Enhanced Case Detection and Improved Diagnosis of PKDL in a Kala-azar-Endemic Area of Bangladesh

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Abstract

Objectives: To support the Bangladesh National Kala-azar Elimination Programme (NKEP), we investigated the feasibility of using trained village volunteers for detecting post-kala-azar dermal leishmaniasis (PKDL) cases, using polymerase chain reaction (PCR) for confirmation of diagnosis and treatment compliance by PKDL patients in Kanthal union of Trishal sub-district, Mymensingh, Bangladesh.

Methods: In this cross-sectional study, Field Research Assistants (FRAs) conducted census in the study area, and the research team trained village volunteers on how to look for PKDL suspects. The trained village volunteers (TVVs) visited each household in the study area for PKDL suspects and referred the suspected PKDL cases to the study clinic. The suspected cases underwent physical examinations by a qualified doctor and rK39 strip testing by the FRAs and, if positive, slit skin examination (SSE), culture, and PCR of skin specimens and peripheral buffy coat were done. Those with evidence of *Leishmania donovani* (LD) were referred for treatment. All the cases were followed for one year.

Results: The total population of the study area was 29,226 from 6,566 households. The TVVs referred 52 PKDL suspects. Probable PKDL was diagnosed in 18 of the 52 PKDL suspect cases, and PKDL was confirmed in 9 of the 18 probable PKDL cases. The prevalence of probable PKDL was 6.2 per 10,000 people in the study area. Thirteen PKDL suspects self-reported from outside the study area, and probable and confirmed PKDL was diagnosed in 10 of the 13 suspects and in 5 of 10 probable PKDL cases respectively. All probable PKDL cases had hypopigmented macules. The median time for PKDL development was 36 months (IQR, 24–48). Evidence of the LD parasite was documented by SSE and PCR in 3.6% and 64.3% of the cases, respectively. PCR positivity was associated with gender and severity of disease. Those who were untreated had an increased risk (odds ratio = 3.33, 95%CI 1.29–8.59) of having persistent skin lesions compared to those who were treated. Patients' treatment-seeking behavior and treatment compliance were poor.

Conclusion: Improved detection of PKDL cases by TVVs is feasible and useful. The NKEP should promote PCR for the diagnosis of PKDL and should find ways for improving treatment compliance by patients.

Introduction

Post-kala-azar dermal leishmanisis (PKDL), is a skin disorder, usually develops in 10–20% and about 60% of patients with visceral leishmaniasis (VL)/kala-azar after treatment, respectively, in the Indian subcontinent and Sudan [1]. It has also been reported in individuals without prior history of VL and those undergoing treatment for VL [1–6]. The protozoan parasite *Leishmania donovani* (LD) is the only causative agent. Clinical manifestations of PKDL are macular, maculo-papular, and nodular rash in people who are otherwise well [1] and may be confused with leprosy. Since PKDL is the only interepidemic reservoir of anthroponotic VL, the existence of a few cases is

sufficient to trigger a new epidemic of VL in a given community [1,4,7]. Thus, identification and treatment of PKDL is an essential strategy in eliminating VL.

In 2005, Health Ministers of Bangladesh, India, and Nepal signed a Memorandum of Understanding for the elimination of VL from the Indian subcontinent by 2015 [8]. Active VL and PKDL case detection and their proper management are two important strategies of the elimination program [8]. Until now, no definite method has been identified for active VL and PKDL case detection but one proposed plan includes a house-to-house search for cases by public-health workers. Such a method is expensive and requires alternative strategies for VL and PKDL active case detection [9].

