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European multi-centre study to establish MIC and zone diameter epidemiological cut-off values for *Bacillus anthracis*

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ABSTRACT

Objectives: *Bacillus anthracis* clinical breakpoints, representing a systematic approach to guide clinicians in selecting the most appropriate antimicrobial treatments, are not part of the guidance from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). This is because defined distributions of MIC values and of epidemiological cut-off values (ECOFFs) have been lacking. In this study, a Europe-wide network of laboratories in collaboration with EUCAST, aimed at establishing standardized antimicrobial susceptibility testing methods, wild-type MIC distributions, and ECOFFs for ten therapeutically relevant antimicrobials.

Methods: About 335 *B. anthracis* isolates were tested by broth microdilution and disc diffusion methodologies. MIC and inhibition zone diameters were curated according to EUCAST SOP 10.2 and the results were submitted to EUCAST for ECOFFs and clinical breakpoint determination.

Results: Broth microdilution and disc diffusion data distributions revealed putative wild-type distributions for the tested agents. For each antimicrobial agent, ECOFFs were defined. Three highly resistant strains with MIC values of 32 mg/L benzylpenicillin were found. MIC values slightly above the defined ECOFFs were observed in a few isolates, indicating the presence of resistance mechanisms to doxycycline, tetracycline, and amoxicillin.

Discussion: *B. anthracis* antimicrobial susceptibility testing results were used by EUCAST to determine ECOFFs for ten antimicrobial agents. The MIC distributions were used in the process of determining clinical breakpoints. The ECOFFs can be used for the sensitive detection of isolates with resistance mechanisms, and for monitoring resistance development. Genetic changes causing phenotypic shifts in isolates displaying slightly elevated MICs remain to be investigated. **Flavia Dematheis, Clin Microbiol Infect 2024;30:1170**

Introduction

Bacillus anthracis has been recognized as a life-threatening infectious agent for humans and animals across various countries worldwide [1]. This nonmotile Gram-positive bacterium forms endospores and is the causative agent of the zoonotic disease

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anthrax. It predominantly affects herbivorous mammals, since these animals regularly come into contact with pathogens in their environment, often through grazing on soil contaminated with endospores released from the carcasses of previously infected animals [2]. The disease is commonly transmitted to humans through contact with infected animals or their spore-contaminated products. These spores can endure in the environment for many years [1]. Given their remarkable resilience and facile dissemination, coupled with their notable pathogenicity, *B. anthracis* is classified by the CDC as a Category A bioterrorism agent [3].

Clinical breakpoints represent a systematic approach to assist clinicians in selecting the most appropriate antimicrobial therapy while minimizing the risk of therapeutic failure and the emergence of antibiotic resistance. In particular, they are used to interpret the results of antimicrobial susceptibility testing (AST) allowing the categorization of a microorganism as susceptible, susceptible with increased exposure, or resistant to a given antimicrobial agent [4].

In Europe, clinical breakpoints are established by European Committee on Antimicrobial Susceptibility Testing (EUCAST) assigned by the European Medicines Agency. EUCAST employs a comprehensive approach that integrates various types of studies encompassing laboratory data, clinical outcomes, statistical analysis and expert consensus. One of the main differences in the assessment of clinical breakpoints between EUCAST and other agencies lies in the integration of studies regarding the distribution of MIC values, inhibition zone diameters in the microbial population against specific antimicrobial agents, and the assessment of epidemiological cut-off values (ECOFFs) [5]. The ECOFFs represent, for a given microbial species and antimicrobial agent combination, the highest MIC or the lowest inhibition zone diameter, devoid of phenotypically detectable acquired resistance mechanisms. It defines the upper end of the wild-type (WT) MIC distribution or the lower end of the WT inhibition zone distribution, respectively [6].

For *B. anthracis* ECOFFs are not defined yet, therefore in this study a European network of laboratories aimed (a) at establishing, in collaboration with EUCAST, standardized AST methods; (b) at determining the WT MIC distributions for ten therapeutically relevant antimicrobial agents and (c) at establishing ECOFFs for these substances.

