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Clinical Presentation and Diagnosis of Anthrax in Iranian Goats

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Running title: Anthrax in Iranian Goats

Abstract

Background: Anthrax is considered one of the most important zoonotic diseases due to its widespread distribution, significant economic losses, and potential risks to public health. The manifestation of anthrax in goats is comparable to its presentation in other small ruminants.

Objectives: To enhance understanding of the clinical signs and post-mortem findings of anthrax and improve its diagnosis in the goat population of Iran, this study examined the occurrence of anthrax in goats in some provinces of Iran between 2016 and 2021.

Methods: Among a population of 3,465 goats, 50 cases of sudden death were documented. Samples were collected from recently deceased animals, including bone marrow and blood from ear veins. These samples were sent to the laboratory for direct examination, culture, and molecular assay (PCR).

Results: Out of the collected samples, 36 cases were attributed to anthrax. Among these deaths, 27 animals were found dead, while nine goats were slaughtered before succumbing. *Bacillus anthracis* was confirmed in all 36 suspicious samples using direct examination and PCR. Additionally, 11 samples (30%) were found to be culture positive for anthrax. It is worth noting that classic signs of anthrax, such as unclotted blood oozing from natural orifices and the absence of rigor mortis, were not observed in 19 dead goats.

Conclusion: This study reveals that there are instances of anthrax in goats where the typical clinical signs, such as blood oozing from natural body orifices, are absent. This finding should be considered during the differential diagnosis, especially when the classic signs are used as a criterion.

Keywords: Anthrax, *Bacillus anthracis*, Goats, PCR, Sudden death.

Introduction

Anthrax is a widely known infectious disease that primarily affects livestock and can also be transmitted to humans. Anthrax is a peracute, acute, or subacute, often fatal disease of animals, including goats. In severe cases, animals can succumb to the disease suddenly, without premonitory clinical signs. One notable sign observed at the time of death is the presence of blood, which may be oozing from the mouth, nostrils, and anus of infected animals. While goats can contract anthrax, reported cases in goats are relatively uncommon, perhaps because they are not naturally ground grazing animals (Jula, 2011; Pooyanmehr & Barzanuni, 2019; Smith & Sherman, 2023). Cattle are infected more than sheep and goats because cattle pull pasture out of the ground with roots, whereas sheep and goats bite plants off the ground level or browse on shrubs (Alam *et al.*, 2022).

Nevertheless, outbreaks have been documented in goats in various regions such as Nigeria, Texas, China, and Ethiopia and can occur wherever anthrax is endemic in other species. Anthrax is prevalent in many tropical and subtropical areas worldwide and continues to pose challenges to livestock production in numerous countries. It is considered enzootic in several Asian and African nations, and sporadic cases have been reported in Australia, certain parts of Europe, and America (Sardar *et al.*, 2023; Smith & Sherman, 2023).

The history of anthrax in animals in Iran dates back to 1905, when a French military veterinarian reported cases of the disease in royal horses (Jula, 2011; Tizard, 2020). In 1945, a devastating outbreak of anthrax resulted in the death of over one million sheep in Iran.

