Diagnoses of Chytridiomycosis on Dominica

ASHLEY M. BROOKS
Texas A&M University, College Station, Texas
Study Abroad Dominica 2008
Dr. Thomas Lacher and Dr. Jim Woolley

ABSTRACT

Six *Eleutherodactylus martinicensis* on Dominica were caught on two different transects. They were swabbed for DNA, and then those swabs were tested for Chytridiomycosis. I carried out the three parts of diagnoses: extraction, amplification, and visualization. A PCR was run with two different programs, Chytrid A and B. Finally, agarose gel electrophoresis was carried out, and a picture of the developed gel was taken. The results all came back negative.

INTRODUCTION

Chytridiomycosis is a disease of amphibians which affects the keratinized layer of their skin. Although we are beginning to learn more about this disease, the reason why and the mechanism how it kills the amphibians is still unknown. There are efforts all over the world to figure out those questions. In Dominica, Chytrid was reported on 3 December 2002. The local people started to see that the frogs all over the island were showing up dead. They contacted the Forestry Division, and the next day, researchers began to try to figure out what was happening to these animals.

Since Chytrid was first reported on the island, there has been an enormous effort made by numerous collaborators.. The government enacted new legislation, e.g. The Hunting of Frogs Prohibition in 2004 and the Importation of Frogs Prohibition in 2004. There are now captive breeding programs, and there is a heightened awareness all over the island. Posters, brochures, radio broadcasts, and interviews have all been used to try to inform the people of what is happening

Today, there are two steps to test frogs for Chytridiomycosis. Researchers conduct transects across the island to collect visual and auditory samples. Frogs are collected and samples are taken from each frog. These swabs are then shipped to the Molecular laboratory where the DNA samples are tested.

METHODS AND MATERIALS

Currently, the most accurate and best way to diagnose Chytridiomycosis, which is caused by the fungus *Batrachochytrium dendrobatidis* (*Bd.*), is by completing a PCR assay. "PCR" stands for Polymerase-Chain Reaction. This is a technique that identifies the infection level of each sample taken. What PCR detects is the presence or absence of the DNA from *Bd.* The reasons that PCR is still the best means of diagnosis are the following: one can collect many samples rapidly without harming the specimen, PCR is able to detect even the smallest amount of infection, it has a minimal amount of cross-reactions with other fungi, and numerous samples can be tested at once.

The purpose of carrying out the PCR is to test for the presence of the pathogen from the frogs we swabbed. By testing for *Bd.*, it helps to look at how the overall populations of different species on the island are being affected. Every two months researchers take samples from all over the island to test different frogs. Each frog gets swabbed twice (one for the Dominican forestry department's records and one for the Zoological Society of London). By looking at the samples and where frogs are turning up positive, more can be known about what parts of the island are infected with *Batrachochytrium dendrobatidis,* and the severity of the infection is also seen.

When completing the diagnosis, there are three main parts. These include the extraction, the amplification, and the visualization steps. The extraction step involves removing the DNA from the swab that was taken. The next step, amplification, multiplies the cuts of DNA that were taken,

and the final step, visualization, uses gel electrophoresis to observe the final results. Each of these steps has different components. The following section outlines the entire process.

The extraction of *Batrachochytrium dendrobatidis* DNA involves a heat bath, centrifugation, and bead beating. All of this is used in a Taqman Assay. Every person who is involved with the lab work must wear gloves to avoid contamination. To begin the extraction, 0.03g-0.04g of .5mm zirconium/silica microbeads must be measured. These can then be placed into a 1.5ml centrifuge tube. Then one must pipette 60µl PrepMan Ultra into a centrifuge tube. The only time to use 60µl is when a swab sample is being tested. At other times, 50µl of PrepMan Ultra are used. Next, the swab is toe clipped so that only 1-4mm is used (depending on the thickness of the swab), and the swab should be added to the sample.

In our tests, we ran 12 samples total. This means we tested 6 frogs with 2 swabs for each. Also, we set up one negative control, one positive control, and one extra. This extra helps avoid contamination and false results. If something gets messed up then the extra can be used. In our tests, our number 8 sample was mixed wrong and so we were lucky to have the extra so that we could properly test number 8.

