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CURRENT OPINION

What pre-Columbian mummies could teach us about South American leishmaniases?

Denis Sereno[1,2,*,†], Mohammad Akhoundi[3], Franck Dorkeld[4], Bruno Oury[1], Hooman Momen[5] and Pascale Perrin[2]

[1]IRD UMR 177 (IRD, CIRAD), Centre IRD de Montpellier, Montpellier 34394, France, [2]MIVEGEC/Université de Montpellier CNRS/UMR 5244/IRD 224-Centre IRD, Montpellier 34394, France, [3]Service de Parasitologie-Mycologie, Hôpital de l'Archet, Centre Hospitalier Universitaire de Nice, Provence-Alpes-Côte d'Azur, Nice 06003, France, [4]INRA-UMR 1062 CBGP (INRA, IRD, CIRAD), Montpellier SupAgro, Montferrier-sur-Lez, Languedoc-Roussillon 34988, France and [5]Instituto Oswaldo Cruz, Fiocruz, 21040-360 Rio de Janeiro, Brazil

[*]**Corresponding author:** IRD UMR 177 (IRD, CIRAD), Centre IRD de Montpellier, Bureau 164, Montpellier 34394, France. Tel: 0033467416328.
E-mail: denis.sereno@ird.fr
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[†]Denis Sereno, http://orcid.org/0000-0002-0034-9291

ABSTRACT

A recent report on the taxonomic profile of the human gut microbiome in pre-Columbian mummies (Santiago-Rodriguez *et al*. 2016) gives for the first time evidence of the presence of *Leishmania* DNA (sequences similar to *Leishmania donovani* according to the authors) that can be reminiscent of visceral leishmaniasis during the pre-Columbian era. It is commonly assumed that *Leishmania infantum*, the etiological agent of American visceral leishmaniasis (AVL) was introduced into the New World by the Iberian conquest. This finding is really surprising and must be put into perspective with what is known from an AVL epidemiological and historical point of view. Beside *L. infantum*, there are other species that are occasionally reported to cause AVL in the New World. Among these, *L. colombiensis* is present in the region of pre-Columbian mummies studied. Other explanations for these findings include a more ancient introduction of a visceral species of *Leishmania* from the Old World or the existence of a yet unidentified endemic species causing visceral leishmaniasis in South America. Unfortunately, very few molecular data are known about this very long pre-Columbian period concerning the circulating species of *Leishmania* and their diversity in America.

Keywords: pre-Columbian mummies; pathogenic *Leishmania* species; American visceral leishmaniasis

ABBREVIATIONS

HIV: Human immunodeficiency virus
DNA: Deoxyribonucleic Acid
Mya: Million years ago
Kya: Kilo years

Santiago-Rodriguez *et al*. (2016) recently gave evidences of the presence of *Leishmania* DNA in the gut of two pre-Columbian mummified individuals from Cuzco. They use the complete genome of a species closely related to *Leishmania infantum* (*L. donovani*), as template, to assign DNA fragments from next

