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Genetic markers for antimony resistant clinical isolates differentiation from Indian Kala-azar

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ABSTRACT

Visceral Leishmaniasis or Kala-azar is caused by the protozoan parasites belonging to the Genus *Leishmania*. Once thought eradicated from the Indian subcontinent, the disease came back with drug resistance to almost all prevalent drugs. Molecular epidemiological studies revealed the polymorphic nature of the population of the main player of the disease, *Leishmania donovani* and involvement of other species (*L. tropica*) and other genus *(Leptomonas)* with the disease. This makes control measures almost futile. It also strongly demands the characterization of each and every isolate mandatory which is not done. In this background, the present study has been carried out to assess the genetic attributes of each clinical isolates (n = 26) of KA and PKDL patients from India and Bangladesh. All the isolates were characterized through Restriction Fragment Length Polymorphism (RFLP) analysis to ascertain their species identity. 46.2% of the isolates were found to be Sodium Stibogluconate (SSG) resistant by amastigote-macrophage model. When the clinical isolates were subjected to Single Stranded Conformation Polymorphism (SSCP) of Internal Transcribed Spacer 1 (ITS1), Internal Transcribed Spacer 2 (ITS2) and some anonymous markers, the drug resistant *Leishmania* isolates of SSG can be distinguished from the sensitive isolates distinctly. This study showed for the first time, the genetic markers for SSG drug resistance of Indian Kala-azar clinical isolates.

1. Introduction

Leishmaniases are a spectrum of protozoan diseases spanning a variety of disease forms that differ in their epidemiology, pathogenesis and clinical manifestations. The visceral form of the disease known as Visceral Leishmaniasis (VL) or Kala-azar (KA) occurs in India, Bangladesh, Brazil, Iran, Nepal and Sudan (Sundar, 2001a) and is most fatal, if left untreated. A proportion of apparently cured KA patients (6–10%) may expand to Post Kala-azar Dermal Leishmaniasis (PKDL) (Thakur and Kumar, 1992). Along with immune suppression and co – infection with HIV, another serious health problem related to it is the emergence and progression of resistance to the commonly used antimonial drugs (Desjeux and Alvar, 2003; Dujardin, 2006). Approximately eight decades ago, the pentavalent antimonial drug, urea stibamine was brought to the Indian subcontinent (Brahmachari, 1922) for the treatment of KA but the effectiveness of this drug had reduced with time, even with increasing the dose and period of treatment (Lira et al., 1999; Croft et al., 2006). It was reported that Leishmania parasites resistant to antimonials is related to Sodium Stibo Gluconate (SSG) treatment breakdown in the Indian province of Bihar (Lira et al., 1999; Dube et al., 2005) and accounts for 60%-70% treatment failure in this region (Sundar et al., 2000). On the other hand, in the neighboring country Nepal, VL patients from endemic regions, infected with SSG-resistant parasites were found to have only a 25% unsuccessful SSG treatment (Rijal et al., 2007). Interestingly, recent report suggested the development of *Leishmania* antimonial resistance in the population of Bihar occurred in some cases was through the distribution of arsenic contamination of ground water (Perry et al., 2015). Antimony and arsenic are the two important elements as they have a long therapeutic history and in the periodic table, they are related to each other through distribution of numerous similar chemical properties (Yan et al., 2005). The naturally occurring triva-

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lent arsenic is able to contaminate the groundwater (Chakraborti et al., 2003) and in the Bihar state of India, the people were at risk of arsenic exposure from naturally occurring trivalent arsenic contamination in the groundwater accessed by them from the some shallow tube wells as a source of drinking water. Historically, when the antimonial resistance of *Leishmanial* parasite was suspected for the first time in 1980s, trivalent arsenic was used in the laboratory to make antimonial cross-resistance in order to divulge the mechanisms of resistance (Dey et al., 1994). In ten out of thirty districts in Bihar, the arsenic exposure, endemic Visceral Leishmaniasis (VL) and antimonial resistance are coexisted. Perry et al. hypothesized that the selection processes are similar in case of VL patients who have been chronically exposed to environmental arsenic and parasite resistance to arsenic and as a consequence of this, the cross resistance to antimonial drugs may be obtained (Perry et al., 2013). Second line treatment with Amphotericin B (AmB), though highly efficacious (Jha et al., 1995), is associated with serious side effects and can only be administered in hospital settings. Other alternative drugs remain largely unsatisfactory due to high cost (AmBisome) (Sundar, 2001a), high toxicity (Pentamidine) (Sundar, 2001a) or side effects encompassed with ototoxicity, injection site pain and increased level of liver enzymes (Paromomycin) (Moore and Lockwood, 2010). Resistance against AmB has also been shown by the clinical isolates of KA (Purkait et al., 2012). Miltefosine (ML), the first oral anti cancer drug which is an alkyl phospholipid compound, also known as hexadecylphosphocholine (Sundar, 2001b; Sundar et al., 2002) had been approved in India for the treatment of Indian VL patients, including cases unresponsive to antimonial and has achieved more than 97% cure rate (World Health Organization, 2010; Sundar et al., 2012). Within few years, unresponsiveness to MIL in VL (Arif et al., 2008; Das et al., 2013) was reported. Thus, insensitivity to the current drugs and lack of alternative, effective drugs are the dual troubles that have made control of KA an extremely hard task. From epidemiological point of view, association of other species, *L. tropica* (Sacks et al., 1995; Khanra et al., 2012) and other genus, *Leptomonas* (Srivastava et al., 2010) with Indian Kala-azar makes the scenario more complicated.

