Reply to Bastien et al

To the Editor—We thank Bastien et al for their comments on our recent article [1]. However, we completely disagree with them since our findings concerning the presence of *Leishmania* in the feces of wild gorillas represent a major point of concern for public health in Cameroon. The application of modern diagnostic tools can determine information about emerging pathogens and can document spatial changes in the distribution of these agents and theirs hosts and vectors.

Before responding scientifically to the comments from Bastien et al, we need to highlight some points. First, our work underwent extensive peer review before it was accepted and published as an article containing original research findings. Moreover, our laboratory is considered one of the pioneering laboratories in the world, providing advanced diagnostic laboratory services (with >400 000 polymerase chain reaction [PCR] analyses/year). Additionally, it is a reference laboratory for many of the world's emerging and neglected infectious diseases.

We are confident in the PCR results from our article [1] because we performed 3 different PCR analyses on 3 different sequences of *Leishmania* organisms (18S ribosomal DNA, internal transcribed spacer region, and Cytb gene), in addition to the first screening of the stool samples via real-time PCR. Furthermore, we excluded the possibility of contamination of these fecal samples by *Leishmania* DNA from our laboratory, as we did not use any positive control during the PCR procedure, and until recently, we had never performed PCR on this organism. Moreover, feces from gorillas have previously been shown to contain other bloodborne pathogens, such as *Plasmodium falciparum* [2], *Rickettsia felis* [3], and human immunodeficiency virus [4]. For these reasons, it is also possible that these great apes may be reservoirs for *Leishmania* organisms, which is how other neglected or emerging human pathogens spread in Africa. Finally, both *Leishmania*-positive and *Leishmania*-negative stool samples from gorillas, as well as DNA, were sent to Switzerland for external, blind molecular validation by Dr G. Greub (a coauthor on this reply). His laboratory results are in agreement with our findings. Indeed, in his laboratory, using their accredited *Leishmania*-specific PCR used for diagnostic purposes (ie, a PCR adapted from Mary et al [5]), he amplified all 5 native samples blindly submitted, with high parasite load of about 500–18 000 copies/5 μL. These positive results are unlikely due to PCR contamination, since in the setting of the diagnostic laboratory of Lausanne's university hospital, which processes >25 000

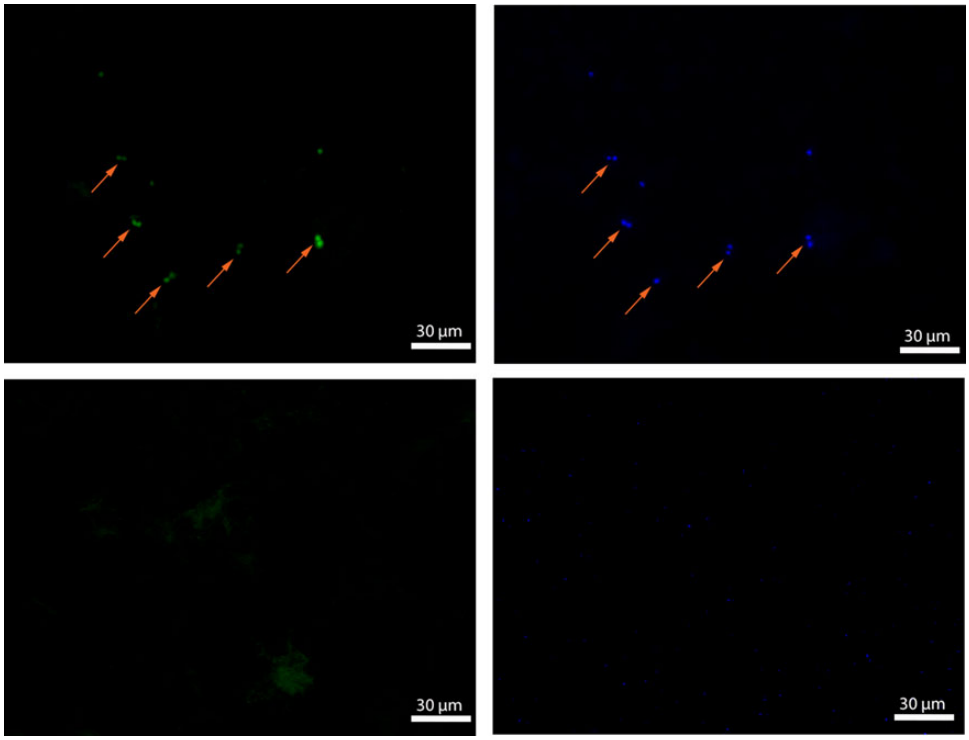

Figure 1. Confocal microscopy images of a stool sample stained with the FAM-labeled 18S ribosomal RNA *Leishmania*-specific probe Leish_18S_651 (left panels) and the nuclear dye DAPI (right panels). The top panels show a fecal sample positive for *Leishmania* organisms by polymerase chain reaction (PCR) analysis. The bottom panels show a sample negative for *Leishmania* species by PCR.

