Chapter 10
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Study on Simple Detection of *Bacillus anthracis* Spores by Precipitation Method with Goat Antibody Anti Anthrosa

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ABSTRACT

Background: *Bacillus anthracis* has the potential to be used as a biological weapon or as a form of bioterorism. *Bacillus anthracis* attacks are very lethal, and spore transmission is simple and inexpensive. The goal of this research was to detect *Bacillus anthracis* spores.
Methods: To encourage capsule formation, *Bacillus anthracis* isolates were cultured on serum agar and then sheep blood medium.
Spores which formed painted using the method of Schaefer and Fultton. *Bacillus anthracis* spores were detected using precipitation and immuno-chromatography techniques.
Results: *Bacillus anthracis* spores are green, as seen by painting with the Schaeffer and Fulton method. A silver white tint resulted from a precipitation interaction between *Bacillus anthracis* spores and goat antibody anti anthrosa.
Anti-anthrosa goat antibody was used to identify Anthrosa of Bacillus anthracis spores using immuno-chromatography. The molecular weight of anthrosa is ±148 kDa.
Conclusions: *Bacillus anthracis* spores can be detected utilising the methods of precipitation and immuno-chromatography using goat antibody anti anthrosa. Anti-anthrosa antibodies from goats have been found to react favourably with *Bacillus anthracis* spores.

Keywords: Bacillus anthracis; bioterrorism; biological weapon; anthrosa; goat antibody anti anthrosa.

1. INTRODUCTION

Some biological agents have been used, among others, anthrax, plaque, smallpox, tularemia and viral haemorrhagic fever [1]. Bacillus anthracis causes anthrax, a disease with bioterrorism potential [2]. Bacillus anthracis spores could be used as a biological weapon in the future.

Bacillus anthracis spores were chosen as a biological weapon due to their high killing power, short incubation period, low cost, and ability to be packaged as an aerosol form of biological agents (aerosolize bioagents). It has been proven that *Bacillus anthracis* spores then packaged in a letter sent by post, giving rise to fears that biological warfare agents (biological weapon agents = BWAs) used by terrorists to attack civilians [3].

The United States Centers for Disease Control and Prevention (CDC) states that *Bacillus anthracis* were subjected for "biological warfare research programs" over 60 years ago in some countries. CDC classifies that anthrax including category A bioterrorism agent, because it can kill people in large numbers and the large area [4].

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Because that the mechanism of biological attacks/threats prevention should comprise: primordial, primary, secondary and tertiary levels of prevention of biological attacks/threats [5]. Anthrax disease including zoonotic disease that is potentially lethal to humans [6,7]. *Bacillus anthracis* found in two forms, namely vegetative and spores form [8]. The principle of anthrax virulence factor is the production of capsules and 2 exotoxin during vegetative form. Endospores is an infectious agent and are resistant to heat, drying, chemicals or irradiation, and can survive in the long term [9].

The aerosol of *Bacillus anthracis* spores are selected as a weapon for bioterrorism. *Bacillus anthracis* spore infections affect different parts of the human body such as the intestines, skin and lungs, causing gastrointestinal, skin and lungs diseases [7]. Anthrax spores that infect into a person's body will grow tobecome deadly anthrax disease.

To counteract biological attack such as anthrax bacteria, including anthrax and biological weapons bioterorism anthrax can be done by detecting the presence of anthrax spores. Anthrax spores can be recognized in various ways such as by recognizing anthrosa compounds contained in the walls of spores. The outermost layer of *Bacillus anthracis* spores called exosporium, which contains a number of proteins. The protein constituent exosporium called Bacillus collagen-like protein of anthracis (BclA), which is a glycoprotein which contain short chain sugars with the O-glycosylation. The protein is known as anthrose [10].

BclA arrangement can be seen using mass spectroscopy (MS) and nuclear magnetic resonance spectroscopy (NMR). Glycosyl part of BclA are oligosaccharides consisting of 2-O-methyl-4- (3-hydroxy-3-methylbutanamido)-4.6-dideoxy-D-glucose, hereafter referred anthrose, and three residues rhamnose [11].

