



## NOTE

Wildlife Science

## A case of Anthrax in two captive pumas (*Puma concolor*)

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**ABSTRACT.** In this study, we aimed to report anthrax cases in two pumas, brought to the Pathology Department, Faculty of Veterinary Medicine, Erciyes University for suspected poisoning upon their sudden death at the Kayseri Zoo, in Turkey. In the necropsy, enlargement and malacia were observed in the spleens. The cut surfaces of the spleens were in extreme red-blackish color. *Bacillus anthracis* was isolated as a pure culture from both samples which belong to dead pumas. *B. anthracis* isolates had pXO1 and pXO2 plasmids. Both isolates were found to be sensitive to eight antibacterials tested. This study demonstrates that feeding of the wild carnivorous kept in any zoo with the appropriate meats which belongs to healthy animals is extremely important.

**KEY WORDS:** Anthrax, *Bacillus anthracis*, histopathology, puma

Anthrax, which is a significant zoonosis threatening the life of humans and domestic and wild herbivore and carnivore animals, is caused by *Bacillus anthracis* that is a spore forming bacterium which quite resistant to environmental conditions [5, 7]. Anthrax cases have been reported in many animal species in natural parks and zoos in the various locations of the world [12, 13, 15, 17, 19]. The disease occurs in wild animals mostly by the consumption of food and water contaminated with *B. anthracis*. However, the sporulation of *B. anthracis* and resistance of these spores to pH, high and low temperatures and chemical substances enables it to stay in the soil for a long time. Therefore, it is very important to understand the ecology of Anthrax and the geographical distribution of *B. anthracis* [5, 6, 27, 28]. While the lesions differ in lions and leopards among predatory animals, head and neck regions are edematous, and edema and hemorrhage are seen in the lymph nodes through these regions [27]. Except observing anthrax bacilli among tissue and erythrocyte clusters microscopically, necrosis and edema are seen in all organs [27].

While anthrax is observed sporadically in the mid and northern regions of Europe nowadays, it has been reported more frequently in Mediterranean countries, particularly Turkey, Greece, Balkan States, Italy and Spain [21–23]. There have been studies which reports anthrax cases seen especially in the eastern part of Turkey. In these studies, it was reported that *B. anthracis* was isolated from cattle, sheep and human [1, 22].

Although cultural examination is considered as the golden standard for the diagnosis of anthrax, Giemsa (capsule), Gram staining (cell wall) and lysis test by gamma phage are required for final definition. In addition, virulent isolates of *B. anthracis* have pXO1 and pXO2 genes which are encoded on plasmids, and PCR (Polymerase Chain Reaction) method revealing the presence of these virulence genes provides the confirmation and rapid identification of *B. anthracis* isolates [2]. Nucleotide array is significant for family typing and molecular epidemiological studies [27].

In this case, we aimed to report macroscopic–microscopic and bacteriological findings of anthrax case found in two pumas which were brought to the Pathology Department, Faculty of Veterinary Medicine, Erciyes University for suspected food poisoning upon their death.

The materials of this study were two pumas, both were 5-year-old, of which one was male (Puma 1) and one was pregnant female (Puma 2), brought from Kayseri Zoo to the Pathology Department, Faculty of Veterinary Medicine, Erciyes University for necropsy and suspected poisoning upon their death. The zoo officials reported that the pumas were shared the same cage and fed with the same food. They also reported that the pumas did not show any clinical symptoms before they died. Additionally, no outstanding symptom was observed during the external inspection during necropsy.

After the systemic necropsy examination, the tissues were fixed in 10% neutral formalin solution, routine procedure was followed and immersed in paraffin. The tissues were cut in 4–5  $\mu$  and examined under light microscope by following Hematoxylin-

