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Case Report

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An atypical localization of *Bacillus anthracis* in a cow's milk and udder

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Abstract: A 6-year-old Simental-Swiss Brown cross-bred cow, which aborted in the first trimester of the gestation and involuntarily presented anthrax bacilli around the udder, was subjected to this study. The cow was vaccinated with an attenuated anthrax vaccine (*Bacillus anthracis* 34 F2) 6 months ago and had normal physical examination findings. Samples taken 10 days after the abortion were representative of all possible spaces that could carry the bacteria such as milk, blood, environment, and swabs from rectum, udder and tits of an infected cow. Microbiological examination was carried out by staining the smears with Azure and in vitro culture of the samples on some enriched and selective media such as 7% sheep blood agar (SBA), *Brain Heart Infusion* (BHI) Broth media with 7% sheep blood, and polymyxin-lysozyme-ethylenediaminetetraacetic acid-thallous acetate (PLET) agar. On a molecular basis, the virulence plasmid-targeted PCR analysis of the isolates was performed. In addition, MLVA-8 and MLVA-25 typing of *B. anthracis* isolates were conducted. Upon the isolation of *B. anthracis* with a concentration of 6×10^2 cfu/mL in milk sample and different subtypes obtained from the isolates by MLVA-8 and MLVA-25 typing, the present study enables us to instantiate the presence and unusual spread of bacteria around the udder of cows.

Key words: *Bacillus anthracis*, milk, udder, culture, PCR, MLVA

1. Introduction

In order for a pathogen to successfully cause a disease, it must spread between different hosts. In the case of *Bacillus anthracis*, disease progression is partly controlled by the presence of 2 virulence plasmids, while long-term survival is ensured through the ability to form spores. While the spore form of the organism is more resistant to chemical biocides and conventional antibiotics and unable to replicate, the more susceptible vegetative form can actively grow in a variety of media including milk [1,2].

Milk is an externally secreted fluid designed specifically to nourish the young. Microorganisms are rarely shed into both human and animal milk and this can be an important source of infections such as HIV, cytomegalovirus and human T-cell lymphotropic virus 1 (HTLV- 1) of human [3] and *Brucella* spp., *Campylobacter* spp., and *Mycobacterium bovis* in animals [4,5]. However, *B. anthracis*-contaminated milk is rarely encountered and infrequently reported [6–8]. However, in general it is difficult to isolate the bacteria from milk once clinical symptoms appear due to high fever and reduction or cessation of milk production [9,10].

This case report investigates the history of a dairy cow that involuntarily secreted an unusual *B. anthracis* cell via its milk and presented it around the udder.

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2. Case history

A 6-year-old Simmental-Brown Swiss cross-bred cow was reported to have aborted in her first-trimester period and 10 days before microbiological sampling. Unfortunately, the aborted fetus and associated materials were destroyed before reporting. The cow was bred by the same owner since birth in a single barn with the remaining 20 cattle. The cow was farmed extensively on open pasture as much as possible during the autumn season and was barn-fed on hay and straw for the remainder of the year. A spore-based vaccine of *Bacillus anthracis* 34 F2 strain (Vetal, Turkey) was administered to all the cattle in the herd 6 months ago (October 2015). It was initially claimed by the farmer that the abortion was a result of common infectious agents and that milk contamination was coincidental.

The cow was resampled after 3 days on the unexpected result of the first sampling. During sampling, the cow had normal physical findings (body score 2, body temperature 38 °C, normal pulse rate and appetite, no change in milk, and no signs of inflammation in the udder). The first batch sample was a milk cocktail taken from all the teats by the farmer. The second batch samples were obtained by the expert staff from the Veterinary Faculty at Kafkas University (Kars, Turkey). Swab samples were obtained

from the dirty udder (lobe and teats). The udders were cleaned with soap, water, and subsequently alcohol and additional milk samples were aseptically obtained from individual teats. A rectal swab was also obtained with additional samples from the local environment such as barn floor and feeder. As the cow was still feeding a 7-month-old calf, blood samples were obtained from both animals.

