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Detection of *Leishmania* (*Mundinia*) *macropodum* (Kinetoplastida: Trypanosomatidae) and heterologous *Leishmania* species antibodies among blood donors in a region of Australia with marsupial *Leishmania* endemicity

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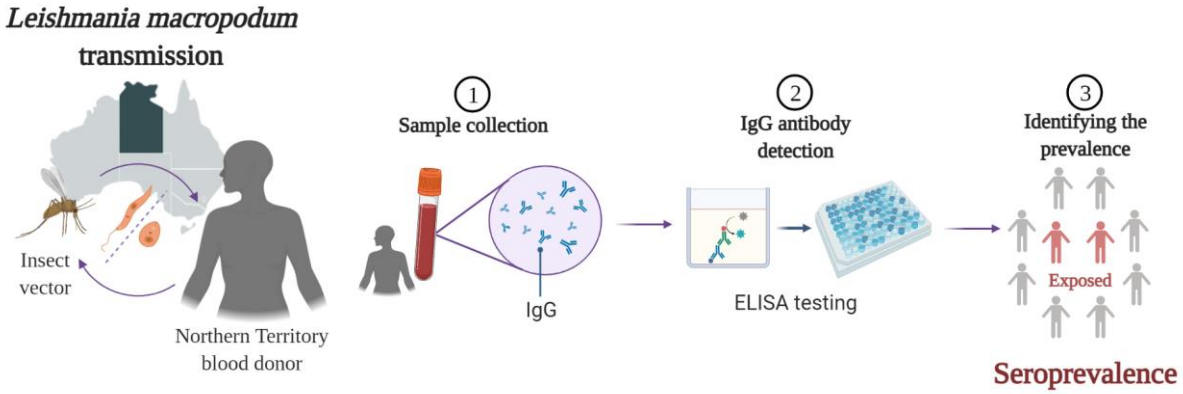
Graphical Abstract



Abstract

Objectives: The Australian *Leishmania* (*Mundinia*) *macropodum* parasite causes cutaneous leishmaniasis (CL) among marsupial species. While CL is a major public health burden globally, it is not clear if humans are naturally exposed to the unique *L. macropodum*. To assess whether humans have an IgG response to *L. macropodum*, we examined anti-*Leishmania* antibodies among humans residing in a region of marsupial *Leishmania* endemicity in Australia.

Methods: Employing a serological enzyme-linked immunosorbent assay, we characterized *Leishmania*-specific IgG and IgG subclass responses to soluble *Leishmania* antigen (SLA) from *L. macropodum* and other *Leishmania* species *(L. donovani, L. major and L. mexicana*) in 282 blood donor samples.

Results: We found 20.57% of individuals demonstrated a positive total IgG response to *L. macropodum*. For individuals with antibodies to SLA from one *Leishmania* species, there was no increased likelihood of recognition to other *Leishmania* species. For samples with detectable *L. macropodum* IgG, IgG1 and IgG2 were the prevalent subclasses detected.

Conclusions: It is not yet clear whether the IgG antibody detection in this study reflects exposure to *Leishmania* parasites or a cross-reactive immune response that was induced against

an unrelated immunogen. Future studies should investigate whether *L. macropodum* can result in a viable infection in humans.

Keywords: Serology; IgG subclasses; Leishmania parasites; Human; Cutaneous leishmaniasis

Introduction

Cutaneous leishmaniasis (CL) is one of the three main clinical forms of the leishmaniases, a complex group of vector-borne diseases with a diverse spectrum of clinical presentation (Burza et al., 2018). Infection is acquired through the bite of phlebotomine sandflies carrying *Leishmania* (Kinetoplastida: Trypanosomatidae) parasites (Bates, 2007). Approximately 20 *Leishmania* parasite species can cause CL in humans with varying degrees of clinical severity ranging from sub-clinical (often referred to as asymptomatic) and self-resolving lesions, to chronic infection that results in severe tissue destruction and disfigurement. CL causes major public health burden across the world; estimated to affect more than 1 million people annually (Burza et al., 2018).

