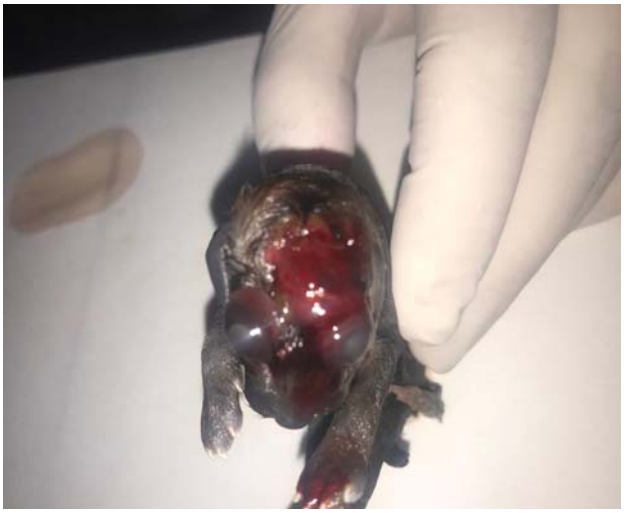

Figure 1. Presence of a membrane in place of the skull bones.

Figure 2. Protrusion (in the eyes) and anencephaly.

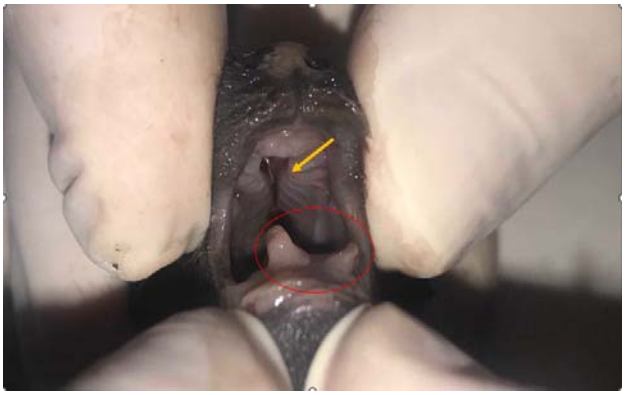

Figure 3. Cleft palate (palatoschisis) (yellow arrow) and cleft tongue (bifid tongue) (red circle).

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The medulla spinalis, ganglia, meninges from the nervous system, and the bony vertebrae were distinguished on the H&E-stained sections (Figure 4). No structures belonging to the brain or cerebellum tissues were found. Immunohistochemistry staining for GFAP was positive in the astrocytes of the grey matter around the canalis centralis and the glia limitans under the meninges (Figure 5-6). Neurons in the medulla spinalis and spinal ganglions were positive for NeuN (Figure 7-8). Retinal layers were not completely formed on the eye sections stained with H&E and NeuN (Figure 9-10).

In this case, the absence of the brain and cerebellum tissues, the skull is covered with a membrane, and the presence of only the medulla spinalis, both macroscopically and microscopically, are coherent with anencephaly in humans. Protrusion of the eyes, which develop from the forebrain during the embryonic stage (2), and the deficits in the retinal layers observed on histologic sections are consistent with anencephalic findings in humans and animals.

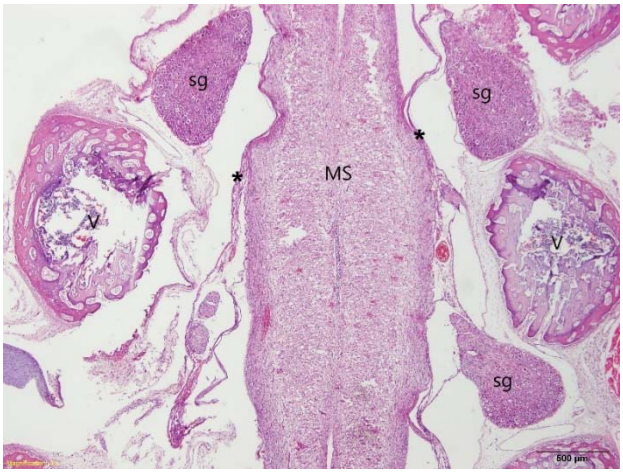

Figure 4. Medulla spinalis (MS), bony structures (V), meninges (*), and ganglia (sg) H&E. 4x objective.

