The leishmaniasis are a collection of diseases caused by parasites of the genus *Leishmania* and spread via sand fly bites. Different species of *Leishmania* cause different types of disease: visceral, cutaneous and mucocutaneous.

Visceral leishmaniasis (VL) is a potentially fatal systemic disease caused by *L. donovani* and *L. infantum* that kills up to 20,000 people each year, mainly in South Asia, East Africa and Brazil (Figure 1). Treatment options are limited and there is currently no vaccine. Disease is aggravated and treatment options even more limited in people with HIV.

**Project Aims**

**Key Question:** Can we identify novel immune mechanisms controlling parasite spread in the host using population dynamics?

1. Develop molecular, mathematical and evolutionary/ecological methods to identify parasite population bottlenecks in cells and animal models of infection.
2. Investigate the immunological mechanisms responsible for bottlenecks in VL infection and their impact on parasite dissemination.
3. Use mathematical models to describe and predict how immune interventions affect parasite dissemination.

**What is a bottleneck?**

- **Ecological/evolutionary biological concept:** Bottlenecks are obstacles which apply pressure to a population, e.g. immune responses.
- **This reduces the population size, which we can measure using genetics and evolutionary biology theory.**
- **Diversity in the surviving (Founder) population can be calculated using mathematical models to estimate the “size” of the bottleneck (Figure 3).**
- **Identifying where and how narrow bottlenecks occur can help identify ways to kill the parasite.**

**How do we find these bottlenecks?**

A line of *L. donovani* parasites were engineered to express the cas9 enzyme, with a hygromycin resistance marker; in their ribosomal locus (using the method described by Beneke et al., 2017), allowing CRISPR-cas9 editing of the parasite genome (Figure 4).

Using CRISPR-cas9 genome editing, a library of parasite lines, each with a unique genetic “barcode” but no other changes to parasite genotype or phenotype, was produced by replacing the hygromycin resistance with a new DNA sequence. These parasite lines can then be used in experiments, both in vivo and in vitro, to assess bottleneck sizes in different tissues and under different conditions; this involves analysing the input vs output proportions of parasites with each barcode, using Illumina DNA sequencing. This method is adapted from that described in Abel et al., 2015 [2].

Initial work will look at how barcode proportions differ between tissues known to harbour *Leishmania* parasites, such as the liver, spleen, lymph nodes and bone marrow, to assess where bottlenecks are impacting dissemination in the mouse host.

In vitro work could look at whether these bottlenecks happen at the cell level, by taking cells from these organs and infecting them in a lab environment.

**Zombie: In Situ Barcode Imaging**

The barcodes are under T3 promoter control, meaning the barcode is only expressed when the T3 polymerase enzyme is present. Barcode amplification and detection in fixed cells and tissues is possible via Fluorescence *In Situ* Hybridisation (FISH), using “coloured” probes specific for each barcode (Figure 5).

Parasites can be tracked within tissues and single cells to understand infection dynamics.

**Potential Factors and Mechanisms Affecting Dissemination**

Further experiments to understand the factors involved in parasite dissemination (Figure 6) and their underlying mechanisms could use a range of methods including:

- In vivo models
- Immunological assays
- Innovative cellular imaging (Figure 7)

**Figure 3: An Illustration of a population bottleneck and its impact on genetic diversity. Based on Abel et al., 2015 [1]. Created with Biorender.**

**Figure 4: Experimental methodology for inserting genetic barcodes into *L. donovani* to investigate parasite dissemination via population dynamics. This figure shows how barcodes can be inserted into parasite genomes using CRISPR-cas9 genome editing, to produce a library of parasites, each with a unique barcode, but otherwise identical. This library can then be used for experimental work at the whole organism, tissue, and single cell levels. The proportions of barcodes in the input vs output proportions of parasites with each barcode, using Illumina DNA sequencing. This method is adapted from that described in Abel et al., 2015 [2].**

**Figure 5: Schematic illustration of the Zombie method (Askary et al., 2020) for in situ barcode imaging. T3 polymerase is added to fixed samples to produce barcoded mRNA. FISH probes with different fluorophores indicate different barcodes. Created with Biorender.**

**Figure 6: Potential influencing factors on dissemination of parasites in the host. Created with Biorender.**

**Figure 7: Murine bone marrow macrophages infected with *L. donovani* cas9-L. donovani. This figure shows a confocal microscope image of macrophages infected with *L. donovani* for 1hr at 37°C, to establish parasite localization within the cell. Magenta staining shows LAMP3, a lysosomal marker; cyan indicates DAPI staining.**

**Key References**

5. Wiesch, F., & Alfaro, S. (2018). In vivo and in vitro.  A vaccine to treat PKDL is currently being tested in Sudan, but a better understanding of how the parasite spreads in the body is needed to aid development of new treatments and vaccines to prevent infection and improve health and wellbeing for those at risk of VL and PKDL.

**Acknowledgements**

Many thanks to all Kaye and Mottram lab members, particularly Juliana Brambilla, Jeremy Mottram and Paul Kaye. Thanks also to Hull York Medical School for funding this project.