SHORT COMMUNICATION

The epidemiological investigation and control of an anthrax outbreak in a village in Central Anatolia, Turkey

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Introduction

Bacillus anthracis, the etiologic agent of anthrax primarily infects grazing herbivores and can be transmitted to humans through contact with infected animal products. In humans, the most commonly occurring form is cutaneous anthrax, an occupational hazard of workers who handle processed animal products or handle infected animals. Two other forms of anthrax exist in humans, inhalational and gastrointestinal, which occur less frequently but are more serious if left untreated. These forms occur from the ingestion or inhalation of *B. anthracis* spores [1,2].

Grazing animals are exposed to *B. anthracis* spores from direct contact with contaminated soil, standing water, and pasture grass or livestock feed. Upon death, the infected animals contain vast amounts of bacteria (estimated to be up to 10^{12} from a 550 kg cow) [3] that can be released into the surrounding environment from the slaughtering process or through the action of carnivores and scavengers [4]. Once released, the vegetative cells convert to spores which contaminate the soil, grass, and local water sources. Spores are well-known to persist in the environment for long periods of time (usually more than 60 years [2,5]) as well as provide resistance to environmental climate such as temperature and humidity [6], as well as chemical decontamination strategies [7]. Once ingested, spores can then germinate and reestablish the infection cycle. Anthrax outbreaks are usually observed in susceptible livestock deaths in a pasture [2,8].

Although, there has been a general decrease in the number of anthrax outbreaks, the infection is still endemic or hyperendemic in some provinces of Turkey including Kars, Erzurum, and Van [1,9]. Human cases are reported to the Public Health Office at the Ministry of Health and are annually decreasing, while animal cases are reported to the Regional Office at the Ministry of Agriculture. Eastern provinces such as Kars, Erzurum, and Van have reported several epizootic outbreaks while

other provinces in Central Anatolia including Kayseri have reported sporadic outbreaks in humans [1,9]. This study sought to review the epidemiological features and infection control strategies of an anthrax outbreak in a novel location while fully characterizing the biological agent responsible.

Materials and methods

Background information

Kayseri is located in Central Anatolia and has a human population of approximately 1.3 million. The city has one university hospital and a referral tertiary care hospital. A total of 58 human anthrax cases were recorded by the Regional Public Health Department between 2005 and 2014 in Kayseri (Table 1). Sixteen different anthrax epizootic outbreaks were also recorded by the Regional Office at the Ministry of Agriculture between 2000 and 2014. All human cases during this period were cutaneous anthrax as the result of contact with sick / dead animals or contaminated animal products. The source of infection in animals was noted as contaminated pasture. In the previous three decades, Karakimse village had a single reported anthrax outbreak (August–October 2013).

Design of epidemiological investigation

Karakimse is an agricultural village located in the north-western part of Kayseri and is 40 km from the city of Kayseri at an altitude of 1162 m above sea level. In 2013, the population of the village was 459 humans, approximately 200 cattle and 3000–3500 sheep and goats (mostly sheep). During the previous three decades, there were no recorded cases of human and animal anthrax and as such no routine animal vaccination program exists. Provincial agricultural officers noted that an animal died from anthrax in Duver, a neighboring town

(<7 km from Karakimse) in June 2013; however, no movement of animals between Duver and Karakimse could be confirmed.

Notification of the index case

The primary report of anthrax infection was made by a physician from the City Hospital to the Public Health Service on 4 October 2013. This notification was for two patients from Karakimse, who were diagnosed with cutaneous anthrax. Following the diagnosis, public health officials and provincial directorate of agriculture visited Karakimse. Investigators had discovered that a number of animals had died of unknown causes since mid-August. The owners of these animals then slaughtered and butchered additional sick animals. Although the animal meat had been consumed by the owners and their neighbors, some of the meat was sold to a butcher and transferred to the nearby town of Kalaba (15–20 km far from Karakimse).

After notification of suspected anthrax from Karakimse, an investigatory team consisting of researchers from the Public Health Office at the Ministry of Health, the Regional Office of the Ministry of Agriculture and Erciyes University (Kayseri) was established. A visit to the outbreak area was planned to investigate and to implement an infection control program.

The team reviewed anthrax notification forms from the Public Health Office and the Regional Office at the Ministry of Agriculture. The location of animal outbreaks were also recorded and analyzed. The study was approved by the Erciyes University Local Ethics Committee.

