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A Novel Protocol for Washing Environmental Microbes from Amphibian Skin

A key derived feature of amphibians is their unique skin morphology, which includes a perpetually moist surface associated with mucous and granular (e.g., poison) glands (Duellman and Trueb 1986). The skin morphology of different amphibian species varies considerably (Heatwole and Barthalmus 1994), ranging from the highly toxic alkaloids sequestered onto the surface of dendrobatid frogs (Saporito et al. 2004) to the highly vascularized surface of the lungless plethodontid salamanders (Czopek 1962). Associated with this diversity of skin morphologies is an array of microorganisms, which when mutually beneficial, represent an important line of defense against pathogens (Belden and Harris 2007; Ramsey et al. 2010). The moist, thin, bare surface of amphibian skin allows gas exchange and is permeable to water (Heatwole and Barthalmus 1994), and therefore could be less effective at preventing pathogen transmission than the more impervious skin surfaces of other tetrapods. In addition, the skin of amphibians has been documented in the past to have a poor or non-existent cell-mediated and humoral immune response (Berger et al. 1998; Pessier et al. 1999; Woodhams et al. 2006). Recent evidence indicates that amphibians can acquire behavioral and immunological resistance to emerging fungal pathogens like *Batrachochytrium dendrobatidis* (McMahon et al. 2014). Amphibians have also developed an armory of potent antimicrobial peptides (Nicolas and Mor 1995; Simmaco et al. 1998; Rollins-Smith et al. 2002) that may somewhat compensate for their lack of scales, feathers, and hair. Evidence suggests

DONALD M. WALKER*
Tennessee Technological University, Department of Biology, 1100 N. Dixie Ave., Cookeville, Tennessee 38505, USA
BRANDY R. LAWRENCE
Department of Pharmacy, The University of Findlay, Findlay, Ohio 45840, USA
DAKOTA ESTERLINE
MICHAEL McANDREW
JULIA EDELBROCK
Department of Natural Sciences, The University of Findlay, Findlay, Ohio 45840, USA
SEAN P. GRAHAM
Department of Biology, Geology, and Physical Sciences, Sul Ross State University, Alpine, Texas 79832, USA
CRYSTAL KELEHEAR
Smithsonian Tropical Research Institute, Apartado 0843-03092, Balboa, Ancon, Panama, Republic of Panama

** Corresponding author: dmwalker@tntech.edu*

Table 1. Standardized washing protocol developed and tested in this study.

Protocol Step	Researcher #1 Description	Researcher #2 Description
Squirt bottle	Begin the protocol with clean nitrile or latex gloves. Hold the salamander while researcher #2 washes it.	Wash the salamander for 15 seconds (25 mL sterile ddH_2O) using a squirt bottle.
Wash bath #1	Change into fresh gloves at this time to prepare for transfer of the salamander into the second wash bath after the first wash is complete.	Place the salamander into a sterile 90 mm petri dish containing 25 mL sterile ddH_2O and swirl the plate in a circular motion for 30 seconds.
Wash bath #2	Wearing fresh gloves, place the salamander into a sterile 90 mm petri dish containing 25 mL sterile ddH_2O and swirl the plate in a circular motion for 30 seconds.	Change into fresh gloves at this time to prepare for transfer of the salamander into the third wash bath after the second wash is complete.
Wash bath #3	Change into fresh gloves at this time to prepare for swabbing of the salamander.	Wearing fresh gloves, place the salamander into a sterile 90 mm petri dish containing 25 mL sterile ddH_2O and swirl the plate in a circular motion for 30 seconds.
Swabbing	Hold the salamander while researcher #2 performs the swabbing technique.	Swab the salamander on the left, right, dorsal, and ventral sides for a total of 15 strokes from the head to the tip of the tail.
Storage	Store swabs at -20°C within one hour of being collected for lab specimens or in 99.9% acetone for field collections.	

that the cutaneous antimicrobial peptides and microbiota associated with amphibians may play a key role in maintaining amphibian health and mediating the effects of emerging infectious diseases, which have been implicated as a major cause of amphibian declines worldwide. For example, species-specific differences in both antimicrobial peptides (Rollins-Smith et al. 2002; Rollins-Smith 2009) and microbiota (Harris et al. 2009) are associated with susceptibility to *B. dendrobatidis*, the fungal pathogen responsible for amphibian population crashes in Australia, western North America, and the American tropics. Specifically, cutaneous microbiota and secreted antimicrobial peptides of amphibians can kill or inhibit the growth of *B. dendrobatidis* (Rollins-Smith and Conlon 2005; Harris et al. 2006; Brucker et al. 2008a; Brucker et al. 2008b; Becker et al. 2009; Harris et al. 2009). Therefore, a better understanding of amphibian microbial communities may increase our understanding of this devastating wildlife disease.