Methods

Participants and study design

Under the framework of the EU-funded Joint action SHARP, a European network of six laboratories was defined and included: the Bundeswehr Institute of Microbiology (Munich, Germany), Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata (Foggia, Italy), Robert Koch Institute (Berlin, Germany), Friedrich-Loeffler-Institut (Institute of Bacterial Infections and Zoonoses, Jena, Germany), Belgian Institute for Health Sciensano (Brussels, Belgium), and the National Veterinary Research Institute (Pulawy, Poland). The EUCAST Development Laboratory (Växjö, Sweden) and the Bundeswehr Institute of Microbiology coordinated the study and validated the quality of the data produced. Every laboratory was asked to perform broth microdilution (BMD) and disc diffusion (DD) methods using their own *B. anthracis* strain collections, ranging from 17 to 146 isolates per laboratory.

SOPs for MIC determination and DD testing were defined and disseminated within the consortium. Before testing the target microorganism, the AST methodologies were harmonized within the laboratory network performing a consensus training using quality control strains. *B. anthracis* isolates were tested against ten therapeutically relevant antimicrobial agents. BMD and DD were performed in parallel from the same inoculum suspension at each laboratory site to ensure consistency of the results and to assess the correlation between MIC and zone diameters clinical breakpoint. The resulting MIC and DD distribution values were collected at the Bundeswehr Institute of Microbiology, curated, and submitted to EUCAST for ECOFFs determination.

B. anthracis collection and culture preparation

A total of 335 nonduplicate *B. anthracis* isolates from human, animal and environmental sources, isolated in different geographical areas between 1947 and 2020 were selected for AST. Bacteria were grown under biosafety level 3 (BSL-3) conditions on Columbia blood agar at 35 ± 1°C in air for 16 to 18 hours and subcultivated once under the same conditions before testing.

AST

All isolates were tested against amoxicillin, benzylpenicillin, ciprofloxacin, clindamycin, doxycycline, levofloxacin, linezolid, rifampicin, tetracycline and vancomycin using both BMD and DD methodologies. User-defined commercial BMD plates (Merlin Diagnostika) and antimicrobial discs from Oxoid (Basingstoke) were used to perform BMD and DD testing, respectively. The MIC ranges (mg/L) covered by the BMD panels and the disc potency (μg) used for the DD method were summarized in Tables 1 and 2. Mueller-Hinton agar from BD Diagnostic Systems, either ready-to-use or in-house prepared was used for DD. Since one of the investigators used two types of agar plates for testing different samples, two types of distributions were accounted for this laboratory and described in the text as '3_1' and '3_2'.

AST was performed according to the ISO 20776-1 standard for BMD [7] and the EUCAST instructions for DD [8] with slightly reduced incubation time (16–18 hours instead of 16–20 hours). Zone edges and MIC were red according to EUCAST reading guidelines [8,9]. The validation of the methodologies was performed by each study centre using quality control strains during each AST. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were selected as quality control (QC) strains for BMD and DD testing, while *Enterococcus faecalis* ATCC 29212 was additionally used for DD.

Consensus training using QC strains

Before testing the target microorganism, the DD methodology was trained within and between the laboratories using QC strains. In particular, each study centre was asked to test each microbial-agent combination over 10 days to train the ability to report inhibition zone diameters within the EUCAST ranges and as close as possible to the EUCAST target values. Differently, given the experience gained by the participating institutes during the previous European Joint Action EMERGE (efficient response to highly dangerous and emerging pathogens at the EU level), the execution of BMD on two different days per each microbial-agent combination was considered sufficient to train the method.

Pre-submission data curation

The data produced at each laboratory centre were collected at the Bundeswehr Institute of Microbiology and inspected before submission to EUCAST as follows. Prior data aggregation, a DD graph, partitioned by the level of MIC and coloured by institute, was performed for each antimicrobial agent to verify that the modes of the individual MIC distributions in the dataset were equal to or within one two-fold dilution of the most common mode observed in the seven collected distributions. A similar DD graph but

Table 1
MICs of *Bacillus anthracis* isolates in response to selected antimicrobial agents (aggregated data from six centres)

	MIC value (mg/L)											
Antimicrobial agent	BMD panels (mg/L)	≤0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	32	Isolates
Benzylpenicillin	0.001–32	7	20	94	97	84	20	8	2			3	335
Amoxicillin iv	0.004–0.5	3	18	145	140	23	1	2	**3**				335
Ciprofloxacin	0.008–1			2	250	78	5						335
Levofloxacin	0.008–1			2	85	238	5	5					335
Vancomycin	0.25–32							9	196	120	10		335
Clindamycin	0.031–4			1	4	60	123	136	7	4			335
Doxycycline	0.001–2[a]	**55**	144	126	6	3	1						335
Tetracycline	0.001–2[a]	1	1	135	150	45	2		1				335
Linezolid	0.125–8						1	42	266	24	2		335
Rifampin	0.031–4				2	9	109	191	24				335