The first batch milk sample was subjected to cultural processing in terms of routine analysis of eugenic agents. Hence, the milk cocktail was cultured directly onto 7% sheep blood agar (SBA) and incubated aerobic and microaerobic conditions at 37 °C for 24–48 h.

Bacteriological analysis of the second batch samples was performed more specifically (agent-specific) as a result of the unexpected culture of the first attempt. In brief, smear slides were prepared from the samples and stained with Azure staining method as previously described [11]. The swabs were suspended in sterile phosphate-buffered saline (PBS) and 150 µL of the suspension was plated directly onto 7% SBA and polymyxin-lysozyme-ethylenediaminetetraacetic acid-thallous acetate (PLET) agar plates. The plates were incubated aerobically at 37 °C for 24–48 h. All the samples were also enriched in sheep blood-enriched Brain Heart Infusion (BHI) broth and plated on SBA and PLET agar plates.

Suspected *B. anthracis* colonies were subcultured on SBA plates and confirmed using phenotypic methods as previously described [12]. Phenotypically confirmed isolates were subjected to PCR confirmation of the virulence plasmids as previously reported [12].

Confirmed isolates of *B. anthracis* were subjected to MLVA-8 and MLVA-25 typing at the Public Health Institution, Ankara, Turkey. The data were analyzed as previously described [13,14]. Dendrograms were produced using the Genemapper software (BioNumerics version 6.1, Applied Maths, Sint-Martens-Latem, Belgium).

3. Results and discussion

Microorganisms have a variety of mechanisms for transmission between hosts. Nearly all microbes are shed from body surfaces, survive transmission in the environment, enter a susceptible person or animal, and develop and/or multiply in the newly infected host [15]. The secretions of infected animals such as blood, saliva, sweat, and milk are the main routes of microbial exit to the outside world. While milk appears to be a suitable medium for the successful transfer of various microorganisms, the fate and impact of *B. anthracis*-contaminated milk is relatively unknown [6–8] and difficult to examine even in artificially contaminated milk [9]. This is assumed to be because of high fever, low milk secretion, or stopping lactation with the onset of illness or lack of lactation due to sudden death [9,10]. In this study, *B. anthracis* at a concentration of approximately 6×10^2 cfu/mL was isolated and phenotypically confirmed from the first batch milk sample by susceptibility to penicillin and Gamma phage and production of a visible capsule (Figure 1, Table 1). However, since the infectious dose needed to cause anthrax via ingestion is believed to be equal or greater than 10^8 spores [16], a huge volume of unpasteurized milk would need to be ingested to produce a threat to the health of humans or animals. However, the contamination of the surrounding environment or the infection of individuals who come into close contact with animals may be a viable risk [17].

Although milk is considered as a complex fluid with a high protein content and is exposed to microbiological growth, it is known that bovine milk inhibits the metabolic activity of some agents. Inhibition is dependent on both xanthine oxidase (XO) activity and the presence of nitrite, implying that XO-generated nitric oxide functions as an antibacterial agent [18]. Furthermore, milk contains several antimicrobial activities, including lactoferrin, lactoperoxidase, lysozyme, and possible N-acetyl-B-D-glucosaminidase, which may be involved in protecting against mastitis, bacterial growth postharvest, and protecting the consumer of the milk product. In this study, further sampling revealed no culturable *B. anthracis* in all the samples (Table 1). The reasons for the failure

