

Evaluation of *in vitro* antimicrobial susceptibility of *Bacillus anthracis* strains isolated during anthrax outbreaks in Italy from 1984 to 2017

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Anthrax, caused by *Bacillus anthracis*, is a non-contagious infectious disease that affects a wide range of animal species (primarily ruminants) including humans. Due to the often-fatal outcome in humans, quick administration of definitely effective antimicrobials is crucial either as prophylaxis or as a clinical case therapy. In this study, 110 *B. anthracis* strains, temporally, geographically, and genetically different, isolated during anthrax outbreaks in Italy from 1984 to 2017, were screened using a broth microdilution method to determine their susceptibility to 16 clinically relevant antimicrobial agents. The strains were isolated from various matrices (human, animal, and environmental samples) and were representative of thirty distinct genotypes previously identified by 15-loci multiple-locus variable-number of tandem repeats analysis. The antimicrobials tested were gentamicin, ceftriaxone, streptomycin, penicillin G, clindamycin, chloramphenicol, vancomycin, linezolid, cefotaxime, tetracycline, erythromycin, rifampin, amoxicillin, ciprofloxacin, doxycycline, and trimethoprim. All isolates were susceptible to most of the tested antimicrobials, with the exception of trimethoprim for which all of them showed high minimal inhibitory concentration values. An intermediate level of susceptibility was recorded for ceftriaxone and cefotaxime. Although the Centers for Disease Control and Prevention recommend the use of doxycycline, ciprofloxacin, penicillin G, and amoxicillin for treatment of human cases and for post-exposure prophylaxis to anthrax spores, this study shows a high degree of *in vitro* susceptibility of *B. anthracis* to many other antimicrobials, suggesting the possibility of an alternative choice for prophylaxis and therapy.

Keywords: *Bacillus anthracis*; Antimicrobial susceptibility testing; Anthrax treatment; Minimum inhibitory concentration

Introduction

Anthrax is a serious infectious disease, that mainly affects ruminants (farmed and wild animals) and can be transmitted to humans exposed to infected animals or infected products of animal origin (farmers, vets, butchers, carders, workers in the textile industry, etc.) [7]. The etiological agent of anthrax is *Bacillus anthracis*, a Gram-positive rod-shaped bacterium able to produce extremely resistant spores that, under unfavorable environmental conditions, can survive in the environment for several decades.

In particularly receptive animals (cattle, buffalo, sheep, goats, deer, and other wild ungulates), anthrax develops without

apparent symptoms but has a fatal outcome characterized by sudden death due to acute or hyperacute septicemia and leakage of uncoagulated blood from natural openings.

In humans, anthrax can occur in three different forms with clinical signs dependent on the penetration route of the pathogen: cutaneous (generally non-fatal), pulmonary, and gastrointestinal [7]. Moreover, another fatal form has been described in drug users occurring after the intravenous injection of drugs contaminated with anthrax spores [2].

B. anthracis lives most of its existence in the form of a spore in soil, until ideal conditions develop allowing it to enter the reproductive cycle, which is presented mainly in domestic and wild ruminants. In animals, *B. anthracis* expresses its

Received 20 Aug. 2018, Revised 10 Nov. 2018, Accepted 30 Nov. 2018

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This study was presented in part at the 16th Medical Biodefense Conference; October 28–31, 2018; Munich, Germany.

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pISSN 1229-845X
eISSN 1976-555X

pathogenic activity primarily through the antiphagocytic activity of the capsule (whose genes are localized on the pXO2 plasmid) and the production of a toxic complex consisting of three proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF) (whose genes are localized on the pXO1 plasmid) [7].

