Species-specific escape of *Plasmodium* sporozoites from oocysts of avian, rodent, and human malarial parasites

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Abstract

Background: Malaria is transmitted when an infected mosquito delivers *Plasmodium* sporozoites into a vertebrate host. There are many species of *Plasmodium* and, in general, the infection is host-specific. For example, *Plasmodium gallinaceum* is an avian parasite, while *Plasmodium berghei* infects mice. These two parasites have been extensively used as experimental models of malaria transmission. *Plasmodium falciparum* and *Plasmodium vivax* are the most important agents of human malaria, a life-threatening disease of global importance. To complete their life cycle, *Plasmodium* parasites must traverse the mosquito midgut and form an oocyst that will divide continuously. Mature oocysts release thousands of sporozoites into the mosquito haemolymph that must reach the salivary gland to infect a new vertebrate host. The current understanding of the biology of oocyst formation and sporozoite release is mostly based on experimental infections with *P. berghei*, and the conclusions are generalized to other *Plasmodium* species that infect humans without further morphological analyses.

Results: Here, it is described the microanatomy of sporozoite escape from oocysts of four *Plasmodium* species: the two laboratory models, *P. gallinaceum* and *P. berghei*, and the two main species that cause malaria in humans, *P. vivax* and *P. falciparum*. It was found that sporozoites have species-specific mechanisms of escape from the oocyst. The two model species of *Plasmodium* had a common mechanism, in which the oocyst wall breaks down before sporozoites emerge. In contrast, *P. vivax* and *P. falciparum* sporozoites show a dynamic escape mechanism from the oocyst via polarized propulsion.

Conclusions: This study demonstrated that *Plasmodium* species do not share a common mechanism of sporozoite escape, as previously thought, but show complex and species-specific mechanisms. In addition, the knowledge of this phenomenon in human *Plasmodium* can facilitate transmission-blocking studies and not those ones only based on the murine and avian models.

Keywords: Sporozoite escape, Oocyst, Mosquito vector, *Plasmodium*, Human, Murine, Avian

Background

Malaria remains a life-threatening disease that threatens approximately 3.4 billion people in 104 tropical countries, mainly in Africa, Asia, and South America, with an estimated 207 million cases and half a million deaths reported per year [1]. This vector-borne disease is caused by protozoa of the genus *Plasmodium*, of which *Plasmodium falciparum*, endemic to Africa, is the most prevalent species, followed by *Plasmodium vivax* in Asia and the Americas [1]. Other *Plasmodium* species infect other animal species, such as *Plasmodium gallinaceum* and *Plasmodium berghei*, responsible for avian and murine...
malaria, respectively [2, 3]. Many experimental studies have used P. berghei and P. gallinaceum as laboratory models to investigate the interactions between the parasites and their vectors. These two Plasmodium species are easily maintained in experimental animals, facilitating investigative research in laboratories [4–8].

The Plasmodium life cycle begins in a permissive vector when a female mosquito takes a blood meal from an infected vertebrate host that contains gametocytes, the stage of the parasite that can infect the invertebrate vector. Only a few minutes after the infective blood meal enters the midgut lumen of the susceptible mosquito, these gametocytes undergo activation to generate micro- and macro-gametes that fertilize to produce a diploid zygote. After DNA replication and the production of a 4N parasite, the zygote will differentiate into an ookinete over the next 18–24 h depending on the respective parasite species. Ookinetes are a motile form of the parasite that invade and pass through the midgut epithelium until they reach the midgut basal lamina towards the haemocoel of the mosquito. At this location, between the epithelial cells of the midgut and the basal lamina, the ookinete differentiates into a protruding rounded oocyst facing the mosquito haemocoel [8–12]. The presence of well-developed protruding oocysts in the midgut wall is indicative of infection by Plasmodium [13–15], and is a reliable measurement to determine the infection rate and the susceptibility of a mosquito species to a particular Plasmodium species. In the midgut wall, the oocysts progress to the asexual phase of multiplication known as sporogony, which is completed in approximately 1–2 weeks, the longest phase of the Plasmodium life cycle in the mosquito vector. Ultimately, this biological process produces thousands of sporozoites, the final form of Plasmodium in the vector. The sporozoites are motile sickle forms that escape from the oocysts into the mosquito hemocoel and invade the salivary gland. Once inside the salivary gland, the sporozoites are ready to be injected into a new vertebrate host via a mosquito bite, completing the Plasmodium life cycle in the invertebrate vector [16–18].

