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Identification of Virulence of *Bacillus anthracis* from Soil by Multiplex PCR Technique in the South Sulawesi Province of Indonesia

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Abstract Anthrax is a disease caused by bacteria that attack herbivores and acute. The disease is caused by *Bacillus anthracis* is a gram-positive bacteria can form endospores and capable dormants for decades in the soil. This study aims to detect the presence of germs that cause anthrax in the soil at several locations ranch scene the disease in six districts / cities in South Sulawesi Province, Republic of Indonesia. Then determine the virulence of *Bacillus anthracis* were found. The method in this research is by taking 32 samples of soil in the farm ever anthrax disease. The land taken from test using multiplex polymerase chain reaction (m-PCR), which uses three pairs of primer, namely (Bac R, Cap and Lef) to detect the presence of *Bacillus anthracis* same time know the status of the virulence of the bacteria. The results of this study found two positive samples containing *Bacillus anthracis* and is known that both these types of agents were found to be virulent.

Keywords: *multiplex polymerase chain reaction, virulence, Bacillus anthracis*

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1. Introduction

One of the diseases that are zoonotic fairly dreaded anthrax. The disease is caused by the bacterium *Bacillus anthracis*. This disease primarily affects herbivores animals, such as cows, goats and horses. Infection in humans typically with the inclusion of *B. anthracis* spores through the skin or mucous membrane, with spore inhalation into the lungs or eat the meat of infected animals. Spores will grow in tissues in the body, and the vegetative growth of the organism starts that resulted in the formation gelatinous edema and congestion. The bacteria then spread through the lymph into the bloodstream and multiply freely in the blood and tissue immediately before and after the death of the animal [10].

Indonesia is endemic anthrax, according to the Ministry of Health of the Republic of Indonesia in 2012 reached 11 provinces endemic anthrax in animals, including West Sumatra, Jambi, West Java, Central Java, South Sulawesi, Central Sulawesi, Southeast Sulawesi, North Sulawesi, West Nusa Tenggara, East Nusa Tenggara and Papua. Until now these areas is still an area that has a tendency to appear periodically anthrax outbreak. Frequent outbreaks of disease, requires a planned effort to control the disease, including how to diagnose diseases quickly and accurately so that the disease can be overcome [17].

Indonesian population living mostly in rural areas and doing business in agriculture. So keep cows, buffaloes, goats or horses are an integral activity of enterprises in the agricultural field. The threat of anthrax disease course will greatly unsettle the farmers. Besides, anthrax are zoonotic disease that can easily be transmitted to humans. So that the disease can be life-threatening society and becomes a very scary thing [17].

Diagnosis of anthrax generally still use bacterial culture techniques or by staining the capsule in preparation of blood pillowcase. Although the capsule staining on the blood pillowcase preparations for the identification of anthrax can give an accurate result, but still needed confirmation test. One technique confirmation of the test results anthrax is by culture and/or identifying the bacteria that cause anthrax through inoculation of isolates in experimental animals, typically mice. This confirmation test contains safety and security risks (biosafety and biosecurity risk) related to the microorganism used, so we need a method other alternatives that can provide confirmation of the test results quickly, accurately, and safely [20].

Molecular methods such as polymerase chain reaction (PCR) in addition to not damage the environment, it also

provides the results confirm that much more quickly and precisely than conventional tests. Multiplex PCR method is a variant of PCR that utilize more than one pair of primers multiple target sequences simultaneously in a single reaction [6]. Multiplex PCR method for the diagnosis of anthrax has been developed, among others, by utilizing three pairs of primer [12].

This study is to detect the presence of the bacteria that cause anthrax in the neighborhood farms in some districts/ cities in the south province of Sulawesi region, as well as to know the status of the virulence of the bacteria.

2. Materials and Methods

In this study conducted soil sampling at the farms, stables or burial of animal carcasses were attacked by anthrax. Done taking 32 samples weighing approximately 100 grams of soil at a depth of 10 cm and put it in a sterile container

2.1. Identification of *Bacillus anthracis* with Culture Method

Soil samples were weighed as much as 25 grams, is mixed with 100 ml physiological saline sterile in a sealed tube and shaked for 2 hours, then incubated at room temperature overnight. Subsequently, 7 ml of the supernatant taken carefully inserted a sterile tube, and then heated in a water bath with a temperature 62,5^{o}C for 15-30 minutes, then centrifuged at 3000 rpm for 15 minutes. Supernatant carefully discarded in a safe place. The suspension is then cultured in blood agar plate and incubated at 37°C for 24 hours. Then observed morphologically. Suspect colonies were cultured again in blood agar and incubated at 37°C 24 hours.