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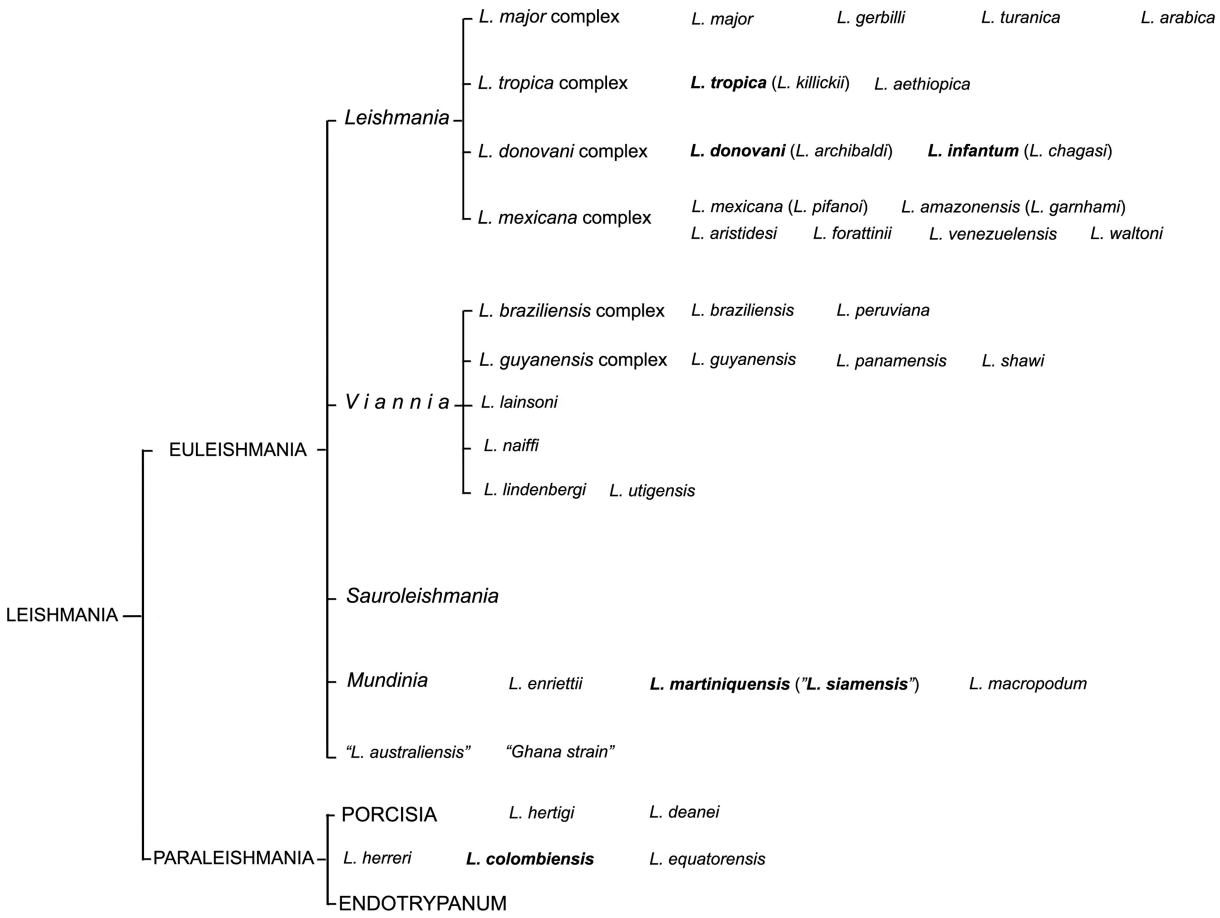

Figure 1. Updated classification of human pathogenic *Leishmania* species. Species known to cause visceral form of the disease are highlighted in bold.

generation sequencing (NGS) to *Leishmania*. They identified 19 DNA fragments of 50–434 bp matching with 16 contigs and gave no information on the reads (Santiago-Rodriguez *et al.* 2016). The circulation of parasites with visceralization capabilities, prior to the Iberian colonization, has never been mentioned before this report. In this viewpoint, we discuss the implication that this finding has on our current knowledge concerning the evolutionary history of the *Leishmania* genera and subgenera. We further address questions regarding the use of ancient microbial (*Leishmania*) DNA studies to follow the spread and prevalence of visceral leishmaniasis within human populations through time.

LEISHMANIA AND LEISHMANIASES

Leishmaniases are vector-borne diseases caused by obligate parasites (unicellular eukaryote) from the genus *Leishmania* (Trypanosomatida: Trypanosomatidae), which is subdivided into two major phylogenetic lineages, referred as the *Euleishmania* and *Paraleishmania* sections (Cupolillo *et al.* 2000). The section *Euleishmania* comprises five subgenera: *Leishmania sensu stricto*, *Viannia*, *Sauroleishmania*, *Mundinia* and the *Leishmania australiensis*-related species (see Akhoundi *et al.* 2016; Espinosa *et al.* 2016; Barratt *et al.* 2017). The subgenus *Viannia* is restricted to the Neotropics, while the subgenus *Leishmania sensu stricto* occurs in both the New and the Old World. The subgenus *Sauroleishmania* encompasses *Leishmania* species that are lizards infecting parasites (see Akhoundi *et al.* 2016) (see Fig. 1). The genus *Leishmania* includes 53 named species (without considering the synonyms) of which 29 are present in the Old World and 20 in the New World. Twenty-one species are pathogenic to humans.

The clinical manifestations of leishmaniases range from simple cutaneous lesions (CL) to diffuse cutaneous (DCL), mucocutaneous (MCL), mucosal (ML), post Kala-azar dermal (PKDL) and visceral (VL) or American visceral (AVL) forms.

