Parasite load and risk factors for poor outcome among children with visceral leishmaniasis. A cohort study in Belo Horizonte, Brazil, 2010-2011

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Clinical and laboratory risk factors for death from visceral leishmaniasis (VL) are relatively known, but quantitative real-time polymerase chain reaction (qPCR) might assess the role of parasite load in determining clinical outcome. The aim of this study was to identify risk factors, including parasite load in peripheral blood, for VL poor outcome among children. This prospective cohort study evaluated children aged ≤ 12 years old with VL diagnosis at three times: pre-treatment (T0), during treatment (T1) and post-treatment (T2). Forty-eight patients were included and 16 (33.3%) met the criteria for poor outcome. Age ≤ 12 months [relative risk (RR) 3.51; 95% confidence interval (CI) 1.89-6.52], tachydyspnoea (RR 3.46; 95% CI 2.19-5.47), bacterial infection (RR 3.08; 95% CI 1.27-7.48), liver enlargement (RR 3.00; 95% CI 1.44-6.23) and low serum albumin (RR 7.00; 95% CI 1.80-27.24) were identified as risk factors. qPCR was positive in all patients at T0 and the parasite DNA was undetectable in 76.1% of them at T1 and in 90.7% at T2. There was no statistical association between parasite load at T0 and poor outcome.

Key words: visceral leishmaniasis - parasite load - risk factors - children - Brazil

It is estimated that there are between 200,000-400,000 cases of visceral leishmaniasis (VL) in the world annually. Over 90% of these occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Alvar et al. 2012). In Brazil, there has been an increase in the number of the VL cases over the past few decades, with an annual average of 3,484 cases between 2000-2010 (MS 2011a, b). Over the last decade, a high lethality rate has been observed, ranging from 3.2-8.5% (MS 2011a, b).

Belo Horizonte, the capital of the state of Minas Gerais, an endemic region for VL, also showed an increase in VL cases, with an annual average of 83 cases per year between 2000-2004 and 132 cases per year between 2005-2010. The lethality rate in Belo Horizonte between 2006-2011 was higher than the national average, ranging from 8.1-23% (PBH/SMS 2012).

Previously identified risk factors for death are prolonged course of the disease, very old and very young age, malnutrition, vomiting, diarrhoea, jaundice, bleeding, dyspnoea, oedema, coinfection with human immunodeficiency virus, bacterial coinfection, severe anaemia, leucocytosis, neutrophilia and neutropaenia (Seaman et al. 1996, Lyons et al. 2003, Werneck et al. 2003, Collin et al. 2004, Rey et al. 2005, Mueller et al. 2009, Costa et al. 2010, Sampaio et al. 2010).

Various infectious diseases, such as acquired immune deficiency syndrome and hepatitis C, have a clear correlation between microorganism load and disease outcome (Ghany et al. 2011, Panel on Antiretroviral Guidelines for Adults and Adolescents 2013). Two studies assessed the association between pre-treatment parasite counts and VL outcome. These studies, from Sudan and Brazil, showed that higher number of parasites in the spleen or bone marrow (BM) were associated with a higher death rate (Seaman et al. 1996, Costa et al. 2010). Both studies used microscopic methods for parasite quantification, which have limitations due to the invasive procedures required to obtain the spleen and BM aspirates and the excessive time required for the analysis of these samples (da Silva et al. 2005). Quantitative real-time polymerase chain reaction (qPCR) is a technique that can quantify parasite load in an accurate manner in different biological samples, including peripheral blood. Moreover, it provides results more rapidly than microscopic techniques, with less DNA contamination and fewer false positive results than standard PCR (Kubista et al. 2006).

Considering VL severity and lethality rate, the early identification of factors associated with poor outcome and death is relevant to the premature establishment of appropriate treatment. The objective of this study was to identify risk factors, including parasite load quantified by qPCR in the peripheral blood, for VL poor outcome among children.