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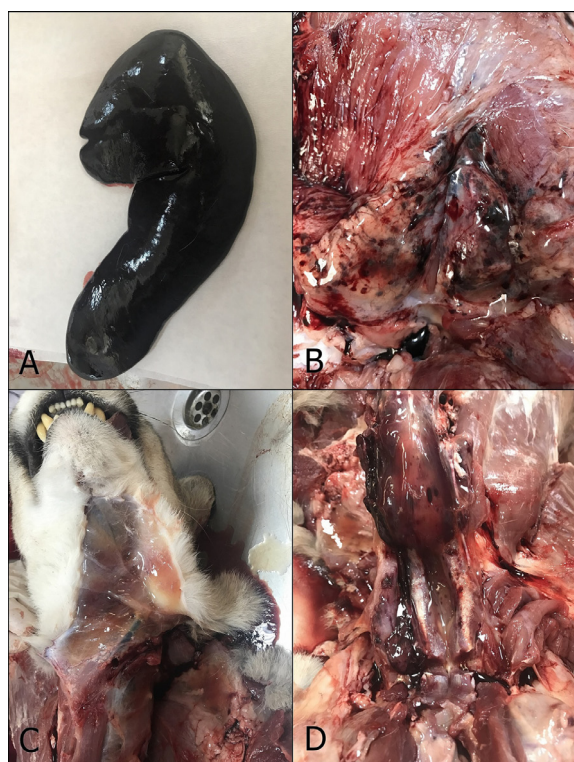
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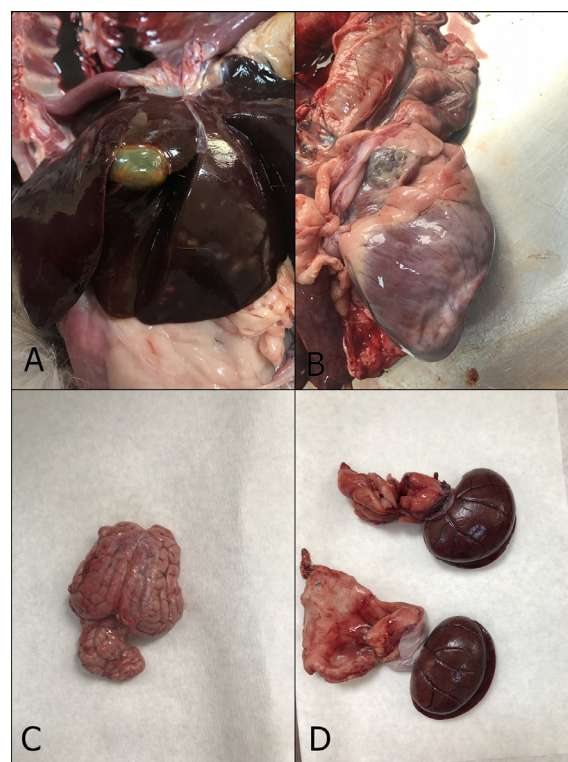
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**Table 1.** A table containing a list of the primers used for this purpose is provided below

| Plasmid target | Size (bp) | Sequence 5'-3'                               | Primer         | Reference                    |
|----------------|-----------|--|----------------|------------------------------|
| pXO1           | 596       | TACTGACGAGGAGCAACCGA<br>GGCTCAGTGTAACCTCTAAT | CAP6<br>CAP103 | Beyer <i>et al.</i> 1995 [4] |
| pXO2           | 1,035     | GAGGTAGAAGGATATACGGT<br>TCCTAACACTAACGAAGTCG | PA8<br>PA5     |                              |



**Fig. 1.** A. Appearance of growth and malacia were observed in the spleen of Puma 1. B–C. Appearance of growth, hemorrhage and edema on retropharyngeal lymph nodes as well as accumulated gelatinous fluid in Puma 2. D. Appearance of petechias on submandibular region in Puma 1.



**Fig. 2.** A. Appearance of gray-whitish color with subserosal location in liver of Puma 1. B. Appearance of myocardium was in dull color with loose consistency in Puma 1. C–D. Kidneys and brain were in hyperemic appearance in Puma 2.

Eosin and Gram staining [18] procedures.

After the necropsy, swab samples were taken from the liver and spleen, and were stained with Giemsa. Also, swab samples were inoculated onto Blood Agar (Blood Agar Base No. 2, Oxoid) containing 7% sheep blood, and plates were incubated for 24 hr at 37°C under aerobic condition. PCR which has been reported by Buyuk *et al.* [9] with minor modifications was used for detecting the presence of pXO1 and pXO2 in *B. anthracis* isolates. Amplified products were resolved by 1.5% agarose (Prona) gel electrophoresis and visualized under a UV transilluminator (G:BOX Chemi XRQ, Syngene).

