Rev Soc Bras Med Trop 51(4):537-541, July-August, 2018
doi: 10.1590/0037-8682-0348-2017

Short Communication

Alterations induced in the ileum of mice upon inoculation with different species of *Leishmania*: a preliminary study

***Amanda Gubert Alves dos Santos*[1], *Jéssica de Paula Ferlini*[2], *Suellen Lais Vicentino*[1],[3], *Maria Valdrinez Campana Lonardoni*[4], *Debora de Mello Gonçales Sant'Ana*[1],[5] *and Gessilda de Alcântara Nogueira de Melo*[1],[4]**

[1]. Programa de Pós-Graduação em Biociências e Fisiopatologia, Universidade Estadual de Maringá, Maringá, PR, Brasil.
[2]. Departamento de Farmácia, Universidade Estadual de Maringá, Maringá, PR, Brasil.
[3]. Instituto de Ciências Biológicas, Médicas e da Saúde, Universidade Paranaense, Umuarama, PR, Brasil.
[4]. Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, PR, Brasil.
[5]. Departamento de Ciências Morfológicas, Universidade Estadual de Maringá, Maringá, PR, Brasil.

Abstract

Introduction: *Leishmania* species cause skin, mucosal, and disseminated lesions. We studied the effects of three *Leishmania* species on ileal morphology in mice. **Methods:** BALB/c mice were intraperitoneally inoculated with *Leishmania (Leishmania) amazonensis*, *Leishmania (Viannia) braziliensis*, and *Leishmania (Leishmania) major* (4 animals/group). After 72h, the ilea were collected and histologically processed. **Results:** Following inoculation, the goblet cell and intraepithelial lymphocyte populations increased, while Paneth cell number and crypt width decreased. In addition, enterocyte size, villi height, and mucosa, submucosa, and muscular tunic thickness increased. **Conclusions:** *Leishmania* modified the quantity of cells in and morphology of mice ilea.

Keywords: Leishmaniasis. Small intestine. Innate response.

Leishmaniasis is a parasitic disease that manifests in cutaneous, mucosal, and visceral forms and causes several clinical sequelae, including disseminated lesions that depend on the parasitic species and parasite-host interactions[1]. Several species of *Leishmania*, including *Leishmania (Leishmania) amazonensis*is (LLA), are able to induce cutaneous and visceral leishmaniasis[2]. *Leishmania (Viannia) braziliensis* (LVB) and *Leishmania (Leishmania) major* (LLM) are associated with the mucocutaneous form of the disease[3]. The different forms of leishmaniasis occur from migration of the parasite from the skin to other sites far from the inoculated area via lymphatic or hematogeneous dissemination[4]. The parasite is also able to invade and remain in organs associated with the phagocyte-mononuclear cell system[5].

In addition to cutaneous lesions, the parasite is found in lymph nodes, liver, and spleen[6]. However, little is known about the effects of the *Leishmania* infection on the gastrointestinal tract. Thus, the present study investigated the effects of inoculation with three *Leishmania* species on the morphology and morphometry of the ileum in mice.

Corresponding author: Dra. Gessilda de Alcântara Nogueira de Melo.
e-mail: gessilda.melo@gmail.com
Received 24 October 2017
Accepted 28 December 2017

Ethical considerations

The present study was approved by the *Comitê de Conduta Ética no Uso de Animais* of the State University of Maringá [*Universidade Estadual de Maringá* (UEM); 039/2013] and performed in accordance with the Guidelines of the Brazilian Control and Experimentation. Sixteen female Balb/c mice (25-40g) from the central animal facility of UEM were used. The animals were kept under a controlled temperature (22ºC) and 12h/12h light/dark cycle with food and water available *ad libitum*. The mice were randomly divided into four groups with four animals per group: the control group and three groups that were inoculated with 0.1ml (2×10^8 parasites/ml) of *L. (L.) amazonensis* (MHOM/BR/1989/166MJO; LLA), *L. (L.) major* (LV39; LLM), and *L. (V.) braziliensis* (MHOM/BR/1987/M11272; LVB).

Promastigotes were obtained from the parasite bank of the Leishmaniasis Laboratory of UEM. The parasites were cultured in 199 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 1% L-glutamine, 1% human urine, and 10% fetal bovine serum. The parasites were then harvested in the stationary phase of culture and inoculated into the peritoneal cavity to ensure the intestine was not contaminated.

