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ANTIMICROBIAL SUSCEPTIBILITY OF *BACILLUS ANTHRACIS* STRAINS FROM HUNGARY

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The susceptibility of 29 *Bacillus anthracis* strains, collected in Hungary between 1933 and 2014, was tested to 10 antibiotics with commercially available minimum inhibitory concentration (MIC) test strips. All strains were susceptible to amoxicillin, ciprofloxacin, clindamycin, doxycycline, gentamicin, penicillin, rifampicin, and vancomycin. Intermediate susceptibility to erythromycin and cefotaxime was detected in 17.2% (5/29) and 58.6% (17/29) of the strains, respectively. Correlations were not observed between the isolation date, location, host species, genotype, and antibiotic susceptibility profile of strains.

Key words: Antibiotic susceptibility, anthrax, *Bacillus anthracis*, Hungary

Bacillus anthracis, the causative agent of anthrax, is a spore-forming, Gram-positive, zoonotic bacterium, a potential biological warfare agent which primarily infects herbivores, but can cause serious disease in humans with cutaneous, gastrointestinal, pulmonary, and injectional forms (WHO, 2008; Fasanella et al., 2010; Hanczaruk et al., 2014). The extremely resistant spore is able to survive in the soil for decades (Fasanella et al., 2010). *Bacillus anthracis* is susceptible to most antibiotics used in therapy (Lightfoot et al., 1990; Doğanay and Aydin, 1991; Odendaal et al., 1991; Cavallo et al., 2002; Coker et al., 2002; Mohammed et al., 2002; Turnbull et al., 2004; Luna et al., 2007; Caplan et al., 2009; Habrun et al., 2011; Ortatatli et al., 2012; Quinn et al., 2011). The main recommended antibiotics to treat human anthrax cases are ciprofloxacin and doxycycline, apart from penicillin (WHO, 2008). Animals showing clinical signs of anthrax are also treated with different antibiotics, mainly penicillin and oxytetracycline (Quinn et al., 2011).

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Table 1: Epidemiological data and minimum inhibitory concentration values of 10 antibiotics and details of 29 Hungarian *Bacillus anthracis* strains and *Staphylococcus aureus* ATCC 29213^{T} reference strain. Colour codes: white – susceptible, grey – intermediate

Strain ID	Location of isolation	Date of isolation	Host	Genotype	MIC (mg/L)									
					Amo	Cef	Cip	Cli	Dox	Ery	Gen	Pen	Rif	Van
BA1	Nógrádmegyer	1990s		A.Br.Ba1168	0.032	12	0.032	0.125	0.023	0.25	0.094	< 0.016	0.125	0.75
BA2	Ladánybene	1990s		A.Br.Ba1168	0.023	8	0.047	0.125	0.023	0.5	0.094	< 0.016	0.25	0.75
BA3	Szűgy	2003	cattle	A.Br.Ba1168	0.023	8	0.047	0.125	0.023	0.5	0.094	< 0.016	0.125	0.5
BA4	Jászladány	2013	sheep	A.Br.A0362	0.023	12	0.064	0.25	0.047	0.75	0.125	0.023	0.19	0.75
BA5	Debrecen	2014	lion	A.Br.Ba1168	0.032	8	0.032	0.125	0.023	0.5	0.125	< 0.016	0.38	1
BA7	Tiszafüred	2014	cattle	A.Br.A0362	0.023	16	0.032	0.19	0.032	0.5	0.094	0.023	0.125	0.5
BA8	Egyek	2014	cattle	A.Br.A0362	0.023	12	0.047	0.19	0.032	0.38	0.125	< 0.016	0.25	1.0
BA11	Tiszafüred	2014	cattle	A.Br.A0362	< 0.016	0.25	0.032	0.125	0.032	0.125	0.064	< 0.016	0.004	1.0
BA12		1989		A.Br.008	0.023	6	0.023	0.094	0.023	0.38	0.047	< 0.016	0.19	0.75
BA15		1997		A.Br.A0060	0.023	16	0.047	0.19	0.032	0.5	0.125	< 0.016	0.19	1
BA19				A.Br.008	0.032	16	0.064	0.25	0.032	0.5	0.125	< 0.016	0.19	1
BA20				B.Br.CNEVA	0.032	16	0.023	0.19	0.032	0.75	0.125	0.023	0.19	1
BA21	Tasnád*			A.Br.A0362	0.032	12	0.032	0.19	0.032	0.5	0.19	< 0.016	0.25	0.75
BA23				A.Br.A0362	0.023	16	0.047	0.19	0.047	0.75	0.19	0.023	0.19	0.75
BA25				A.Br.008	0.032	16	0.032	0.125	0.023	0.5	0.094	< 0.016	0.19	0.75
BA27				A.Br.008	0.023	16	0.047	0.25	0.023	0.5	0.094	< 0.016	0.25	0.75
BA28		1936	sheep	A.Br.Ba1168	< 0.016	1	0.047	0.125	0.032	0.25	0.094	< 0.016	0.19	1.5
BA29	Piricse	before 1938		A.Br.Ba1168	0.023	6	0.032	0.25	0.032	0.5	0.19	< 0.016	0.25	1.5
BA31	Keszthely	before 1938		A.Br.Ba1168	0.023	6	0.032	0.094	0.023	0.5	0.25	< 0.016	0.38	0.75
BA32	Alcsút			A.Br.Ba1168	0.023	8	0.032	0.094	0.023	0.38	0.125	< 0.016	0.25	1
BA33		1939	sheep	A.Br.Ba1168	0.016	12	0.047	0.125	0.032	0.25	0.25	< 0.016	0.19	0.75
BA34	Keszthely	before 1938		A.Br.008	0.016	12	0.047	0.19	0.032	0.5	0.25	< 0.016	0.25	1
BA36				B.Br.CNEVA	0.023	12	0.032	0.19	0.047	0.75	0.19	0.023	0.094	1
BA38				A.Br.008	0.023	8	0.047	0.25	0.023	0.38	0.094	< 0.016	0.25	2
BA39	Kartal			A.Br.Ba1168	0.032	16	0.047	0.25	0.047	0.5	0.38	0.023	0.38	1.5
BA44				A.Br.008	0.064	2	0.064	0.125	0.032	0.5	0.19	0.023	0.5	1.5
BA46	Mád			A.Br.Ba1168	0.016	1	0.032	0.19	0.023	0.25	0.047	< 0.016	0.25	1
BA47				B.Br.CNEVA	0.032	16	0.047	0.19	0.032	0.5	0.125	< 0.016	0.094	1
BA49	Tiszafüred	2014	cattle	A.Br.A0362	0.023	12	0.064	0.19	0.032	0.75	0.25	< 0.016	0.19	0.75
S. aureus ATCC29213^{T}					0.5	0.75	0.125	0.125	0.19	0.38	1.5	0.094	0.004	0.38