Author Summary

PKDL is a skin disorder which usually develops in 10–20% and about 60% of patients with visceral leishmaniasis (VL) after treatment respectively in the Indian subcontinent and Sudan. However, cases among people without prior VL have also been reported. Except skin lesion, PKDL patients are healthy and usually do not feel sick. However, persistence of a few PKDL cases is sufficient to initiate a new epidemic of anthroponotic VL. Thus, identifying and treating people with PKDL is a key strategy for the elimination of kala-azar. Diagnosis of PKDL relies upon clinical criteria and a serological test which is not specific for PKDL. The use of the existing laboratory diagnostic tools for confirmation of PKDL among PKDL suspects is unknown. In the Indian subcontinent, PKDL is not self-limited and needs to be treated with sodium stibogluconate injections for 4–6 months. No data are available relating to treatment compliance by patients, particularly in Bangladesh. The results of the present study showed that trained village volunteers were useful for identifying PKDL suspects, and diagnostic confirmation improved with the use of PCR. However, patients' adherence to prescribed treatment was poor.

In the Indian subcontinent, PKDL was first described by Brahmchari in 1922 [10]. Since then, nearly 90 years have passed and no gold standard diagnostic method could be developed for PKDL; diagnosis, thus, relies on clinical criteria [1–6]. PCR of slit skin scraping specimens has demonstrated high sensitivity for diagnosing PKDL in a laboratory-based study [11]. However, its use in clinically-diagnosed PKDL patients is unknown. PCR testing of peripheral blood buffy coat has been found to be a highly-sensitive method for the diagnosis of VL [12,13], and theoretically, it may help also confirm PKDL.

In Bangladesh, in the post-malaria-eradication era, the first reports on PKDL were from hospital- based studies [14,15]. Until now there has been limited information on the burden of PKDL in the VL-endemic communities of Bangladesh [4]. Preliminary results of an ongoing surveillance study of PKDL in Fulbaria, Mymensingh, Bangladesh, showed that the burden of PKDL was high and presents a challenge for the National Kala-azar Elimination Programme (NKEP) [4]. More information on the burden of PKDL will help the NKEP to develop adequate national strategies for controlling PKDL.

We therefore studied the feasibility of using trained village volunteers (TVVs) for detecting suspected PKDL cases; estimated the prevalence of PKDL in Kanthal Union, Trishal, a VL-endemic area of Bangladesh; described the clinical features of these patients; evaluated the contribution of PCR for the confirmation of PKDL diagnosis in clinically-diagnosed PKDL cases; and investigated the patients' compliance to treatment.

Methods

Study area and population

We conducted a study in Kanthal union, Trishal sub-district under Mymensingh district, Bangladesh. During January-March 2008, trained field research assistants performed a census in Kanthal union. During the census, field research assistants assessed household number and the number of people in each household. They also asked and recorded whether any member within the family suffered from kala-azar in the past and if any of them currently had skin rash.

Trained village volunteers

After consulting the local community leaders and obtaining their consent, one community volunteer was selected from each ward. The research team trained the volunteers on what was PKDL, how did a PKDL case looks like, and how to look for the suspected PKDL case (see definitionbelow). A two-day training was imported to the volunteers, and pictures of skin lesions from PKDL patients from published literature and textbooks were used. During April-May 2008, nine TVVs visited each household at least once searching for suspected PKDL cases and, if found, referred the case to the study clinic. Household members who had a history of VL but were not present during the home-visits were invited to visit to the study clinic for assessment. Additionally, the study physician assessed patients with a history of VL and skin rash who lived in villages outside the study area. Most of these patients were directed to our study clinic from nearby public union health posts where rK39 tests were not available.

Evaluation and procedures

The study physician examined the suspected PKDL cases and requested an rK39 strip test (Kala-azar Detect [TM] Rapid Test, InBios International, Seattle, WA, USA) as needed. A trained field research assistant performed the rK39 strip test as per the manufacturer's instructions. If the results were positive, the patients were considered probable PKDL cases (see definition below) and were requested to undergo slit skin scraping and blood collection. A physician-microbiologist from the Bangabandhu Sheikh Mujib Medical University (BSMMU) in Dhaka performed slit skin scraping and collected skin specimens for staining, culture, and PCR test in the study clinic. The study physician collected up to 5 mL of venous blood in EDTA tubes and gently shook. After collection, the blood and skin specimens were transported to the Parasitology Laboratory of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) within 3–4 hours maintaining cold chain. PCR tests were performed in the Parasitology Laboratory of ICDDR,B on the following day, and the study physician was informed about PCR results immediately after testing.