Exosporium of the spores of *Bacillus cereus* group (*Bacillus cereus, Bacillus anthracis and Bacillus thuringiensis*) that plays a major role in spore adhesion and virulence. BclA is a major constituent of its hair-like surface [12,13]. The BclA is known to be used for developing diagnostics of *Bacillus anthracis* spores and thus targeted therapeutic interventions [12].

Various ways have been conducted to identify the spores of *Bacillus anthracis*, among others, with poly chain reaction (PCR) [2,14,15,16] spectrometry, flow cytometry and transmission electron microscopy [17,18]. Methods developed to detect anthrax spores contained in an envelope that is by engineering mikrospectroscopy [19]. Amperometric technique was also used for detecting *Bacillus anthracis* [20].

Besides these ways, the technique of enzyme-linked immunosorbent assay (ELISA) was developed to detect anthrax spores [9]. The purpose of this study wanted to detect vegetative cells and spores of *Bacillus anthracis* using a microscope, biochemical tests, precipitation and immunochromatography using goat antibody anti anthrosa. Using the principle of immunochromatography, anthrosa strip can be made to detect the presence of *Bacillus anthracis* spores.

2. METHODS

2.1 Isolation of *Bacillus anthracis*

Bacteria isolated from vesicular fluid, then cultured on polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar base medium (Sigma-Aldrich). Isolates were then cultured in nutrient broth (Sigma-Aldrich) was then performed Gram. Motility test used tryptic soy broth medium (Sigma-Aldrich) and incubated at 37° C for 24 hours.

2.2 Biochemistry Test for Vegetetive cells of *Bacillus anthracis*

Bacteria isolates were cultured in a nutrient broth (Sigma-Aldrich) and nutrient agar (Sigma-Aldrich). Culture in nutrient broth carried out to nutrient broth test, citrate test, and glucose test. Culture on nutrient agar carried out to 7% Na Cl test and blood agar test.

2.3 Induction and Staining Spores of *Bacillus anthracis*

Bacillus anthracis isolates were cultured on blood agar (Sigma-Aldrich) to induce sporulation. Spores are formed painted with malachite green as primary stain and the counterstain is safranin.

2.4 Anthrosa Detection

Anthrosa detection is done by two ways, namely precipitation and electrophoresis. Precipitation reactions conducted on anthrosa using goat antibody anti anthrosa. Goat antibody anti anthrosa obtained by purification of the blood serum of goats previously vaccinated with anthrax vaccine. Electrophoresis was performed to detect of anthrosa using protein marker.

3. RESULTS

The results of Gram staining and biochemical tests on the *Bacillus anthracis* is presented in Fig. 1.

The results of Gram staining and biochemical tests on the *Bacillus anthracis*. A=the results of Gram staining; B=broth test (B1 and B2=*Bacillus anthracis* in the brooth medium, B3=*Bacillus cereus* in the brooth medium, B4=*Bacillus thuringiensis* in the brooth medium); C=nutrient broth were shaken test (C1=*Bacillus anthracis*, C2=*Bacillus cereus,* C3=*Bacillus thuringiensis*); D=citrate test (D1 and D2=*Bacillus anthracis*); E=glucose test (E1-8=*Bacillus anthracis*); F=blood agar test (F1=*Bacillus anthracis* on agar medium with 7% Na Cl, F2=*Bacillus anthracis* on blood agar, F3=*Bacillus subtilis* on blood agar medium) (Fig. 1).

The results of *Bacillus anthracis* painting with paint Gram, showed a purple color that react positively. *Bacillus anthracis* including Gram positive, bacilli shaped and non motile bacteria.

Bacillus anthracis cultured in nutrient broth is not made turbid (Fig. 1 (B1 and B2)), while *Bacillus cereus* (Fig. 1 (B3)) and *Bacilluc thuringiensis* (Fig. 1 (B4)) were cultured in a nutrient broth becomes cloudy.