The transmission dynamics of *Leishmania* are complicated and highly dependent on the infecting *Leishmania* species. Humans can act as the primary reservoir host (anthroponotic transmission), or when non-human species are involved, the transmission is zoonotic. The main *Leishmania* species responsible for CL are *Leishmania major*, *L. tropica* and *L. aethiopica* (Old World species), and *L. amazonensis*, *L. mexicana*, *L. braziliensis* and *L. guyanensis* (New World species). With several etiological *Leishmania* species causing variable clinical manifestations, the epidemiology of CL is complicated (Panahi et al., 2022).

In 2001 a novel species, *Leishmania* (*Mundinia*) *macropodum,* was identified as endemic in the tropical region of the Northern Territory (NT), Australia, and shown to cause clinical manifestations of CL in marsupial species (Dougall et al., 2009; Rose et al., 2004). *L. macropodum* is suspected to be transmitted by *Forcipomyia* (*Lasiohelea*) Kieffer (Diptera: Ceratopogonidae) (Dougall et al., 2011; Panahi et al., 2020), a day-biting phlebotomine sandfly. *F*. (*Lasiohelea*) has not been implicated in the transmission of other medically important *Leishmania* species (Ready, 2013). Locally acquired human cases of CL have not been reported or diagnosed in Australia, leading to the assumption that humans are not infected by this parasite. To date, the possibility of human exposure to *L. macropodum* in the NT had not been formally investigated.

The gold standard for diagnosis of clinical CL in any vertebrate is based on direct microscopic examination of skin lesion smears or biopsies, in combination with culturing parasites from lesions of infected hosts (Aronson et al., 2016). It has been reported that aysmptomatic human CL is the most common outcome following the bite of an *Leishmania* -infected sandfly (Andrade-Narvaez et al., 2016). The absences of clinical lesions in these individuals makes diagnosis a challenge. Molecular methods (polymerase chain reaction; PCR) and/or serological methods (enzyme-linked immunosorbent assay (ELISA), immunochromatography test, direct agglutination test and western blot) have all played a supportive role in detecting asymptomatic infection or prior exposure to leishmania parasites (Mannan et al., 2021, Ibarra-Meneses et al., 2019). There is no consensus on the best method to formally diagnosis asymptomatic CL. Currently, a postivitve response to the the Montenegro skin test (MST) is considered the most practical marker for asymptomatic CL and the term asymptomatic CL infection is used with caution requiring an individual to have a positive MST and be living in an endemic area of CL without clinical signs/symptoms (Andrade-Narvaez et al., 2016).

Leishmania parasites are known as persistent parasites, i.e. they can reside indefinitely within phagocytic cells, even when symptoms of CL have resolved (Mendonça et al., 2004). The challenge of identifying asymptomatic individuals and the potential role of these individuals in the *Leishmania* transmission cycle makes transfusion-transmissible *Leishmania* a possibility in endemic areas (Aliaga et al., 2019; Cardo, 2006; Ferreira-Silva et al., 2018; França et al., 2013; Fukutani et al., 2014; Jimenez-Marco et al., 2018; Pérez-Cutillas et al., 2015; Riera et al., 2008; Sarkari et al., 2015).

Given serological assays are not able to delineate current asymptomatic infection, previous now cleared symptomatic infection, or merely previous parasite exposure, serological surveys alone are not recommended as the sole method for diagnosing asymptomatic CL, regardless of whether an individual is from an endemic region (Aronson et al., 2016). However, the complexity of validated tests such as the MST, raises concerns that asymptomatic infections are going undiagnosed and asymptomatic infection is viewed as a concern for global *Leishmania* elimination programs (Mannan et al., 2021). Given the simplicity of serological assays such as ELISAs, further research into their role in supporting CL diagnosis is being investigated (Deepachandi et al., 2020; Sarkari et al., 2014). ELISAs have been developed using a range of *Leishmania* antigens such as SLA, recombinant proteins and synthetic peptides, all which can influence the sensitivity and specificity of the assay.