To date, studies on the skin microbiota of amphibians have been variable, with protocols differing substantially among studies. For example, the two most cited papers (Culp et al. 2007; Lauer et al. 2007) describing the methodology for removing environmental microbes prior to skin sampling and characterization of the resident microbial communities differ in methodology (e.g., the number of times each animal was washed and the volume of water used for each wash). However, these two protocols are often cited in a single study, and citing studies do not precisely follow the same protocols (McKenzie et al. 2012; Fitzpatrick and Allison 2014; Kueneman et al. 2014). A standardized washing protocol is necessary to allow for comparison of independent studies on the skin microbiota of amphibians.

Any conclusions regarding resident symbionts depend on the ability of the researcher to separate environmental microbiota from strict host/symbiont associations. To contribute to the study of amphibian skin microbiota, we set out to develop a standardized sampling protocol for biologists studying this topic. We conducted identical protocols using plethodontid salamanders in both the laboratory and the field, to ensure that this protocol can be used in both settings. Moreover, we used an experimental approach to determine if our washing protocol worked. We used a plate-based assay to count colony-forming units to show a sequential reduction of cultureable bacteria in our four-step protocol and molecular confirmation using PCR to show resident microbial community presence after the decontamination protocol. Here, we present evidence for our ability to retrieve microbial communities from amphibian skin after washing off transient environmental microbes.

Materials and Methods

Study species.—Red-backed Salamanders (*Plethodon cinereus*; N = 23) were collected at Jolliffe Nursery in Wetzel Co., West Virginia, USA (39.657°N, 80.555°W), on 20 December 2013. Red-backed Salamanders were kept individually in microcosms (40 × 25 × 18 cm) on natural soil substrate (depth = 10 cm), misted daily with water, and maintained at 21.5°C on a 12 h dark: 12 h light cycle in the laboratory (as a component of another research project). After 12 weeks salamanders were subjected to the washing protocol described below. The washing protocol was also field tested on Northern Gray-cheeked Salamanders (*Plethodon montanus*; N = 6) in Nantahala National Forest (35.700°N, 082.399°W), North Carolina, USA. Northern Gray-cheeked Salamanders were collected by hand from beneath logs on 12 May 2014, stored with moist substrate in plastic ziplock bags, and subjected to the washing protocol within 8 h of collection.

Washing protocol.—This procedure required two researchers beginning the protocol with clean nitrile or latex gloves and is outlined in Table 1. Researcher #1 held the amphibian while

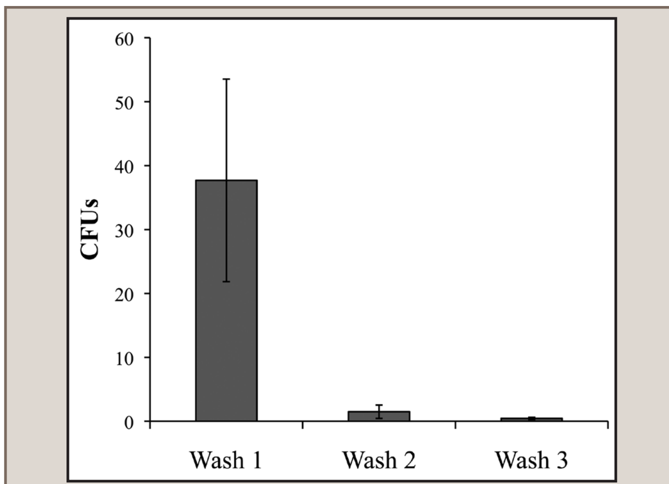

Fig. 1. Colony forming units (CFUs) for three sequential washes of Red-backed Salamanders (*Plethodon cinereus*). Mean (± 1 S.E.) CFUs.

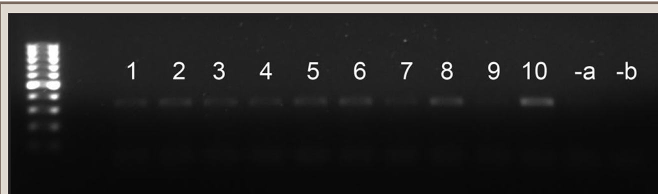

Fig. 2. Gel electrophoresis of laboratory-tested Red-backed Salamander (*Plethodon cinereus*) skin swabs (lanes 1–10) and negative controls (–a, –b).