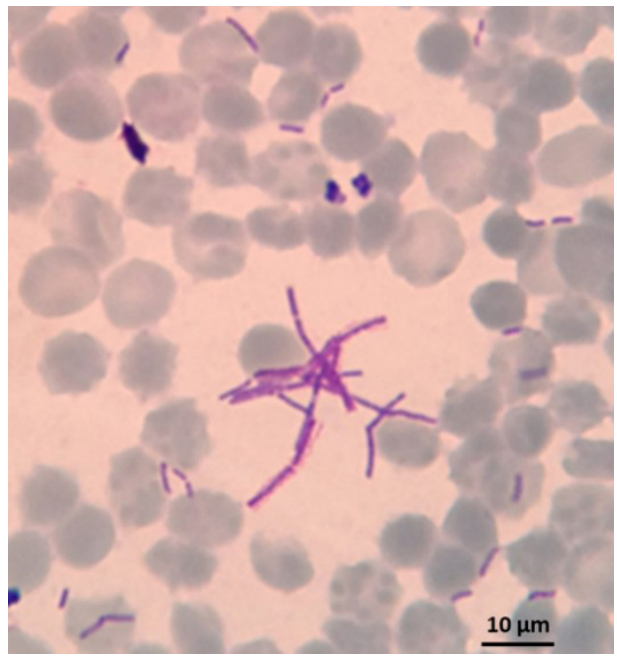

Figure 1. Azure staining images of the first sampled milk-cocktail before enrichment. *B. anthracis* was found surrounded by a visible pink capsule.

Table 1. Properties of the samples which were microbiologically examined.

Sample number	Sample origin	Sampling area	Total count (cfu/mL)	Spore count (cfu/mL)	Smear		Culture	Characterization tests			PCRs		Genotyping results		
					Direct	Enriched	SBA/PLET	BC agar	γ	Pen	PA	Cap 6/103	MLVA cluster	MLVA- 8 genotype	MLVA- 25 genotype
1	Milk	Cocktail	6×10^2	-	+	ND	+*	+	+	+	-	-	A3.a	33, 40, 43, 45	1–4
2	Milk	Left front teat	ND	ND	?	+	-	ND	ND	ND	-	-	-	-	-
3	Milk	Left back teat	ND	ND	+	+	-	ND	ND	ND	-	-	-	-	-
4	Milk	Right front teat	ND	ND	?	-	-	ND	ND	ND	-	-	-	-	-
5	Milk	Right back teat	ND	ND	-	-	-	ND	ND	ND	-	-	-	-	-
6	Feces	Rectal swab	ND	ND	?	?	-	ND	ND	ND	-	-	-	-	-
7	Floor	Barn floor	ND	ND	?	?	-	ND	ND	ND	-	-	-	-	-
8	Provender	Fodder	ND	ND	-	-	-	ND	ND	ND	-	-	-	-	-
9	Eluate	Dirty udder lobe	ND	ND	+	+	-	ND	ND	ND	-	-	-	-	-
10	Eluate	Dirty inter-teats	ND	ND	?	-	-	ND	ND	ND	-	-	-	-	-
11	Blood	Whole blood of cow	ND	ND	-	-	-	ND	ND	ND	-	-	-	-	-
12	Blood	Whole blood of calf	ND	ND	-	-	-	ND	ND	ND	-	-	-	-	-

ND: Not determined; ?: Suspicious; SBA: Sheep blood agar; PLET: polymyxin-lysozyme-ethylenediaminetetraacetic acid-thallous acetate agar
BC Agar: Bicarbonate agar; γ: Gamma phage; Pen: Penicillin; *: This positivity belongs to SBA agar plates.

of *B. anthracis* isolation from the samples of the second period are considered to be the adverse effects of the above factors. Though some smears are positive for *B. anthracis* morphology, we could not successfully isolate the agents. Moreover, all the isolates were vegetative cell-substituted for spores. These findings are in parallel with Howie [19], who reported that *B. anthracis* vegetative cells failed to form spores and died between 4 and 6 days in sterile milk. Similarly, Bowen and Turnbull [1] stated that the vegetative forms of bacteria die quite rapidly in milk. Contrary to this, Baxter [20] reported that *B. anthracis* may multiply and sporulate in milk, but the growth conditions were unspecified. In this study, the cultivation of the milk samples in a short period just after the sampling is considered to restrict the multiplication or sporulation of the agent. Besides that, the PCR reactions applied to direct milk DNA extracts of this study failed expectedly to amplify specific regions of *B. anthracis.* This may be due to the interference from various food components [21] and the low amount and heterogeneous distribution of the pathogen [22]. The assays of relatively high detection limits (real-time PCR, lateral flow assay) may generally fail to detect milk contaminated with a low level of bacteria. Thus, it was suggested that milk cannot be directly used as a medium for PCR reaction without prior DNA extraction to eliminate calcium and other potential reaction inhibitors [23,24].

