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RESEARCH ARTICLE

Phenotypic and genotypic characteristics of *Bacillus anthracis* associated with the occurrence of anthrax cases in East Java, Central Java, and Yogyakarta, Indonesia

Ully Indah Apriliana[1], Soedarmanto Indarjulianto[2], Hendra Wibawa[1], Tri Untari[3], Endang Ruhiat[1], Basuki R. Suryanto[1], Lestari Lestari[1], and Fatah Nugroho[4]

1. Disease Investigation Center Wates, Wates, Indonesia.
2. Department of Internal Medicine, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.
3. Department of Microbiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.
4. Department of Internal Medicine, Graduate Student of Veterinary Sciences, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

ABSTRACT

Background and Aim: Anthrax is a zoonotic disease caused by *Bacillus anthracis*, a spore-forming bacterium capable of long-term environmental persistence. In Indonesia, anthrax has been reported in 22 provinces, with East Java, Central Java, and Yogyakarta identified as persistent endemic regions. Understanding the phenotypic and genetic characteristics of local *B. anthracis* isolates is critical for informing targeted control strategies under the One Health framework. This study aimed to characterize the phenotypic and genotypic profiles of *B. anthracis* isolates collected between 1990 and 2021 from three anthrax-endemic provinces in Java, Indonesia, and to identify potential environmental and epidemiological risk factors influencing transmission.

Materials and Methods: A total of 28 isolates obtained from environmental and animal sources across 12 districts were examined using conventional phenotypic methods and confirmed by multiplex polymerase chain reaction (PCR) targeting Ba813, *lef* (pXO1), and *capC* (pXO2) genes. Laboratory data were interpreted alongside epidemiological and environmental information within a One Health framework.

Results: All isolates displayed classical phenotypic traits of *B. anthracis*: Gram-positive morphology, non-hemolytic, non-motile, capsule formation, and sensitivity to penicillin, tetracycline, and ciprofloxacin. PCR results confirmed the presence of both chromosomal and plasmid virulence markers. Notably, consistent traits across isolates indicated genetic homogeneity among circulating strains. Risk factors contributing to anthrax persistence included inadequate vaccination coverage, livestock movement through trade routes, the slaughter of infected animals, poor carcass disposal, environmental spore survival, and traditional practices such as “Purak” slaughter.

Conclusion: This study provides novel insights into the virulence and genetic stability of *B. anthracis* in three Indonesian provinces. The findings emphasize the need for integrated control measures that include enhanced surveillance, public education, vaccination campaigns, and environmental decontamination. A robust One Health approach is essential for the sustainable management and eventual eradication of anthrax in endemic regions.

Keywords: anthrax, *Bacillus anthracis*, endemic regions, Indonesia, multiplex polymerase chain reaction, One Health, phenotypic characteristics.

Corresponding Author: Soedarmanto Indarjulianto
E-mail: indarjulianto@ugm.ac.id

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Co-authors: UIA: ullyindah82@mail.ugm.ac.id, HW: hi.wibawa@gmail.com, TU: t_untari@ugm.ac.id, ER: endru284@gmail.com, BRS: bsuryanto3@gmail.com, LL: lestari.dvm@gmail.com, FN: fatah.nugroho@mail.ugm.ac.id

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INTRODUCTION

Anthrax is a zoonotic disease caused by *Bacillus anthracis*, a Gram-positive, rod-shaped bacterium capable of forming both capsules and spores. The bacterium exhibits two distinct life stages: A vegetative phase and a dormant spore phase. *B. anthracis* spores are notably resistant to heat, desiccation, and several disinfectants, enabling them to persist in harsh environmental conditions for extended periods. Their survival is influenced by both biotic and abiotic environmental factors [1]. While vegetative cells develop within susceptible hosts and produce a lethal toxin complex, the spores remain as long-term contaminants in soil [1, 2]. Anthrax affects all mammalian species, including humans, thus posing significant zoonotic risks [3]. Herbivores are commonly infected through ingestion of spores present in contaminated pastures or environments [2]. From a One Health perspective, human transmission may occur through four primary routes: cutaneous, gastrointestinal, inhalational, and injectional [1, 4, 5], highlighting the necessity for coordinated surveillance across human, animal, and environmental health sectors.