The diseases are endemic in large areas of the tropics, subtropics and in the Mediterranean basin, globally spanning more than 98 countries and territories. There are about 350 million people at risk and some 12 million cases, with an estimated worldwide annual incidence of 0.7–1.2 million cases of CL and 0.2–0.4 million cases of VL (Alvar *et al.* 2012). *Leishmania donovani* in the Old World and *L. infantum* in both the Old and the New World are responsible for a large majority of visceral forms of the disease. Other cases of VL are caused occasionally by *L. siamensis* (Old World), *L. martiniquensis* (New World) and *L. colombiensis* (New World) (Cnudde *et al.* 1994; Akhoundi *et al.* 2016) (Fig. 2).

SOUTH AMERICAN LEISHMANIASES

In South America, all clinical forms of leishmaniasis are encountered. About 33% of CL cases occur in Brazil, but CL also have a great health impact on the entire subcontinent. More than 90% of VL cases occur in Brazil. In other countries (Alvar *et al.* 2012; Ready 2014), a very low endemicity is observed whereas

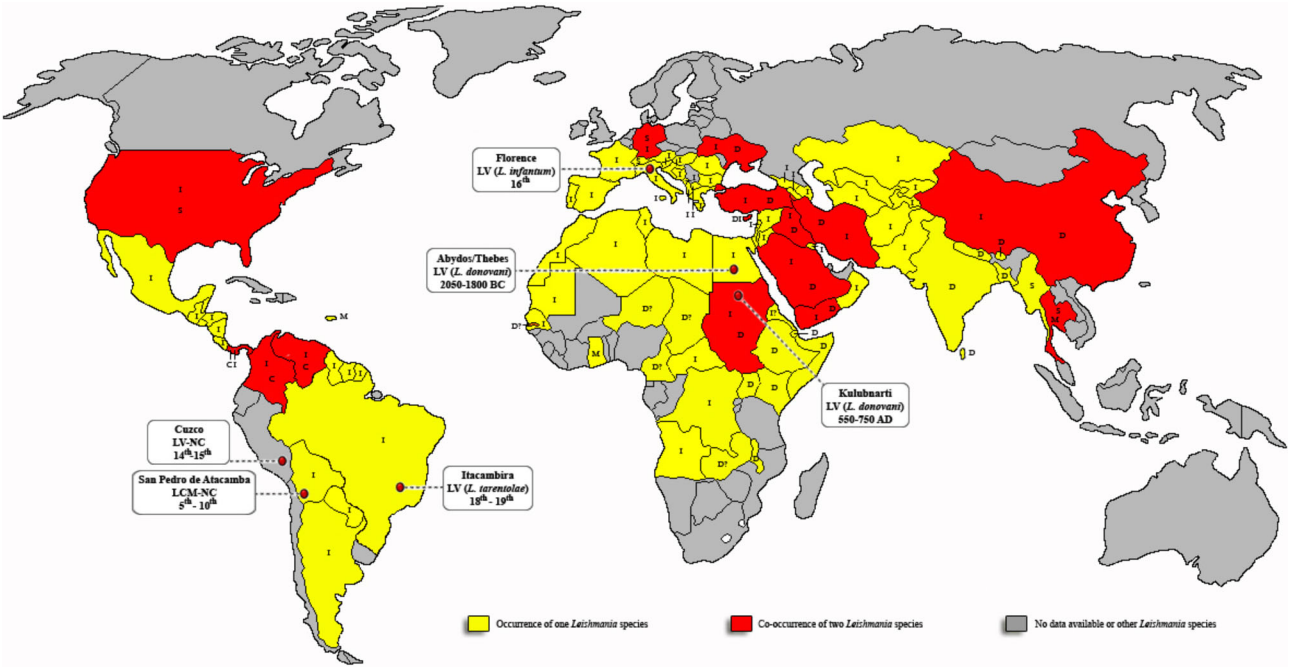

Figure 2. Worldwide distribution of visceralizing human *Leishmania* species and location of human remains where the presence of *Leishmania* DNA were investigated. NC: Identification of the *Leishmania* at the species level not sufficiently precise. I: *L. infantum*, D: *L. donovani*, C: *L. colombiensis*, S: *L. siamensis*, M, *L. martiniquensis*.