In Australia, symptomatic cases of *L. macropodum* CL have been identified and the parasites have been isolated from marsupial species (Dougall et al., 2009; Rose et al., 2004). Additionally, we have shown that several marsupial species from the *L. macropodum* endemic

region of Australia are IgG positive to SLA derived from *L. macropodum*, despite showing no clinical signs of an active infection (*Panahi et al., data not published*). Given that humans are known hosts of medically important *Leishmania* species, it is important to determine whether humans may be infected with *L. macropodum*.

As the first step to this end, plasma samples were tested for the presence of antibodies that recognized SLA derived from *L. macropodum* and three other *Leishmania* species. Where IgG was detected, IgG subclass responses were also examined.

Materials and Methods

Study design and sample collection

This study was carried out using archived samples from ARC Lifeblood donors (n=282) residing in the NT, which is considered a marsupial *L. macropodum*-endemic region. Samples were collected at the capital city (Darwin) blood collection centre in 2016 and were provided deidentified for this study. Demographic characteristics of blood donors included their age and sex. Previous clinical history was unavailable.

Negative controls (NC; n=12) were recruited from South East Queensland (SEQ), a region in Australia that is non-endemic for *L. macropodum* as *i)* no local cases of CL have been reported in humans or animals, and *ii*) to our knowledge it is a region where the suspected transmitting-vector, *F.* (*Lasiohelea*) sp., is absent. Eligibility criteria for NC were: *i*) no travel history to the NT or to a recognised *Leishmania*-endemic country and *ii*) no previous clinical history of leishmaniasis.

For donor samples, whole blood was collected in plasma preparation tubes (BD Vacutainer PPT plasma preparation tube 5 mL, Becton Dickinson) followed by centrifugation at 1000 × g for 10 minutes. Plasma was collected and stored in aliquots at –80°C. For SEQ NC, whole

blood was collected in heparin plasma collection tubes (BD Vacutainer lithium heparin tube 6 mL, Becton Dickinson) and centrifuged at 1000 × g for 10 minutes. Plasma was collected and stored in aliquots at –80°C.

Preparation of Soluble Leishmania Antigen (SLA)

SLA was prepared from cultured *L. macropodum, L. major, L. donovani* or *L. mexicana* promastigotes (Dougall et al., 2011; Rose et al., 2004). *Leishmania* promastigotes were grown at 26°C in Grace's Insect medium (Gibco) containing 20% heat-inactivated foetal bovine serum (iFBS), 2mM L-glutamine, 100 u/mL penicillin and 100 ug/mL streptomycin. SLA was prepared and standardised across *Leishmania* species using 1×10^8 promastigotes per mL at their stationary phase. Briefly, promastigotes were washed three times in cold 1 × phosphate buffered saline, pH 7 (PBS; Gibco) at 3000 × g for 15 minutes at 4°C. Following washing, the parasite suspension was submitted to five freeze-thaw cycles between dry ice and a 37°C water bath. The parasite suspension was subsequently sonicated on ice with three pulses of 30 seconds (Sonicator 150). Finally, the suspension was centrifuged at 8000 × g for 20 minutes at 4°C. The supernatant was collected and stored at –80°C.

Enzyme-linked immunosorbent assay (ELISA)

For the detection of anti-*Leishmania* antibodies, a three-step detection in-house enzyme-linked immunosorbent assay (ELISA) was developed. Briefly, 96-well polystyrene plates (Costar, Thermo fisher Scientific) were coated with diluted SLA in bicarbonate coating buffer, pH 9.6, and incubated overnight at 4°C. After washing with 0.05% Tween20/PBS (Sigma), plates were blocked with 200 µL 3% BSA/0.05% Tween20/PBS buffer and incubated for one hour at 37°C. Following three washes, plasma (diluted 1:50 in 3% BSA/0.05% Tween20/PBS) was added to the plates in duplicate and incubated for one hour at 37°C. The plates were washed three times