SUBJECTS, MATERIALS AND METHODS

Patients and clinical assessment - This was a prospective cohort study conducted at João Paulo II Hospital (HJPII) of Hospital Foundation of Minas Gerais State (FHEMIG) from June 2010-June 2011.

The inclusion criteria were hospitalisation at HJPII, age ≤ 12 years old and laboratory-confirmed VL by one of the following methods: BM smear, PCR (k-DNA) in BM aspirate or in peripheral blood, indirect immunofluorescence antibody test or rk39 immunochromatographic antigen test (Kala-azar Detect™ or Diamed IT-Leish™). All patients with confirmed VL in the study period were invited to participate the study.

The main endpoint was poor outcome, characterised by at least one of the following interventions: admission to the intensive care unit (ICU), administration of vasoactive amines, mechanical ventilation or transfusion of blood products (red blood cells, platelets or fresh frozen plasma). Demographic and clinical characteristics and laboratory findings were assessed before the initiation of treatment.

The patients underwent three clinical evaluations: pre-treatment (T0), between 10-15 days of treatment (T1) and between 40-60 days after initiation of treatment (T2). Spleen and liver sizes were measured, considering their extensions below the costal margin at midclavicular line. Then, the measures were divided by child body surface, in order to obtain an adjusted measure, comparable between all children, despite the weight and height variation observed among these patients. In each clinical evaluation, peripheral blood samples were withdrawn for parasite load assessment. For the continuous measures, such as laboratory values, cut points were defined according to Brazilian and international guidelines.

Parasite load by qPCR quantification - Total DNA from peripheral blood samples was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Two independent assays for the detection and quantification of *Leishmania* spp and human DNA were performed using the StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). For the *Leishmania* assay, the target DNA was the small-subunit ribosomal RNA (SSU rRNA) gene, which is conserved among all *Leishmania* species. The *Leishmania* assay consisted of the primers LEIS.U1 (5'-AAGTGCTTTCCCATCGCAACT-3') and LEIS.L1 (5'-GACGCACTAAACCCCCTCCAA-3'), designed to amplify a 67-bp fragment and the fluorogenic probe LEIS.P1 (FAM 5'-CGGTTCGGTGTGTGGCGCC-3'TAMRA). The protocol described by Gomes et al. (2012) was applied. For the human assay, the *ACTB* reference gene was used as the target and the primers Aco1 and Aco2 (Musso et al. 1996), which generate 120-bp fragments, were used in this assay. The reaction mixtures contained 12.5 µL of Syber® Green PCR Master Mix 2X (Life Technologies), 0.1 µM of each primer and 3 µL of DNA template in a final volume of 25 µL. The cycling parameters were universal and the melting analysis was conducted based on the parameters of the StepOnePlus™ Real-Time PCR System.

Standard curves were prepared for each assay using known quantities of pCR™ 4 TOPO™ vector (Life Technologies) containing the cloned human gene (*ACTB*, 120 bp) and the 67 bp *Leishmania infantum* SSU rRNA fragment. The recombinant plasmids were serially diluted 1:10 to create each standard curve. The quality parameters of the standard curves, including PCR efficiency, linear dynamic range and correlation coefficient, were obtained by software analysis and were accurate and similar to those obtained in previous studies (Gomes et al. 2012, Marques et al. 2012). The parasite load was expressed by the *Leishmania* DNA load (relative copy number of the 67 bp SSU rRNA fragment) normalised against the reference gene *ACTB*, according to Overbergh et al. (1999). *ACTB* copy numbers for the target samples were divided by the highest *ACTB* value obtained by the experiment, resulting in a correction factor used for normalisation.

Statistical analysis - The Statistical Package for Social Sciences (SPSS®) 14.0 (IBM Corporation, New York, NY, USA) was used for data management and statistical analysis. Categorical variables were analysed by the chi-square test and, when appropriate, the Fisher exact test was used. For continuous variables, the Student t test and Wilcoxon test were utilised. The relative risk (RR) and the 95% confidence interval (CI) were calculated as a measure of effect of the dichotomous and categorical variables on the outcome. The variables with missing values above 50% were excluded from the analysis. A significance level of 5% was used.

