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Standardization of a Polymerase Chain Reaction Protocol for Detecting Antimicrobial Peptides in *Anopheles Darlingi* (Diptera: Culicidae) Mosquitoes from the Brazilian Amazon

Erian Santos (eriansantos.bio@gmail.com)
Universidade Federal do Para https://orcid.org/0000-0001-6121-1766
Ana Cecília Feio dos Santos
Evandro Chagas Institute: Instituto Evandro Chagas
Luciana Letícia da Costa Pires
Evandro Chagas Institute: Instituto Evandro Chagas
Samir Mansour Moraes Casseb
Evandro Chagas Institute: Instituto Evandro Chagas
Gustavo Moraes Holanda
Evandro Chagas Institute: Instituto Evandro Chagas
Izis Mônica Carvalho Sucupira
Evandro Chagas Institute: Instituto Evandro Chagas
Ana Cecília Ribeiro Cruz
Evandro Chagas Institute: Instituto Evandro Chagas
Eduardo José Melo dos Santos
Universidade Federal do Pará: Universidade Federal do Para
Marinete Marins Póvoa
Evandro Chagas Institute: Instituto Evandro Chagas

Methodology

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Abstract

Background: Antimicrobial peptides (AMPs) are proteins of the innate immune system that can limit infections of the malaria-carrying parasite *Plasmodium,* which develops inside anopheline mosquitoes, the human malaria vectors. Despite this, studies on Brazilian Amazon species of anopheline mosquitoes are still needed. The aim of the study is to develop a standard molecular polymerase chain reaction (PCR) technique to detect the AMPs cecropin A (CecA) and defensin from *Anopheles darlingi* to support studies involving their detection and amplification and better understanding of the roles of these peptides in the *Anopheles-Plasmodium* interaction.

Methods: The collection of anopheline mosquitoes was carried out in three municipalities in the Brazilian Amazon: Altamira and Peixe-Boi, in the state of Pará, and Cruzeiro do Sul, in the state of Acre. The primers were built based on the sequences available in GenBank, and PCR followed standard protocols with different annealing temperatures tested. The PCR products were purified and then sequenced by the dideoxy chain termination method.

Results: CecA and defensin amplification were standardized with annealing temperatures of 59ºC and 55ºC, respectively. The amplified products and sequencing demonstrated the good quality of both primer sets.

Conclusions: For the first time, a standardized molecular technique for detecting AMPs was described in *An. darlingi*, a mosquito species from the Brazilian Amazon, supporting future studies aiming to understand the interactions of this species and the action of these peptides during infection and providing important molecular markers for the control of human malaria.

Background

The Brazilian Amazon is the site of almost all human malaria cases in Brazil, with approximately 98% of cases distributed in several municipalities that are endemic for the disease. This high percentage of malaria cases is related to the environmental changes that have occurred in the Amazon with agriculture, livestock, extraction activities and urban development works that resulted in a significant impact on its ecosystem and favour the development of vector mosquitoes[1,2,3]. The vector mosquitoes belong to the family Culicidae, subfamily Anophelinae, order Diptera, genus *Anopheles.* In Brazil, the most important subgenus is *Nyssorhynchus*, and its main representative is the species *Anopheles (Nys.) darlingi* Root, 1926[3].

An. darlingi is considered the main vector of malaria given its behavioural characteristics, such as anthropophilia and endophagy, and because it is susceptible to human malaria parasites that circulate in Latin America. In addition, even when not abundant, this mosquito can maintain *Plasmodium* transmission locally[3,4,5]. Infection rates in this vector can be high, as has already been reported in the Brazilian Amazon, where an infection rate of more than 18% was observed in the state of Pará[6], a value

comparable to that of other anopheline mosquitoes of epidemiological importance such as *Anopheles gambiae* (11.1%) and *Anopheles funestus* (13.1%)[7,8].

The vectorial competence of these mosquito species is influenced by the vector's immune system. However, the molecular mechanism of immune system responses is still unclear[9]. In this context, studies have been performed to identify possible molecular markers for human malaria control. Included among the targets of interest are the main antimicrobial peptides (AMPs) of the innate immunity of mosquitoes, such as cecropins and defensins[10,11].

AMPs are proteins that act in the protection against invading organisms such as bacteria, viruses, fungi and protozoa, forming the host's first line of defence[12,13,14]. In mosquitoes, the involvement of these peptides has been demonstrated in studies involving Cecropin A (CecA) in *An. gambiae* against *P. berghei*[11,15] and defensin in *Ae. aegypti* against *P. gallinaceum*[16].

