Absence of *Trypanosoma* infection among *Hoplobatrachus tigerinus* (Amphibia: Dicroglossidae) from Boeny, western Madagascar

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Abstract

We investigated the presence of *Trypanosoma* and other blood parasites in a species of frog, *Hoplobatrachus tigerinus*, from the Boeny Region, and introduced to Madagascar. Blood samples collected from 134 living *H. tigerinus* were examined. Results from microscopy and PCR analyses did not find any evidence of *Trypanosoma* infection. Furthermore, other blood parasites such as *Hepatozoon* or *Lankesterella* were screened but not one of the *H. tigerinus* was infected. Our findings are in contrast with native Asian populations that are infected by these different blood parasites. This study provides a baseline to assess the circulating blood parasites in the amphibian fauna, as well as in other vertebrates of Madagascar.

Résumé

La présence de *Trypanosoma* et d'autres hémoparasites a été recherchée chez une espèce de grenouille introduite *Hoplobatrachus tigerinus*, provenant de la région du Boeny, dans la partie occidentale de Madagascar. Des échantillons sanguins provenant de 134 individus de *H. tigerinus* ont été collectés puis analysés. Les résultats de microscopie et les analyses moléculaires n'ont révélé aucune infection à *Trypanosoma*. D'autres hémoparasites comme *Hepatozoon* et *Lankesterella* ont également été recherchés mais aucun des *H. tigerinus* n'en était infecté. Nos résultats sont en contraste avec les observations faites dans les populations asiatiques qui sont infectées par divers parasites sanguins. Cette étude fournit une base de référence pour évaluer les hémoparasites circulants aussi bien chez les amphibiens que chez d'autres groupes de vertébrés de Madagascar.

Introduction

Madagascar is well-known for its considerable amphibian fauna (Glaw & Vences, 2007), of which ≥ 99% are known to be endemic (Vieites *et al.*, 2009). The highly diverse amphibian fauna is threatened by different ecological and biological pressures, including habitat loss, habitat fragmentation, pathogens, and introduced species (Andreone *et al.*, 2014; Kolby *et al.*, 2014; Mecke *et al.*, 2014; Moore *et al.*, 2015). One amphibian species introduced to the island at the beginning of 20th century from India is the bullfrog *Hoplobatrachus tigerinus* (Dicroglossidae) (Poynton, 1999), which is currently widespread on Madagascar (Glaw & Vences, 2007) and is locally exploited for food (Kosuch *et al.*, 2001). In Asia, *H. rugulosus* is known to host different parasites such as *Trypanosoma*, *Hepatozoon*, and *Lankesterella* (Sailasuta *et al.*, 2011), and if these organisms are present in introduced *H. tigerinus* found on Madagascar, this may constitute a potential infectious threat particularly for endemic amphibian species. Information about parasites found in Malagasy amphibian species is poorly known and needs further documentation to evaluate different risks associated with the conservation of Malagasy amphibians. With this intent, we studied protozoan blood parasites in *H. tigerinus*, employing both morphological and molecular screening methods to determine parasitism rates of *Trypanosoma*.

Methods

Hoplobatrachus tigerinus sampling

For this study, 134 living *Hoplobatrachus tigerinus*, including 95 females and 39 males, from near the villages of Amboromalandy, Ambato Boeny, Anjiajia, and Andranofasika in the Boeny Region of northwestern Madagascar, were purchased from a single market vendor in Antananarivo between February and July 2014. *Hoplobatrachus tigerinus*

Maeder, M. N., Raherinjafy, R., Andriamahefa, H., Ramasindrazana, B. & Randrianarivelojosia M. 2019. Absence of Trypanosoma infection among *Hoplobatrachus tigerinus* (Amphibia: Dicroglossidae) from Boeny, western Madagascar. *Malagasy Nature*, 13: 173-175.

from the Boeny Region, especially those collected near rice fields, are sold for human consumption. A blood sample was collected from each animal via cardiac puncture and stored in EDTA at -20°C before analysis.

Blood parasite screening by microscopy and nested PCR amplification

In order to detect the presence of *Trypanosoma* in the samples, 10 µl of blood was collected from each frog and immediately used to prepare a blood smear. The smears were air-dried at room temperature, fixed with methanol, and then stained with Giemsa stain. The complete blood smear was examined using a binocular microscope under 400x and 1000x magnification using immersion oil.

For molecular analysis, genomic DNA was extracted from 200 µl of blood by using the phenol chloroform method (Contamin *et al.*, 1995). Amplification of a portion of ssRNA was undertaken with a nested PCR using 609F/706R for the primary PCR (609F: CACCCGCGGTAATTCCAG/706R: TTGAGGTTACAGTCTCAG) and SSU450F/SSU450R for the nested PCR (SSU450 F: TGGGATAACAAAGGAGCA/SSU450 R: CTGAGACTGTAACCTCAAAGC) (Noyes *et al.*, 1999). The primary PCR was conducted in a 25 µl reaction volume containing 1.5 µl of $MgCl_2$ 25 mM, 0.2 µl of dNTP, 1.5 µl of buffer 10x, 0.5 µl of Taq Polymerase, 1 µl of each primer (609F/706R), 16.3 µl of distillated water, and 3 µl of total nucleic acid as template. The cycling conditions were 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C for 35 cycles. Nested PCR was performed using 25 µl reactions containing 1.5 µl of $MgCl_2$ 25 mM, 0.2 µl of dNTP, 1.5 µl of buffer 10x, 0.5 µl of Taq Polymerase, 1 µl of each primer (SSU450F/SSU450R), 16.3 µl of distillated water, and 3 µl of the product of the first step PCR. The cycling conditions were 1 min at 95°C, 1 min at 54°C, and 1 min at 72°C for 30 cycles. All PCR reactions were initiated for 5 min at 95°C (denaturation) and a 10 min at 72°C (final extension). The PCR amplification was verified by positive and negative controls.

Results and discussion

Screening of 134 *Hoplobatrachus tigerinus* blood smears using 10 µl of blood under a binocular microscope revealed no evidence of *Trypanosoma* infection. Further, the presence of *Lankesterella* and *Hepatozoon* protozoan parasites were assessed and no positive smear was identified. PCR amplification of the ssRNA for *Trypanosoma* was conducted and no evidence of this parasite was detected. Based on these samples, *H. tigerinus* from the Boeny Region were free of *Trypanosoma* infection. This result is in contrast with findings from Asia, where *H. rugulosus* are infected with *T. rotatorium*-like organism and *T. chattoni* with a total infection rate of 33.6% (Sailasuta *et al.*, 2011).

We describe methods for detecting circulating blood parasites in amphibians, specifically those present in Madagascar. This approach can be extended to other hosts including bats and birds that might also be infected by *Trypanosoma* spp. Studies using blood smear screening have already been conducted on a variety of Malagasy vertebrates (Brygoo, 1963; Savage *et al.*, 2000; Raharimanga *et al.*, 2002, 2003; Laakkonen *et al.*, 2003) and molecular techniques will be an important component to elucidate the taxonomy and diversity of *Trypanosoma* species occurring on Madagascar.

Acknowledgments

We would like to thank Prof. Camargo from the University of Sao Paulo, Brazil, for providing positive control DNA of *Trypanosoma* sp. We are also grateful to Steven M. Goodman for his comments on earlier versions of this note. This research was financially supported by the Institut Pasteur de Madagascar.

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