Field visits and sampling

The investigatory team visited the outbreak area to investigate and to implement a control program on 7 October. The team performed face-to-face interviews with various residents to establish a timeline for the outbreak and to ascertain if any animals or villagers exhibited cutaneous lesions, diarrheal, or acute respiratory disease. Family physician files and Regional Public Health records were also reviewed for any clinical form of anthrax. Anthrax was suspected if an animal exhibited symptoms of infection such as: bloated carcass, bleeding of unclotted blood from natural orifices, subcutaneous hemorrhages, and extensive hemorrhagic swelling of spleen. Investigators also tried to identify animal slaughtering places and sought to determine the following: what happened to

the carcasses of sick or dead animals? Who was involved in the slaughtering or butchering process? If an animal was butchered, who came into contact with the infected animal meat? What process was made for meat storage (storage in deep freeze, making sausage, etc.)?

Investigators also asked local physicians, if any patients from the Karakimse area exhibited classical symptoms of anthrax between 2013 and 2016. Field visits and soil sampling were performed between October 2013 and March 2016.

Samples of meat or meat products (raw or cooked meat and/or sausage) were collected from various animal carcasses. During the first field visit in October 2013, soil samples (about 200–250 g) were taken from the surface of identified slaughtering places because the land was dry and hard. Amid the second visit in March 2016, soil samples were taken at 10–15 cm in depth and 20–25 cm in depth at the contaminated sites. The soil samples were stored in sterile sampling containers until they were examined in the laboratory. Serum samples were also obtained from villagers with cutaneous lesions or who complained of diarrhea or respiratory disease.

Laboratory investigation

B. anthracis isolation

Meat and meat products were cultured on 7% sheep blood agar (SBA) and incubated aerobically at 37 °C overnight and checked for growth. Suspected *B. anthracis* colonies were identified according to WHO Anthrax Guidelines [2] using classical phenotypic criteria i.e. classical colony morphology (ground glass appearance, flat, opaque, tenacious, and grayish-white), penicillin G (10 Units; Oxoid, Basingstoke, UK), and gamma phage (~10⁹ PFU/mL) activities.

Forty grams of soil were suspended in 200 ml sterile distilled water (SDW) and mixed by hand. Suspensions were incubated for 20 min at room temperature to allow large particulates to sediment and a 1 ml aliquot of the supernatant was removed and heat treated at 62.5–63.0 °C for 15–20 min in a circulating type water bath (Nüve, Turkey) to remove vegetative cells. Heat-treated samples were then diluted 1:10 in SDW and 150 µl spread over the surface of duplicate SBA plates. Plates were incubated aerobically at 37 °C for 24 or 48 h, assessed for 'classical' *B. anthracis* criteria as described above and the total viable spore (TVS) content per gram of soil was calculated.

PCR analysis of virulence plasmids

The presence of the *B. anthracis* virulence plasmids, pXO1 and pXO2 were assessed using PCR as described by Buyuk et al. [10] In brief, a 25 µl reaction mixture containing 12.5 µl Taq PCR Master Mix (Qiagen, UK), 2.5 µl primer mix (Cap6/103 or PA5/8), 2.5 µl template DNA, and 7.5 µl dH₂O (supplied as part of the Taq PCR Master

Table 1. Age and gender distributions of anthrax cases recorded in Kayseri between 2005 and 2014.

Age	Female	Male	Total
≤19	2	3	5
20–29	3	15	18
30–44	3	17	20
45–64	3	9	12
≥65	2	1	3
Total	13	45	58

Mix Kit) was prepared and run following PCR conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 40 s, with a final elongation step of 72 °C for 5 min. Amplicons were analyzed on a 1.5% (w/v) agarose gel stained with SafeView nucleic acid stain (NBS Biologicals, Huntingdon, UK) using a horizontal gel electrophoresis system (BioRad, USA).

Genotyping analysis

MLVA-8 and MLVA-25 analysis were performed at the Public Health Institution of Turkey (Ankara, Turkey) and VNTR sizes were normalized to the related sizes for genotypic comparisons as previously described [11,12].

ELISA for testing anti-PA and anti-LF responses

The recombinant antigens; Protective antigen (Lot 17115A5B), and Lethal Factor (Lot 1722B11B) were purchased from Quadrantech Diagnostics Ltd (Epsom, Surrey, UK). An optimized ELISA method was employed to determine the IgG specific responses of each individual. In brief, 100 µl recombinant antigen at 2 µg/ml in PBS and 100 µl human IgG standards (Sigma Aldrich, UK) with concentration 0.5, 0.25, 0.125, and 0.0625 µg/ml were added onto the first 10 and the last 2 columns, respectively, and incubated at 37 °C for 2 h. Following coating, plates were automatically washed with 300 µl of PBS + 0.1% v/v Tween-20 (PBST) (Wellwash Microplate Washer, Thermo Scientific) and incubated at 37 °C for 1 h to block the wells. Test serum samples, positive, and negative controls were serially diluted 1 in 2 down the plate with PBST, with the first well containing a 1:10 dilution. Following incubation at 37 °C for one hour, plates were washed with PBST. One hundred µl anti-human IgG mouse monoclonal antibody (Jackson Immuno Research Labs, USA) was diluted 1:1000 in PBST and added to each well. Plates were then incubated for 1 h at 37 °C and again washed with PBST. Substrate solution was prepared by dissolving 0.7 g sodium phosphate dibasic (Sigma Aldrich, UK), 0.5 g citric acid (Sigma Aldrich, UK), one ABTS substrate tablet (Sigma Aldrich, UK), and 25 µl H₂O₂ (Sigma Aldrich, UK) in 100 mL of SDW. One hundred µl of substrate was added to each well and plates were incubated at 37 °C for 30 min. The reaction was stopped by the addition of 100 µl 2% SDS (Sigma Aldrich, UK) in SDW and the absorbance determined at 405 nm (Spectra Max Plus 384 Absorbance Microplate Reader, Molecular Devices). Positive serum controls taken from individuals vaccinated with the Anthrax Vaccine Precipitated and negative serum controls taken from individuals with no history of infection or vaccination were also performed.

