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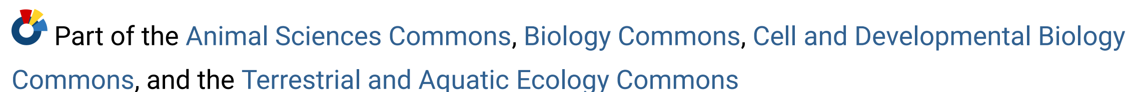
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Presence and Prevalence of Bd (*Batrachochytrium dendrobatidis*) in Picado's Bromeliad Treefrog (*Isthmohyla picadoi*)

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Abstract

***Batrachochytrium dendrobatidis* (Bd) is a virulent amphibian pathogen that invades the keratinized skin cells of juvenile and mature amphibians and the mouthparts of tadpoles, and causes the disease chytridiomycosis. By causing regions of outer layers of the skin to unevenly thicken, osmoregulation and respiration are disrupted. Considering the important role of skin in amphibian respiration, chytridiomycosis has high mortality rates. And because Bd is an exotic pathogen, introduction to new sites- and naïve populations- quickly leads to epizootic outbreaks and high losses in susceptible populations. However, despite amphibian declines and extinctions in the Cerro Punta region of western Panama, anuran species *Isthmohyla picadoi* has persisted. It is known that some amphibians harbor epidermal bacterial flora that serve to protect from chytridiomycosis, and hypothesized that *I. picadoi* does as well. In this study, presence and prevalence of Bd in a sample of *I. picadoi* individuals (n=9) will be determined using genomic extraction and PCR. *I. picadoi* individuals were captured and swabbed near Guadalupe Arriba, Chiriquí Province, in the Republic of Panama. Eight individuals were captured within Finca Dracula, owned by Andrés Maduro, and one was captured near a lodge owned by Los Quetzales Lodge and Spa. Captures were made on 26, 28 and 29 May 2012. Genomic extraction for *I. picadoi* DNA and Bd DNA was performed on swabs in Set 1 on 15 November 2013, and on swabs in Set 2 on 28 April 2014. Quantitative PCR for Bd DNA in Sets 1 and 2 was performed on 26 and 28 April 2014, respectively. None of the samples tested positive for Bd DNA.**

Introduction

Though prominent issues such as deforestation, increasing pollution levels and human induced climate change are often cited as significant factors in species decline and extinction,

fungus *Batrachochytrium dendrobatidis* (Bd) appears to play a prominent role in the recent mass extinction of many amphibian species worldwide. The associated skin disease, chytridiomycosis, has been found among many amphibian populations in Africa, Europe, New Zealand, Australia, North America, Central America and South America (Boyle, 2004). Upon encountering an uninfected population, Bd can drastically reduce these amphibian populations: abundance can fall by roughly 80%, and species richness by about 50% in less than 5 months. Chytridiomycosis is associated with at least 93 amphibian species worldwide, 43 of which are in Central America (Lips *et al.*, 2006).

On a regional scale, Bd dispersal appears virtually uninhibited, within its relatively broad environmental constraints. But locally, the fungus must rely upon physical contact between amphibian conspecifics, as well as transfer between individuals and their environment in order to spread. Events such as breeding, that necessitate the presence of many individuals in the same locality, provide Bd with a multitude of potential naïve hosts, furthering dispersal. These infected individuals become potential vectors for further spread to other tadpoles, to adult frogs and to other bodies of water, if they reach maturity and disperse.

Bd is an aquatic fungus that grows optimally at pH 6-7, between 17-25°C (Piotrowski *et al*. 2004). These parameters allow flourishing of Bd in many of the most amphibian species-rich regions of the world, particularly in the Neotropics (Ron 2005). However, though anuran *Isthmohyla picadoi* resides within these regions, sometimes in areas of known Bd presence, populations have not declined in recent years.

