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Letter to the Editor

Remarks on identification of amplified fragment length polymorphisms linked to SAG resistance in *Leishmania*

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Dear Editor,

In a previous issue of Acta Tropica, Kumar et al. (2009) report on the use of AFLP to document genetic polymorphisms between SAG resistant and sensitive *Leishmania* isolates. Even though AFLP is a powerful tool for random screening of DNA polymorphisms in an organisms genome (Vuylsteke et al., 2007), the results presented in this paper raise some serious issues on the quality of the generated data for the following reasons:

(1) Many studies on *Leishmania donovani* have reported on the clonal spread and homogenous nature of the parasite population in the Indian subcontinent, evidenced from both coding and non-coding sequences (Mauricio et al., 2001, 2004; Manna et al., 2005; Pandey et al., 2007; Alam et al., 2009). In view of this extensive similarity, a random screening such as AFLP would typically result in a vast majority of monomorphic fragments, with only minor polymorphism. However, the authors report on 55.35% polymorphic AFLP fragments. In other words, only 44.65% of the *L. donovani* genome of the studied isolates would be conserved, which is far less than one would expected from analyzing different species of the *Leishmania* genus (Peacock et al., 2007).
(2) Given that the genome size of *L. donovani* is around 32 Mbp (Peacock et al., 2007), one can estimate the average number of AFLP fragments expected from a given enzyme and primer combination. The authors use EcoRI as a 6-cutter, which would generate around 7800 fragments. The frequent cutter used is Tru9I, which is expected to cut within each of these fragments, yielding about 15,600 EcoRI–Tru9I templates. When using 3 selective nucleotides from each side, these 15,600 EcoRI–Tru9I fragments are amplified by 4096 primer combinations, yielding on average about 4 fragments per AFLP run. Yet the authors report a multitude of this. This calculation is even overestimating the expected number of fragments, as the EcoRI/Tru9I enzyme combination is most useful in AT-rich genomes. In GC-rich genomes such as *Leishmania* (60% GC; Peacock et al., 2007), this primer combination would result in an even lower number of bands. The above calculations make abstraction of the kinetoplast DNA, which as evidenced from kDNA diversity studies (Singh et al., 1999; Laurent et al., 2007) accounts for a negligible fraction of the organisms genetic complexity.
(3) The authors claim to have identified polymorphisms linked with SAG resistance, but these have not been validated for more than the 6 isolates studied. Statistically, in view of the fact that each polymorphic band is either present or absent in a particular isolate, there are 2 to the power 6 possible combinations for each polymorphic marker (000001, 000010, 000011, etc.), being 64. Two of these combinations (or 1 in 32) separate 3 random strains from the remaining 3, e.g. 111000 and 000111. Taking into account the 1294 polymorphic fragments reported (Table 1 in Kumar et al., 2009), one would expect purely by chance that 1294 divided by 32, or about 40 polymorphic markers are characteristic for any 3 combinations of isolates. The fact that the authors identify 42 markers that separate SAG resistant from sensitive isolates (Table 3 in Kumar et al., 2009) is thus nothing more than what one would statistically expect from studying only 6 isolates, and hence has no meaning unless validated on a larger panel.
(4) AFLP fingerprinting is a highly sensitive technique, influenced by minor experimental variations. The authors are quite vague about the exact strategy that was followed, and no data on repeatability and reproducibility are given.

In view of these restrictions, much care should be taken not to over interpret the data presented in this publication. Essential quality controls for every AFLP approach are the presence of more monomorphic than polymorphic bands, certainly when dealing with closely related organisms; and the repeatability of the experiments, criteria not met by the paper here discussed. Only by using the correct experimental conditions, we believe that AFLP can contribute significantly to the search for markers related to particular phenotypes of *Leishmania* parasites.

References

Alam, M.Z., Kuhls, K., Schweynoch, C., Sundar, S., Rijal, S., Shamsuzzaman, A.K.M., Raju, B.V.S., Salotra, P., Dujardin, J.C., Schönian, G., 2009. Multilocus microsatellite typing (MLMT) reveals genetic homogeneity of *Leishmania donovani* strains in the Indian subcontinent. Infect. Genet. Evol. 9, 24–31.
Kumar, A., Boggula, V.R., Sundar, S., Shasany, A.K., Dube, A., 2009. Identification of genetic markers in Sodium Antimony Gluconate (SAG) sensitive and resistant Indian clinical isolates of *Leishmania donovani* through amplified fragment length polymorphism (AFLP). Acta Trop. 110, 80–85.
Laurent, T., Rijal, S., Yardley, V., Croft, S., De Doncker, S., Decuypere, S., Khanal, B., Singh, R., Schönian, G., Kuhls, K., Chappuis, F., Dujardin, J.C., 2007. Epidemiological dynamics of antimonial resistance in *Leishmania donovani*: genotyping reveals a polyclonal population structure among naturally-resistant clinical isolates from Nepal. Infect. Genet. Evol. 7, 206–212.
Manna, M., Majumder, H.K., Sundar, S., Bhaduri, A.N., 2005. The molecular characterization of clinical isolates from Indian Kala-azar patients by MLEE and RAPD-PCR. Med. Sci. Monit. 11, BR220–BR227.
Mauricio, I.L., Gaunt, M.W., Stothard, J.R., Miles, M.A., 2001. Genetic typing and phylogeny of the *Leishmania donovani* complex by restriction analysis of PCR amplified gp63 intergenic regions. Parasitology 122, 393–403.
Mauricio, I.L., Stothard, J.R., Miles, M.A., 2004. *Leishmania donovani* complex: genotyping with the ribosomal internal transcribed spacer and the mini-exon. Parasitology 128, 263–267.

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Pandey, K., Yanagi, T., Pandey, B.D., Mallik, A.K., Sherchand, J.B., Kanbara, H., 2007. Characterization of *Leishmania* isolates from Nepalese patients with visceral leishmaniasis. Parasitol. Res. 100, 1361–1369.
Peacock, C.S., Seeger, K., Harris, D., Murphy, L., Ruiz, J.C., Quail, M.A., Peters, N., Adlem, E., Tivey, A., Aslett, M., Kerhornou, A., Ivens, A., Fraser, A., Rajandream, M.A., Carver, T., Norbertczak, H., Chillingworth, T., Hance, Z., Jagels, K., Moule, S., Ormond, D., Rutter, S., Squares, R., Whitehead, S., Rabbinowitsch, E., Arrowsmith, C., White, B., Thurston, S., Bringaud, F., Baldauf, S.L., Faulconbridge, A., Jeffares, D., Depledge, D.P., Oyola, S.O., Hilley, J.D., Brito, L.O., Tosi, L.R.O., Barrell, B., Cruz, A.K., Mottram, J.C., Smith, D.F., Berriman, M., 2007. Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. Nat. Genet. 39, 839–847.
Singh, N., Curran, M.D., Middleton, D., Rastogi, A.K., 1999. Characterization of kinetoplast DNA minicircles of an Indian isolate of *Leishmania donovani*. Acta Trop. 73, 313–319.
Vuylsteke, M., Peleman, J.D., van Eijk, M.J.T., 2007. AFLP technology for DNA fingerprinting. Nat. Protoc. 2, 1387–1398.

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