In Indonesia, anthrax has been documented in 22 of the country's 34 provinces [6]. However, epidemiological studies remain scarce in several high-risk regions. This study seeks to bridge this knowledge gap by concentrating on provinces with both high livestock densities and endemic anthrax – specifically Central Java, East Java, and the Special Region of Yogyakarta (D.I. Yogyakarta), which fall under the authority of the Balai Besar Veteriner Wates [Disease Investigation Center (DIC), Wates]. These provinces are characterized by substantial livestock populations, and certain districts continue to report anthrax cases. The earliest confirmed case of anthrax in this region occurred in 1990 in dairy cattle imported from the United States and introduced to the districts of Semarang, Salatiga, and Boyolali. Historical records also indicate outbreaks in Tegal, Pekalongan, Surakarta, and Banyumas between 1906 and 1957 [6]. Despite ongoing surveillance and control initiatives, anthrax remains endemic, with recurrent cases in districts such as Blitar, Pacitan, Wonogiri, Sragen, Kulon Progo, Sleman, and Bantul [7]. Numerous isolates of *B. anthracis* obtained through outbreak investigations, surveillance, and monitoring have been archived at DIC, Wates; however, many were only identified based on phenotypic features without further molecular characterization.

This study investigates anthrax cases occurring in East Java, Central Java, and Yogyakarta between 1990 and 2021. It focuses on the phenotypic and genetic characterization of *B. anthracis* isolates and evaluates their transmission patterns and associated risk factors. By integrating phenotypic, molecular, and epidemiological data, this research offers novel insights into the dynamics of anthrax transmission and provides essential evidence to inform future public health and veterinary control strategies in Indonesia.

MATERIALS AND METHODS

Ethical approval

B. anthracis isolates from freeze-dried and glycerin media were used as samples. All experiments were conducted in a BSL-2 plus facilities laboratory (DIC Wates) with accreditation number: LP-618-IDN in accordance with the guide for *B. anthracis* identification [8, 9] recommended by the Ministry of Agriculture, Indonesia.

Study period and location

The study was conducted from January 2019 to July 2021. The samples were processed at the Zoonosis Laboratory of DIC Wates using BSL-2 plus facilities.

Origin of isolates

This study analyzed 28 *B. anthracis* isolates, comprising 13 archived isolates from the DIC Wates collection (1990–2017) and 15 recently obtained isolates identified between 2019 and 2021 (Table 1). The isolates were sourced from 12 districts located in anthrax-endemic regions across three provinces: Central Java, East Java, and the Special Region of Yogyakarta (Figure 1). The samples included both animal-derived specimens (such as ethylenediaminetetraacetic acid blood, nasal swabs, and organs) and environmental materials (including soil, sawdust, and straw). All isolates were stored at −20℃ before analysis. Avirulent reference strains – *B. anthracis* Sterne 34F2 and *B. cereus* ATCC 11778 (Culti-Loops™; Thermo Scientific, USA) – were employed as controls. All laboratory procedures adhered to the standards set forth by the World Organization for Animal Health [8] and World Health Organization guidelines [9].

Phenotypic and genotypic characterization

Conventional microbiological techniques were employed for bacterial isolation using agar media, assessment of motility, antibiotic susceptibility testing (penicillin, tetracycline, and ciprofloxacin), Giemsa staining, capsule staining, and spore staining, following established protocols described in earlier studies by World Health Organization [9] and Apriliana *et al*. [10]. The studies by Apriliana *et al*. [10] and Ramisse *et al*. [11] presented a novel methodological framework by integrating phenotypic analysis with multiplex polymerase chain reaction (PCR) targeting the Ba813 chromosomal marker along with the *lef* and *capC* virulence genes [10, 11], offering a new diagnostic perspective for anthrax detection in Indonesia. All experimental procedures were conducted at the Biotechnology Laboratory of DIC Wates Yogyakarta. Descriptive statistical analysis of laboratory findings was performed to explore associations between isolate characteristics and known risk factors contributing to anthrax transmission.

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Table 1: Sample origin and phenotypic–genetic characteristics of the studied isolates.