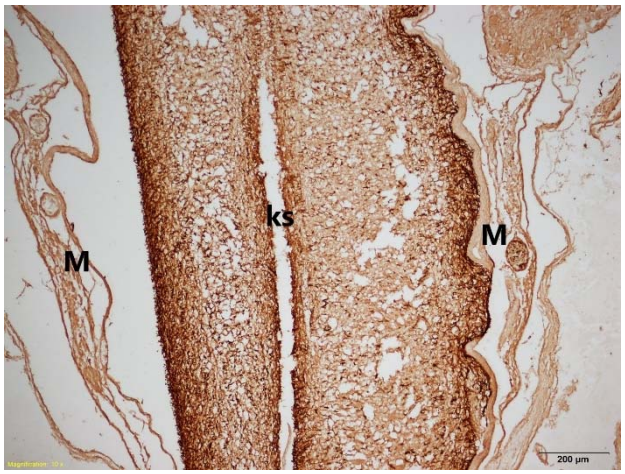

Figure 5. Dense GFAP positivity around the canalis centralis (ks) and in the glial limitans under the meninges (M) of the medulla spinalis. 10x objective.

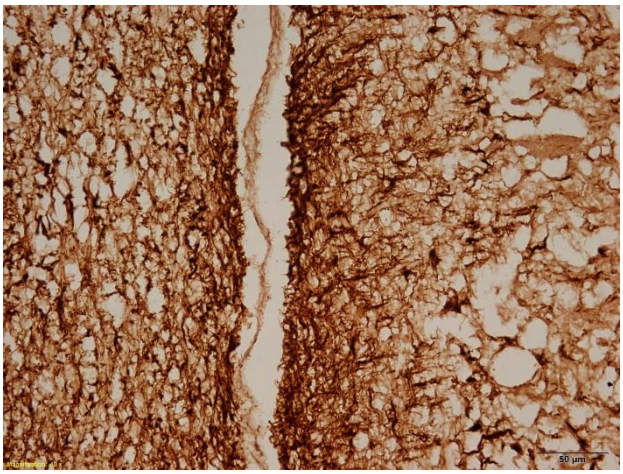

Figure 6. Dense GFAP immunoreactivity in the astrocytes around the canalis centralis. 40x objective.

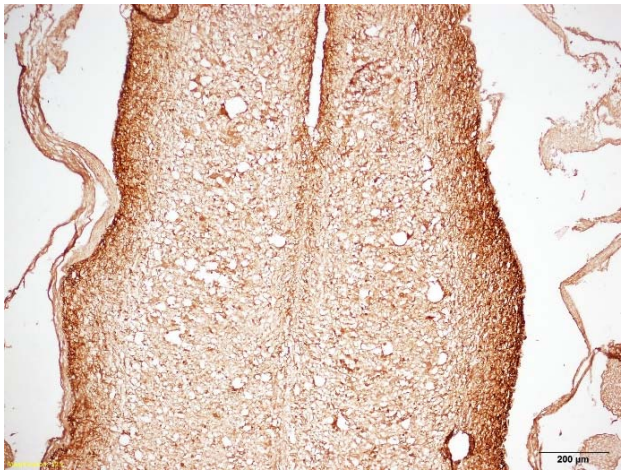

Figure 7. NeuN positivity in the neurons of the medulla spinalis. 10x objective.

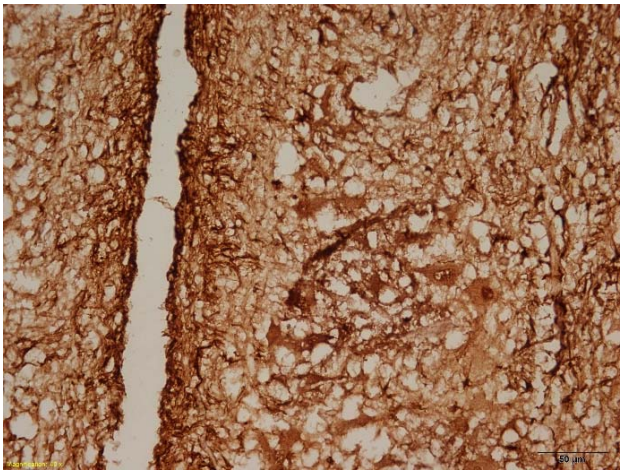

Figure 8. NeuN immunoreactivity in the neurons of the medulla spinalis. 40x objective.

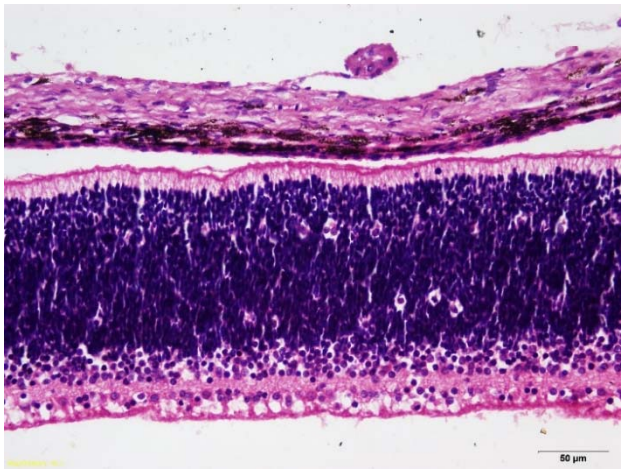

Figure 9. Incomplete retinal layers of the eye, H&E. 10x objective.