On the other hand, all the first batch milk isolates were found not to have the expected-size PCR products specific to both PA and the cap region, which are encoded by the pX01 and pX02 plasmids, respectively. However, the MLVA typing yielded that all the isolates possessed the pX01 and pX02 plasmid markers. It may be due to an inadequacy of primers used in in-house PCR for specific target amplification in both plasmids. The isolates were already found as encapsulated in bicarbonate medium when cultured in a CO_2 incubator (Table 1). Several genes are carried on the *capBCADE* gene cluster on the pXO2 plasmid, which is responsible for the expression of the poly-D glutamic acid capsule [25,26]. Further studies are required to determine the reasons for this discrepancy between PCR and MLVA typing. Moreover, it raises the possibility that the primers used in this study may not be as qualified as first thought.

Bacillus anthracis is one of the highly monomorphic species among bacteria. Thus, newer molecular methods such as MLVA typing may be one of the reasonable methods to study the molecular epidemiology of this pathogen. The first report on global MLVA typing of *B. anthracis* strains was conducted by Keim et al. [13]. They described 2 lineages (A and B) containing 6 branches (A1, A2, A3, A4, B1, and B2) and 89 genotypes. A great majority of the *B. anthracis* isolates from Turkey were determined to be genetically identical to the A3.a cluster [27]. Similarly, Durmaz et al. [14] studied 251 *B. anthracis* strains from Turkey and found a total of 12 distinct A.3.a subtypes. More recently, a canonical SNP analysis and MLVA typing that was carried out on *B. anthracis* strains found that Turkish strains belonged to the *B. anthracis* group A.Br.003 and the Australian 94 lineages [28]. In this study, all the 6 isolates belonged to different subtypes of the A3.a major cluster (Figure 2A) by MLVA-8 and 4 different subtypes (Type 1 to 4) by MLVA-25 typing (Figure 2B). Furthermore, all the isolates possessed the pX01 and pX02 plasmid markers with the MLVA typing. The genotypes that emerged coincide with the given *B. anthracis* genotype scale in Turkey [13,14,27,28]. It should come as no surprise that the circulating *B. anthracis* strains are in the major cluster (A3.a cluster) and not diverse from that found countrywide. All the more amazing, these 6 colonies which were obtained from a common ancestor (from unique milk sample) provided different subtypes in both MLVA typing methods. It is believable that the cow was infected with different subtypes or the udder was environmentally exposed to *B. anthracis* mixture. Nevertheless, further studies are required to clarify the composite face of this bacteria.

The Sterne strain 34F2 livestock vaccine has been in use for animal welfare and pregnant animals are frequently among those vaccinated without any adverse effects. In a study, no isolation of *B. anthracis* was made in milk samples collected from each of 49 vaccinated cows twice daily for 10 postvaccination days [29]. Similarly, the crosscheck of milk sample isolates was also made and they

Figure 2. A) MLVA-8 typing. GK: genotype of Keim et al. [13]. B) MLVA-25 typing GT: Genotype N: Number of the isolates.

were found to be different from the vaccine strain used in Turkey, which was already genotyped as GK61 (Sterne) and located within the A3.b cluster. It is assumed that the cow survived although she excreted substantial amount of *B. anthracis* based on the vaccination made about 6 months ago. The vaccine was a spore-based vaccine which provides at least 12 months of protection according to the information given by the manufacturer. The calf was also found negative for the presence of *B. anthracis* both in cultural and molecular methods. It is assumed that milk can be a source of infection for offspring, but the bacterial load was too low to cause this effect. The protection face of vaccination is considered effective for the survival of the calf.

In conclusion, *B. anthracis* was detected atypically localized in a cow's milk and udder. The bacterium may have been acquired through an ascending route from the teats following the environmental contamination of udder, rather than its classic (descending) excretion with milk. Considering the expectation that the host will be infected with a single bacterial subtype, the presence of different MLVA types in this study strengthens the environmental exposure possibility caused by the *B. anthracis* bacterial mixture. Further studies are needed to clarify the composite face of this bacterium as it is a mutant model that does not cause typical anthrax. Prophylaxis and disinfection procedures were recommended to the owner. In the following days, no progressive infection in the aforementioned cow and no new cases were reported.

Conflict of Interest

The authors declare that they have no conflict of interest.

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