Resistance finding of *Bacillus anthracis* towards penicillin in East Java, Central Java, and Yogyakarta Provinces, Indonesia

U I Apriliana[1,2], E Ruhiat[2], Mariyono[2], H Wibawa[2], T Untari[3], and S Indarjulianto[3*]

[1]Veterinary Science Doctoral Study Program, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Jl. Fauna 2, Karangmalang, Yogyakarta, 55281, Indonesia
[2]Balai Besar Veteriner Wates Yogyakarta, Jalan Raya Jogja-Wates Km 27 Po Box 18, Wates, Yogyakarta, 55602, Indonesia
[3]Faculty of Veterinary Medicine, Universitas Gadjah Mada, Jl. Fauna 2, Karangmalang, Yogyakarta, 55281, Indonesia

E-mail: indarjulianto@ugm.ac.id

Abstract. Anthrax is a worldwide distributing zoonotic disease, caused by *Bacillus anthracis*, which occurs sporadically in Indonesia, particularly in the provinces of East Java, Central Java, and Yogyakarta, which are the working areas of the Disease Investigation Center (DIC) Wates. Penicillin has been the primary antimicrobial treatment recommended for anthrax since there has never been a report of resistance to this antibiotic in Indonesia. The objective of this research was to assess the sensitivity of *B. anthracis* isolates from Central Java, East Java, and Yogyakarta to penicillin and tetracycline. Sixteen *B. anthracis* isolates from DIC Wates collected between 1990-2021 recovered from environmental samples were used in this study. All isolates were identified by phenotype, then tested for sensitivity to penicillin and tetracycline by agar diffusion (Kirby-Bauer) and broth dilution method. The data obtained were compared with the standard and analyzed descriptively. The results showed that all isolates were *B. anthracis*. One of 16 isolates (6,25%) consistently showed resistance to penicillin, but was sensitive to tetracycline, while 15 isolates (93,75%) showed sensitive to both antibiotics. A penicillin-resistant isolate was soil sample from anthrax endemic area. In conclusion, there was *B. anthracis* isolate that was found resistance to penicillin. Therefore, tetracycline can be used as an alternative for anthrax treatment.

Keywords: Anthrax, Antibiotic sensitivity, *Bacillus anthracis*, Penicillin resistance, Tetracycline.

1. Introduction

Anthrax emerges by spore-producing, rods-encapsulated Gram positive bacteria, *Bacillus anthracis*. Mammals, particularly herbivores and even humans, are susceptible to anthrax infection. More than 95% of anthrax patients have the cutaneous form, which is rarely lethal when treated with antibiotics. In contrast, anthrax inhalation has a significant fatality rate in humans [1]. The gastrointestinal forms of anthrax are brought on by spore ingestion and a novel method, namely injectable route, has been found in many European countries [2-3]. In addition to contaminating soil and animal feed, spores can also harm animal by products including hair, wool, skins, and bones [4]. The nature of the disease agent and the epizootiology of anthrax, which includes the situation in the area, the kinds of animals that are susceptible, the effects, and the modes of transmission, are technically related to the eradication and control of anthrax. [5-6]. In addition to vaccination, antimicrobial drug therapy is another component of those efforts in various parts of the world [7-8].

Bacillus anthracis is a bacterium that is sensitive to most antibiotics, but early therapy is crucial so that it can eliminate the bacterium before releasing toxins into the bloodstream [8-9]. Beta-lactam antibiotics, such as penicillin, are recommend as prophylactic therapy for anthrax by the WHO [10] and CDC [11]. Penicillin was administered intramuscularly (IM) for 4-5 consecutive days. Tetracycline or its derivatives are another antibiotic utilized for the treatment of anthrax in the field in addition to penicillin [12]. Currently, 10-25% of *B. anthracis* isolates apart from the environment, animals, and human have been found to be resistant to penicillin antibiotics [13–15].