Subsequently, anthrax has been documented repeatedly in cattle and sheep. The signs observed in small ruminants in these reports include nausea, anorexia, bloating, blood seepage from natural body cavities, lack of blood clotting, vomiting, fever progressing to severe abdominal pain, hematemesis, and almost always bloody diarrhea. As a preventive measure, a vaccination campaign targeting sheep and goats was initiated in 1932, which later expanded into a government-funded large-scale vaccination program covering national herds of sheep, goats, and cattle. Despite these extensive vaccination efforts, the World Organization for Animal Health (OIE) continues to receive reports of both small and large anthrax epidemics in Iran, with outbreaks occurring throughout the country on an annual basis (Jabbari & Moazeni Jula, 2007; Jula, 2011; Tadayon *et al.*, 2016). Anthrax remains prevalent in Iran, with a broad geographic distribution, posing a significant threat to both domestic and wild animals, as well as public health concerns (Ghaderi *et al.*, 2020). Several previous studies have focused on the epidemiology of *Bacillus anthracis*, the bacterium responsible for anthrax, in Iran (Bower & Cook, 2022; Esmaeili *et al.*, 2017; Khandia *et al.*, 2021). In 2007, Vahedi successfully isolated *B. anthracis* from soil samples collected across 14 provinces in Iran (Jula, 2011; Vahedi Darmian, 2007). Another study conducted in 2017 by Esmaeili et al. assessed the effectiveness of the anthrax control program implemented in Iran's livestock population between 1990 and 2015. The study revealed a total of 452 anthrax outbreaks in cattle and 761 outbreaks in sheep and goats during that period, resulting in the unfortunate deaths of 666 cattle and 5,775 sheep and goats (Esmaeili *et al.*, 2017). These studies highlight the significant impact of anthrax on Iran's livestock industry and emphasize the ongoing need for disease monitoring, control, and prevention efforts.

Despite the global presence of anthrax and its significant impact on animal and human health, there is a lack of knowledge regarding its clinical signs and accurate diagnosis. This knowledge gap contributes to the continued transmission of *B. anthracis*. It is crucial to address these gaps to prevent outbreaks and improve anthrax surveillance and management (Sardar *et al.*, 2023). Furthermore, there is a scarcity of studies specifically focusing on anthrax in goats, highlighting the need to understand the disease's effects on this species. Anthrax can cause sudden death in goats, and other common diseases in Iranian herds, such as peracute pneumonia or enterotoxemia, can also lead to sudden death. It's worth noting that not all anthrax cases involve blood oozing from the body's natural orifices. Therefore, an accurate diagnosis of anthrax is essential. The current study aims to enhance our understanding of anthrax in Iranian goats, including its clinical manifestations, carcass traits, and diagnostic methods. This study aims to provide valuable insights into the incidence and specific characteristics of anthrax in goats in Iran.

Materials and Methods

Study Areas and Sampling Strategy

From 2016 to 2021, a total of 50 cases of sudden death were reported among a population of 3,465 goats in Isfahan, Kerman, South Khorasan, Gilan, and Yazd provinces. The herds had received vaccinations for foot-and-mouth disease and Enterotoxemia as per the guidelines of the

veterinary organization. There were no changes in the animals' feeding practices, and they had undergone parasitic treatment according to standard protocols.

The sample collection included 50 cases of sudden death in goats, specifically focusing on those that exhibited signs indicative of potential anthrax, such as sudden death with or without blood oozing from natural orifices. These animals exhibited clinical signs such as high fever, respiratory difficulties, cardiovascular disorders, and sepsis. Samples, including bone marrow and ear veins blood, were collected from recently deceased animals and sent to the laboratory for direct examination and culture. The samples were pooled into sterile transport tubes and transported to the laboratory in ice boxes at 4 °C.

Sample Processing

To confirm the presence of *B. anthracis* in suspected animal samples, thin blood smears were prepared and subjected to Giemsa staining and the M'Fadyean's reaction using polychrome methylene blue. These methods were used to identify Gram-positive rod-shaped bacteria with square ends and capsules. The samples were cultured on Polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar, a selective medium designed explicitly for *B. anthracis*. The cultures were incubated aerobically at 37°C for 24 to 48 hours (Markey, 2013). Additionally, samples were cultured on blood agar to test for hemolysis, and motility testing was performed using the hanging drop method. Furthermore, colonies were inoculated in nutrient gelatin and incubated at 25°C for eight days to observe the growth characteristics. The bacteria's culture growth, colony morphology, and microscopic appearance were evaluated. The bacteria from the cultures were subjected to Gram staining for microscopic examination (Green, 2021; Jula, 2011).