Treatment and follow-up

We referred all cases positive for the LD parasite or positive for LD DNA by PCR to the Upazila Health Complex for treatment as per the national guideline for kala-azar elimination [16]. After referral, we followed up patients to find out whether they went to the hospital for admission, and if admitted, whether they completed full treatment courses. Further, we followed all the probable PKDL patients after one year from the date of their referral by household-visit and collected information on the status of their skin rash. Patients who were rK39 test-negative were referred to the Mymensingh Medical College Hospital for further medical consultation.

Laboratory methods

Collection of slit skin scraping for staining, culture, and PCR. The affected area of the skin was cleaned with 70% v/v alcohol and allowed to dry completely. The edge of the lesion was squeezed firmly between the finger and the thumb to drain the area of blood. Using a sterile scalpel blade, a small incision was made into the dermis; any blood was blotted away. The cut surface was then scraped in an outward direction to obtain the tissue fluid and cells for the following procedures:

Preparation of slides and process of staining

The materials were thinly spread on three-clean glass slides using a circular motion working outwards to avoid damaging any

parasites. When the smears dried, two slides were fixed with a few drops of absolute methanol for 2–3 minutes. The remaining slide was heat- fixed by holding it, smear side up, over the flame of a spirit lamp for a few seconds. After fixation, the slides were stored and transported to the laboratory of BSMMU and stained with modified Ziehl-Neelsen (Z-N) staining (using 5% sulphuric acid, H_2SO_4). It was then examined under oil immersion lens using 100x oil immersion objectives to look for *Mycobacterium leprae*. The methanol-fixed slides were stained with Giemsa stain and examined for LD bodies.

Collection of slit skin scrapings for PCR

Collection of slit skin scraping specimens for PCR was done as described previously [17], with little modification which included collection of skin scrapings using a sterile cotton swab to maximize the collection of tissue fluid and cells. After cutting the edge of the lesion, a sterile cotton swab was used for absorbing the tissue fluid and placed into an appendorf tube containing 500 µL of NET buffer (150 Mm Nacl, 15 Mm Tris-HCl [pH-8.30]). The cotton swab was kept in the tube for one hour before removal; and the buffer solution was preserved at $-20°C$ at the Parasitology Laboratory of ICDDR,B until PCR was performed.

Collection of slit skin scrapings for culture

Slit skin scrapings were collected with sterile stainless steel wire loops and inoculated in the Novy-MacNeal-Nicolle medium which was transported to the Microbiology Laboratory of BSMMU, in a air-conditioned car maintaining the temperature at 22°C and was incubated at 22–24°C in the laboratory. Culture was examined weekly and was kept up to four weeks before discarding as negative.

Preparation of buffy coat. After arrival at the laboratory, blood samples were centrifuged at 8000 rpm for 10 minutes at room temperature, and 500 µL of buffy coat was collected from the middle layer of the tube containing concentrated leukocytes. The buffy coat was kept in a 1.5-mL sterile microcentrifuge tube and preserved at $-20°C$ for DNA extraction and PCR amplification.

DNA extraction from buffy coat and skin slit. Buffy coat DNA was extracted for PCR using QIAamp DNA blood mini kit (Qiagen, Hilden, Germany, Cat. no. 51106) as per the manufacturer's instructions. The DNA was eluted in 0.2 mL of AE buffer (supplied with the Qiagen kit). DNA was extracted from the skin slit also following the manufacturer's instructions, except that the proteinase K digestion was carried out for 60 minutes at 50°C. The DNA was eluted in 0.2 mL of AE buffer (supplied with the Qiagen kit). The purity of the DNA was satisfactory since a ratio of OD at A260/A280 was within 1.7–1.9 for all DNA samples. We used molecular-grade water instead of blood as an extraction control for checking for carry-over contamination in every run of DNA extraction and PCR amplification.

