

USE OF rK39 FOR DIAGNOSIS OF POST KALA-AZAR DERMAL LEISHMANIASIS IN NEPAL

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Abstract. A recently developed nitrocellulose-based dipstick test, rK39, has been widely used for the diagnosis of kala-azar. In this study, we evaluated its use for the diagnosis of post kala-azar dermal leishmaniasis (PKDL). We also investigated the time taken by patients to develop PKDL after apparent cure of kala-azar (visceral leishmaniasis, VL) and the time taken by patients to come to the hospital after the appearance of symptoms of PKDL. A majority of patients developed the disease within three years after the apparent cure of kala-azar (KA). A majority of patients sought treatment within five years after the onset of PKDL. The amastigotes of *Leishmania donovani* bodies (LDBs) were demonstrated in 70, 20, and 20% of slit-skin smears (SSS) prepared, respectively, from nodular, papular, and macular forms. The presence of highest density (6+) LDBs in the SSS of 20% of nodular PKDL patients indicated that they may have acted as reservoir in the community. Other reservoirs are not known in Nepal. Only 8% cases were detected by aldehyde test. Although this test is obsolete it is still used in rural parts of Nepal. The dipstick (rK39) was 96% sensitive and 100% specific to diagnose PKDL. Its positive predictive value, negative predictive value, and diagnostic efficacy were 100, 91, and 97% respectively. Due to the advantage of cost compared with the direct agglutination test (DAT), and being easy to use and store in field conditions, rK39 is a good tool to diagnose PKDL in rural situations. All the PKDL patients were cured of the disease after treatment by SAG.

INTRODUCTION

Post kala-azar dermal leishmaniasis (PKDL) is a recognized complication of unknown cause that may arise after the apparent cure of visceral leishmaniasis (VL), or kala-azar (KA). After the apparent cure of KA, it takes 1-7 years in India (Thakur and Kumar, 1990) but only 0-6 months in Sudan (Zijlstra *et al*, 1995) for the appearance of PKDL. It develops in 20% treated cases of KA in India (Thakur and Kumar, 1992), although in Sudan

it has been reported to develop in about 36% (Osman *et al*, 1998) to 56% (Zijlstra *et al*, 1995) of treated cases.

In 1977, there was a massive epidemic of kala-azar in Bihar state of India, which constituted 25% (100,000 cases) of the total number of leishmaniasis cases in the world. In 1993, a major epidemic occurred in India that caused an estimated 250,000 cases (Boelaert *et al*, 1999). Southern parts of eastern and central regions of Nepal, which are endemic areas for kala-azar, are adjacent to Bihar state of India. Blanket coverage of the villages of Nepal by spraying DDT for the malaria eradication program in the 1960s also controlled kala-azar. KA cases were reported from Nepal for the first time in 1980 (HMG, 2001).

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PKDL is characterized by the presence of hypopigmented or erythematous macules, papules, and/or nodules that appear on the face and different parts of the body. These different forms take different time intervals after the apparent cure of KA. PKDL rarely causes discomfort to the patients, therefore, they attend hospitals years after the appearance of symptoms. Multiple lesions may coalesce to form larger lesions and may lead to the gross disfigurement of facial features, such as the nose and lips, giving an appearance similar to leprosy. PKDL may be confused with diffuse cutaneous leishmaniasis (DCL), lepromatous leprosy, fungal infections, secondary syphilis, and other skin disorders (Ramesh and Mukherjee, 1995; WHO, 1996). Leishmaniasis has gained renewed prominence in the Middle East, following the emergence of *L. donovani* as an organism that affects patients who are already infected with HIV. Definitive diagnosis of PKDL requires demonstration of the parasite in a slit-skin smear or in culture, which is very complex, even under the most favorable circumstances.

The direct agglutination test (DAT), like many other serological tests for KA, employs an antigen preparation based on whole leishmania promastigotes (Harith *et al*, 1987). Although the results differed in various epidemiological situations, the sensitivity and specificity of DAT for diagnosis of kala-azar is very high (Bern *et al*, 2000; Chappuis *et al*, 2003).

A recently developed nitrocellulose dipstick test that detects the antibody to recombinant amastigote antigen K39 (rK39) is highly sensitive and specific for the diagnosis of acute KA (Badaro *et al*, 1996; Sundar *et al*, 1998; Bern *et al*, 2000; Chappuis *et al*, 2003).

