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CASE REPORT

Bacillus anthracis Isolated from a Dog in Turkey

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A B S T R A C T

An Anatolian Akbash shepherd dog died suddenly, without any clinical signs in Konya, Turkey. Performing necropsy, pathological examination, a culture test, laboratory tests and a multiplex-PCR of bacteria isolated from the dog revealed an anthrax and identified the bacteria as *Bacillus anthracis* (*B. anthracis*). The chromosomal gene sequence of bacteria was 99% identical in the GenBank under accession numbers CP002091 (*B. anthracis* str. H9401), CP001598 (*B. anthracis* str. A0248) and CP001215 (*B. anthracis* str. CDC684). Antimicrobial susceptibility test was performed and cefuroxime, cefquinome, sulfamethoxazole-trimethoprim, chloramphenicol, tetracycline and rifampicin resistance were seen in isolate. In this case, how the dog was infected with *B. anthracis* could not be determined.

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INTRODUCTION

Anthrax is an acute infectious bacterial disease affecting many species of domestic animals, wild animals and humans, though it is particularly important among herbivores (Hugh-Jones and de Vos, 2002). The disease is caused by *B. anthracis*, a spore-forming, Gram-positive, nonmotile, aerobic, large (1 to 1.5 µm by 4 to 10 µm) and square-ended rod in the family of Bacillaceae. Animals become infected with the bacteria by ingestion, inhalation spores or possibly by being bitten by flies that have fed on an infected animal or carcass (Anonymous, 2012). *B. anthracis* has two virulence plasmids: pXO2 encoding poly-D-glutamic acid capsule and pXO1 encoding, a tripartite toxin consisting of protective antigen, edema factor and lethal factor. Virulent strains of *B. anthracis* produce toxins and are encapsulated (Rasheed *et al*., 2013). Infected, septicemic animals are likely to have large numbers of bacteria in the blood. Hemorrhage before death and the opening of carcasses cause sporulation and environmental contamination. Anthrax is generally rare in dogs and cats, but it can occur when they ingest contaminated carcasses or animal products (Hugh-Jones and de Vos, 2002).

The aim of the present case was to diagnose the anthrax by pathological and microbiological methods in a dog and identify the bacteria isolated from the animal using classical bacteriological methods, multiplex-PCR and genome sequence. Also, aimed to determine the antimicrobial susceptibility of *B. anthracis* strain.

Case history and findings: A female Anatolian Akbash shepherd dog died suddenly, without any clinical signs, at the Research Farm of the Faculty of Veterinary Medicine in Konya, Turkey. A degree of enlargement of the dog's body as well as some tarry and unclotted bloody fluid were observed on the animal's natural orifices while no rigor mortis was detected. A gastrointestinal hemorrhageas well as a dark red and enlarged spleen which contained an abundant amount of unclotted blood was seen in necropsy (Fig. 1A and B). Furthermore, rod-shaped, capsule-forming bacilli were present in the hematoxylin-eosin-stained sections of spleen (Fig. 2A) and the Giemsa-stained spleen smear (Fig. 2B).

Samples from spleen were inoculated onto Blood Agar Base (Oxoid, CM0854) supplemented with 7% defibrinated sheep blood at 37ºC for 24 hours. The colonies were irregular, greyish-white, non-hemolytic in the blood agar, and 3-4 mm in diameter, with undulate margins. Gram-positive, spore-forming, long filamentous chains of bacilli were seen in Gram staining (Fig. 2C). Following, colony morphology, hemolysis, motility, string of pearls formation grown with penicillin, capsule production in bicarbonate agar, susceptibility to penicillin

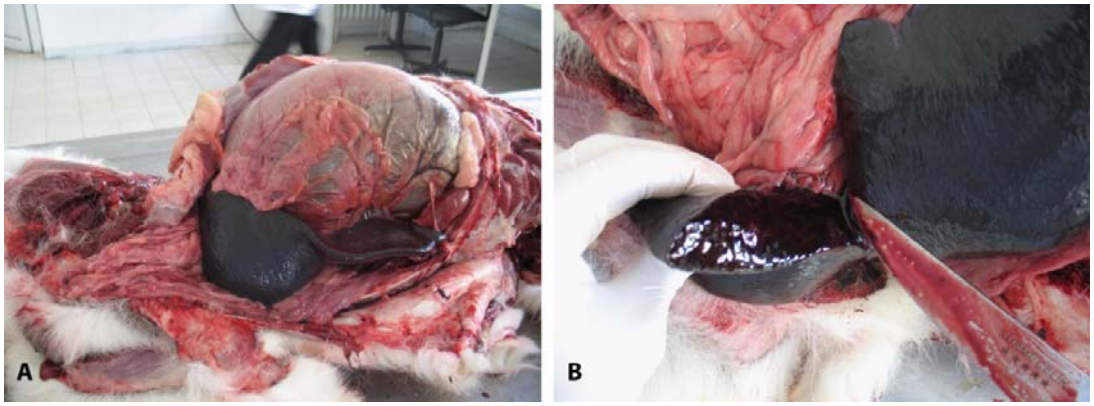

Fig. 1: A) Gastro-intestinal hemorrhages and splenomegaly and B) bulging on the cut surface of the spleen and unclotted blood in dog affected from anthrax.