In Italy, animal anthrax is an endemic disease and most of the recorded animal outbreaks are characterized as sporadic episodes occurring usually during summer and after a rainy spring. The occurrence of new cases is generally linked to climate changes (with major incidences under warmer and extreme weather) or human activities that interfere with soil surfaces (drainage- or excavation-related work). Cases typically involve a low number of animals (one to three on average) grazing on contaminated pastures. With the exception of rare outbreaks, the disease is predominantly diffused in southern and island regions of Italy, especially in Basilicata, Apulia, and Sicily [9]. In Italy, over the last 50 years, about 200 outbreaks of animal anthrax have been recorded, while, more recently, between 2006 and 2017, 5 human cases of cutaneous anthrax occurred (unpublished data).

Furthermore, *B. anthracis* is considered one of the most important agents of bioterrorism. Because of its characteristics, it can be potentially misused as a biological weapon, as was confirmed by bioterrorism events in the United States in 2001 [10]. Due to the often-fatal outcome of human cases, quick administration of definitely effective antimicrobials is crucial either in prophylaxis, after presumptive exposure, or for therapy of clinical cases.

Materials and Methods

B. anthracis strains

The *B. anthracis* strains selected for this study are members of the strain collection of the Anthrax Reference Institute of Italy (Ce.R.N.A.) located at the Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata. The selected strains were isolated in Italy between 1984 and 2017 from different outbreaks and various sources: 2 strains were isolated from two different cases of human cutaneous anthrax, 102 were isolated from animals (50 from cattle, 34 from sheep, 10 from goats, 5 from horses, 1 from donkey, 1 from deer, and 1 from a pig), and 6 were isolated from environmental samples (1 from water and 5 from soils) (Table 1). In addition, 3 *B. anthracis* vaccine strains were included: *B. anthracis* Sterne 34F2 (pXO1⁺, pXO2⁻), Carbosap (pXO1⁺, pXO2⁺), and Pasteur I type (pXO1⁻, pXO2⁺).

All strains have been identified and characterized to assess the presence of plasmids pXO1 and pXO2 by polymerase chain reaction (PCR) as previously described [8]. Moreover, all strains were genotyped by 15-loci multiple-locus variable-number of tandem repeats analysis (MLVA) as reported by Van Ert *et al.* [13]. All work involving live *B. anthracis* was

Table 1. Characteristics and genotypes of the *Bacillus anthracis* strains tested in this study

Isolate	Year	Source	pXO1	pXO2	MLVA 15 genotype
1	1984	Sheep	+	+	25
2	1984	Sheep	+	+	1
3	1984	Sheep	+	+	11
4	1984	Sheep	+	+	2
5	1984	Sheep	+	+	4
6	1984	Sheep	+	+	1
7	1984	Sheep	+	+	27
8	1984	Cattle	+	+	27
9	1984	Cattle	+	+	28
10	1984	Horse	-	+	8
11	1993	Sheep	-	+	21
12	1993	Sheep	+	+	23
13	1993	Goat	-	+	8
14	1993	Sheep	+	+	10
15	1993	Sheep	+	+	21
16	1995	Sheep	+	+	27
17	1995	Cattle	+	+	29
18	1996	Donkey	+	+	2
19	1996	Goat	+	+	16
20	1997	Cattle	+	+	18
21	1997	Sheep	+	+	20
22	1997	Sheep	+	+	20
23	1997	Goat	+	+	16
24	1998	Goat	+	+	21
25	1999	Cattle	+	+	10
26	1999	Cattle	+	+	24
27	1999	Human	+	+	2
28	1999	Sheep	+	+	13
29	1999	Cattle	+	+	5
30	2000	Sheep	+	+	21
31	2000	Sheep	+	+	7
32	2000	Cattle	+	+	22
33	2001	Sheep	-	+	12
34	2001	Cattle	+	+	21
35	2001	Sheep	+	+	1
36	2002	Cattle	+	+	11
37	2002	Sheep	+	+	2
38	2002	Sheep	+	+	21
39	2002	Goat	+	+	21
40	2002	Cattle	-	+	2
41	2002	Cattle	+	+	2
42	2004	Cattle	+	+	15
43	2004	Horse	+	+	1
44	2004	Deer	+	+	1
45	2004	Goat	+	+	1
46	2004	Cattle	+	+	1
47	2004	Sheep	+	+	1
48	2004	Goat	+	+	1
49	2004	Goat	+	+	1