Bacillus anthracis culture in nutrient broth when shaken showed the typical image that is like tendrils of silkworms (Fig. 1 (C1)), while *Bacillus cereus* (Fig. 1 (C2)) and *Bacillus thuringiensis* (Fig. 1 (C3)) in nutrient broth when shaken remain murky. *Bacillus anthracis* cultured in medium enriched citrate to show negative (Fig. 1 (D1)) and positive reaction (Fig. 1 (D2)).

Bacillus anthracis cultured in medium enriched glucose will not form acid and gas (Fig. 1E). *Bacillus anthracis* cultured on agar medium with 7% NaCl showed good growth (Fig. 1 (F1)). Culture of *Bacillus anthracis* did not hemolysis on blood agar medium (Fig. 1 (F2)), while the *Bacillus subtilis* cultures do hemolysis on blood agar medium (Fig. 1 (F3)).

Results of painting vegetative cells and spores of *Bacillus anthracis* presented in Fig. 2. Fig. 2. Results of painting vegetative cells and spores of *Bacillus anthracis*. A=vegetative cells of *Bacillus anthracis* seem rod-shaped chain; B, C and D=spores of *Bacillus anthracis* seem solitary and green. Gram staining on cells of *Bacillus anthracis* showed purple. Results of precipitation reactions on the anthrose using goat antibody anti anthrosa and electrophoresis is presented in Fig. 3.

Fig. 3 Anthrosa precipitation of *Bacillus anthracis* with goat antibody anti anthrosa and electrophoresis. A=anthrosa precipitation of *Bacillus anthracis* with goat antibody anti anthrose; B=precipitation reaction of *Bacillus cereus* with goat antibody anti anthrose. C=Electrophoresis of anthrose that reaction with goat antibody anti anthrose (1=protein marker, 2 and 3=*Bacillus anthracis*, 4=*Bacillus cereus*, 5 and 6=*Bacillus subtilis*, 7=*Bacillus thuringiensis*, 8 and 9=buffer sample); µM=micro Mol; kDa=kilo Dalton.

