Genotyping of *Bacillus anthracis* Strains Circulating in Albania

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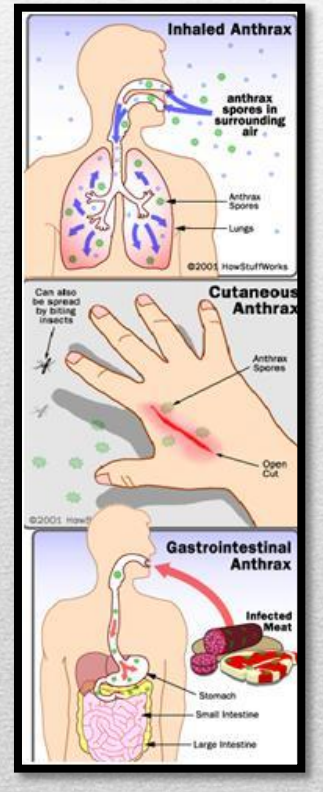
Anthrax

- Anthrax, whose causative agent is *Bacillus anthracis*, is a non-contagious infectious disease that affects several animal species, the humans included.



- In animals anthrax generally has a fatal outcome characterized by sudden death and leakage of blood coming out from the natural openings.

- In humans the disease develops in three ways and forms: pulmonary, gastrointestinal and cutaneous.

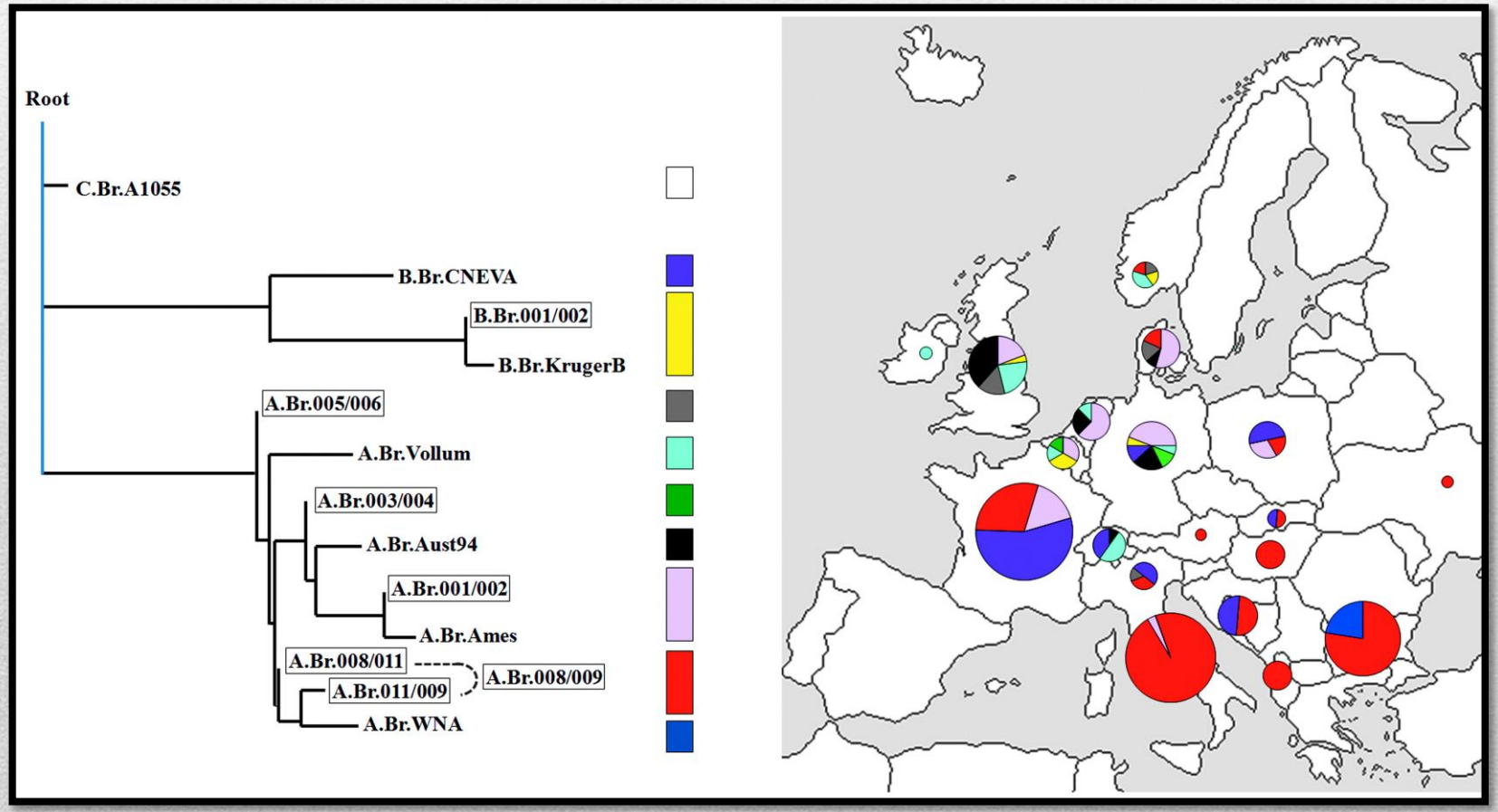
Major tasks of the *National Reference Center for Anthrax*