The mice were euthanized under deep anesthesia with halothane vapor (Tanohalo, Cristália, Itapira, Brazil). After laparotomy, 1cm of the distal ileum was collected and processed

for histology. Semi-serial 4-µm transverse sections were cut and stained with hematoxylin and eosin for morphometric analysis of the intestinal wall and enterocytes and quantification of Paneth cells and intraepithelial lymphocytes (IELs). Periodic acid Schiff (PAS) and Alcian blue (AB) pH 1.0 and 2.5 were used to stain and evaluate goblet cells.

For cellular quantification, each section was divided into four quadrants and Paneth cells that were present in eight crypts in each quadrant per animal per group were quantified. Goblet cells and IELs were quantified by counting a total of 2,500 epithelial cells from each animal and the ratios of goblet cells and IELs to 100 epithelial cells were calculated.

Morphometric analysis was performed using images captured with a digital camera (Pro series 3CCD camera) coupled to an optical microscope (Olympus BX50). Measurements were made using Image-Pro Plus software (Media Cybernetics). The width and height of villi, width and depth of crypts, thickness of the mucosa, submucosa, and muscular tunic, and total thickness of the ileum wall were measured from the images. We performed 80 measurements for each parameter throughout the circumference of the ileum for each animal. We evaluated the width and height of enterocytes and also captured images of 10 Paneth cells per quadrant per animal per group and counted the number of granules inside the Paneth cells. Using these same images, we measured the areas of the Paneth cells and their granules.

The data were analyzed using BioEstat 5.3 software. The data distributions were evaluated using D' Agostinho-Pearson normality tests and the Bartlett test was used to check the homogeneity of the variances. Data with normal distributions were statistically analyzed using analysis of variance (ANOVA) followed by Tukey's multiple-comparison tests. Data with non-normal distributions were analyzed using Kruskal-Wallis followed by Dunn's test. A $p < 0.05$ was considered to indicate statistically significant differences. Data are presented as mean ± standard error.

The LVB group displayed an increase in enterocyte width and height. The LLM group presented a decrease in enterocyte height. The number of Paneth cells decreased in the LLA and LVB groups. Further, there was an increase in the area of the Paneth cells in the LVB group and in the number of granules inside them in the LLM and LVB groups. There was also an increase in the number of sialomucin-producing goblet cells, based on AB staining (pH 2.5), in the LLA, LLM, and LVB groups. The LLM group also presented with an increase in the number of sulfomucin-secreting cells, as shown by AB staining (pH 1.0). The LLA and LVB groups had more neutral mucin cells based on PAS staining. The number of IELs increased in all three inoculated groups (**Table 1** and **Figure 1**).

Morphometric analysis of the ileal wall (**Figure 1** and **Figure 2**) indicated that all infected groups exhibited an increase in the height of the villi and thickness of the mucosa, submucosa, and muscular tunic, reflecting an increase in the total thickness of the ileal wall. The LVB group also had increased villi width. A decrease in the width of the crypts was observed in all of the infected groups. The depth of the crypts decreased in the LLM group.

Leishmaniasis that is caused by infection with *L. (V.) braziliensis* and, more rarely, *L. (L.) major*, has been reported to involve the oral and nasal cavities[3]. *L. (L.) amazonensis* can also migrate to the liver[6]. Previous studies have reported histopathological changes caused by species associated with cutaneous leishmaniasis that are similar to changes observed in visceral leishmaniasis[7].

TABLE 1: Cellular alterations in the ilea of Balb/c mice inoculated with different species of *Leishmania*.