Ethics - This work was conducted in accordance with the Declaration of Helsinki and with Brazilian rules on human research. The study was approved by the Ethical Committees of the Research Centre René Rachou/Oswaldo Cruz Foundation and of FHEMIG. All parents or guardians signed an informed consent form prior to the study.

RESULTS

Forty-eight patients were included in the study. The average age was 3.3 years, ranging from three-nine years old. Thirty-seven children (77.1%) were under five years old and 25 (52.1%) were male. The median time between the onset of symptoms and hospital admission was 26 days, ranging from four-180 days.

In T0 evaluation, the most frequent symptoms were as follows: fever (48 patients, 100%), pallor (42, 87.5%), malaise (39, 81.2%), abdominal distension (39, 81.2%), hyporexia (32, 66.7%), weight loss (27, 56.2%), vomiting (26, 54.2%) and abdominal pain (23, 47.9%). The most frequently observed signs on physical examination were the following: pallor (48, 100%), splenomegaly (47, 97.9%) and hepatomegaly (46, 95.8%).

The initial treatment was meglumine antimoniate in 26 children (54.2%), amphotericin B deoxycholate in 15 (31.2%), liposomal amphotericin B in two (4.2%) and a combination of a single dose of liposomal amphotericin followed by meglumine antimoniate in five (10.4%). In six patients (12.5%), the initial treatment was changed. In 37 patients, drug choice was based on assistant physi-

cian decision, according to Brazilian Ministry of Health recommendations. In other 11 patients, the initial drug was chosen based on a concomitant clinical trial that was taking place at the same hospital. Four patients who initially received meglumine antimoniate finished the treatment with amphotericin B deoxycholate, due to an initial inadequate treatment response. In two other patients, amphotericin B deoxycholate was replaced by liposomal amphotericin B, due to acute renal lesion. The mean treatment duration was 22 days.

During hospitalisation, 16 patients (33.3%) met the criteria for poor outcome: 11 underwent blood transfusion, three underwent blood transfusion and were admitted to the ICU, one was admitted to the ICU and required mechanical ventilation and one underwent blood transfusion, was admitted to the ICU and required mechanical ventilation and vasoactive amine treatment. There was no death related to VL in the study period.

The risk factors for poor outcome identified at T0 were the following (Table): age ≤ 12 months (RR 3.51; 95% CI

TABLE

Association between demographic, clinical and laboratory findings and poor outcome
in children with visceral leishmaniasis hospitalised at João Paulo II Hospital, June 2010-June 2011