Completion of the Plasmodium life cycle in the vector requires passage through several barriers inside and outside the midgut. One important and poorly studied barrier is the exit of sporozoites from the oocyst, a critical step that allows sporozoite release into the haemolymph and subsequent invasion of the mosquito salivary gland. Knowledge of the escape mechanism of various Plasmodium species is largely unknown for the human malaria parasites, and only a few reports using the laboratory models have previously been published. Studies of the development of P. berghei oocysts using a scanning electron microscope (SEM) showed a single small hole in the oocyst wall, inside which sporozoites could be seen [19]. Sinden and Strong reported a torn oocyst from which several P. falciparum sporozoites had been released [20]. Meis and collaborators studying the sporogony of P. falciparum and P. berghei, reported some details of sporozoite escape and concluded that the two species showed similar mechanisms of escape, i.e., the oocysts burst and sporozoites were released into the hemocoel of the mosquito vector [21]. Although published studies have provided some details, knowledge of sporozoite escape from the oocysts of distinct Plasmodium species remains incomplete and is primarily based on P. berghei, a classical murine malarial parasite used as an experimental model in several laboratories. Moreover, most of the studies on the molecular mechanism of oocyst formation and sporozoite escape have been done using murine P. berghei mutant parasites, resulting in conclusions that have been generalized to human Plasmodium species without further morphological study.

Understanding the mechanisms of sporozoite escape in various Plasmodium species as well as correlations with molecular findings, may contribute to our knowledge of the parasite life cycle in the mosquito vector. Scanning electron microscopy analysis of the external side of the dissected midguts of infected mosquitoes is a valuable tool for studying sporozoite escape from oocysts and has not been well explored. Here, this study provides comprehensive insight into the microanatomy of the mechanism of sporozoite escape from oocysts in four species of Plasmodium: the two laboratory models, avian P. gallinaceum and rodent P. berghei, and the two primary causative agents of human malaria, P. vivax and P. falciparum. It was showed that sporozoite escape is not a common biological process, as previously thought, but the mechanism is complex and species-specific.

Methods
Mosquito rearing
Mosquitoes of Anopheles gambiae, Anopheles aquasalis and Aedes aegypti were reared at 27 °C with 80 % humidity on 12 h light/dark cycle under insectary conditions. They were provided with 10 % sucrose solution ad libitum until 1 day before the infective blood meal, as described previously [8, 15].

Infection of mosquitoes with Plasmodium
Susceptible female mosquitoes (4–5 days old) were chosen to be experimentally infected with one of the four Plasmodium species through a membrane feeder device at 37 °C for 30 min, as described previously [8]. Anopheles gambiae were infected with stage IV and V gametocytes of the cultured P. falciparum NF54 strain. The mature gametocytes were mixed with type O2 blood and
offered to the mosquitoes [22–24]. Anopheles gambiae were also infected with *P. berghei* by direct skin feeding on infected Swiss Webster female mice with a parasitaemia level of 4–8 % and containing 2–3 gametocyte exflagellations per field when observed at 400× under a light microscope. *Aedes aegypti* were infected with *P. gallinaceum* by direct skin feeding on an infected chicken (*Gallus domesticus*) with a 10 % parasitaemia level and at least 2 % circulating gametocytes [25]. *Anopheles aquasalis* were fed on *P. vivax*-infected blood collected from patients diagnosed with malaria, as described in the Ethics statement.

**Ethics statement**
For the acquisition of *P. vivax* infected human blood, patients were selected among the people visiting the Hospital at the Foundation of Tropical Medicine located in Manaus, Brazil looking for malaria diagnosis and treatment during outbreaks. Diagnosis was performed by Giemsa stained blood smear. After positive diagnosis and visualization of gametocytes, patients were interviewed and inquired about the possibility of volunteer donation of a small amount of blood for research purposes. After verbal agreement, a term of consent was first read to the potential volunteers, with detailed verbal explanation, and, after final consent, signed by the patient. After this, one 200 ml sample of venous blood was drawn from each patient and placed in heparinized tubes. Blood samples were kept under refrigeration in an icebox (at approximately 15 °C) for about 15 min, taken to the laboratory. The infected *P. vivax* blood samples were offered to mosquitoes through membrane feeder devices. Patient selection criteria were: to be *P. vivax* positive, to have about 4–8 % of circulating gametocytes as determined by the National Institutes of Health international protocols, and to consent to be part of the research consent form that was approved by the Brazilian Ministry of Health, National Council of Health, National Committee of Ethics in Research (CONEP—Approval Number 3726). All patients were treated in accordance with the Brazilian Malaria National Control Programme guidelines.