I. picadoi is found mainly in the montane forests of Panama and Costa Rica. Members spend each stage of their life cycle- and likely much of their lives- in epiphytic bromeliads (Stuckert *et al.* 2009). These bromeliads are situated mainly in trees and provide habitat for a

variety of organisms including amphibians, snails, insects and plants (pers. obs.). The arboreal nature of *I. picadoi* also means that they may only rarely share bodies of water with other amphibians. With most of their time spent in the canopy and little in zones with higher *Bd* zoospore counts, *I. picadoi* may have as yet avoided the full force of this pathogen. (Stuckert *et al.,* 2009). Additionally, certain flora have been seen to inhibit Bd (Harris *et al*. 2006), and some amphibian populations with high proportions of these 'anti-Bd bacteria' have persisted for years in regions known to harbor Bd (Lam *et al*., 2010). As *I. picadoi* has exhibited this characteristic, its arboreal and anti-Bd tendencies merit further investigation. Using modified methods outlined by Hyatt *et al*., we will determine whether Bd is present on any of our swabbed *I. picadoi* individuals (n=9). In a study performed by Lindquist *et al*. (2011), only one *I. picadoi* individual out of 32 swabbed tested positive for Bd. A low presence of Bd in this closely related study, in addition to no sign of chytridiomycosis at the time of swabbing, has led us to hypothesize that our sample of nine individuals will not show infection by Bd.

Materials and Methods

Procedure 1: Specimen Capture and Sampling; Environmental Data Sampling

Materials

VWR Powder Free Nitrile Examination Gloves
Sterile Medical Wire Dry Swab
Sandwich Ziploc Bag
Fisher Scientific Digital Calipers
Rite-in-the-Rain Field Notebook
70% Isopropyl Alcohol
Kimwipes
1.5mL Microcentrifuge Vial
Maglite® Flashlights
Greenbeam 50 Green Laser Pointer
Dry Ice

Methods

Eight *Isthmohyla picadoi* individuals were captured within Finca Dracula, owned by Andrés Maduro, and the ninth was captured near a lodge owned by Los Quetzales Lodge and Spa. Collections were performed after nightfall; flashlights, headlamps and laser pointers were used to triangulate male vocalizations and locate individuals within bromeliads (Bromeliaceae). Frogs were captured by hand with VWR Powder Free Nitrile Examination Gloves (nonsterile) and transferred to a new Sandwich Ziploc Bag. Gloves were changed between each specimen to prevent cross-contamination of individual samples.

Specimens were then transferred to a lodge owned by Los Quetzales where they underwent sample processing. Each individual was arbitrarily assigned a successive individual code including the first two letters of the genus and species names and a three-digit number, which corresponded to the number of the given species collected. Each individual was measured inside the Ziploc bag for SVL using Fisher Scientific Digital Calipers. Individuals were assessed for signs of chytridiomycosis, including red coloration on the pelvic girdle and lethargy. Individuals were sexed based on presence of nuptial pads and darkened skin under the chin, indicating males. Individuals were then thoroughly swabbed with Medical Wire Dry Swabs (sterile) on ventral and dorsal surfaces, as well as the limbs and digits. Individual sex, code, collection location, and capture date were noted on swab vials. Swabs (Set 1) were then returned to vials and kept in a cooler on dry ice, until transferred to a -20°C freezer until extraction. All individuals were then returned to their respective bags for the night and returned within 24 hours to their site of capture. Shed epithelial skin fragments were collected and stored in sterile Microcentrifuge vials (Set 2) after frog release.

