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Original Article

In Vitro Low Responsiveness of *Leishmania donovani* Towards Sodium Antimony Gluconate

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Abstract

Kala-azar has been uprising concomitantly with drug-resistant strains of the causatinve agent, particularly in the neighbouring India. The actual perspective of drug resistance in *Leishmania donovani* in Bangladesh is yet to be explored. So, this prospective study, as a preliminary one, was done to observe *in vitro* drug responsiveness against Sodium Antimony Gluconate (SAG) and Amphotericin B of 41 strains of *L. donovani* isolated from Kala-azar cases. The cases (n=41) were selected from 45 clinically suspected febrile patients those who were positive for Kala-azar by immunochromatographic test (ICT). The selected cases were subsequently confirmed as Kala-azar by detection of Leishmania Donovan (LD) bodies from bone marrow aspirates (n=38) by microscopy and/or showing promastigotes in modified McNeal, Nicole and Novy (NNN) media (n=41). Minimum Inhibitory Concentrations (MICs) of SAG and Amphotericin B were seen in relation with history of previous SAG therapy of the patients. Among 08 strains with previous SAG therapy, MICs of SAG were 500 μg in 05 (62.5%) and 250 μg in 03 (37.5%) cases. In remaining 33 strains with no previous SAG therapy, MIC of the drug was 250 μg. In all 41 strains, MIC of Amphotericin B was 05 μg irrespective of the history of previous SAG therapy. The study revealed that strains of *L. donovani* with low responsiveness to standard dose of pentavalent antimonials have been started to appear in our community that needs further study at community level in a larger population.

Key words: *Leishmania donovani*, Low responsiveness, Sodium antimony gluconate

Introduction

Sodium antimony gluconate (SAG) was first introduced in the decade of 1940 and used as a first-line drug for the treatment of Visceral Leishmaniasis (VL). The drug is administered parenterally at a dose of 20 mg/kg body weight daily for 20-40 days.[1] If treatment with SAG is unsuccessful, Pentamidine is used, administered parenterally at a dose of 2-4 mg/kg body weight 1-3 times a week.[2] Amphotericin B is another drug of second choice administered as a slow intravenous infusion of 2 mg/kg body weight on alternate days for 20-40 doses. Lipid associated drug (Ambisome) is the Amphotericin B incorporated into Liposomes and has been used successfully to treat Kala-azar patients unresponsive to standard drugs.[3] The Ambisome is administered intravenously at 3 mg/kg body weight daily for 30 days in a 5% glucose solution as a slow drip. Ambisome can be used for all ages.[4] Recently, Miltefosine appeared to be the first orally effective agent for the treatment of VL given at a dose of 100 mg /day for 28 days for adults weighing more than 25 kg.[5] The drug has already been registered in India since March, 2001. Other drugs under investigation are Paromomycin, Imidazoles, Triazoles and Purine analogue-Allopurinol. Paromomycin, an antibiotic of the

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Aminoglycoside family was used in India and Kenya in combination with Sodium Stibogluconate (Sb) and found effective in treating unresponsive cases of VL.[6,7]

Major limitations considered in the treatment of Kala-azar are various side effects of the drugs and treatment failure. Up to 15% therapeutic failure occurs with antimonials worldwide. But, one study in Bihar, India documented treatment failure rate of 65% and two other studies recorded treatment failures at a rate of 37% and 42%.[8-10] Explanations of Antimony treatment failures include under-treatment due to inadequate supply of the drug and immunologic or pharmacokinetic defects in the host.[11] In Bangladesh, rate of treatment failure is roughly estimated as 5%,[12] which does not simulate with the clinical experiences and is expected to be higher. The hypothesized event of treatment failure is supposed to be due to emergence of resistance properties among strains of *L. donovani* against SAG. This would be a serious threat for public health in near future since incidence of Kala-azar has been uprising concomitantly with drug resistant strains of *L. donovani*.[11,12] So, it is an essential demand of time to elucidate the actual perspectives of drug-resistance in *L. donovani*. In this context, no study yet has been done or reported from Bangladesh.

Considering the background described above, any preliminary study on drug responsiveness of VL cases would stand rational for overall public health ground. And further advanced studies would be possible taking information from a base-line study. Therefore, the present study was designed to find out drug responsiveness among *L. donovani* of VL cases.