Isolate number	Isolate source	Province	Year	Hemolyze	Mucoid colony	Motility	Capsule	Sensitivity to penicillin G*	PCR amplification		
									*Ba*813	*lef*	*capC*
1	Freeze dried	Central Java, Semarang	1990	No	Mucoid	No	Yes	S	+	+	+
2	Cow	Yogyakarta, Sleman	2003	No	Mucoid	No	Yes	S	+	+	+
3	Soil	Central Java, Pati	2007	No	Mucoid	No	Yes	S	+	+	+
4	Soil	Central Java, Sragen	2010	No	Mucoid	No	Yes	S	+	+	+
5–6	Soil	Central Java, Sragen	2011	No	Mucoid	No	Yes	S	+	+	+
7–8	Soil	Central Java, Boyolali	2011	No	Mucoid	No	Yes	S	+	+	+
9	Cow	East Java, Blitar	2014	No	Mucoid	No	Yes	S	+	+	+
10	Cattle	Central Java, Wonogiri	2016	No	Mucoid	No	Yes	S	+	+	+
11	Sawdust	Yogyakarta, Kulon Progo	2017	No	Mucoid	No	Yes	S	+	+	+
12	Sheep	Yogyakarta, Bantul	2017	No	Mucoid	No	Yes	S	+	+	+
13	Soil	Yogyakarta, Gunungkidul	2019	No	Mucoid	No	Yes	S	+	+	+
14–15	Cattle	Yogyakarta, Gunungkidul	2019	No	Mucoid	No	Yes	S	+	+	+
16–21	Soil	Yogyakarta, Gunungkidul	2020	No	Mucoid	No	Yes	S	+	+	+
22–23	Cattle	Yogyakarta, Gunungkidul	2020	No	Mucoid	No	Yes	S	+	+	+
24–25	Soil	East Java, Pacitan	2021	No	Mucoid	No	Yes	S	+	+	+
26	Goat	East Java, Pacitan	2021	No	Mucoid	No	Yes	S	+	+	+
27	Cattle	East Java, Tulungagung	2021	No	Mucoid	No	Yes	S	+	+	+
28	Soil	East Java, Tulungagung	2021	No	Mucoid	No	Yes	S	+	+	+
29	*Bacillus anthracis*	Sterne 34F2-Vaccine strain		No	Non-mucoid	No	No	S	+	+	-
30	*Bacillus cereus*	ATCC-11778		Yes	Non-mucoid	Yes	No	R	-	-	-

*S=Sensitive, R=Resistant, PCR=Polymerase chain reaction

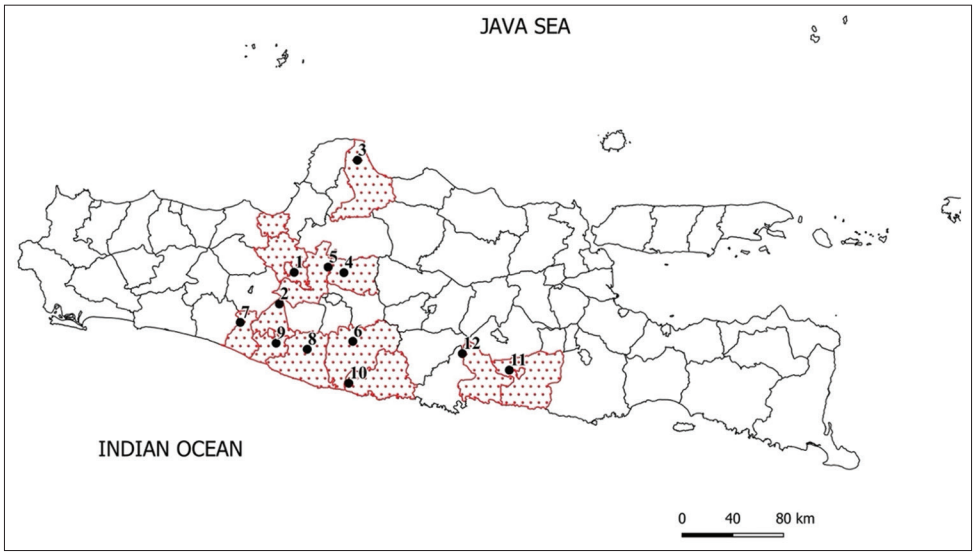

Figure 1: District of anthrax cases in Central Java, East Java, and Yogyakarta from 1990 to 2021 with sequence of events: (1) Semarang, (2) Sleman, (3) Pati, (4) Sragen, (5) Boyolali, (6) Wonogiri, (7) Kulon Progo, (8) Gunungkidul, (9) Bantul, (10) Pacitan, (11) Blitar, and (12) Tulungagung.