Variables	n		Poor outcome (n) Yes	No	p	Relative risk (95% CI)
Age ≤ 12 months	48	Yes	6	1	0.01[a]	3.51 (1.89-6.52)
		No	10	31		
Symptoms ≥ 60 days	48	Yes	2	3	1.00[a]	1.23 (0.39-3.90)
		No	14	29		
Moderate or severe malnutrition	48	Yes	1	2	1.00[a]	1.00 (0.19-5.22)
		No	15	30		
Diarrhoea	47	Yes	5	9	0.74[a]	1.18 (0.49-2.82)
		No	10	23		
Vomiting	47	Yes	9	17	0.66	1.21 (0.51-2.86)
		No	6	15		
Bleeding (except cutaneous)	48	Yes	1	6	0.40[a]	0.39 (0.60-2.50)
		No	15	26		
Jaundice	48	Yes	2	0	0.11[a]	3.29 (2.12-5.09)
		No	14	32		
Oedema	48	Yes	6	5	0.14[a]	2.02 (0.95-4.30)
		No	10	27		
Tachydyspnoea	48	Yes	3	0	0.03[a]	3.46 (2.19-5.47)
		No	13	32		
Bacterial infection	48	Yes	11	9	0.01	3.08 (1.27-7.48)
		No	5	23		
Liver enlargement ≥ 10 cm	48	Yes	8	4	0.01[a]	3.00 (1.44-6.23)
		No	8	28		
Spleen enlargement ≥ 10 cm	48	Yes	13	19	0.13	2.17 (0.72-6.53)
		No	3	13		
Leukocytes ≤ 1,500 cells/mm³	48	Yes	1	2	1.00[a]	1.00 (0.19-5.22)
		No	15	30		
Neutrophils ≤ 500 cells/mm³	48	Yes	3	2	0.32[a]	1.98 (0.85-4.63)
		No	13	30		
Prothrombin activity ≤ 70%	41	Yes	10	16	0.74	1.15 (0.49-2.74)
		No	5	10		
AST or ALT ≥ 100 UI/L	44	Yes	4	10	1.00[a]	0.86 (0.32-2.26)
		No	10	20		
Total bilirubin ≥ 1.0 mg/dL	40	Yes	3	2	0.32[a]	1.91 (0.80-4.54)
		No	11	24		
Albumin ≤ 2.5 g/dL	44	Yes	14	8	< 0.0001	7.00 (1.80-27.24)
		No	2	20		
Creatinine clearance < 60 mL/min/1.73 m²	43	Yes	1	1	1.00[a]	1.58 (0.38-6.77)
		No	13	28		

a: Fisher's exact test; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CI: confidence interval.

1.89-6.52), tachydyspnoea (RR 3.46; 95% CI 2.19-5.47), bacterial infection (RR 3.08; 95% CI 1.27-7.48), liver enlargement ≥ 10 cm (RR 3.00; 95% CI 1.44-6.23) and serum albumin ≤ 2.5 g / dL (RR 7.00; 95% CI 1.80-27.24).

The qPCR was positive in all patients at T0. At T1 and T2, the parasitic loads were assessed in 46 and 43 patients, respectively. Two children who were not assessed at T1 were assessed at T2. There was a wide variation in qPCR at each time point (Fig. 1). As expected, a statistically significant difference was observed between the number of SSU rRNA copies (in $\log_{10}$) between T0-T1 (p < 0.0001) and T0-T2 (p < 0.0001), but no difference was observed between T1-T2 (p = 0.12).

Among the 46 patients assessed at T1, 35 (76.1%) had undetectable parasite DNA, in four (8.7%), SSU rRNA copies decreased and in the last seven (15.2%) the parasite load remained stable. Considering the 43 patients with a parasite load assessment at T2, 90.7% (39/43) had an undetectable parasite DNA; 29 of them were negative at T1, nine were positive at T1 and became negative and one was not tested at T1. Two children (4.7%) showed a decrease in the number of SSU rRNA copies (in $\log_{10}$) compared to the previous evaluations, but the parasite DNA did not become undetectable (1 of them was not tested at T1). Two other children (4.7%) had an undetectable parasite DNA at T1 and a positive test at T2, with a number of copies similar to that at T0 (Fig. 2).

There was no statistical association between the number of SSU rRNA copies (in $\log_{10}$) and the demographic, clinical and laboratory findings at T0. The mean parasite load was 2.94 SSU rRNA copies (in $\log_{10}$) (95% CI 2.55-3.33) in the poor outcome group and 3.56 SSU rRNA copies (in $\log_{10}$) (95% CI 3.07-4.06) in the other group. Despite a low p value, there was no statistical correlation between poor outcome and high parasite load at T0 (p = 0.08) (Fig. 3).