- Anthrax vaccine production;
- Molecular epidemiology and genotyping;
- Disease control;
- Bioterrorism emergency (Italian Laboratory Reference);
- Animal model;
- Research;
- National and international cooperation.



Anthrax in Europe

In Europe it has almost entirely disappeared except for some Eastern Europe and Mediterranean countries such as Italy, Albania, Greece, Romania, Georgia and Turkey in particular.
The population of *B. anthracis* in Europe is phylogenetically heterogeneous and the most prevalent subgroups are A.Br.008/009 (Trans Eurasian group-TEA-), B.Br.CNEVA and A.Br.001/002. (Derzelle and Thierry, 2013).



Anthrax in Albania

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 control programs.

Albania (such as other Balkan Countries) could be considered the «bridge» between Europe and Middle East.

It is important region for movement of animals and therefore strains.

For these reasons it is important to keep under control the situation and the distribution of *Bacillus anthracis* strains in this area.

Anthrax in Albania: OIE reporting history

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Protecting animals, preserving our future

From 2009 to 2011, 36 anthrax outbreaks have been confirmed and during the outbreaks were also reported human cases of cutaneous anthrax.

An emergency of the disease was observed in 2012, when 54 anthrax outbreaks were notified to the OIE.

- The **peak** was recorded between July and October and the disease has mainly affected goats and sheeps.
- The OIE website reports that the most affected **sites** were in the districts of Gjirokastër, Vlorë and Kukës.

Anthrax in Albania: Animal vaccination

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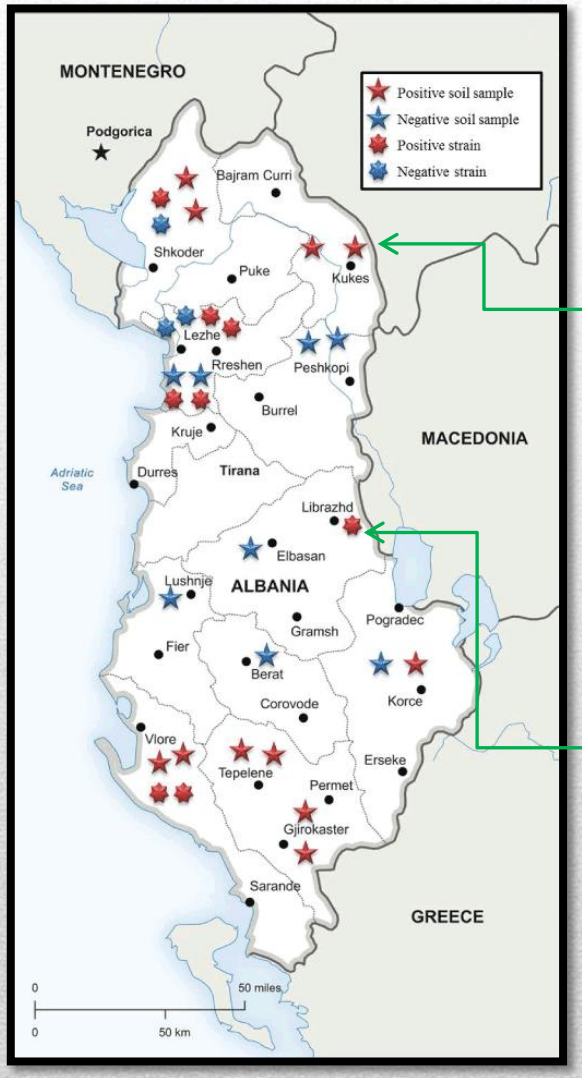
For the prophylaxis against animal anthrax, the live attenuated anthrax **Sterne vaccine** is used, produced in Albania.