It was reported that the origin of SSG resistant clinical isolates of KA were polyclonal (Laurent et al., 2007; Downing et al., 2011). At the same time, heterogeneity was present among SSG sensitive and resistant clinical isolates of KA (Kumar et al., 2009). It is currently not known whether any putative marker is common to all antimony resistant parasites (Decuypere et al., 2012).

As stated earlier, unresponsiveness to MIL in VL (Arif et al., 2008; Das et al., 2013) in addition to resistance against AmB (Purkait et al., 2012) and development of resistance to the commonly used antimonial drugs (Desjeux and Alvar, 2003; Dujardin, 2006) have already been reported (Rijal et al., 2013). Thus, whichever drug we choose for the treatment, after some years of its use, resistance will appear. The only way to move away from the curse is to understand the genetic attributes of each of the isolate so that the clue to control the parasite would come in to existence.

The prime aim of our study was to characterize each clinical isolate of Indian KA (here, n = 26) for phenotypes and genotypes towards the development of markers for drug resistance, if any. Various sophisticated molecular biological methods have been developed for the identification of *Leishmania* species on the basis of the genomic information of the parasites (Schönian et al., 2000; Manna et al., 2005; Khanra et al., 2011, 2012). We had divided our work into two directions: one, after ascertaining their species identity, characterize them on the basis of their drug sensitivity towards the drug SSG. The other attempt was comparative genotyping by Single Stranded Conformation Polymorphism (SSCP) of some marker genes or intergenic spacers present in the genomes of the clinical isolates (n = 26) collected in the recent past.

Our study revealed that 12 isolates (46.2%) out of 26 were SSG resistant. It further corroborated that significant polymorphisms exists in the population of *L. donovani* in India. Finally, it may be concluded that the antimony resistant clinical isolates of VL could be differentiated from sensitive ones by SSCP of ITS1, ITS2 and one anonymous marker, L0114. This is the first observation of its kind.

2. Materials and methods

2.1. Ethics statements

Collection of Bone marrow aspirates from Kala-azar patients were approved by the Ethical Committee of the Calcutta National Medical College, Kolkata. The written consents were obtained from every patient and guardians (in case of minors) prior to the study.

2.2. Leishmania parasites culture and reference strains

We have characterized twenty six (n = 26) clinical isolates from KA and PKDL patients of India and Bangladesh including some (n = 12) of our previous study (Khanra et al., 2011, 2012). The clinical isolates were collected in the years 2006 to 2013. Information concerning the patients' age, sex, clinical status and country of some of the isolates (n = 21) from KA and PKDL patients were previously reported (Mukhopadhyay et al., 2011; Khanra et al., 2011, 2012; Das et al., 2014) and the rest were illustrated in Supplementary Table S1. World Health Organization (WHO) reference strains for *L. donovani* (DD8), *L. tropica* (K27) and one *L. donovani* isolate (MHOM/IN/1983/AG83) were included in the study as reference strains. The existence of amastigotes in the bone marrow of the patients was confirmed by Giemsa staining as well as transformation into promastigote forms in culture medium M199 (St. Louis, MO, USA) with 10% Fetal Bovine Serum (FBS) (Gibco, USA).

2.3. Genomic DNA isolation

Genomic DNA was prepared from the parasite samples as previously described (Khanra et al., 2012).

2.4. Characterization of the isolates by PCR-RFLP method

ITS1 region was amplified from parasite genomic DNA of all samples with the following primers: LITSR (5′-CTGGATCATTTTCCGATG-3′)/L5.8S (5′- TGATACCACTTATCGCACTT-3′) (Eurofins Scientific GmbH, Germany) and the Hsp70 PCR amplification was performed with the primers HSP70sen (5′-GACGGTGCCTGCCTACTTCAA-3′) and HSP70ant (5′-CCGCCCATGCTCTGGTACATC-3′) (Eurofins Scientific GmbH, Germany) as described previously (El Tai et al., 2000; El Tai, 2004; Montalvo et al., 2010). The amplified products were subjected to ITS1 PCR-RFLP and Hsp70 PCR-RFLP analysis individually for species characterization, as described earlier (El Tai et al., 2000; Montalvo et al., 2010). PCR-RFLP patterns of some of the *Leishmania* parasites (n = 12) were reported earlier (Khanra et al., 2012). The ITS1 and Hsp70 PCR product of nuclear DNA of rest of the clinical isolates (n = 14) were digested with 5U HaeIII enzyme (Bioenzyme, USA) at 37 °C for 4 h, followed by analysis on 3% Agarose gel.

2.5. Cell line used for in vitro study

Murine Macrophage (MØ) like tumor cell, RAW 264.7 was obtained from American Type Culture Collection. Cells were maintained in complete RPMI 1640 medium with 10% FBS at 37 °C with 5% CO_2 in a humidified atmosphere.