Procedure 2: Swab DNA Extraction via Qiagen Mini Extraction Kit

Materials

Qiagen Mini Extraction Kit
- ATL Buffer
- Proteinase K
- AL Buffer
- AW1 Buffer
- AW2 Buffer
- AE Buffer (1/10 dilution)
- MinElute Spin Column
- Collection Tubes

100% Ethanol
2mL Eppendorf tubes (sterile)
1.5mL Microcentrifuge tubes (sterile)
Water bath (56°C)
Multiblock Heater® (70°C)
Vortex Genie 2
Denville Scientific 260D Brushless Microcentrifuge
Denville Scientific Tips: 200µL, 1000µL
Labnet Labpette P200, P1000
VWR Powder Free Nitrile Examination Gloves

Methods

Swabs were removed from -20°C freezer and transferred to a labeled 1.5 Microcentrifuge tube. Each swab was handled with clean gloves and broken off from the swab handle in order to fit into the respective tube. The nine swabs in Set 1 and nine swabs in Set 2 were processed following a modified Qiagen Mini Extraction protocol. 20µL of Proteinase K and 200µL ATL Buffer were added to each vial, using Denville Scientific Tips (200µL and 1000µL, respectively) and Labnet Labpette (P200 and P1000, respectively), pulse-vortexed for 10" using Vortex Genie 2, and incubated in a water bath at 56°C for 1 hour, with 10" vortexing every 10'. Samples were then briefly centrifuged in Denville Scientific 260D Brushless Microcentrifuge to spin down drops from lids.

200µL of Buffer AL was added to each sample with 15" pulse-vortexing after addition. Samples were incubated in Multiblock Heater® at 70°C for 30', with 10" vortexing at 3'

intervals. Samples were briefly centrifuged to remove drops from lids. 200µL of 100% Ethanol was added to each sample with 15" pulse-vortexing. Entire lysate was then transferred to a labeled MinElute Spin Column with collecting tube and was centrifuged at 6000 x g (8000 rpm) for 1'. MinElute Spin Columns were then transferred to new collecting tubes and the flow-through was discarded. 500µL of Buffer AW1 was added to MinElute Spin Column and then centrifuged for 1'at 6000 x g (8000 rpm). MinElute Spin Column was again transferred to a new collecting tube and the flow-through was discarded. 500µl of Buffer AW2 was added to MinElute Spin Column and was centrifuged at 6000 x g (8000 rpm). MinElute Spin Column was transferred to a new collecting tube once more, and the flow-through was again discarded. Then the MinElute Spin Column was centrifuged for 3 min at full speed (20,000 x g or 14000 rpm) for 3' to completely dry the membrane. MinElute Spin Column was transferred to a sterile, labeled 2mL Eppendorf tube. 50µL of a 1/10 dilution of Buffer AE was added to the MinElute Spin Column and the samples were left to incubate at room temperature for 5'. Samples from Set 1 were then centrifuged for 1' at 6000 x g (8000 rpm) and MinElute Spin Columns were discarded. Samples from Set 2 were centrifuged for 1' at full speed (20,000 x g or 14000 rpm) and MinElute Spin Columns were discarded. All Eppendorf tubes were closed and stored at -20°C until PCR.

Procedure 3: Basic Standard Real-Time qPCR Set-Up

Materials:
Laminar Flow Hood
Labnet Labpettte P20, P200, P1000
Denville Sharp Precision Barrier Tips, PCR-Ready: 20µL, 200µL, 1000µL
1.5 mL microcentrifuge tube
Applied Biosystems (ABI) StepOne Plus™ Real-time qPCR Machine
Applied Biosystems (ABI) MicroAmp Optical 8-cap strips
Applied Biosystems (ABI) MicroAmp Optical 8-well strips (100µL)
Vortex Genie 2
Applied Biosystems (ABI) Taqman Standard Mastermix 2x

PCR-ready DH_20
200 µM ITS1-3 Chytr primer
200µM 5.8S Chytr primer
100µM ChytrMGB2 probe
Bd Standards: Concentrations of 10,000, 1000, 100, 10 1 and 0.1 zoospore equivalent/ µL
Target DNA

Methods:

The basic final mastermix recipe included 10µL ABI Taqman Standard Mastermix 2x, 1µL of primer/probe and 4µL of PCR-ready DH_20 for each reaction in the plate. Following ~10" vortexing, 15µL of final mastermix was applied to ABI MicroAmp Optical 8-well 100µL strips (hereafter, wells). 5µL of target DNA was then added, with the non-template controls (NTC) for each plate containing 5µL of DH_20 instead of the DNA. After filling a strip, an ABI MicroAmp Optical 8-cap strip was used to seal the wells. They were then placed in the ABI StepOne Plus™ machine and the specific cycle was selected for that plate.