Anthrax occurs sporadically in Indonesia, especially in DIC Wates service region, which includes East Java, Central Java and Yogyakarta provinces. The most recent case occurred in 2021 in Tulungagung District, East Java province (DIC Wates, unpublished). Multivitamins, antipyretics, and antibiotic therapy are used to treat anthrax in the field. The preferred antibiotics are penicillin G and tetracycline. Penicillin and tetracycline antibiotic resistance in anthrax infections has not yet been identified in Indonesia. According to findings from earlier investigations, there were *B. anthracis* isolates from straw and ground soil which were sensitive to penicillin in the agar diffusion test but the dilution method to identify the lowest inhibitory concentration (MIC value) had not yet been used to test them [16]. The objective of this research was to assess the tetracycline and penicillin susceptibility of environmental isolates of *B. anthracis*. The results of this study should give a general overview of the antibiogram of isolates of *B. anthracis*, particularly in the DIC Wates service area.

2. Materials and Methods

2.1. Ethical approval

All tests were carried out in BSL-2 plus facilities at the DIC Wates Zoonosis Laboratory in the Special Region of Yogyakarta, Indonesia, with accreditation number LP-618-IDN, and in line with the Ministry of Agriculture of Indonesia's suggested guidance for *B. anthracis* identification [10,17]. The samples used in this study obtained from areas where anthrax-related deaths were thought to have occurred.

2.2. Bacillus anthracis isolates

The study was conducted from September 2020 to February 2021. Sixteen *B. anthracis* isolates were recovered from environmental samples in the form of soil (n = 14), sawdust (n = 1), and straw (n = 1) from 1990-2021. The samples were collected from 4 districts in the province of Central Java, 2 districts in Yogyakarta, and 1 district in the province of East Java while the region was experiencing an anthrax outbreak (Table 1). *Bacillus anthracis* was isolated and identified from those samples following the standart procedure outlined by WHO [10] and OIE [17] including Gram staining, motility, hemolysis on blood agar,

and capsule visualization (Table 1). The reference isolates were *Bacillus cereus* ATCC 11778 (Culti-LoopsTM, Thermo Scientific, USA) and the Sterne 34F2 strain of the *Bacillus anthracis*.

Prior to the susceptibility tests, the bacteria were cultivated on agar with 5% sheep red blood cells. All samples performed overnight incubation at 37°C. All work was performed at a Biocontainment Cabinet class II type A2 (ESCO, Singapore). Nitrile gloves, safety goggle, as well as other necessary personal protective equipment (PPE) were also utilized. Bleach-based solutions and 70% ethanol were employed to clean and sterilize the equipment and surfaces. All of the solid biohazardous material was autoclaved before removal and burning.

2.3. Antibiotic susceptibility testing

The tetracycline antibiotic 30 µg (CT0054B) and the penicillin G antibiotic 10 U (CT0043B) were examined for antibiotic sensitivity adopting Kirby-agar Bauer's disk diffusion technique (Oxoid, Thermo Scientific, USA). One ose of pure culture, was standardized to Mc Farland 0.5, equivalent to $< 3 \times 10^8$ CFU/ml (CFU: Colony Forming Unit), by dissolving it in 0.5 ml of sterile distilled water. The suspension was plated on Mueller Hinton agar (MHA) media, an antibiotic disc was placed on top of the media, then kept overnight at 37°C. The zone of resistance is measured in millimeters (mm) using a ruler/caliper. By contrasting the zone of inhibition in standard cultures of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 against penicillin and tetracycline antibiotics, it was possible to determine quality assurance (QA) and susceptibility breakpoint. The zone of inhibition of *B. anthracis* in the agar diffusion method has not been determined by the Clinical Laboratory Standards Institute (CLSI). Therefore, the diameter of the *Staphylococci* inhibition zone is used to interpret the zone of inhibition to penicillin and tetracycline antibiotics [18-20].

The outcomes of the sensitivity test were compared using the broth microdilution method with the agar diffusion technique and to determine the MIC value according to CLSI guidelines [18]. This method was carried out using a pure culture suspension in 2 ml of physiological NaCl (0.85%) which was equivalent to 0.5 Mc Farland. A total of 5 µl of the bacterial suspension was put into 5.5 ml of Mueller Hinton broth (MHB) media and then slowly resuspended using 1 ml microtips. A total of 50 µl of dilution was put into a test plate (100 µl /well) that had been coated with graded dilution of antibiotics: 0.06; 0.12; 0.25; 0.5; 1; 2; 4; 8 for penicillin and 0.25; 0.5; 1; 2; 4; 8 for tetracycline antibiotics. The microplate was then homogenized by gently shaking, covered with a microplate plastic sheet to prevent the suspension from drying up, and incubated at 37°C overnight. The analysis of the research was conducted by reading from the bottom of the plate. The presence of sediment (+) was resistant to antibiotics, and no sediment/clear (-) was sensitive to antibiotics. The MICs for penicillins and tetracyclines were then determined by comparing the lowest concentration of bacterial growth inhibition (MIC) observed for each antibiotic tested to the CLSI standard [21-22]. Standard culture of *Staphylococcus aureus* ATCC 25923 were utilized as a reference [19].

