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Manuscript Including References (Word document) Click here to download Manuscript Including References (Word document) Mucosome inhibition

Amphibian skin defences show variation in ability to inhibit growth of *Batrachochytrium dendrobatidis* isolates from the Global Panzootic Lineage

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Keywords: chytridiomycosis, microbial symbionts, pathogen susceptibility, fungal pathogens, innate defences, mucosome, skin products

Abbreviations:
GPL: Global Panzootic Lineage

Abstract

The fungal pathogen *Batrachochytrium dendrobatidis* has caused declines and extinctions in hundreds of amphibian species across the world. Virulence varies among and within lineages; the Global Panzootic Lineage (GPL) is the most pathogenic, although there is also variation in lethality between GPL isolates. Amphibians have a number of defences against pathogens, and skin products including the microbiota and host peptides have been shown to have considerable influence over disease progression. Here we show the collective skin products (the mucosome) of two amphibian species show significant variation in their ability to inhibit different globally-distributed isolates of GPL. This may in part explain the variation in disease susceptibility of hosts to different strains of *Batrachochytrium dendrobatidis*. More work is required to identify particular traits associated with mucosomes that confer broad-spectrum inhibition across GPL in order to facilitate the development of prophylaxis and/or treatments for chytridiomycosis *in situ*.

Main article

Although there are a number of emerging infectious diseases that are devastating wildlife populations globally, chytridiomycosis is unique in its ability to infect amphibian hosts across an unprecedented diversity of genera and species within a given class of vertebrates [1]. This disease has been linked to the decline and extinction of hundreds of amphibian species worldwide, and it is the most devastating wildlife disease of vertebrates in recorded history [1]. Amphibian chytridiomycosis is caused by fungal Chytridiomycetes of the genus *Batrachochytrium,* of which two have been identified to date; *B. dendrobatidis* and *B. salamandrivorans* [2,3]. Declines from *B. salamandrivorans* are thought to be recent and restricted to salamander populations in Northern Europe, although its' spread to other geographical regions are predicted to cause additional population declines and extinctions [4, 5]. *Batrachochytrium dendrobatidis*, on the other hand, has been causing declines across the whole class of amphibians on a worldwide scale since the 1970's [1]. Although there are a number of globally distributed endemic lineages of *B. dendrobatidis* that do not appear to cause mass mortality events within their range, the hypervirulent Global Panzootic Lineage (GPL) continues to cause amphibian declines and extinctions in the Americas, Australia and Europe [1]. In addition, there is variation in the virulence of different GPL isolates for a given host species, however little is known about factors that influence host susceptibility across the genetic and pathogenicity variation exhibited by GPL [6-9]. Amphibians, like all vertebrates, have evolved a number of defences to protect them from infectious diseases. Of particular interest are skin-associated products found in the mucus of amphibians, which form the first line of defence on contact with pathogens such as *Batrachochytrium spp*. These products include peptides, lysozymes, alkaloids, antibodies, symbiotic bacteria and bacterial metabolites, and are collectively known as the 'mucosome' [10]. The *in vitro* anti-*B. dendrobatidis* function of the mucosome has been shown to correlate directly with *in vivo* susceptibility and pathogen prevalence across a number of amphibian species [10]. It has previously been shown that individual bacteria isolated from the skin of amphibians show variation in their ability to inhibit across the range of genetic variation shown by GPL [11-13], but whether this is also true for the mucosome has not yet been tested.

Here we determine whether mucosomes collected from two host amphibian species show variation in their inhibitory capabilities across a suite of eight globally-distributed *B. dendrobatidis* GPL isolates (Table 1). *Batrachochytrium dendrobatidis* isolates were selected that appear in different parts of the *B. dendrobatidis* GPL phylogenetic tree (O'Hanlon, pers. comm.) and that represent an international distribution, including four isolates from South Africa where the frogs used in the study were collected. Isolates originated from a range of different host species (Table 1) and had been passaged between 7 and 12 times. For this study, eight sub-adult African bullfrogs (*Pyxicephalus adspersus*) and eight adult common river frogs (*Amietia delalandii*) were collected from Potchefstroom, North-West Province, South Africa and transported individually in sterile plastic bags to the lab, where mucosomes were immediately collected from each individual according to Woodhams et al. [10]. Briefly, frogs were placed in individual sterile cups and a given volume of sterile water added to each cup according to the surface area of each frog. Animals were held in the cups for one hour, after which the mucosome

rinse water was collected and filtered through a 0.22µm sterile filter (Millipore, Ireland) and kept on ice until challenge assays were conducted. Mucosomes were challenged against eight *B. dendrobatidis* GPL isolates using an *in vitro* spectrophotometer assay method adapted from Bell et al. [14], Woodhams et al. [10] and Becker et al. [15]. Three flasks of each *Batrachochytrium dendrobatidis* isolate were grown in 1% tryptone broth at 21°C until maximum zoospore production was observed (~3-4 days; ~1 x 10^6 zoospores ml^{-1}). The three flasks of each isolate were combined and zoospores separated from sporangia by filtering through 20µm sterile filters (Millipore, Ireland). To conduct the spectrophotometer assays, 50µl of mucosome and 50µl of *B. dendrobatidis* suspension were pipetted into 96 well plates. Each *B. dendrobatidis*-mucosome combination was run with six replicates. Positive controls were included using 50µl sterile water instead of mucosome filtrate. Negative controls were included using 50µl sterile water and 50µl of heat-treated *B. dendrobatidis* for each isolate.