*formerly part of Hungary, now Romanian territory

Commercially available minimum inhibitory concentration (MIC) test strips (Etest, BioMérieux Inc., Marcy l'Etoile, France) proved to be a simple and flexible method to determine the antibiotic susceptibility profiles with MIC values of *B. anthracis* strains. The effectiveness of the strips is comparable to that of conventional methods used for MIC value determination (Turnbull et al., 2004; Luna et al., 2007).

The aim of this study was to determine the susceptibility of *B. anthracis* strains from Hungary to 10 antibiotics with commercially available MIC test strips.

Materials and methods

The study involved 29 *B. anthracis* strains isolated from diverse host species in various parts of Hungary between 1933 and 2014 (Table 1). The strains were identified and genotyped as described previously. Briefly, the strains were identified on species level with a dual-probe TaqMan assay targeting a single nucleotide polymorphism (SNP) in the *plcR* gene (Easterday et al., 2005). The genotypes of the isolates were determined with melt mismatch amplification mutation assays according to Birdsell et al. (2012) targeting the SNPs identified by Van Ert et al. (2007).

The susceptibility of the strains to 10 antimicrobial agents (amoxicillin, cefotaxime, ciprofloxacin, clindamycin, doxycycline, erythromycin, gentamicin, penicillin, rifampicin, and vancomycin) was determined with commercially available MIC test strips (Etest, BioMérieux Inc., Marcy l'Etoile, France). *Staphylococcus aureus* ATCC 29213^T served as a positive control. The strains were cultured on Mueller-Hinton agar plates overnight at 37 °C. Colony suspensions in 3 ml of physiological saline, equalled to a density of 0.5 McFarland, were consistently taken on 5 mm-thick Mueller-Hinton agar with sterile swab. An E-test strip was placed on each plate after approximately 10 min, to allow absorption of excess moisture into the agar. The plates were incubated at 37 °C for 20 h and MIC values were read according to the manufacturer's instructions. We interpreted the breakpoints according to Clinical and Laboratory Standards Institute (CLSI, 2011) standards for *B. anthracis* and CLSI standards for *Staphylococcus* sp. where standards were unavailable for *B. anthracis* (Table 2). All *B. anthracis* manipulations were performed in a biosafety level 3 laboratory using class III biosafety cabinet.

Results

Based on the MIC_{90} values all Hungarian *B. anthracis* isolates were susceptible to penicillin (0.023 mg/L), amoxicillin (0.032 mg/L), ciprofloxacin (0.064 mg/L), doxycycline (0.047 mg/L), rifampicin (0.38 mg/L), vancomycin

(1.5 mg/L), gentamicin (0.25 mg/L), and clindamycin (0.25 mg/L) (Tables 1 and 2). Most of the strains (24/29, 82.8%) were susceptible to erythromycin, but intermediately susceptible strains (5/29, 17.2%) also occurred. The strains were susceptible (12/29, 41.4%) or intermediately (17/29, 58.6%) susceptible to cefotaxime.

