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Combination of cytochrome b heteroduplex-assay and sequencing for identification of triatomine blood meals

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ABSTRACT

The identification of blood meals in vectors contributes greatly to the understanding of interactions between vectors, microorganisms and hosts. The aim of the current work was to complement the validation of cytochrome b (*Cytb*) heteroduplex assay (HDA) previously described, and to add the sequencing of the *Cytb* gene of some samples for the identification of blood meals in triatomines. Experimental feedings of reared triatomines helped to clarify the sensitivity of the HDA. Moreover, the sequencing coupled with the HDA, allowed the assessment of the technique's taxonomic level of discrimination. The primers used to produce DNA fragments of *Cytb* genes for HDA had a very high sensitivity for vertebrate DNAs, rather similar for mammals, birds and reptiles. However, the formation of heteroduplex depended on blood meal's quality rather than its quantity; a correlation was observed between blood meals' color and the positivity of HDA. HDA electrophoresis profiles were reproducible, and allowed the discrimination of blood origins at the species level. However, in some cases, intraspecific variability of *Cytb* gene generated different HDA profiles. The HDA based on comparison of electrophoresis profiles is a very useful tool for screening large samples to determine blood origins; the subsequent sequencing of PCR products of *Cytb* corresponding to different HDA profiles allowed the identification of species whatever the biotope in which the vectors were captured.

1. Introduction

Triatomines are hematophagous insects of epidemiological importance, because most of them are vectors of *Trypanosoma cruzi*, the agent of Chagas disease. The identification of blood meal origins in triatomines contributes to the understanding of their feeding habits, both in natural and human habitats (Carneiro Freitas et al., 2005; Bosseno et al., 2006; Caranha et al., 2006). It gives an insight into vector-host interactions in relation to transmission cycles of the parasite (Brenière et al., 2004). In the 1980's immunological techniques were developed to identify blood meals in hematophagous vectors, such as the complement fixation test based on the detection of host antibodies (Staak et al., 1981) and enzyme-linked immunosorbent assay (ELISA) that specifically detects host immunoglobulins, through the development of specific conjugates of potential host genus or species (Chow et al., 1993). These techniques are laborious and need the elimination of undesirable cross-reactions (Hunter and Bayley, 1996). Moreover, they have low applicability in studies with wild vectors because of the difficulty of obtaining specific conjugates for wild host species. More recently, several tools based on DNA analysis techniques have been developed to identify blood meals origins. Many of them consist in multiplex-PCR that can rapidly discriminate between potential hosts by molecular weights of PCR products (Mota et al., 2007). Nevertheless, these techniques assume the development of specific primers for species, genera or other taxonomic levels based on known DNA sequences. Other techniques such as cytochrome b heteroduplex-assay (*Cytb*-HDA) (Boakye et al., 1999; Lee et al., 2002; Kirstein and Gray, 1996) or partial sequencing of cytochrome oxidase I (*COI*) and *Cytb* genes (Townzen et al., 2008), have the advantage of being exhaustive. Previous studies showed that the *Cytb*-HDA was very useful to discriminate blood meals (Njiokou et al., 2004; Bosseno et al., 2006) because it detects differences between DNA sequences of species closely related or not (Tang and Unnasch, 1995). Moreover, using this technique and subsequent cloning and sequencing of PCR products, Bosseno et al. (2009) identified multiple blood meals in triatomines.

In the present study we further studied the *Cytb*-HDA combined with the sequencing of PCR products to identify blood meals of triatomines collected in any environment. The specificity and sensitivity of the technique were investigated. Experiments with artificial feeding of triatomines also helped to clarify the limits of

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the method. The interpretation of HDA profiles was strongly highlighted by the analysis of current sequences with GenBank ones to examine the taxonomic level of HDA discrimination. Also recommendations are suggested for the processing of triatomines in the field to facilitate these molecular analyses in the laboratory.

2. Materials and methods

2.1. Harvesting blood meals

Blood meals were obtained in different ways: (i) by abdomen dissection and removing the entire digestive track with forceps, (ii) abdominal pressure to get the blood contents, after cutting the terminal part of the abdomen when the bugs are fully engorged. Instruments (scissors, forceps) must be cleaned with a 10% sodium hypochlorite solution and rinsed with distilled water between each sample. Samples were stored at −20 °C or diluted V/V with a solution of 6 M Guanidine-HCl, 200 mM EDTA for storage at room temperature or at 4 °C.