PCR methods. *Leishmania*-specific nested PCR (Ln-PCR) was performed to detect LD DNA in peripheral buffy coat and skin specimens using 2 µL of extracted DNA by the method described previously [13], with primers targeting the parasite's SSU-rRNA region [12]. For the first PCR run, we used Kinetoplastida-specific primers (R221 5′-GGTTCCTTTCCTGATTTACG-3′ and R332 5′- GGCCGGTAAAGGCCGAATAG-3′). For the second PCR, 1 µL of 1:50 dilution of the first PCR product was used as a template in the presence of 0.15 µmol/L of the *Leishmania*-specific primers R223 and R333.

Microscopy. *Microscopy for LD bodies:* At least two experts examined the Giemsa stained slit skin slides for amastigotes and also examined the wet film of culture for promastigotes.

Examination for M. leprae: At least two experts also looked for *M. leprae* in the Z-N stained slit skin slides.

Definitions

Case definitions. A suspected PKDL patient was a person from a kala-azar-endemic area with a past history of kala-azar and a skin lesion. A probable PKDL case was a patient with suspected PKDL who also had a positive rK39 test result. A confirmed PKDL case included those probably PKDL cases who also had the LD parasite identified by slit skin examination or culture, or had a PCR test positive for LD DNA. All the confirmed PKDL cases were referred to the Upazila Health Complex for treatment as per the national guideline for kala-azar elimination [16]. However, all analyses presented in this paper are based on clinically-defined PKDL with positive rK39 dipstick test, i.e. probable PKDL cases.

Age-groups. The study population was divided into three age-groups: <15 years; 15–43 years; and ≥44 years.

Duration of onset of PKDL. Duration was defined in months between the past treatment history of VL and the onset of skin lesion as reported by the patient.

Grading of PKDL. Skin lesions were graded into three major categories as described previously [1]. Briefly, grade I included scattered maculopapular or nodular rash in the face with or without some involvement in the upper chest or arm; grade II included maculopapular or nodular rash mostly of the face and extending to the chest, back, upper arms, and legs; and grade III included maculopapular or nodular rash covering most parts of the body, including hands and feet.

Progression of skin lesions. Based on case history given by the patients, the study physician categorized progression of skin lesions into rapid (conspicuous/obvious progression every month from onset), gradual (clear, steady progression from onset but at less detectable pace), and slow/stagnant (appearance, with little or no progression throughout early months).

Treatment categories. Complete treatment was defined when a patient completed the full treatment course of 20 consecutive days per month for consecutive six months with sodium stibogluconate at a dose of 20 mg/kg/day and with a gap of 10 days between courses. Termination of treatment before its completion was defined as incomplete/partial treatment, and if a patient refused treatment with sodium stibogluconate, it was defined as refusal of treatment.

Cure of PKDL. Cure was defined as complete disappearance of skin lesion(s) after treatment, as reported by the patient and assessed by the trained Field Research Assistant.

Calculation of sample-size and statistical analysis

Assuming a PKDL prevalence of 0.04% (95% CI 0.03–0.05), the required sample-size needed to search for PKDL cases was 25,098. A total population of 29,226 in the study area was sufficient for our study. All data were computed using the EpiInfo software (version 3.2.2.) and were analyzed using the SPSS software (version 11.0) through descriptive and analytical methods. Comparison between proportions was done by chi-square with Fisher's exact correction. Means were compared by ANOVA or Kruskal-Wallis where applicable. We used Kaplan-Meier survival curves to identify the median time for onset of PKDL after the completion of VL treatment. All p values were two-tailed, and a p value of ≤0.05 was considered significant.