Despite the importance of AMPs in reducing parasite development inside the mosquito, there are no exploratory studies on the mechanism of action of these peptides in species of anopheline mosquitoes in the Brazilian Amazon, such as *An. darlingi*. Thus, this work aims to standardize a molecular polymerase chain reaction (PCR) technique to detect the AMPs CecA and defensin from *An. darlingi* mosquitoes to support studies involving their detection and amplification and understanding of their role in the *Anopheles-Plasmodium* interaction.

Methods

Anopheline mosquito study and collection area

Anopheline mosquitoes were collected in three municipalities in the Brazilian Amazon, two in the state of Pará (PA) (Peixe-Boi, 47º 18' 44” W; 01º 11' 31” S and Altamira, 03º 12' 12" S; 52º 12' 23" W), and one in the state of Acre (AC) (Cruzeiro do Sul, 7° 37' 51" W; 72° 40' 12 "S) (Fig. 1).

Figure 1 Anopheline mosquito collection areas in three municipalities in the Brazilian Amazon.

The municipality of Peixe-Boi (PA) has an abundance of anopheline mosquitoes but no transmission of human malaria. Until August 2020, the number of cases of human malaria in the municipality of Altamira (PA) was 553 (4.1% of the total of Pará State), and the number of cases in Cruzeiro do Sul (AC) was 4482 (61.1% of the total of Acre State)[17]. The average annual rainfall in Peixe-Boi (PA), Altamira (PA) and Cruzeiro do Sul is 2,471 mm, 1,844 mm and 2,139 mm, respectively. The rainy season occurs mainly between November and April, as in other municipalities in the Brazilian Amazon, and the average annual temperature is 26ºC[18,19].

Mosquito collection was conducted between July and December 2018 for four hours (6–10 pm) in the municipality of Peixe-Boi (PA) in the extra-home environment (carried out stably without negatively affecting the welfare of the animals) and 12 hours (6 pm-6 am) using the protected human attraction

method in Altamira (PA) and Cruzeiro do Sul (AC), both in peri/intradomicile environments. For identification, the females were anaesthetized in a freezer (for approximately 5 minutes), at which point they were morphologically identified using entomological keys[20, 21].

Primer design

Alignments were made to compare the sequences of CecA and defensina in all anopheline species available to verify regions that present greater similarity between species for primer design. The alignment of the sequences was performed in the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/)[22].

After comparing the sequences, primers were constructed based on the GenBank genetic sequence databases of *An. darlingi* for CecA (ADMH02001013.1) and defensin (ADMH02000296.1) (Table 1).

Table 1
Primers of the two AMPs for PCR sequencing

Primers	Sequence (5' – 3')	*Amplicon*
Cecropin A (F)	TACCGACCAGCATCAGTCAG	434 bp
Cecropin A (R)	AAAGCATAAGGGGCAGGAGT	
Defensin (F)	GCTCTTCACTCGAACAGTGC	497 bp
Defensin (R)	CTTCCGGTCTTTCATGTTGC	

Primers to amplify the gene sequences of *An. darlingi* CecA and defensin were designed with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/). The parameters used were melting temperature between 55–60ºC, amount of GC% up to 55% and no dimer or hairpin formation.

DNA extraction, PCR assay and sequencing

The anopheline mosquito DNA was extracted following the Collins method[23], with a final volume of approximately 50 µl. The extracted DNA was run on a 0.8% agarose gel (Ultra-pure Agarose, BRL 155517-014) at 100 volts for 1 hour stained with GelRed and methylene blue for viewing under ultraviolet light (Fluo-Link, FlowGen) and photographed in a Kodak® Edas 290 photo documentation system.

The PCR for each anopheline mosquito AMP was prepared in a 1.5 ml Eppendorf tube and contained nuclease-free water, Tris (10 mM, pH 8.3, 50 mM KCl), MgCl2 (1.5 mM), dNTPs (10 mM), primers (10 pmol), 2 U of Taq DNA polymerase (Ludwig Biotech) and 1.0 µl of the target DNA, for a final volume of 25 µl. The PCR conditions in the thermal cycler (Veriti 96 Well Thermal Cycler-Applied Biosystems®) followed denaturation steps at 95ºC for 30 sec, and annealing temperatures that were tested between 54ºC and 61ºC for 40 sec and extension at 72ºC for 40 sec, repeated 35 times.