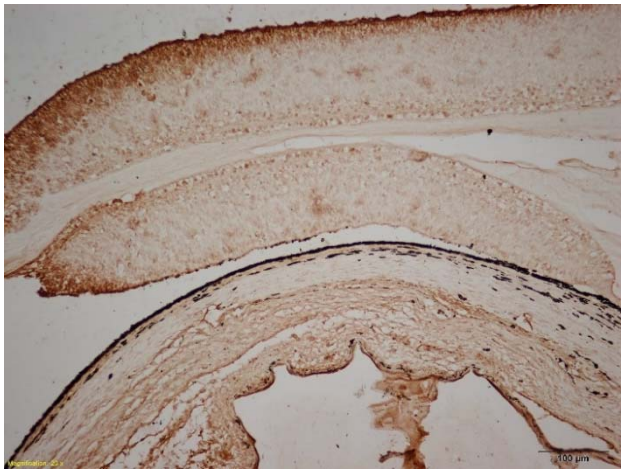

Figure 10. NeuN immunoreactivity in retinal layers of the eye. 20x objective.

In gross and microscopical examination, no cerebrum and cerebellum tissue were found. Immunohistochemical analyzes showed GFAP positive glial fibrils. This positive staining is as expected; it is intense in the glial limitans around the spinal canal and under the meninges, and it is lighter in gray matter. The immunohistochemically reactivity of glial cells which were positive for glial fibrillary acidic protein matches the expression profile of mature astrocytes of adult animals. Based on the presence of NeuN positive neurons and the positions of these neurons, this tissue was thought to be medulla spinalis tissue belonging to the central nervous system. In addition, there are spinal ganglia around the spinal canal and between the vertebral bones, where the presence of NeuN positive neurons is observed. The main purpose of these stainings is to show that the tissue in the area below the neck-cranial border is a tissue belonging to the central nervous system. The area of interest was stained with hematoxylin and eosin, but staining with the neuron marker NeuN and the astrocyte marker GFAP was necessary to clearly show the tissue of the central nervous system. We showed that in central nervous system malformations such as anencephaly, the histology of the spinal cord is normal and cells that can be stained with neuron and astrocyte specific antibodies are present in this tissue.

The development of the neural tube is a multi-step process controlled by various genes and environmental factors. NTDs are a group of complex and heterogenic central nervous system anomalies, including anencephaly, spina bifida, and encephalocele (17) and may occur under the influence of nutritional and environmental factors (15). Folic acid intake has an important role in preventing the development of NTD in humans and until the third month of pregnancy is reported to reduce NTDs significantly, and widespread folic acid consumption reduced the frequency of these cases by 25% to around 1/1500 (6, 14, 15, 20). While folic acid consumption is known to prevent NTDs in humans, only one study shows that folic acid intake reduces NTDs in dogs (4). Zinc deficiency is also reported as a possible cause of anencephaly (22). Safra et al. (21) discovered an NTD-related gene in dogs for the first time and reported that NTDs might be associated with a genetic disorder.

Toxic plants, viral infections, teratogenic agents, and genetic factors are thought to be the causes of malformation in animals (10). Some anticonvulsant drugs like valproic acid consumed in the first trimester have been reported to cause NTDs (15). While anencephaly is observed sporadically in cattle, it is seldom observed in other species (8). NTDs are rare in dogs (23).

The bifid tongue is a bifurcation formed in the tongue and is generally uncommon in animals. Millard et al. (13) reported that there had been only 46 cases of bifid tongue in the last 150 years. Rifai et al. (19) reported that while cleft lower lips were observed in mules, only one mule had a bifid tongue. Bifid tongue and mandibular cleft are usually observed together in animals. Bifid tongue composes nutritional problems for the animals born with it and requires surgery for reconstruction (10). In our case, accompanying anencephaly is also noted in addition to the bifid tongue and cleft palate (palatoschisis). The literature review reveals no previous cases of this combination of malformations in dogs, so it is concluded that this case is the first.

In our case, it is known that the parents did not have any previous health issues, and they both were fully vaccinated. Folic acid and zinc analyze could not performed in our case unfortunately. The birth is the first birth of the mother. The detected anencephaly, bifid tongue, and cleft palate formation are thought to result from an environmental insult the mother was exposed to.

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In conclusion, studies about fetal malformations and their etiologies in dogs are not sufficient. More advanced future studies are required.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

OY, SE, SEY, ERY and DB conceived and planned the experiments. OY, SEY, SE, MOO planned and stained histochemically and immunohistochemically staining. OY, SEY, DB, SE and ZAK contributed to sample preparation. OY, SEY, ERY, AS and MOO contributed to the interpretation of the results. OY, ERY and SEY took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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