Stuck in the Throat: Dissecting the Role of the Leishmania Biofilm in Parasite Development and Transmission

Introduction:

Leishmaniasis, caused by *Leishmania spp* parasites, poses a significant public health threat in various regions as a vector-borne disease. Depending on the species of *Leishmania,* the disease can take on three forms, Cutaneous which primarily causes skin lesions, Muco-cutaneous which affects mucosal membranes and Visceral which is the most severe form of the disease. The transmission dynamics involve a crucial interaction between Leishmania and its sandfly vector. Briefly, upon taking an infected bloodmeal, the ingested amastigotes undergo development in the sandfly midgut, where they become procyclic promastigotes, then metacyclic promastigotes. During this development the parasites will migrate towards the midgut and then the proboscis where it is ready to be transmitted. It is during the later stages of development that leads to the formation of a glycan-rich biofilm, often referred to as Promastigote Secretory Gel (PSG). This biofilm plays a pivotal role in safeguarding and regurgitating infective metacyclic forms into the vertebrate host. Notably, PSG is believed to influence sandfly biting behaviour, inducing multiple probes and incomplete feeds due to its accumulation in the midgut lumen, consequently increasing the risk of further infections [(1)]. The presence and function of Proteophophoglycans (PPGs) in the PSG is only just being understood, notably studies have shown that the main effects of PPGs are protective, and therefore allow establishment in the sandfly and further infection into a definitive host. In essence, this behaves in a similar fashion to *Yersinia spp*, the plague causing bacteria. A drop in temperature that is detected by the bacteria following the fleas bloodmeal stimulates the production of an Extracellular Matrix. This matrix, similar to the PSG of *Leishmania spp* is rich in polysaccharides and functions to protect the bacteria from the flea immune response as well as increase transmission in a similar fashion to sandflies and *Leishmania*, i.e Blocked fly theory [(2)] whereby the biofilm blocks the flea proventriculus, causing an incomplete bloodmeal and multiple bites.

Additionally, observations have indicated that C-Reactive Protein (CRP) found in human, rat, and mouse sera exhibits a strong avidity for specific PPGs such as filamentous proteophosphoglycan (fPPG) and Secreted Acid Phosphatase (ScAP) [(3)]. The binding of CRP to these ligands, along with lipophosphoglycan (LPG), triggers activation and subsequent depletion of the immune complement system. This raises questions as to whether PPGs also play a role in influencing a hosts immune response.
This research project is designed to unravel the developmental, biophysical, and biochemical intricacies of the PSG biofilm, with a specific focus on understanding the roles played by PPGs and their interactions with sand fly physiology and CRP. The investigation aims to shed light on the mechanisms underlying biofilm formation, sandfly biting behaviour modulation and the impact of CRP on the physiological and Biochemical relationship of PSG and the sand fly. By delving into these aspects, the study seeks to contribute valuable insights that could inform targeted interventions and innovative strategies for disrupting the transmission cycle of Leishmania parasites.

Objectives:

The primary objectives of this research are as follows:

Development in sand flies:

Investigate the impact of specific PPGs on Leishmania development in sand flies. Assess midgut attachment, migration in the biofilm, valve attachment, and metacyclogenesis.

Transmission from sand flies:

Examine the relationship between biofilm composition and infectious dose parameters (parasite number, promastigote forms, and co-regurgitated biofilm). Explore the influence of biofilm biogenesis on the blood feeding behaviour of the sand fly vector.

Distribution and ultrastructural arrangement in the biofilm:

Track parasite distribution within the biofilm using advanced microscopy techniques. Employ serial block face electron microscopy to explore the in situ ultrastructure of the interaction between Leishmania and the biofilm.

Investigate CRP modification:

Explore how C-reactive protein (CRP) modifies biofilm features when infected sand flies take a second bloodmeal. Investigate CRP's potential to alter biofilm and parasite distribution, influencing transmission dynamics.

Methodology:

Assessing parasite development and transmission.

The Cre-Lox mediated deletion method will be utilised to generate mutants lacking specific Proteophosphoglycans (PPGs). With experience in design and implementation of qPCR, I can employ this assay technique to confirm gene knockout in mutant strains. These mutants, along with their complemented strains, will undergo evaluation both *in vivo* using the sandfly model and *in vitro* through parasite culture. Prior experience with bacterial, fungal and mammalian cell cultures will help with the continuous successful culturing of parasites under aseptic conditions, thus reducing the chance of contaminants impacting results.