PCR analyses/year, false-positive results due to PCR contamination are rarely experienced (<1 case/10 000 PCR analyses), and when they occurs, only very low numbers of DNA copies are amplified, a situation contrasting with the high parasite load observed here in all 5 positive samples received. Please note that the primers used here (primer 1, CTTTTCT GGTCCTCCGGGTAGG; primer 2, CCA CCCGGCCCTATTTTACACCAA; and probe, TTTTCGCAGAACGCCCCTACC CGC [Fam-MGB]) are relatively specific since they do not amplify all *Leishmania* species, but they have a sequence 100% similar to that of the kinetoplast of *Leishmania major*. Finally, the minor-groove format of the probe further increases the specificity of this PCR.

We also disagree with Bastien et al in their contention that the ingestion of sandflies by primates seems unlikely and has never been reported. Recent studies showed that insect consumption by apes could be achieved either directly (through insect predation) or indirectly (incidentally, when apes feed on different plant parts containing insects that burrow into it or feed on them) [6, 7]. Indeed, DNA sequences that belong to small insects, such as sandflies and mosquitoes, have been detected in the feces of nonhuman primates through extensive molecular analysis [6, 7]. For this reason, we speculated that the detected promastigote form of *Leishmania* organisms in the fecal samples might result from the fact that the vectors are part of the diets of these wild animals. However, we agree that sand flies do not feed on feces; nevertheless, we do not exclude the possibility that these flies may be trapped in feces. A very recent study showed that the presence of animal dung is considered a factor that is associated with *Phlebotomus argentipes* density [8].

In their letter, Bastien et al comment on the experimental results obtained via fluorescence in situ hybridization (FISH) and their doubts concerning the amastigote form of *Leishmania* species. The FISH staining of the stool samples performed in our work used the specific Leish_18S_651 probe in combination with DAPI staining. This *Leishmania*-specific probe was adapted from Frickmann et al [9] and correctly identified all tested *Leishmania* species and excluded closely related species (*Trypanosoma* species). In parallel with this probe, DNA staining with DAPI allowed the authors to reliably differentiate specific probe binding from autofluorescing cells. We used the same strategy (ie, DAPI counterstaining with a *Leishmania*-specific probe) to perform this discrimination, and we are confident in our results. We have also used FISH to analyze stool samples from gorillas that were *Leishmania* negative by PCR. Figure 1 shows no cells that are double positive by means of the Leish_18S_651 probe and DAPI staining

in these samples, which confirms the specificity of the amastigote staining observed in Figure 3*D* and 3*E* from our earlier article [1]. This pattern was similar in all 3 PCR-negative stool samples tested, and in each sample, 3 different areas were analyzed. Moreover, we could further confirm the presence of the amastigote form of *L. major* in gorilla stool samples from the sand fly PCR results obtained in this study (Table 1 from our earlier article [1]). FISH staining indicated an absence of the promastigote form of *L. major* in stool sample number 69 (Table 1 from our earlier article [1]), while results of sand fly PCR on the same sample also indicated the absence of sand flies in the stool sample. This result confirms that the positive signal from the FISH staining of sample 69 belongs to the amastigote form, rather than the promastigote form, because the amastigote form is not found in sand fly hosts (Table 1 from our earlier article [1] and Figure 1).

We agree that a serological survey would be much more convincing and might answer many questions: Are these wild gorillas really infected with *Leishmania* species or are they only carriers? If they are infected, is it only a local infection (cutaneous leishmaniasis) or a systemic infection (due to the presence of *Leishmania* amastigotes in gorilla feces)? In addition, how was *L. major* introduced to the stool samples? Unfortunately, it is impossible to collect blood specimens from theses endangered animals, and even the observation of these protected animals requires difficult-to-obtain authorization.

Finally, many factors, such as global climate change, urbanization, and immigration, have effects on different microorganisms, their vectors, and their reservoir. These interactions lead to changes in the incidence and natural distribution of infectious diseases far from their areas of endemicity [10, 11]. Furthermore, Dr B. Dondji, who is among the authors who commented on our article, referred to 326 cutaneous leishmaniasis cases; most of these cases were seen in immigrants from the southern forest of Cameroon [12]. He has also commented on the necessity of identifying new probable foci in the southern forest and determining the repertory of this parasite in that area [12]. Thus, we think that application of new tools, such as molecular biology, in the field of ecological parasitology could shed light on previously unrecognized zoonotic risks for the local population and pave the way to new discoveries regarding parasite life cycles, epidemiological data, and the detection and identification of parasites, their transmission, and colonization.

Notes

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Ibrahim Hamad,[1] Claire-Lise Forestier,[1] Gilbert Greub,[2] Katia Jaton,[2] Didier Raoult,[1] and Fadi Bittar[1]

[1]Aix Marseille Université, URMITE, UM63, CNRS 7278, IRD 198, Inserm 1095, France; and [2]Institute of Microbiology, University of Lausanne and University Hospital Center, Switzerland

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Correspondence: Fadi Bittar, PhD, Aix Marseille Université, URMITE, UM63, CNRS 7278, IRD 198, Inserm 1095, 27 BD Jean Moulin, Marseille 13005, France (fadi.bittar@univ-amu.fr)

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