Methods

This prospective study was conducted in the Department of Microbiology, Mymensingh Medical College during the period from March, 2004 to February, 2005. Cases were selected from Mymensingh Medical College Hospital, Community Based Medical College Hospital and nearby Trishal Thana Health Complex. Relevant history, clinical findings, laboratory records and findings of follow-up of every case was recorded in a pre-designed data sheet and subsequently analyzed by computer programme SPSS version 12.0.

Clinically suspected febrile patients on the basis of prolonged low-grade fever, weight loss, hepato-splenomegaly, anemia with or without skin pigmentation, and history of previous SAG therapy (treatment failure) (n=45) were investigated for anti-leishmanial antibodies using K-39 antigen-based Immunochromatographic test (ICT). Cases showing ICT-positive results were subsequently confirmed as Kala-azar by detection of LD bodies from bone marrow aspirates by microscopy and/or showing growth of promastigotes in culture (n= 41).

Collection of bone marrow and culture into NNN media: Up to 1 ml of bone marrow was collected from iliac crest of each of the study cases at Microbiology departments of the study sites following standard procedure. Immediately after aspiration of bone marrow, thin films of the aspirate were made on at least three microscopic slides. At the same time, one drop of the marrow materials was inoculated into the water of condensation of two sets of tubes of the NNN media: one set of drug free media and another containing specific drugs following standard procedure.[13] After inoculation of marrow material into 2 drug free NNN tubes and 3 tubes of different strengths of each SAG (0.25 mg/ml, 0.5 mg/ml and 1.0 mg/ml) and Amphotericin B (05 µg, 10 µg, and 15 µg/ml) were incubated at 24°C in a thermostat-regulated refrigerator in the range of 18-25°C.[14]

Staining and microscopic examination of marrow film: The bone marrow films were stained with Leishman stain according to the previously described method.[15] At least 1000 fields per slide, preferably around the edges of the preparation, were examined to detect amastigote of *Leishmania donovani*. If any amastigote form was found in 1000 fields, the slide was reported as positive. Grading of the positive smears was done according to previously developed standard chart.[16]

Observation for Promastigotes: Promastigotes of *L. donovani* were detected from water of condensation of each of properly inoculated and incubated culture media by observing typical morphology and motility. On finding promastigotes in any such preparation, the corresponding

culture result was reported as positive. This procedure was started from the 3rd day of incubation onwards up to 7 days. Tubes showing no promastigotes within 7 days, kept incubated further upto 10 days. On 10th day, if no promastigote was found again, the tubes were reported as negative and discarded.[16]

Reporting of drug sensitivity: If any promastigote was seen in any drug-containing tube, the parasite was designated as unresponsive to that particular concentration of the drug.[14]

Results

The present study was conducted on a total of 45 subjects. Of them, 41 (91.11%) were confirmed as Kala-azar cases having parasite-positive laboratory findings. Majority of the cases (29, 64.4%) were in the age group of 2-12 years. Number of cases were found to decline gradually with increase in age. A minimum of 01 (2.44%) case was in the age group of 57 years and above. Total males were 27 (60.0%) and females 18 (40.0%) (Table I) giving a male to female ratio of 1.5:1.

The relationship between history of previous SAG therapy and *in vitro* MIC of the isolates of *L. donovani* promastigotes was considered and it was seen that MIC was higher among previous SAG history-positive cases. Out of 08 history-positive cases, the MIC of SAG was 500 µg/ml in 05 cases and 250 µg/ml in 03 cases, whereas in the remaining 33 history-negative cases, the MIC of SAG was 250 µg/ml. (Table II)

Table I: Age and gender distribution of study subjects

Age in years	Gender of the cases	
	Male	Female
2-12 (n=29)	17	12
13-23 (n=07)	04	03
24-34 (n=03)	01	02
35-45 (n=03)	02	01
46-56 (n=02)	02	00
> 56 (n=01)	01	00
Total (n=45)	27	18

Table II: Relationship between treatment failure and MIC of SAG

Treatment failure	MIC of the *L. donovani* promastigotes		
	250 µg/ml	500 µg/ml	1000 µg/ml
Yes (n=08)	03 (37.5%)	05 (62.5%)	00 (00%)
No (n=33)	33 (100.0%)	00 (00%)	00 (00%)
Total (n=41)	36 (87.8%)	05 (12.2%)	00 (00%)

No history of previous Amphotericin B therapy was recorded. In all 41 cases, the MIC of Amphotericin B was 05 µg/ml.