Ethical consideration

The Ethical Review Committees of both ICDDR,B and Tropical Disease Research/WHO approved the study. Written informed consent was obtained from each study participant and from parents/legal guardian for all child participants. Written informed consent also was obtained from the head of each household when performing the census.

Results

Prevalence of PKDL in study area

The census performed by the Field Research Assistants revealed a total population of 29,226 people from 6,566 households: 11,298 people (38.7%) were aged ≤15 years, and the median family size within each household was 5 (IQR 4–6). The Field Research Assistants also listed a total of 235 individuals with a past history of VL and 21 suspected PKDL patients based on the information obtained during their visits to households at the beginning of the study. However, the TVVs referred 52 suspected PKDL patients, including the 21 cases, listed by the Field Research Assistants from the study area. No cases from the study area were self- reported but 13 patients were self-reported from villages outside the study area. No new cases of suspected PKDL from the study area were found in the following months after the initial survey by the TVVs.

Of the referred and self-reported suspected PKDL cases, 18 of 52 (34.6%) and 10 of 13 (76.9%) were respectively found to be probable PKDL cases. The referred and self-reported probable PKDL cases did not differ regarding age [mean years ±standard error (SE), 24.1±3.7 for referred cases, 18.5±3.3 for self-reported cases, p = 0.33], sex (% of male patients, 55.6% for referred vs 40.0% self-reported, p = 0.69), and duration of onset of PKDL (mean years±SE, 46.0±5.1 for referred vs 36.0±23.1 for self-reported cases, p = 0.29). PKDL was confirmed in 50% of the patients from each group (9/18 for referred and 5/10 for self-reported suspected PKDL patients). The calculated prevalence of probable PKDL in the study area was 6.2 per 10,000 people, slightly more common among the age-group of 15–43 years and without any relation with gender [Table 1].

Characteristics of PKDL patients

We pooled all the 28 probable PKDL cases for further analysis. The median age of the patients was 21.5 years (IQR, 10.7–29.0), and the large majority (67.9%) was aged ≥15 years. All the patients had previously been treated for VL with sodium stibogluconate for 28 consecutive days, except one (3.6%) who had interruption in treatment. The median duration of onset of PKDL was 36 months (IQR, 24–48) (Fig. 1). The duration of onset of PKDL was not related to gender (38.57±5.9, n = 14 for females vs 46.71±5.9, n = 14 for males, p = 0.34) but the younger group

developed PKDL significantly earlier compared to the older age-group (26.67±3.8 months, n = 9, vs 50.21±5.1 months, n = 19, p = 0.006).

Hypopigmented macules were the most common skin lesions and most frequently appeared first on the face (Table 2). In most (94.4%) cases, the skin lesions progressed gradually and were associated with itching in 25% of the cases. Eight of the 28 (29%) cases, skin lesions were classified as grade I and the remaining 20 (71%) as grade II at the time of presentation to the study clinic. The severity of the disease, as estimated by grading, showed no association with sex: 11 of the 14 females (78.6%) and 9 of the 14 males (64.3%) had grade II PKDL, p = 0.34. Similarly, the severity of the disease had no association with age-group (78% vs 68% grade II respectively in the younger and older age-groups, p = 0.48) nor the duration of onset of PKDL (r = 0.03, p = 0.9).