Underlined numbers represent the statistical mode within each respective distribution; bold values include truncated values (below or above the concentrations on the MIC panel).
[a] Note, during the study the concentrations of tetracycline and doxycycline were slightly adjusted to improve the MIC scoring of these antimicrobial agents.

partitioned by the institute and coloured by MIC was also created to verify that the individual distributions cover the same DD range. A density function of the aggregated DD data and a bar plot of the MIC aggregated data were created to inspect visually the distribution of these data types. A normal and a log-normal distribution (right skewed continuous probability distribution) of aggregated MIC and DD data, respectively, were regarded as good distributions. The correlation between MIC and DD data was illustrated through histograms of inhibition zone diameters, colour-coded according to the MIC clinical breakpoints reported here in Table 3 [10]. Specifically, shades of green and yellow were employed to depict a WT population classified as susceptible (green) or susceptible with increased exposure (yellow), respectively. Tones of red were used for samples belonging to the resistant population. Box plots were used to quickly identify outliers in the data sets.

ECOFFs determination

For each antimicrobial agent, the aggregated MIC data were analysed using the freeware statistical program "ECOFFinder" [11] following the methodology described by Turnidge et al. [12]. Differently, DD ECOFFs were assessed based on the lower limit of the WT distribution, and previous expert consultation. Benzylpenicillin and tetracycline discs were used to infer amoxicillin i.v. and doxycycline susceptibility, respectively.

Results

Harmonization AST testing using QC strains

The consensus training on QC strains allowed the introduction of the EUCAST DD methodology in the laboratory centres and the refinement of the reading procedure (Fig. S1).

Data analysis

The inspection of the DD graphs partitioned by the level of MIC and coloured by the institute revealed the same modal MIC for each individual distribution or at least a modal MIC with one two-fold dilution apart from the commonest modal value (Figs S2–S9). DD histograms partitioned by the institute and coloured by MIC revealed that the individual distribution produced by the different laboratory centres covers the same DD range per each antimicrobial agent (Figs S10–S17). Based on these results, the seven MIC or DD distributions produced by the six investigators were pooled together and inspected as shown in Fig. 1 for ciprofloxacin. Similar graphs were prepared for each antimicrobial agent and are reported in the Supplementary Material (Figs S18–S21). Few discrepancies between MIC values and inhibition zone diameter for benzylpenicillin, amoxycillin i.v., clindamycin and rifampicin were observed and referred to as outliers (Figs S20a, b, e, i). The MIC and DD results from the six laboratories were aggregated and frequency tables of the MIC and DD distributions were displayed in Tables 1 and 2, respectively.

ECOFFs determination and data interpretation

For each drug investigated, ECOFFs were defined based on 330 to 335 observations and listed in Table 3, together with the recently published EUCAST clinical breakpoints [10]. The ECOFFs determination revealed a WT phenotype for the majority of the isolates. Three strains with benzylpenicillin MIC values of 32 mg/L and inhibition zone diameters of 6 mm (and thus clearly outside the WT distribution) were found (Fig S20a), indicating the expression of a resistance mechanism. MIC values slightly above the defined ECOFFs were also observed in a few isolates for amoxicillin, doxycycline and tetracycline (Fig S20b, f, g).

Discussion

An agreement on governing international standardized AST procedures for *B. anthracis* is needed to elicit an effective therapy outcome. Unfortunately, for the time being, there is a lack of internationally accepted criteria for the interpretation of such tests for *B. anthracis*. Currently, the Clinical and Laboratory Standards Institute (CLSI) simply recommends the utilization of the BMD method. The results obtained through this method are to be interpreted based on the clinical breakpoints for MICs outlined in the CLSI guideline M45, 3rd edition [13]. These clinical breakpoints are currently exclusively available for amoxicillin, penicillin, doxycycline, tetracycline, ciprofloxacin and levofloxacin. Furthermore, prior to this investigation, the EUCAST had not issued recommendations for *B. anthracis* due to the absence of ECOFF values. Therefore, the major aim of this study, was to validate the use of BMD and DD methodologies for AST of *B. anthracis* and determine MIC ECOFFs for ten antimicrobial agents, which served as background data for EUCAST when determining clinical MIC breakpoints and corresponding zone diameter breakpoints [10].

