Title of dissertation				
Development of Molecular and Antibody-based Approaches for Diagnosis and Risk Mapping of Cutaneous Leishmaniasis(CL) in Sri Lanka				
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Sri Lanka is facing a surge in cutaneous leishmaniasis (CL), with the routine slit skin smear (SSS) diagnostic method missing over 30% of cases. This study aimed to improve CL diagnosis, especially for doubtful cases, and to develop user-friendly methods for CL risk mapping in Sri Lanka. The objective was to introduce highly sensitive molecular tests to enhance case detection and explore serum and urine-based techniques to boost diagnostic capabilities in hospital clinics. Immunological methods were also tested to identify vector biting patterns, crucial for developing CL risk maps to guide targeted prevention and control. The experiments included developing nested PCR and RFLP for detection and species identification from clinical samples, real-time quantitative PCR to estimate parasite burden, in-house ELISA to detect serum and urine IgG against the rKRP42 antigen of *Leishmania donovani*, in-house ELISA to detect serum and urine IgG against the sandfly salivary gland antigen 747 of Phlebotomus argentipes, and an antibody capture direct agglutination test (Abc-DAT) to detect serum and urine IgG against *L. donovani*.

A cohort of 194 patients with cutaneous leishmaniasis were recruited. Sociodemographic and clinical data were collected along with clinical samples including punch biopsy specimens of skin lesions, slit skin smears (SSS) serum and urine. They were examined clinically by a dermatologist and by SSS microscopy performed by both study investigators and hospital technicians. A *Leishmania* genus-specific modified nested PCR-RFLP method using two novel inner primers targeting Internal Transcribed Spacer 1(ITS1) region was tested on DNA extracted from biopsy specimens of CL patients' skin lesions. Nested-ITS1 PCR was used as the reference standard. Real-time ITS1 quantitative PCR was carried out to detect and quantify parasite burden in the skin lesions of the same cohort of patients using novel *Leishmania* genus specific primers. Parasite quantification on SSS was also performed using a modified version of the WHO (Word Health Organization) smear grading system. The new in-house ELISA to detect IgG against the recombinant antigen rKRP42 of *L. donovani* was tested on both serum and urine of CL cases (n=190). Serum ELISA was also tested on a Japanese control group which was considered as the non-endemic control (JC; n=80). Urine ELISA was tested in Japanese controls and in endemic controls (EC; n=255). The same cohorts were examined using recombinant salivary gland antigen 747 of *Phlebotomus argentipes* to assess vector biting. Antibody capture Direct Agglutination Tests (abcDAT) to detect IgA and IgG against *L. donovani* was tested using urine of CL cases and endemic control group. Serum of 100 CL cases were tested with rK39 rapid test.

Nested ITS1-PCR was sensitive enough to detect 2.5fg of parasite DNA and detected a significantly higher percentage (94%) of CL cases compared to SSS (hospital technicians:77.6%, study investigators:80.9%). Nested Its1-PCR showed 95% sensitivity in detecting lesions which were difficult to diagnose clinically whereas SSS performed poorly in detecting this type of lesions. RFLP analysis identified all nested PCR positive cases as *L. donovani*. Anti-rKRP42 serum ELISA was 94.4% sensitive for case detection. Those who had high serum ELISA titers showed a 9% positive rate with rK39 strip test. Combination of SSS by hospital technician and Anti-rKRP42 serum ELISA improved sensitivity of case detection up to 99%. Anti-rKRP42 urine ELISA did not provide expected results; it needs further optimization. The qPCR assay successfully detected and quantified parasites in 86.8% of samples. Parasite burden quantification through qPCR correlated well with quantification by SSS. However, no significant correlation was observed between the magnitude of the anti-rKRP42 IgG response and parasite burden. Parasite burden was higher in early lesions, smaller lesions, and certain clinical forms. Both serum and urine anti-747 ELISAs identified >70% of CL cases as positive. A positive correlation was demonstrated between the spatial distribution of positive rates by anti-747 urine ELISA and

nested PCR. Therefore, anti-747 urine ELISA demonstrated its utility as a community accepted method to map the risk of CL transmission.

ITS1-PCR, is especially useful for detecting Old World *Leishmania* species. The present study enhanced the sensitivity of classical ITS1-PCR primers (LITSR and L5.8S) by tenfold with novel inner primers. The nested PCR approach improved specificity by avoiding non-specific amplification but required stringent contamination control. Although nested PCR is more time-consuming, it offers increased sensitivity, aligning with previous findings. RFLP analysis requires one further step from nested PCR and offers a straightforward, rapid, and accurate method for species determination. In this study, *L. donovani* was the exclusive cause of CL in the southern focus, which was consistent with prior research. However, the risk of introducing new parasites remains due to increased travel, emphasizing the importance of monitoring imports. Quantitative PCR was less sensitive for case detection than nested PCR but still detected a substantially higher case number compared to SSS. Real-time PCR does not necessarily offer higher sensitivity over conventional PCR but carries many advantages such as speed, reproducibility, quantification ability and does not require post-PCR analysis. The study revealed that routine diagnostic methods missed over 20% of clinically diagnosed CL cases. SSS had limited accuracy, particularly for certain lesion types and chronic cases with low parasite density. In contrast, nested ITS1 PCR performed well across all lesion categories, highlighting the need for highly sensitive tests. Combining SSS and serum ELISA achieved nearly 99% sensitivity in case detection when compared to nested PCR. The method can be cost-effective, particularly in resource-poor settings, despite lower specificity and negative predictive value. We could not demonstrate a correlation between the parasite burden by qPCR and the IgG response of the host. Therefore, further studies are required to establish a protective or disease exacerbating role of IgG or whether it can accurately predict disease resolution role in Sri Lankan CL. Understanding the immune response dynamics and IgG subclasses are important to develop immune diagnostics as prognostic and diagnostic tools. Spatial distribution analysis using urine 747-ELISA showed a positive relationship between exposure intensity and CL prevalence, suggesting the potential use of urine ELISA for disease distribution mapping. Urine is a friendly tool for epidemiological surveys because it a non-invasive, easily collectable sample. Seasonal variations in sandfly biting may however influence the antibody responses.

In conclusion, this study introduced innovative diagnostic methods for CL in Sri Lanka, significantly enhancing the accuracy of detection. Novel primers are introduced for nested ITS1-PCR and ITS1-qPCR for detection and quantification of parasites from skin lesions with high sensitivity. Additionally, RFLP analysis enabled species-level identification. The anti-rKRP42 serum ELISA achieved over 94% sensitivity and proved valuable as a supplementary diagnostic tool. The sensitivity of the combination of SSS and anti-rKRP42 serum ELISA was 99% and can be used to enhance diagnostic capacities of hospital clinics in Sri Lanka. Anti-747 urine ELISA, although less sensitive, demonstrated its potential for large-scale screening and spatial risk mapping, with a clear correlation between sandfly exposure and CL prevalence. The study highlighted the limitations of the routine diagnostic method, skin slit smear (SSS), missing over 20% of CL cases. Furthermore, the finding of CL cases testing positive for the rK39 rapid diagnostic test for visceral leishmaniasis (VL) warrants further investigation.

Photos

Engaging in research work at the laboratory of the Division of Medical Zoology of the Department of Infection and Immunity of Jichi Medical University.

With my Japanese Supervisor, Prof. Hirotomo Kato, after the thesis defense viva.