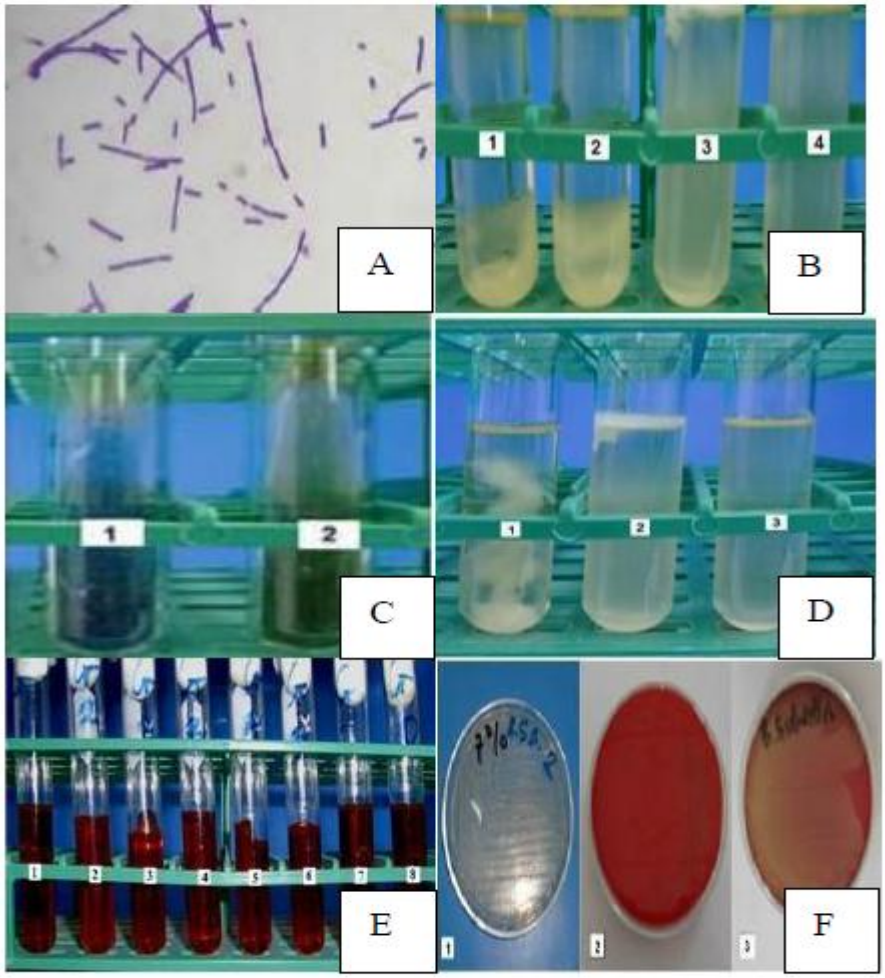

Fig. 1. The results of gram staining and biochemical tests on the *Bacillus anthracis*