Our experimental data align with EUCAST Standard Operating Procedures for defining WT distributions and determining ECOFFs SOP 10.2 [6]. Therefore, MIC and DD ECOFFs could be determined by EUCAST based on 330 to 335 isolates for amoxicillin,

Table 2
Zone diameter distribution for *Bacillus anthracis* isolates for the selected antimicrobial agents (aggregated data from six centres)

Antimicrobial agent	Inhibition zone diameter (mm)																																						
	Disc potency (μg)	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	Isolates
Benzylpenicillin	1	3									1				1		1	11	11	12	40	46	58	49	36	34	5	7	3			1	2						321
Ciprofloxacin	5																			1	1	4	8	5	12	21	24	34	53	45	48	41	25	10	2				334
Levofloxacin	5																		1	4	9	10	15	17	20	26	41	54	62	46	20	4	5						334
Vancomycin	5					1	3	25	36	69	100	68	20	9	2	1																							334
Clindamycin	2				1		1				1			3		31	28	29	43	49	50	29	31	21	8	2	1		1										329
Tetracycline	30																	1					1	7	13	33	43	42	48	52	43	32	12	4	1	1	1		334
Linezolid	10																3	5	7	21	36	31	33	66	53	47	17	7	3					1					330
Rifampin	5				1			1	1	1	5	21	39	27	37	70	70	31	18	7	2	1		1															333

Underlined values represent the statistical mode within each respective distribution.

benzylpenicillin, ciprofloxacin, clindamycin, doxycycline, levofloxacin, linezolid, rifampicin, tetracycline and vancomycin (Table 3). The majority of the tested isolates belong to the WT population, expected to exhibit a susceptible phenotype (Fig. S20). These data are consistent with most studies reported so far [14]. The stability of antibiotic susceptibility observed in *B. anthracis* might be attributed to its peculiar biological trait, characterized by the alternating vegetative phases in host bodies with long metabolic dormancy periods in the form of spores. This lifestyle limits the potential for acquiring novel antimicrobial resistances through horizontal gene transfer and governs a notable genetic and phenotypic homogeneity of this species [15].

Although many studies demonstrate *B. anthracis* susceptibility to penicillin [14], the presence of resistance to this antimicrobial agent is well known [16,17]. In our study, we identified three isolates with benzylpenicillin MIC values of 32 mg/L and 6 mm inhibition zone diameter (i.e. growth up to the disc), indicating the expression of a resistance mechanism to benzylpenicillin. This underscores the imperative for AST when considering benzylpenicillin as the primary treatment option. Several studies demonstrated that mutations affecting the expression of the two chromosomal β-lactamase genes, *bla1* (which encodes a penicillinase) and *bla2* (which encodes a cephalosporinase), as well as mutations in genes encoding for the sigma factor *SigP* or its anti-sigma factor *RsiP,* controlling expression of *bla1*, contribute to resistance to penicillin [17–19]. However, the genetic changes causing the observed phenotypic shifts in the few isolates displaying slightly elevated MICs remain to be investigated.

The analysis of the MIC and DD distributions in Fig. S20, reveals that certain *B. anthracis* strains with elevated MIC values do not correlate with the inhibition zone diameter values. A meticulous review of the outliers (data not shown) suggests that this discrepancy is attributable to sporadic reading errors. However, a resistant phenotype could be observed in a few samples for doxycycline, tetracycline and amoxicillin. In particular, the samples that exhibited resistance to benzylpenicillin exhibit cross-resistance to amoxicillin i.v. (Fig. S20a,b). For doxycycline and tetracycline, only one isolate displayed a MIC of 0.125 mg/L and a DD value of 22 mm (Figs S20f,g), indicating a non-WT phenotype. Naturally occurring multidrug resistance *B. anthracis* strains to penicillin, doxycycline, chloramphenicol, macrolides and rifampicin have been reported in various laboratories [19]. To our knowledge, there have been no reported instances of *in vivo* resistance to quinolones in *B. anthracis*. However, it is noteworthy that *in vitro* resistance towards quinolones can be induced through subculturing with sub-inhibitory concentrations [20].