3. Results

All isolates in this study were identified phenotypically and showed gray-white colonies with fibrous edges such as caput medusa, non-hemolytic and tacky on blood agar media, non-motile, mucoid and encapsulated colonies on bicarbonate media, except for 2 isolates (id 15 and id 24) were non-mucoid and not encapsulated like the control isolate *B. anthracis* vaccine strain (Sterne 34F2) (Table 1). Gram-positive bacteria are shown by the large, lenghtwise stretched square-ended rods that make up the purplish-blue bacterial cells. All of the isolates in this investigation could be classified as *B. anthracis* based on the phenotypic test and comparison to the literature [10,16].

Table 1. Isolate origin and phenotype identification of *Bacillus anthracis* (*n* = 16)

No	Isolate (code)	Province	District-Sub District	Years	Hemolyze	Tackyness	Motility	Bicarbonate media	Capsule
1	Freeze dry (Id1)	Central Java	Semarang	1990	No	Yes	No	Mucoid	Yes
2	Soil (Id3)	Central Java	Pati	2007	No	Yes	No	Mucoid	Yes
3	Soil (Id4)	Central Java	Sragen	2010	No	Yes	No	Mucoid	Yes
4	Soil (Id5)	Central Java	Sragen	2011	No	Yes	No	Mucoid	Yes
5	Soil (Id6)	Central Java	Boyolal	2011	No	Yes	No	Mucoid	Yes
6	Soil (Id7)	Central Java	Boyolal	2011	No	Yes	No	Mucoid	Yes
7	Soil (Id8)	Central Java	Sragen	2011	No	Yes	No	Mucoid	Yes
8	Soil (Id15)	Central Java	Boyolali	2019	No	Yes	No	Non-mucoid	No
9	Straw (Id24)	Central Java	Pati	2020	No	Yes	No	Non-mucoid	No
10	Sawdust (Id11)	Yogyakarta	Kulonprogo	2017	No	Yes	No	Mucoid	Yes
11	Soil (Id18)	Yogyakarta	Gunungkidul	2020	No	Yes	No	Mucoid	Yes
12	Soil (Id19)	Yogyakarta	Gunungkidul	2020	No	Yes	No	Mucoid	Yes
13	Soil (Id20)	Yogyakarta	Gunungkidul	2020	No	Yes	No	Mucoid	Yes
14	Soil (Id23)	Yogyakarta	Gunungkidul	2020	No	Yes	No	Mucoid	Yes
15	Soil (Id25)	East Java	Pacitan	2021	No	Yes	No	Mucoid	Yes
16	Soil (Id27)	East Java	Pacitan	2021	No	Yes	No	Mucoid	Yes
17	*B. anthracis*Sterne 34F2-Vaccine *strain* (control)	-	-	-	No	Yes	No	Non-mucoid	No
18	*B. cereus* ATCC-11778 (control)	-	-	-	Yes	No	Yes	Non-mucoid	No

Antibiotic sensitivity test using agar diffusion and broth dilution techniques was carried out at 37°C and incubate overnight. *B. anthracis* was tested for antibiotic sensitivity in this study, and the results showed that 15 isolates (93.75%) were susceptible to tetracycline and penicillin, but one isolate (6.25%) (id 15) was resistant to penicillin with a zone of inhibition diameter of 19 mm by the agar diffusion method (Figure 1).