2.2. DNA extraction and polymerase chain reaction (PCR)

The extraction of DNA from blood meal samples was performed with the QIAamp DNA mini kit (Quiagen, Courtaboeuf, France), according to the recommended protocol for blood samples with minor modifications: a maximum volume of 200 μl of blood meal was processed and samples <200 μl were diluted in PBS to a final volume of 200 μl. In the final step, DNA was eluted with distilled water in a final volume of 50 or 30 μl for blood meal samples <20 μl. DNAs from animal blood or tissue were extracted using the same kit according to the manufacturer's recommendations. The quantification and purity of DNA was performed by measuring the absorbance at 260 and 280 nm, using a Biomate 3 spectrophotometer (Thermo Electron Corporation, Madison, WI, USA). A first PCR amplification of a 355 bp *Cytb* fragment (PCR1-*Cytb*) was achieved with the set of primers previously described (Lee et al., 2002): F-5′-CCCCTCAGAATGATATTTGTCCTCA-3′ and R-5′-CCATCCAACATCTCAGCATGATGAAA-3′, in 50 μl of reaction mixture containing 50 mM of Tris–HCL (pH 8.5), 50 mM of NaCl, 1.5 mM of $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer, 2.5 U of Taq polymerase (Gotaq Flexi, Promega, Madison, Wisconsin, USA), and 5 μl of DNA template. Amplification was performed on a Mastercycler (Eppendorf, Hamburg, Germany) under the following conditions: starting step of 95 °C for 3.5 min, 36 cycles (95 °C, 30 s; 55 °C 50 s; 72 °C, 40 s) followed by an extension step at 72 °C for 5 min. The PCR products were then used for HDA. A second multiplex PCR amplification of *Cytb* (PCR2-*Cytb*) proposed to discriminate human (315 pb) and other mammal (420 pb) blood feedings was applied to animal DNAs to test their sensitivity and specificity with the following set of primers: DC-*Cytb*-UP 5′-CRT GAG GMC AAA TAT CHT TYT-3′, DC-*Cytb*-DW 5′-ART ATC ATT CWG GTT TAA TRT-3′ y H-*Cytb*-DW 5′-AGG AGA GAA GGA AA GAA AT-3′ (Mota et al., 2007) under the same conditions previously described for PCR1-*Cytb*. Five microliter of each PCR product were analyzed by electrophoresis in a 1% agarose gel using EZ-Vision™ as staining solution and visualized under UV light. The intensity of each amplified products was also estimated.

2.3. Sensitivity and specificity of PCR

To evaluate the sensitivity and specificity of the PCR assays, different amounts of DNA templates (1 fg to 100 ng) obtained from several vertebrate species, mammal, birds, reptiles and triatomines species (*Triatoma infestans* and *Triatoma sordida),* were tested.

2.4. Heteroduplex-assay (HDA)

The heteroduplex DNA chains were generated by mixing 5 μl of PCR1-*Cytb* products of each sample with 5 μl of PCR1-*Cytb* products obtained from known DNA chosen as a driver (human DNA), and 8 μl of distilled water. The heteroduplex formation was performed either by using a thermocycler Mastercycler (Eppendorf, Hamburg, Germany) under the following conditions: 94 °C for 5 min, 28 °C for 1 h and 4 °C for 10 min, or by DNA denaturation (5 min boiling at 94 °C) and cooling at laboratory temperature (bellow 25 °C). The heteroduplex products were diluted in EZ-Vision™ buffer (2 μl) and separated on a pre-cast NuPAGE 5% Bis–Tris acrylamide gel in Tris-Borate EDTA buffer according to the manufacturer's instructions (Invitrogen, Carslbad, California, USA). The electrophoresis conditions were 100 V for 3 h and the banding was examined under UV light. Blood meal origins were identified by comparing the DNA patterns between them and with those formed with the DNAs of known vertebrate species.

2.5. Sequencing of PCR1-Cytb products

The PCR products were directly sequenced by the company Macrogen (Seoul, Korea). Then Blast *n*-searches in GenBank permitted the identification of the level of identity with known sequences, and subsequently the species corresponding to the blood meals.

2.6. Experimental feeding of reared T. infestans

Colonies of *T. infestans* were reared in the insectarium under controlled conditions (26 °C and 60–70% humidity). A first protocol was designed to assess the minimum amount of blood meal that can be identified by testing the PCR1-*Cytb*-HDA. Thirty 5th nymphal instars of *T. infestans* were fed on mouse (*Mus musculus*) during 1–49 min after 45 days of fasting. The bugs numbered from 1 to 20 had received partial feedings ranging between 10 and 70 mg of blood. The ten others (21–30) were left on the mice until they no longer wanted to eat and got 182–524 mg of blood. Eight days post feeding, all bugs were processed.

A second protocol was designed to evaluate how long after feeding blood meals can be identified by PCR1-*Cytb*-HDA. Thirty 5th nymphal instars of *T. infestans* were fed on mouse for partial meals during 5–10 min. Twenty, 30 and 45 days post feeding, 10 specimens were processed in each group. For each specimen, the volume of blood ingested was estimated by the difference between the weight before and after feeding. Similarly, before processing each bug was weighed and the color of the food source was registered.

2.7. Origin of natural populations of triatomines

A total of 258 blood meals of *T. infestans* captured in domestic (151) and wild environments (107) in various Bolivian departments were processed by PCR1-*Cytb* and by HDA for PCR positive samples. The details of the results (geographic origin of the samples, relationship between ecotopes where the bugs were collected and blood meal origins) of the total sample will be exposed elsewhere; here 2–5 PCR1-*Cytb* products corresponding to the different HDA profile detected among the total sample were sequenced in order to analyze the relationship between HDA patterns and blood meal origins.

2.8. Data analysis

Correlations between HDA results (positive/negative) and the insects' weights before feeding, weight increase after feeding expressed by the ratio weight after feeding/weight before feeding, and weight at sacrifice expressed by the ratio weight at sacrifice/