Also, mice and chickens were maintained at the Animal Care Facility of the FIOCRUZ-MG under specific pathogen-free conditions and were used in accordance to a study protocol approved by the FIOCRUZ Ethical Committee for Animal Use (CEU; license number LW30/10). It was followed the Public Health Service Animal Welfare Assurance #A4149-01 guidelines according to the National Institutes of Health (NIH) Office of Animal Care and Use (OACU) since these studies were done according to the NIH animal study protocol (ASP) approved by the NIH Animal Care and User Committee (ACUC), with approval ID ASP-LMVR5.

**Scanning electron microscopy of infected mosquito midgut**
The mosquito midguts were dissected daily, from day 8 to day 16 after the infective blood meal. The dissected midguts were fixed for 2 h in 4 % glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.2 and then post-fixed with 1 % osmium tetroxide for 2 h. The fixed samples were dehydrated using a graded acetone series, CO₂-dried in a critical-point drying device (Emitech K850, USA) and gold-coated in a sputter coater (Emitech K550, USA) as detailed previously [26]. The samples were analyzed and imaged using a JSM-5600 scanning electron microscope (Jeol USA, Inc).

**Results**
Careful comparative SEM analyses of infected midguts dissected from susceptible mosquito vectors, containing distinct *Plasmodium* species, revealed several new details of the oocyst surface and the sporozoite escape process that are unique to each *Plasmodium* species.

**Escape of Plasmodium gallinaceum sporozoites from oocysts**
Magnification of dissected midguts showed hundreds of rounded avian *P. gallinaceum* oocysts on the midgut surface of the infected *Ae. aegypti*. Most of the oocysts formed small groups on the midgut surface (Fig. 1a, b). Flattened oocysts and completely smooth oocysts were observed side by side, some with haemocytes attached to the surface (Fig. 1c). On the 14th day after infection, it was possible to observe sporozoites escaping from oocysts in the dissected midguts. These dissected midguts were carefully scrutinized for the presence of oocysts, in order to observe the details of sporozoite escape. Several cracked oocysts of *P. gallinaceum* were observed at distinct stages, from some with small cracks in the surface, to some that were completely broken, exposing hundreds of escaping sporozoites (Fig. 1d–f). The completely cracked oocysts liberated thousands of sporozoites into the mosquito haemocoel (Fig. 1d, e). In empty oocyst shells, it was possible to observe the porous surface of the internal side of the oocyst wall (Fig. 1e, f).

**Escape of Plasmodium berghei sporozoites from oocysts**
At 13 and 14 days after infection of *An. gambiae* with *P. berghei*, several oocysts were observed to be protruding between the muscle fibers covering the midgut surface, at different stages of rupture (Fig. 2a–d). The upper surface of these oocysts was wrinkled, and the basal surface, inserted in the midgut tissue, was smooth; it was also possible to observe some flattened oocysts (Fig. 2a). In most of the oocysts, the wall showed distinct stages of “decortication” until the sporozoites were liberated. This
holes where sporozoites had escaped, were occasionally observed (Fig. 3g), along with some undeveloped flattened oocysts immediately adjacent to completely smooth oocysts (Fig. 3g).

**Escape of Plasmodium falciparum sporozoites from oocysts**

The dissected midgut sections of the infected *An. gambiensis* revealed approximately ten to a few hundred *P. falciparum* oocysts (data not shown), most of them of similar size, located on the midgut surface. Some oocysts protruded, isolated or in groups of 4–6 individuals, on the basal midgut surface. *Plasmodium falciparum* oocysts could be classified into two distinct types according their surface: completely smooth and wrinkled surfaces (Fig. 4a, b). At 14 days after infection, detailed analysis revealed the initial process of a single sporozoite actively escaping through a unique hole, always from a completely smooth oocyst. These escaping single sporozoites also presented the “pointing finger” shape similar to those seen with *P. vivax*, leading with the anterior tip (Fig. 4c). At 13 and 14 days after infection some completely smooth oocysts showed small broken areas from which a few sporozoites were escaping. These oocysts were beginning to show folded areas on the surface (Fig. 4d). During *P. falciparum* sporozoite escape, it was possible to observe a flattened oocyst with a lateral opening, showing a cluster of escaping sporozoites inside (Fig. 4e, f). Notably, only completely smooth oocysts appeared to produce escaping *P. falciparum* sporozoites, and were never observed escaping from wrinkled oocysts.
The Table 1 shows the proportion of the distinct Plasmodium oocysts according to their main microanatomical aspects of their surfaces, as described in details above.