2.2. Multiplex PCR Assay

2.2.1. DNA Extraction and m-PCR

DNA extraction using Pure Link Genomic DNA Kit (Invitrogen, catalog no. K1820-01). Combine 180 mL of Genomic Digestion Buffer to 25 mL and 200 mL Proteinase K specimens. The suspension is placed on a vortex and incubated at 55^{o}C for 15 minutes. Then the suspension is centrifuged at maximum speed for 3 minutes. After it was added 20 mL of RNase and back incubated at room temperature for 2 minutes. The suspension was transferred to a new tube and added 200 mL of Genomic Lysis / Binding buffer and placed on a vortex. Then added 200 mL of absolute ethanol, and placed again on a vortex and centrifuged. The suspension was transferred to Qiamp Spin Column. Then centrifuged for 5-10 minutes at a speed of 12000 rpm, all the liquid being stored in the Collection Tube discarded. Then added 500 mL of Wash Buffer 1 to wash the spin column membrane, and then re centrifuged for 5-10 minutes at a speed of 8000 rpm. All of the liquid being stored in the Collection tube is removed, then added 500 mL of Wash Buffer 2 to wash the spin column membrane, and then re-centrifuged for 15 minutes at a speed of 8000 rpm. Then the supernatant was removed and centrifuged again for 1 minute at a speed of 8000 rpm (without fluids). Then replaced recovery collection tube with 1.5 ml tube and added 50-100 mL of Genomic elution buffer and allowed to stand at room temperature for 1 minute. Suspension back centrifuged at a speed of 12000 rpm for 3 minutes. Qiamp disposed Spin Column and collection tube labeled. Eluted liquid is total DNA to be stored at -80°C [8].

Multiplex PCR method used could detect and identify to distinguish strains of *B. anthracis* and the possibility of contamination of other *Bacillus* species. This technique uses three pairs of primer (Bac R, Cap and Lef) for detecting genus, strain virulent and avirulent strains of *B. anthracis* [15] with the primary detail as follows:

Table 1. The primers used to amplify the gene of Bacillus anthracis

Primers	Sequens	Size	Gen Target
Bac R1	5'-TTA ATT CAC TTG CAA CTG ATG GG-3'	152 bp	Chromosome
Bac R2	5'-AAC GAT AGC TCC TAC ATT TGG AG-3'		
Cap-57	5'-ACT CGT TTT TAA TCA GCC CG-3'	264 bp	pXO2
Cap-58	5'-GGT AAC CCT TGT CTT TGA AT-3'		
Lef-3	5'-CTT TTG CAT ATT ATA TCG AGC-3'	385 bp	pXO1
Lef-4	5'-GAA TCA CGA ATA TCA ATT TGT AGC-3'		

2.2.2. Visualization by Gel Electrophoresis

Making TBE 10% (in volume 500 ml) by adding 10 x TBE 50 ml to 450 ml in sterile distilled water in order to obtain a final volume of 500 ml. The solution was thoroughly mixed. Making the 2% agarose gel by weighing the agarose according to the desired volume, then the solution TBE agarose heated together to boiling and a solution looks clear (may use microwave heating). Ethidium bromide is added as much as 2-5 mL or can also with SYBRR Safe DNA gel stain (Cat. No. S33102). Agarose subsequently poured into the mold and wait until it hardens. Once hardened inserted into the electrophoresis chamber. TBE 1X solution was added to agarose submerged completely. Samples were inserted coupled with loading dye 1.5 - 2 mL into each well. Each test is always included DNA electrophoresis ladder (commonly used 100 bp DNA ladder). Agarose and running sample using 90 volt power supply, 400 A for 90 minutes. Then the reading of the results is done by UV illuminator Viewer or Gel Doc System (Bio Rad) [8].

3. Result

3.1. Culture Method

Based on observations of the culture that is done just acquired two (2) pieces of soil samples positive for *Bacillus anthracis*. Formed colonies on blood agar which distinguish, that is gray white to gray with a diameter of 0.3 - 0.5 mm, which is smooth but sticky mucoid like thick paint (tacky). Characteristics of mucoid colony that

was soft due to the substances produced by the *B. anthracis* capsule [18]. Both of these samples derived respectively from Buae Village, Watang Pulu District, Sidenreng Rappang Regency and from the Allaere Village, Tanralili District, Maros Regency. Additional samples (30 samples) negative *Bacillus anthracis*.