		CG	LLA	LLM	LVB
Enterocytes	Height (µm)	16.16±2.71^{a}	15.80±2.81^{ab}	14.30±2.40^{c}	16.64±2.72^{d}
	Width (µm)	4.40±0.90^{a}	4.30±0.96^{ab}	4.34±0.94^{abc}	4.60±0.96^{d}
Paneth cells	Number	87.33±12.23^{a}	53.75±10.42^{b}	68.33±16.63^{abc}	66.00±8.21^{bc}
	Area (µm^2)	70.76±3.60^{a}	83.05±4.29^{ab}	68.39±3.60^{abc}	88.71±3.43^{b}
	Number of granules	10.37±5.68^{a}	11.62±7.33^{ab}	12.67±7.68^{bc}	13.35±7.30^{c}
	Area of granules (µm^2)	21.16±1.32^{a}	23.82±1.61^{ab}	18.48±1.48^{ac}	25.38±1.24^{b}
Goblet cells	PAS	10.59±0.89^{a}	13.66±1.04^{b}	12.67±1.05^{ab}	14.08±1.06^{b}
	AB pH 1.0	11.06±0.26^{a}	12.09±0.11^{ab}	12.73±0.15^{b}	11.65±0.49^{ab}
	AB pH 2.5	11.08±0.11^{a}	13.69±0.49^{b}	13.21±0.58^{b}	12.50±0.28^{b}
IELs		0.48±0.07^{a}	1.11±0.13^{b}	0.99±0.13^{b}	0.98±0.04^{b}

Mean ± standard error followed by different letters (a-d) in the same row indicate significant differences ($p < 0.05$, ANOVA). $n = 4$ animals/group. **CG:** control group; **LLA:** *Leishmania (Leishmania) amazonensis*; **LLM:** *Leishmania (Leishmania) major*; **LVB:** *Leishmania (Viannia) braziliensis*; **IELs:** intraepithelial lymphocytes; **PAS:** periodic acid Schiff; **AB:** Alcian blue; **ANOVA:** analysis of variance.

