RESEARCH ARTICLE (Open Access)

Genotyping *Bacillus anthracis* Strains of Albania

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Abstract

Anthrax in Albania is an endemic disease characterized by few outbreaks involving a very low number of animals. 11 strains of *Bacillus spp* isolated from died animals of different regions of Albania were examined. The analysis revealed that only 8 strains were confirmed as *Bacillus anthracis*. 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 / 2 and Albania GT/3.

Keywords: *Bacillus anthracis*, Real time PCR, MLVA, CanSNPs.

1. Introduction

Anthrax, whose causative agent is *Bacillus anthracis*, is a non-contagious infectious disease that affects several animal species, human included. Domestic and wild ruminants represent the most susceptible categories [5]. The bacterial agent has the characteristic of forming spores that can survive in the environment for several decades [5]. In susceptible animals anthrax generally has a fatal outcome characterized by sudden death and leakage of blood from the natural openings. In humans the disease develops in three forms depending on the route of penetration of the bacterium: cutaneous, pulmonary and gastrointestinal. Recently, a fourth fatal form has been reported in drug users as a result of injection of heroin contaminated with anthrax spores [13]. 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 [2].

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. The population of *B. anthracis* in Europe is phylogenetically heterogeneous and the most prevalent subgroups are A.Br.008 / 009, B.Br.CNEVA and B.Br. 001/002 [3].

In Albania anthrax is an endemic disease characterized by few outbreaks involving a small number of animals. The disease was widespread in the past, but now the number of outbreaks has been drastically reduced thanks to the controls programs carried out in accordance with the guidelines of WHO and OIE. For the prophylaxis against animal anthrax is used the live attenuated anthrax Sterne vaccine, produced in Albania. The prophylaxis 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.

2. Material and Methods

Bacterial strains. From the of Food Safety and Veterinary Institute of Tirana were sent to the Italian Reference Center for Anthrax at the Istituto Zooprofilattico of Puglia and Basilicata (Foggia - Italy) eleven strains of Bacillus spp isolated from sheep and cattle died in different areas of the Albanian

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territory and identified on the basis of morphological and biochemical profile as Bacillus anthracis. All shipments were made in accordance with the international standards of biosafety

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 DNeasy 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) [7].

CanSNP analysis. We Utilized 13 TaqMan-Minor Groove Binding (MGB) allelic discrimination assays with oligos and probes as desbribed by Van Ert et al. [14] for each of 13 canSNPs. Each 5 l reaction contained 1 × TaqMan Genotyping Master Mix (Applied Biosystems), 250 nM of each probe, and 600 nM each of forward and reverse primers and 1.0 µl Approximately 1 ng of template DNA. For all assays, thermal cycling parameters were 95 ° C for 10 min., Followed by 50 cycles of 95 ° C for 15 s and 60 ° C for 1min. Endpoint fluorescent data were Measured on the ABI 7900HT. CanSNPs profiles were Compared to the 12 Recognized worldwide sublineages and sub-groups [14].

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. [14]. The four specific primer pairs were selected for SNR reactions Following Garofolo et al. [10]. 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 HotStarTaq 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 mM vrrA, vrrC2 0.1); and 1 ng of template DNA. The thermocycling conditions were as follows: 95 ° C for 3 min; 35 cycles at 94 ° C for 30 s, at 60 ° C for 45 s, and at 72 ° C for 1 min; and finally, 72 ° C for 5 min. The SNR was performed in a multiplex PCR reaction in a final volume of 15 l containing 1 × PCR reaction buffer (Qiagen); 1 U of HotStarTaq Plus DNA polymerase (Qiagen); dNTPs (0.2 mM each); 3.5 mM MgCl2, dNTPs; and appropriate Concentrations of forward and reverse primers (0.2 mM HM1, HM2 0.2 mM, 0.2 mM HM6, HM13 0.1 mM); and 1 ng of template DNA. The thermocycling conditions were as follows: 95 ° C for 5 min; 35 cycles at 94 ° C for 30 s, 60 ° C for 30 s, and 72 ° C for 30 s; and finally, 72 ° C for 5 min.

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.

3. Results and Discussion

The analysis of 11 strains of *Bacillus spp* showed that only 8 were confirmed as *Bacillus anthracis*. 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.

The MLVA test at 15 loci showed three different genotypes: Albania GT/1, Albania GT/2 and Albania GT/3.

The analyses of 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 confirm the hypothesis that all of them are the result of the evolution of a local common ancestral strain. However, the research should be extended to other parts of Albanian territory and especially in the southern regions more closed to Turkey, where the genetic variability of the circulating strains of *Bacillus anthracis* is very high.[6]

The SNR test performed on all isolates showed the existence of five sub genotypes within the Albania GT/1, two sub genotypes in Albania GT/2 and four sub genotypes within the Albania GT/3.

The research of sub genotypes, carried out through the analysis of SNRs, can be of great help in the epidemiology studies of anthrax outbreaks because it allows researchers to be able to make a detailed analysis of the evolutionary infectious episodes.

The CanSNPs analysis confirms that *B. anthracis* strains circulating in Albania belong to the large family of TEA (Trans Eurasian). However it is not excluded that in the course of further investigation can be isolated strains belonging to other lineages as has been observed in Italy, where in addition to the dominant genotype TEA there are ecological niches of B.Br.CNEVA and A.Br.005 / 006 in the regions Northern Italy [10].

Table 1. List of bacterial strains analyzed

Sample	Geographic area	Nr strains
Bac. strain	Kukes	2
Bac. strain	Lezhe	2
Bac. strain	Lezhe	2
Bac. strain	Librazhd	1
Bac. strain	Shkoder	1
Bac. strain	Shkoder	1
Bac. strain	Vlore	2

Table 2. List of results for bacterial strains

Geographic area	Results	CanSNP	Genotype
Kukes	2 positive	A.Br.008/009	Albania GT/3
Lezhe	2 positive	A.Br.008/009	Albania GT/3
Lezhe	2 negative	-	-
Librazhd	1 positive	A.Br.008/009	Albania GT/2
Shkoder	1 negative	-	-
Shkoder	1 positive	A.Br.008/009	Albania GT/2
Vlore	2 positive	A.Br.008/009	Albania GT/1

4. Conclusions

The results of this study shows that the technique of Real Time PCR is a quick and safe method for the real detection of the pathogen and its family genotype.

The data of anthrax circulating in different regions of Albania can now be compared with data from other countries, and modern analyzing techniques should be applied in our labs.

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6. References

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