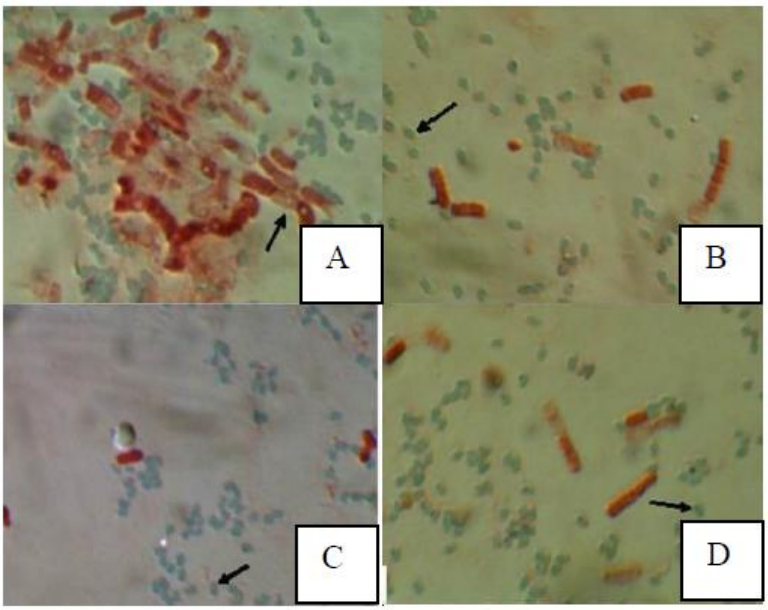

Fig. 2. Results of painting vegetative cells and spores of *Bacillus anthracis*

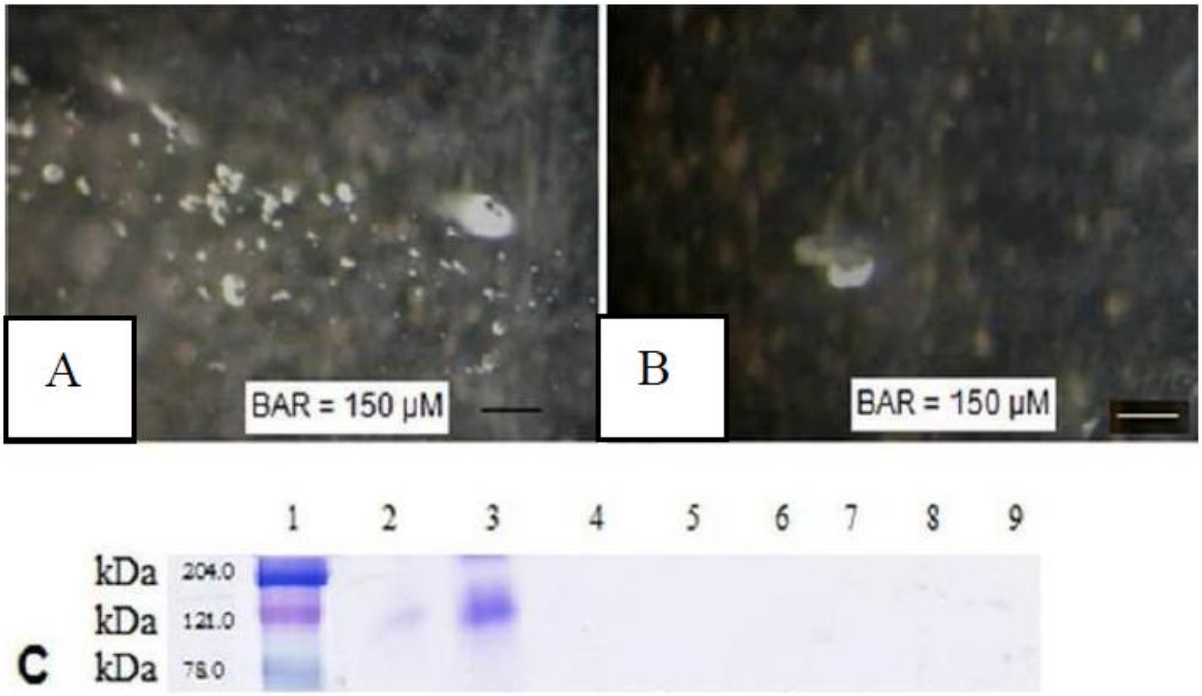

Fig. 3. Anthrosa precipitation of *Bacillus anthracis* with goat antibody anti anthrosa and electrophoresis

Precipitation reaction between anthrosa of *Bacillus anthracis* with goat antobody anti anthrose showed a lot of whitish, while the *Bacillus cereus* are not many shows a whitish color (Fig. 3A and B).

The result meant that the precipitation test on the *Bacillus anthracis* reacted positively, whereas on the *Bacillus cereus* showed negatively. Electrophoresis results on *Bacillus* spore suspension shows that anthrosa in *Bacillus anthracis* is detected with a molecular weight ± 148 kDa (Fig. 3. C).

4. DISCUSSION

In this study, isolates of *Bacillus anthracis* cultured on PLET agar medium. The medium is used as a selective growth medium [21]. In many countries, the use of PLET agar medium that contains highly toxic thallium acetate at a high concentration, excludes its use due to work-safety regulations [22]. Gram staining on cells of *Bacillus anthracis* showed purple so reacted positively. *Bacillus anthracis* including Gram positive bacteria, bacillus shaped according to research confirming that anthracis, the causative agent of anthrax disease, is a Gram-positive spore-forming bacterium [23,24]. Previous research showed that the composition of the cell wall of *Bacillus anthracis* is dominated by carbohydrates and very specific [25].

Bacillus anthracis found in two forms, namely vegetative and spores form. However, vegetative cell of *Bacillus anthracis* have very specific nutrient and physiological requirements survive outside a host [26]. Subsequent research showed that *Bacillus anthracis* the causative agent of anthrax secretes a tri-partite exotoxin that exerts pleiotropic effects on the host. The purification of the exotoxin components, protective antigen, lethal factor, edema factor and allowed the rapid characterization of reviews their physiologic effects on the host [27].

The other result of study show that poly-gamma-D-glutamic acid capsule antiphagocytic properties confers on *Bacillus anthracis* and is essential for virulence [8]. Another study show that sporulation was induced by growth on nutrient agar plates at 30°C with appropriate antibiotic selection. Sporulation was essentially complete (>95%) after 72 hours [28]. Some spore proteins play roles in

assembly of both the coat and exosporium. CotE of *Bacillus anthracis* directs assembly of at least one coat protein other than itself [29].

Results of previous studies show that gene expression in *Bacillus anthracis* during growth and sporulation. Large portion (~36%) of the *Bacillus anthracis* genome is regulated in a growth phase-dependent manner, and this regulation is marked by five distinct waves of gene expression as cells proceed from exponential growth through sporulation. More than 750 proteins present in the spore. Also reported that the genes responsible for the assembly and maturation of the spore are tightly regulated in discrete stages. The spore also contains an assortment of specialized, but not obviously related, metabolic and protective proteins [30].