Table 1. Continued

Isolate	Year	Source	pXO1	pXO2	MLVA 15 genotype
50	2004	Pigs	+	+	1
51	2005	Goat	+	+	21
52	2005	Cattle	+	+	19
53	2005	Cattle	+	+	19
54	2005	Cattle	+	+	19
55	2005	Cattle	+	+	19
56	2006	Sheep	+	+	4
57	2006	Soil	+	+	1
58	2006	Cattle	+	+	1
59	2006	Cattle	+	+	2
60	2007	Cattle	+	+	30
61	2008	Sheep	+	+	21
62	2008	Cattle	+	+	2
63	2008	Water	+	+	2
64	2008	Soil	+	+	2
65	2009	Cattle	+	+	3
66	2009	Soil	+	+	10
67	2009	Cattle	+	+	17
68	2009	Sheep	+	+	21
69	2009	Cattle	+	+	17
70	2009	Cattle	+	+	17
71	2009	Cattle	+	+	17
72	2009	Cattle	+	+	17
73	2009	Sheep	+	+	1
74	2009	Cattle	+	+	1
75	2011	Sheep	+	+	21
76	2011	Cattle	+	+	6
77	2011	Horse	+	+	21
78	2011	Horse	+	+	6
79	2011	Cattle	+	+	6
80	2011	Sheep	+	+	6
81	2011	Cattle	+	+	6
82	2011	Cattle	+	+	21
83	2011	Horse	+	+	21
84	2011	Sheep	+	+	21
85	2011	Sheep	+	+	21
86	2012	Cattle	+	+	17
87	2012	Cattle	+	+	17
88	2012	Cattle	+	+	17
89	2012	Human	+	+	26
90	2012	Soil	+	+	1
91	2012	Cattle	+	+	2
92	2012	Cattle	+	+	2
93	2012	Cattle	+	+	2
94	2012	Cattle	+	+	2
95	2013	Soil	+	+	1
96	2014	Cattle	+	+	1
97	2014	Cattle	+	+	6
98	2014	Cattle	+	+	18
99	2016	Sheep	+	+	21

Table 1. Continued

Isolate	Year	Source	pXO1	pXO2	MLVA 15 genotype
100	2016	Goat	+	+	1
101	2016	Sheep	+	+	4
102	2016	Sheep	+	+	4
103	2016	Sheep	+	+	4
104	2016	Cattle	+	+	19
105	2016	Cattle	+	+	19
106	2016	Cattle	+	+	19
107	2017	Cattle	+	+	15
108	2017	Cattle	+	+	15
109	2017	Cattle	+	+	15
110	2017	Cattle	+	+	15

MLVA, multiple-locus variable-number of tandem repeats analysis.

performed in a biosafety level 3 laboratory within a class II safety cabinet.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by applying a broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [4,5]. The following most-common broad-spectrum antimicrobials, all from Acros Organics (Thermo Fisher Scientific, USA), were used at the indicated concentrations: gentamicin (0.008–16 µg/mL), streptomycin (0.015–32 µg/mL), penicillin G (0.001–2 µg/mL), amoxicillin (0.008–16 µg/mL), clindamycin (0.008–16 µg/mL), chloramphenicol (0.06–128 µg/mL), vancomycin (0.06–64 µg/mL), linezolid (0.06–64 µg/mL), erythromycin (0.008–16 µg/mL), tetracycline (0.008–16 µg/mL), ciprofloxacin (0.004–8 µg/mL), doxycycline (0.004–4 µg/mL), rifampin (0.004–8 µg/mL), cefotaxime (0.25–512 µg/mL), ceftriaxone (0.25–512 µg/mL), and trimethoprim (0.125–128 µg/mL). Each molecule was dissolved in the appropriate solvent and two-fold diluted in cation-adjusted Mueller Hinton broth (CAMHB) (BD Biosciences, USA) using microplates (100 µL/well).