PCR assays were performed to molecularly confirm the bacteria isolated from the culture and the number of 25 positive smears observed for *B. anthracis*. The presumptive *B. anthracis* samples were confirmed by PCR assays as described in WHO guidelines (Jiranantasak *et al.*, 2022; Thompson, 2022). The smear from each slide was scraped off (Aminu *et al.*, 2020). Genomic DNA was purified following the conventional Phenol–chloroform method (Kingston *et al.*, 2015). Briefly, an adequate amount of sample was resuspended in 300 µL lysis buffer (50 mM Tris-Cl, 50 mM EDTA, 1 % sodium dodecyl sulfate, 10 mM NaCl, pH 8.0 ± 0.2) and 50 µL of 100 mg/mL lysozyme solution then incubated at room temperature for 45 min. DNA was isolated by phenol–chloroform extraction followed by ethanol precipitation. The DNA pellet was dissolved in 100 µL distilled water and stored at −20 °C until further use.

The PCR reaction was performed in a 0.2 microliter microtube with a final volume of 25 microliters using oligonucleotide primers for *Bac* (*B. anthracis* specific fragment), *pag* (Protective antigen), and *Cap* (Capsule) genes (Table 1) (Jula, 2011). *pag* and *Cap* genes are located on virulence plasmid pX01 and pX02, respectively, and are distinguished from non-virulent *Bacillus* spp., which lack these plasmids (Jula, 2011; Kingston *et al.*, 2015).

The reaction solution contained 4mM MgCl2, 200 µM of each dATP, dCTP, dGTP, and dTTP, 0.4 µM of each primer, 2.5 µM of 10× reaction buffer, and 3µl of DNA template. The cycling parameters were an initial denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 50sec, annealing at 55°C for 50sec, and extension at 72°C for 30sec. The final cycle was followed by an extension at 72°C for 1min. The PCR products were analyzed by electrophoresis on a 1% agarose gel stained with 0.5 µg/ml ethidium bromide.

Results

Cases Observed and Clinical Findings

Among the 50 instances of sudden death recorded in a population of 3,465 goats, 36 deaths were attributed to anthrax (as shown in Table 2). Among these cases, 27 goats were found dead, likely due to the peracute stage of the disease characterized by bacteremia and toxemia. Nine goats were slaughtered before succumbing to the disease. In 8 cases, affected goats exhibited signs such as excessive salivation severe depression with drooping heads, followed by progressive recumbency and imminent death within one to two days. Other clinical signs included bloating, swelling, ataxia, recumbency, and subcutaneous edema. However, Classic signs of anthrax, such as unclotted blood oozing from the nostrils, mouth, and anus, and absence of rigor mortis, were not observed in 19 deceased goats. Only in four cases were unclotted blood observed oozing from natural orifices (Figure 1). In slaughtered animals, blood did not clot, and the spleen was significantly enlarged. Bleeding was observed in the serous layers, indicating septicemia, and there was gelatinous infiltration of the subcutaneous tissue (Figure 2).

Bacteriological & Molecular Confirmation

The presence of *B. anthracis* bacteria was confirmed in 36 samples through Giemsa staining and M'Fadyean's reaction. The results of M'Fadyean's reaction showed large Gram-positive rods with square ends, where blue bacilli were observed in short chains surrounded by a pink capsule. Giemsa staining also confirmed the presence of gram-positive, square-ended bacilli

with evident capsules. Out of these 36 positive samples, 11 (30%) exhibited suspected colonies of *B. anthracis* on selective media (PLET agar). These colonies were characterized as grayish, circular, and dome-shaped. To further confirm the presence of *B. anthracis*, these suspected samples were sub-cultured on blood agar to detect non-hemolytic colonies. Ultimately, 11 samples displayed non-hemolytic colonies with flat, dry, large white to grey appearance and irregular margins resembling Medusa's head (jointed bamboo- rod). After eight days of inoculation in gelatin medium, these 11 isolates exhibited an inverted fir-tree growth pattern. Gram staining of bacteria grown on blood agar revealed Gram-positive rods in long chains. All confirmed isolates tested negative for motility.