Procedure 4: Standard Real-time qPCR of Swab DNA with Chytrid primers/probe

The plates were set up according to the instructions found in Procedure 3, using the DNA obtained from Procedure 2 (Tables 1 & 2). Plate 1, containing DNA from Set 1, used ABI Taqman Universal Standard Mastermix (2x). Plate 2, containing DNA from Set 2, used ABI Taqman FastStart Standard Mastermix. The 1µL primer/probe mix was 9% 200 µM ITS1-3 Chytr primer, 9% 200µM 5.8S Chytr primer, 5% 100µM ChytrMGB2 probe and 77% DH_20, which utilized FAM dye and NFQ-MGB quencher to determine DNA concentration. A standard curve was also used on these plates, with each reaction containing the standard 15µL of mastermix and 5µL of DNA from known zoospore concentrations. After plate set-up, the ABI StepOne Plus™ ran a standard curve test with the following cycles: Holding Stage at 50°C for 2'

followed by 95°C for 10', then 40 cycling stages at 95°C for 15", each followed by an annealing step at 60°C for 1' at which the dye reading was taken.

Results

This experiment was designed to test for *Bd* presence and prevalence in all swab samples collected. Each sample was tested with ChytrMGB2 primer/probe, and none of the samples amplified detectable quantities of *Bd* DNA, before (Figure 1) or after skin shedding (Figure 2). Bd standards had C_T values approximately 10-fold lower than Bd standards used in similar research conducted by Neal and Sicher during the summer of 2013. Additionally, not all wells of Bd standard amplified: in Set 1 two wells from Standard 6 (2×10^{-2} zoospore equivalent/µL), one well from Standard 5 (2×10^{-1} zoospore equivalent/µL) and one from Standard 1 (2×10^{3} zoospore equivalent/µL) did not amplify detectable quantities of Bd DNA. In Set 2, two from Standard 6 (2×10^{-2} zoospore equivalent/µL) and all 3 from Standard 5 (2×10^{-1} zoospore equivalent/µL) did not amplify detectable quantities of Bd DNA (Figure 3).

Discussion

This experiment expanded on research performed by Lindquist *et. al*. (2011) and others concerning the presence and prevalence of *Batrachochytrium dendrobatidis* (Bd) in Picado's Bromeliad Treefrog (*I. picadoi*). Stuckert *et al.* (2009) determined that *I. picadoi* live primarily in epiphytic bromeliads and have little contact with bodies of water inhabited by other amphibians. This may have largely protected them from exposure to Bd zoospores, but does not completely exclude them from carrying and/or becoming infected with Bd. In addition to possible microbial resistance on the skin of *I. picadoi,* its arboreal habitat suggests the possibility of low Bd infection rates among this anuran species.

The results of the qPCR runs show that 0% of the nine individuals swabbed tested positive for Bd DNA, confirming our hypothesis (Figures 1 & 2). This is not unexpected, as only one out of 32 individuals from the same location tested positive for Bd DNA presence in the study performed by Lindquist *et. al*. (2011). A sample more than three times smaller in size would be expected to contain little to no evidence of Bd DNA. Next, we will perform qPCR with Rhodopsin primers and probe as a positive control. Since errors may occur in swabbing technique and genomic extraction, a positive control is needed to identify any false negative and false positive results in Bd detection and quantification.