AVL is absent in Bolivia. In South America, the first descriptions of lesions reminiscent to those observed for current cutaneous forms are described as earlier as 1000 BC. The notification of a disease named 'valley sickness' or 'Andean sickness' or 'white leprosy' is mentioned during the 15th and 16th centuries AD that correspond to the Inca period. Proofs of a *Leishmania* infection are documented in a 6-year-old pre-Columbian female mummy (700–800 AD) (Gerszten, Allison and Maguire 2012). All these reports are likely to correspond to South American CL or MCL. The current cases of AVL diagnosed are all caused by *Leishmania infantum* initially named *L. chagasi*. Until now, AVL has never been documented in pre-Columbian individuals.

Three major hypotheses are proposed concerning the evolutionary history of the *Leishmania* genera and subgenera. Nevertheless, there are still missing elements from the puzzle to choose between the three evolutive scenari, which are as follows:

i. The Palearctic hypothesis proposes the entrance of the *Leishmania* ancestor (evolved from lizard *Leishmania*) in the Nearctic across the Beringia land bridge during the Oligocene (33–23 Mya), spreading across the Northern continent until it reaches the Neotropics (Kerr, Merkelz and MacKinnon 2000) via the isthmus of Panama around 2.8 Mya (O'Dea *et al.* 2016).
ii. The Neotropic hypothesis assumes that the genus *Leishmania* has probably evolved in South America during the Paleocene or Eocene era (46–36 Mya). Then it has migrated via Beringia into Asia and dispersed towards India and Africa where major diversifications have occurred during the Miocene (24–14 Mya), giving rise to the various *Leishmania* complexes (Croan, Morrison and Ellis 1997; Noyes *et al.* 2000; Lukes *et al.* 2007). *Leishmania infantum* has split from the early *L. donovani* lineage about 1 Mya ago (Kerr, Merkelz and MacKinnon 2000).
iii. The supercontinent hypothesis is based first on the division of *Leishmania* into two lineages on Gondwana (*Euleishmania* and *Paraleishmania*) and followed by the progressive separation of South America after the breakup of Gondwana during the late Cretaceous (100–90 Mya) (Harkins *et al.* 2016). A subsequent *Leishmania* migration back to the New World would be required during the mid-Miocene via the Nearctic and Beringia.

Regarding the origin of *L. infantum* in South America, it is believed that 500 years ago, *L. infantum* has crossed the Atlantic Ocean through Iberian colonization via infected dogs and reached Central America during the 16th century, causing AVL (Momen, Grimaldi and Deane 1987; Mauricio, Stothard and Miles 2000; Alvar *et al.* 2008; Kuhls *et al.* 2011; Leblois *et al.* 2011). As mentioned above, beside *L. infantum*, *L. colombiensis* (*Paraleishmania* section) causes VL in South America, although only few cases have been reported and documented (Kreutzer *et al.* 1991; Delgado *et al.* 1993; Rodriguez-Bonfante *et al.* 2003). In addition, *L. martiniquensis*, which belongs to the Mundinia section (Fig. 1), is the causative agent of VL in Martinique island (Cnudde *et al.* 1994). Unusual and rare clinical presentations, with a dissemination of parasites into internal organs, are well documented in some individuals infected by a *Leishmania* species that usually causes cutaneous forms. These are documented in the Old and New World (Barral *et al.* 1986, 1991; Barro-Traoré *et al.* 2008; Sarkari *et al.* 2016; Spotin and Parvizi 2016). Such unusual clinical presentation is frequently but not exclusively associated with the immune-suppression caused by human immunodeficiency virus co-infections for instance (Hernández *et al.* 1993, 1995; Ramos-Santos *et al.* 2000; Alvar *et al.* 2008; Zijlstra 2014). The dysfunction of the T-helper 1-mediated immune response for example is also likely to interfere with the progression, severity and clinical features of leishmaniases (Fishman 2011; Floral *et al.* 2014; Geiger *et al.* 2016).

AVL: WHAT PRE-COLUMBIAN MUMMIES TEACH US?

For the first time, Santiago-Rodriguez *et al.* (2016) gave evidences for the existence of *Leishmania* parasites with visceralization capabilities, prior to the Iberian colonization. Using as template the complete genome of *Leishmania donovani,* a species closely related to *L. infantum,* they assigned DNA fragments extracted from the gut of two pre-Columbian mummified individuals from Cuzco, as *Leishmania*. The post-Columbian origin of *L. infantum* in South America and the absence of its proven or suspected vectors (i.e. *Lutzomyia almerioi*, *Lu. atunesi*, *Lu. cruzi*, *Lu. forattenii*, *Lu. longipalpis*, *Lu. migonei*, *Lu. olmeca*, *Lu. ovallesi*, *Lu. pseudolongipalpis*, *Lu. sallesi*) in the area of Cuzco also argue against *L. infantum* as the *Leishmania* species infecting the mummies.

