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Mari Friman and Heli Simojoki did the clinical work, Leila Kakko is an expert in cleaning and wrote the disinfection part, Camelia Constantin participiated in the writing and contributed with the picture of the reference B. antracis strain Maria A. Andersson made the first suggestion of antrax, sent the strain for official identification and collected the preliminary data. Szabolcs Nagy wrote the final version of the paper, submitted the paper and is the corresponding author. Heidi Salonen participiated in editing the manuscript and administrative tasks. Magnus Andersson was responsible for the clinical investigation of he bull and aiding in making the diagnosis of antrax.

An atypical *Bacillus anthracis* infection in a bull - a potential occupational health hazard

Running title: Scrotal anthrax in a bull

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Contents

Bacillus anthracis infecting cattle is usually identified based on the typical symptom: sudden death. *B. anthracis* causing atypical symptoms may remain undiagnosed and represent a potential occupational health hazard for i.e. veterinarians and producers, butchers and tanners. In the year 2004 one case of sudden death in a dairy farm in southern Finland was diagnosed as bovine anthrax. Four years later 2008, an atypical case of anthrax was diagnosed in the same holding. The bull was taken to the Production Animal Hospital of the Faculty of Veterinary Medicine, University of Helsinki because of fever, loss of appetite and a symmetrically swollen scrotal sac. Penicillin treatment cured the fever but not the swollen scrotum. Before the intended therapeutic castration, a punctuate consisting of 10 ml fluid collected into a syringe from the scrotal sac was cultivated on blood agar at 37 ^{o}C. After 24 h an almost pure culture of a completely non-hemolytic *Bacillus cereus* like bacteria was obtained. The strain was identified as *B. anthracis* using Ba specific primers by the Finnish Food Safety Authority (RUOKAVIRASTO). After the diagnosis the bull was euthanized and destroyed, the personnel were treated with prophylactic antibiotics and the clinic was disinfected. In this particular case, treatment with water, Virkon S and lime seemed to be effective to eliminate endospores and vegetative cells since no relapses of anthrax have occurred in 10 years. This case is the last reported anthrax case in Finland.

Key words

Bacillus anthracis, Bos taurus, occupational hazard

Introduction

Anthrax is a deadly zoonotic infection caused by the facultative anaerobic endospore forming bacterium *Bacillus anthracis (B. anthracis;* Watson and Keir 1994, Erikson and Kornacki 2003*).* If spread, anthrax may cause major financial losses and represent a treat for human health (Wilson et al 2016). Herbivores such as cattle are particularly susceptible to anthrax infections, humans are less susceptible, and pigs, carnivores and birds are even more resistant (Lewerin et al 2010). The typical symptom recorded in anthrax infected cattle is sudden death within 1-2 hours of illness, possibly without clinical symptoms. The natural reservoir of anthrax is considered to be the soil contaminated with endospores originating from carcasses infected by *B. anthracis*. Herbivores get the infection when grazing but do not serve as a reservoir because they rapidly die (Lewerin et al 2010, Schmid and Kaufmann 2002).

B. anthracis is closely related to, and is in many respects indistinguishable from the *Bacillus cereus (B. cereus)* group bacteria, which includes food poisoning strains and opportunistic pathogens (Watson and Keir 1994, Erikson and Kornacki 2003, Apetroaie et al 2005). *B. anthracis* and *B. cereus* exist in vegetative form as rod shaped bacteria and as endospores.

Sporulation of *B. anthracis* is regulated by CO_2 levels; low levels of CO_2 in the open air induce sporulation, whereas the high levels of CO_2 in infected tissues inhibit sporulation (Lewerin et al 2010). The vegetative cells are poor competitors in the environment and are rapidly outcompeted and may loose virulence (Green et al 1985, Braun et al 2015). Endospores are resistant to desiccation, high temperature (up to 100 ^{o}C), antimicrobial chemicals, extreme pH, irradiation and survive for years, possibly decades in hostile

environments and may accumulate in desiccated indoor dust. Use of detergents increased respiratory virulence of anthrax spores. This makes cleaning and disinfection of contaminated buildings difficult (Schmid and Kaufmann 2002).