Discussion

Chemotherapy is critically important in reducing the burden of disease, and antimonials (SbV) are the first-line drugs for all clinical forms. Treatment by SbV is long, expensive and not devoid of adverse side effects. In many states of India, treatment failure is already well documented due to low responsiveness of parasites to sodium antimony gluconate (SAG). In Bangladesh, resurgence of Visceral Leishmaniasis (VL) has been noticed first in the decade of 1970 that has gradually increased to an epidemic form in many localities. Currently, VL appears at a rate in excess of 15,000 cases per year.[12,17]

Kala-azar occurs among various age groups depending on the infecting species, geographic location, disease-reservoir and host immunocompetence. In Indian type of VL, children between 5 and 15 years of age were affected more.[17] In the present study, majority of cases (64.4%) were in the 2-12 years age group. Almost similar findings were also found in many studies. Ali and Ashfold in a study found 142 cases of VL, where 58% were children below 15 years.[18] In another study by Mittal *et al* observed that majority patients of VL were in the age group of 5-15 years.[19] Higher incidence of KA in children might be due to the observation that children of poor family generally suffer from malnutrition. Consequently, their immunity is hampered, increasing the risk of getting KA infection and developing severe disease. Moreover, adult population in the endemic locality might develop protective immunity from previous infection that reduces the chance of re-infection.[20]

In the present study, male to female ratio was 1.5: 1. Other two reports in home from the ICDDR,B also reported almost similar ratio between male to female and found the ratio of 1.2:1 and 1.04:1.[21,22] Several local studies were also consistent as showing male to female ratio of 1.38:1, 2:1, and 2.4:1.[17,23] Similar finding was reported also from abroad where ratio between male to female was 1.3: 1.[24] In a study from Sudan, it was reported that like many other countries males are almost twice (1.8:1) as likely to be affected by VL than females.[25] Males are infected more often than females, most likely because of their increased exposure to sand flies due to professional activities.

In the present study, 41 strains of Leishmanial parasites were isolated in culture. Out of which 36 strains did not yield promastigotes at a SAG concentration of 250 μg/ml (MIC). Of 8 strains isolated from treatment failure KA cases with previous SAG therapy, 05 did not yield promastigotes at SAG concentration of 500 μg/ml (MIC).

As a whole, about 12.2% (05/41) strains showed higher inhibitory concentration of SAG in the present study. On the contrary, none of 41 strains yielded promastigotes at an MIC of Amphotericin B of 5 μg/ml. We could not compare this data with that of other studies at home. Because, no data in this respect is as yet published or reported. But studies from India indicated that unresponsiveness to pentavalent antimonials had increased from 34% to 64% recently.[10,14] Parasites causing Kala-azar among those who were clinically unresponsive also showed resistance in *in vitro* sensitivity test method using standard concentration of pentavalent antimonials. Besides these, it was further observed that pentavalent sensitive parasites could be made resistant to the drug by repeated passage in experimental animals followed by incomplete treatment with suboptimal doses of the drug.[12] Considering above-mentioned observations, it was suggested that the steady rise in pentavalent antimony unresponsiveness of KA patients as found in India were due to infection with resistant parasites, generated as a result of irregular and often incomplete treatment of the patients. Since the present study could not investigate all those parameters, no comment could be made. But irregular and incomplete treatment by SAG in the poor KA patients in Bangladesh is a common phenomenon due to non-availability and high cost of the drug.[12] Considering the statement, higher MIC for Leishmanial isolates from treatment failure KA cases obtained in the present study might be logical. But the issue needs critical and urgent exploration on a large-scale study to encounter prevailing and future surgence of KA all over the country.

Analyzing the findings of the study, it can be concluded that KA affected more males than females and majority of the infections occurring among children were under 12 years of age. Strains of *L. donovani* unresponsiveness to standard dose of pentavalent antimonials have been started to appear in Bangladesh.

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