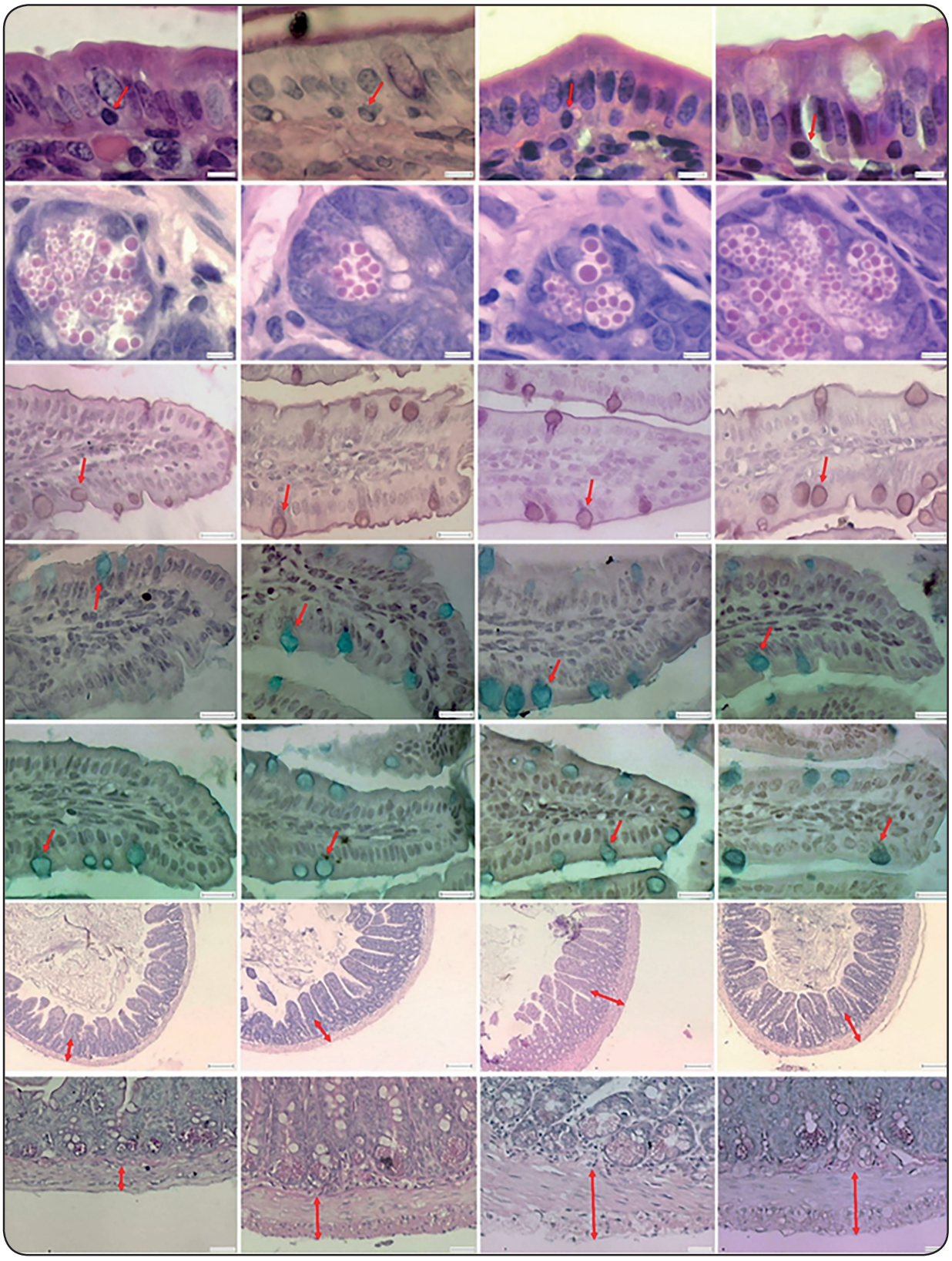

FIGURE 1: Photomicrographs of cross-sections of the ileum mucosa of the control group (first column) and 72 hours after intraperitoneal inoculation of *Leishmania (Leishmania) amazonensis* (second column), *Leishmania (Leishmania) major* (third column), or *Leishmania (Viannia) braziliensis* (fourth column) promastigotes. Enterocytes and intraepithelial lymphocytes stained with hematoxylin and eosin (first row, 1,000X magnification, scale bar = 5μm). Paneth cells at the base of crypts (second row, hematoxylin and eosin, 1,000X magnification, scale bar = 5μm). Goblet cells in villi stained with periodic acid Schiff and Alcian blue pH 1.0 and 2.5 (third, fourth, and fifth row, respectively; 400X magnification, scale bar = 25μm). Arrows indicate the total wall thickness and mucosa (sixth row) stained with hematoxylin and eosin (40X magnification, scale bar = 125μm). Arrows indicate muscular thickness (seventh row, 200X magnification, scale bar = 50μm).