This part of the extraction involves many steps that repeat. First, one must homogenize the solution using the bead beater for 45 seconds. Then you take the homogenized sample and centrifuge it for 30 seconds at 14,500 revolutions per minute (rpm). Repeat the bead beating, and then repeat the centrifugation. All the centrifuge tubes should be removed, and then placed in a rack. This will then be set in a 100°C water bath for 10 minutes. When the time is complete, remove the rack and allow it to cool. Another round of centrifugation should then occur at 14,500rpm for 3 minutes. Finally, collect the solution and store as much supernatant as possible in a 1.5ml sterilized Eppendorf appropriately labeled.

The second part of the diagnosis is the amplification. This is the actual part that uses the PCR machine. This involves two runs. Each run is comprised of different amounts of the mixture, and has a different PCR program. The first run is a 50µl reaction by volume. This includes the following substances: 40µl of PCR grade water, 1µl of the DNTP mixture (2'-deoxynucleoside 5'-triphosphate), 1.25 µl of the Forward Primer (1), 1.25 µl of the Reverse Primer (1), 5 µl of the Buffer, 0.5 µl of the Taq enzyme, and 1 µl of each DNA Sample.

The PCR cycle for the first run is called Chytrid A. This program runs for 35 cycles. The chart below shows the temperature that the samples are run at for the specified length of time.

Temperature	Length of Time
95°C	3 minutes
95°C	0.3 minutes
50°C	0.3 minutes
72°C	0.45 minutes
72°C	7 minutes
4°C	∞

Table 1: Chytrid A Program on the PCR

On the other hand, the second run has different amounts of some of the ingredients. It is still a 50µl reaction by volume. This includes the following substances: 49 µl of PCR grade water, 1 µl of the DNTP mixture (2'-deoxynucleoside 5'-triphosphate), 1.25 µl of the Forward Primer (2), 1.25 µl of the Reverse Primer (2), 5 µl of the Buffer, 0.5 µl of the Taq enzyme, and 2 µl of each DNA Sample from the first run. For the second run, the PCR cycle's program is Chytrid B. This has the same components as Chytrid A (see Table 1.) except for the number of cycles. Chytrid B is run for 30 cycles instead of 35.

Finally, the visualization step finishes the diagnostic process. The visualization step involves gel electrophoresis. The gel (which is only 2% gel) is comprised of 2g of agarose and 200ml 1 x TAE (tris acetate) buffer. This buffer offers the best resolution for

bigger DNA. On the other hand, this provides the lowest amount of buffering capacity which means that it will take longer to develop because a lower voltage must be used. When the gel is prepared, it must be stirred, heated, and then cooled to room temperature. While it is cooling, the tray that the gel will be poured in should be prepared. The following is a picture of the tray and apparatus used in agarose gel electrophoresis:

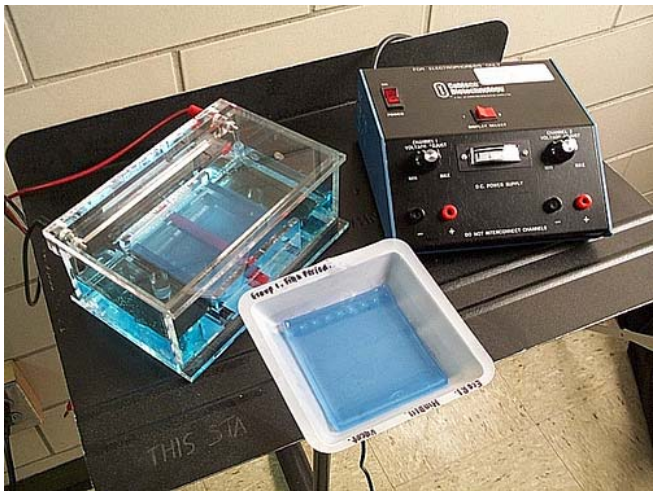


Figure 1: Gel Electrophoresis machine with tray.

Each side of the tray was covered in tape that created a makeshift wall for the gel to harden against, and the comb(s) should be added. Once the gel is cooled to room temperature, it should be poured into the tray to harden. This process takes about a half hour.

Once the gel is in solid form, the comb can be removed and the entire gel is placed into the gel electrophoresis apparatus. More buffer solution is added so that the entire gel is under liquid. The samples are then prepared. Each PCR completed sample needs 2µl of loading dye. This is blue, and it allows the movement up the gel matrix to be seen. Once 2µl of dye has been dropped into each sample, all of the samples are spun in a small centrifuge to evenly distribute the dye.