**Discussion**

The longest developmental stage of the Plasmodium life cycle in the mosquito vector is sporogony, the process of formation of thousands of sporozoites. A single parasite invades the epithelium of the midgut of a mosquito vector and remains in the gut wall for several days. This single-celled protozoan remains outside the mosquito cells, and grows into a large-lobed syncytial nucleus by mitotic division, inside a structure named the oocyst, which forms mature sporozoites. These mature sporozoites escape from the oocysts into the mosquito cavity, after which they invade the salivary gland in preparation for injection in a new vector. The duration of this stage of the Plasmodium life cycle varies according to the species, but usually lasts 8–14 days after the mosquito vector has ingested the infective blood meal [7–9, 13, 27].

In this study, to examine the microanatomy of sporozoite escape from oocysts of the four Plasmodium species, 10–20 midgut sections were dissected daily from infected mosquito vectors, 6–16 days after the infective blood meal. The midgut samples were dissected, fixed, and processed in the same laboratory, following an identical rigorous protocol to facilitate comparative analyses. The microanatomical analyses presented here clearly and accurately show the ultrastructural aspects of the oocyst surfaces and the processes of sporozoite escape. Preliminary analyses revealed that in all Plasmodium species, the oocysts are rounded structures that protrude individually or in small groups from the exterior of the midgut wall of the mosquito vector. However, the oocysts of the four Plasmodium species differ in surface features of the external wall and in the process of sporozoite escape.

In the avian parasite *P. gallinaceum*, the outer surfaces of all oocysts were completely smooth. During the process of sporozoite escape, *P. gallinaceum* oocysts were cracked, suggestive of internal forces disrupting the oocyst wall from the inside. The broken oocysts were similar to broken eggs, exposing their internal surface, with subsequent release of large groups of sporozoites into the mosquito cavity. In contrast, both murine *P. berghei* oocysts showed a hybrid surface, wrinkled on the top and smooth on the base. Compared to *P. gallinaceum*, *P. berghei* sporozoites appear to have a less violent mechanism of escape from the oocysts. On the upper, wrinkled surface of the oocysts, a small part of the wall begins to decorticate, creating a small opening, followed by progressive dissolution of the oocyst wall. Then, the highly structured clusters of sporozoites detach from the internal oocyst wall. In the murine and avian species of Plasmodium, the final steps of the sporozoite escape process, no empty oocysts were observed, distinct from the species of Plasmodium that infect humans. Only one comparative study of *P. gallinaceum* and *P. berghei* oocysts has been published [19]. In both Plasmodium species, both completely smooth
oocysts and rare, wrinkled oocysts were observed, which
the authors considered matured oocysts or sample prepa-
ration artifacts. Although they only showed two images,
they suggested these two Plasmodium species have simi-
lar sporozoite escape mechanisms.

All P. vivax oocysts showed similar completely smooth
surfaces, and in this respect, they are morphologically
similar to P. gallinaceum oocysts. In contrast, two types
of P. falciparum oocysts were observed: completely
smooth and wrinkled oocysts. These oocysts were ran-
domly distributed, sometimes side-by-side, in the mos-
quito midgut at a 50:50 ratio. Previous studies found that
infected P. falciparum mosquitoes contained only wrink-
led oocysts, but no escaping sporozoites were observed
[20, 21]. The authors suggested that the wrinkled sur-
face was characteristic of mature oocysts. However,
although we also observed two types of oocysts in P. falci-
parum, sporozoites were only observed escaping from
completely smooth oocysts, indicating that completely
smooth oocysts contain mature sporozoites. The wrink-
led oocysts may be immature oocysts or oocytes that
cannot produce healthy, mature sporozoites.