Bacillus anthracis in a nutrient broth medium does not make turbid (Fig. 1 (B1 and B2)), this means that *Bacillus anthracis* does not ferment glucose, peptone and yeast extract that are components of the medium. Instead, *Bacillus cereus* (Fig. 1 (B3)) and *Bacillus thuringiensis* (Fig. 1 (B4)) were cultured in a nutrient broth becomes cloudy, it means that the bacteria are able to ferment glucose, peptone and yeast extract in a medium so as to produce metabolites. Results of research have shown that transcription of the major virulence factors, namely: toxin and capsule, triggered by bicarbonate which is becoming a major compound in the mammalian body. Further also been shown that glucose is an additional signaling molecule recognized by *Bacillus anthracis* for toxin synthesis [31].

Bacillus anthracis cultured in nutrient broth when shaken showed the typical image that is like tendrils of silkworms (Fig. 1 (C1)), while *Bacillus cereus* (Fig. 1 (C2)) and *Bacillus thuringiensis* (Fig. 1 (C3)) remain murky. The fact is the hallmark of *Bacillus anthracis*. *Bacillus anthracis* cultured in medium enriched citrate react negatively (Fig. 1 (D1)) and positively (Fig. 1 (D2)). The varied reactions may be due to genetic variation of *Bacillus anthracis* causes the difference in response to the formation of siderophore citrate.

The results showed that asbABCDEF gen of *Bacillus anthracis* responsive to petrobactin (catecholate siderophore that functions in both iron acquisition and virulence in a murine models of anthrax) biosynthesis. Results of in vitro analysis showed that each asb gen mutant grew to a very limited extent as vegetative cells in iron-depleted medium. In contrast, none of the *Bacillus anthracis* asb mutant strains were able to outgrow from spores under the same culture conditions. Further data showed that asbA gen also play role in the step of petrobactin biosynthesis, while asbB role in catalyzes condensation of a second molecule of 3,4-dihydroxybenzoyl spermidine with 3,4-dihydroxybenzoyl spermidinyl citrate to form the mature siderophore [32]. Based on the facts in this study that there are variations in results of citrate test to *Bacillus anthracis*, it needs further research.

Bacillus anthracis cultured in medium enriched glucose did not form acid and gas (Fig. 1. E). The results of another study showed the presence of glucose increased the expression of the protective antigen toxin component-encoding gene (pagA) by stimulating induction of transcription of the AtxA virulence transcription factor. We know that glucose is a critical element in human and animal cells used as a primary source of energy. It has been shown that induction of *Bacillus anthracis* toxin gene expression by glucose and determined that CcpA transcription factor plays a positive role by indirectly regulating the transcription of the gene encoding AtxA [31].

Bacillus anthracis cultured on agar with 7% NaCl showed good growth (Fig. 1 (F1)). We suspect, the nature of which is caused by the structure of the cell membrane that are typical of *Bacillus anthracis*. The assumption is based on the fact that the study results showed that the lipoprotein cell membrane function and regulate multiple cellular processes in Gram-positive bacteria. Has been observed that *Bacillus anthracis* has BA0330 and BA0331 lipoproteins that interact with peptidoglycan. BA0330 lipoprotein plays an important role in the adaptation of bacteria to grow in high salinity, whereas BA0331 contribute arranged to form uniform cells. The data show that the BA0330 and BA0331 play a role in the regulation of *Bacillus anthracis* cell wall structure to be stable [33].

Bacillus anthracis did not do hemolysis on blood agar medium (Fig. 1 (F2)), while the *Bacillus subtilis* cultures do hemolysis on blood agar medium (Fig. 1 (F3)). However, the ability of *Bacillus anthracis* to express β-hemolysis was reported [34,35]. The production of strong β–hemolysis on human blood

agar plates by the *Bacillus anthracis* strains was unexpected, as this organism has been considered traditionally non hemolytic [36].