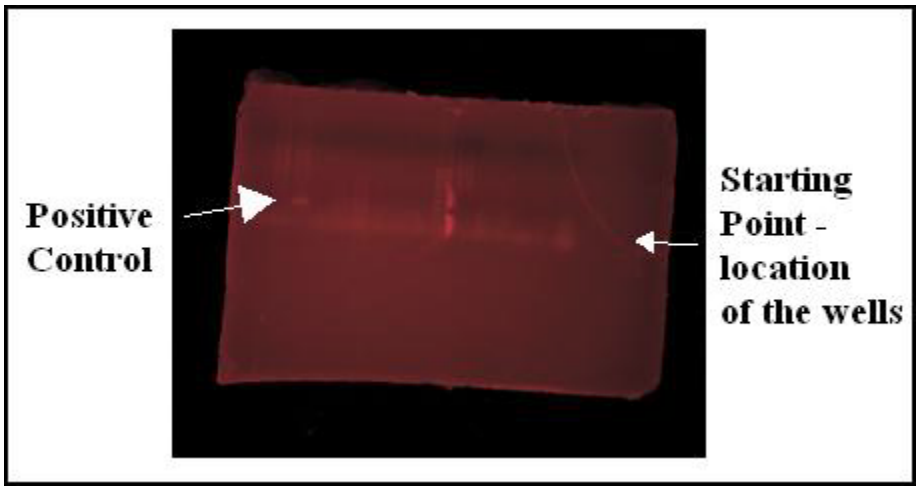
When the gel and the DNA samples are prepared, it is time to fill the wells. The comb that was added to the gel created little pockets in it called wells. Each well has 12µl of sample inside. When each well is filled, the machine is turned on. The gel fronts should be set up to move from negative to positive. This is because DNA is negatively charged due to its sugar-phosphate backbone. The machine is run for 40 minutes at 150 volts. It is the electromotive force (EMF) that moves the molecules through the matrix. The molecules will move in the gel toward the positive anode at different speeds according to their mass. After 40 minutes, the gel is removed and placed into a different container. This is then filled with buffer so that the gel is pretty much covered. Then 7µl of dye (called SYBR gold) is added to the buffer. This is light sensitive, so we kept it under the cabinet in the dark. This made sure that the gel was not over exposed. The gel is stained for 45 minutes with the SYBR gold dye. We made sure to stir it by hand every 10 minutes because we did not have an automatic stirrer. Next, the gel was de-stained for 30 min. The SYBR gold dye was removed and buffer was added. Finally, the gel is completely prepared. The only steps left are to place the finished gel in a light box under ultraviolet light so that the fluoresced light is able to be seen. A picture can be taken to record the results.

RESULTS

The data collected in the end of this entire process is in the form of a picture. This picture is of the finished gel with the molecules that have moved across the matrix. Because the sample that was run was made up of a combination of components, each one is made up of bands. These bands correspond to each element in the mixture. The

distance that each band travels is almost inversely proportional to the logarithm of the molecule's mass.

Each band corresponds with a number. They are labeled at approximately "100" variables. *Batrachochytrium dendrobatidis* shows as a band slightly above 200. When testing a sample to see if they have Chytridiomycosis, one must look to see if there is a band that appears at that specific height on the gel. The following is a picture of the gel that we developed inside of the Molecular Biology Laboratory in the Forestry Department.



This gel shows that there is no Chytrid present in any of the samples that were taken. There are no bands that fall right above the 200 line, and so all the results are negative. If it seems confusing to see the bands, the results should be compared to the positive control that shows Chytrid.

Throughout the entire process of diagnosing Chytrid, it is important to always think about contamination. Because PCR is so ultra sensitive, it only takes a couple of zoospores for the results to show positive. If incorrect or misleading results show up, this is called a false positive. Because it is pretty easy to contaminate other samples, the first

thing that should be noted is whether or not both of our negative controls showed actually tested negative. Our results did indeed show negative, so this means that there is a high chance that our tests were free of contaminants.

DISCUSSION

Chytridiomycosis has had a dramatic affect on Dominica. This fungus has devastated populations of frogs all over the island. By keeping a constant eye out, and continuing to test for Chytrid, the government is doing the best it can to try to save as many animals as possible.