The PCR products were run on a 2% agarose gel (Ultra-pure agarose, BRL 155517-014) at 100 volts for 1 hour and 30 minutes, stained with GelRed and methylene blue to be viewed under ultraviolet light (Fluo-

Link, FlowGen) and photographed in a Kodak® Edas 290 photo documentation system.

PCR products were purified by the PureLink® PCR Purification kit (Invitrogen, Carlsbad USA), sequenced by the dideoxy chain termination method using the ABI PRISM Dye Terminator kit (Applied Biosystems) and analysed using a 3130 Genetic Analyser (Thermo Fisher, Santa Clara, CA) according to the manufacturer's instructions. Afterwards, the samples were assembled in the BioEdit Sequence Alignment Editor program (version 7.0.5, Borland Software Corporation), and the sequence quality was verified by Chromas Lite software (version 2.6.5, Technelysium Pty. Ltd., Tewantin, Queensland, Australia).

Results

The PCR standardization of the two AMPs of interest (Fig. 2–3) was described following the conditions in a thermocycler with annealing for 40 sec at 59ºC for CecA and 55ºC for defensin.

The proposed methodology allowed the detection of *An. darlingi* peptides from three different municipalities in the Brazilian Amazon. In addition, the samples showed good quality in relation to sequencing (Fig. 4).

Discussion

This is the first study involving the standardization of a molecular technique for detecting peptides from the innate immune system of the main vector of human malaria in Brazil, *An. darlingi*. The role of these AMPs in the defence against pathogens makes these peptides possible targets for molecular studies for the control of human malaria, whether in studies that aim to explore their mechanisms of action, genetic structure, or pattern of gene expression under certain environmental conditions or during infection[13,14].

We chose CecA and defensin AMPs because they are the main molecules of the mosquito immune system and have important activities against different pathogens, including *Plasmodium*[11,15,16]. Other studies have shown that cecropin and its derivatives have an action against pathogens such as *Trypanosoma*[24], *Leishmania*[25], *Candida albicans*[26] and *Brugia pahangi*[27]. In anopheline mosquitoes, these AMPs, especially CecA, have a strong influence on the primary immune response in both pupae and adults, acting against infection of the malaria parasite[11]. In addition, the levels of gene expression of members of the CecA and CecB cecropin families have the potential to reduce the number of *P. berghei* oocysts in the midgut of *An. gambiae by* up to 60%[15].

The defensin peptide also demonstrated an influence on *An. gambiae*, as evidenced by markers that showed high expression in the anterior midgut when females were fed blood infected with *P. berghei*, inducing a significant immune response in this vector[28]. Additionally, in *An. gambiae*, the antiparasitic activities of defensin showed an important role in the immune defence against sporozoites of *P. gallinaceum*[16].

The search for new tools that can help in malaria control or eradication is still a challenge for those who aim to protect populations at risk of acquiring malaria. The lack of a *Plasmodium* vaccine, the parasite's resistance to drugs and the appearance of insecticide-resistant mosquitoes show the priority of searching for new methods that can provide understanding and elucidation of the parasite-host relationship, i.e., the parasite's biological cycle[29].

In the case of *Anopheles* mosquitoes, the immune system tries to eliminate the parasites, mainly in the midgut and/or on the way to the salivary glands as a way to defend against invasion/infection by pathogens. Thus, effector molecules that are expressed in these locations, acting in defence of the host, can be important targets in controlling the parasite. In this context, the AMPs CecA and defensin can be used due to their fast action, solubility, and resistance to proteolytic digestion, in addition to being expressed in *Plasmodium* development sites, aiming for parasite elimination[11,30,31].

Previously, most studies on AMPs have been in *An. gambiae*, the main vector of human malaria on the African continent, which highlights the importance of studies on the species *An. darlingi*, the main vector of human malaria in the Brazilian Amazon region. The epidemiological importance of *An. darlingi* is related to its behavioural characteristics, susceptibility to human malaria parasites and adaptation. It was found that this species has genetic and even phenotypic variations within the same population and between different populations, mainly due to climatic (seasonal variation) and environmental factors (degradation of the environment and geographical barriers such as biomes) [32,33]. These issues are of concern for public health because the vector can maintain transmission in different regions in Brazil.

In this sense, the standardization of molecular techniques may assist studies exploring the functions of AMPs and their relationships with *Plasmodium* infection in the species *An. darlingi*, since AMPs are harmful molecules for a variety of microorganisms, causing lysis and cell death by integrating with the cell membrane of the pathogen[11,30,31,34].