again, and 100 µL of a goat anti-human IgG (Fc specific) secondary antibody (Merck Millipore, Australia) was added at 1:10,000 in 3% BSA/0.05% Tween20/PBS buffer. Plates were incubated for one hour at 37°C. Following further washing, 100 µL of a tertiary antibody, an anti-goat IgG (whole molecule) horseradish peroxidase (HRP) conjugate (Merck Millipore, Australia) was added at 1:30,000 in 3% BSA/0.05% Tween20/PBS buffer and plates were incubated for one hour at 37°C. Finally, plates were washed four times and 100 µL of 3,3k,5,5k-tetramethylbenzidine substrate solution (TMB; Invitrogen, Thermo Fisher Scientific) was added to the plates for 5 minutes. The reaction was stopped by the addition of 100 µL 2N HCL and absorbance was measured at 450 nm using a Tecan infite2000 PRO plate reader. NC samples were included in triplicate on each plate and the seropositivity cut-off was established as the mean optical density (OD) of NC plus 3 standard deviations above the mean. The serological experiments were repeated three times, yielding comparable results.

Samples that were categorized as IgG positive (in all three assays) for each *Leishmania* parasite were further assessed for IgG subclass responses with the three-step detection ELISA as described above but with modifications. Following plasma sample incubations, mouse anti-human IgG1, 2, 3 or 4 (1:1,000; IgG1 clone HP6069; IgG2 clone HP6002; IgG3 clone HP6047; IgG4 clone HP6025; Invitrogen, Thermo Fisher Scientific) was added and plates were incubated for one hour at 37°C. Bound IgG subclasses were detected with a goat anti-mouse IgG-HRP conjugated antibody (1:5,000; Abcam) for one hour at 37°C. Plates were developed with TMB for 20 minutes prior to reading. Again, NC samples were included in triplicates on each plate and serological experiments were repeated thrice.

Statistical analysis

To examine relationships between seropositivity, age and sex, a number of statistical tests were undertaken. First, a chi-squared test was used to determine whether males or females were more likely to be positive to any parasite species. Then an analysis of variance (ANOVA) was used to test relationships between age and seropositivity for any parasite species. Finally, to determine whether an individual was more likely to be seropositive for a given parasite species, an ANOVA was used. We also examined whether there was significant variation in the IgG subclass response within parasites (e.g., for *L. macropodum* IgG1-4) and between parasites (e.g., IgG1 across all parasites). To do so we used a generalised linear model (GLM) to assess the number of IgG subclass responses either above or below the OD threshold for each parasite species and subclass. To determine the most prevalent IgG subclass detected amongst IgG positive blood donors a cut-off for positivity was based on NC. All analyses were undertaken in R and we considered a relationship to be significant if p-value = <0.05.

Results

Detection of IgG that recognises SLA derived from Leishmania species

A total of 282 blood donors residing in the NT were screened for IgG against SLA derived from the following four *Leishmania* parasite species, *L. macropodum*, *L. major*, *L. mexicana* and *L. donovani*. Plasma from ninety-six (34.04%) blood donors contained IgG that recognised SLA from at least one *Leishmania* species. IgG positivity was highest for SLA derived from *L. macropodum* (20.57%), followed by *L. donovani* (13.12%), *L. major* (9.57%) and *L. mexicana* (6.03%) (Table 1). Of the 58 individuals IgG positive for *L. macropodum*, 34 tested positive for *L. macropodum* alone (Table 2), whereas 20 showed positive IgG for one or more other species. Twenty-four blood donors (8.51%) were IgG positive for ≥ two *Leishmania* species, five blood donors (1.77%) were IgG positive for ≥ three *Leishmania* species and four blood donors (1.42%) were IgG positive for all four *Leishmania* species (Table 1).

There was no significant difference between males and females in terms of the presence of IgG that recognised SLA, except for *L. mexicana*, where males had significantly higher IgG positivity rates than females (p-value = 0.04). There was also no significant relationship between age and IgG positivity for any *Leishmania* species (Table 1).