In the current study, 11 culture samples and 25 smears were positive for *B. anthracis*, and the amplification of the expected species-specific DNA band (152 bp) by PCR confirmed the accuracy of the conventional bacteriological methods for *B. anthracis* identification in all of the samples. All *B. anthracis* samples, identified as encapsulated using M'Fadyean's reaction, exhibited amplification of a specific fragment of the capsular gene. This fragment measured 209 bp in length. Therefore, the amplification of the 209 bp capsular gene fragment confirms the presence of encapsulation in all the *B. anthracis* isolates examined. The capsular fragment was not amplified in the vaccine *B. anthracis* strain (34F2 Sterne). In all of the *B. anthracis* samples, The expected fragment of the *Pag* target gene was successfully amplified. This fragment had a length of 330 bp. The amplification of the 330 bp fragment in all of the *B. anthracis* isolates indicates the presence of the Pag gene, which is specific to *B. anthracis*.

Discussion

In Iranian small ruminants herds, diseases like anthrax, peracute pneumonia, and enterotoxemia often lead to sudden death in animals. Therefore, it is crucial to comprehend the clinical signs and carcass characteristics to promptly diagnose and manage these diseases (Jula, 2011). In the current study, out of 50 cases of sudden death in goats, 36 cases were attributed to anthrax. However, accurate diagnosis was challenging due to the absence of classic signs. Lack of classic signs has significant implications for the diagnosis of anthrax, as it can potentially result in misdiagnosis and confusion with other diseases. During investigations conducted in the current study, numerous typical farmers did not consider the possibility of anthrax disease. A previous report by Esmaeili et al. in 2010 highlighted that the absence of blood oozing from natural orifices in cases of sudden death caused by anthrax in small ruminants had led to misdiagnosis of the disease (Esmaeili *et al.*, 2010). These observations emphasize the need for vigilance and a comprehensive diagnostic approach considering factors beyond classic signs.

In the present study, the predominant clinical finding in goats was sudden death and the lack of blood clotting, which aligns with findings reported in previous studies. In a study by Mongoh et al. 2005, sudden death was the most common clinical sign observed in 243 anthrax-positive cases across different species, accounting for 38% of cases. Blood seepage from natural orifices was the second most common sign, only observed in 17% of cases (Mongoh *et al.*, 2008). Another study by the same researcher in 2007 reported similar findings, with sudden death being the predominant clinical sign in 116 out of 243 diagnosed anthrax cases. Blood oozing from natural orifices was observed in only 51 animals, and flatulence was observed in 18

animals (Mongoh *et al.*, 2007). Mongoh suggests that while many literature sources mention blood oozing from natural orifices in anthrax carcasses, this sign is not commonly observed (Esmaeili *et al.*, 2010; Mongoh *et al.*, 2008). The findings of the present study align with this claim and support the notion that blood oozing from natural orifices is not a perpetual sign in anthrax-infected carcasses.

A recent study by Omodo in 2023 examined the incidence of anthrax in livestock in Uganda from 2017-2018. The study included animal species, such as cattle, sheep, goats, pigs, and dogs. Among the 339 animals exhibiting clinical signs consistent with anthrax, a mortality rate of 66.4% (225 out of 339) was reported. The clinical signs observed in this study were similar to those documented in the present study. The most common sign was sudden death (49%). Goats accounted for 8% of the affected animals. Interestingly, like in the present study, the classic signs of anthrax, such as blood oozing from orifices and absence of rigor mortis, were not consistently observed in all cases. Omodo's study utilized a similar method to detect *B. anthracis* but differed in the diagnostic approach. Omodo's study relied on smear preparation and M'Fadyean's reaction for confirmation. In contrast, unlike in the present study, bacterial isolation, biochemical tests, and molecular confirmation (PCR) were not performed (Omodo *et al.*, 2023).

