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ORIGINAL ARTICLE

Early Detection of *Bacillus anthracis* From Saliva in Anticipation of a Bioterrorism Attack

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Abstract

Objective: To assess potential for early detection of oral infection by *B. anthracis* spores for preparedness of a bioterrorism attack. **Material and Methods:** The laboratory study used saliva with a range of initial anthrax concentrations, to compare detection by direct observation from conventional blood agar culture and by anthrax-specific PCR after a shorter culture in BHI broth. Three types of saliva were collected: stimulated saliva, unstimulated/whole saliva, and unstimulated/whole saliva with antibiotic treatment (for negative control). Using bivariate Kruskal-Wallis and Mann-Whitney tests for statistical analysis for factors that could affecting anthrax detection, significant differences between the test groups was assumed at $p<0.05$. **Results:** From unstimulated whole saliva heat shock treated at 62.5^{0}C, *B. anthracis* growth was detected with both methods. PCR detection from a BHI broth culture could shorten the time to diagnosis in comparison to conventional culture in blood agar. **Conclusion:** Saliva can provide useful samples for diagnosis of oropharyngeal anthrax. In comparison to conventional culture on blood agar, shorter-term culture in BHI broth provides potential for earlier detection and diagnosis.

Keywords: Bioterrorism; Biological Warfare; Anthrax; Saliva.

Introduction

Bioterrorism involves terrorists or extremists, who apply microorganisms (bacteria, viruses, fungi) or toxins as weapons causing disease and/or death in humans, animals and/or plants [1-4]. One famous example of such an attack was the letter containing anthrax spores [1]. In a few days, 22 victims were hospitalized, 12 of them identified with cutaneous anthrax and 10 with inhalation anthrax. Four victims died due to respiratory failure [1].

Anthrax is an acute disease caused by *Bacillus anthracis*, categorized by World Health Organization as bioterrorism type A agent [5]. The disease is easy to disseminate and transmit from human to human, with a high mortality rate and potential as a challenge to be prepared for in the society. The infection route defines predilection, type and clinical manifestation. Ingested spores will develop as oropharyngeal and gastrointestinal anthrax. According to the Centers for Disease Control and Prevention (CDC), to define the diagnosis of anthrax requires stepwise laboratory testing first in a local laboratory, referral laboratory and final validation by a national laboratory. The laboratory testing will typically take 12-48 hours and the defining diagnosis 1-3 days [2-4,6,7].

In case of infection by ingestion, saliva provides promising diagnostic sample material, because it is the first body fluid in contact with the environment. Saliva is common in diagnostic sampling with high sensitivity, specificity and reliability for other purposes, but uncommon for defining the diagnosis of anthrax. Previous laboratory studies have shown that in military personnel that received anthrax vaccination through nasal and oral mucosal membranes, specific antibody and specific IgG are detectable from saliva [8,9].

As the conventional route of laboratory testing and diagnosis is relatively slow and potential for transmission high, there is need for methods of early detection. Potential detection from saliva is promising as samples of saliva are fast, easy and noninvasive to collect at lower cost than other samples of body fluids, and can be collected by personnel without general healthcare training. The present study therefore aimed to assess the potential for detecting *B. anthracis* from samples of saliva.

Material and Methods

The study was using laboratory testing to assess the potential usefulness of saliva for diagnostic sampling of anthrax. As no saliva from real human anthrax infection was available, saliva from healthy individuals was used with added *B. anthracis*.

Three types of saliva were collected: stimulated saliva, unstimulated/whole saliva, and unstimulated/whole saliva with antibiotic treatment (for negative control). Culturing in blood agar was used to confirm that saliva was initially free from *Bacillus* bacteria. Spores of *B. antrachis* strain 34F2 from collection of Research Center for Veterinary Science, Bogor, Indonesia; were then injected through spike process. Anthrax stock was prepared in concentrations of 100 to 108 colony forming unit (CFU)/ml, at intervals of one decade (order of magnitude), with and without heat shock treatment in a water bath at 62.5ºC for 15 min. The subsequent incubation was conducted in blood agar for 18 hours at 37ºC and in BHI broth for 7 hours at 39ºC, in all cases in duplicate. For blood

agar cultures the results were obtained as direct CFU counts, and for BHI broth cultures using Geneaid Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) and visualizing the PCR product in UV illumination after electrophoresis. Figures 1 and 2 show examples of test results.

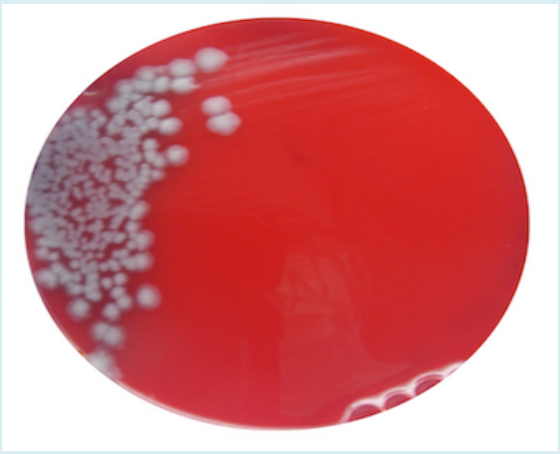

Figure 1. *B. anthracis* growth in blood agar (300 CFU).