IgG subclass responses to SLA among blood donors with detectable IgG from a marsupial Leishmania endemic region of Australia

Donors that were IgG positive to SLA from a given *Leishmania* species were further tested to determine their IgG subclass profile (Figure 1). IgG1 and IgG2 were the predominant subclasses detected for *L. macropodum* (26/58 IgG1, 26/58 IgG2) and *L. donovani* (15/37 IgG1, 14/37 IgG2) whereas IgG2 was solely predominant for *L. major* (10/12 IgG2) and *L. Mexicana* (9/17 IgG2).

Using an ANOVA, *L. macropodum* was the only parasite species that had a significant difference in percentage positivity between antibody subclass responses (p-value = 0.0151). There was no significant difference in the remaining subclass responses between *Leishmania* species.

Comparing between species within each subclass, IgG3 and IgG4 showed significantly lower OD values to IgG1 (p-value = 0.049, p-value = 0.011) across all species. IgG2 was not different to IgG1 (p-value = 1). There was no significant difference within antibody subclass responses for *L. donovani* (p-value = 0.5), *L. major* (p-value = 0.413) or *L. mexicana* (p-value = 0.669).

Irrespective of parasite, IgG3 responses were significantly below the optical density (OD) threshold (p-value = 0.0452) indicating limited IgG3 response to any Leishmania species. IgG4 appeared show higher OD readings for *L. macropodum* when comparing between species, though this was not statistically significant (p-value = 0.08394).

Discussion

This is the first serosurvey conducted in Australia examining the prevalence of IgG responsiveness and the IgG subclass profile to SLA derived from four *Leishmania* species among blood donors residing in a region of Australia known to be endemic for marsupial CL. Serosurveys are routinely used to evaluate and confirm *Leishmania* parasite exposure in humans (Ibarra-Meneses et al., 2022). In this study we examined anti-*Leishmania* antibodies among blood donors residing in a region of marsupials *Leishmania* endemicity in Australia to assess whether humans have an IgG response to *L. macropodum*. We observed a 20.57% IgG positivity to *L. macropodum*, 13.12% to *L. donovani*, 9.57% to *L. major* and 6.03% to *L. mexicana.*

Our results in blood donors represent a cross-section of the adult population residing in the NT, and therefore this study suggests that approximately 20% of this NT population may have IgG to *L. macropodum*. It was reported that the seroprevalence of anti-*Leishmania* (*Viannia*) *braziliensis* IgG among healthy blood donors residing in endemic countries of the New World was up to 11.4%, with 15.3% among the general population (Mannan et al., 2021). It is important to note that antibody detection is not necessarily a confirmation of current infection.

Further limitations of the current study must also be considered. In endemic areas of human disease, surveys are regularly conducted to estimate the prevalence of asymptomatic

Leishmania infection, which is currently estimated to be 20-60% (Ibarra-Meneses et al., 2022; Singh et al., 2014). However, there is no unified approach to define asymptomatic infection (reviewed in (Ibarra-Meneses et al., 2022)). The only currently accepted method of confirming human infection in areas of *Leishmania* endemicity, is parasitological diagnosis through direct parasite examination (Aronson et al., 2016). Thus, the clinical significance of IgG positivity to SLA derived from *L. macropodum* in this study must remain uncertain.

Currently, there is no commercially available, validated serological test for *L. macropodum.* We developed an in-house ELISA using SLA by adapting previously published protocols (Dougall et al., 2011; Rose et al., 2004). As our SLA preparation is produced by disrupting whole promastigotes it is possible that it may contain antigens that cross-react with other pathogens with phylogenetic proximity (Carvalho et al., 2018). Cross-reactivity of *Leishmania* with pathogens such as *Babesia canis* (Oliveira et al., 2008), *Ehrlichia canis* (Costa et al., 2017), and *Trypanosoma cruzi* (Menezes-Souza et al., 2015), and cross-reactivity between *Leishmania* species is widely acknowledged to cause false-positive results. Additionally, during the recruitment of healthy controls, we were only able to recruit 12 participants with no travel history to leishmania endemic areas. Whereas we acknowledge that the lower number may have impacted the specificity of the assay, the OD values for the 12 controls demonstrated close grouping adding reassurance our results.