There are three official forms of anthrax infection, pulmonary, gastrointestinal and cutaneous (Watson and Keir 1994, Erikson and Kornacki 2003). In humans, inhalation anthrax may represent a risk in contaminated urban buildings such as industrial environments and laboratories. Cutaneous anthrax is connected to contact with infected animals and gastrointestinal anthrax is the consequence of consumption of contaminated meat (Watson and Keir 1994, Schmid and Kaufmann 2002). Symptoms of typical anthrax infections in cattle are well recognized, rapid diagnosis and prophylactic vaccination have prevented large bovine epidemics in northern Europe since the 1950s. However, pulmonary anthrax, the most lethal form of infection, transmitted by inhalation of endospores may still represent an indoor air related health hazard, especially if the transmission source is not recognized (Lewerin et al 2010, Schmid and Kaufmann 2002, Watson and Keir 1994). Pathologists and veterinarians could be exposed to the vegetative bacteria during necropsies of anthrax victims and during analyzing anthrax infected specimen and media. Laboratory acquired infections are usually cutaneous, but also inhalation anthrax has been reported (Schmid and Kaufmann 2002). Atypical bovine anthrax, if not diagnosed rapidly may still represent an occupational health hazard.

Bovine anthrax was common in Finland in the 1950s, but declined after the 1960s (Kivelä 1993). In 2004 one case of sudden death in cattle was diagnosed as anthrax in southern Finland. Four years later an atypical case of anthrax was diagnosed in the same holding (Andersson et al 2011). This study is a case report describing the atypical anthrax infection treated at the Production Animal Hospital of the Faculty of Veterinary Medicine, University

of Helsinki. Cleaning, decontamination and disinfection of facilities contaminated by the anthrax infected bull, and post prophylactic treatment of the personnel are also described.

Materials and Methods

Case description

In September 2008 a young (age 14 months, weight 310 kg) *Bos taurus* bull of Ayrshire breed was brought to the Production Animal Hospital of the Faculty of Veterinary Medicine, University of Helsinki as a patient used for education of veterinary students. On the farm the bull had suffered from high fever, anorexia and symmetrically swollen scrotal sac. The bull had been examined by a veterinarian and was suspected to suffer from orchitis.

The puncture sample from the scrotal sac was taken for bacteriological examination. The liquid was found to be yellow and transparent. The sample was cultured in the laboratory of the Department of Production Animal Medicine (Saari, Finland); 0.01 mL of liquid was streaked onto blood agar and incubated at 37 °C. The suspected orchitis was medicated with procaine penicillin (19 000 IU/kg SID, Penovet®, Vetcare Oy, Salo, Finland) five days and non-steroidal anti-inflammatory drugs, meloxicam (0,2 mg/kg, Metacam® Vetcare Oy, Salo, Finland) once. All other symptoms except the swelling of scrotum disappeared during the antimicrobial treatment (Fig. 1A). Fever appeared again after ending of antimicrobial therapy. The bull was brought to the hospital for therapeutic castration six days after the start of the antimicrobial treatment (procaine penicillin 19 000 IU/kg SID, Penovet®, Vetcare Oy, Salo, Finland). The antimicrobial treatment was continued for four days on the hospital. Before the intended castration, the bacterial culture from the scrotal punctuates was analyzed for aerobic bacteria and was suspected to be *B. anthracis*. Based on these bacteriological findings in combination with the apparent sensitivity to penicillin of the infection, an atypical local *B. anthracis* infection in the scrotum was suspected. All further laboratory investigations and

surgery was cancelled to avoid further contamination of the facilities. The strain was transported to the Finnish Food Safety Authority (RUOKAVIRASTO, Helsinki) and identified as *B. anthracis* using Ba specific primers. The strain was named BA2968, a new sublineage of *B. anthracis*, which is thought to be autonochthous for Finland; its identification and characterization are described in Andersson et al (2011) and Lieneman et al (2018). When the case was officially declared as anthrax, the bull was euthanized (Fig. 1B), and the carcass was destroyed by burning in a hazardous waste facility. The clinic was disinfected and the exposed personnel treated with prophylactic antibiotics according to Finnish legislation.All people potentially exposed to bacteria and/or spores were given post prophylactic treatment with doxymycine according to the guidelines for occupational health care.

Disinfection of the bull's pen

The pen was first cleaned with water with a hose and detergent. Afterwards the surfaces were let to dry until next day. The dry surfaces were covered with a lime pap of calcium hydroxide $Ca(OH)_2$ and Virkon S (1%) alternately, to create rapid changes of pH on the surfaces. The procedures were repeated 4 times. Finally, the walls of the pen were painted with lime, which was left on the surfaces. The manure collected during hospitalization was treated with lime.

Results and Discussion

This study describes an atypical anthrax infection in a bull and illustrates the challenges of the diagnostic process. This is to our knowledge the first described case of a bovine anthrax infection localized in the scrotum. There is only one other documented scrotal anthrax infection reported, a human case from Zimbabwe (Latif and Nathoo 1983).