DISCUSSION

Age less than or equal to one year was related to increased lethality and poor response to pentavalent antimony use in other Brazilian studies (Santos et al. 2002, Rey et al. 2005, Costa et al. 2010). According to Braga et

al. (2013), presence of respiratory abnormalities at hospital admission was a risk factor for poor VL outcome in children. Tachydyspnoea was described as a risk factor for death by other authors (Costa et al. 2010, Sampaio et al. 2010) and its relation to disease severity can have multiple causes: interstitial pneumonitis secondary to VL, pneumonia or thoracic restriction due to increased sizes of the liver and of the spleen. Thus, its aetiology should be investigated carefully, including the use of X-ray images, to enable specific diagnosis and prompt treatment of the complication.

The presence of bacterial infection in patients with VL is a recognised cause of death (Rey et al. 2005, Oliveira et al. 2006, Costa et al. 2010, Sampaio et al. 2010, Araújo et al. 2012) and was associated with poor outcome in this study. In HJPII, febrile neutropaenic patients (neutrophil < 500 cells/mm³) with VL receive broad-spectrum antibiotics routinely, which could have positively influenced the disease course and may have contributed to the absence of deaths during the study period.

Increased liver size was not associated with mortality in previous studies. However, a Brazilian study suggested that the size of this organ is proportional to the duration of the illness (Pedrosa & da Rocha 2004). Thus, larger hepatomegaly may be associated with longer duration of the disease, which has already been associated with adverse VL outcome (Seaman et al. 1996, Collin et al. 2004).

The reduction of albumin level, as observed in our study, has been associated with the severity of VL only in a study in Tunisia (Abdelmoula et al. 2003). Oedema and malnutrition, which are related to hypoalbuminaemia and are associated with higher lethality (Collin et al. 2004, Rey et al. 2005), were not identified as risk factors for poor outcome in this study. Therefore, once the change in albumin levels most likely precedes the clinical manifestations of malnutrition and oedema, monitoring of albumin levels may be relevant for early identification of potentially severe cases.

All patients with VL showed a positive pre-treatment qPCR, indicating that it may be used as a diagnostic tool, in agreement with other studies in France and Italy (Mary et al. 2006, Antinori et al. 2009). This study also showed a good association between qPCR and treatment response: 76.1% (95% CI 60.9-87) showed an undetectable parasite DNA during treatment and 90.7% (95% CI 78.4-96.3) had an undetectable parasite DNA at the end of the treatment, which has also been described in studies in France (Mary et al. 2004, 2006), Italy (Bossolasco et al. 2003), Tunisia (Aoun et al. 2009) and India (Sudarshan et al. 2011).

The four children who were positive by qPCR at T2 were considered clinically cured and did not relapse during the period of the study, which suggests that parasitaemia persistence or reemergence may not be associated with disease relapse, as described for immunosuppressed patients (Bossolasco et al. 2003, Mary et al. 2006). The positivity of qPCR at T2 could be explained by the detection of free DNA instead of viable parasites (Fisa et al. 2002) or by reinfection after return to endemic areas at T2.
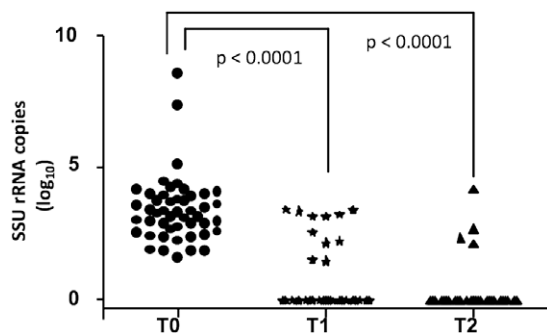

Fig. 1: number of small-subunit ribosomal RNA (SSU rRNA) copies (in $\log_{10}$) before starting treatment (T0), between 10-15 days after initiation of treatment (T1) and between 40-60 days after initiation of treatment (T2) in children with visceral leishmaniasis hospitalised at João Paulo II Hospital, June 2010-June 2011.

The finding of two patients with negative qPCR at T1 and a positive test at T2 reinforces this hypothesis, but it can also be explained by a fluctuation in detectable DNA.

There was no association between any of the symptoms, signs, laboratory exams and the parasite load. This finding indicates that it is not possible to predict parasite load by any parameter of easier evaluation or lower cost than qPCR.