Cross-reactivity as a possible explanation for our results cannot be ruled out. However, from human serosurveys across Australia, neither *B. canis*, *E. canis*, *T. cruzi* nor other *Leishmania* species are circulating and locally acquired among humans in the Northern Territory of Australia (Faddy et al., 2019; Jackson et al., 2014). Whereas *Trichomonas vaginalis* is present in the NT, the prevalence in women is reported far higher than men, and our results show no

gender bias, suggesting cross reactivity with *Trichomonas is unlikely to have occurred. L. macropodum* is known to circulate endemically in marsupials in this area and thus exposure to this species is more plausible than exposure to those with known serological cross-reactivity. Further, cross-reactivity with other *Leishmania* species in this sample is unlikely to be significant. We screened all blood donor samples against three additional *Leishmania* species and although we detected antibodies to all three *Leishmania* species within the study samples, samples were not statistically likely to be IgG positive to more than one species and therefore no correlation between IgG positivity to *L. macropodum* and the other *Leishmania* species was identified. The possibility for cross-reactivity to non-related antigens was not investigated in this study.

The detection of IgG that recognized SLA from non-endemic *Leishmania* species in individual samples probably reflects overseas exposure. As this study used archived and unidentified blood donor samples, we were unable to obtain data on clinical history, occupation or travel history of donors and it was therefore not possible to confirm the likelihood of an overseas exposure. Without the ability to collect further information, it was also not possible to determine risk factors nor timeline for exposure to *L. macropodum* for individual donors.

Previous studies on *Leishmania* species have examined IgG subclass profiles to explain the diverse clinical manifestations by identifying specific elevated and suppressed antibodies as potential markers in early diagnosis as well to understand subclinical CL. In this study, *L. macropodum* IgG1 and IgG2 were the most prevalent subclasses. From murine models infected with *L. major* and *L. donovani*, a clear relationship has been demonstrated between T_H2 interleukin induced (IL)-4/IgG1 with disease progression, and T_H1 induced interferon (IFN)-γ/IgG2 with resistance and protective immunity (Afrin and Ali, 1998; Anam et al., 1999;

Bretscher et al., 1992), however this relationship is not well understood in humans or may not be applicable during infection with *L. macropodum*. The presence of specific IgG subclasses has been suggested to be associated with different clinical forms of CL (Magalhães et al., 2021). Patients with CL (caused by *L. mexicana* and *L. braziliensis*) have shown to have a strong cell-mediated immunity with a predominance of IgG1 and IgG2 subclasses whereas patients with mucocutaneous leishmaniasis (MCL) have a predominance of IgG3, but in both cases, they have been linked with a T_H1 reactivity. In comparison, during diffuse leishmaniasis (DL) an elevated IgG4 antibody response has been demonstrated to be correlated with a T_H2 response (Anam et al., 1999; Castes et al., 1988; Rodríguez et al., 1996). Here we detected a predominance of IgG1 and IgG2 among blood donors with detectable IgG to *L. macropodum*. However, without evidence of clinical history of blood donors, an association between IgG subclass responses and *L. macropodum* CL remains inconclusive.

This is the first study of its kind assessing the prevalence of anti-*L. macropodum* IgG and IgG subclass responses in a healthy human population from a region of marsupial *L. macropodum* endemicity in Australia. This study suggests humans are exposed and mount an immune response to *L. macropodum* but cannot conclusively rule out an impact of SLA cross-reactivity on results. Future studies should therefore *i*) develop an assay using a recombinant protein that is validated for *L. macropodum* to increase the sensitivity and to confirm the specificity of IgG responses, and ii) evaluate whether IgG positivity indicates infection with *L. macropodum* in human hosts. In Australia, a clear link between symptomatic CL of marsupials and *L. macropodum* infection has been demonstrated. Therefore, the ongoing circulation of *L. macropodum* in the NT and the implication to public health is important to further investigate.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Study design: E.P, D.I.S, M.K.Y, H.M.F, and L.J.H.

Data collection: E.P, D.I.S, M.K.Y, H.M.F, and L.J.H.