After the assessment of the ECOFFs, these values have been used in the EUCAST process to establish both MIC and zone diameter clinical breakpoints for *B. anthracis* (EUCAST breakpoint tables version 14.0 or Table 3 in this study). The analysis of our data based on these clinical breakpoints reveals that the WT population of *B. anthracis* at large is generally susceptible to the majority of the tested antimicrobial agents. For benzylpenicillin, ciprofloxacin and levofloxacin, EUCAST has established breakpoints at arbitrary "off-scale" levels, categorizing them as "susceptible with increased exposure". This prompts clinicians to consider higher dosing regimens, aligning with treatment guidelines for *B. anthracis* [21]. Interestingly, the clinical breakpoints defined by EUCAST in this study, derived from the ECOFFs identified herein and aligned with *B. anthracis* treatment guidelines [21], differ from CLSI values outlined in the CLSI guideline M45, 3rd edition [13], with the exception of amoxicillin i.v. (Table S1). This discrepancy underscores the effect of the ECOFFs in defining the EUCAST clinical breakpoints.

In conclusion, this multi-centre study validated the use of standardized and internationally agreed BMD and DD

Table 3
Epidemiological cut-off values for *Bacillus anthracis* based on DD data from 330 to 335 observations for each antimicrobial agent. EUCAST clinical breakpoints (EUCAST Breakpoints Tables version 14.0., valid from 2024.01.01) are also listed

Antimicrobial agent	MIC and zone diameter ECOFFs of *B. anthracis* determined in this study			EUCAST MIC and zone diameter clinical breakpoints for *B. anthracis*			
	ECOFF (mg/L)	Observations	Zone diameter ECOFF (mm)	MIC breakpoints		DD breakpoints (mm)	
				S ≤	R >	S ≥	R <
Benzylpenicillin	0.5	335	18	0.001	0,5	50	18
Amoxicillin i.v.	0.125	332	18[a]	0.125	0.125	50[a]	18[a]
Ciprofloxacin	0.25	335	24	0.001[b]	0.25	50	24
Levofloxacin	0.25	335	23	0.001[b]	0.5	50	23
Vancomycin	4	335	10	(4)[c]	(4)[c]	(10)[c]	(10)[c]
Clindamycin	1	335	17	1	1	17	17
Doxycycline	0.06	330	26[a]	0.06	0.06	26[a]	26[a]
Tetracycline	0.125	335	26	0.125	0.125	26	26
Linezolid	2	335	20	2	2	20	20
Rifampicin	1	335	12	(1)[c]	(1)[c]	(12)[c]	(12)[c]

DD, disc diffusion; EUCAST, European Committee on Antimicrobial Susceptibility Testing; ECOFFs, epidemiological cut-off values.
[a] In this study both, benzylpenicillin and tetracycline disc diffusion were used to infer amoxicillin i.v. and doxycycline susceptibility, respectively.
[b] An MIC breakpoint of S ≤ 0.001 mg/L is an arbitrary "off-scale" breakpoint that categorizes wild-type organisms (organisms without phenotypically detectable resistance mechanisms to the agent) as "Susceptible, increased exposure" (I).
[c] Breakpoints in brackets are used to warn against the use of specific antimicrobial agents without the use of additional therapeutic measures.