Conclusion

For the first time, the standardization of a molecular technique for detecting AMPs was described for *An. darlingi*, a mosquito species from the Brazilian Amazon, supporting future studies aiming to understand the interactions of this species and the action of these peptides during an infection and providing important molecular markers for the control of human malaria.

Abbreviations

AMPs - Antimicrobial peptides

CecA - Cecropin A

PCR – Polimerase chain reaction

PA - Pará state

AC - Acre state

Declarations

AcknowledgEments

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AUTHOR'S CONTRIBUTIONS

EAS performed the technical methods and wrote the main manuscript text. LLCP and ACFS performed the technical methods. GMH, SMMC and ACRC performed the sequencing methods. EJMS revised the manuscript. IMCS and MMP performed the mosquitoes collection and revised the manuscript. All authors have read and approved the final version of the manuscript.

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AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare no conflict of interest.

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Figures

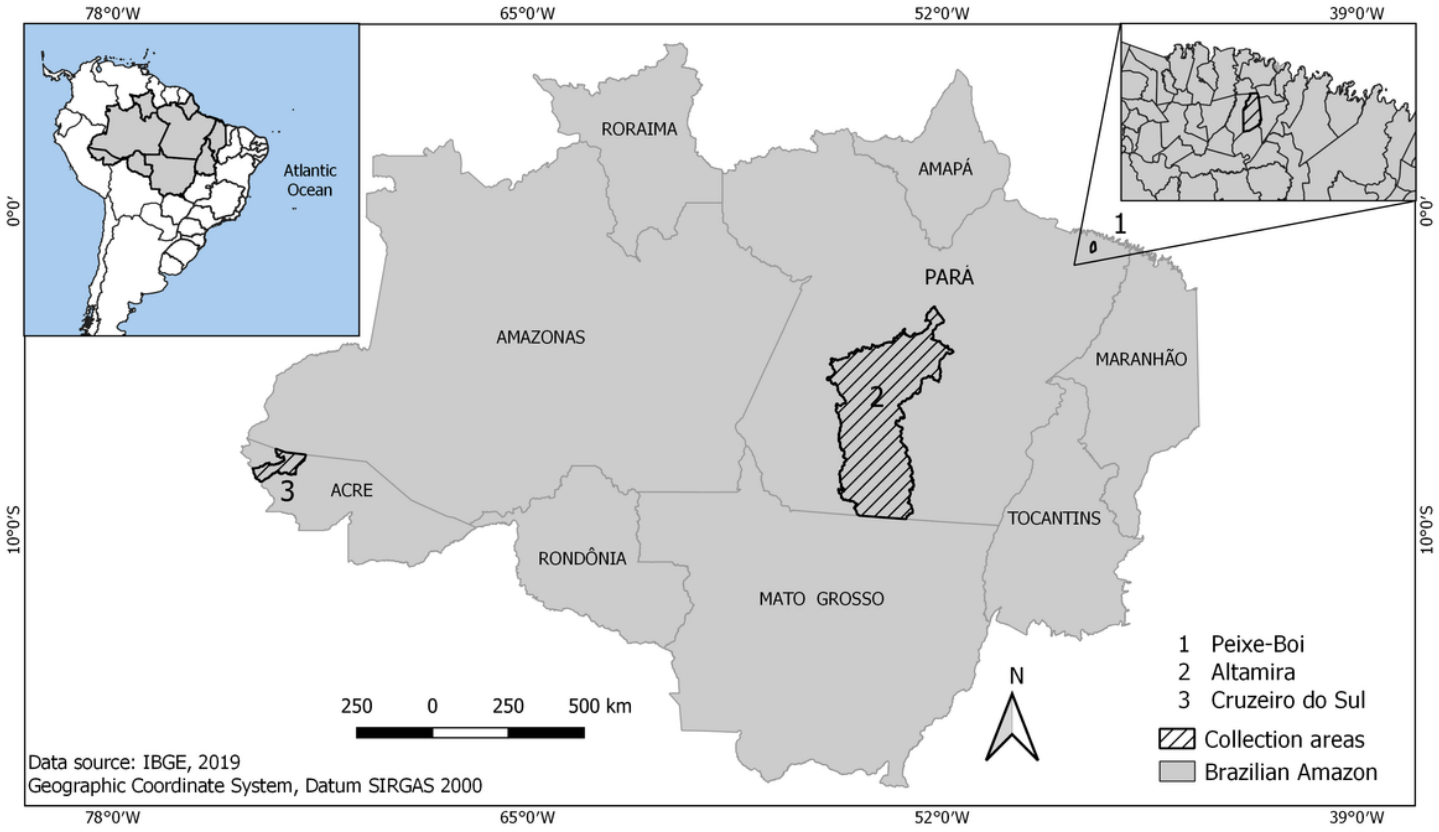

Figure 1

Anopheline mosquito collection areas in three municipalities in the Brazilian Amazon. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