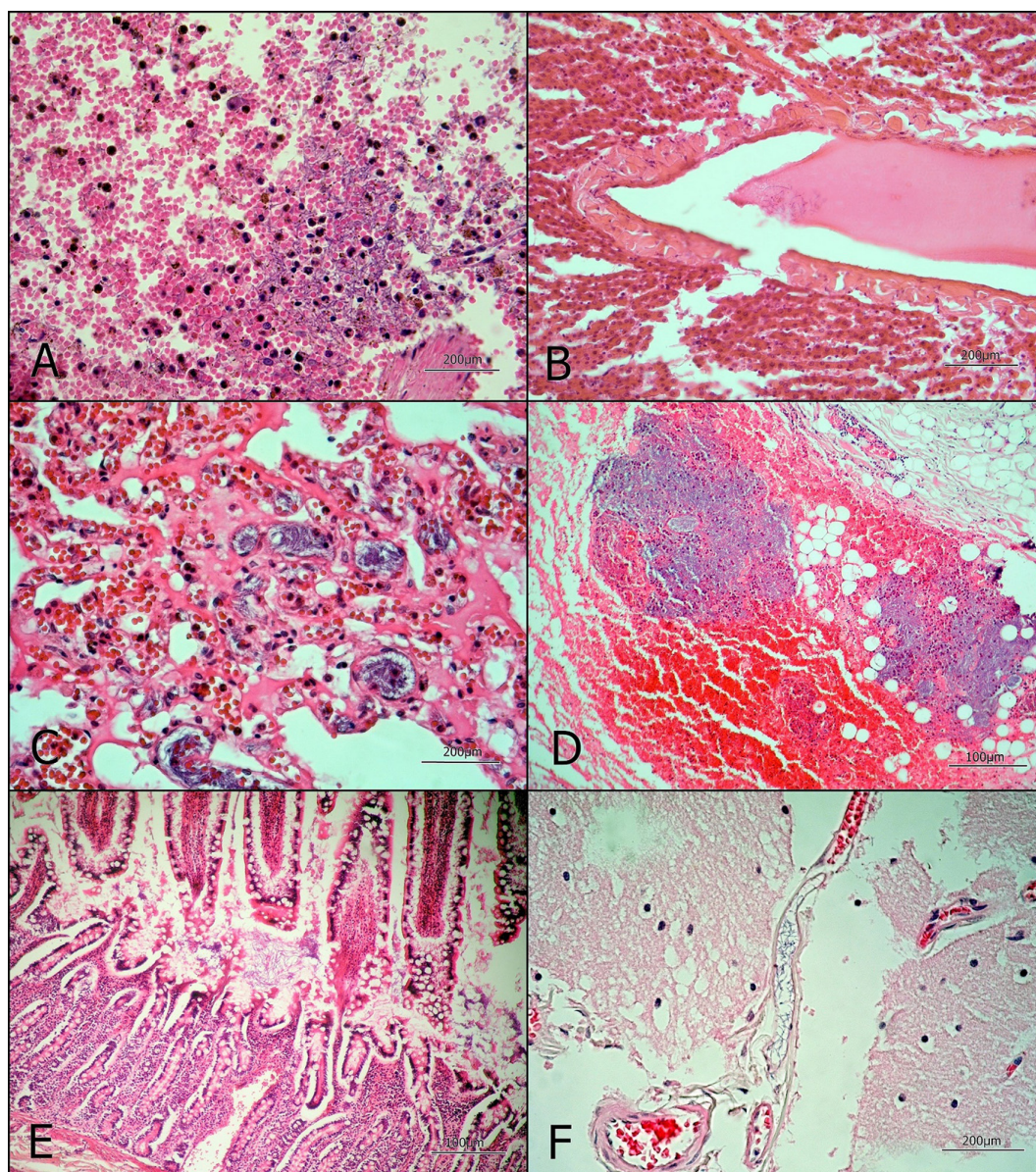
A table containing a list of the primers [4] used for this purpose is provided below (Table 1).

*Bacillus anthracis* K125 isolate (pXO1 and pXO2, from the culture collection of Dept. of Microbiology, Faculty of Veterinary Medicine, Kafkas University) was used throughout the study. DNA extraction from *Bacillus anthracis* isolates was carried out according to the method reported [9].

In addition, antibiotic susceptibility testing of the isolates obtained was performed by using Kirby-Bauer disc diffusion method [14]. The following antibiotic discs were used: penicillin (10 U, Oxoid), amoxicillin 20 µg/clavulanic acid 30 µg, (Oxoid), gentamicin (10 µg, Oxoid), ampicillin (10 µg, Oxoid), tetracycline (30 µg, Oxoid), erythromycin (15 µg, Oxoid), enrofloxacin (5 µg, Oxoid), levofloxacin (5 µg, Oxoid) and ciprofloxacin (5 µg, Bioanalyze).