In the *in vivo* setting, sandflies will be infected with the modified strains, and their behaviour will be compared to those infected with unmodified strains. This will demonstrate what effect each investigated PPG has on the development of the parasite as well as the behaviour in the sand fly. In order to accomplish this, samples will be dissected at various time points and the PSG subjected to Biochemical assays such as ELISA as well as microscopy. To assess midgut attachment, migration in the biofilm, valve attachment, and metacyclogenesis, various microscopy techniques, including light confocal, and fluorescence microscopy will be employed alongside any required staining such a Giemsa. Upon infection with blood containing these parasites, sandflies will be maintained using a sugar solution. They will then periodically undergo dissection to expose parasites at the various developmental stages. The dissection will also allow for the visualisation of any physiological changes that PPGs may cause to the sand fly as well as allowing for other assays to be carried out, for example ELISA, western blot, SDS PAGE and qPCR. The techniques mentioned above will enable the observation of structures necessary for parasitic attachment to the midgut and stomodeal valve. Additionally, this approach will facilitate the monitoring of the attachment process under different conditions, shedding light on the factors driving attachment and conditions that may inhibit it, such as the reduction or absence of specific PPGs or a calcium depleted environment [(4)]. This multifaceted approach looking at modified and wildtype strains will allow us to compare phenotypic changes that occur as the parasites develop, thus allowing us to make links between various PPGs and the biochemical effects on the parasite development within the PSG and sand fly, such as whether the absence of PPGs will affect sandfly ability to survive and escape the peritrophic matrix.

Fluorescent and light microscopy will provide visualisation of both modified and unmodified parasites during various developmental stages, allowing for the tracking of parasite behaviour within the sandfly and biofilm. This microscopy will also offer insights into how the

presence of CRP impacts parasitic load and survival post-ingestion after a sandfly bite. Moreover, parasitic load within the sand fly can be tracked this way, with the various life stages being tracked in addition to parasite numbers (Fluorescence Assisted Cell Sorting, (FACS), can be used to confirm parasite numbers). Feeding behaviour can be assessed before dissection and analysis to determine a link between sand fly behaviour and parasite load comparing mutated strains to their wild type counterparts, thus providing further evidence on the importance of understanding PPGs.

Assessing distribution and ultrastructure

Building on this, the use of serial block face scanning electron microscopy (SBFSEM), will be employed. This is a novel technique used to generate highly accurate 3D image slices of a sample. As the slices used in this technique are small (25 nm), this will allow for detailed understanding of the interactions between the different PPGs found in the PSG and the midgut/stomodeal valve within the sandfly. Additionally, the use of SBFSEM will allow detailed investigation into the different developmental stages within the sandfly, and therefore how PPGs influence these stages. With this method, further investigations into parasite density can be looked at in greater detail, shedding light on how parasitaemia could influence sand fly behaviour. Additional investigations using SBFSEM as well as biochemical techniques can shed light on the mechanism of attachment and can confirm work seen before whereby it was hypothesised that the location of attachment could be indicative of different developmental stages [(3)].

CRP and Biofilm modification

In parallel, *in vitro* experiments will be conducted using parasite culture to investigate the impact of various PPGs on biofilm formation and host infection. Previous research has indicated that parasites in culture form aggregates with fibrous material at the centre. These aggregates can be purified and tested using western blot, Gel electrophoresis and ELISA to confirm presence of PPGs. These can then be added to PSG deficient in those PPGs. This process aims to determine whether PPGs have a protective effect on parasites and whether the addition of C-reactive protein (CRP) during the developmental stages influences parasite and biofilm development *in vitro*. Upon a second bloodmeal, it has been observed that metacyclics undergo a process called reverse metacyclogenesis, whereby metacyclic promastigotes become prolific promastigotes, thus allowing for amplification of parasite numbers [(5)]. While this has been observed in an uninfected bloodmeal, it would be prudent to see if the effect remains true with an infected bloodmeal, and therefore, whether this will impact how infective progressive bloodmeals become. Furthermore, the effect of CRP on parasite survival after a second bloodmeal, especially an infected bloodmeal, can be assessed as it is likely higher levels of CRP will be found in infected blood.

Biochemical techniques like ELISA, western blot, and Surface Plasmon Resonance will be utilised to investigate the impact of CRP on parasite survival following ingestion, drawing upon my experience in these methods. By assessing mutant strains alongside their complemented strains, the research aims to elucidate whether the removal of PPGs adversely affects parasite survival in the presence of CRP within the sandfly. This integrated approach involving molecular, microscopic, and serological techniques holds promise for a comprehensive understanding of the intricate dynamics between PPGs, CRP, and Leishmania survival throughout its life cycle.

Expanding on these concepts, it would be valuable to explore the effects across multiple Leishmania species, considering that some species share the same PPGs, but not all produce PPGs that react with CRP.

Significance and Expected Outcomes:

This research will provide crucial insights into the molecular mechanisms governing *Leishmania* biofilm biogenesis and its impact on transmission through sandflies. Understanding the role of specific PPGs and their effect on the parasite lifecycle, both in the sandfly and in the host will be crucial to the development of methods we can employ to interrupt transmission. Furthermore, the interplay with CRP in shaping the biofilm will contribute to the development of targeted interventions to further disrupt the transmission cycle, potentially offering new avenues for Leishmaniasis control. This understanding could pave the way towards the development of a vaccine against all forms of *Leishmania* infection.