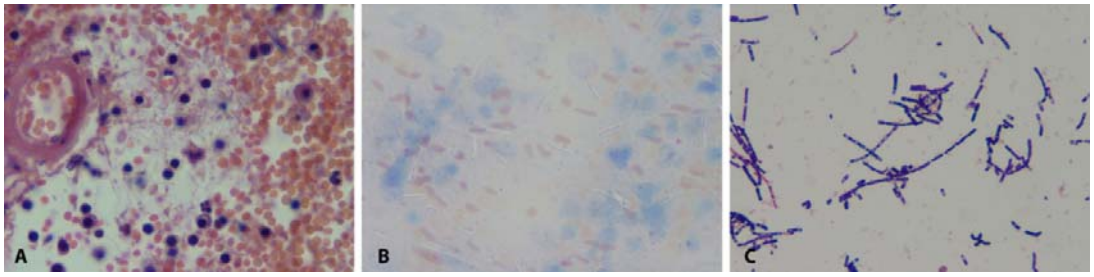

Fig. 2: Rod-shaped bacilli in spleen of anthrax affected dog. A) (H & E); X 400; B) Encapsulated bacilli (Giemsa) and C) Spore-forming bacilli (Gram); X 1000.

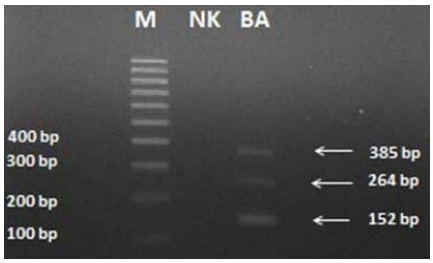

Fig. 3: Agarose gel electrophoresis of multiplex-PCR products (M: 100 bp molecular size marker (Sigma, P1473), NK: Negative control (distilled water), BA: *Bacillus anthracis* isolated from dog.

G and animal test in mice were performed, according to Anonymous (2003). The bacteriological characteristics of the *B. anthracis*-suspicious strain are given in Table1.

According to the pathological and bacteriological findings, this was a case of *B. anthracis* infection, and the bacteria isolated from the dog were identified as *B. anthracis* according to classical bacteriological methods. A multiplex-PCR assay was performed for the detection of pXO1 and pXO2 plasmids and, the chromosomal gene of *B. anthracis* by *lef*, *cap* and *Ba813* primer, respectively (Table 2). The multiplex-PCR was carried out in an Eppendorf thermocycler (Matercycler *gradient*, 5331 000.010, Germany) according to the protocol described by Fasanella *et al*. (2001). The multiplex-PCR analysis of the isolate gave positive bands at the 152 bp sequence by*Ba813* primer, 264 bp sequence by *cap* primer and 385 bp sequence by *lef* primer which specifically matched the chromosomal gene, the pXO2 virulence plasmid and the pXO1 virulence plasmid of *B. anthracis*, respectively (Fig. 3). The isolate was thus confirmed as *B. anthracis*.

For the gene sequences analysis of *B. anthracis* isolate, the 152 bp fragment of the chromosomal gene amplified by the *Ba813* primer and sequences of the amplified PCR products were determined with forward and reverse primers by using an ABI 3130 XL (USA) automated sequencer and a BigDye Terminator v3.1 cycle sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom), according to the kit protocol. Basic Local Alignment Search Tool (BLAST) analysis was used to identify the DNA of the sequence data (www.ncbi.nlm.nih.gov/BLAST). The chromosomal gene sequence was 99% identical in the GenBank under accession numbers CP002091 (*B. anthracis* str. H9401), CP001598 (*B. anthracis* str. A0248) and CP001215 (*B. anthracis* str. CDC 684). According to the GenBank results, these isolates further confirm the bacteria as *B. anthracis*.

Antimicrobial susceptibility testing of *B. anthracis* isolate was performed to the following antimicrobial agents using the disc diffusion method according to Anonymous (2008) recommendations (M31-A3): penicillin-G (10 U, Oxoid), amoxicillin (10 µg, Oxoid), ampicillin (10 µg, Oxoid), ampicillin-sulbactam (20 µg, Oxoid), amoxicillin-clavulanic acid (30 µg, Oxoid), cephalexin (30 µg, Oxoid), ceftiofur (30 µg, Oxoid), cefuroxime (30 µg, Oxoid), cefepime (30 µg, Oxoid), erythromycin (15 µg, Oxoid), azithromycin (15 µg, Oxoid), clarithromycin (15 µg, Oxoid), danofloxacin (5 µg, Bioanalyse, Turkey), enrofloxacin (5 µg, Oxoid), neomycin (30 µg, Oxoid), kanamycin (30 µg, Oxoid), streptomycin (25 µg, Oxoid), sulfamethoxazole-trimethoprim (25 µg, Oxoid), chloramphenicol (10 µg, Oxoid), tetracycline (10 µg, Oxoid), rifampicin (5 µg,