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Tables and Figures

	1	2	3	4	5	6	7	8	9	10	11	12
A	ISPI 001	ISPI 003	ISPI 005	ISPI 007	ISPI 009	NTC	X	X	X	X	X	X
B	ISPI 001	ISPI 003	ISPI 005	ISPI 007	ISPI 009	NTC	X	X	X	X	X	X
C	ISPI 001	ISPI 003	ISPI 005	ISPI 007	ISPI 009	NTC	X	X	X	X	X	X
D	ISPI 002	ISPI 004	ISPI 006	ISPI 008	Std. 5	Std. 6	X	X	X	X	X	X
E	ISPI 002	ISPI 004	ISPI 006	ISPI 008	Std. 5	Std. 6	X	X	X	X	X	X
F	ISPI 002	ISPI 004	ISPI 006	ISPI 008	Std. 5	Std. 6	X	X	X	X	X	X
G	Std. 1	Std. 1	Std. 1	Std. 2	Std. 2	Std. 2	X	X	X	X	X	X
H	Std. 3	Std. 3	Std. 3	Std. 4	Std. 4	Std. 4	X	X	X	X	X	X

Table 1. Plate 1. Amphibian swab DNA used to test for Bd amplification. Samples obtained before skin-shedding. No swab sample wells showed Bd DNA amplification. *Bd* Standard wells with amplification highlighted in green.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ISPI 001	ISPI 003	ISPI 005	ISPI 007	ISPI 009	Std. 2	X	X	X	X	X	X
B	ISPI 001	ISPI 003	ISPI 005	ISPI 007	ISPI 009	Std. 2	X	X	X	X	X	X
C	ISPI 001	ISPI 003	ISPI 005	ISPI 007	ISPI 009	Std. 2	X	X	X	X	X	X
D	ISPI 002	ISPI 004	ISPI 006	ISPI 008	Std. 1	Std. 3	X	X	X	X	X	X
E	ISPI 002	ISPI 004	ISPI 006	ISPI 008	Std. 1	Std. 3	X	X	X	X	X	X
F	ISPI 002	ISPI 004	ISPI 006	ISPI 008	Std. 1	Std. 3	X	X	X	X	X	X
G	Std. 4	Std. 4	Std. 4	Std. 5	Std. 5	Std. 5	X	X	X	X	X	X
H	Std. 6	Std. 6	Std. 6	NTC	NTC	X	X	X	X	X	X	X

Table 2. Plate 2. Amphibian swab DNA used to test for Bd amplification. Samples obtained after skin shedding. No swab sample wells showed Bd DNA amplification. *Bd* Standard wells with amplification highlighted in green.