Because of the multiplicity of hypotheses regarding the *Leishmania* species involved, it is crucial to identify accurately the *Leishmania* found in the digestive tract of the pre-Columbian mummies:

i. On the one hand, the infection could be caused by a *Leishmania* species, usually known to be responsible for cutaneous forms, associated or not with concomitant infection or disease. It would be interesting to gather more information regarding the presence of parasitic, viral or bacterial co-infection(s) that may cause immunosuppression. The microbiome analyses should give us some leads.
ii. On the other hand, these findings could be indicative of the presence of an active indigenous transmission cycle of AVL during the pre-Columbian period. The only other known species of *Leishmania,* which causes AVL in continental South America, endemic to this geographic area, is *L. colombiensis*. Its active transmission cycle may have occurred either in Cuzco or in the neighboring countries, which were, at that time, part of the Inca Empire. Although the proven vector for *L. colombiensis* is currently unknown, this species has been isolated in the endemic area from *Lu. hartmanni* and *Lu. shannoni* (Kreutzer *et al.* 1991; Ramírez *et al.* 2016), and is able to develop in colony-reared *Lu. trapidoi* (Kreutzer *et al.* 1991). Interestingly, the presence of *Lu. hartmanni* has been reported in the area of Cuzco (Sanchez-Saldaña *et al.* 2004), which adds further evidences supporting this hypothesis.
iii. Another possibility about the origin and the identity of the infection in these pre-Columbian mummies would involve another *Leishmania* species not yet identified. The Amazon basin contains a large diversity of *Leishmania* species. Only a small proportion of them have been described as formal species. The current restrictions on the capture and sacrifice of sylvatic animals for research purposes and the difficulties to conduct field works make that most of this diversity remains underexplored. It is possible that further indigenous parasites, not yet identified, capable of causing VL could be present. Therefore, the discovery, in the area of Cuzco or in the neighboring Amazonian basin, of an active transmission cycle involving a *Leishmania* species, which possesses the capacity to disseminate into internal organs, cannot be ruled out.
iv. The last hypothesis implies that the etiological agent of the VL identified in pre-Columbian mummies by Santiago-Rodriguez *et al.* (2016) was introduced in South America together with the dogs brought by early human migrants (Van Ash *et al.* 2013) from East Asia by 14.6 Kya/13 Kya (date of the split between Northern and Southern branches) or by these migrants themselves (Raghavan *et al.* 2015). This agent could be related to *L. donovani* or *L. infantum*. Nevertheless, traces of such event have not been found in genetic analyses of isolates from South American patients (Lukes *et al.* 2007).

Therefore, studies performed on pre-Columbian mummies require the identification of the exact causative agent (if not *L. infantum/L. chagasi*). It could be rather related to an indigenous species and/or to *Leishmania* parasites whose identity and origin have to be investigated. This would not be incompatible with the common hypothesis of an introduction of *L. infantum* through the Iberian conquest but would simply imply that *Leishmania* species with visceralization capabilities were already present before the arrival of the Spanish and Portuguese conquistadores.

QUEST FOR ANCIENT LEISHMANIA DNA IN HUMAN REMAINS

Ancient microbial DNA (amDNA) like ancient DNA (aDNA) fragments are characterized by highly fragmented DNA (more or less 100 nt) and DNA damages such as cytosine deaminated DNA molecules (Dabney, Meyer and Pääbo 2013). In the aDNA field, such short DNA fragments are often present in environmental DNA samples which make difficult the authentication of DNA fragments extracted from ancient remains. Before the emergence of the NGS, a very stringent procedure, including a replication of the results in an independent laboratory, was used to identify the genetic material. Clearly, the advent of NGS allowed for a massive output of aDNA sequences, giving the opportunity to explore in more detail the features of ancient DNA fragments like deamination toward the ends of the fragments (Briggs *et al.* 2007; Overballe-Petersen, Orlando and Willerslev 2012). But three things complicate the analysis of amDNA: the short length of aDNA fragments, the possible lack of reference genomes to match with and the fact that there is a high-sequence similarity within and among taxa when short genetic sequences are used. The genus *Leishmania* is a slow evolving monophyletic group (Marcili *et al.* 2014). Comparative genomic analysis of *Leishmania* revealed divergence at the gene or pseudogene level with genes that are restricted to individual species (Peacock *et al.* 2007).