Serum samples were repeated in duplicated and data analysis was performed using Microsoft Excel as a macro to batch process the results. The mean values of the triplicate human IgG standards were used to prepare a standard curve of Absorbance versus antibody content and the antibody titer (µg/ml) of samples were calculated.

Results

Characteristics of the Karakimse outbreak and villager interviews

Sick animals were slaughtered by their owners or shepherds. The meat from the sick animals was consumed mainly by their family members and some was delivered to neighboring families. Some of the contaminated meat was processed into sausage and sold to a butcher. By the beginning of October a total of 6 cattle and 17 sheep had died. Four people (two animal owners, one shepherd, and one helper) diagnoses were confirmed as cutaneous anthrax. The incubation period varied between five and seven days. All had been involved in the animal slaughtering and butchering processes. Nine individuals (5 adults and 4 children age ranging from 4 to 14) complained of mild diarrhea and had consumed sausage made from contaminated meat were suspected of intestinal anthrax. These people were closely observed for 10 days by the family physician. The diagnosis could not be confirmed. Diarrhea improved within two to three days in all cases without any antibiotic administration. There was no suspected or confirmed pulmonary anthrax during the outbreak period.

B. anthracis isolation results

B. anthracis was isolated from meat (5×10^5 cfu/g) samples. Six of the seven soil samples which were collected during the first visit to the village (October 2013) were positive for the presence of *B. anthracis* spores with a varying spore content (1.33×10^2 to 9.32×10^5 spore/g soil). All isolates exhibited 'classical' *B. anthracis* phenotypes and were PCR positive for pXO1 and pXO2 (Table 2).

A total of 12 soil samples from 2 foci were taken in March 2016 during the second sampling season. From these samples, the presence of *B. anthracis* was confirmed phenotypically and genotypically (Table 2) in two samples taken from a depth of 20–25 cm at a concentration of between 1.66×10^2 and 5×10^2 spore/g. The follow up study confirmed that *B. anthracis* spores remained viable within the areas in which cattle had been previously slaughtered.

MLVA results

Five different genotypes were observed following MLVA-8 typing. All genotypes, genotype 43 (4 isolates), genotype 33 (2 isolates), genotype 36 (1 isolate), genotype 40 (1 isolate), and genotype 45 (1 isolate) were in the A3.a major cluster and found to be consistent with those reported by Keim et al. [12]. Six different genotypes were observed with MLVA-25 typing; genotype 1 (3 isolates), genotype 2 (2 isolates), and genotype 3–6 (1 isolate for each) (Table 2). The dendrograms were constructed from the MLVA analysis data using GeneMapper software and

Table 2. Characteristics of *B. anthracis* strains isolated from soil samples.

Specimen Code	Penicillin susceptibility	Gamma phage susceptibility	Capsule formation in NaHCO ₃ medium	PA-PCR	CAP-PCR	Genotyping results		
						MLVA cluster	MLVA-8 genotype	MLVA-25 genotype
First visit sampling 2013 positive cultures 6/7 samples								
MD2	+	+	+	+	+	A3.a	43	1
MD3	+	+	+	+	+	A3.a	33	2
MD4	+	+	+	+	+	A3.a	45	3
MD5	+	+	+	+	+	A3.a	40	4
MD6	+	+	+	+	+	A3.a	36	5
MD7	+	+	+	+	+	A3.a	43	6
Meat samples	+	+	+	+	+	A3.a	33	2
Control visit sampling 2016 positive culture 2 / 12 samples								
MD2-5	+	+	+	+	+	A3.a	43	1
MD2-10	+	+	+	+	+	A3.a	43	1

are shown in Figures 1 and 2. The emerged genotypes of this study coincide with the given *B. anthracis* genotype scale in Turkey [11,12].