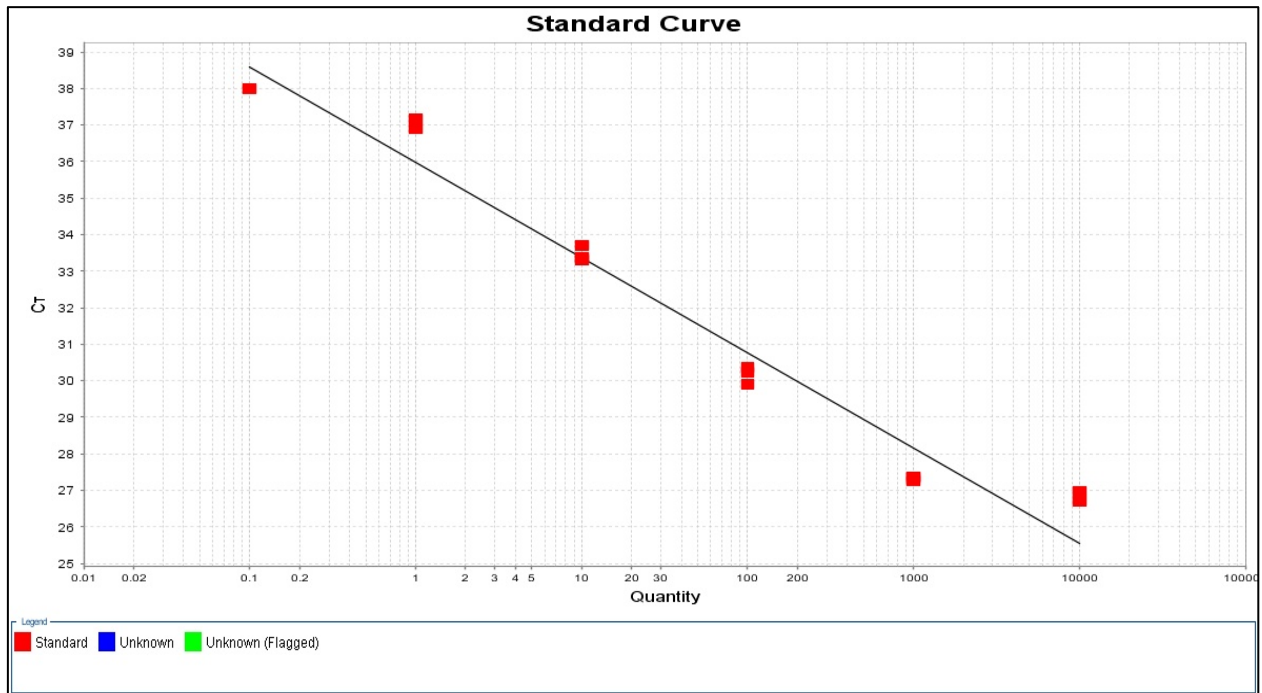

Figure 1. Bd Standard Concentration Curve (Plate 1). Bd standards in concentrations of 10,000, 1000, 100, 10 1 and 0.1 zoospore equivalent/ µL were used in triplicate to quantify the amount of Bd DNA present in any samples that tested positive for Bd DNA. R^2 value: 0.957.