Overnight cultures of each *B. anthracis* strain were suspended in sterile saline to match a 0.5 McFarland standard using a densitometer. The cell suspensions were further diluted 1:100 in CAMHB to obtain a bacterial concentration of approximately $1-5 \times 10^4$ CFU/mL (CFU, colony-forming unit), and 100 µL bacterial aliquots were added to each well of the microplate.

Two microliters of a resazurin solution (6.75 mg/mL) (Sigma-Aldrich, USA) were added to each well to assess bacterial growth [11], and the plates were incubated overnight at 37°C. The color change caused by the resazurin was observed in the plate wells: a change from purple to pink/colorless indicated cell growth. The lowest concentration of each antibiotic that prevented bacterial growth was deemed the

minimal inhibitory concentration (MIC). The use of resazurin allowed easy detection of microbial growth in extremely small solution volumes in microtiter plates; thus, there was no need for spectrophotometry of the plate samples.

The reported CLSI breakpoints ($\mu\text{g/mL}$) for *B. anthracis* were used for penicillin, amoxicillin, ciprofloxacin, doxycycline, and tetracycline while the breakpoints for *Bacillus* spp. were used for the cephem antibiotics [4]. Interpretative criteria for *Staphylococcus* spp. were used for gentamicin, clindamycin, chloramphenicol, vancomycin, linezolid, erythromycin, rifampin, and trimethoprim, according to CLSI guidelines [5]. As CLSI breakpoints for streptomycin were not available, it was not possible to give any comparable interpretation to the activity of this antimicrobial.

To check the validity of the antimicrobial susceptibility testing method, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were tested in parallel as control strains.

Results

The *B. anthracis* pXO1 plasmid was not detected by PCR in five of the examined strains, including the Pasteur I type vaccine strain, whereas the pXO2 plasmid of *B. anthracis* was present in all examined strains except the Sterne vaccine strain (Table 1). In total, 30 different genotypes were identified by the 15-loci MLVA (Table 1), and to each genotype, an identification

number was arbitrarily attributed by Ce.R.N.A. (data not shown). The concentration that inhibited the growth of 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates, the MIC range of each antimicrobial substance tested, and the susceptibility percentages are shown in Table 2.

Discussion

Antimicrobial susceptibility testing has a key role in the management of anthrax infections. In this study, a total of 110 *B. anthracis* strains, isolated in Italy between 1984 and 2017 were studied to determine their susceptibility to antimicrobials. The presence of virulence factors, which were evaluated by PCR, showed that all isolates, except the Sterne vaccine strain, harbored the *capC* gene (on plasmid pXO2). The *pagA*, *lef*, and *cya* genes (on plasmid pXO1 and encoding PA, LF, and EF, respectively) were not detected in five of the tested isolates. However, it appeared that the susceptibility to antimicrobials of isolates lacking plasmids was comparable to those with both pXO1 and pXO2 plasmids.

All isolates were susceptible to most of the tested antimicrobial with the exception of trimethoprim, as expected [1], for which a high level of resistance was observed, whereas for ceftriaxone and cefotaxime intermediate susceptibility levels were recorded. The results are in agreement with other previously published studies [6]. Moreover, all 30 investigated genotypes showed no difference in antimicrobial susceptibility,

Table 2. Antibiotic susceptibilities of 110 *Bacillus anthracis* strains isolated during anthrax outbreaks in Italy

