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Laboratory Confirmation Human Specimens Suspected of Anthrax in Kulonprogo District of Yogyakarta Special Region

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Abstract

Anthrax is an acute zoonoses infectious disease caused by *Bacillus anthracis* bacteria. *Bacillus anthracis* had ability to form endospores for self-defense. Anthrax infection could be divided into four types namely skin anthrax, gastrointestinal anthrax, anthrax of the respiratory tract (lung) and meningitis anthrax. The examination was performed to confirm the diagnosis of patients suspected of being infected with *Bacillus anthracis*. The examination methods consisted of culture for whole blood spesimens, konvensional polymerase chain reaction (PCR) on growing colonies and whole blood specimens without growing colony, and enzyme-linked immunosorbent assay (ELISA) for blood serum spesimens. The 6 cultured specimens, there were 2 colony-growing specimens with 1 identical colony with *Bacillus anthracis* colonies. The colonies confirmed with PCR and only 1 positive as *Bacillus anthracis* pathogens. The 6 specimens examined there was a positive specimen of *Bacillus anthracis* pathogen.

Keywords: anthrax, laboratory examination, Yogyakarta Special Region

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1. Introduction

Anthrax or commonly known as inflammatory of spleen diseases, miltbrand, miltvuur or splenic fever is an acute infectious disease considered as one of the major zoonoses in the world.[1, 2] This infection is caused by *Bacillus anthracis* bacteria that can deadly anthrax (meningitis anthrax) or causing pandemic (pulmonary anthrax).[2] *Bacillus anthracis* is a bacteria had ability to form endospores for self-defense and caused the bacteria to be difficult to eradicate. Anthrax most commonly affected animals, but can also infected to humans. Animals that were the main source of transmission in humans were buffalo, cattle, and sheep. Infections in dogs and other carnivore animals are rare even though those animals were vulnerable as well. Anthrax also can infect birds, especially ostriches. Other sources of transmission might come from environments contaminated by *Bacillus anthracis* spores such as soil, vegetables and water.[1, 3]

Humans infected anthrax by exposure to sick animals, infected animal tissues, or high-grade anthrax spores. Until now, no report of the occurrence of anthrax transmission

through touch or direct contact between humans.[1, 3] Infections in humans caused in deaths that are generally caused by exposure or consuming meat or livestock products were infected.[4]

The incidence of anthrax in Indonesia involved economic losses and affected human safety. Indonesia is still anthrax endemic area.[5] Until now, the Ministry of Agriculture had recorded 11 provinces of anthrax endemic namely West Java, Central Java, Special Region of Yogyakarta, West Nusa Tenggara, East Nusa Tenggara, West Sumatera, Jambi, South Sumatra, Lampung, Southeast Sulawesi, and the Special Capital Region of Jakarta. Five provinces of West Java, Central Java, West Nusa Tenggara, East Nusa Tenggara and Yogyakarta Special Region recorded anthrax case in humans.[5, 6]

Anthrax divided into four types namely skin anthrax, gastrointestinal anthrax, anthrax of the respiratory tract (lung) and meningitis anthrax.[3] In skin anthrax, *Bacillus anthracis* entered through the wounded skin, abrasions, or through insect bites with an incubation period 2-7 days. Gastrointestinal antraks divided into intestinal anthrax and oropharyngeal anthrax. Bacillus anthracis in gastrointestinal anthrax entered through contaminated food with an incubation period of 2-5 days and mortality rate 25-60%. Anthrax in the respiratory tract occured due to inhalation of *Bacillus anthracis* spores with with an incubation period 2-6 days and mortality rate can reach 86% within 24 hours. Meningitis anthrax is was a complication of symptoms of high fever, headache, muscle aches, cough, difficulty breathing or continued from the 3 forms of anthrax mentioned above. The mortality rate of meningitis anthrax reached 100% with clinical symptoms of cerebral hemorrhage.[7, 8] This study aims to confirm the diagnosis of patients suspected of being infected with *Bacillus anthracis*.

2. Methods

2.1. Sample

The specimens examined were sent from where the case of anthrax case happened in Kulonprogo District Yogyakarta Special Region. Samples from 6 patients each consisting of blood serum and whole blood. The number of specimens examined is 12 with details as shown in Table 1.