The objective of this study was to evaluate the rK39 antigen-based dipstick for the diagnosis of PKDL in clinically suspected patients in Nepal. Although the aldehyde test is obsolete due to the lack of specificity, it is still

in use for the diagnosis of KA in Nepal. Therefore, we included slit-skin smear (SSS) for direct demonstration of the parasite, aldehyde test, direct agglutination test (DAT), and rK39 in this study. Our study also focused on the effect of a time interval between KA and PKDL, and the time taken by the patients to come to hospital after onset of PKDL.

MATERIALS AND METHODS

Study station and patients

The study was carried out from April 1998 to July 2000 with patients who had morphological features of PKDL and who attended the Outpatient Department (Dermatology) of the BP Koirala Institute of Health Sciences (BPKIHS), Dharan, a 650-bed university hospital situated in the Eastern Region of Nepal. Thirty-five persons who were not on treatment during the previous six months were included in this study. Twenty-five had suffered previously from KA and were treated with sodium antimony gluconate (SAG). Another five had skin conditions that looked like PKDL. Five villagers from similar endemic communities who had no personal or household history of KA were used as control. Symptoms of the patients were categorized in macular, maculopapular, and nodular forms. Macular patients were divided into two groups, one with those who had a past history of KA, and the other with those who had not suffered from KA before. Before the collection of samples, informed consent was obtained, after explaining the details of the study, from each patient. The institutional research committee approved the study.

Smear

Slit-skin smears (SSS) were prepared aseptically from different types of the dermal lesions using surgical knives on three microslides and were coded. One independent slide was used for each one of Giemsa staining, Ziehl-Neelsen staining, and KOH mount-

ing (Cheesbrough, 1993) to demonstrate *Leishmania donovani* bodies (LDBs), *Mycobacterium leprae*, and pityriasis versicolor, respectively. In the Giemsa-stained smears, the density of LD bodies was scored under an objective 100x eyepiece 10, using a scale ranging from 0 (no parasite per 1,000 oil immersion fields) to +6 (> 100 parasites per field), a method originally developed for splenic aspirate (WHO, 1984), which has been successfully applied for quantification of SSS.

Serological tests

A 5-ml blood sample was collected in a plain vial from each patient, and the serum was separated. Aldehyde and rK39 dipstick tests were performed the same day. The remaining serum was preserved at -70°C for DAT testing and for future reference.

Aldehyde test

Five hundred µl of serum was placed into a 3 ml glass test tube and mixed with 50 µl (10% of the serum) of marketed formaldehyde (40% concentration). If the serum turned white like that of hard-boiled egg within 20 minutes, the test was considered positive otherwise negative.

rK39 antigen based dipstick test

This is a non-invasive, nitrocellulose dipstick immunoassay that uses a recombinant leishmania antigen, K39 (39 amino acid repeats found in a kinecin-like gene of visceral leishmaniasis species), for the detection of IgG antibodies (Qu *et al*, 1994). Twenty µl of serum was added on the nitrocellulose strip of the dipstick and inserted in the 3 ml test tube, held vertically, with two drops of chase buffer solution (supplied with the dipstick kit by the manufacturer, InBios International, Seattle, WA). The appearance of two visible red bands means one control band (the test was valid) and one test band (rK39 antibody was present) which indicated that the test was positive. The test was negative if only the control band appeared. The results were read after five min-

utes and if still negative, after 10 minutes. Even a weak band in the test region was considered as a positive result. The test was repeated if the control line remained negative after 10 minutes.

Direct agglutination test (DAT)

DAT was performed in commercially available v-bottomed microtiter plates with a two-fold dilution of serum samples from 1:100 to 1:12,800. Positive and negative controls were used for every plate. A sample diluent was prepared by mixing 0.2% w/v gelatin + 0.9% w/v NaCl + 0.78% v/v 2- mercaptoethanol in distilled water. The DAT was considered negative at a titer of < 1:3,200, borderline at 1:3,200, and positive at ≥ 1:6,400 (Bern *et al*, 2000; Chappuis *et al*, 2003; Koirala *et al*, 2004). The test was read visually against a white background, and the end-point titer was taken as the last well where agglutination was observed.

Reference standard

We used a combination of parasitology (slit-skin smear) and DAT as the reference standard for evaluation of the rK39. Those with LD bodies in microscopy in the SSS and/or DAT titer ≥ 6,400 were confirmed as PKDL cases. A non-PKDL case was an individual with negative parasitology and a negative DAT (≤ 3,200).

Treatment of patients

The patients with PKDL were treated with intramuscular sodium antimony gluconate (SAG) 20mg/kg body weight/day, and others were prescribed needed medication. Patients with PKDL and *M. leprae* were provided free medication.

