

FINAL REPORT: Surveillance for anthrax (*Bacillus anthracis*) in water buffalo (*Bubalus bubalis*) sympatric with the Javan rhinoceros (*Rhinoceros sondaicus*) population in Ujung Kulon National Park, Indonesia

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Abstract: This purpose of this study was to conduct a survey of anthrax (*Bacillus anthracis*) in the 19 buffer villages that surround Ujung Kulon National Park in Java, Indonesia. Soil specimens were collected from water buffalo terminals in each village, and when possible specifically from areas with a history of sudden water buffalo death. Twenty-three samples were collected and analyzed in a microbiology laboratory at the Institut Pertanian Bogor. Colony description, tryptic soy agar, brain heart infusion broth, and microscopic analysis were used to support a suspected diagnosis of *B. anthracis*. Six samples were suspected and brought to Balai Besar Penelitian Veteriner Bogor for PCR analysis. All samples were negative although we are not completely confident in this report and will pursue one further step for confirmation.

Report: This study is part of a two-year health assessment of the water buffalo populations in the villages surrounding Ujung Kulon National Park that will focus on hemorrhagic septicemia (caused by *Pasteurella multocida*). The health assessment is an essential step in the process of establishing a second population of Javan rhinoceros (*Rhinoceros sondaicus*) in the park as they are susceptible to many of the same diseases and can succumb quite quickly if infected. Likewise, the villagers depend on their water buffalo as a living and equal health precautions will be taken to screen the rhinoceroses for diseases that could sicken the water buffalo.

The objective of this study was to conduct a survey of anthrax (*Bacillus anthracis*) in the 19 buffer villages that surround Ujung Kulon National Park in Java, Indonesia. Soil specimens were collected from water buffalo terminals in each village, and when possible specifically from areas with a history of sudden water buffalo death. Soil samples will also be collected at a later date from sites of historic rhinoceros deaths within the peninsular region of Ujung Kulon National Park.

An essential part of working with these water buffalo is gaining the owners' trust. Without their cooperation, we would have no animals to take blood samples from, and no terminals to take soil from. Socializations were planned ahead of time in each village through communication with the head of the village (Kades). During our sample collection, we first held socialization, where over the course of one to two hours, the project was explained to the owners. These visits were relatively informal, encouraging owners to ask questions and allowing them the opportunity to get to know us and bridge the gap in culture and language. A card with the phone numbers of the two veterinarians and the local agricultural ministry liaison was handed to each person in attendance. A packet of multivitamins was given for each buffalo we took a blood sample from.

As stated, the soil samples were taken from buffalo terminals, the locations where they return at the end of a day of grazing. Per the guidelines of the method, samples were collected 8cm from the surface and placed into a labeled Ziploc bag.

After our time spent in the field, I worked with the technicians at the Anthrax Diagnostic Laboratory at the Institut Pertanian Bogor (Bogor Agricultural University) to analyze the soil samples. We collected 23 soil samples from the 19 buffer villages. From four villages we took two samples as they had larger populations of water buffalo, they were larger geographically, and we had the money in our budget to do so.

Our original proposal indicated that we would use the novel GABRI method to analyze our soil samples. Due to a miscommunication we discovered that the proper materials necessary to use this method were not obtainable

locally in Indonesia and it would take months to order from an international source. In addition, the novel method is still unpublished and our results would be relatively meaningless until cited in a peer-reviewed journal. Therefore we decided to use the standard method for anthrax analysis that is routinely used at this diagnostic laboratory. The technicians are very comfortable and familiar with the standard anthrax diagnostic method and it seemed like the best option under the circumstances.

The standard method protocol is as follows:

Materials (per sample):

- Bottle containing 90ml of saline
- Water bath set to 80°C
- Six 9ml saline dilution blanks
- Five plates of blood agar
- Pipettes
- Spread/hockey stick

Procedure:

1. Prepare 10g of soil in a bottle containing 90ml of saline and mix well.
2. Prepare five, serial decimal dilutions of the soil suspension. Completely immerse the tube in the 80°C water bath for 15 minutes. Heating + cooling constitutes the *heat-shocking* procedure to release spores.
3. Inoculate 0.1ml from each of the five dilutions onto a separate spread plate of blood agar.
4. Incubate the plates at 30-37°C for 1-2 days.
5. After 24-48 hours incubation, examine the blood agar plates for colonies of *Bacillus* species, which often are characterized by a flat and chalky appearance.
6. Note: *Bacillus anthracis* produces non-hemolytic colonies on blood agar.

Colonies found on the blood agar were described and recorded. Those colonies that showed characteristics of *Bacillus anthracis*, namely a non-hemolytic colony, were then subcultured onto Tryptic Soy Agar and incubated overnight to produce a separate colony. These were further subcultured into Brain Heart Infusion broth (two test tubes per sample) to assess motility and the bacterial growth. *Bacillus anthracis* should be non-motile, and should not cloud the broth due to the chain formation of the bacilli creating a denser population that rests at the bottom of the test tube instead of creating a homogeneous solution. Slides were also prepared for each suspected colony using the gram stain. Bacteria morphology and characteristics were recorded.

With four different characterization techniques (gram (+) bacillus, non-hemolytic on blood agar, non-motile, clear BHI broth), we were able to narrow down our findings into six individual samples highly suspected to contain *Bacillus anthracis*. The Institut Pertanian Bogor (IPB) did not have the primers necessary to perform the PCR analysis on site so the six suspected samples were brought to Balai Besar Penelitian Veteriner Bogor (BBALITVET) for PCR analysis to confirm the presence or absence of anthrax. All six samples came back negative from BBALITVET. Unfortunately, we are uncertain how reliable the results are for both logistical and political reasons and are exploring one further step to confirm once and for all the presence or absence of *Bacillus anthracis* in our suspected samples.

I presented our project and findings to veterinary students at IPB. This was a very rewarding experience and I was pleased with the long question session following the presentation. It was great to see how eager the students were to learn and even better to hear the suggestions to improve future work.

In summary, this project was an essential preliminary survey for anthrax in the buffer villages surrounding Ujung Kulon National Park. In order to safely translocate Javan rhinoceroses in the future, it is imperative to know the diseases that are common in the water buffalo populations that will ultimately share common habitat. It is recommended that a larger soil survey with many more samples be conducted for a more comprehensive analysis of the region. This was an incredible experience to work with Indonesian veterinarians, the local government and most importantly, the villagers. The significance and necessity of working locally in the field became clear and has further shaped how I believe conservation work should be conducted in the future.