Table 1: Specimen type.

No.	Specimen code	Specimen type
1.	1015802079	Blood serum, whole blood
2.	1015802080	Blood serum, whole blood
3.	1015802081	Blood serum, whole blood
4.	1015802082	Blood serum, whole blood
5.	1015802083	Blood serum, whole blood
6.	1015802084	Blood serum, whole blood

2.2. Procedure

All laboratory tests were conducted in microbiology laboratory of Veterinary Research Center of the Ministry of Agriculture. Laboratory examination with culture are carried out on whole blood specimens. Culture is gold standard in isolation process and bacteria identification. The growing colonies and whole blood specimens without growing colony, then subjected to further examination with biomolecular methods using Polymerase Chain Reaction (PCR). All blood serum was used for serological examination with Enzyme-linked Immunosorbent Assay (ELISA).

3. Results

3.1. Culture

All of whole blood specimens were inoculated into blood agar medium and then incubated at 37°1C for 12 hours. Of all cultures specimens, two specimens showed growth of bacterial colony were code 1015802083 and 1015802084. Of the two growing specimens, the code 1015802084 had characteristics of grayish white color, uneven edges and regular, rough, gloomy, non hemolytic, non motile and clay consistency. The description of the colony were shown in Figure 1. When noted, this colony were identical to *the Bacillus anthracis* colony. Growing colonies were not subjected to biochemical tests, but continued with biomolecular examination.

3.1.1. Polymerase chain reaction (PCR)

This examination aimed to determine the virulence of bacteria that grow on blood agar media and whole blood. Growing colonies in both specimens, namely 1015802083 and 1015802084 and whole blood specimens without growing colony were extracted and the other specimens are extracted from the whole blood. The extraction result

Figure 1: The colonies in the specimen code 1015802084..

were continued with biomolecular examination using the polymerase chain reaction (PCR) method. This method was done to identify the colony more quickly without going through biochemical tests that take longer. The PCR product was electrophoresised and the results showed that the code 1015802084 which had a very identical colony with *Bacillus anthracis* colonies was positive as *Bacillus anthracis* pathogen.

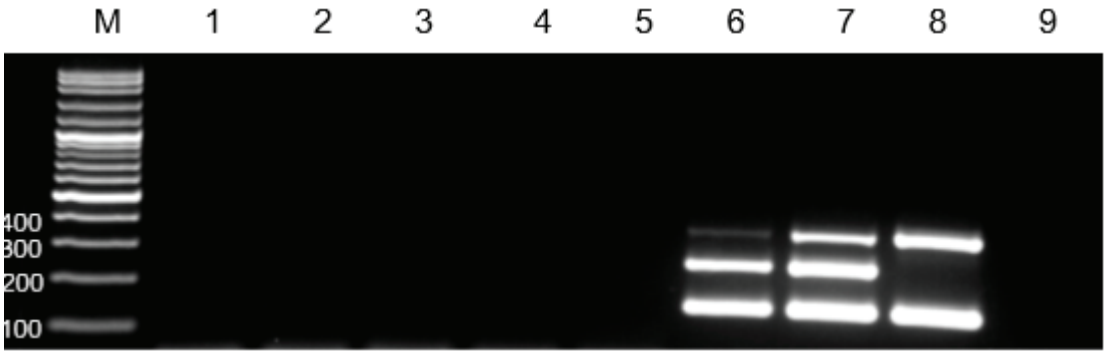

Figure 2: PCR product (M : marker, 1: Whole blood 1015802079, 2: Whole blood 1015802080, 3: Whole blood 1015802081, 4: Whole blood 1015802082, 5: The coloni code 1015802083, 6 : The coloni code 1015802084, 7: Positive control of pathogen *Bacillus anthracis*, 8: positive control of non pathogen *Bacillus anthracis*, 9: Negative control.).

3.1.2. Enzyme-linked immunosorbent assay (ELISA)

The materials used for the Enzyme-linked Immunosorbent Assay (ELISA) examination only blood serum specimens. The ELISA test for all blood serum specimens showed negative results.