The prophylaxis program provides the vaccination of animals for at least ten years since the last outbreak.

From 2009 to 2011, 3.008.278 doses of vaccine were administrated covering the 25,03% of the cattle population, 2,33% of the equine, 34,08% of the sheeps/goats and 0,29% of the pigs.

Anthrax in Albania: SAMPLES



Soil samples were collected in 19 burial sites of dead animals with suspicion of anthrax in the districts of: Shkodër, Vlorë, Lushnjë, Kurbin, Tepelenë, Dibër, Kukës, Elbasan, Korçë Berat and Gjirokastër.

Instituti i Sigurisë Ushqimore dhe Veterinarisë of Tirana sent to us 11 ***strains*** isolated from sheeps died in several districts of the Albanian territory.

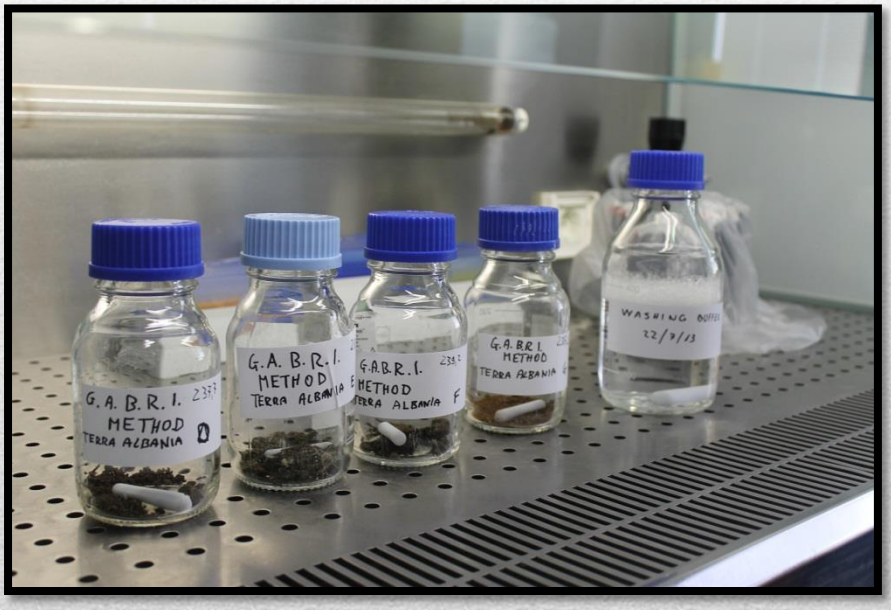
Anthrax in Albania: SAMPLING of soils samples

Soil samples were collected in 19 burial sites in which dead animals with suspicion of anthrax were buried.

Isolation of *B. anthracis* from soil samples

Ground Anthrax Bacillus Refined Isolation - G.A.B.R.I.- (Fasanella et al., 2013)

Briefly: 7.5 grams of soil were added to 22,5 ml of a 0.5% aqueous solution of Tween 20.

