**Research Article**

**Healthcare**

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# Infected soil samples by Bacillus anthracis, confirmed by PCR

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## Abstract

Anthrax in Albania is an endemic disease characterized by few outbreaks involving a very low number of animals. 19 samples of soil coming from burial sites of different regions of Albania were examined. The analysis of soil samples revealed that 11 of them were contaminated with anthrax spores.The analysis of CanSNPs showed that all isolates belong to lineage A major subgroup A Br. 008/009 (TransEurasian or TEA strains). The MLVA test at 15 loci showed three different genotypes: Albania GT/1, Albania GT / 2and Albania GT/3.

## Introduction

Anthrax, whose causative agent is *Bacillusanthracis*, is a non-contagious infectious disease that affects several animal species, human included. Domestic and wild ruminants represent the most susceptible categories (Dragon and Rennie, 1995). The bacterial agent has the characteristic of forming spores that can survive in the environment for several decades (Hugh-Jones and Blackburn, 2009). In susceptible animals anthrax generally has a fatal outcome characterized by sudden death and leakage of blood from the natural openings. Thanks to the robust ability of spores to maintain viability and pathogenicity for many decades and the lower production, *B. Anthracis* is considered one of the pathogens of greatest interest as a bacteriological weapon in a possible bioterrorist attack (Christopher et al., 1997). Currently in Europe anthrax is almost entirely disappeared except for some East Europe and Mediterranean countries such as Italy, Albania, Greece, Romania, Georgia, Russia and Turkey in particular.

In Albania anthrax is an endemic disease characterized by few outbreaks involving a small number of animals. For the prophylaxis against animal anthrax is used the live attenuated anthrax Sternevaccine, produced in Albania. The prophylax is program provides for the vaccination of animals for at least ten years since the last outbreak. In Albania in the case of a suspected anthrax outbreak, the veterinarians are obliged to inform the Ministry of Agriculture which alert the veterinary services of the area that implement the appropriate security procedures and control. For the diagnosis is not always used the isolation and the biomolecular tests that permit to obtain a certainty identification of the agent. The diagnosis is often based on the observation to the microscope of the slides prepared with blood from dead animals and stained with methylene blue. This paper reports the results of an epidemiological survey carried out in areas considered at higher risk of anthrax.

## Materials and Methods

*Soil samples*

Soil samples were collected in 19 burial sites of dead animals with suspected of anthrax in the regions of Shkodër, Vlorë, Lushnjë, Fushë-Kuqe, Tepelenë, Dibër, Kukës, Elbasan, Korçë, Berat, Gjirokastër.

| Samples | Geographic area | Results |
|---|---|---|
| Soil sample | VLORË | Positive |
| Soil sample | VLORË | Positive |
| Soil sample | GJIROKASTËR | Positive |
| Soil sample | TEPELENË | Positive |
| Soil sample | TEPELENË | Positive |
| Soil sample | VLORË | Positive |
| Soil sample | SHKODËR | Positive |
| Soil sample | SHKODËR | Positive |
| Soil sample | KUKËS | Positive |
| Soil sample | GJIROKASTËR | Positive |
| Soil sample | KORÇË | Positive |
| Soil sample | MILOT | Negative |
| Soil sample | LUSHNJË | Negative |
| Soil sample | FUSHË-KUQE | Negative |
| Soil sample | DIBËR | Negative |
| Soil sample | ELBASAN | Negative |
| Soil sample | KORÇË | Negative |
| Soil sample | BERAT | Negative |
| Soil sample | DIBËR | Negative |

Isolation of *B anthracis* from soil samples.

The soil samples were analyzed by the Ground Anthrax Bacillus Refined Isolation method (Fasanella et al, 2013). Briefly to 7.5 grams of ground were added 22.5ml of a 0.5% aqueous solution of Tween 20. After washing in magnetic stirrer for 30 minutes, the suspension was centrifuged for 5 minutes at 2000rpmand the supernatant incubated for 20 minutes at 64 ° C.

After incubation the suspension was diluted 1:1 with tryptosebroth containing 75mg of fosfomycin. 1ml of the diluted suspension was seeded in TSMP plates and incubated at 37 °C for 24 hours. After this period, the dishes were subjected to the reading.

*Extraction of DNA*

Each suspect colony was transferred to a blood agar plate and after 24 hours of incubation was taken adequate rate of bacterial patina and subjected to DNA extraction using the DN easy Blood and Tissue kit Kit-Qiagen.