No pathological lesion was found in the external inspection during necropsy while enlargement and malacia were observed in the spleens of two pumas during internal inspection, and the section surfaces were in extreme red-blackish color (Fig. 1A). Enlargement of retropharyngeal lymph nodes associated with edema and accumulated gelatinous fluid (Fig. 1B and 1C) and petechias on submandibular region were seen (Fig. 1D). Similarly, 1 × 1.5 cm foci in gray-whitish color with subserosal location in liver (Fig. 2A)





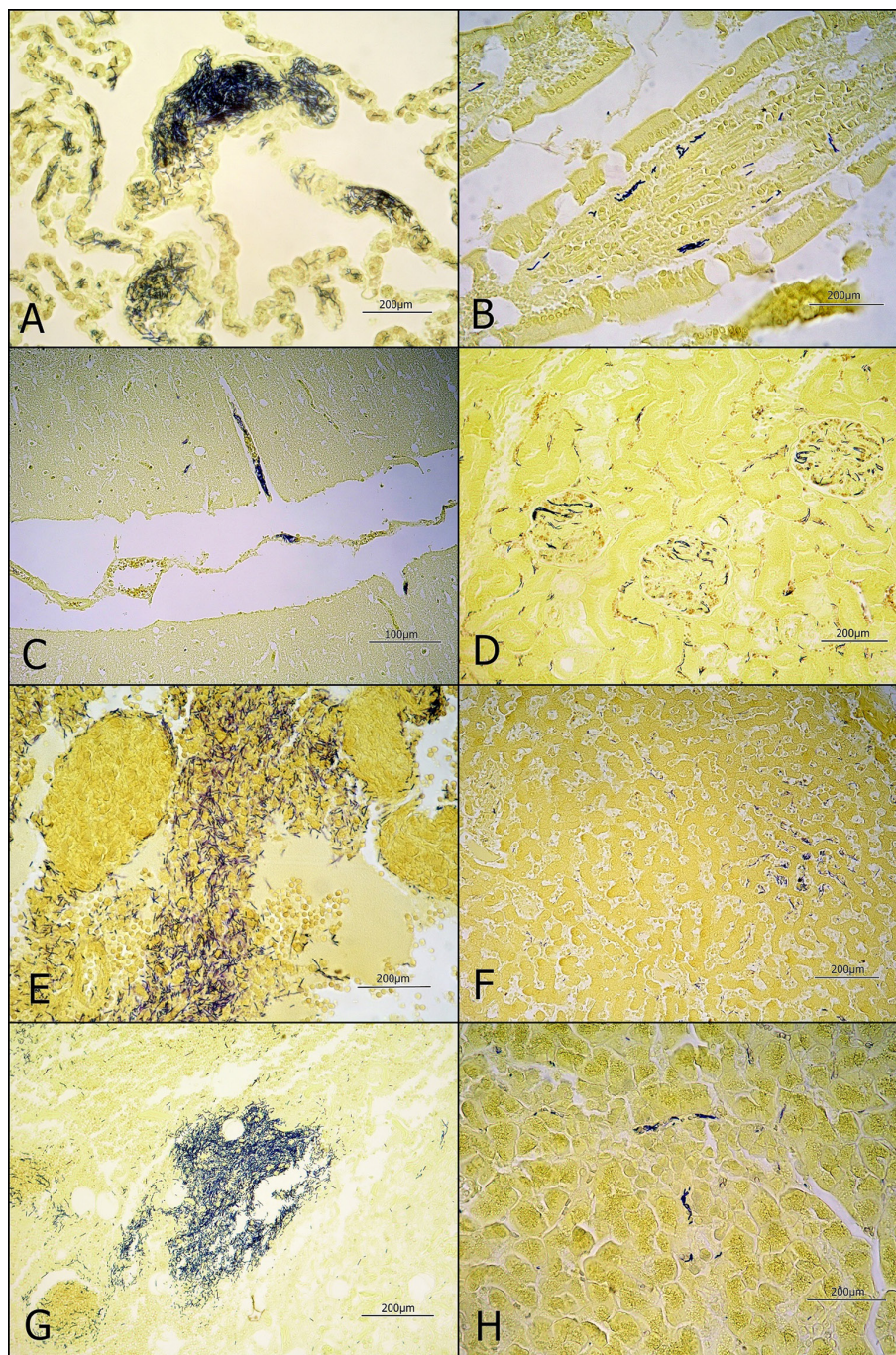
**Fig. 3.** A. Appearance of hemorrhagic splenitis with lymphocytosis in Puma 1, HxE,  $\times 200$ . B. Appearance of congestion and perivascular cell infiltration in the liver of Puma 1, HxE,  $\times 200$ . C. Vegetative form of *B. anthracis* in the lung with intra-alveolar edema and hemorrhage in Puma 1, HxE,  $\times 200$ . D. Appearance of hemorrhagic esophagitis with extensive bleeding area in Puma 2, HxE,  $\times 100$ . E. Appearance of *B. anthracis* in the lamina muscularis mucosae in the intestine in Puma 1, HxE,  $\times 100$ . F. Hyperemia in brain vessels in Puma 2, HxE,  $\times 200$ .