The puncture sample from the scrotal sac aerobically cultivated on blood agar at 37° C revealed an almost pure culture of a *B. cereus*-like colonies. A pure culture was prepared from one of the colonies on the primary isolation plate, and the strain was compared to selected *B. cereus* and *B. anthracis* strains (Fig. 2.). The strain was found to be sensitive to penicillin and exhibited white non-hemolytic colonies with curly edges. The colonies isolated from the scrotal puncture were similar to the non-hemolytic penicillin sensitive *B. anthracis* reference strain NC0823402 (Apetroaie et al 2005; obtained from National Collection of Type Cultures, Public Health Laboratory Service, London UK; Fig. 2A), but differed from the four penicillin resistant *B. cereus* reference strains (shown in Fig. 2B and C). Microscopic examination of the strain revealed endospore forming rod shaped bacteria with oval spores in non-swollen sporangia, similar to the *B. cereus* type strain ATCC 14579 and the emetic strain NC 7401 (data not shown). Before the bacteriological finding in the puncture sample (from the scrotal sac) no indication of anthrax existed and the bull was transported to the university veterinary clinic for castration.

The most common symptoms of bovine anthrax infections such as sudden death, high fever, blood around the body orifices are characteristic and easy to diagnose. This report shows that anthrax may cause quite different symptoms in cattle as unexpected local infection with low morbidity.

It is possible that cases of bovine scrotal anthrax infections have been missed. However, even atypical and mild anthrax infections are important to diagnose to avoid environmental, especially indoor contamination with endospore and to prevent occupational health risks.

The non-hemolytic *B. cereus* like bacteria found in the puncture sample in combination with penicillin sensitivity of the infection (the decline of fever) rose the suspicion of anthrax. The emetic toxin, cereulide, producing food poisoning causing *Bacillus cereus* strains may be completely non-hemolytic as shown in Fig. 2, indicating that not all non-hemolytic *B. cereus*

like isolates are *B. anthracis*. However, all cereulide producing non-hemolytic *B. cereus* strains tested so far were penicillin resistant (Apetroaie et al 2005).

Anthrax was a common disease in Finland until 1960 (Kivelä 1993). Reported anthrax cases were 111 in the 1950s, and 27 in 1960s. In the 1970s and the 1980s one case per decennium were reported. In the 2000s one case was reported in 2004 and another in 2008 from the same farm (Andersson et al 2011). *B. anthracis* vegetative cells have been shown to survive poorly outside of a viable host. The vegetative cells are suspected to be highly susceptible to antagonism from other environmental bacteria, and may rapidly lose virulence (Dragon and Rennie 1995, Green et al 1985, Braun et al 2015).

It is tempting to speculate a connection between the two outbreaks of anthrax on the same farm in 2004 and in 2008, however, isolates are not available from the 2004 outbreak for genomic comparison, unfortunately. The suspected, but not confirmed, source of infections in 2004 and 2008 were contaminated pasture or silage. According to the farm owners memory and hearsay, the silage was collected from fields where unexpectedly dead cows where buried centuries ago. No relapses of anthrax, bovine or human, have been recorded in the farm or in Finland since 2008. A hypothetical explanation would be that the scrotal infection was caused by *B. anthracis* endospores from the deadly case (2004) in the farm, which had survived in the environment. These bacteria had partly lost their virulence and were unable to cause typical clinical anthrax cases. The scrotum is communicating with the abdominal cavity. It is possible that fodder contaminated with *B. anthracis* caused an undiagnosed abdominal infection, explaining the loss of appetite. This infection responded to penicillin treatment, but some bacteria may have reached the scrotum causing a local infection. Other possibility is that the bacteria entered directly the scrotum through a local wound or skin lesion which was in contact with the contaminated environment. However, the physical

inspection of the bull did not reveal any kind of scar, skin lesion, therefore in our opinion this latter possibility is unlikely.

According to Finnish legislation (MMMa24/2013 and MMMa 843/2013) any possible environmental contamination with anthrax spores has to be eliminated to prevent further outbreaks; buildings that harbored sick animals have to be cleaned and disinfected. In the case described here anthrax was suspected in time to prevent operation or necropsy of the anthrax infected bull. After anthrax was suspected no further investigation were performed in the clinic to avoid indoor contamination with blood or secretions.