RESULTS

Among the 35 persons of the study, a history of KA and treatment with SAG was present in 25 of them. No hepatosplenomegaly was found in any of the patient. The details of

Table 1
Result of different diagnostic methods for different PKDL stages.

| Stages | No | Duration in months | | SSS positive for | | | Serum test positive | | |
|---------------|----|--------------------|------------------|------------------|------------------|------------|---------------------|-----|------|
| | | KA to PKDL | PKDL to hospital | LDBs | <i>M. Leprae</i> | Pityriasis | Aldehyde | DAT | rK39 |
| Macular | 5 | 15-26 | 18-48 | 1 | 0 | 0 | 0 | 5 | 4 |
| Maculopapular | 10 | 12-36 | 1-84 | 2 | 0 | 0 | 0 | 10 | 10 |
| Nodular | 10 | 23-60 | 1-96 | 7 | 0 | 0 | 2 | 10 | 10 |
| No symptom | 10 | | | | | | | | |
| Total | 35 | | | 10 | 2 | 3 | 2 | 25 | 24 |

KA = Kala-azar; PKDL= Post kala-azar dermal leishmaniasis
SSS = Slit skin smear; LD Bs= Leishmaniasis donovani bodies

Table 2
Diagnostic accuracy of rK39 dipstick.

| | | DAT | | |
|------|---|-----|----|----|
| | | + | - | |
| rK39 | + | 24 | 0 | 24 |
| | - | 1 | 10 | 11 |
| | | 25 | 10 | 35 |

Sensitivity: 96%; specificity: 100%; positive predictive value: 100%; negative predictive value: 91%; efficacy: 97%

the time interval for appearance of symptom of PKDL after apparent cure of KA, the time taken by the patients to come to the hospital after development of different forms of PKDL, and the results of the smear serum tests are presented in Table 1. Z-N staining showed lepra bacilli in two patients. KOH preparations were positive for pityriasis in 3 patients. Among the 25 cases having past history of KA, 24 were positive for both rK39 and DAT (titer were $\geq 1:12,800$). One patient had DAT titer =1:6,400 but was negative for rK39, *M. leprae*, and pityriasis. The performance of rK39 is presented in Table 2.

DISCUSSION

The time interval between treatment of KA and appearance of macular form of PKDL was

15-26 months, with a mean \pm SD of 22.8 ± 4.44 months. For the maculopapular form, the time interval was 12-36 months, with a mean \pm SD of 23.8 ± 9.77 months. For nodular form, it was 23-60 months, with a mean \pm SD of 34 ± 12.64 months. Usually, more than one type of lesions were present. The majority of patients developed this disease within 2-3 years after an apparent cure of KA, which was similar to a previous finding (Thakur and Kumar, 1990). In a KA endemic area of eastern Sudan, the interval between treatment of KA and development of PKDL was typically six months or less (Zijlstra *et al*, 1995). Five patients in our study took three years or more to develop PKDL after an apparent cure of KA. Three of them had developed nodular forms by the time they attended the hospital. After the beginning of the symptoms of PKDL, patients took about three years time to develop nodular forms, with the exception of three patients who took 6-8 years. In a previous observation, it took eight years to develop nodular forms (Sharma *et al*, 2000). In that study, the number of the patients was only three, which might have influenced the result.

After the appearance of PKDL, patients attended hospital after 18-48 months with macular form, with a mean \pm SD 28.4 ± 11.46 months; after 1-84 months with the maculo-

popular forms, with mean \pm SD of 26.1 ± 26.1 months; and after a period of 1-96 months with nodular forms, with a mean \pm SD 39.5 ± 33.4 months. The majority of patients sought treatment within five years after the onset of PKDL. During a study conducted by Koirala *et al* (1998), pictures of PKDL patients were displayed to the health workers of health posts, other medical professionals, and villagers. That is why the first case of PKDL was reported (Karki *et al*, 2003) from that area, and why five PKDL patients of that area attended hospital for treatment within six months of the appearance of symptoms.