Data analysis: E.P, E.B.S and L.J.H.

Writing: E.P, D.I.S, M.K.Y, H.M.F, and L.J.H.

Ethical approval

The study was conducted in accordance with Australian ethical guidelines and approved by the Griffith University Human Research Ethics Committee (GU HREC; GU Ref No: 2019/805, 24th October 2019) for the involvement of participants from the Southeast Queensland (SEQ) region and approved by the Australian Red Cross (ARC) Lifeblood Human Research Ethics Committee for access to archived donor plasma samples (Ref No: 08102019, 14th October 2019). Written informed consent was obtained from all subjects involved in the study.

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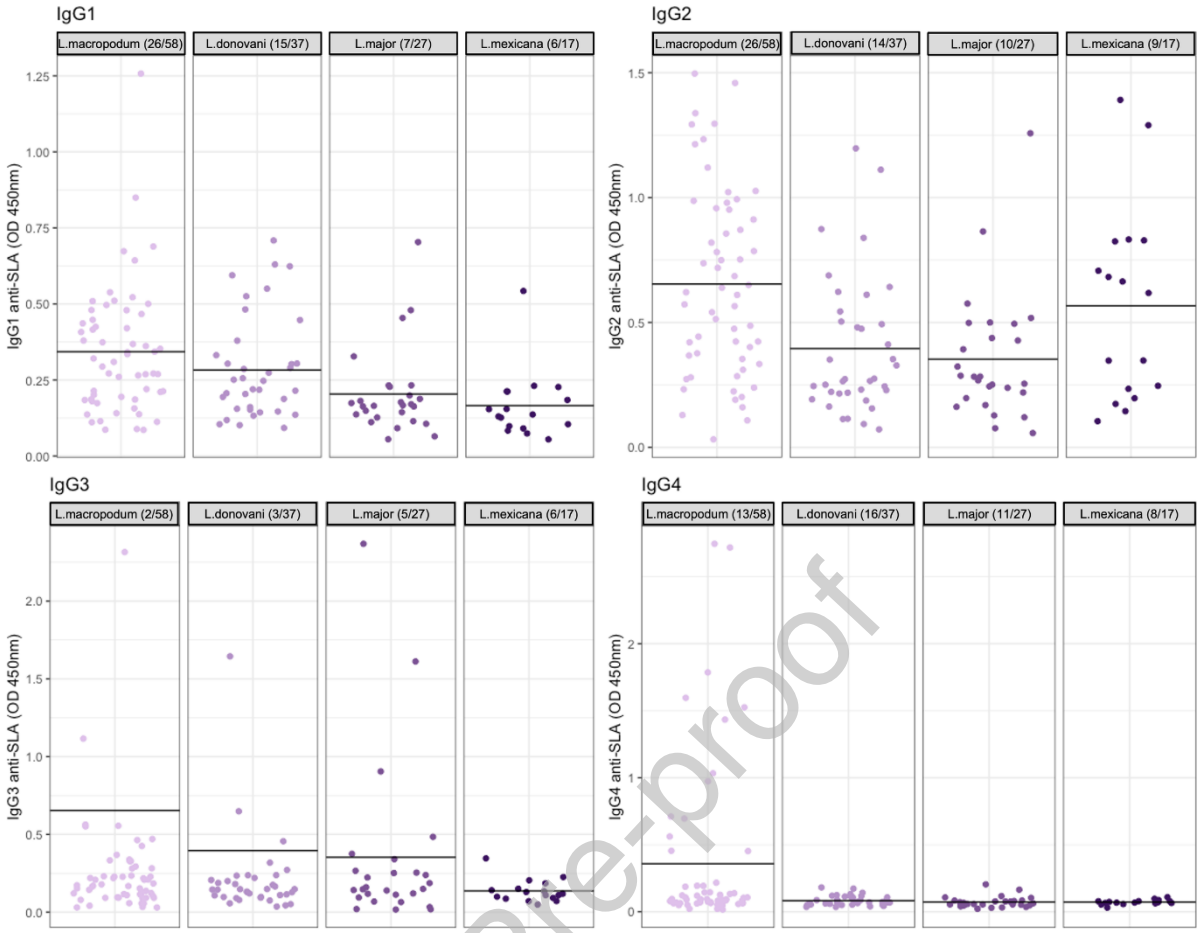