B. anthracis endospores are resistant to disinfectants and cleaners. Quaternary ammonium compounds, acid, alkali, and ionic and nonionic detergents do not completely inactivate *B. anthracis* endospores (Brachman et al 1960; Erikson and Kornacki 2003). A detergent has even been shown to increase virulence of *B. anthracis* endospores (Watson and Keir 1994). Toxic disinfectants as sodium hypochlorite, formaldehyde, and phenols which are highly effective against *Bacillus s*pores are not well suited for decontamination of the indoor environment (Hamouda et al 1999). There a two reports on products reducing viability of *B. anthracis* endospores that are not harmful to human health and the environment: per acetic acid (Candielere et al 2016) and a surfactant nano emulsion BCTP (Hamouda et al 1999).

New disinfecting methods using less chemicals have been introduced recently and there are two main methods: using high-intensity 405 nm light might be one solution to combat *B. anthracis* spore exposure (Helgason et al 2000, Nicholson et al 2003, Sagripanti et al 2007, St Denis et al 2013). Hydrogen peroxide used as "Dry gas" vaporized hydrogen peroxide (VHP) system has been shown to be effective against for example *Mycobacterium tuberculosis, Mycoplasma, Acinetobacter, Clostridium difficile –B. anthracis,* as well as for viruses and prions when hydrogen peroxide is used as a 30% solution. Using it as a dry gas form does not

harm surfaces as much as using hydrogen peroxide as a liquid (Boyce 2016, Galvin et al 2012, Fichet et al 2004, Heckert et al1997, Rogers et al 2005, Pottage et al 2010).

B. anthracis may cause atypical symptoms which may remain undiagnosed and represent a potential occupational health hazard for i.e. veterinarians and producers, butchers and tanners. Therefore, early detection of *B. anthracis* and appropriate risk management measures are essential. In this particular case, washing with water and treatment with Virkon S and lime seemed to be effective to eliminate endospores and vegetative cells since no relapses of anthrax have occurred in 10 years.

Ethical statement

This was a clinical case, no animal experiments were performed in this study, and, therefore, approval from an ethics committee was not required. The decision to cull the bull was done according to the demands of the Finnish authorities.

Conflict of interest statement

The authors declare no conflict of interest.

Data Availability Statement

No data are available.

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FIGURE LEGENDS

Fig. 1. A young Ayrshire bull suffering from atypical anthrax. Panel A shows the bull after penicillin treatment which cured the fever and loss of appetite but not the swollen scrotal sac (arrows). Panel B shows the bull euthanized after anthrax diagnosis.

Fig. 2. Diversity of hemolytic pattern and microscopic views of *B. anthracis* and *B. cereus* strains. Hemolysis was recorded on blood agar incubated for 24 h at 37 °C (panels A, B, C and D). Reference strains are shown in panels A, B and C: the nonvirulent non-hemolytic *B. anthracis* strain NC0823402 (A), the hemolytic *B. cereus* type strain ATCC 14579 (B) and three food poisoning strains (C), F-528, (Hemolytic) NC7401(weak hemolysis) and RIVM67000 (non-hemolytic (C). Panel D shows the completely non-hemolytic white colonies with curled edges of the strain isolated from the scrotal punctuate.

Figure 1.

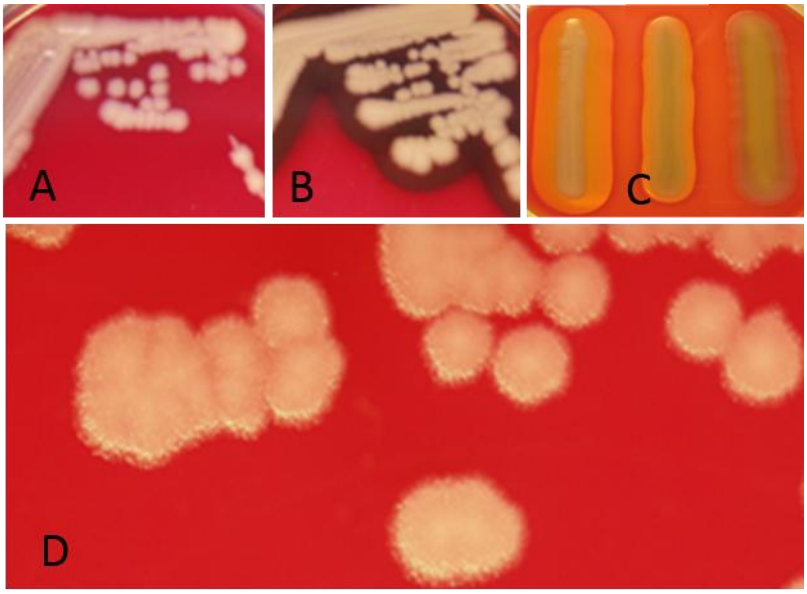

Figure 2.