RESULTS

Phenotypic characteristics

All 28 *B. anthracis* isolates, along with the Sterne 34F2 strain, exhibited no hemolysis when cultured on 5% sheep blood agar, in contrast to *B. cereus* ATCC 11778, which displayed hemolytic activity. The colonies of *B. anthracis* appeared grayish in color, measured between 2 and 3 mm in diameter, and had flat to slightly convex, dull, and opaque surfaces with irregular margins, commonly described as having a "frosted glass" appearance. Some colonies presented with a distinctive comma-shaped extension. When lifted with a loop, the colonies exhibited a sticky consistency.

These phenotypic traits of *B. anthracis*, interpreted through a One Health lens, underscore the necessity for integrated disease control measures that account for both environmental persistence and zoonotic transmission. When cultured on media enriched with

0.8% sodium bicarbonate under 5% carbon dioxide (CO_2) at 37℃, nearly all isolates produced mucoid colonies. Exceptions included the *B. anthracis* Sterne 34F2 strain and *B. cereus* ATCC 11778, both of which remained non-mucoid under these conditions.

Antibiotic susceptibility testing revealed that all *B. anthracis* isolates were sensitive to penicillin, tetracycline, and ciprofloxacin. In contrast, *B. cereus* ATCC 11778 demonstrated resistance to penicillin while retaining sensitivity to tetracycline and ciprofloxacin.

Microscopic morphology

All examined isolates were Gram-positive bacteria exhibiting a large, rod-shaped morphology with square ends and elongated cells, resembling *B. anthracis* strain Sterne 34F2 (Figure 2a). In contrast, *B. cereus* ATCC 11778 appeared as large rods with rounded ends, typically arranged in short chains of two to three cells. Polychrome methylene blue staining of isolates cultured on bicarbonate-enriched medium (Figure 2b) and defibrinated sheep blood medium (Figure 2c) revealed the presence of a pink amorphous zone surrounding the cells, indicating capsule formation in all 28 isolates. Conversely, no capsule formation was observed in *B. anthracis* strain Sterne or *B. cereus* ATCC 11778. Spore staining produced consistent results across both test and control isolates, revealing centrally located pink spores or unstained gaps within the bacterial cells (Figure 2d).

Molecular testing using multiplex PCR

Multiplex PCR successfully amplified the species-specific Ba813 chromosomal marker, along with the *lef* gene from the pXO1 plasmid and the *capC* gene from the pXO2 plasmid in all 28 tested isolates, confirming their identity as *B. anthracis*. The Sterne 34F2 strain, serving as a positive control for the avirulent form of *B. anthracis*, produced amplification of only the Ba813 and *lef* targets (Figure 3). Each of the 28 isolates (sample codes 1–28) was fully characterized with respect to its phenotypic and virulence profiles and confirmed to be *B. anthracis* (Table 1).

DISCUSSION

B. anthracis exhibits two distinct life cycle stages: A transient vegetative phase and a dormant spore phase, with the latter capable of prolonged environmental persistence [2]. The spore stage supports long-term survival and facilitates an extended evolutionary process, contributing to the genetic monomorphism observed in *B. anthracis* populations [12]. The findings of this study, interpreted through a One Health framework, highlight the complex interrelationship between environmental reservoirs, livestock management practices, and public health challenges in sustaining anthrax endemicity across Central Java, East Java, and Yogyakarta.

All isolates derived from clinical and environmental samples across 12 districts consistently demonstrated phenotypic characteristics typical of classical *B. anthracis*.

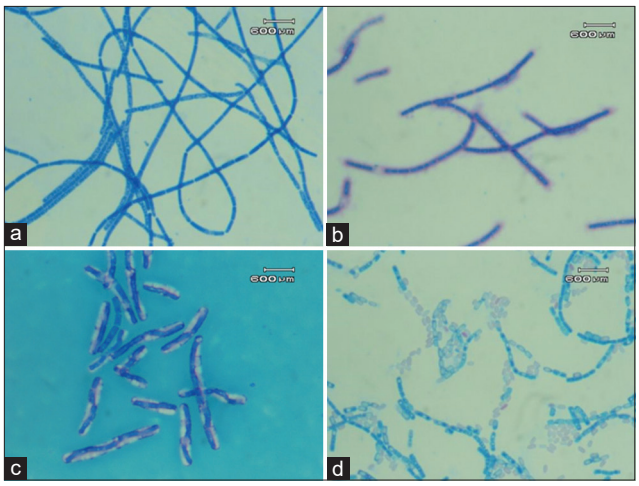

Figure 2: (a) Gram staining and polychrome methylene blue staining showing capsule formation (b) from bicarbonate media, (c) from defibrinated sheep blood media, and (d) modified Ziehl-Neelsen staining for spores (pink).