Plate readings were taken every 24 hours for four days using a 492nm filter. Data were transformed using the equation Ln(OD/(1-OD)), and regression analysis used to gain the slope values for each sample over time. Total *B. dendrobatidis* inhibition was calculated using the following formula; Inhibition (%) = [1-(slope of sample/slope of control)] x 100, where a positive number represents inhibition of *B. dendrobatidis* growth and a negative number indicates enhanced growth of *B. dendrobatidis*. The average inhibition percentage was calculated for each individual sample, and the eight samples acted as replicates for a given host species in subsequent analyses.

Overall, most *B. dendrobatidis* isolates were inhibited in the presence of mucosomes from both species (Figure 1). A Mann-Whitney U test indicated significant differences in mucosome inhibition between the two species for the UK1 isolate of *B. dendrobatidis* ($W = 20$, $p = 0.015$), but there were no significant differences between host species for all other isolates (all $p > 0.05$). Almost all *B. dendrobatidis* isolates were inhibited when challenged with mucosome from *A. delalandii*, with the exception of two isolates that showed negligible growth or inhibition (South Africa 1a and UK2; Figure 1). There were significant differences in *A. delalandii* mucosome inhibition between *B. dendrobatidis* isolates (Kruskall-Wallace chi-squared = 21.686, d.f. = 7, $p = 0.003$) and a Dunn post-hoc analysis indicated significant differences between a number of isolates (Table 2). Almost all isolates were different to 2-4 other isolates, with no discernible relation to geographical origin of isolate. The isolate from Spain was not statistically different to any other *B. dendrobatidis* isolate, with intermediate growth inhibition in comparison to all others (Figure 1; Table 2). As with *A. delalandii*, the growth of most isolates of GPL was inhibited when challenged with mucosome collected from *P. adspersus*, with the exceptions of South Africa 1b (negligible growth or inhibition), South Africa 3 (high level of variation in its response) and UK1, which exhibited very high levels of enhanced growth in the presence of *P. adspersus* mucosome (Figure 1). The overall model for differences in growth of *B. dendrobatidis* isolates in the presence of *P. adspersus* mucosome was significant (Kruskall-Wallace chi-squared = 21.596, d.f. = 7, $p = 0.003$). The Dunn pairwise comparisons (Table 2) show that UK1 was significantly different to all other isolates of GPL with the exception of South Africa 1b, which was significantly different to the Spain and Sardinia isolates.

Together these results show that the growth of different isolates of *B. dendrobatidis* GPL varies significantly in the presence of amphibian mucosomes, and that there is some variation in mucosome inhibition between host species across the range of isolates. This suggests that the response of the pathogen is linked to traits associated with the host mucosome as well as inherent traits of the various *B. dendrobatidis* isolates. It has previously been shown that individual bacteria isolated from amphibian skin also show variation in their ability to inhibit across a range of *B. dendrobatidis* isolates [11-13], suggesting that the bacteria or their metabolites within the mucosome play a role in determining inhibition of a given isolate of *B. dendrobatidis*. A number of recent studies show that the composition of the bacterial community associated with the skin of amphibians is correlated with infection probability of *B. dendrobatidis* [16-19]. Although the role of the microbiome composition in determining susceptibility across GPL variation has not yet been tested *in vivo,* the *in vitro* data presented here along with that of Antwis et al. [11], Muletz et al. [12] and Bletz et al. [13] indicates strong potential for variation in the response of the host to different isolates of the fungal pathogen, both in terms of changes in the host microbiome and the infection outcome for the host. Other mucosome traits aside from bacteria (e.g. peptides, lysozymes) may also account for the variation in mucosome-pathogen responses in our data. Amphibians show variation in their susceptibility to different isolates of *B. dendrobatidis* [6-9], and the data presented here suggest this may be related to interactions between *B. dendrobatidis* and some aspect(s) of the mucosome defences of amphibians. Additionally, this pathogen has a highly complex genome with widespread aneuploidy [5, 24]; the variation in mucosal inhibition between different *B. dendrobatidis* isolates demonstrated here may be linked to differential phenotypic or genotypic traits associated with these isolates as has been suggested in other studies [6-9].