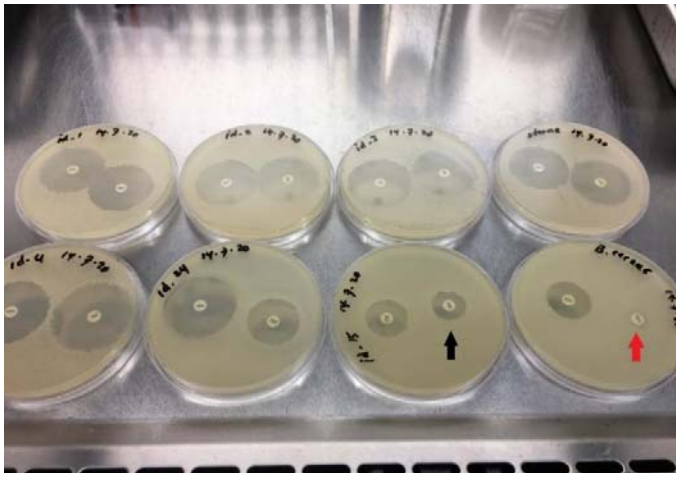

Figure 1. Antibiotic sensitivity test by agar diffusion method (Kirby-bauer technique). *Bacillus anthracis* isolate (id 15) (black arrow) and *B. cereus* ATCC 11778 (red arrow) were resistant to pencillin.

The same results were obtained using the microbroth dilution method, with isolate id 15 showed resistance to penicillin (MIC = 2 µl/ml; range 0.06 – 8 µl/ml), but sensitive to tetracycline (MIC = 1 µl/mL; range 0.25 – 8 µl/ml). The interpretation of the antibiotic sensitivity test results from the two methods can be seen in Table 2. Isolate id 15 which is penicillin-resistant, but sensitive to tetracycline comes from soil collected during anthrax monitoring in Ampel sub-district of Boyolali district in 2019, which is an anthrax endemic area.

Table 2. Antibiotic susceptibility test (*n* = 16)

Antibiotic	Break point						Result		
	Agar Diffusion *(Kirby-Bauer)* (mm)[a]			Broth Dilution (µl/ml)[b]			*n* isolate		
	S	I	R	S	I	R	S	I	R
Penicillin	≥29	-	≤28	≤0.5	-	≥1	15	0	1
Tetracycline	≥19	15-18	≤14	≤1.0	-	-	16	0	0

[a]according to the *Staphylococcus aureus* inhibition zone from CLSI M100-S23 (2013) [18].
[b]according to the *Bacillus anthracis* MIC from CLSI updated document 45 (2016) and CLSI M100-S17 (2007) [21-22].

4. Discussion

Bacillus anthracis could be isolated in all 16 samples from this study. Samples of loam, sawdust, and straw that were gathered between 1990 and 2021 in various areas within DIC Wates' working area, which had a radius of 53-236 km, are entirely derived from the environment. However, there were isolates from the same district over a lengthy period of time, specifically from Pati district (2007 and 2020) and Boyolali district (2011 and 2019). Most of these samples were drawn from collection of related cases that happened close together (Table 1). This shows that anthrax still exists in regions where it has been detected, that it can reemerge years later, and even spread to other areas that were previously anthrax-free.

Anthrax outbreaks continue to occur frequently, including in Ampel sub-district, Boyolali district of Indonesia [23–25]. Boyolali experienced its first outbreak in 1990, followed by instances in 1992, 1993, 1998, 2001, 2002, and 2012 [23]. The reported human cases occurred in 2008, whereas in 2012, cases occurred only in cattle, goats and sheep [24]. Lewerin reported a case of recurrent anthrax after 27 years at the same location in Sweden [25]. The resurgence of anthrax in Boyolali may be due to the region being a hotspot for anthrax epidemics, facilitated by low vaccination coverage, which ranged from 8% to 11% of all cattle in 2011-2012 [23].

Anthrax control and eradication in Boyolali district is carried out by antibiotic treatment (tetracycline) of live cattle both healthy and sick around the anthrax location, disinfection using formalin at the case location (abattoir, dead cattle location, dead cattle graves), and vaccination [23]. Due to limited resources and time in the field, the selection of antibiotics used in DIC Wates service areas is not always based on the results of *B. anthracis* antibiotic isolation and susceptibility testing. There have never been any previous antibiotic sensitivity tests against *Bacillus anthracis* isolates from environment in the service area of DIC Wates, including Boyolali district.