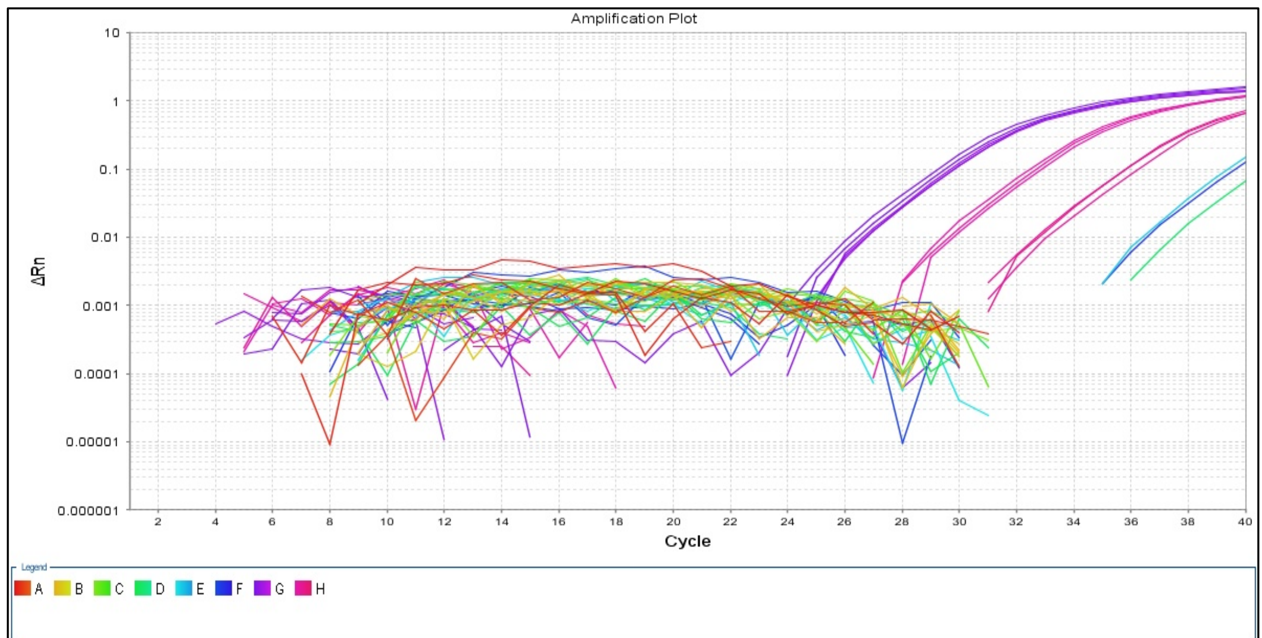

Figure 2. Bd Amplification Before Skin Shedding (Set 1). No amplification of Bd DNA was detected in any of the nine *I. picadoi* samples. Detectable curves (where ΔRn > 0.01) indicate successful amplification of most Bd standards.

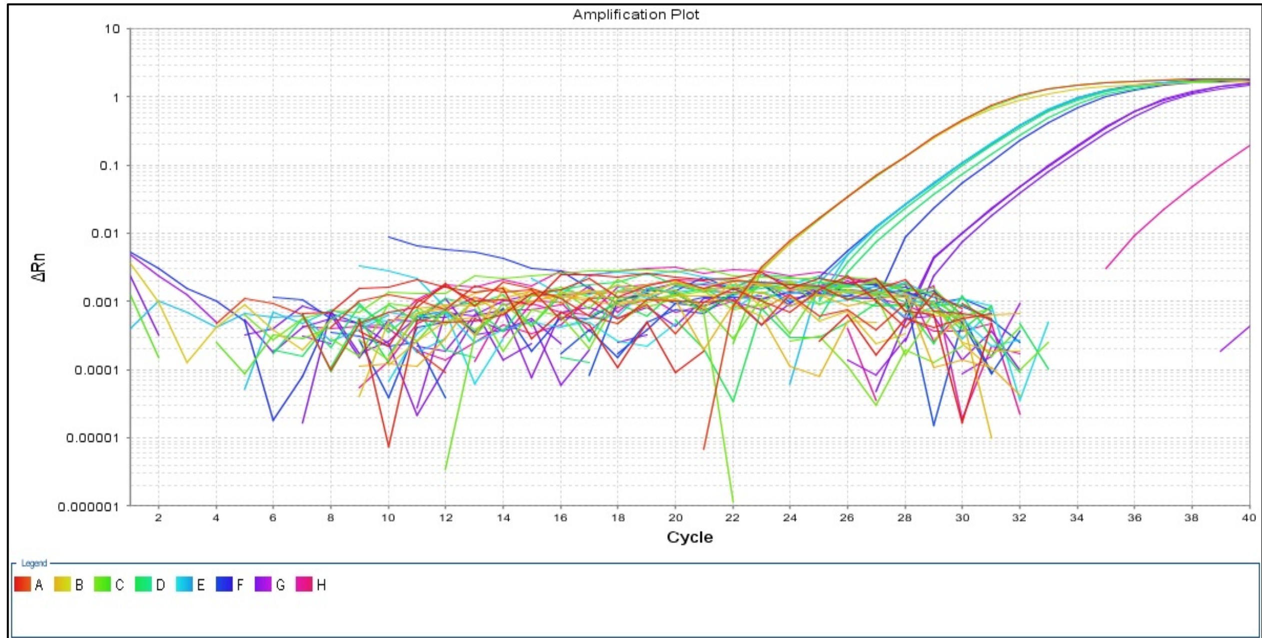

Figure 3. Bd Amplification After Skin Shedding (Set 2). No amplification of Bd DNA was detected in any of the nine *I. picadoi* samples. Detectable curves (where $\Delta Rn > 0.01$) indicate successful amplification of most Bd standards.

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