Antimicrobial agent	MIC ($\mu\text{g/mL}$)			MIC breakpoints ($\mu\text{g/mL}$)			Percent of isolates with the following interpretation		
	Range	50% *	90% *	S (\leq)	I	R (\geq)	S	I	R
Gentamicin	0.008–16	0.125	0.25	4	8	16	100	–	–
Streptomycin	0.015–32	0.5	1	–	–	–	–	–	–
Penicillin G	0.001–2	0.015	0.03	0.5	–	1	100	–	–
Amoxicillin	0.008–16	0.06	0.06	0.12	–	0.25	100	–	–
Clindamycin	0.008–16	0.125	0.25	0.5	1–2	4	100	–	–
Chloramphenicol	0.06–128	4	4	8	16	32	100	–	–
Vancomycin	0.06–64	2	2	2	4–8	16	100	–	–
Linezolid	0.06–64	1	2	4	–	8	100	–	–
Erythromycin	0.008–16	0.125	0.25	0.5	1–4	8	100	–	–
Tetracycline	0.008–16	0.06	0.125	1	–	–	100	–	–
Ciprofloxacin	0.004–8	0.03	0.03	0.25	–	–	100	–	–
Doxycycline	0.004–4	0.015	0.03	1	–	–	100	–	–
Rifampin	0.004–8	0.125	0.25	1	2	4	100	–	–
Ceftriaxone	0.25–512	16	16	8	16–32	64	–	100	–
Cefotaxime	0.25–512	16	16	8	16–32	64	–	100	–
Trimethoprim	0.125–128	> 128	> 128	8	–	16	–	–	100

MIC, minimal inhibitory concentration; S, susceptible; I, intermediate; R, resistant. *MIC at which 50% or 90% of tested isolates are inhibited.

probably due to the high genetic homogeneity of *B. anthracis*.

There are few reports on the antimicrobial susceptibility profiles of *B. anthracis*. This can be probably explained by the rarity of human anthrax in industrialized and developed countries, where the most of the scientific research is concentrated, by the elaboration of effective control programs over the last 50 years and by efficient therapies with well-known antimicrobials such as amoxicillin, doxycycline, ciprofloxacin, and penicillin [3,12]. The latter are the antibiotics of first choice, as recommended by the Centers for Disease Control and Prevention, for the treatment of anthrax in human cases.

In order to be effective, an antimicrobial treatment should be timely, starting immediately after infection. Healing is possible if you intervene in the initial stages of the disease, and the late administration of antibiotics is often useless. The results in the present study suggest a high degree of *in vitro* susceptibility of *B. anthracis* to many antimicrobials. The effective activity of gentamicin, streptomycin, clindamycin, chloramphenicol, vancomycin, linezolid, tetracycline, erythromycin, and rifampin, on all tested *B. anthracis* strains demonstrates several appropriate alternative antimicrobials for *B. anthracis* prophylaxis and/or treatment. All of the tested *B. anthracis* strains showed intermediate susceptibility to cepheims (cefotaxime and ceftriaxone) and resistance to trimethoprim. The resistance of *B. anthracis* to trimethoprim supports a previous report of an intrinsic resistance of *B. anthracis* to this compound [1].

The relationship between the indications obtainable from *in vitro* tests and the real clinical efficacy of an antimicrobial is obviously not absolute and depends on a complex set of factors including the effective *in vivo* role of the microorganism and the correct modality and timing of administration of the antimicrobial, as well as the pharmacokinetic and pharmacodynamic characteristics of the antimicrobial.

Interestingly, in this study, we demonstrated that the *B. anthracis* isolates, belonging to different genotypes and isolated from different sources over a long period (1984–2017) showed the same levels of susceptibility to the tested antimicrobials. Further monitoring of the antibiotic susceptibility of *B. anthracis* is important for the identification of resistant clones and for choosing the best therapeutic strategy when an anthrax outbreak occurs.

Acknowledgments

The authors would like to thank Michela Iatarola, Angela Aceti, Elena Poppa and Francesco Tolve for the skillful technical support. This study was funded by the Ministry of Health of Italy, Current Research 2017, project “IZSPB 03/17”, which supported the work of Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata. The funders had no role in study design, data collection, and interpretation, or the decision to submit the work for publication.

Conflict of Interest

The authors declare no conflicts of interest.

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