Laboratory findings

All the patients were negative for *M. leprae* (Table 3). All the 28 cultures were negative for Leishmania parasites. Slit skin microscopy found LD bodies only in one of the 28 patients. Of the 28 patients 12 (42.9%) were positive by slit skin scraping specimen PCR and 14 (50%) by peripheral blood buffy coat PCR. The PCR method was positive in 64% of the patients, defined by any PCR test-positive (Table 3). The buffy coat PCR results were positively associated with the severity of the disease: 65% (13/20) of grade II PKDL patients were positive for peripheral buffy coat PCR compared to 12.5% (1/8) of the PKDL patients with grade I disease (p = 0.012). However, skin specimen PCR results did not show a similar relationship with PKDL grades: 45% (9/20) and 37% (3/8) respectively with grade II PKDL and grade I PKDL were positive by skin specimen PCR (p = 0.72). The female patients had significantly more peripheral buffy coat PCR-positive results compared to the male patients [10/14 (71%) for female vs. 4/14 (29%) for male patients, p = 0.023]. The female patients were also more likely to have a positive skin specimen by PCR (50%, 7/14) compared to the male patients (36%, 5/14) but the difference was not statistically significant (p = 0.45).

Treatment and follow-up

Eighteen patients positive for the evidence of the LD parasite (1 by both LD body and LD DNA and 17 by LD DNA only) by any laboratory method were referred to the sub-district hospital for treatment. Of these patients, 7 completed treatment, 6 partially completed treatment, and 5 refused treatment. The common reasons for incomplete treatment or not to be treated were concern about loss of daily wages, loss of school days, and insolvency (Table 4). All the patients with complete treatment, half of those with incomplete treatment, and none who refused treatment were cured at follow-up. Compared to those with complete treatment, the risk of not being cured with incomplete treatment or treatment refusal was three times higher (relative risk 3.33, 95% CI 1.29–8.59, p = 0.004). Ten of the 28 patients were not referred for treatment due to lack of evidence of the LD parasite in their skin or blood. At follow-up after one year, 8 of 10 patients had persistent skin lesions; skin lesions had deteriorated in 1 patient, and 1 was spontaneously cured.

Discussion

The major findings of the present study were: the TVVs were useful for the active detection of PKDL case; the prevalence of PKDL was high in the study area; PCR was useful for the confirmation of PKDL diagnosis; and low treatment compliance and current treatment-seeking behavior of PKDL patients present

Table 1. Distribution and prevalence of probable PKDL patients by sex and age group in Kanthal.

	Total number of people in the study area	Number of probable PKDL patients	Prevalence of probable PKDL per 10,000 people
Sex			
Male	14999	10	6.7 [a]
Female	14227	08	5.6
Age group			
<15 years old	10569	05	4.7
15–43 years old	13535	11	8.1 [b] [c]
≥44 years old	5122	02	3.9
Total	29226	18	6.2