This study was not able to show any association between parasite load and clinical outcome. This finding differs from the results of previous studies in Sudan and Northeast Brazil (Seaman et al. 1996, Costa et al. 2010), which showed that a large number of parasites in the splenic aspirate and the BM aspirate, respectively, were related to a higher risk of death. These differences could be explained by the absence of association between the parasite counts in spleen, BM and peripheral blood and also by the different techniques used (optic microscopic vs. qPCR). The difference in study endpoints, death (Sudan and Northeast Brazil) and poor outcome (current study) could also explain this difference. It is important to emphasise that no deaths were observed in this study. The wide variation in SSU rRNA copies (in $\log_{10}$), as reported by other authors in France (Mary et al. 2004, 2006), Italy (Bossolasco et al. 2003), Tunisia (Aoun et al. 2009) and India (Sudarshan et al. 2011) and the limited sample size may also have contributed to this finding.

Although not statistically significant, there was a tendency of association between high parasite load and good outcome. This finding is in accordance with the high influence of individual immune status and other cofactors in disease manifestations (Murray et al. 2005).
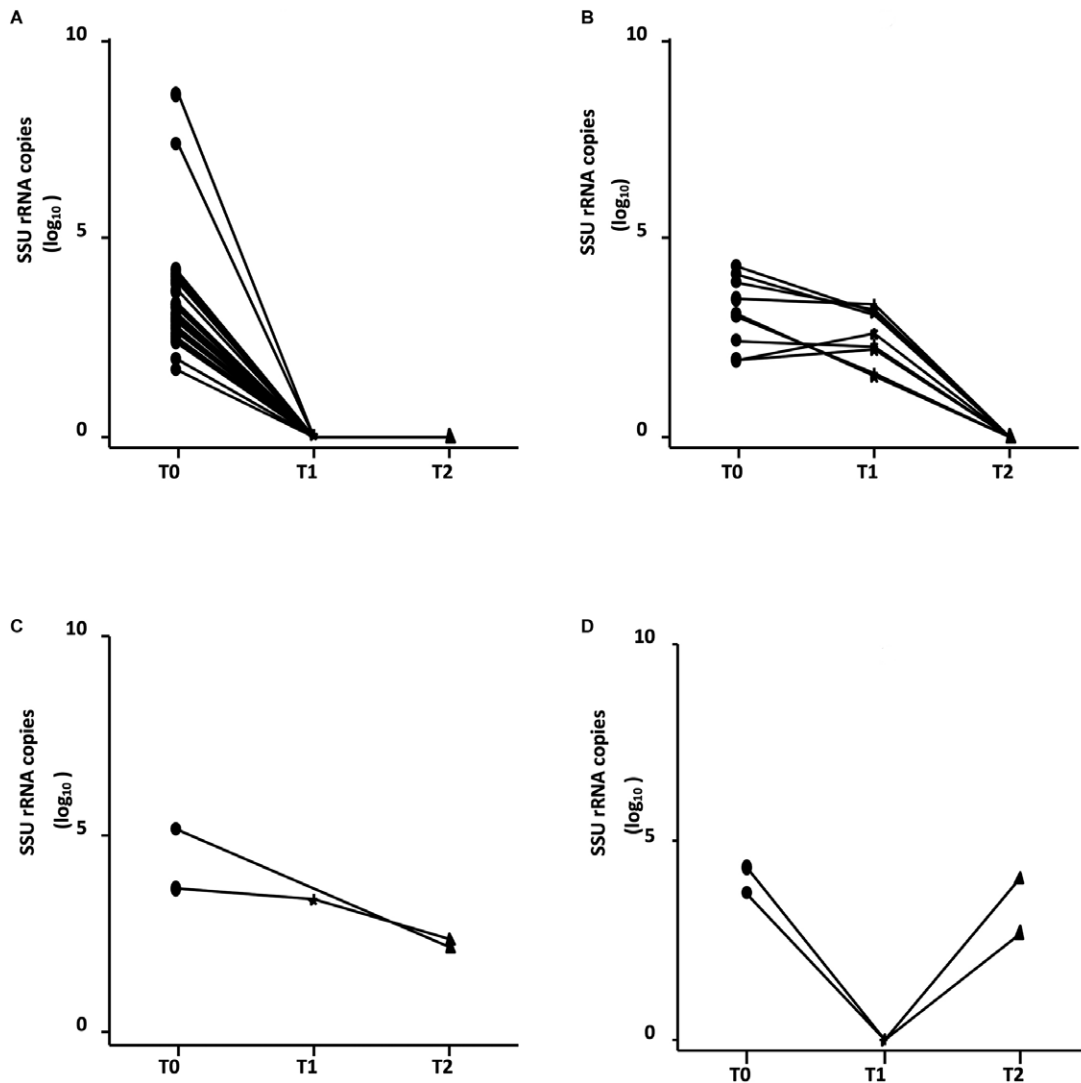