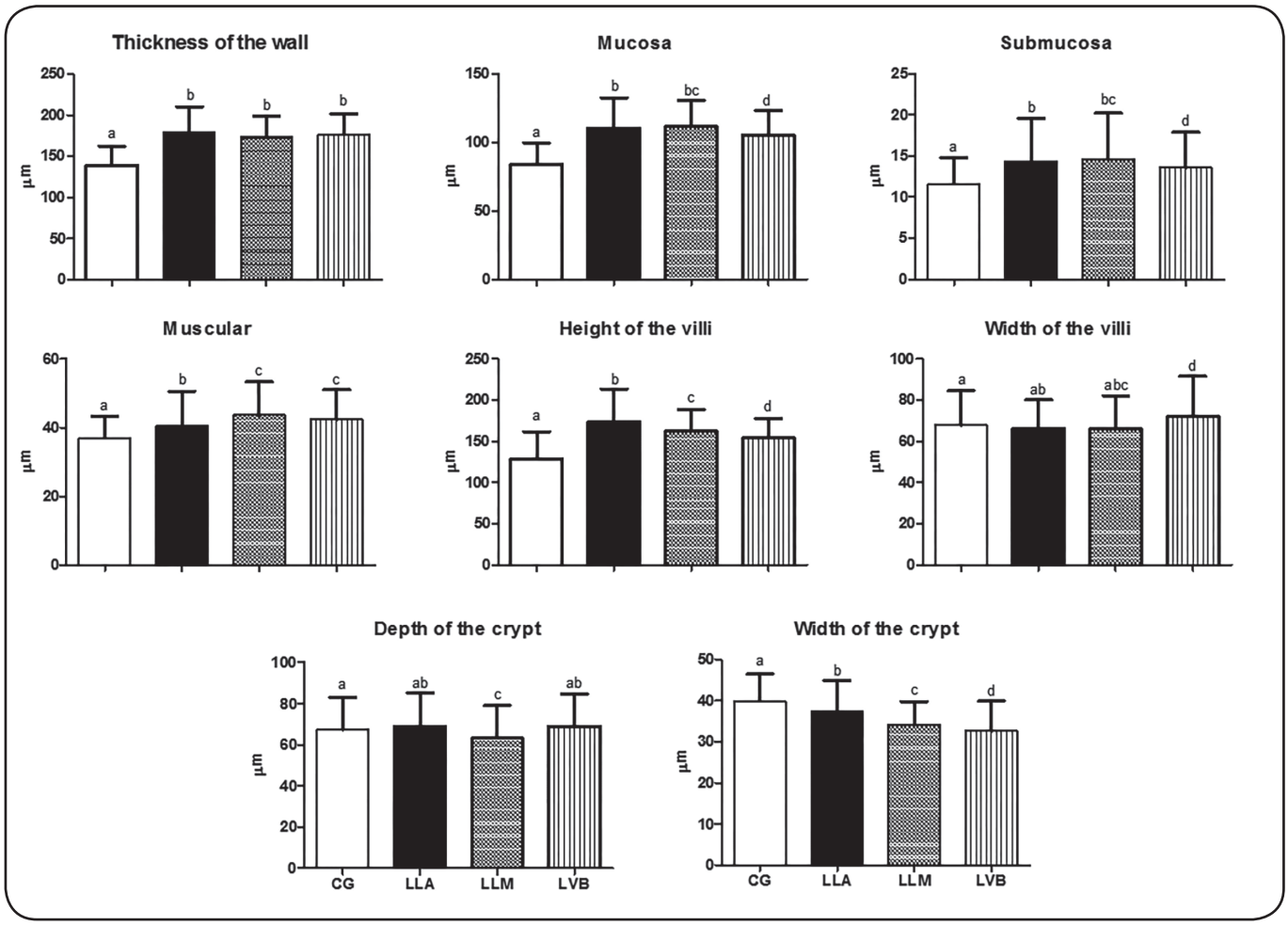

FIGURE 2: Morphometry of the ileal wall in Balb/c mice 72h after intraperitoneal inoculation with LLA, LLM, and LVB promastigotes. Data are expressed as mean ± standard error. Different letters indicate significant differences ($p < 0.05$, ANOVA). $n = 4$ animals/group. **CG:** control group; **LLA:** *Leishmania (Leishmania) amazonensis*; **LLM:** *Leishmania (Leishmania) major*; **LVB:** *Leishmania (Viannia) braziliensis*; **ANOVA:** analysis of variance.

In the peritoneal cavity, immune cells, some of which are phagocytic, can recognize pathogens and initiate an early response against parasites[8]. In response to infection or chemical stimuli, these phagocytic cells can migrate from the peritoneal cavity to the lamina propria[9], perhaps transporting the parasite.

During the innate immune response, the release of several mediators can cause tissue injury depending on the intensity of the inflammatory response. The intestinal response depends on the infection and inflammation in remote locations and may endure after treatment[10]. Although we did not detect the parasite in the ileum, inflammation triggered by *Leishmania* inoculation may be responsible for the alterations observed in this study. In a previous study, histopathological changes caused by some isolates of *L. (V.) braziliensis* were observed even when no amastigotes were found in the tissue of interest[7].

Enterocytes play an important role in the mucosal immune response and may control local inflammation[11].We observed variable changes in enterocytes in the different inoculated groups. These differences may be related to the susceptibility of Balb/c mice to different species of *Leishmania*.

Paneth cells play an important role in the intestinal innate defense system by releasing substances stored within their granules[12]. A decrease in the number of Paneth cells may be related to the inflammatory state triggered after parasite inoculation. By contrast, an increase in the area and number of granules may reflect a compensatory response to the reduction in the number of Paneth cells.

Goblet cells are responsible for mucin production and release, which provides lubrication and protection against pathogens[13]. We observed an increase in the number of goblet cells, possibly reflecting a defense mechanism to protect the intestinal epithelium.

Intraperitoneal inoculation of *Leishmania* species increased the number of IELs, indicating that more immune cells migrated to the epithelium. These cells can produce cytokines, which are important for controlling infections[14]. Furthermore, the proliferation and recruitment of immune cells to the intestine may have contributed to the increase in the thickness of the submucosa and villi[15]. Additionally, according to Mawe[10], local inflammation alters the metabolism of intestinal cells,

especially smooth muscle cells and epithelial cells, which may have contributed to the observed hypertrophy.

In summary, the present study found that inoculation with different species of *Leishmania* induced alterations in the small intestine in mice. *L. (V.) braziliensis* provoked the most pronounced alterations. These findings may spur further studies on host-parasite interactions that are not limited to the skin, but also likely involve distal organs such as the intestine.