Bacillus anthracis vegetative cells seem rod-shaped chain (Fig. 2A). *Bacillus anthracis* spores seem solitary and green (Fig. 2 (B, C and D)). Each *Bacillus anthracis* vegetative cells produce a spore [37]. Nutrient-poor medium that is known to promote sporulation [38,39]. The other study show that *Bacillus anthracis* sporulation is triggered under growth-limiting conditions and enables the organism to remain dormant and highly resistant to degradation [40]. Rapid germination of *Bacillus anthracis* spores is induced by serum that is commonly supplemented in cell culture growth formulations; however, some serum-free growth media formulations can also induce *Bacillus anthracis* germination [41,42].

Anthrosa precipitation of *Bacillus anthracis* with goat antibody anti anthrosa showed a lot of whitish, while the *Bacillus cereus* are not many shows a whitish color (Fig. 3). The result meant that test positive precipitation occurs in *Bacillus anthracis*, while the *Bacillus cereus* precipitation test negative. Study result previously showed that to obtain protein samples for immunoprecipitation, BclA-eGFP fusion-expressing sporulating cultures in Tiger broth at T_5 were harvested by centrifugation, and the cells lysed by bead-beating using 0.1 milli meter glass beads (Biospec Products) [28].

Other studies show that there are similarities endospores surface antigen between *Bacillus cereus* and *Bacillus anthracis*. Result of analysis showe that anthrose is monosaccharide, who compiled tetrasaccharide on endospre of *Bacillus anthracis*. Anti tetrasaccharide monoclonal antibodies and anti-anthrose-rhamnose disaccharide monoclonal antibodies were tested for their fine specificities in a direct spore ELISA. The tested with inactivated spores of a broad spectrum of *Bacillus anthracis* strains and related species of the *Bacillus* genus [9].

Electrophoresis results of *Bacillus anthracis* spore suspension in this study shows that BclA detected with molecular weight about 148 kDa (Fig. 3C), whereas

and *Bacillus thuringiensis* was not found. Other investigators have also been demonstrated BclA. BclA protein is located on exosporium shown by monoclonal antibody labeling.

The peptide backbone has a predicted size of approximately 39 kDa, but the intact protein migrates with an apparent mass of 250 kDa for the Sterne strain, which is consistent with its being heavily glycosylated [43]. Glycoprotein purified from *Bacillus thuringiensis* exosporium it was identical for both 205-kDa and 70-kDa monomeric forms. At *Bacillus cereus*, the protein is shown in band 205-kDa stains heavily as a glycoprotein. The protein does not correspond to the BclA glycoprotein, although independent evidence of the presence in *Bacillus cereus* exosporium [44].

It is proposed that the tetrasaccharide of the main exosporium protein (BcLA) from *B. anthracis* could be developed for diagnosis, therapy or a vaccine [45]. However, it should be remembered that the cell wall polysaccharide characteristics of *Bacillus anthracis* are similar to those of Bacillus cereus. The similarity of the epitopes possessed by the two types of Bacillus can cause cross reactions in the antigen-antibody test [46].

5. CONCLUSION

Data from our study shows that isolates is *Bacillus anthracis*. Isolates were characterized as Gram positive, bacilli and non motile bacteria. Bacteria that cultured in nutrient broth is not made turbid, when shaken showing typical images such as tendrils of silkworms, react negatively or positively towards the medium-enriched citrate, did not form acid and gas in the medium-enriched glucose, grow well on agar enriched 7% NaCl, did not do hemolysis on blood agar medium. Painting to spores of *Bacillus anthracis* are green.

Precipitation reaction between spores of *Bacillus anthracis* with goat antibody anti anthrosa showed in a silver white color. Anthrosa of *Bacillus anthracis* spores detected by means of immunochromatography using goat antibody anti anthrosa. We therefore conclude that the methods of precipitation and immunochromatography using goat antibody anti anthrosa can be used to detection of *Bacillus*

anthracis spores. Goat antibody anti anthrosa react positively with *Bacillus anthracis* spores. Anthrosa on the spores of *Bacillus anthracis* size of ± 148 kDa.

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ETHICAL APPROVAL

The study was approved by the Institutional Ethics Committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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