were also observed in the myocardium. Myocardium was in dull color with loose consistency (Fig. 2B). The brain and kidneys were hyperemic in appearance (Fig. 2C and 2D). No remarkable pathological lesion was found in the necropsy of two fetuses removed from the uterus.

In the microscopic examination, intact and fragmented erythrocytes were found in cortex and medullary sinuses of the spleen as well as hemorrhagic splenitis with lymphocytosis (Fig. 3A). Hemorrhagic lymphadenitis was found in the lymph nodes. There were congestion and perivascular cell infiltration in the liver (Fig. 3B), focal hyalinized glomerules in the kidneys, wide edema, hemorrhage and emphysema areas in the lungs (Fig. 3C), hemorrhagic esophagitis with extensive bleeding in the esophagus (Fig. 3D), vasculitis in the pancreas, and hyperemic and small bleeding areas in the intestinal mucosa. Lymphoplasmatic enteritis (Fig. 3E) and focal non-suppurative myocarditis with degenerative-necrotic areas in myocardium and severe hyperemia were seen in brain vessels (Fig. 3F). *B. anthracis* was observed within the vessels in all organs associated by septicemia with gram staining (Fig. 4).

Rough and non-hemolytic colonies, which are specific to *B. anthracis*, were observed on the blood agar plates. The isolates were purified by streaking on blood agar and were then examined using the following tests; Gram staining, oxidase test, catalase test, motility test and lysis by gamma phage [25].



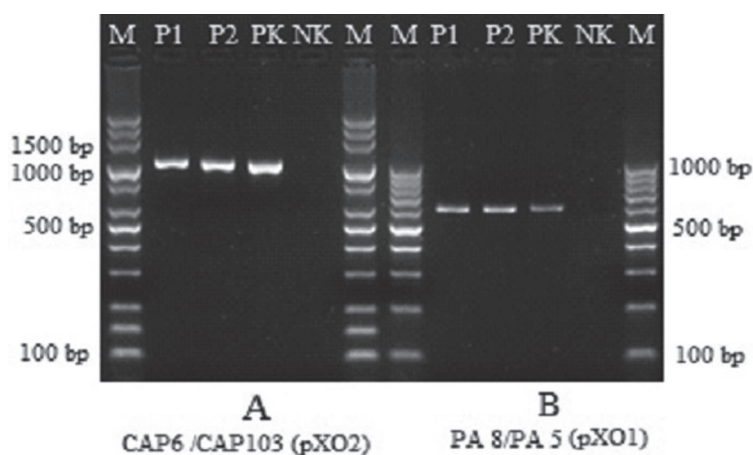


**Fig. 4.** A. Vegetative form of *B. anthracis* in the lung in Puma 1, Gram staining,  $\times 200$ . B. *B. anthracis* in the Lamina propria of intestinal mucosae in Puma 1, Gram staining,  $\times 200$ . C. Appearance of *B. anthracis* in brain vessels in Puma 2, Gram staining,  $\times 100$ . D. Vegetative form of *B. anthracis* in kidney of glomerulus in Puma 2, Gram staining,  $\times 200$ . E. Vegetative form of *B. anthracis* in spleen in Puma 1, Gram staining,  $\times 200$ . F. Appearance of *B. anthracis* in hepatic sinusoids in Puma 1, Gram staining,  $\times 200$ . G. Appearance of *B. anthracis* in the esophagus in Puma 2, Gram staining,  $\times 200$ . H. Appearance of *B. anthracis* between the pancreatic acinus in Puma 1, Gram staining,  $\times 200$ .

Encapsulated bacilli, specific for *B. anthracis* were observed in Giemsa staining slides prepared from the liver and spleen. The result of gamma phage susceptibility testing was positive. The isolates were identified as *B. anthracis* in the result of cultural examinations.

In the molecular analysis, pXO1 and pXO2 plasmids were detected in *B. anthracis* isolates obtained from both pumas (Fig. 5). *B. anthracis* isolates were found to be susceptible to all antibiotics tested.