Figure 1. IgG subclass profiles for *Leishmania* species among SLA-IgG positive blood donors residing in Northern Territory, Australia. IgG subclass responses were determined by *Leishmania* species soluble *Leishmania* antigen (SLA)-specific ELISA. The y-axis represents the optical density (OD) measured at 450nm. The cut-off value for positivity is indicated as a horizontal line (black) and represents the mean+3 standard deviation of negative control samples from a non-endemic region (Southeast QLD). Each circle represents a single individual screened for IgG subclass (IgG1-4) responses to SLA derived from four *Leishmania* species. Experiments were repeated thrice, and this figure represents the results from one assay.

Table 1. IgG positivity for soluble *Leishmania* antigen derived from *L. macropodum, L. major, L. mexicana and L. donovani* among blood donors residing in the Northern Territory, Australia[1].

	Number tested	Positive		
		Number	%	95% CI
***L. macropodum* IgG**				
Overall	282	58	20.57	16.26—25.67
Age group				
18 - 24	51	11	21.57	12.49—34.63
25 - 34	52	15	28.85	18.33—42.27
35 - 44	53	12	22.64	13.45—35.53
45 - 54	50	9	18.00	9.77—30.80
55 - 64	47	6	12.77	5.98—25.17
≥ 65	29	5	17.24	7.60—34.55
Sex				
Male	149	26	17.45	12.20—24.34
Female	133	32	24.06	17.59—31.99
***L. major* IgG**				
Overall	282	27	9.57	6.66—13.57
Age group				
18 - 24	51	2	3.92	1.08—13.22
25 - 34	52	4	7.69	3.03—18.17
35 - 44	53	8	15.09	7.85—27.05
45 - 54	50	8	16.00	8.34—28.51
55 - 64	47	4	8.51	3.36—19.93
≥ 65	29	1	3.45	0.61—17.18
Sex				
Male	149	18	12.08	7.78—18.29
Female	133	9	6.77	3.60—12.36
***L. mexicana* IgG**				
Overall	282	17	6.03	3.80—9.44
Age group				
18 - 24	51	1	1.96	0.35—10.30

25 - 34	52	4	7.69	3.03—18.17
35 - 44	53	4	7.55	2.97—17.86
45 - 54	50	3	6.00	2.06—16.22
55 - 64	47	4	8.51	3.36—19.93
≥ 65	29	1	3.45	0.61—17.18
Sex				
Male	149	13	8.72	5.17—14.35
Female	133	4	3.01	1.18—7.48
***L. donovani* IgG**				
Overall	282	37	13.12	9.67—17.56
Age group				
18 - 24	51	4	7.84	3.09—18.50
25 - 34	52	10	19.23	10.80—31.90
35 - 44	53	7	13.21	6.55—24.84
45 - 54	50	9	18.00	9.77—30.80
55 - 64	47	4	8.51	3.36—19.93
≥ 65	29	3	10.34	3.58—26.39
Sex				
Male	149	22	14.77	9.96—21.34
Female	133	15	11.28	6.95—17.78

[1]IgG positivity was determined by *Leishmania* species SLA-specific ELISA

Table 2. Total positive blood donors for each *Leishmania* species and number of blood donors showing positive results to other species[1].

First / Second	***L. macropodum***	***L. major***	***L. mexicana***	***L. donovani***
L. macropodum	NA	11/27	5/17	20/37
L. major	11/58	NA	17/17	10/37
L. mexicana	4/58	9/27	NA	7/37
L. donovani	20/58	10/27	7/17	NA
Total positive	58	27	17	37

[1]IgG positivity was determined by *Leishmania* species SLA-specific ELISA. Columns list first species positive. Rows the subsection of IgG positive to other species.