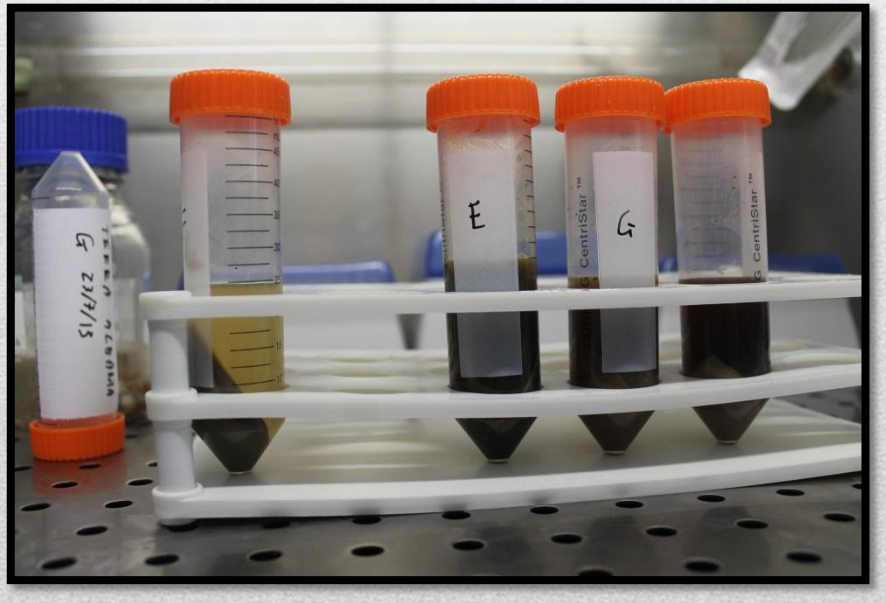


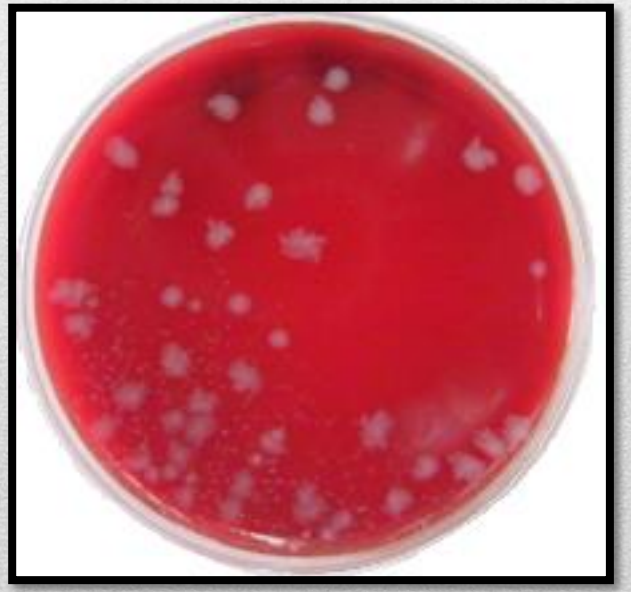
After washing in magnetic stirrer for 30 minutes, the suspension was centrifuged for 5 minutes at 2000 rpm and the supernatant incubated for 20 minutes at 64°C.