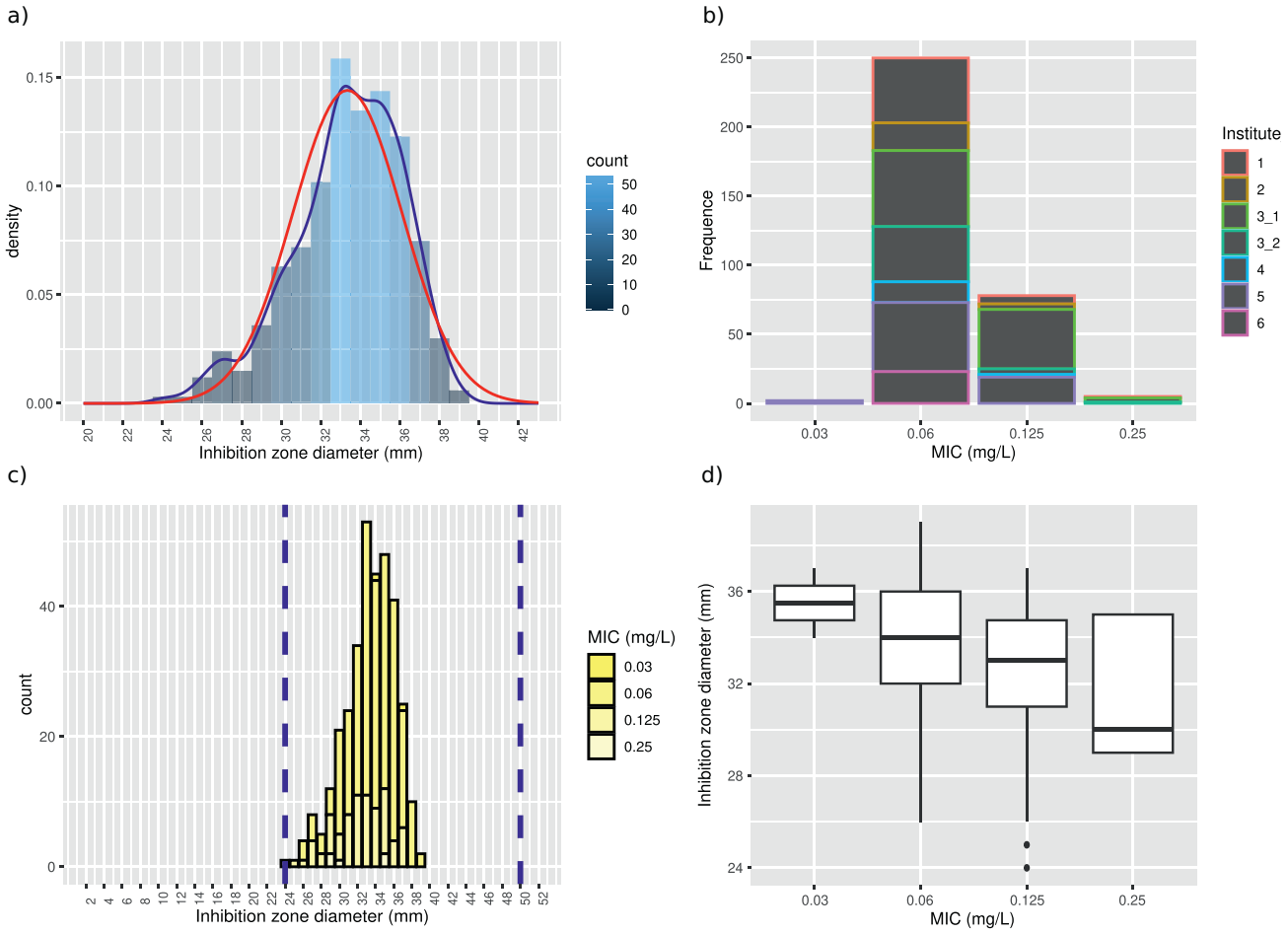

Fig. 1. Aggregated MIC and inhibition zone distribution of *Bacillus anthracis* against 5 µg ciprofloxacin. (a) Density function (blue line) showing a normal distribution (red line) of the DD aggregated data; (b) Bar plot showing the MIC aggregated data distribution for ciprofloxacin; (c) Histogram from aggregated data: inhibition zone diameters and corresponding MIC values for ciprofloxacin; blue dashed lines represent DD clinical breakpoints correlated to MIC clinical breakpoints; (d) Box plot from aggregated data, showing the correlation between MIC and DD values together with outliers, represented by individual data points. The graphs are based on 334 observations. DD, disc diffusion.

methodologies for *B. anthracis* and determined ECOFFs for ten antimicrobial agents. The ECOFFs can now be used to detect emerging resistance development in *B. anthracis* and provide the groundwork for assessing EUCAST clinical breakpoints for this highly pathogenic microorganism [10].

Author contributions

Conceptualization: F.D., S.Z. and E.M.; Methodology: F.D., V.M., A.K-J., D.J., T.F. and S.B.; Validation: F.D., E.M., V.M., D.J., F.M., M.E., R.G., D.G. and M.M.; Formal analysis: F.D.; Data curation: F.D. and E.M.; Writing—original draft preparation: F.D. and V.M.; Writing-review and editing: F.D., V.M., E.M, G.K., G.G. and S.Z.; Visualization: F.D.; Supervision, G.K. and E.M.; Project administration: D.J. and S.Z. All authors have read and agreed to the published version of the manuscript.

Transparency declaration

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2024.05.019.

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