3.2. Multiplex PCR Assay

From the appearance of the electrophoresis results of the soil samples were positive (culture) shows a virulent strain of *B. anthracis*. It is identified with the discovery of the DNA fragment of the plasmid pXO1 that encodes the toxin lethal factor (Lef) along the 385 bp DNA fragments from plasmid pXO2 that encodes the capsule anthrax (Cap) along 264 bp, as well as a common marker chromosomes genus *Bacillus* (Bac R) along 152 bp. While soil samples there negative culture not shows presence of *B. anthracis*.

Table 2. Data culture and PCR results

Regency	code of Sampel	culture	PCR
Pinrang	K-1	-	-
	K-2	-	-
	K-3	-	-
	K-4	-	-
	K-5	-	-
Parepare	K-6	-	-
	K-7	-	-
	K-8	-	-
	K-9	-	-
	K-10	-	-
Sidenreng Rappang	K-11	-	-
	K-12	-	-
	K-13	+	+
	K-14	-	-
	K-15	-	-
	K-16	-	-
	K-17	-	-
Maros	K-18	-	-
	K-19	-	-
	K-20	-	-
	K-21	-	-
	K-22	+	+
Takalar	K-23	-	-
	K-24	-	-
	K-25	-	-
	K-26	-	-
	K-27	-	-
Gowa	K-28	-	-
	K-29	-	-
	K-30	-	-
	K-31	-	-
	K-32	-	-

4. Discussion

This study showed that soil samples taken positive *Bacillus anthracis* only in two places. And both the positive samples, either with the same culture techniques as well as the multiplex PCR technique. And both B. anthracis found a virulent strain, known after testing by multiplex PCR method.

Detection and identification using culture techniques provide good results, *Bacillus anthracis* can be grown on a blood agar which becomes the medium. However, this technique requires a long time. Confirmation of the presence of both plasmids virulent *B. anthracis* is a standard procedure in the diagnosis of alleged anthrax (WHO, 1998; Inoue et al., 2004). In the past, efforts to confirm this by inoculating experimental animals such as mice or guinea pig and observed symptoms of illness or death of the animal for at least 24 hours. But along with the strengthening of animal welfare issues and the risk of excessive propagation of infectious agents that potentially pollute the environment, the use of experimental animals in the pathogenicity trials have started to be avoided and replaced with other methods that are more environmentally friendly [14].

Confirm using multiplex PCR becomes satisfactory alternative, because it requires a relatively faster and does not pollute the environment. However, this method requires a higher cost.

Virulence and pathogenicity of *B. anthracis* is determined by the molecular determinants, namely poly-D-glutamyl acid which is a major component of the bacterial capsule synthesized by plasmid pXO2. These capsules have anti phagocytosis and play a role in invasive stages of *B. anthracis* infection [11]. While multicomponent-toxin anthrax namely protective antigen (PA), lethal factor (LF) and edema factor (EF) produced by plasmid pXO1 and play a role in the stage of toxigenic bacteria [7,18,20].

All virulent strain of *B. anthracis* has a capsule in which the capsule material production associated with the formation of mucus that are specific to smooth colonies of virulent bacteria. While the anthrax toxins, virulence is strongly influenced by the synergy and combination activity each factor. Each will have no impact lethal toxin (lethal) on the host's body when working alone. For example, the combination of PA + LF produces toxins lethal activity, EF + PA produce edema, EF + LF is inactive, and EF + PA + LF + EF produces edema and necrosis that are lethal [18].

Production of the anthrax toxin is mediated by a temperature-sensitive plasmid, pX01, of 110 megadaltons. The toxin consists of three distinct antigenic components. Each component of the toxin is a thermolabile protein with a mw of approximately 80kDa.

Factor I is the edema factor (EF) which is necessary for the edema producing activity of the toxin. EF is known to be an inherent adenylate cyclase, similar to the *Bordetella pertussis* adenylate cyclase toxin. Factor II is the protective antigen (PA), because it induces protective antitoxic antibodies in guinea pigs. PA is the binding (B) domain of the anthrax toxin which has two active (A) domains, EF (above) and LF (below). Factor III is known as the lethal factor (LF) because it is essential for the lethal effects of the anthrax toxin. Apart from their antigenicity, each of the three factors exhibits no significant biological activity in an animal.

The ability of the PCR test is very accurate, sensitive and specific in detecting the presence of marker gene plasmid pXO1 and marker gene plasmid pXO2 of samples

such as the detection of toxin PA and capsules [1,9], detection of toxin lethal factor and capsules [15], or the detection of anthrax spores in a sample from the ground with the identification marker chromosomes and plasmids pXO1 / pXO2 [2,3,5].

Bacillus anthracis virulence determination by multiplex PCR techniques have become indispensable to determine the degree of virulence of the bacteria in the field. So that these techniques need to be developed further so that the easier we make and the faster test taken action on the case.