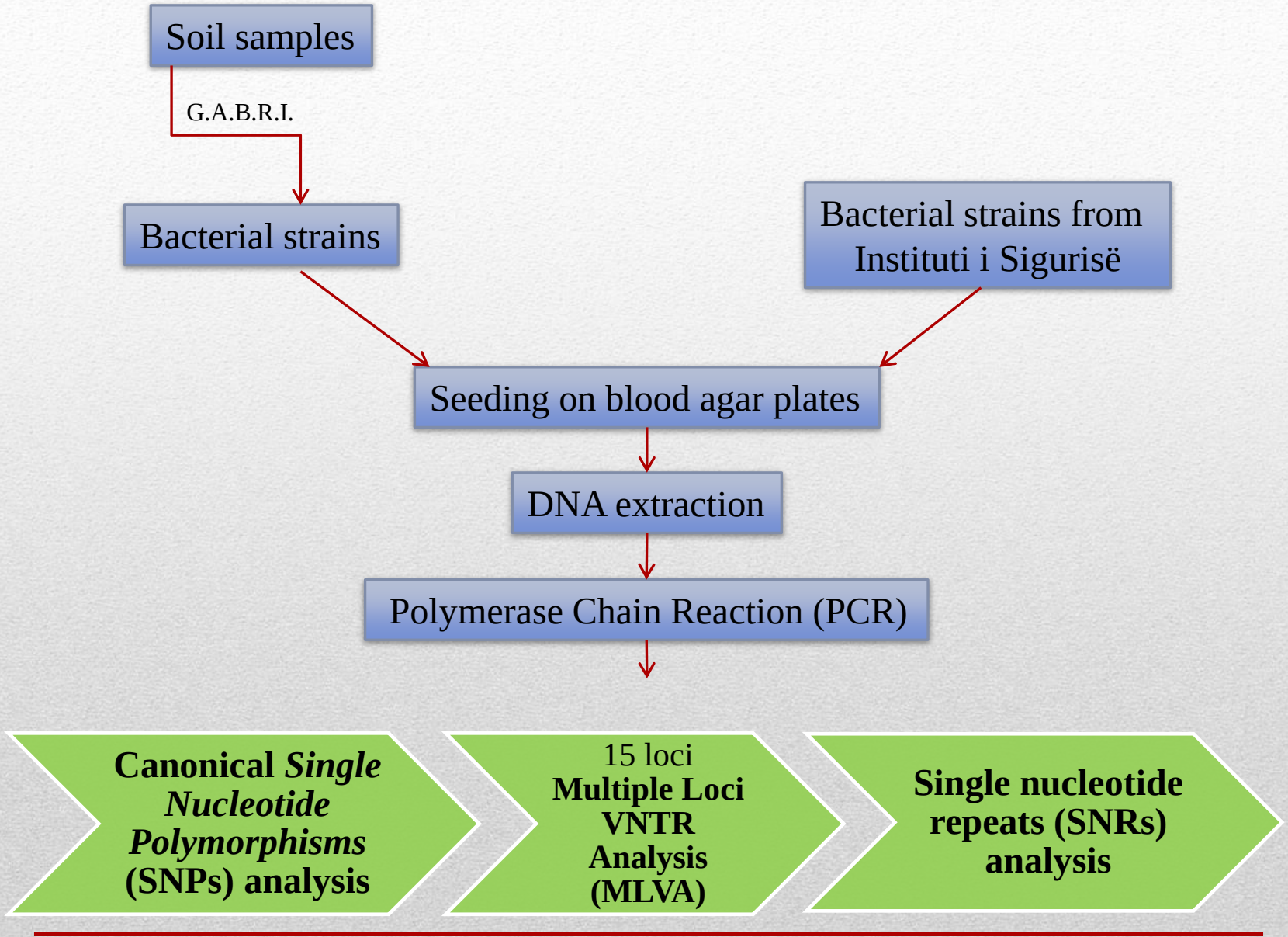
Isolation of *B. anthracis* from soil samples

G.A.B.R.I method (Fasanella et al., 2013)

After incubation the suspension was diluted 1:1 with Tryptose Phosphate Broth containing 125 µg/ml of Fosfomycin and 1 ml 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.






Soil samples
G.A.B.R.I.
Bacterial strains
Bacterial strains from Instituti i Sigurisë
Seeding on blood agar plates
DNA extraction
Polymerase Chain Reaction (PCR)
Canonical *Single Nucleotide Polymorphisms* (SNPs) analysis
15 loci
Multiple Loci VNTR Analysis (MLVA)
Single nucleotide repeats (SNRs) analysis

Anthrax in Albania: results on soil samples

- 11/19 samples were positive

- 13 **CanSNPs** → A.Br.008/009

MLVA test → 3 genotypes → Albania GT/1, Albania GT/2, Albania GT/3

Brief description of the results obtained on soil samples.