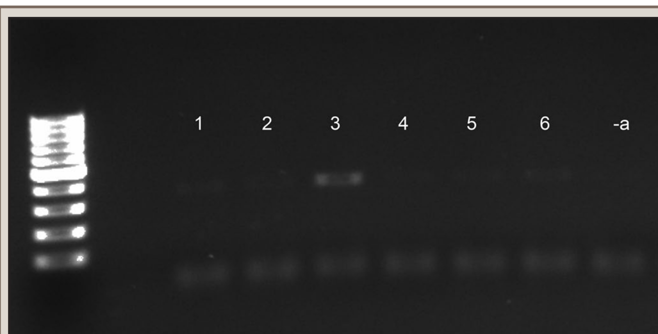

Fig. 3. Gel electrophoresis of field-tested Northern Gray-cheeked Salamanders (*Plethodon montanus*) skin swabs (lanes 1–6) and negative control (lane –a).

researcher #2 washed the animal for 15 sec (25 ml sterile ddH_2O) with a squirt bottle. The animal was then placed into a sterile 90 mm Petri dish with 25 ml sterile ddH_2O and gently swirled in a circular motion for 30 sec. With a new set of gloves researcher #1 picked up the animal and carried out two additional (sequential) Petri dish washes repeating the aforementioned steps. The squirt bottle was only used once at the beginning of the protocol. With a fresh set of gloves, researcher #1 then picked up the animal while researcher #2 swabbed it 15 times on the right, left, dorsal, and ventral sides of the salamander. A total 15 strokes with the swab was performed from the head to tail tip of each salamander. Swabs were stored at -20°C within one hour of being collected (Red-backed Salamanders) or stored in 99.9% acetone (Fukatsu 1999) for field collections (Northern Gray-cheeked Salamanders); differences in storage conditions allowed assessment of the feasibility of field *vs* lab-based applications of this protocol.

Plate-based assay.—An aliquot of the Petri dish water from the three sequential salamander washes was sampled using a plate-based method by quantifying bacterial colony forming units (CFUs). Specifically, 150 µl of wash water was transferred to a 90 mm Tryptic Soy Agar (Fisher Scientific) plate and spread across the surface of the medium using a glass L-shaped rod. Plates were then inverted and incubated for 48 h at 32°C. CFUs were counted and recorded for each of the three sequential washes. To evaluate whether our washing protocol reduced the quantity of bacteria on the salamander's skin we performed a repeated measures ANOVA on ln-transformed CFUs using JMP Pro 9.0.0.

Molecular methods.—To confirm the presence of resident microbial communities a subset of the salamanders (N = 10, *P. cinereus*; N = 6, *P. montanus*) subjected to the washing protocol were swabbed with a sterile cotton swab autoclaved for two hours (Gefrides et al. 2010) and DNA extracted with the Qiagen DNA Micro Kit according to the manufacturers protocol for isolating genomic DNA from small volumes of blood. The following alterations were made to the DNA extraction protocol: extraction was performed directly from the swab after an incubation period of 7 h (with proteinase K - step 5 of protocol). PCR conditions were as follows for a 25 µl reaction: 12.5 µl New England Biolabs Taq 2X Master Mix, 1 µl (10 µM) forward primer 515F, 1 µl (10 µM) reverse primer 806R (Caporaso et al. 2011), 5 µl DNA, 5.5 µl sterile ddH_2O. Electrophoresis was performed in a 1% agarose gel at 140 volts for 40 min. Five µl of each PCR reaction was added to each well. PCR amplicon fluorescence was interpreted as the presence of resident microbial communities from the salamander skin.

Results

There was a significant reduction in the amount of environmental bacteria (CFUs) with washing ($F_{1,45} = 34.89$; $P < 0.0001$; Fig. 1). DNA extraction, PCR, and gel electrophoresis of both field and lab tested skin swabs indicated the presence of PCR amplicons targeting resident bacterial communities on the skin of these salamanders (Figs. 2– 3). For *P. cinereus,* 9 of 10 skin swabs showed PCR amplicons (Fig. 2), whereas for *P. montanus* 4 of 6 skin swabs had dull PCR amplicons (Fig. 3).