4. Discussion

Laboratory tests on specimens aimed to establish a diagnosis based on existing clinical symptoms. Examination was done to detect the source of infection or detection of antibodies in patients by using several appropriate specimens. In skin anthrax, the examination material were taken from a new lesion with a cotton swab. If the lesion had become eschar, the edge of the lesion were removed and the specimen taken from below the lesion. Eschar excision is not allowed because it facilitates systemic anthrax. In intestinal anthrax, examination material were taken from faeces or blood if needed. The blood used for examination were taken before the patient was given antibiotics. Not only for bacterial culture, whole blood or blood serum from patients were used for serological examination. Serological examination requires paired sera was taken at intervals of at least 10 days. In pulmonary anthrax, the required examination material was blood which were carried out by direct examination, culture or serology. Diagnosis of meningitis anthrax were done by examination of cerebrospinal or blood fluid specimens.[1, 9]

Several methods were used in laboratory tests to detect *Bacillus anthracis* including microscopic examination of peripheral blood smear preparations, culture and biochemical tests, virulence confirmation by polymerase chain reaction (PCR), and serological testing. Serological examination had done with Ascoli precipitation test to determine infected tissue, ELISA for antibody detection and hypersensitivity test (Anthraxin) as a reflection of the presence of cell-mediated immunity. [9, 10]

The conventional method of cultur bacteria was the gold standard in detecting bacteria were suspected to be the source of infection. This method has been recommended by the World Health Organization (WHO) and the Central for Disease Control and Prevention (CDC). The conventional method was done with various techniques according to the type of specimen were : (1) specimens from new animals or humans without preservatives, (2) specimens from new animals or humans with preservatives, and (3) old specimens, decayed carcasses, material that has been processed or from the environment (including soil).[11]

Bacillus anthracis resembled a chain stem with a blue elbow end and a pink capsule. The specimens were inoculated on the agar medium and incubated at 37 ˚C for 16-24 hours. *Bacillus anthracis* morfology colonies were grayish white, irregular and irregular edges, rough, gloomy, non hemolytic, non motile and clay consistency. *Bacillus anthracis* was growth in broth media would look like cotton with clear-looking media.[12] Conventional methods with culture and biochemical tests were needed for identification

of *Bacillus anthracis*, but the examination procesed took longer, i.e 24 to 48 hours or more.[11, 12]

One of the advantages of the PCR method was the sensitivity was more than 90% and the specificity was more than 99%. Because of these advantages, this method was very useful for detecting the presence of organisms in low concentrations and in patients without symptoms. The PCR technique began to be used widely to detect the presence of virulence factor genes and determine whether or not a virulent isolate. This method is relatively fast with high sensitivity and specificity.[13, 14]

One cell of *Bacillus anthracis* were detected by PCR and were examined from the results of isolation on blood agar based on colony morphology. Detection of *Bacillus anthracis* with PCR was the most reliable method to detect blood specimens that have been long enough (15-17 days) and also succeed in diagnosing anthrax on blood smears that have been stored for 6 years and blood samples that have been stored for 18 months at -20°C. Although the PCR method is was faster to get results, conventional methods with *Bacillus anthracis* culture on 7% sheep blood agar were usually more reliable than blood smears.[13–16]

The advantage of laboratory examination for the diagnosis of *Bacillus anthracis* using the PCR method was the speed of time required for the examination process. In addition, the method was a sensitive and specific method when compared to the breeding method which is the gold standard method for bacterial identification. Identification using the PCR method, the sample that we checked or identified the infection-causing bacteria was known in one day. This is very different from conventional inspection methods which must took more than one day to find out the results. When compared with conventional methods, the PCR method required quite expensive costs and equipment.[11, 16]

The ELISA method were used to detect and measure antibody titers in human specimens infected with *Bacillus anthracis*. This technique were used to confirm and retrospectively assess anthrax cases that occur in endemic areas, especially cases that occur in humans.[17] The ELISA method was widely used to evaluate vaccinations, epidemiological studies in humans, livestock and wild animals. If this test was used for diagnosis, laboratory tests with other methods must also be carried out.[1, 7, 18, 19]

The choice of method for laboratory examination was based on the target detected. Diagnosis that was done quickly and accurately could reduced the risk of death and took steps to control the disease, both decontamination of the affected area, vaccination and closure of the area and monitoring of livestock traffic. Determination of a slow diagnosis

provides a risk of wider spread and contamination of the area and handling of animals and affected humans will also experience delays so that it can cause death..[20]

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