*PCR*

DNAs were subjected to Polymerase Chain Reaction (PCR) using anthrax-specific primers (pXO1 and pXO2 plasmids, and chromosome) (Fasanella et al., 2001).

*Can SNP Analysis*

We utilized 13 TaqMan-Minor Groove Binding (MGB) all elic discrimination assays with oligosand probes as described by VanErt et al. (VanErt et al., 2007) for each of 13can SNPs. End point fluorescent data were measured on the ABI 7900HT. Can SNPs profiles were compared to the 12 recognized world wide sublineages and sub-groups (VanErt et al., 2007).

*15-loci MLVA and SNR analyzes*

We utilized 5 'fluorescent-labeled oligos, deprotected and desalted, specifically selected for the Variable Number Tandem Repeats (VNTR) and Single Nucleotide Repeats (SNR) used.

The 15 specific primer pairs for the Multilocus Variable Tandem Repeat Analysis (MLVA) were selected as described by Van Ert et al. (Van Ert et al., 2007). The four specific primer pairs were selected for SNR reactions Following Garofolo et al. (Garofolo G. et al., 2011).

MLVA PCRs were performed in two multiplex reactions in a final volume of 15 l. The reaction mixture contained 1 × PCR reaction buffer (Qiagen), 1 U of Hot Star Taq Plus DNA polymerase (Qiagen); dNTPs (0.2 mM each); 3 mM MgCl2 and appropriate concentrations of each primer (multiplex 1: vntr12 0.37μM, vntr35 0:37 uM, vntr23 0.2 mM, 0.2 mM vntr16, vntr17 0.2μM, 0.2μM vrrB2, vrrC1 0.1 mM, 0.1 mM vntr32; multiplex 2: vntr19 0.2 mM , CG3 0.2 mM, vrrB1 0.2 mM, 0.2 mM PX01, pX02 0.2 mM, 0.1 mMvrrA, vrrC2 0.1); and 1 ng of template DNA.

*Automated Genotype Analysis*

The MLVA PCR products were diluted 1:80 and subjected to capillary electrophoresis on ABI Prism 3130 genetic analyzer (Applied Biosystems) with 00:25 μl of GeneScan 1200 and sized by GeneMapper 4.0 (Applied Biosystems Inc.) SNR Amplified PCR products were diluted 1:80 and subjected to capillary electrophoresis on ABI Prism 3130 genetic analyzer (Applied Biosystems) with 12:25 μl of GeneScan 120 Liz, and sized by GeneMapper 4.0 (Applied

Biosystems Inc.). In all the analyzes the samples were processed in triplicate to allow the correct sizing of the fragments.

**Results**

The analysis of soil samples of 19 suspected burial sites showed that 11 were contaminated with anthrax spores.The analysis of CanSNPs showed that all isolates belong to lineage A major subgroup A Br. 008/009 which is widely represented in Europe and Asia: TransEurasian or TEA strains.