Although just one anthrax case which has been confirmed by both histopathological and PCR methods in a puma was reported in Turkey [11], this is the first report of anthrax case in pumas diagnosed by cultural and histopathological examination. In addition,



**Fig. 5.** pXO2 and pXO1 specific PCR results. A) M: Marker Mid Range DNA Ladder 100 bp to 3 kb, Jena Bioscience, P1, P2: Puma 1 and Puma 2. *B. anthracis* isolates (1,035 bp), PK: Positive control (*B. anthracis* K125 isolate (pXO2), NK: Negative control. B) M: Marker 100 bp DNA Ladder 100 bp to 1 kb, Jena Bioscience, P1, P2: Puma 1 and Puma 2, *B. anthracis* isolates (596 bp), PK: Positive control (*B. anthracis* K125 isolate (pXO1), NK: Negative control.

recovered isolates were confirmed by molecular analysis (PCR) and tested for their antibacterial susceptibility.

In many natural parks and zoos across the world, anthrax disease has been reported for various carnivore animals such as cheetah, puma, leopard, lion, jaguar, tiger, fox, weasel and wolf [13, 28]. Carcass and rendering products of dead animals due to anthrax plays an important role as a source of infection [5, 6, 27, 28]. Although human anthrax has three forms called as cutaneous, gastrointestinal and inhalational, septicemia occurs frequently in animals [8, 20, 24, 27]. The most prominent findings in our case were limited with intestines and lung, while encapsulated bacteria associated with septicemia were observed in multiple organs. Edema and hemorrhage observed in the ligament in pharynx region indicates that *B. anthracis* was taken through gastrointestinal tract, and it makes us think that the transmission has occurred from infected animal carcasses to the pumas during feeding, and this supports other study results [5, 27, 29].

In animals affected by anthrax in natural parks and zoos, the clinical symptoms such as exhaustion, respiratory distress, and difficulty in eating and drinking were reported in a limited number of studies [13, 15, 27]. In our case, in addition to the similar clinical findings reported for pumas, it was particularly stated in the anamnesis that these animals did not participate in the feeding session before their death. While the necropsy findings of the affected animals were similar to those reported by researchers [12, 13, 15, 17, 19, 27] which were splenomegaly, hepatomegaly, edema and congestion accompanying to necrosis in axillary-inguinal-mesenteric lymph nodes, lungs, mediastinum, meninges-brain and gastrointestinal system, effusion in pleural and pericardial serosa, epicardial hemorrhage, peritesticular and periovarium bleedings, they were different than necrosis on liver and heart, and bleeding focuses similar to petechia in the esophagus.

In the experimental studies for the pathogenesis of anthrax [10, 16, 20, 29], and microscopic findings of the tissues taken from animals died of anthrax in natural parks and zoos [13, 15, 19, 27] showed that there were lymphocytosis in the spleen; edema as well as necrosis in axillary, inguinal and mesenteric lymph nodes and subcapsular sinuses; extension of the alveoles in lungs as a result of fibrin accumulation, macrophage and neutrophil infiltration; demyelination in cerebellum, gliosis, hyperemia and vasculitis in the vessels, neutrophil infiltration and meningitis; neutrophil infiltration with lowered severity compared to other organs in addition to hemorrhagic areas in the interstitial tissue in kidneys; and diffuse congestion in liver, hyperemia and neutrophil infiltration in portal intervals and vena centralis. In our case, lymph nodes were consistent with the findings of lungs, liver and kidneys, and unlike other researchers, there were lesions in the esophagus, but no information was obtained in the literature for these tissues. Especially fibrino-necrotic haemorrhagic esophagitis suggests that the agent was taken orally.

*B. anthracis* have pXO1 and pXO2 plasmids which encode *lef*, *cya*, *pag*, and *cap* virulence genes respectively and these plasmids have been used in the molecular analysis for diagnosis of anthrax by some authors [3, 26]. In our study, the laboratory diagnosis of *B. anthracis* was established by both phenotypic and molecular tests (PCR specific to pXO1 and pXO2 genes), and the results obtained by conventional methods were confirmed by molecular method. *B. anthracis* isolates, in the current study were found to be sensitive to penicillin, amoxicilline clavulanic acid, gentamicine, ampicillin, tetracycline, erythromycin, enrofloxacin, levofloxacin and ciprofloxacin.