In this study, *B. anthracis* was tested for susceptibility to penicillin and tetracycline, and the results revealed that 15 isolates (93.75%) were susceptible to these antibiotics. One isolate (6.25%) showed resistance to penicillin, using both the agar diffusion test (zone of inhibition diameter = 19 mm) and the microbroth dilution method (MIC = 2 µl/ml). The resistant isolates came from soil in 2019 from Boyolali district, which is an anthrax endemic area. Additionally, penicillin-resistant of *B. anthracis* isolates have been reported in earlier studies [26–29].

Agren et al. [26] have suggested penicillin-resistant of *B. anthracis* with a MIC of 4 µl/ml and the bacterium produces beta-lactamase. The isolates came from samples of anthrax-infected animals that grazed in the area of an anthrax burial site in 1940 [26]. Kutmanova found 24.64% human-derived *B. anthracis* in the Kyrgyz Republic were penicillin-resistant, 78.26% to gentamicin, 51.44% to chloramphenicol, and 64.50% to ampicillin [13]. In France, Cavallo revealed that 11.5% of *B. anthracis* isolates from both animal and environmental specimens were penicillin-resistant [14]. Another study, found beta-lactamase activity in 1 penicillin-resistant isolate with MIC value of 128 µl/ml from historical *B. anthracis* isolate from human specimen [28]. Coker found different things, including up to 3 isolates from animals that were resistant to penicillin but not those that produced beta-lactamases. In Turkey, Durmaz discovered 155 animal isolates, 93 human isolates and 3 environments that were all resistant to ceftriaxone but susceptible to quinolones, vancomycin, tigecycline, and linezolid antibiotics, and one animal-derived isolate was resistant to doxycycline, erythromycin, and penicillin [30].

The results of this study, which revealed one isolate from soil that was penicillin-resistant, differed from most other studies. Penicillin antibiotic sensitivity allows classical *Bacillus anthracis* to be distinguished from *B. cereus*, which has many traits like *B. anthracis* [10,28–29]. Research by Azarkar and Bidaki obtained isolates from the blood and sputum of patients infected with anthrax that are sensitive to penicillin [1]. According to Dragon, all *B. anthracis* isolate from soils was penicillin sensitive [31]. *Bacillus anthracis* isolated from different locations during the period 1984 – 2017 with different genotypic groups, was also reported to be penicillin sensitive, but resistant to trimethoprim [32].

Bacillus anthracis resistance to penicillin has been reported either naturally or by induction. Exposure to broad-spectrum beta-lactamase antibiotics to *B. anthracis* can lead to resistance to penicillin antibiotics [33]. The synthesis of one or more beta-lactamase enzymes, which hydrolyze the beta-lactam ring thus rendering bacteria inactive, promotes this resistance [34]. *Bacillus anthracis* has 2 beta-lactamase genes namely *bla*1 (penicillinase) and *bla*2 (cephalosporinase), but both are not expressed due to truncation of *plcR* positive regulator [15,35]. Additionally, penicillin resistance has been linked to changes in the *rsiP* and *sigP* genes, which influence the development of beta-lactamases [26,36].

Antibiotic-resistant strains of *B. anthracis* in an area can be caused by the use of a particular type of antibiotic or overuse of the antibiotic which is also influenced by the origin of the sample (clinical or environmental). Bruce and colleagues classified *B. anthracis* strains based on ten genes involved in antimicrobial resistance (AMR) namely *mph*L, *bla*1, *fos*B, *bla*2, *vml*R, *bcII*, *tem*-116, *cfr*C, *dfr*G, *oxa*-59 which were identified from the Comprehensive Antibiotic Resistance Database (CARD) of 350 *B. anthracis* genomes from 35 countries [37]. Based on the absence of the *fosB* resistance gene and the absence of the *vmlR* resistance gene, the study estimated that branch C clusters and branch B clusters might co-cluster [37].

Bacillus anthracis, a soil-borne penicillin-resistant strain may have developed its resistance through horizontal gene exchange with other soil microbes. Sahl [38] claimed that recombinant DNA or plasmid vectors can cause antibiotic resistance. Genetic transmission is made possible by *B. anthracis* germination in the surrounding environment (soil). Saile and Koehler reported the germination of *B. anthracis* in the plant rhizosphere, supported by diverse microbial populations in the soil, allowing horizontal AMR gene transfer [39]. This is consistent with the presence of the antibiotic resistance genes *metS2* and *ileS2* in *B. anthracis*, which share significant similarity (61% and 65%) with the genomic loci of *Staphylococcus aureus* and *Streptococcus pneumoniae*, respectively [40]. Invivo resistance can be caused by the heterogeneity of the *B. anthracis* population in the environment as well as the possible high number of bacteria that infect the host [26].