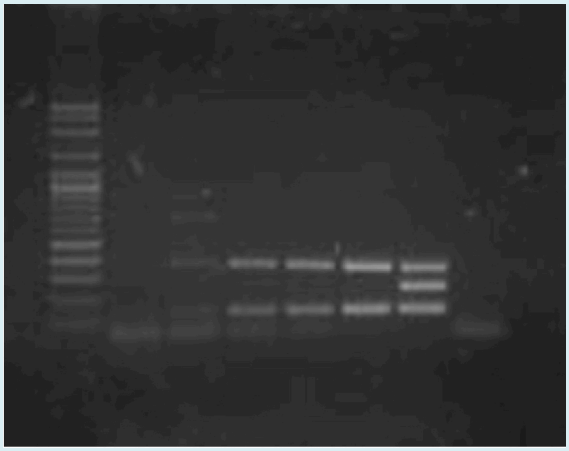

Figure 2. PCR detection of *B. anthracis* from BHI broth at initial concentrations of 10^3, 10^4 and 10^5.

Data Analysis

Data were analyzed using IBM SPSS Statistics Software, version 23 (IBM Corp., Armonk, NY, USA). Kruskal-Wallis and Mann-Whitney tests were used for factors that could affecting anthrax detection. Significant differences between the test groups was assumed at $p<0.05$.

Results

The results showed generally higher bacterial growth rates in blood agar with increasing initial anthrax concentration and with preceding heat shock treatment at 62.5°C. Without the heat shock treatment, at initial concentration of 10^5, the PCR method of BHI broth failed to detect anthrax indicated by direct observation from blood agar culture (Table 1).

Table 1. *B. anthracis* growth from saliva with initial concentration of 10^5, without heat shock.

Saliva	Detection by PCR of BHI Broth	CFU Count of Blood Agar			p-value
		Mean	Minimum	Maximum	
Stimulated	Not Detected	9.5	0	19	
Unstimulated/whole	Not Detected	51	2	100	0.234
Unstimulated/whole with AT*	Not Detected	0	0	0	

*Antibiotic treatment for negative control.

Judging from the observed CFU count in blood agar culture, unstimulated whole saliva was a more robust source carrier than stimulated saliva. The heat shock treatment significantly promoted bacterial growth and detectability, so that the minimum initial concentration was similar (about 10^3) for both detection methods (Table 2).

Table 2. *B. anthracis* growth from unstimulated whole saliva, according to initial concentration, with heat shock.

Initial Concentration	Detection by PCR of BHI Broth	CFU Count of Blood Agar			p-value
		Mean	Minimum	Maximum	
10^5	Detected	319	288	350	0.006
10^4	Detected	84	54	95	
10^3	Detected	6	4	11	
$<10^3$	Not Detected	0	0	0	

To intervene a bioterrorism attack, fastest method is of interest. The comparison is shown in Table 3.

Table 3. Comparison time according to saliva type and incubation time.

Growth Medium	Saliva and Treatment	Incubation Time
Blood Agar	Provocated without Heat Shock	18 Hours
Blood Agar	Unprovocated without Heat Shock	18 Hours
Blood Agar	Unprovocated with Heat Shock	18 Hours
BHI Broth	Unprovocated with Heat Shock	7 Hours

Discussion

Ingestion of anthrax spores can initiate an infection to develop into oropharyngeal and gastrointestinal anthrax. Saliva is then the first body fluid in contact with the spores. Here the anthrax ingestion was simulated by spike introduction of *B. anthracis* spores to saliva at a wide range of concentrations, with and without heat shock treatment at 62.5^{0}C. Subsequent anthrax detection was tested by conventional direct observation of bacterial CFUs from blood agar incubated for 18 hours, and by PCR from BHI broth incubated for 7 hours. The shorter incubation of the latter approach can provide a potentially faster method of detecting anthrax infection, and is of interest for intervening bioterrorism attacks involving anthrax.

Without the heat shock treatment and at an initial concentration sufficient for conventional detection from blood agar culture, PCR of BHI broth failed to detect anthrax (Table 1). The heat shock treatment significantly promoted bacterial growth and reduced the minimum initial concentration to similar levels for both methods of detection (Table 2).

Judging from the observed CFU counts in blood agar culture, unstimulated whole saliva appeared to be a more robust source carrier than stimulated saliva, although the difference was not statistically significant (p=0.234). In spite of suggested easier detection of microbial signatures from stimulated saliva in some previous studies, unstimulated whole saliva provided satisfactory anthrax detection in the present work, when combined with preceding heat shock treatment. This in agreement with other previous studies to detect antigen and IgG indications of anthrax exposure from unstimulated whole saliva [8,9,12-14].

Saliva is the product of plasma ultrafiltration and includes more than 2300 identified proteins, 20-30% of which also appear in blood. From the identification point of view, any disease

such as anthrax can add its specific proteome signature [10]. Also, the anthrax pathogenesis will involve recruitment of macrophages of the non-specific immune system, and about 1.0-1.5% of the leukocytes in saliva are macrophages [11].

Direct observation of growth in blood agar show that for *B. anthracis*, higher rate of growth was found with than without a preceding heat shock treatment (Tables 1 and 2). Both direct observation of CFU count in blood agar and PCR of BHI broth showed that *B. anthracis* can be detected starting from an initial concentration of 10^3 CFU/ml. Significant differences between concentration groups (p=0.006) were found. BHI broth required 7 hours for incubation, less than blood agar that needed 18 hours.

Conclusion

B. anthracis can be detected particularly well from unstimulated whole saliva heat shock treated at 62.5°C for 15 minutes, starting from initial concentration of at least 10^3. To shorten the time to diagnosis, the incubation time with PCR of BHI broth is shorter than the gold standard using culture in blood agar. Detection of *B. anthracis* is therefore possible from saliva after infection, and faster methods of detection will facilitate improvements in national preparedness, to limit the spreading the disease in case of bioterrorism attacks applying anthrax.

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Conflict of Interest: The authors declare no conflicts of interest.

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