*Bacillus anthracis* is generally susceptible to antibacterials and we performed antibacterial susceptibility testing to detect whether resistance occurs in our isolates. Resistance observed in the *B. anthracis* isolates is an important issue for the treatment of human infections due to *B. anthracis*. To our knowledges there is limited publication [1] about the antibacterial resistance of the animal isolates in Turkey. It is pleasing that antibacterial resistance was not detected in our two isolates.

Anthrax is a notifiable disease in Turkey. If an anthrax case occurs, it is alerted authorities of the ministry of food, agriculture,



and livestock. Quarantine procedures are carried out by the authority for 20 days on the herds or zoo in which anthrax has occurred. The site where the animals died is thoroughly scorched with a down-directed flame and disinfected with 10% formalin. Burying the carcass deep (at least 6 feet) and covering with quick lime are valid procedure. Healthy animals are isolated and also monitored carefully for signs of the disease. The healthy domestic animals found in the infection area are annually vaccinated with *B. anthracis* non-encapsulated, Max Sterne 34F2 strain during a five-year period.

Consequently, anthrax should be considered in cases where sudden death occurs in zoo animals.

## REFERENCES

1. Aydın, F., Atabay, H. I., Genç, O., Atahan, H. and Bölük, M. 2000. The epizootiology and epidemiology of anthrax in Kars District, assessment of anthrax cases recorded between 1995 and 2000, some characteristics of *B. anthracis* strains isolated from various sources. *Kafkas Univ. Vet. Fak.* **6**: 55–59.
2. Bell, C. A., Uhl, J. R., Hadfield, T. L., David, J. C., Meyer, R. F., Smith, T. F. and Cockerill, F. R. 3rd. 2002. Detection of *Bacillus anthracis* DNA by LightCycler PCR. *J. Clin. Microbiol.* **40**: 2897–2902. [Medline] [CrossRef]
3. Berg, T., Suddes, H., Morrice, G. and Hornitzky, M. 2006. Comparison of PCR, culture and microscopy of blood smears for the diagnosis of anthrax in sheep and cattle. *Let. Appl. Microbiol.* **43**: 181–186. [Medline] [CrossRef]
4. Beyer, W., Glöckner, P., Otto, J. and Böhm, R. 1995. A nested PCR method for the detection of *Bacillus anthracis* in environmental samples collected from former tannery sites. *Microbiol. Res.* **150**: 179–186. [Medline] [CrossRef]
5. Blackburn, J. K., McNyset, K. M., Curtis, A. and Hugh-Jones, M. E. 2007. Modeling the geographic distribution of *Bacillus anthracis*, the causative agent of anthrax disease, for the contiguous United States using predictive ecological [corrected] niche modeling. *Am. J. Trop. Med. Hyg.* **77**: 1103–1110. [Medline] [CrossRef]
6. Blackburn, J. K. and Goodin, D. G. 2013. Differentiation of springtime vegetation indices associated with summer anthrax epizootics in west Texas, U.S.A., deer. *J. Wildl. Dis.* **49**: 699–703. [Medline] [CrossRef]
7. Blackburn, J. K., Odugbo, M. O., Van Ert, M., O’Shea, B., Mullins, J., Perrenten, V. and Hadfield, T. 2015. *Bacillus anthracis* diversity and geographic potential across Nigeria, Cameroon and Chad: further support of a novel West African lineage. *PLoS Negl. Trop. Dis.* **9**: 1–14.
8. Brachman, P. S. 1991. Anthrax. p. 75. *In: Bacterial Infections of Humans, Epidemiology and Control* (Evans, A. S., Brachman, P. S. eds.), Plenum Medical, New York.
9. Buyuk, F., Sahin, M., Cooper, C., Celebi, O., Saglam, A. G., Baillie, L., Celik, E., Akca, D. and Otlu, S. 2015. The effect of prolonged storage on the virulence of isolates of *Bacillus anthracis* obtained from environmental and animal sources in the Kars Region of Turkey. *FEMS Microbiol. Lett.* **362**: fmv102. [Medline] [CrossRef]
10. Duong, S., Chiaraviglio, L. and Kirby, J. E. 2006. Histopathology in a murine model of anthrax. *Int. J. Exp. Pathol.* **87**: 131–137. [Medline] [CrossRef]