District	N° of collected soil samples	Result	CanSNP	Genotype
BERAT	1	1 negative	--	--
DIBËR	2	2 negative	--	--
ELBASAN	1	1 negative	--	--
GJIROKASTËR	2	2 positive	A.Br.008/009	Albania GT/1-GT/2
KORÇË	2	1 positive	A.Br.008/009	Albania GT/3
		1 negative	--	--
KUKËS	2	1 positive	A.Br.008/009	Albania GT/2
		1 positive*	A.Br.008/009	Albania GT/2-GT/3
KURBIN	2	2 negative	--	--
LUSHNJË	1	1 negative	--	--
SHKODËR	2	2 positive	A.Br.008/009	Albania GT/2
TEPELENË	2	2 positive	A.Br.008/009	Albania GT/1
VLORË	2	2 positive	A.Br.008/009	Albania GT/1

* Sample in which two different genotypes were found.

- The analysis of additional CanSNP (14th) revealed that all isolates belong to sublineage A.Br.008/011.

Anthrax in Albania: results on strains

- 8/11 samples were positive

- 13 **CanSNPs** → A.Br.008/009

MLVA test → 3 genotypes → Albania GT/1, Albania GT/2, Albania GT/3

Brief description of results obtained on bacterial strains.

District	N° of collected strains	Animal	Result	CanSNP	Genotype
KURBIN	2	sheep	2 positive	A.Br.008/009	Albania GT/3
LEZHË	2	sheep	2 positive	A.Br.008/009	Albania GT/3
LEZHË	2	sheep	2 negative	--	--
LIBRAZHD	1	sheep	1 positive	A.Br.008/009	Albania GT/2
SHKODËR	1	sheep	1 negative	--	--
SHKODËR	1	sheep	1 positive	A.Br.008/009	Albania GT/2
VLORË	2	sheep	2 positive	A.Br.008/009	Albania GT/1

- The analysis of additional CanSNP (14th) revealed that all isolates belong to sublineage A.Br.008/011.

Anthrax in Albania: MLVA test

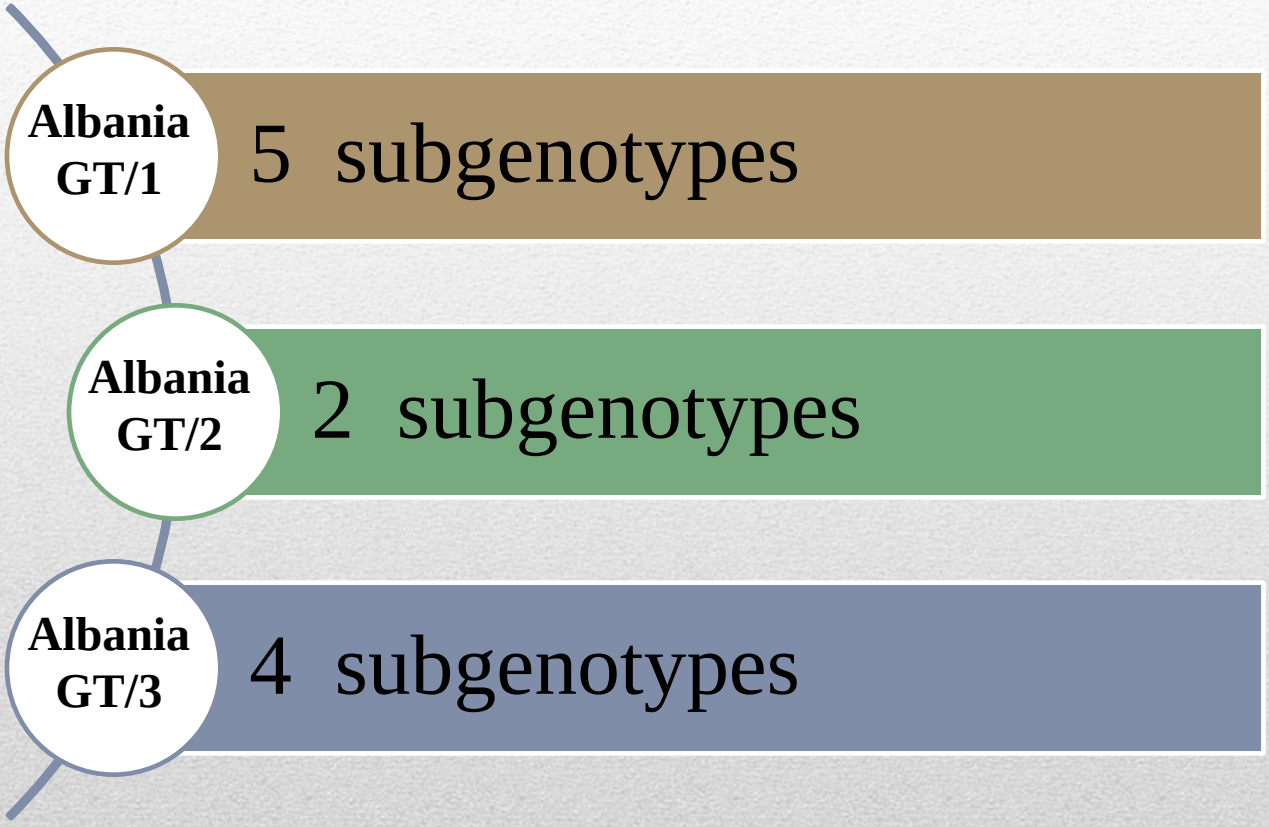
15-loci MLVA typing panel of each genotypes in the MLVA bank (http://mlva.u-psud.fr/) revealed that these three genotypes have never been reported in the literature.