Fig. 2: number of small-subunit ribosomal RNA (SSU rRNA) copies (in $\log_{10}$) before starting treatment (T0), between 10-15 days after initiation of treatment (T1) and between 40-60 days after initiation of treatment (T2) in 43 children with visceral leishmaniasis hospitalised at João Paulo II Hospital , June 2010-2011. A: 29 patients with an undetectable parasite DNA at T1 and T2; B: 10 patients with an undetectable parasite DNA only at T2; C: two patients with a decreased but detectable parasite DNA at T2; D: two patients with an undetectable parasite DNA at T1 and a detectable parasite DNA at T2.
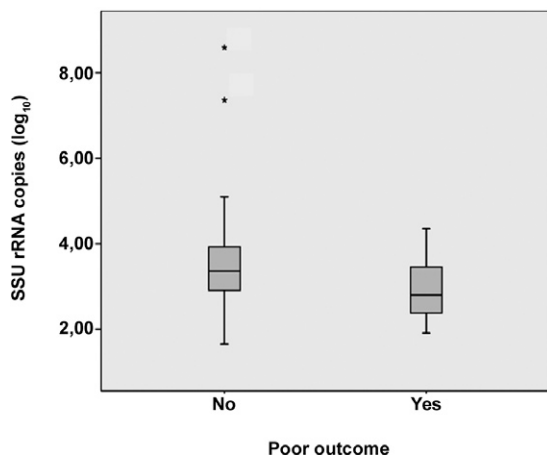
Fig. 3: association between pre-treatment average number of small-subunit ribosomal RNA (SSU rRNA) copies (in $\log_{10}$) and poor outcome among 48 children with visceral leishmaniasis hospitalised at João Paulo II Hospital, June 2010-June 2011.

A higher parasite load might reinforce the specific immune response, which could enhance the host response and the disease resolution. But, considering the small sample size and large variation of parasite load, new data are needed to confirm these findings.

The small number of patients is also a limitation of the present study, once it impaired a more extensive statistical analysis, including multivariate analysis for poor outcome predictors.

In this study, the definition of poor outcome may be a limitation, but it was necessary to redefine the endpoint once there are no deaths during the study period. Clinical outcome is influenced by the access to advanced medical resources, including an ICU, mechanical ventilation and blood transfusions and by medical staff decisions and experience with the disease. The advantages of this study were the prospective design and the uniform assessment performed by a small number of doctors.

In summary, age ≤ 12 months old, tachydyspnoea, bacterial infection, liver size ≥ 10 cm and serum albumin ≤ 2.5 g/dL were associated with poor outcome. Patients with these pre-treatment findings should be carefully monitored to offer proper assistance to those with a greater risk of complications. Pre-treatment parasite load in peripheral blood showed a good association with diagnosis and treatment response, but was not associated with poor outcome in paediatric patients.

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