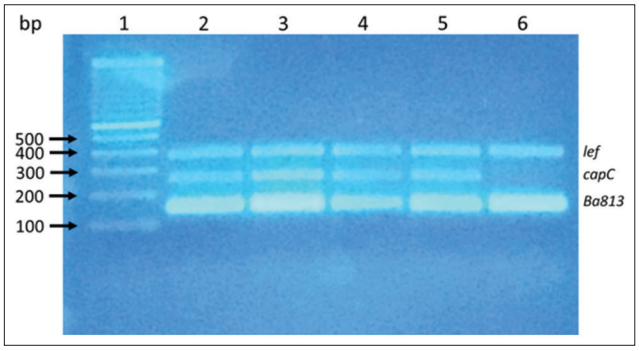

Figure 3: Lanes 2–5, *B. anthracis* isolate; lane 1, 100-bp DNA size marker; lane 6, *B. anthracis* strain Sterne 34F2. *B. anthracis=Bacillus anthracis.*

These included Gram-positive staining, large rods with square ends, absence of hemolysis, non-motility, sensitivity to penicillin, and capsule formation when cultured on media supplemented with bicarbonate under a 5% CO_2 atmosphere or on defibrinated blood agar, consistent with previously reported observations by Doganay and Demiraslan [13].

Multiplex PCR analysis of all isolates revealed amplification of three target genes: the chromosomal marker Ba813 (152 bp), the *lef* gene located on plasmid pXO1 (385 bp), and the *capC* gene on plasmid pXO2 (264 bp) [10, 11]. These results provide novel molecular evidence of the virulence profiles of *B. anthracis* isolates from Indonesia and contribute valuable data on the genetic composition of strains circulating within Southeast Asia.

At present, molecular methods are widely adopted for virulence assessment, offering an alternative to traditional approaches that require specialized animal containment facilities and skilled personnel and raise ethical concerns related to the use of vertebrate animals in experimentation [14]. PCR-based virulence

testing offers several advantages: it reduces diagnostic time, lowers the risk of accidental exposure, permits analysis of small sample volumes, and effectively differentiates between virulent and avirulent strains of *B. anthracis* [15].

B. anthracis possesses two major extrachromosomal plasmids that encode its key virulence factors. The pXO1 plasmid carries genes essential for anthrax toxin production – *pagA*, *lef*, and *cya* – while the pXO2 plasmid encodes the cap gene operon (*capBCADE*), responsible for the synthesis of a poly-γ-D-glutamic acid capsule [16–18]. These plasmids are critical for differentiating *B. anthracis* from other species within the *B. cereus* group. However, atypical *B. cereus* strains have been identified that produce anthrax-like disease in humans and animals, exhibiting chromosomal similarities and harboring plasmids analogous to those of *B. anthracis* [19, 20].

Loss of either plasmid can lead to attenuation of *B. anthracis* virulence [21]. While strains lacking the pXO2 plasmid may retain pXO1 and remain toxigenic in certain mouse models [22], widespread documentation exists of pXO2 plasmid loss in *B. anthracis* strains [10, 11, 23]. For instance, the Pasteur strain was found to lose the pXO1 plasmid after incubation at temperatures between 42°C and 43°C for 10–20 days, though this observation remains under evaluation, as some derivatives continue to express the protective antigen protein [21].

Previous research identified two *B. anthracis* isolates from the Pati and Boyolali districts that, while phenotypically similar to classical strains – exhibiting non-hemolytic, non-motile colonies with sticky and irregular edges – produced non-mucoid colonies on bicarbonate medium and failed to amplify the *capC* gene during multiplex PCR testing. These findings suggest that the isolates may represent avirulent *B. anthracis* strains [10].