Results of 15-loci MLVA analysis on *B. anthracis* strains circulating in Albania.

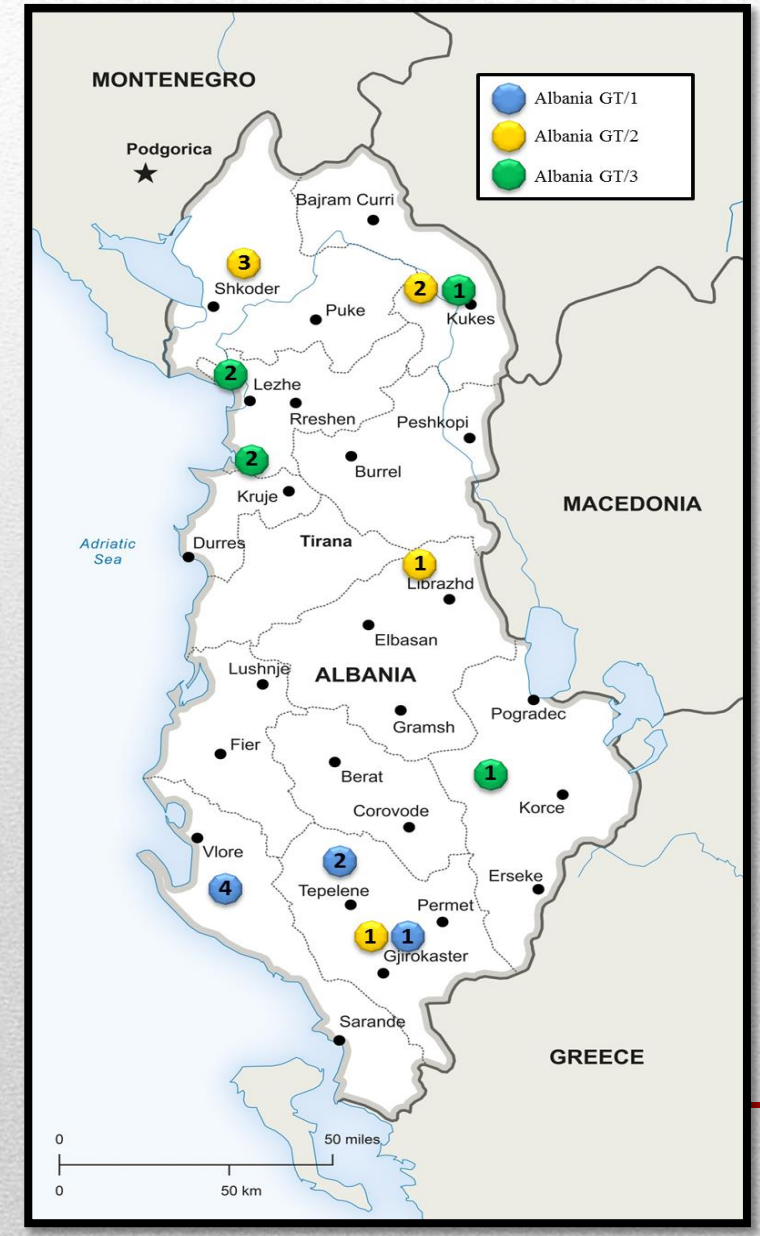
		Fragment size (bp) for each locus														
Genotype	**size**	**vrrA**	**vrrB1**	**vrrB2**	**vrrC1**	**vrrC2**	**CG3**	**pXO1**	**pXO2**	**VNTR12**	**VNTR16**	**VNTR17**	**VNTR19**	**VNTR23**	**VNTR32**	**VNTR35**
Ba001/1FG *Reference strain	obtained	306	223	154	620	594	148	130	133	110	262	382	90	193	501	109
	expected	314	229	162	616	604	153	135	137	115	273	386	96	197	485	110
Albania GT/1	obtained	**306**	223	**154**	620	594	148	130	133	110	262	390	93	193	501	109
	expected	**314**	229	**162**	616	604	153	135	137	115	273	394	99	197	485	110
Albania GT/2	obtained	**283**	223	**154**	620	594	148	130	133	110	262	390	93	193	501	109
	expected	**290**	229	**162**	616	604	153	135	137	115	273	394	99	197	485	110
Albania GT/3	obtained	**306**	223	**145**	620	594	148	130	133	110	262	390	93	193	501	109
	expected	**314**	229	**153**	616	604	153	135	137	115	273	394	99	197	485	110

*Ba001/1FG was analyzed by Van Ert et al. [12] with designation number A0280ITA and Keim et al. [17] with designation number K0021.

Anthrax in Albania: SNR



Distribution of Anthrax in Albania



First preliminary data:

Albania GT/1 results the most **geographically restricted** in the southern part of the country → the movements of the animals are much more controlled;

Albania GT/2 and Albania GT/3 result the **most widespread** genotypes;