The colonization of ancient samples after the individual's death until its excavation or laboratory contamination could confound the analysis. Laboratory contamination by *Leishmania* DNA is unlikely to happen without the presence of *Leishmania* cell cultures and/or purified *Leishmania* DNA during samples processing. The vector of transmission of *Leishmania* to their hosts is through insect bite. Therefore, potential contamination of human remains, after the death, is not possible because *Leishmania* has no ability to survive out of its vertebrate (mammals, lizards, etc.) or invertebrate (Phlebotominae sand fly) hosts.

The use of amDNA to address the evolutionary history of the *Leishmania* genera and subgenera will benefit from NGS advances. To perform such analyses, it will be necessary to have more reference genomes, representative of the currently known diversity of the *Leishmania* genera and subgenera. For instance, 16 complete genomes representative of 14 *Leishmania* species are currently available to perform such analysis (http://tritrypdb.org/tritrypdb/). The majority of these genomes refer to species belonging to the *Leishmania* subgenus of the *Euleishmania* section. Within the *Paraleishmania* section, only *Endotrypanum monterogeii* (strain LV88) was sequenced. First, it is now essential to have more reference genomes to implement NGS metagenomic accurate assignation of ancient *Leishmania* DNA. Second, the use of appropriate molecular markers on which a target-enrichment

strategy is amenable (Schuenemann *et al.* 2011) will help to perform confident *Leishmania* species assignation (Akhoundi *et al.* 2017).

Because of the diversity encompassed by leishmaniases (including a spectrum of diseases caused by more than 20 *Leishmania* species), the type of tissue sampled for the analysis will address different questions. CL, the most common *Leishmania* symdrome, affects the skin with lesions often occurring in the area of the bites of infected sand flies. But a minority of cutaneous infections caused by species of the *Vianna* subgenus is associated with concomitant or late ML and can result in destructive lesions of the nasopharyngeal/laryngeal mucosa (Aronson *et al.* 2016). These types of lesions are often observed in mummies of the pre-Columbian era (see Novo *et al.* 2016). Therefore, the body areas exposed to the bites of sand flies, i.e the hand, the face, the ears, the legs, and so on , are most likely to contain ancient *Leishmania* DNA. Unlike CL, VL and AVL do not produce visible lesions. VL shows the dissemination of *Leishmania* parasites throughout the reticulo-endothelial system and their ability to colonize and replicate into various internal organs (spleen, liver, bone marrow, etc.) (see for review Akhoundi *et al.* 2016; Aronson *et al.* 2016).

CONCLUSIONS

Modern techniques aimed at identifying ancient pathogen DNA not only help to identify a disease but also provide information on its frequency and evolutionary origin. They can also trace back sociocultural contacts and their potential role in the transmission and spread of infectious diseases. These techniques have successfully detected *Leishmania* infections in ancient Egyptian and Nubian mummies. They also gave crucial information on contacts between these two populations during the Middle Kingdom of Pharaonic Egypt (2050–1650 BC) (Zink *et al.* 2006) (See Fig. 2). This methodology showed the occurrence of visceral leishmaniasis in remains of Eleonora from Toledo, the wife of Cosimo I de Medicis, a famous person of the Italian Renaissance (Nerlich *et al.* 2012). They have also revealed some unconventional *Leishmania* infections, like the presence of a DNA signature of a non-human pathogenic *Leishmania* species, namely *L. tarentolae*, in the rib, skin and abdominal regions of a 300 -year-old human Brazilian mummy (Novo *et al.* 2015). Finally, it is a way to highlight the past occurrence of leishmaniases in currently leishmaniasis-free areas, but the identification of the exact species was not performed (Costa *et al.* 2009).

Based on what is known about the evolutionary history and epidemiology of AVL, the findings presented by Santiago-Rodriguez *et al.* (2016) emphasize the interest of studying pathogens causing infectious diseases in the light of ancient DNA. It opens new perspectives on the pre-Columbian *Leishmania* epidemiology, the clinical forms present during the pre-Columbian period and the identity of the causative agents.

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AUTHORS' CONTRIBUTIONS

DS and PP drafted the manuscript. All finalized the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest. None declared.

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