The most noteworthy feature of the two human Plas-
modium species, P. vivax and P. falciparum, is the
dynamic mechanism of sporozoite escape from oocysts,
distinct from that of the laboratory model Plasmodium
species. Careful observation showed that the first sig-
nals of sporozoite escape are identical for the two human
Plasmodium species: escape begins with a single sporo-
zoite, in a rigid perpendicular position, forcing an exit
from through the oocyst wall. The rigid perpendicular
sporozoite opens a tiny hole in the oocyst wall with its
anterior end. The oocyst wall is composed of two layers;
the internal layer is of Plasmodium origin and the exter-
nal thick layer that is derived from the basal lamina of
the mosquito midgut [28, 29]. Moreover, in addition to
allowing for growth, the capsule must have an ordered
structure to allow for precursors and nutrients that sup-
port parasite growth and differentiation to enter the
oocyst and metabolites to exit it [30, 31]. Subsequently,
this tiny hole in the oocyst wall grows larger and allows
other sporozoites to escape. Although this first step, with
a single sporozoite making a tiny hole in the oocyst wall,
is identical between the two species, the subsequent steps
of sporozoite escape differ between P. vivax and P. falci-
parum. In P. vivax, a small group of sporozoites continue,
in the same rigid perpendicular position as the first, to
actively move forward to enlarge the hole in the oocyst
wall. In P. falciparum oocysts, small groups of sporo-
zoites escape, and individual sporozoites are flexible
comma shapes, characteristic of random motion of the
parasite [32–34]. A geometrical model of malaria parasite
migration demonstrated that sporozoites could be mod-
eled as self-propelled individuals that can have curved or
rigid structures for motion in distinct environments [35].
This programmed rigidity and flexibility of the human
Plasmodium sporozoites appears to act distinctly in the
two species of Plasmodium, since it plays a role in open-
ing the oocyst wall, allowing escape.

Molecular mechanisms related to oocyst formation and
sporozoite escape have been demonstrated, mainly using
mutants of murine P. berghei, which infects rodents,
but not in Plasmodium species that infect humans. It is
important to note that these analyses demonstrate that P.
berghei sporozoites escape from oocysts by a process that
harms the oocyst wall. The circumsporozoite (CS) pro-
tein, secreted by sporozoites, covers the internal layer of
the oocyst wall [36, 37]. It was demonstrated in P. berghei
that the disruption or deletion of some regions of the CS
protein affects the formation and maturation of sporozo-
ites, escape from the oocyst, and subsequent progression
of the Plasmodium life cycle [38, 39]. Likewise, several
other gene deletions have been described that affect P.
berghei oocyst formation and consequent sporozoite

Table 1 Proportion of the oocysts according to their surface microanatomical details

|                | P. gallinaceum (n = 138) | P. berghei (n = 325) | P. vivax (n = 160) | P. falciparum (n = 162) |
|----------------|--------------------------|----------------------|--------------------|------------------------|
| Flattened      | 7.9 %                    | 2.1 %                | 11.8 %             | 6.8 %                  |
| Smooth         | 83.4 %                   | 20 %                 | 85 %               | 24.8 %                 |
| Wrinkled       | –                        | –                    | –                  | 66.4 %                 |
| Wrinkled + smooth* | –                     | 58 %                 | –                  | –                      |
| Cracked        | 8.7 %                    | –                    | –                  | –                      |
| Small openingsb | –                        | 15.6 %               | 7.5 %              | 2.4 %                  |
| Large openingsb | 8.7 %                   | 4 %                  | 7.5 %              | –                      |

* Oocyst aspects not present in the Plasmodium species

b Wrinkled/smooth oocysts present both characteristic aspects in their surface

Small openings or large openings by our definition was related with small or large fissures considering 1/3 of the oocyst surface

\[ n = 138 \]

\[ n = 325 \]

\[ n = 160 \]

\[ n = 162 \]
escape: an oocyst-specific papain-like cysteine protease, known as the egress cysteine protease (ECP1), oocyst capsule protein (PbCAP380), fertilization gene (PbGEX), lectin adhesive proteins (PbLAPs), protein kinases (PbCDLK), and nuclear forming-like protein (PbMISFIT) [40–48]. The results showed that P. berghei sporozoites are liberated from the oocyst by decortication and subsequent dissolution of the oocyst wall, which is consistent with a mechanism involving a proteolytic activity as has been proposed for P. berghei [42]. Thus, these findings indicate that proteins that act on the oocyst wall, rather than in the sporozoite, should be considered as target candidate molecules to stop transmission.