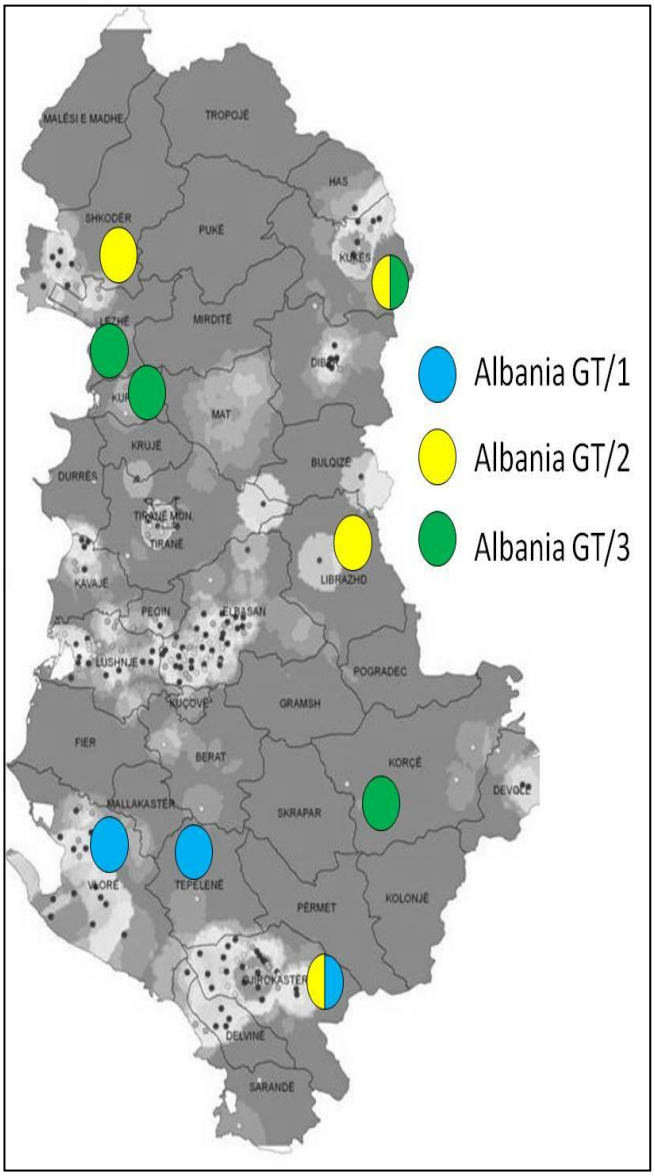
| Region | Result | CanSNPs | MLVA |
|---|---|---|---|
| Vlorë | Positive | A Br. 008/009 | GT ALBANIA 1 |
| Vlorë | Positive | A Br. 008/009 | GT ALBANIA 1 |
| Gjirokaster | Positive | A Br. 008/009 | GT ALBANIA 1 |
| Tepelenë | Positive | A Br. 008/009 | GT ALBANIA 1 |
| Tepelenë | Positive | A Br. 008/009 | GT ALBANIA 1 |
| Vlorë | Positive | A Br. 008/009 | GT ALBANIA 1 |
| Shkodër | Positive | A Br. 008/009 | GT ALBANIA 2 |
| Shkodër | Positive | A Br. 008/009 | GT ALBANIA 2 |
| Kukës | Positive | A Br. 008/009 | GT ALBANIA 2/3 |
| Gjirokaster | Positive | A Br. 008/009 | GT ALBANIA 2 |
| Korçë | Positive | A Br. 008/009 | GT ALBANIA 3 |
| Milot | Negative | -- | -- |
| Lushnje | Negative | -- | -- |
| Fushe-Kuque | Negative | -- | -- |
| Dibër | Negative | -- | -- |
| Elbasan | Negative | -- | -- |
| Korçë | Negative | -- | -- |
| Berat | Negative | -- | -- |
| Dibër | Negative | -- | -- |

The MLVA test at 15 loci showed three different genotypes: Albania GT/1, Albania GT/2 and Albania GT/3. The strains isolated from the burial sites of the Regions of Vlorë, Gjirokastër, Tepelenë belong to genotype Albania GT/1. The strains of *Bacillus anthracis* isolated from soil samples collected in burial sites of the Regions of Shkodër, Kukës and Gjirokastër showed a mutation al the locus ***vrra*** in comparison to the genotype AlbaniaGT/1 and for this reason it belongs to a different genotype than in this work was called Albania GT/2. Finally, from the soil samples collected on the burial sites of the Region of Kukës and Korçë, were isolated *Bacillus anthracis* belong to a genotype different from the previous, characterized by a mutation at the locus ***vrrb2*** from the previous genotypes and at the locus ***vrra*** from the Albania GT/2, and that in work was called Albania GT/3.

## Discussion

The analysis of burial sites and the isolated strains indicate that in the investigated areas of Albania there are 3 different genotypes of *Bacillus anthracis* that were referred as Albania GT/1, GT/2 and GT/3. The MLVA analysis indicated that all the genotypes are genetically very similar to each other which confirms the hypothesis that all of them are the result of the evolution of a local common ancestral strain. *B. anthracis* is a bacterium that from a genetic point of view is highly conserved because under spore form it spends most of its existence in the soil. Nature provides few opportunities to the bacterium for its replication cycle, and the development of a extraordinary pathogenicity is the effective strategy to increase substantially the chances of success against the host immune mechanisms.

In summary, the few cases of anthrax that occur each year are only the result of a natural ecological balance that through these extraordinary events promote the maintenance of a bacterial species that otherwise would have become extinct many time ago (Fasanella et al, 2010). Finally, the CanSNPs analysis confirms that *B. anthracis*strains circulating in Albania belong to the large family of TEA (Trans Eurasian).




Distribution of the genotypes of *Bacillus anthracis* circulating in Albania

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