Slit-skin smears of 40% patients were positive for amastigote of LD bodies. However, their positivity varied in different clinical forms: nodular (70%), maculopapular (20%), and macular (20%). In the nodular stage, 66.6% were found to be positive by Sharma *et al* (2000). The density of LDBs in the smear of two nodular patients of this study was 6+. This indicates that PKDL patients may have served as an ideal reservoir for KA transmission with in the Nepali communities. This suggestion is also strengthened by the finding that those patients who had the highest density of LDBs under their skin not attended hospital up to eight years after the appearance of symptoms. PKDL cases had acted as a reservoir of infection between the epidemics in India (Dhiman and Prasad, 1986; Addy and Nandy, 1992; Thakur and Kumar, 1992; Birley, 1993) and also demonstrated through DNA probe by *P. argentipes* feeding on PKDL patients (Dinesh *et al*, 2000). Therefore, it is of great importance to educate people so they know to treat the PKDL patients of their villages, despite there being no physical problems, thereby reducing the reservoir.

Only two samples were positive by aldehyde test, which were obtained from the nodular stage of PKDL. This test was neither sensitive nor specific.

In this study of 35 persons, 24 had DAT

titer $\geq 1:12,800$. One patient had a DAT titer = 1:6,400, symptoms of PKDL, and responded to SAG treatment. Therefore, we confirmed him as a PKDL patient. Therefore, there were 25 PKDL patients in this study, altogether. All of them were cured of PKDL by intramuscular SAG. The other 10 patients were followed up for one year, without a single one of them developing either KA or PKDL.

We found DAT to be 100% sensitive and 100% specific for the diagnosis of PKDL in Nepal. One study on the diagnosis of kala-azar in Nepal, DAT was found to be 91% sensitive and 69% specific, using a cut-off titer as 1:6,400 in a hospital based study (Chappuis *et al*, 2003). However, in a field-based study using the same cut-off titer, DAT was 100% sensitive and 93% specific (Bern *et al*, 2000); and, in Sudan, a sensitivity of 95.9% and a specificity of 99.4% were found at a 1:8,000 cut-off titer (Boelaert *et al*, 1999). However, as all our patients were PKDL, in whom the parasite remained for the extended period, this increased the sensitivity and specificity of the DAT to 100%.

The rK39 dipstick was able to detect 24 PKDL cases out of 25. It was 96% sensitive and 100% specific; and had a positive predictive value of 100%, a negative predictive value 91%, and diagnostic efficacy 97% (Table 2). For the diagnosis of KA in Nepal, rK39 was 100% sensitive and 100% specific (Bern *et al*, 2000). However, Chappuis *et al* (2003) found it to be 97% sensitive and 71% specific. The study by Chappuis *et al* (2003) was hospital based, and the specificity decreased, as the dipstick was also found to react positive to malaria, enteric fever, and disseminated TB. In other countries, for diagnosis of KA, its sensitivity ratings were 100, 67, and 80%, respectively as reported by various studies (Sundar *et al*, 1998; Zijlstra *et al*, 2001; Iqbal *et al*, 2002). The specificity ratings were 98% and 100%, respectively, as reported by Sundar *et al* (1998) and Iqbal *et al* (2002).

Among the 25 patients of this study, a discordant test result was found in one patient who was positive with a DAT titer = 1:6,400, but who had a negative rK39 dipstick result. Concerning the patient who was not diagnosed by rK39, having had a short history of PKDL after KA, there was no persistence of the parasite for long period. Thus, rK39 test would show positive results because of more persisting antibodies, after previous KA, than that detected by DAT.

In our test, for diagnosis of PKDL both DAT and rK39 were 100% specific. DAT was 100% sensitive, while rK39 was 96% sensitive. The rK39 dipstick would be a useful as a confirmatory test for clinically suspected PKDL patients. The rK39 has several major advantages compared with DAT in a field setting. Ease of use and the rapidity of the rK39 dipstick are especially important in rural Nepal. The cost of rK39 was approximately US\$1.20 per test (Bern *et al*, 2000), and the approximate cost of DAT per test was US\$ 8.00 (Bern *et al*, 2000) or US\$ 4.50 (Chappuis *et al*, 2003).

People having PKDL do not suffer from physical problems and so can continue their daily business (mostly agriculture); therefore, they take 1-5 years to attend hospital and seeking treatment. The persistence of the parasite for that long a period after clinical resolution of KA was the greatest factor resulting in the high titer of DAT and high sensitivity of rK39 in PKDL patients. The only patient who could not be detected by rK39 may be due to a less persistent parasite.

The diagnosis of PKDL by slit-skin smear was disadvantaged by low sensitivity (40%). The aldehyde test (8% positive) was less sensitive. We found that both DAT and rK39 dipstick tests were highly sensitive and specific for the detection of PKDL. A positive rK39 test in presence of the symptom would be a confirmation of PKDL. Due to ease of use in field and the immediacy of diagnosis, rK39 is the best of the four options to diagnose PKDL,

even in rural areas.

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