The provinces of Yogyakarta, Central Java, and East Java remain endemic for anthrax, with complete eradication yet to be achieved. As these provinces are contiguous on the island of Java, bordering one another directly, the movement of livestock between districts is frequent. Phenotypic and genetic assessments of *B. anthracis* isolates from these areas revealed uniform morphological characteristics and consistent virulence profiles, suggesting a degree of genetic homogeneity among circulating strains. The first reported anthrax outbreak in Central Java occurred in 1990 in Semarang, Boyolali, and Salatiga districts [6], after which the disease progressively spread to 12 districts. One of the main factors contributing to this spread is the movement of cattle through livestock markets, where animals originating from anthrax-endemic zones – including goats and sheep – are commonly traded [1].

Most anthrax cases in this region are new; however, recurrent cases have been documented in the Boyolali district after an 18-year hiatus, with re-emergence occurring between 2008 and 2012. Consequently, Boyolali has become a hotspot for anthrax. This district holds the highest population of dairy cattle in Central Java (67.08%) and also contains other anthrax-susceptible livestock – including beef cattle, buffaloes, goats, and sheep – representing 3.23% of the province's total livestock population. The substantial volume of cattle transported out of Boyolali, either through livestock trade or direct purchases from local farmers, facilitates the rapid and widespread transmission of anthrax to neighboring areas. In addition to Boyolali, livestock movement through markets also occurs in the districts of Sragen, Wonogiri, Pacitan, and Gunungkidul [7]. The common practice among farmers of selling sick cattle to markets and local butchers (referred to as "jagal") to avoid financial loss significantly increases the risk of anthrax transmission. Furthermore, the circulation of carcasses from infected cattle extends to markets, as observed in Pacitan and Wonogiri districts, posing additional risks for human infection [24, 25]. Limited regulation and supervision of livestock movement through market channels – especially in high-risk areas such as Boyolali – have played a substantial role in the ongoing spread of anthrax across the three provinces [26].

Beyond livestock traffic, the occurrence of anthrax is influenced by natural factors such as seasonal variation, climate, temperature, and rainfall. Environmental characteristics – including organic matter content in the soil, pH, soil type, vegetation, and topography – also significantly affect spore survival [27]. Outbreaks in Pacitan, Kulon Progo, and Gunungkidul districts occurred in upland areas. In these settings, infected cattle are often slaughtered near their enclosures, with skinning and washing of carcasses taking place on-site or in adjacent rivers. This practice allows spores to spread to downstream regions through rainwater runoff, as previously reported by Otieno *et al*. [27]. Anthrax-related livestock deaths frequently arise at the beginning of the rainy season, following prolonged dry periods during which spores present on soil surfaces become mobilized by rain and come into contact with grazing animals [25]. In Gunungkidul district, anthrax cases between December 2019 and January 2020 were linked to persistent environmental contamination. Spores remained detectable for over 10 months, despite disinfection efforts. Similarly, soil samples from endemic monitoring in Sragen district tested positive 2 years after the last reported animal death. These findings suggest that ineffective disinfection may have enabled *B. anthracis* spores to persist in the environment [27]. According to previous study by Yim *et al*. [28], calcium hypochlorite and quaternary ammonium compounds (QAC) are more effective than sodium hypochlorite in eliminating vegetative cells. Furthermore, a sequential application of calcium hypochlorite followed by sodium

hypochlorite was more efficient in spore elimination compared to QAC alone. The long-term environmental survival of anthrax spores is a key factor contributing to the disease's re-emergence in endemic areas [13].