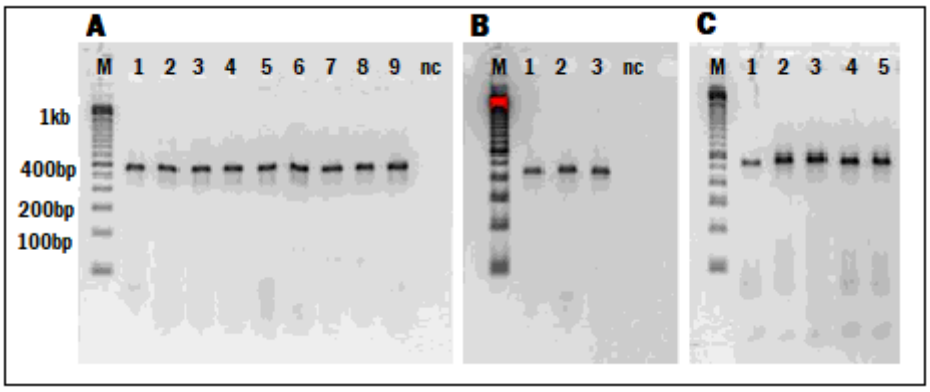

Figure 2

Photo of the 2% agarose gel showing the PCR products for the AMP CecA from An. darlingi (~ 434 bp). A - Samples from Peixe-Boi (PA); B - Samples from Altamira (PA); C – Samples from Cruzeiro do Sul (AC). (M) 100 bp molecular weight marker ladder, (nc) Negative control.

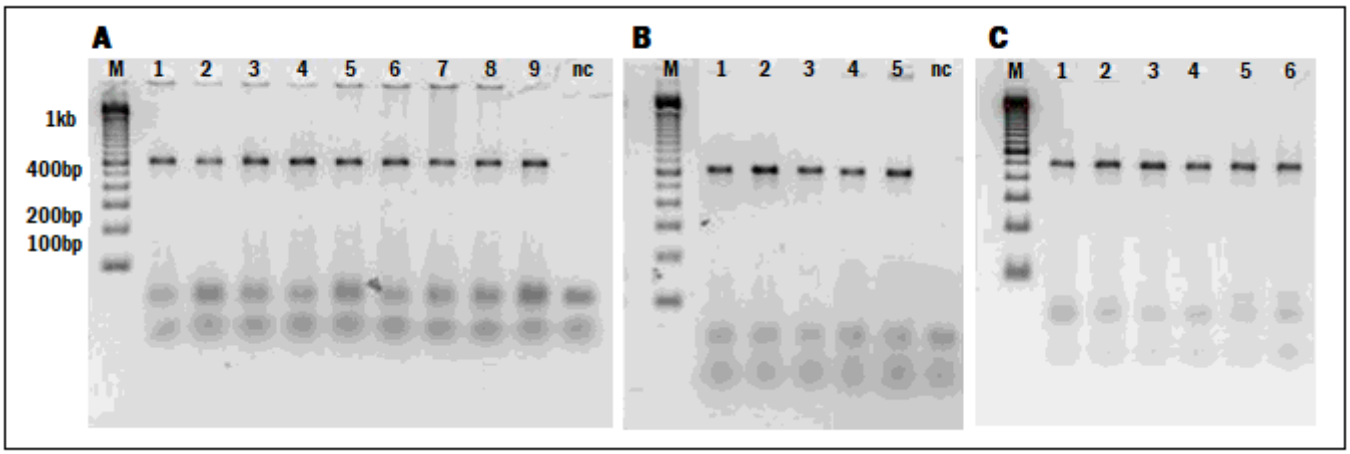

Figure 3

Image of the 2% agarose gel of the PCR products for the AMP defensin from An. darlingi (~ 497 bp). A - Samples from Peixe-Boi (PA); B - Samples from Altamira (PA); C – Samples from Cruzeiro do Sul (AC). (M) 100 bp molecular weight marker ladder, (nc) Negative control.

Figure 4

Visualization of the DNA sequence quality of the two AMPs of An. darlingi in the software Chromas. A – CecA; B - defensin.