Although background information about the tested *B. anthracis* strains was limited, correlations were not observed between the isolation date, location, host species, genotype, and antibiotic susceptibility profile of the 29 *B. anthracis* strains (Table 1).

Table 2

Susceptibility profile of 29 *Bacillus anthracis* strains for 10 antimicrobial agents

Antibiotic	MIC (mg/L)			Breakpoints		% of isolates		
	Range	50%	90%	S (≤)	R (≥)	S	I	R
Amoxicillin	0.016–0.064	0.023	0.032	0.25	0.5[b]	100		
Cefotaxime	0.25–16	12	16	8	64[b]	41.4	58.6	
Ciprofloxacin	0.023–0.064	0.047	0.064	0.5	NA[a]	100		
Clindamycin	0.094–0.25	0.19	0.25	0.5	4[b]	100		
Doxycycline	0.023–0.047	0.032	0.047	1.0	NA[a]	100		
Erythromycin	0.125–0.75	0.5	0.75	0.5	8[b]	82.8	17.2	
Gentamicin	0.047–0.38	0.125	0.25	4	16[b]	100		
Penicillin	< 0.016–0.023	< 0.016	0.023	0.12	0.25[a]	100		
Rifampicin	0.004–0.5	0.19	0.38	1	4[b]	100		
Vancomycin	0.5–2	1	1.5	4	32[b]	100		

MIC: minimum inhibitory concentration; NA: data not available; S: susceptible; I: intermediate; R: resistant; [a]CLSI standard breakpoints for *B. anthracis;* [b]CLSI standard breakpoints for *Staphylococcus* sp.

Discussion

The objective of this work was to study the antibiotic susceptibility profile of *B. anthracis* strains from Hungary. Eight of the ten examined antibiotics also appear on the list of antibiotics recommended for human anthrax treatment (WHO, 2008).

Penicillin G is the primary recommended antibiotic to treat cutaneous anthrax cases without complications. Amoxicillin can be an alternative to penicillin (WHO, 2008). Penicillin and amoxicillin resistance was confirmed in several studies, within a range of 1–12% of the strains examined (Lightfoot et al., 1990; Cavallo et al., 2002; Coker et al., 2002; Mohammed et al., 2002; Turnbull et al., 2004). All Hungarian strains, however, proved to be highly susceptible to penicillin and amoxicillin.

Erythromycin is also a possible alternative in case of penicillin allergy (WHO, 2008). Moderately sensitive strains appeared in several studies (Cavallo et al., 2002; Mohammed et al., 2002; Luna et al., 2007; Ortatatli et al., 2012), and

two resistant strains were described in Turkey (Ortatatli et al., 2012). In the current study, 17.2% of the Hungarian strains were only intermediately sensitive to erythromycin. These data suggest that erythromycin should be avoided in anthrax treatment in Hungary.

Ciprofloxacin and doxycycline are recommended in life-threatening cases and in case of penicillin allergy (WHO, 2008). Ciprofloxacin resistance has not been described yet. Resistance against doxycycline was observed in one strain (MIC 4 mg/L) in Turkey (Ortatatli et al., 2012). All examined strains from Hungary were susceptible to both antibiotics.

Rifampicin is recommended as complementary treatment in anthrax meningoencephalitis, clindamycin and vancomycin in inhalation anthrax, and gentamicin in gastrointestinal anthrax (WHO, 2008). Rifampicin, vancomycin and clindamycin resistance has not been reported yet. Gentamicin resistance was observed in two cases (MIC = 32 mg/L) in Turkey (Ortatatli et al., 2012). All Hungarian strains proved to be susceptible to rifampicin, vancomycin, clindamycin, and gentamicin as well.

Most *B. anthracis* stains are resistant or intermediately susceptible to cefotaxime according to previous findings (Doğonay and Aydin, 1991; Odendaal et al., 1991; Turnbull et al., 2004; Habrun et al., 2011; Ortatatli et al., 2012). Turnbull et al. (2004) found one susceptible strain (MIC 3 mg/L) with the Etest method, while Doğanay and Aydin (1991) described five susceptible strains with the disc diffusion test. In the present study, 41.4% of the Hungarian strains were susceptible to cefotaxime. Additionally, no resistant strains were identified. The MIC_{90} values proved to be lower in our study compared to other studies (Doğanay and Aydin, 1991; Odendaal et al., 1991; Turnbull et al., 2004; Habrun et al., 2011; Ortatatli et al., 2012).

In conclusion, despite the temporal, geographic, host and genetic diversity of the tested *B. anthracis* isolates, their susceptibility profiles were highly similar. According to the present study, penicillin, amoxicillin, ciprofloxacin and doxycycline are the primary choices to treat anthrax, but gentamicin, vancomycin, rifampicin and clindamycin could also be used against *B. anthracis* in Hungary. The application of erythromycin and cefotaxime should be avoided in the treatment of anthrax.

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