Penicillin and tetracycline antibiotics are typically used in Indonesia to treat anthrax. It was crucial to determine the antibiotics' sensitivity to anthrax isolates before treatment since the identification of *B. anthracis* strains that are penicillin-resistant made the use of a single antibiotic to treat anthrax impractical. Treatment using fluoroquinolone antibiotics (ciprofloxacin) as a substitute for penicillin G has been reported [14,41]. Since *B. anthracis* is resistant to trimethoprim/sulfamethoxazole antibiotics, its usage is not advised [41]. Combination of penicillin and linezolid against ciprofloxacin-resistant strains of *B. anthracis* in mice has been investigated [42]. Dassanayake and colleagues investigated the combination of phytochemical-antibiotics as an alternative therapy by utilizing the various pathways in modifying and restoring antibiotic sensitivity for penicillin-resistant strains of *B. anthracis* [43]. Treatment with antibiotics or toxin inhibitors or a combination of the two is an effective means of eradicating anthrax [44]. Antimicrobial therapy must begin promptly after infection in order to be effective. Early disease intervention improves the chances of a cure, while antibiotic medication after the incident frequently has no benefit [32]. Further analysis regarding the genetic material composition of penicillin-resistant of *B. anthracis* isolates reported in this study is urgently needed, so that the cause of resistance can be identified, and can be used as information in investigations of anthrax cases.

5. Conclusion

In conclusion, tetracycline may be utilized as a replacement to penicillin in the treatment of *B. anthracis* resistance. The introduction of *B. anthracis* strains that become penicillin-resistant in soil samples can be induced by both internal and external sources and this can be a threat from improper treatment results so that antibiotic susceptibility testing should be done prior to treatment.

References

[1] Azarkar Z and Bidaki M Z 2016 A case report of inhalation anthrax acquired naturally *BMC Res Notes* **9** 141

[2] Thouret J M, Rogeaux O, Beaudouin E, Levast M, Ramisse V, Biot F V, Valade E, Thibault F, Gorgé O and Tournier J N 2020 Case Report of an Injectional Anthrax in France, 2012 *Microorganisms* **8(7)** 985