Acknowledgments

The authors thank the Coordination for the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES), *Fundação Araucária*, Department of Morphological Sciences, Department of Clinical Analysis and Biomedicine, and the Postgraduate Program in Biosciences and Physiopathology from the State University of Maringá, Brazil.

Conflict of interest

The authors declare that there is no conflict of interest.

Financial support

Universidade Estadual de Maringá, Project number 417/2013. *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) – *Programa de Apoio à Pós-Graduação* (PROAP).

REFERENCES

1. World Health Organization (WHO). Leishmaniasis. Geneve: WHO; 2017. updated 2017 April; cited 2017 Aug 23. Available from: http://www.who.int/mediacentre/factsheets/fs375/en/.
2. Barral A, Pedral-Sampaio D, Grimaldi Jr G, Momen H, McMahon-Pratt D, Ribeiro de Jesus A, et al. Leishmaniasis in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease. Am J Trop Med Hyg. 1991;44(5):536-46.
3. Shirian S, Oryan A, Hatam GR, Daneshbod Y. Three *Leishmania*/*L.* species – *L. infantum*, *L. major*, *L. tropica* – as causative agents of mucosal leishmaniasis in Iran. Pathog Glob Health. 2013;107(5):267-72.
4. Jackson ML, Costa ACRS, Nascimento D, Sampaio G, Carneiro F, Lisboa E, et al. Modalidades clínicas, diagnóstico e abordagem terapêutica da leishmaniose tegumentar no brasil. Gaz Médica da Bahia. 2009;143(79):70-83.
5. Moraes MAP, Correia Filho D, Santos JB. Linfadenopatias na leishmaniose tegumentar americana: considerações sobre dois casos. Rev Soc Bras Med Trop. 1993;26(3):181-5.
6. da Silva SS, Mizokami SS, Fanti JR, Miranda MM, Kawakami NY, Teixeira FH, et al. Propolis reduces *Leishmania amazonensis*-induced inflammation in the liver of BALB/c mice. Parasitol Res. 2016;115(4):1557-66.
7. Pereira CG, Silva ALN, de Castilhos P, Mastrantonio EC, Souza RA, Romão RP, et al. Different isolates from *Leishmania braziliensis* complex induce distinct histopathological features in a murine model of infection. Vet Parasitol. 2009;165(3-4):231-40.
8. Kang S, Jin B, Kim H, Seo G, Jang Y, Kim S, et al. Lactoferrin Combined with Retinoic Acid Stimulates B1 Cells to Express IgA Isotype and Gut-homing Molecules. Immune Netw. 2015;15(1):37-43.
9. Gao J, Ma X, Gu W, Fu M, An J, Xing Y, et al. Novel functions of murine B1 cells: Active phagocytic and microbicidal abilities. Eur J Immunol 2012;42(4):982-92.
10. Mawe GM, Collins SM, Shea-Donohue T. Changes in enteric neural circuitry and smooth muscle in the inflamed and infected gut. Neurogastroenterol Motil. 2004;16(suppl. 1):133-6.
11. Wershil B, Furuta G. 4. Gastrointestinal mucosal immunity. J Allergy Clin Immunol. 2008;121(2):S380-3.
12. Porter EM, Bevins CL, Ghosh D, Ganz T. The multifaceted Paneth cell. Cell Mol Life Sci. 2002;59(1):156-70.
13. Deplancke B, Gaskins HR. Microbial modulation of innate defense: Goblet cells and the intestinal mucus layer. Am J Clin Nutr. 2001;73(6):1131-41.
14. Luangsay S, Kasper LH, Rachinel N, Minns LA, Mennechet FJD, Vandewalle A, et al. CCR5 mediates specific migration of *Toxoplasma gondii*-primed CD8+ lymphocytes to inflammatory intestinal epithelial 44cells. Gastroent. 2003;125(2):491-500.
15. Góis MB, Hermes-Uliana C, Barreto Zago MC, Zanoni JN, da Silva AV, de Miranda-Neto MH, et al. Chronic infection with *Toxoplasma gondii* induces death of submucosal enteric neurons and damage in the colonic mucosa of rats. Exp Parasitol. 2016;164:56-63.