The sampling of soils and strains in the central part of Albania **has proven difficult** because this area is most industrialized albanian region in which are few farms.

Particular case

In the same burial site, it was discovered 2 genotypes.

Brief description of the results obtained on soil samples.

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KURBIN	2	2 negative	--	--
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TEPELENË	2	2 positive	A.Br.008/009	Albania GT/1
VLORË	2	2 positive	A.Br.008/009	Albania GT/1

* Sample in which two different genotypes were found.

Why? Hypothesis:

- mutations during the incubation period of the disease or mixed infections (Beyer and Turnbull, 2013);
- the presence in the same burial site of two or more animals that died at different times and in different outbreaks.

Discussion and conclusions

- All the genotypes are genetically very similar to each other ⟶ hypothesis: all of them are the result of the evolution of a *local common ancestral strain*;
- The research should be extended to *other parts of Albanian territory* ⟶ the southern regions more close to *Turkey*, where the genetic variability of the circulating strains of *Bacillus anthracis* is very high.

Active surveillance
Environmental analysis

⟷

Passive surveillance
Strains analysis

The improvement of the **Public Health laboratories** as well as the **diagnostic procedures** could have a positive impact not only on the prevention and control of the disease but also on the trade of animals and their products in order to ensure the standard quality.

Thank you for your attention!