Table 1: Bacteriological characteristic of the *B. anthracis*-suspicious strain

Bacteriological characteristic	Results
Medusa head appearance	+
Haemolysis	-
Motility	+
String of pearls formation	+
Animal test in mice	+
Susceptibility to penicillin G	+
Capsule production on bicarbonate agar	+

Table 2: Multiplex-PCR primer sets used to identify of *B. anthracis* target

Target	Primer	Sequence	Size
Chromo-somal	Ba813R1	TTAATTCACTTGCAACTGATGGG	152 bp
	Ba813R2	CGATAGCTCCTACATTTGGAG	
pXO1	*lef 3*	CTTTTGCATATTATATCGAGC	385 bp
	lef 4	GAATCACGAATATCAATTTGTAGC	
pXO2	*cap 57*	ACTCGTTTTTAATCAGCCCG	264 bp
	cap 58	GGTAACCCTTGTCTTTGAAT	

Oxoid). *B. anthracis* strain was susceptible to penicillin-G, amoxicillin, ampicillin, ampicillin-sulbactam, amoxicillin-clavulanic acid, cephalexin, ceftiofur, erythromycin, azithromycin, clarithromycin, danofloxacin, enrofloxacin, neomycin, kanamycin, and streptomycin. However, resistance to cefuroxime, cefepime, sulfamethoxazole-trimethoprim, chloramphenicol, tetracycline, and rifampicin was determined.

DISCUSSION

Carnivores such as the dog and cat are quite resistant to *B. anthracis* infection (McGee *et al*., 1994; Anonymous, 2012). Anthrax infection is usually seen in wild carnivores situated in zoological gardens, national parks and game ranching areas because of the difficulty of preventing contact with anthrax-infected animals. A large number of anthrax infections have been reported throughout the world in wild carnivores situated inzoological gardens between the years of 1891-1999 (Hugh-Jones and de Vos, 2002). Ikede *et al*. (1976) reported that the *B. anthracis* infection in sudden died of three captive carnivores (two genet and one caracal) in Nigeria. They could not determine the infection source, though they believed that this carnivore contracted the bacteria through contaminated meat. McGee *et al*. (1994) isolated *B. anthracis* from a 6-year-old male Labrador retriever in 1994 in Mississippi. Because of the gastrointestinal hemorrhage found at necropsy, they believed that the dog was infected orally. Similarly, gastrointestinal hemorrhage was seen at necropsy in the present case suggesting that the dog could likely be infected by oral route. However, the consumption of *B. anthracis*-infected meat could not be determined in the history of this case. Also, culture and PCR analyses did not find *B. anthracis* in the pellet foods used to feed the dog.

To be considered virulent, the *B. anthracis* must not only be encapsulated but must also produce a tripartite toxin (Rasheed *et al*., 2013). A multiplex-PCR analysis of *B. anthracis* isolate showed that the isolate has pXO1 and pXO2 plasmids as well as virulence. In the genome sequence of this isolate, the chromosomal gene sequence was found to be identical to the 3 different *B. anthracis* strains (str. H9401, A0248, and CDC684). According to these findings, this strain is not a new strain of *B. anthracis*.

*B. anthracis*is usually susceptible to a broad range of antibiotics (Doganay and Aydin, 1991). This study revealed that, *B. anthracis* strain was susceptible to beta-lactam antibiotics (penicillin, amoxicillin, ampicillin), beta-lactam+beta-lactamase antibiotics (ampicillin-sulb-actam, amoxicillin-clavulanic acid), first generation and third generation cephalosporins (cephalexin, ceftiofur), macrolides (erythromycin, azithromycin, clarithromycin), fluoroquinolones (danofloxacin, enrofloxacin), and aminoglycosides (neomycin, kanamycin, streptomycin). However, this strain was resistant to second generation and fourth generation cephalosporins (cefuroxime, cefepime), and sulfamethoxazole-trimethoprim. The same results were observed by Doganay and Aydin (1991); Habrun *et al*. (2011). Additionally, chloramphenicol, tetracycline and rifampicin resistance were seen in *B. anthracis* isolate, unlike most of the previous studies (Doganay and Aydin, 1991; Habrun *et al*., 2011).

Conclusion: We believe that the pellet food used to feed the dog was contaminated with *B. anthracis*-infected animal products which are used as protein source, and that the dog was infected orally consuming these foods. Moreover, animal products used as a protein source in animal foods may be contaminated with zoonotic microorganisms, which poses an important health risk for animals and humans. To the authors' knowledge this case is the first report of *B. anthracis* infection in dogs in Turkey.

Authors' contributions: OO performed the necropsy and pathological examination. ZS, OE, HHH, AS and GS were carried out microbiological and PCR analyses of *B. anthracis* isolate. Genome sequencing was carried out by EA. ZS analyzed the data. All authors interpreted the data, critically revised the manuscript and approved the final version.

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