Antibody responses

Serum samples were only obtained from four adults. Anti-PA and anti-LF responses were positive in two people who had been diagnosed with cutaneous anthrax while suspected cases (mild diarrhea; $n = 2$) yielded negative results.

Infection control and prevention studies

Two informative meetings one-on-one with people involved with ill animal slaughtering/butchering or consuming suspected meat took place. An informative meeting was also held in groups as well as with the villagers including animal owners, shepherds, housewives, and people who are involved in the animal slaughter and butchering processes. Information about human and animal anthrax that included potential infection sources, symptoms, and prevention measures was given to the villagers. Contaminated animal slaughtering places (about 2 m²) were burned with diesel fuel. Stored contaminated meat and sausage were destroyed under the supervision of the technicians. Kitchens that were suspected of coming into contact with contaminated meat and meat products were disinfected with hypochlorite and cleaned according to the WHO Guidelines [2].

A livestock vaccination program which administered the Anthrax spore vaccine (34F Sterne spore) was initiated in 2013. During this time, a total of 3322 vaccine doses were provided in 2013, 3847 in 2014, 3045 in 2015, and 5859 in 2016. No additional cases of anthrax in humans or animals were observed during the follow up period.

Discussion

Although the incidence rate of anthrax in humans is decreasing (≤ 150 cases per year between 2011 and 2016) in Turkey, regional outbreaks still present a risk to human

and animal health. In Turkey, anthrax in most commonly observed in rural provinces (particularly Erzurum, Kars, Ardahan, and Van) where agriculture is the primary industry [1,9]. It should also be noted that anthrax is endemic in these regions as well as neighboring regions in Iran and Georgia with the uncontrollable and illegal movement of animals being the likely cause of spreading the infection [9,14–16].

Cutaneous anthrax represents the main presentation of the disease, however gastrointestinal and inhalational cases have been reported in Turkey and neighboring countries [1,9,14,16].

In this outbreak, 23 animals (6 cattle and 17 sheep) died and 4 people were diagnosed with cutaneous anthrax. Nine people complained of mild diarrhea but the infection was not confirmed among the two antibody responses tested. For the confirmation of anthrax with the serological tests, paired blood samples should be taken from patients [2]. Assuming all infected (animals and humans) were detected, the rate of animal infection to human infection was 6/1 (>1). This rate is given as 10/1 (>1) in well-developed industrial countries and 1/10 (<1) in developing agricultural countries [2]. The rate of human infection depends on how many people are involved the slaughtering process; family members being involved from rural areas, as seen in Bangladesh [13], Georgia [14], and the eastern part of Turkey [15,16]. In this outbreak, only shepherds or animal owners were involved the slaughtering processes. However, animals were slaughtered in pastures and near water sources. The slaughtering process then contaminated the surrounding areas to a limited extent (1.33×10^2 and 9.32×10^5 spores/g soil) through the release of infected bodily fluids. The residual level of contamination decreased during the follow up period in 2016. All isolates from the two sampling periods yielded fully virulent *B. anthracis* strains in terms of presence of both plasmids. No genotype change was detected in the isolates sampled twice from unique focus but slightly different genotype profiles were seen among the strains isolated from different foci. Genotyped strains of the present study belong to A3.a branch, which is the major cluster in Turkey. The

Figure 1. A dendrogram of the MLVA-8 typing results of *B. anthracis* isolates. GK: genotype of Keim et al. [11] N: No of the isolates.

Figure 2. A dendrogram of the MLVA-25 typing results of *B. anthracis* isolates. GT: Genotype, N: No of the isolates.

genotype 43 is predominant among the subtypes. The emerged genotypes of this study match the given Turkish *B. anthracis* genotype scale [11,12].

For the prevention of any epizootic outbreak, the vaccination of livestock against anthrax is very important particularly in regions where *B. anthracis* contamination is endemic. In addition to this, animals which have died due to anthrax should be buried far from grazing and water sources. These measures also require a greater level of cooperation and communication between local residents and government officials.

In this outbreak, individuals involved in the slaughtering process did not use personal protective equipment as the authors suggested in agricultural rural areas; animal owners and shepherds should be aware of and educated in the use of personal protective equipment such as gowns, gloves, and goggles when in contact with sick animals or transporting infected carcasses [2,10,13] as this could reduce the risk of human infection. A vaccination program was also initiated against anthrax for livestock using the Sterne vaccine (34F2). During the following three years, there were no new animal case or dead livestock observed.

Conclusion

Although, there are some limitations to this outbreak investigation, this study shows that the control of anthrax in humans depends on the control of infection in animals. Good veterinary practices in endemic areas include close surveillance and reporting, burying or cremation of dead animal carcasses, and decontamination/disinfection procedures. The authors believe that a continued infection control educational program for animal owners and local residents as well as immunization of animals against anthrax control and prevent the infection in endemic areas.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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