Analyses of the sporozoite escape processes in the Plasmodium species that infect humans clearly showed the action of the actively protruding sporozoites is dissimilar from that of murine and avian Plasmodium species. Plasmodium belongs to the phylum Apicomplexa, which is well defined by polarized extracellular stages, which contain specialized secretory organelles named micronemes and rhoptries in their anterior edge. Proteins secreted by these organelles play essential roles in attachment and invasion of target cells, as well as gliding motility, locomotion, and morphological changes [33, 49–52]. The main mode of active locomotion of the sporozoite is an actomyosin-dependent motility that is important for forward locomotion, and penetration and invasion of target cells [53]. In addition, during sporozoite motility, TRAP may coordinate the formation of contact sites and the dissociation of these contact sites from the substrate, including involvement of actin filaments [54, 55]. This raises the possibility that secretory proteins that are involved in the interplay of adhesion molecules and the invasion mechanism, well studied in invasion of host cells, can also play roles in the initial active stage that guides the escape of P. vivax and P. falciparum from the oocyst.

Careful comparative microanatomical analyses of midguts of mosquitoes infected with four distinct Plasmodium species allowed us to make novel observations of sporozoite escape from oocysts. The key findings of this study are the morphological features that reveal for the first time the mechanisms of sporozoite escape from oocysts of four Plasmodium species, including avian, murine, and human malarial parasites. Sporozoites of the four Plasmodium species exit oocysts using different mechanisms. The avian P. gallinaceum and murine P. berghei have been used as experimental models in several laboratories for infection of vertebrates and mosquito vectors. Mice infected with P. berghei have been used as laboratory models for human malaria [56–58] and to investigate interaction of the parasite with vectors of human malaria such as An. gambiae and An. stephensi [59–61]. It is important to state that the findings of the escape of P. berghei and P. falciparum sporozoites from oocysts were obtained from experimental infections of the same mosquito species, the An. gambiae. This fact suggests that the distinct mechanisms of the sporozoite escape is not dependent of the Anopheles species but is regulated by the Plasmodium species. Nevertheless, it is noteworthy to consider that these Plasmodium species differ in the oocyst microanatomical appearance and in the process of the sporozoite escape. Although the molecular mechanism that regulates sporozoite escape remains largely unknown, this study clearly indicates that Plasmodium species do not share a common mechanism, as previously thought.

Conclusions

It was demonstrated that sporozoites of the human malarial parasites P. vivax and P. falciparum escape from the oocyst via a more active process than those of the avian and murine malarial parasites, P. gallinaceum and P. berghei. Detailed analysis showed that all four have distinct escape mechanisms. Sporozoites that infect humans actively create a hole in the oocyst wall, and are not dependent on the breakdown or dissolution of the oocyst wall for escape. These findings provide a strong basis for future studies of how to block sporozoite escape from oocysts in order to prevent transmission of malaria.

Authors’ contributions

ASO, RNP, LCP, KMMC, YTP, BC, RCS carried out the infection experiments of the mosquitoes, participated in the microscopy analysis and drafted the manuscript. LMV, APMD, NBR, MGVB, WMM, AMC participated in the design of the study, performed the analysis and helped to draft the manuscript. PFPP conceived the study, in its design and coordination and write the manuscript. All authors read and approved the final manuscript.

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ASO and RNP are Ph.D. students of the Graduate Program in Health Sciences of the FIOCRUZ-Minas Gerais. KMMC, YTP and BC are Ph.D. students of the Graduate Program in Tropical Medicine of the UEA/FMT-HVD. APMD, LMV, NBR and LCP are pos-doctoral fellows. PFPP is a senior visiting fellow at the FMT-HVD supported by Amazonas State Research Support Foundation (FAPEAM). PFPP, NFCS, MGVB and MVGL are senior fellows from the Brazilian Council for Scientific and Technological Development (CNPq).

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Competing interests
The authors declare that they have no competing interests.

Availability of data and material
All data will be made available upon request to all interested researchers.

Consent for publication
All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
Patient selection criteria were determined by the National Institutes of Health international protocols, and to consent to be part of the research consent form that was approved by the Brazilian Ministry of Health, National Council of Health, National Committee of Ethics in Research (CONEP—approval number 3726).

Also, mice and chickens were maintained at the Animal Care Facility of the FIOCRUZ-MG were used in accordance to a study protocol approved by the FIOCRUZ Ethical Committee for Animal Use (CEUA, License Number LW30/10). It was followed the Public Health Service Animal Welfare Assurance #A4149-01 guidelines according to the National Institutes of Health (NIH) Office of Animal Care and Use (OACU) and NIH animal study protocol (ASP) approved by the NIH Animal Care and User Committee (ACUC), with approval ID ASP-LMVRS.

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