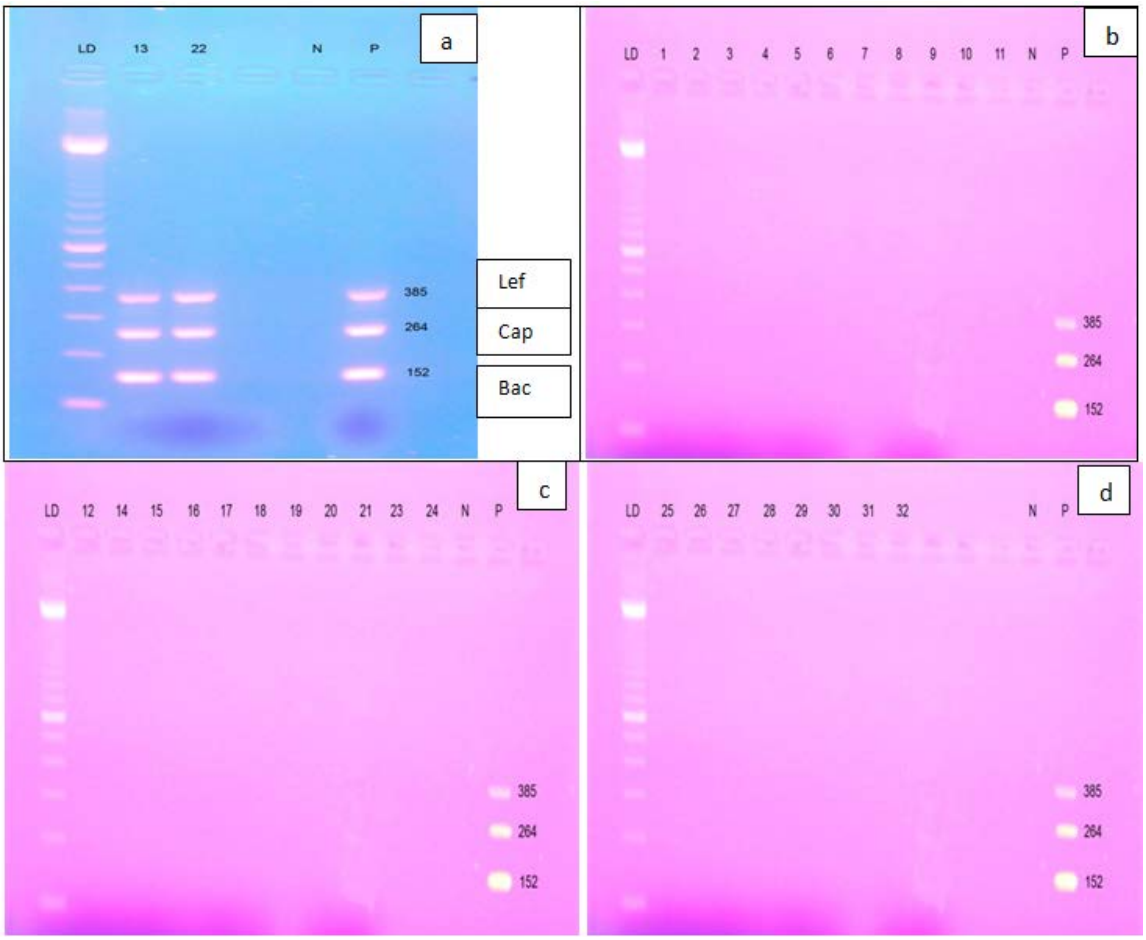

Figure 1. Results of electrophoresis positive samples no. samples 13 and 22 (a), reflecting the bands that show plasmid pXO1 (Lef, 385 bp), Plasmid pXO2 (Cap, 264 bp) and Bacillus anthracis chromosome markers (Bac, 152 bp). Negative samples no samples 1-11 (b), no sample negative samples 12, 14 -21, 23-24 (c) and negative samples sample no. 25 -32 (d)

5. Conclusion

Of the 32 (thirty two) soil samples were taken from the farm site ever attacked by anthrax in several districts / cities found just two (2) samples were positive for the bacteria *Bacillus anthracis*, which is derived from the Buae Village , Watang Pulu District, Sidrap Regency and from Allaere Village, Tanralili District, Maros Regency. Detection of the presence of *Bacillus anthracis* by culture and PCR test showed similar results. However, by using multiplex PCR is known that both samples contained virulent strain of anthrax bacterium, it is characterized by the formation of bands that indicate a toxin lethal factor (LF), anthrax capsule (Cap) and the common genus of bacillus (Bac R).

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