Overall, most *B. dendrobatidis* isolates showed reduced growth in the presence of mucosomes from both species (Figure 1). *Amietia delalandii* are not known to be experiencing chytridiomycosis-related declines in the wild although populations are infected with low levels of *B. dendrobatidis* (38.8% prevalence, *B. dendrobatidis* genomic equivalents < 5.0, n = 464; [23]). Infected wild *P. adspersus* have not been found to date (genomic equivalents = 0.0, n = 10; Weldon, unpublished data). The data presented here suggests the mucosomes of both species may play a role in resisting *B. dendrobatidis* infection, although little is known about the defences of these species and there are many other factors that will also influence susceptibility to *B. dendrobatidis*. In addition, it is not known if the individuals used in this study were infected with *B. dendrobatidis*, which may influence the propensity of the mucosome to inhibit the pathogen.

Experimental work may allow for the prediction and/or identification of particular community traits (e.g. high/low abundance of particular bacterial genera) that confer broad-scale inhibition against the wide genetic and virulence variation shown by *B. dendrobatidis*. The current regimes for treating chytridiomycosis are often laborious and have limited transferability to wild populations [20]. However, the potential use of probiotics is increasingly being researched [21, 22], and it may be possible to

exploit mucosome traits linked to broad scale inhibition across the variation presented by *B. dendrobatidis* in order to develop robust and effective treatments and/or prophylaxis for chytridiomycosis *in situ*. In addition, teasing apart how genomic and transcriptomic factors associated with *Batrachochytrium dendrobatidis* interact with hosts and host-associated mucosomes, and how these factors relate to virulence traits, will provide valuable information about *B. dendrobatidis* epidemiology and ultimately, the mitigation of chytridiomycosis in amphibians.

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Conflicts of interest

There are no conflicts of interest.

Ethical statement

This study was approved by the Biodiversity and Conservation Ecology Scientific Committee and the Animal Research Ethics Committee (NWU-00013-10-S4) of North-West University, and conducted under research permit 028 NW-11 issued by the Department of Economic Development, Environment, Conservation and Tourism, North West Provincial Government, Republic of South Africa.

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Figure 1

Average (± 1 S.E.) inhibition of eight globally distributed isolates of the Global Panzootic Lineage of *Batrachochytrium dendrobatidis* by skin mucosomes collected from two South African host amphibian species. Positive numbers represent inhibition of *B. dendrobatidis* growth and negative numbers indicate enhanced growth of *B. dendrobatidis*. See Table 2 for statistically different pairwise comparisons.

Table 1

Batrachochytrium dendrobatidis isolates used in the study.

Isolate	**Archive code**	**Geographical origin**	**Host species isolated from**
South Africa 1a	MG04	Silver Mine, Western Cape, South Africa	*Amietia fuscigula*
South Africa 1b	MG06	Silver Mine, Western Cape, South Africa	*Amietia fuscigula*
South Africa 2	MG08	Magoebaskloof, Limpopo, South Africa	*Amietia delalandii*
South Africa 3	MG09	Magoebaskloof, Limpopo, South Africa	*Hadromophryne natalensis*
UK 1	CORN 3.1	Penhale Farm, Cornwall, UK	*Ichthyosaurus alpestris*
UK 2	SFBC 014	Sellafield, Cumbria, UK	*Bufo bufo*
Spain	IA 2011	Ibon Acherito, Spain	*Alytes obstetricans*
Sardinia	MODS 28.1	Mont Olia, Sandinia	*Discoglossus sardus*

Table 2

Dunn pairwise comparisons between *Batrachochytrium dendrobatidis* isolate growth in the presence of *Amietia delalandii* (green) and *Pyxicephalus adspersus* (orange) mucosomes. Results in bold and with an * indicate a statistically significant result.

	South Africa 1a	South Africa 1b	South Africa 2	South Africa 3	UK1	UK2	Spain	Sardinia
South Africa 1a		p=0.412	**p=0.033***	**p=0.021***	**p=0.037***	p=0.444	p=0.168	**p=0.038***
South Africa 1b	p=0.131		p=0.067	**p=0.038***	p=0.069	p=0.347	p=0.262	p=0.073
South Africa 2	p=0.474	p=0.134		p=0.378	p=0.488	**p=0.037***	p=0.240	p=0.495
South Africa 3	p=0.468	p=0.132	p=0.478		p=0.405	**p=0.017***	p=0.112	p=0.378
UK1	**p=0.016***	p=0.216	**p=0.016***	**p=0.020***		**p=0.029***	p=0.252	**p=0.476**
UK2	p=0.494	p=0.161	p=0.483	p=0.462	**p=0.020***		p=0.109	**p=0.030***
Spain	p=0.384	**p=0.044***	p=0.373	p=0.434	**p=0.007***	p=0.351		p=0.240
Sardinia	p=0.213	**p=0.018***	p=0.205	p=0.251	**p=0.001***	p=0.175	p=0.382	

Figure

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