The practice of slaughtering sick or recently deceased cattle has been consistently identified as a major risk factor for anthrax transmission in the three provinces [26]. A traditional practice known as "Purak," involving the slaughter of sick livestock in collaboration with local residents and the subsequent sale of the meat at low prices, further facilitates the spread of anthrax, as noted in earlier studies by Islam *et al*. [29] and Kisaakye *et al*. [30]. Human exposure can occur during the handling, skinning, transport, and consumption of meat from infected animals, even when cooked, potentially resulting in cutaneous, gastrointestinal, or inhalational anthrax [29, 31]. Transmission is not limited to animal-to-animal spread through spore contamination; direct human involvement in slaughtering activities also poses a risk. The majority (95%) of human anthrax cases are cutaneous, with the remaining 5% being gastrointestinal or inhalational forms [32]. Makurumidze *et al*. [33] reported that 43% of cutaneous anthrax cases were associated with the slaughtering and skinning of sick animals, and with religious customs permitting the consumption of meat from animals that died under unknown circumstances. Recurrent anthrax incidents often occur in the same location or nearby areas where cattle were forcibly slaughtered during earlier outbreaks [32, 34]. The traditional practice of bathing cattle in rivers is particularly risky, as local residents sometimes wash the internal organs of sick or slaughtered animals in these waterways, contaminating the watershed. PCR testing of three river water samples revealed one positive result for *B. anthracis* [1], and similar waterborne anthrax transmission has been observed in wild animals such as hippopotamuses, which were found dead near contaminated watering holes [35].

Lack of awareness among farmers regarding anthrax – particularly in relation to the management of infected animals – along with delays in case reporting and fear of economic loss due to animal death reports often leads to the concealment of cases and the forced sale of sick livestock. These behaviors further exacerbate anthrax transmission [25, 36]. Musewa *et al*. [37] highlighted that poverty, insufficient access to animal protein, and financial hardship are key factors prompting farming communities to consume meat from infected animals.

Controlling anthrax outbreaks in domestic animals relies on the prompt identification and treatment of infected livestock and the intensification of case surveillance. Effective control strategies include quarantine, chemoprophylaxis, vaccination, restriction of animal access to suspected contamination sources (e.g., infected feed or pastures), proper carcass disposal, and thorough disinfection of affected areas [29, 38, 39]. Vaccination remains a critical preventive measure in endemic zones [31, 40]. However, inadequate vaccination coverage – stemming from improper vaccine administration, insufficient dosing, reduced vaccine immunogenicity, variable host responses, and uneven vaccine distribution – continues to contribute to the recurrence of anthrax in these areas [8, 40].

CONCLUSION

This study provided comprehensive phenotypic and genotypic characterization of *B. anthracis* isolates collected from anthrax-endemic districts in Central Java, East Java, and the Special Region of Yogyakarta between 1990 and 2021. All 28 isolates consistently exhibited classical *B. anthracis* features, including Gram-positive morphology, non-hemolytic and non-motile characteristics, capsule formation under specific conditions, and susceptibility to penicillin, tetracycline, and ciprofloxacin. Multiplex PCR analysis confirmed the presence of chromosomal (Ba813) and plasmid-borne virulence genes (*lef* on pXO1 and *capC* on pXO2), reinforcing the identification and virulence potential of the isolates. These findings suggest genetic homogeneity among strains circulating in the region and underscore persistent anthrax transmission driven by environmental persistence, livestock movement, low vaccination coverage, and traditional animal handling practices.

The primary strength of this study lies in its integration of phenotypic, molecular, and epidemiological data within a One Health framework, enabling a multidimensional understanding of anthrax ecology in Indonesia. The temporal scope (three decades) and geographic coverage across 12 endemic districts add further significance to the dataset.

However, some limitations must be acknowledged. First, the study did not perform whole-genome sequencing or advanced molecular typing (e.g., multilocus variable-number tandem repeat analysis or single-nucleotide polymorphism-based phylogenetics), which would offer deeper insights into strain evolution and transmission networks. Second, while descriptive epidemiological analysis was informative, quantitative risk assessments and spatial modeling were not conducted. In addition, environmental persistence of spores was inferred from historical data and PCR positivity but was not assessed through viability or spore load quantification.

Future studies should prioritize high-resolution genotyping and longitudinal environmental monitoring to delineate the genomic diversity and ecological niches of *B. anthracis*. Incorporating spatial epidemiological tools and assessing vaccine coverage effectiveness will further strengthen anthrax control strategies. A coordinated national surveillance program that bridges veterinary and human health sectors is essential

for mitigating outbreak risks and progressing toward anthrax elimination in Indonesia.

AUTHORS' CONTRIBUTIONS

SI, TU, and HW: Conceptualized, managed, and supervised the study. UIA, ER, and BRS: Performed all experimental procedures and drafted the manuscript. UIA: Data analysis and interpretation. UIA and FN: Collected the data and literature and edited and revised the manuscript. All authors have read and approved the final manuscript.

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COMPETING INTERESTS

The authors report no competing interests to declare.

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