Furthermore, should CRP have an impact on parasite survival, it would be worth considering whether this can have an immunomodulating effect post injection to a host. Expanding on this, as CRP is a important molecule in the complementary immune system, the interplay of other cytokines on parasite survival would be interesting, for example, how do the different PPGs impact Interferon gamma (IFN γ). This data could lead to significant strides towards designing an effective vaccine.

In essence, this research not only contributes to our understanding of the complex biology underlying *Leishmania* transmission but also holds the potential to translate this knowledge into tangible solutions. From targeted interventions disrupting the transmission cycle to the prospect of a broadly effective vaccine, the outcomes of this study could mark a significant stride towards comprehensive control and prevention strategies for Leishmaniasis.

Conclusion:

This research holds the potential to unveil novel aspects of vector-parasite interactions. Through the utilisation of both established and innovative methodologies, an in-depth exploration of various stages of parasite development will be undertaken. This investigation aims to explain the impact of the parasite on the sand fly, considering both physiological and behavioural aspects, and subsequently examining its repercussions on transmission. By delving into fundamental questions regarding biofilm integrity and its modulation by PPGs and CRP, this research has the potential to lay the groundwork for pioneering strategies in the fight against leishmaniasis transmission. The findings derived from this research will not only shed light on a relatively poorly understood segment of parasite development but will also contribute significantly to ongoing efforts aimed at interrupting transmission. Ultimately, these developments hold promise for safeguarding both humans and animals from the transmission of leishmaniasis.

Overview of methods

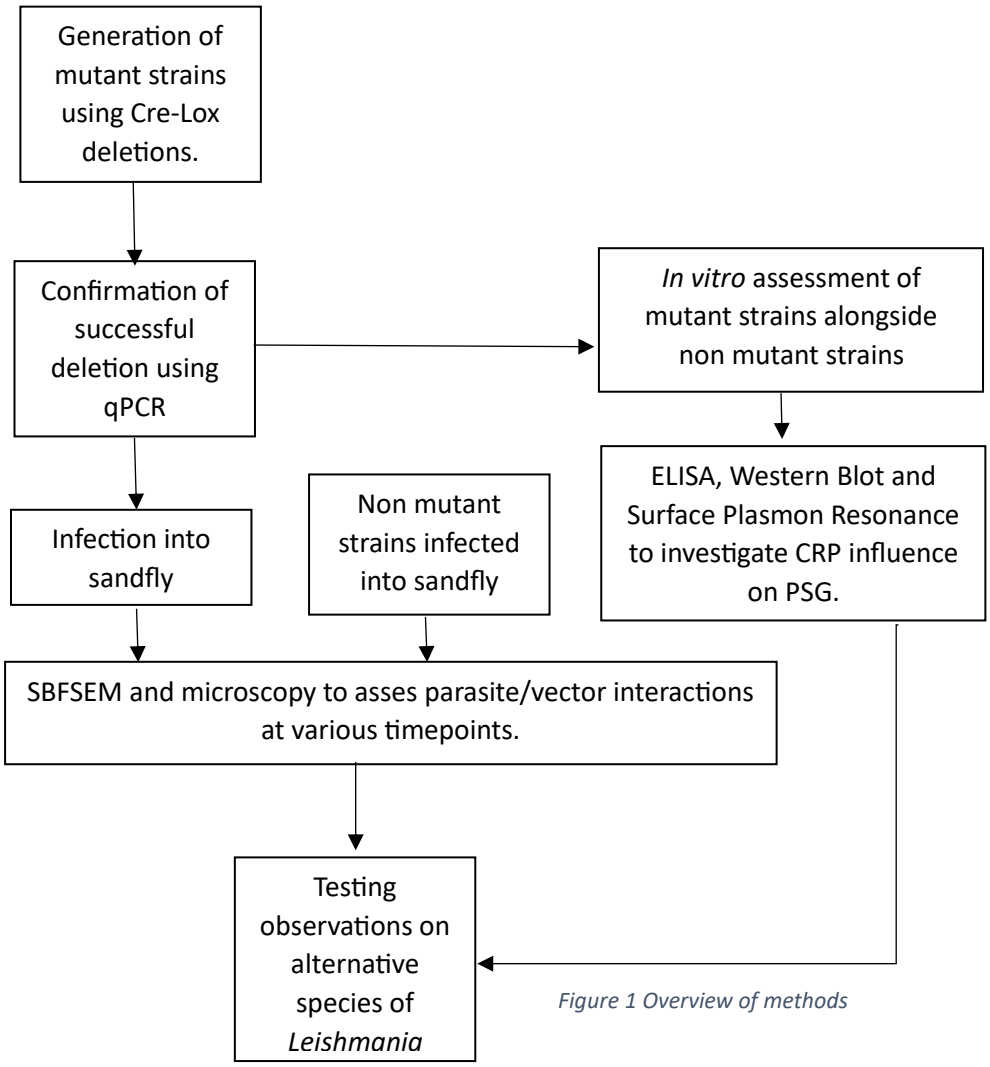

Figure 1 Overview of methods

References:

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