The lab work that was run was fairly simple, and it gives an accurate account of where this fungus is most prevalent. Fortunately, all the work that we did showed to be negative. This gives hope that there are still many creatures that have not yet been affected.

ACKNOWLEDGEMENTS

I would like to thank Diana Warrendorf for working with me throughout this entire project. Together we have tried to make a small difference on the island. I would like to thank the Forestry division of Dominica for going above and beyond what was expected and letting me have hands on experience with these amazing creatures. I am very grateful to Stephen Durand and Ronnie Winston for letting me tag along on the transect of Soufriere Sulfur Springs. Their vast knowledge, willingness to teach, and generosity have made this project a success. Also, I want to especially thank Mr. Arlington James and Mr. Trevorne Douglas. Arlington, you went out of your way to

make this project possible, and I am so grateful. Thank you for sharing your report with us. It helped me better understand how this disease is truly affecting the island. Thank you so much Trevorne for letting me hang around your lab. You opened up your work space for me, and you taught me how to do all the diagnostic work. I truly appreciate the time you put in to help me in every aspect of this project.

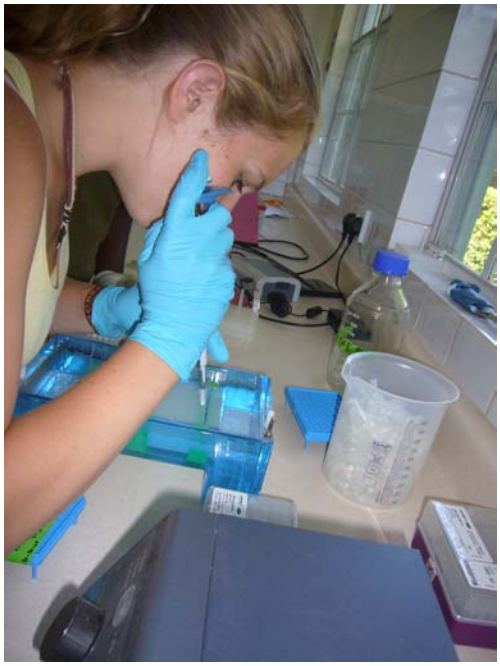
Thank you also to all the students that have helped in any part of my project. Every little input helped, and I am much obliged. Many thanks to the Archbold Tropical Research Educational Center for letting me use their facilities. Thank you to Dr. Lacher and Dr. Woolley. Without the two of you, I would not be writing this today. You believed that this project was possible when it looked like it would never happen. Thank you for the time you spent contacting others, helping me understand Chytrid, and driving me back and forth to Roseau. I appreciate it so much.

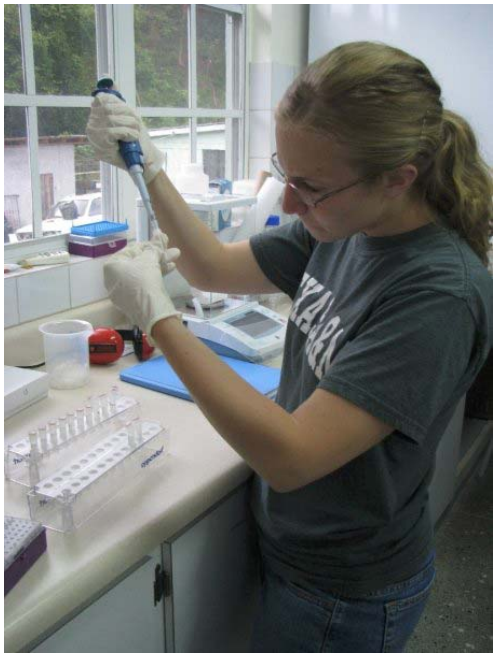
CITATION

BREM, FORREST, J. R. MENDELSON III, K.R. LIPS. Field-sampling protocol for *Batrachochytrium dendrobatidis* from living amphibians, using alcohol preserved swabs. <http://www.amphibianark.org/pdf/Field%20sampling%20protocol%20for%20amphibian%20chytrid%20fungi%201.0.pdf>

JAMES, ARLINGTON. Personal Account of Chytridiomycosis on Dominica, Forestry and Wildlife Division of Dominica. 04 June 2008.

PICTURES


Carrying out the Gel Electrophoresis


Measuring out the samples for extraction


Toe Cutting the sample swabs


Being an automatic stirrer of the gel.