[3] Berger T, Kassirer M and Aran A A 2014 Injectional anthrax - new presentation of an old disease *Euro Surveill* **19(32)** 20877.
[4] Coker P R, Smith K L and Hugh-Jones M E 2002 Antimicrobial susceptibilities of diverse *Bacillus anthracis* isolates. *Antimicrob Agents* Chemother **46(12)** 3843-5
[5] Bakhteeva I and Timofeev V 2022 Some Peculiarities of Anthrax Epidemiology in Herbivorous and Carnivorous Animals. *Life (Basel)* **12(6)** 870
[6] Stella E, Mari L, Gabrieli J, Barbante C and Bertuzzo E 2020 Permafrost dynamics and the risk of anthrax transmission: a modelling study. *Sci Rep* **10(1)** 1–12
[7] Sari I and Apriliana S 2020 General Description, Prevalence, and Prevention of Human Anthrax in Indonesia*BALABA: J. Litbang Pengendalian Penyakit Bersumber Binatang* Banjarnegara **16(2)** 135-148
[8] Savransky V, Ionin B and Reece J 2020 Current Status and Trends in Prophylaxis and Management of Anthrax Disease. *Pathogens* **9(5)** 370
[9] Alam M E, Kamal M M, Rahman M, Kabir A, Islam M S and Hassan J 2022 Review of anthrax: A disease of farm animals. *J Adv Vet Anim Res* **9(2)** 323-334
[10] WHO 2008 *Anthrax in Humans and Animals4th* edition. World Health Organization. Geneva. Switzerland
[11] CDC 2002 Basic diagnostic testing protocols for level A laboratories for the presumptive identification of *Bacillus anthracis*. Environmental Protection Agency, U. (n.d.). *CDC, ASM,* APHL pp 1–22. Retrieved from https://www.epa.gov/sites/production/files/2015-07/documents/cdc-anthrax.pdf
[12] Ditjen PKH 2016 *Pedoman Pengendalian dan Pemberantasan Penyakit Hewan Menular (PHM): Seri Penyakit Anthrax*. Kementerian Pertanian Republik Indonesia. Direktorat Jenderal Peternakan dan Kesehatan Hewan. Jakarta. Indonesia
[13] Kutmanova A, Doganay M and Zholdoshev S 2020 Human anthrax in Kyrgyz Republic: Epidemiology and clinical features. *J Infect Pub Health* **13(8)** 1161–1165
[14] Cavallo J D, Ramisse F, Girardet M, Vaissaire J, Mock M and Hernandez E 2002 Antibiotic susceptibilities of 96 isolates of *Bacillus anthracis* isolated in France between 1994 and 2000. *Antimicrob Agents Chemother* **46(7)** 2307–2309
[15] Turnbull P C, Sirianni N M, LeBron C I, Samaan M N, Sutton F N, Reyes A E and Peruski L F Jr 2004 MICs of selected antibiotics for *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* from a range of clinical and environmental sources as determined by the Etest. *J Clin Microbiol* **42(8)** 3626-34
[16] Apriliana U I, Wibawa H, Ruhiat E, Untari T and Indarjulianto S 2021 Isolation and identification of avirulent strains of *Bacillus anthracis* from environmental samples in Central Java, Indonesia. *Int J One Health* **7(2)** 204-211
[17] OIE 2018 *Anthrax*. Terrestrial Manual. Section 2.1. Multiple Species. Chapter 2.1.1. Office International Des Epizooties (OIE) 1-10
[18] CLSI 2013 Performance standards for antimicrobial susceptibility testing M100-S23; Twenty-Third Informational Supplement. In *Clinical and Laboratory Standards Institute – NCCLS* **33(1)** 73-77
[19] Ortatatli M, Karagoz A, Percin D, Kenar L, Kilic S and Durmaz R 2012 Antimicrobial susceptibility and molecular subtyping of 55 Turkish *Bacillus anthracis* strains using 25-loci multiple-locus VNTR analysis. *Comp Immunol Microbiol Infect Dis* **35(4)** 355–361
[20] Habrun B, Racic I, Kompes G, Spicic S, Benic M, Mihaljevic Z and Cvetnic Z 2011 The antimicrobial susceptibility and virulence factors of *Bacillus anthracis* strains isolated in Croatia. *Vet Med* **56(1)** 23–28