11. Guvenc, T., Onuk, E., Kabak, Y., Yarim, M., Findik, A., Ciftci, A. and Ustunakin, K. 2012. A Case of Anthrax in a Puma (*Puma concolor*). *J. Comp. Pathol.* **146**: 92. [CrossRef]
12. Hugh-Jones, M. E. and de Vos, V. 2002. Anthrax and wildlife. *Rev. - Off. Int. Epizoot.* **21**: 359–383. [Medline] [CrossRef]
13. Ikede, B. O., Falade, S. and Golding, R. R. 1976. Anthrax in captive carnivores in Ibadan, Nigeria. *J. Wildl. Dis.* **12**: 130–132. [Medline] [CrossRef]
14. Kirby, W. M., Yoshihara, G. M., Sundsted, K. S. and Warren, J. H. 1956-1957. Clinical usefulness of a single disc method for antibiotic sensitivity testing. *Antibiot. Annu.* **1956–1957**: 892–897. [Medline]
15. Leendertz, F. H., Yumlu, S., Pauli, G., Boesch, C., Couacy-Hymann, E., Vigilant, L., Junglen, S., Schenk, S. and Ellerbrok, H. 2006. A new *Bacillus anthracis* found in wild chimpanzees and a gorilla from West and Central Africa. *PLoS Pathog.* **2**: e8. [Medline] [CrossRef]
16. Lever, M. S., Stagg, A. J., Nelson, M., Pearce, P., Stevens, D. J., Scott, E. A., Simpson, A. J. and Fulop, M. J. 2008. Experimental respiratory anthrax infection in the common marmoset (*Callithrix jacchus*). *Int. J. Exp. Pathol.* **89**: 171–179. [Medline] [CrossRef]
17. Lindeque, P. M. and Turnbull, P. C. B. 1994. Ecology and epidemiology of anthrax in the Etosha National Park, Namibia. *Onderstepoort J. Vet. Res.* **61**: 71–83. [Medline]
18. Luna, L. G. 1968. *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, McGraw Hill Book Company, New York.
19. Lyon, D. G. 1973. An outbreak of anthrax at the Chester Zoological Gardens. *Vet. Rec.* **92**: 334–337. [Medline] [CrossRef]
20. Lyons, C. R., Lovchik, J., Hutt, J., Lipscomb, M. F., Wang, E., Heninger, S., Berliba, L. and Garrison, K. 2004. Murine model of pulmonary anthrax: kinetics of dissemination, histopathology, and mouse strain susceptibility. *Infect. Immun.* **72**: 4801–4809. [Medline] [CrossRef]
21. Muller, J. D., Wilks, C. R., O’Riley, K. J., Condron, R. J., Bull, R. and Mateczun, A. 2004. Specificity of an immunochromatographic test for anthrax. *Aust. Vet. J.* **82**: 220–222. [Medline] [CrossRef]
22. Özkurt, Z., Parlak, M., Tastan, R., Dinler, U., Saglam, Y. S. and Ozyurek, S. F. 2005. Anthrax in eastern Turkey, 1992-2004. *Emerg. Infect. Dis.* **11**: 1939–1941. [Medline] [CrossRef]
23. Patil, R. R. 2010. Anthrax: public health risk in India and socio-environmental determinants. *Indian J. Community Med.* **35**: 189–190. [Medline] [CrossRef]
24. Quinn, C. P. and Turnbull, P. 1998. Anthrax. p. 799. *In: Topley Wilson’s Microbiology and Microbial Infections, Bacterial Infections* (Collier L. H., Balovas A. and Sussman M. eds.), Edward Arnold, London.
25. Quinn, P. J., Markey, B. K., Leonard, F. C., Hartigan, P., Fanning, S. and FitzPatrick, E. S. 2011. *Veterinary Microbiology and Microbial Disease*, John Wiley & Sons, Oxford.
26. Ramiisse, V., Patra, G., Garrigue, H., Guesdon, J. L. and Mock, M. 1996. Identification and characterization of *Bacillus anthracis* by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA. *FEMS Microbiol. Lett.* **145**: 9–16. [Medline] [CrossRef]
27. Range, R. W. H. 2011. Anthrax in free-ranging wildlife. p. 98. *In: Fowler’s Zoo and Wild Animal Medicine Current Therapy* (Miller, R. E. and Fowler, M. E. eds.), Elsevier Health Sciences, St. Louis.
28. Schmid, G. and Kaufmann, A. 2002. Anthrax in Europe: its epidemiology, clinical characteristics, and role in bioterrorism. *Clin. Microbiol. Infect.* **8**: 479–488. [Medline] [CrossRef]
29. Twenhafel, N. A., Leffel, E. and Pitt, M. L. M. 2007. Pathology of inhalational anthrax infection in the african green monkey. *Vet. Pathol.* **44**: 716–721. [Medline] [CrossRef]