Discussion

This study successfully demonstrates and tests a new standardized protocol for removing environmental microbes for studies on resident microbes composing the amphibian microbiome. We show that successive washes with sterile ddH_2O significantly reduce the amount of CFUs and also that PCR amplifiable microbial DNA is present upon concluding the protocol from salamanders in both the field and the laboratory. Field and laboratory experiments revealed similar results, confirming that our methods can be made portable for biodiversity inventories. Presumably, since no significant change was detected after the third wash in either laboratory maintained or field-collected salamanders, only the endemic microbiota associated with the salamander is sampled after our protocol. This technique is similar to previous studies that showed similar results (Lauer et al. 2007). This protocol will help standardize wash time, water volume, and researcher technique, all of which are critical in the removal of environmental microbes from amphibian skin. Adopting a standardized protocol amongst labs will be imperative for direct comparison of future studies on the amphibian microbiome.

Table 2. Comparison of washing protocols.

	Study species	Number of washes	Total wash time (sec)	Total wash water volume (ml)	Type of wash	Swabbing protocol
This study	*Plethodon cinereus* *Plethodon montanus* *Notophthalamus viridescens* *Lithobates catesbeianus*	4	105	100	sterile ddH_2O	15 swab strokes from head to tail on the right, left, dorsal, and ventral sides
Culp et al. 2007	*Plethodon cinereus*	3	90	60	sterile ddH_2O	Dorsal and ventral sides swabbed; number of strokes unreported
Lauer et al. 2007	*Plethodon cinereus*	2	Not reported	50	sterile dechlorinated H_2O	10 swab strokes on lateral and ventral sides
McKenzie et al. 2012	*Lithobates pipiens* *Pseudacris triseriata* *Ambystoma tigrinum*	3	Not reported	300	sterile H_2O	Swab brushed over entire amphibian for 30 sec
Fitzpatrick & Allison 2014	*Plethodon jordani*	1	Not reported	50	sterile dechlorinated H_2O	10 swab strokes on lateral and ventral sides
Kueneman et al. 2014	*Anaxyrus boreas* *Pseudacris regilla* *Taricha torosa* *Lithobates catesbeianus* *Rana cascadae*	2	Not reported	100	sterile H_2O	Swab brushed over ventral surface and limbs for 30 sec
Walke et al. 2014	*Lithobates catesbeianus* *Notophthalmus viridescens*	2	Not reported	Not reported	sterile dechlorinated H_2O	10 swab strokes along the ventral and 5 strokes along each dorsal/lateral sides

We acknowledge that a plate-based assay is a limitation of this study since it only quantifies microbes capable of growing on synthetic medium at 32°C. In addition, gel electrophoresis of PCR amplicons from salamander skin swabs was used to confirm the presence of skin symbionts. It could be argued that these microbes are environmental in origin. However, based on past research by Culp et al. (2007), Lauer et al. (2007), and the plate-based assay results in this study, we conclude that environmental microbes were successfully removed and the PCR amplicons are representative of skin symbionts of *P. cinereus* and *P. montanus*. We also acknowledge the limitations placed on this washing protocol by the size of the amphibian; studies on larger amphibians will be restricted by Petri dish size. This protocol was designed for small to medium amphibians (< 150 mm length × < 15 mm height) and should prove useful for studies of many similar-sized amphibian species worldwide.

Previous studies have reduced cutaneous microbiota using broad-spectrum antibiotics and/or hydrogen peroxide in salamanders and fish (Lumsden et al. 1998; Russo et al. 2007; Becker and Harris 2010). Our study suggested that these extreme measures might not be necessary to remove environmental microbes. We hypothesize that a volume of 50 ml of sterile water is sufficient to remove the vast majority of environmental microbes, similar to that used in previous studies (60 ml ddH_2O in Culp et al. 2007; 50 ml ddH_2O in Lauer et al. 2007). If this is the case, then results based on next-generation sequencing studies using variations on these washing protocols are likely accurate in the characterization of the amphibian skin microbiome (McKenzie et al. 2012; Fitzpatrick and Allison 2014; Kueneman et al. 2014; Walke et al. 2014).

The need for standardization of such protocols has arisen due to the precarious health of world amphibian populations. This is due in part to emerging infectious pathogens such as *B. dendrobatidis* and the newly discovered *B. salamandrivorans*, which has caused population crashes of salamanders in Europe and has the potential to cause worldwide declines if it spreads to North America and Asia (Martel et al. 2013). Since the associated microbiota of amphibians can be considered an important component of the amphibian immune response, studies focusing on intra- and interspecific differences in microbiota components and diversity are needed to better understand interactions between the skin of amphibians and pathogens. In the future such studies are capable of becoming exceedingly sophisticated with the advent of next-generation sequencing and bioinformatics approaches. However, a necessary first step is a clean sample. We recommend standardization of protocols as studies proceed. This protocol provides researchers with a viable and experimentally tested washing protocol to remove environmental bacteria from the skin of small to medium sized amphibians.

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