[21] CLSI 2016 Methods for antimicrobial dilution and disk susceptibility performance standards for antimicrobial susceptibility testing of infrequently isolated or fastidious bacteria, 3rd ed. CLSI Guideline M45 (updated). In *Clinical and Laboratory Standars Institute – NCCLS* **60-63**
[22] CLSI 2007 Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement. In *Clinical and Laboratory Standars Institute – NCCLS* **27**
[23] Sudarsono I 2013 Pengendalian anthrax dan keterkaitan perilaku masyarakat dengan perkembangan kasus anthrax di kabupaten Boyolali. *Buletin Laboratorium Veteriner* **13(3)** 9–20
[24] Abawi I and Fibriana A I 2019 Analisis spasial faktor lingkungan fisik daerah endemik antraks HIGEIA: *J Pub Health Res Dev* **3(2)** 190-201
[25] Lewerin S S *et al.* 2010 Anthrax outbreak in a Swedish beef cattle herd - 1st case in 27 years: Case report. *Acta Vet Scand* **52** 7
[26] Ågren J, Finn M, Bengtsson B and Segerman B 2014 Microevolution during an Anthrax Outbreak Leading to Clonal Heterogeneity and Penicillin Resistance. *PLos One* **9(2)** e89112
[27] Gargis A S *et al.* 2018 Analysis of Whole-Genome Sequences for the Prediction of Penicillin Resistance and β-Lactamase Activity in *Bacillus anthracis*. *mSystems* **3(6)** e00154-18
[28] Mohammed M J, Marston C K, Popovic T, Weyant R S and Tenover F C 2002 Antimicrobial susceptibility testing of *Bacillus anthracis*: comparison of results obtained by using the National Committee for Clinical Laboratory Standards broth microdilution reference and Etest agar gradient diffusion methods. *J Clin Microbiol* **40(6)** 1902–1907
[29] Odendaal M W, Pieterson P M, de Vos V and Botha A D 1991 The antibiotic sensitivity patterns of *Bacillus anthracis* isolated from the Kruger National Park. *Onderstepoort J Vet Res* **58(1)** 17–19
[30] Durmaz R *et al.* 2012 Anthrax Study Group. Molecular epidemiology of the *Bacillus anthracis* isolates collected throughout Turkey from 1983 to 2011. *Eur J Clin Microbiol Infect Dis* **31(10)** 2783-90.
[31] Dragon D C, Bader D E, Mitchell J and Woollen N 2005 Natural dissemination of *Bacillus anthracis* spores in northern Canada. *Appl Environ Microbiol* **71(3)** 1610-5
[32] Manzulli V *et al.* 2019 Evaluation of *in vitro* antimicrobial susceptibility of *Bacillus anthracis* strains isolated during anthrax outbreaks in Italy from 1984 to 2017. *J Vet Sci* **20(1)** 58-62.
[33] Athamna A, Athamna M, Abu-Rashed N, Medlej B, Bast DJ and Rubinstein E 2004 Selection of *Bacillus anthracis* isolates resistant to antibiotics. *J Antimicrob Chemother* **54(2)** 424–428
[34] Chen Y, Succi J, Tenover F C and Koehler T M 2003 Beta-lactamase genes of the penicillin-susceptible *Bacillus anthracis* Sterne strain. *J Bacteriol* **185(3)** 823-30
[35] Chen Y, Tenover F C and Koehler T M 2004 Beta-lactamase gene expression in a penicillin-resistant *Bacillus anthracis* strain. *Antimicrob Agents Chemother* **48(12)** 4873-7
[36] Pilo P, Rossano A, Bamamga H, Abdoulkadiri S, Perreten V and Frey J 2011 Bovine *Bacillus anthracis* in Cameroon. *Appl Environ Microbiol* **77(16)** 5818-21
[37] Bruce S A, Huang Y H, Kamath P L, van Heerden H and Turner W C 2021 The roles of antimicrobial resistance, phage diversity, isolation source and selection in shaping the genomic architecture of *Bacillus anthracis*. *Microb Genom* **7(8)** 000616
[38] Sahl J W *et al.* 2016 *Bacillus anthracis* Genome Sequence from the Sverdlovsk 1979 Autopsy Specimens. *mBio* **7(5)** e01501-16
[39] Saile E and Koehler T M 2006 *Bacillus anthracis* multiplication, persistence, and genetic exchange in the rhizosphere of grass plants. *Appl Environ Microbiol* **72(5)** 3168-74
[40] Brown J R, Gentry D, Becker J A, Ingraham K, Holmes D J and Stanhope M J 2003 Horizontal transfer of drug-resistant aminoacyl-transfer-RNA synthetases of anthrax and Gram-positive pathogens. *EMBO Rep* **4(7)** 692-8

[41] Luna V A, King D S, Gulledge J, Cannons A C, Amuso P T and Cattani J 2007 Susceptibility of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus thuringiensis* to 24 antimicrobials using Sensititre automated microbroth dilution and Etest agar gradient diffusion methods. *J. Antimicrob. Chemother* **60(3)** 555–567
[42] Heine H S *et al.* 2013 Evaluation of combination drug therapy for treatment of antibiotic-resistant inhalation anthrax in a murine model. *Antimicrob Agents Chemother* **61(9)** e00788-17
[43] Dassanayake M K, Khoo T J and An J 2021 Antibiotic resistance modifying ability of phytoextracts in anthrax biological agent *Bacillus anthracis* and emerging superbugs: a review of synergistic mechanisms. *Ann Clin Microbiol Antimicrob* **20(1)** 79
[44] Head B M, Rubinstein E and Meyers A F 2016 Alternative pre-approved and novel therapies for the treatment of anthrax. *BMC infectious diseases* **16(1)** 621

Ackowledgments

Universitas Gadjah Mada Indonesia provided funding for this study in the form of grant 3143/UN1.P.III/DIT-LIT/PT/2021. The authors also thanked to The Directorate General of Livestock and Animal Health Services of the Ministry of Agriculture of Indonesia, the Disease Investigation Center Wates Yogyakarta, Indonesia, and the Agricultural Human Resources Extension and Development Agency.