In a study conducted by Sardar in the provinces of Khyber Pakhtunkhwa and Balochistan in Pakistan, 19 suspected cases of anthrax were observed over five years. The cases included 11 sheep and goats in Balochistan province and three sheep and five cattle in Khyber Pakhtunkhwa province. The sampling method and bacteriological investigations were similar to the present

study. Out of the suspected cases, 11 were confirmed to be anthrax based on clinical signs, carcass traits, growth characteristics, colony morphology, and positive PCR. Similar to the present study, several infected animals were found dead, and some animals did not exhibit blood oozing from natural orifices. The clinical signs observed in Sardar's study were consistent with those observed in the present study (Sardar *et al.*, 2023). Additionally, a report by Esmaeili et al. in 2010 regarding an anthrax epidemic in the villages of Esfrain city in North Khorasan province noted the absence of blood seepage from the natural orifices of the body in cases of sudden death due to anthrax in light livestock, which further supports the findings of the present study (Esmaeili *et al.*, 2010).

In 2004, a case of suspected anthrax was observed in the Dessie Zuria district of Ethiopia following the sudden death of a goat. The clinical signs observed in this case were similar to those described in the present study. In this particular case, in terms of carcass traits, it was observed that the goat exhibited an absence of rigor mortis, no blood clotting, and blood seepage from the natural orifices of the carcass. The clinical signs observed, along with laboratory confirmation, indicated that *B. anthracis* was the cause of the sudden death. The process of bacteriological examination in this study and the results obtained were consistent with the present study. Further investigations revealed that this disease occurs annually in the region, and the mortality rate among goats due to the disease outbreak was determined to be 32.7% (Shiferaw, 2004).

Despite the regular annual vaccination of livestock carried out by the Iranian Veterinary Organization, cases of disease still occur in the livestock population for several reasons. Firstly,

even with comprehensive vaccination coverage, some animals may not develop adequate immunity due to variations in their immune response and potential human error during vaccination. Secondly, non-cooperation from some livestock farmers with vaccination teams results in the presence of susceptible animals in the region, which can become affected in high-infection situations. The diverse age range of affected animals, including those over one year old, supports this observation. Additionally, animals smuggled and transported without veterinary supervision and lacking a specific anthrax vaccination history may introduce susceptible animals into infected areas. Another factor contributing to the presence of susceptible livestock is the traditional herding system, where bucks are consistently present in the herd, resulting in year-round births and a mix of different-aged kids. According to veterinary organization protocols, kids under two months do not receive the vaccine during vaccination visits. As a result, these kids remain in the herd as susceptible animals that have not received the vaccine and can become infected if they come into contact with the disease-causing agent. In the areas studied in the current research, disease occurrence has been observed in some affected herds, particularly among animals under one year of age, which could be attributed to these factors (Esmaeili *et al.*, 2017; Esmaeili *et al.*, 2010).

Effective prevention and control measures are crucial for reducing the impact of anthrax on public health and the national economy. Controlling anthrax outbreaks in domestic animals relies on prompt identification and treatment of affected animals, enhanced surveillance for additional cases, and the implementation of control measures such as quarantine, prophylaxis, vaccination, and proper disposal of dead animals with decontamination (Olani, Dawo, & Lakew, 2020). It is essential to enhance laboratory surveillance at the regional level to ensure timely detection of

anthrax. Quick and reliable disease detection and confirmation methods are necessary to accurately identify *B. anthracis* and understand its spread and prevalence, particularly in remote areas where anthrax cases are common (Omodo *et al.*, 2023). The present study's findings highlight this disease's significance in the absence of attention, monitoring, and active care. Previous research has given less emphasis to the goat population in anthrax studies. Therefore, there is limited knowledge of this susceptible animal's clinical signs and carcass characteristics in the existing literature.

The present study highlighted the lack of dedicated research explicitly focusing on anthrax in goats. The current study's findings emphasize that the absence of classic signs associated with anthrax does not necessarily exclude the possibility of the disease being present. The study's findings provided valuable insights that can aid in the clinical diagnosis of anthrax in goats during future outbreaks. By documenting and analyzing the clinical manifestations and carcass traits observed in Iranian goats, this study expanded our knowledge of anthrax in this particular species and addressed existing knowledge gaps. The present study reinforces the importance of a comprehensive diagnostic approach that considers typical and atypical signs for accurate diagnosis and effective management of the disease.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Study design, Conceptualization, supervision, and investigation: Hossein Esmaeili; Search the databases, writing, and manuscript preparation: Seyed Mehdi Joghataei. Final approval: All authors.

Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data and materials

The data generated and/or analyzed during the current study are available from the corresponding author on request.

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ارائه بالینی و تشخیص شاربن در بزهای ایرانی

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چکیده:

زمینه مطالعه: بیماری شاربن به دلیل انتشار گسترده، زیان های اقتصادی قابل توجه و خطرات بالقوه برای سلامت عمومی، یکی از مهم ترین بیماری های مشترک بین انسان و دام به شمار می رود. تظاهرات بالینی شاربن در بزها با سایر نشخوارکنندگان کوچک قابل مقایسه است.

هدف: این مطالعه به منظور افزایش درک علائم بالینی و یافته های پس از مرگ و بهبود تشخیص شاربن در جمعیت بزهای ایران، وقوع این بیماری را در برخی از استان های ایران طی سال های 1395 تا 1390 مورد بررسی قرار داد.

روش کار: در جمعیتی شامل 3465 بز، تعداد 50 مورد مرگ ناگهانی ثبت شد. نمونه‌ها از حیوانات تازه تلف شده، از جمله قلم دست و خون از ورید گوش جمع‌آوری شد. این نمونه ها برای بررسی میکروسکپی، کشت و PCR به آزمایشگاه فرستاده شدند.

نتایج: از نمونه های جمع آوری شده، 36 مورد برای شاربن مثبت شدند. 27 حیوان مرده یافت شدند، در حالی که 9 بز قبل از مرگ ذبح شدند. باکتری *باسیلوس آنتراسیس* در تمام 36 نمونه مشکوک با استفاده از بررسی میکروسکپی و PCR تایید شد. علاوه بر این، 11 نمونه (30٪) از نظر کشت مثبت بودند. شایان ذکر است که علائم کلاسیک شاربن، مانند تراوش خون لخته نشده از منافذ طبیعی بدن و عدم جمود نعشی در 19 بز مرده مشاهده نشد.

نتیجه گیری نهایی: این مطالعه نشان می دهد که مواردی از شاربن در بزها وجود دارد که علائم بالینی معمولی مانند خروج خون از منافذ طبیعی بدن وجود ندارد. این یافته باید در هنگام تشخیص افتراقی در نظر گرفته شود، به ویژه هنگامی که علائم کلاسیک به عنوان معیار تشخیص استفاده می شود.

کلید واژه ها: *باسیلوس آنتراسیس*، بز، شاربن، مرگ ناگهانی، PCR.

Figure 1. Unclotted blood was oozing from the natural orifice of a dead goat due to anthrax.

Figure 2. Gelatinous infiltration of subcutaneous, one of the carcass traits in dead goats following anthrax.

Table 1. Oligonucleotide primers were used in the present study for PCR assay.

Target gene	Primer sequence (5′-3′)	Amplified size (bp)	Reference
Bac	AAT GAT AGC TCC TAC ATT TGG AG	330	(Moazeni Jula, Jabbari, & Malek, 2004; Organization & Epizootics, 2008)
Pag	GAG GTA GAA GGA TAT ACG GT	152	(Moazeni Jula, Jabbari, & Malek, 2004; Organization & Epizootics, 2008)
Cap	GTA CCT GGT TAT TTA GCA CTC	209	(Moazeni Jula, Jabbari, & Malek, 2004; Organization & Epizootics, 2008)

Table 2. The number of goats by province and the cases of anthrax observed.

Provinces	Total goats	Affected goats	Casualties	Absence of classic signs
Esfahan	1850	15	15	9
Southern Khorasan	250	3	3	1
Kerman	500	2	2	2
Guilan	65	2	2	2
Yazd	800	14	14	5
Total	**3465**	**36**	**36**	**19**