[a]P = 0.72 compare to Female group;
[b]P = 0.31 vs. <15 years old and
[c]P = 0.53 vs. ≥44 years old.
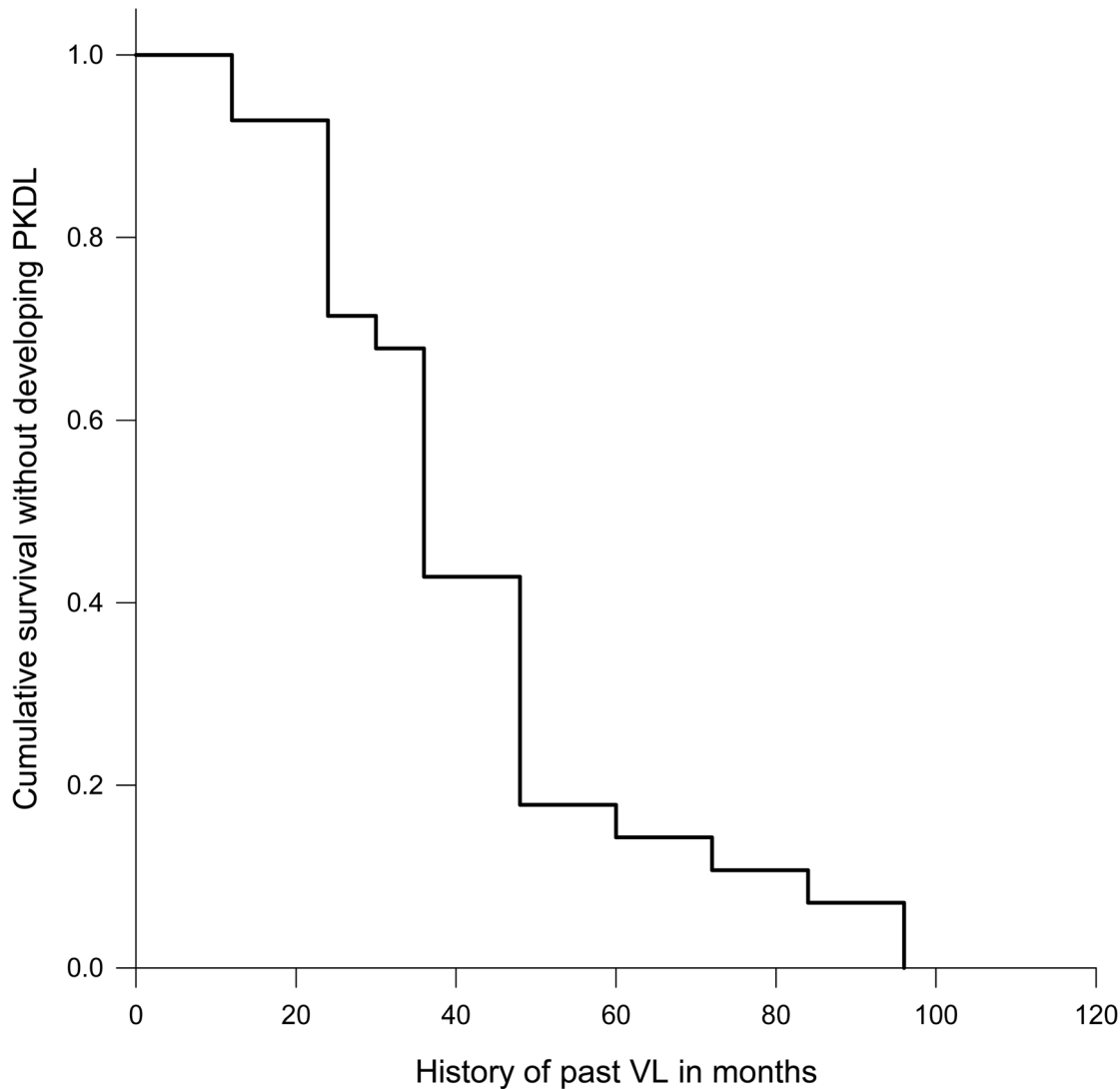doi:10.1371/journal.pntd.0000832.t001

Figure 1. Time for developing PKDL after completion of treatment for visceral leishmaniasis.
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Table 2. Clinical information of probable PKDL patients, n = 28.

Indicators	% (n)
First involvement of rash	
Face	60.7 (17)
Arms	28.6 (8)
Other part of the body	10.7 (3)
Rash progression	
Gradual	96.4 (27)
Slow/stagnant	3.6 (1)
Type of rashes	
hypopigmented macules/patches/confluent plaques	100.0 (28)
hypopigmented papules	10.7 (3)
nodule	3.6 (1)

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a new challenge for the national kala-azar elimination programme in Bangladesh.

The Government of Bangladesh is committed to eliminate VL by 2015, and active detection of VL and PKDL cases is one of the main pillars of this elimination programme [8,16]. The active

Table 3. Laboratory findings in 28 probable PKDL patients.

Laboratory Method	Positive % (n/28)
Microscopy for M. Leprae	0 (0)
Culture for Leishmania promastigote	0 (0)
Microscopy of slit skin scraping specimens	3.6 (1)
PCR	
Slit skin scraping specimens	42.9 (12)
Peripheral buffy coat	50.0 (14)
Any positive	64.3 (18)

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Table 4. Laboratory diagnosis, referral and treatment compliance of probable PKDL patients.

Patient	Age (years)	Sex	PKDL Grade	Slit skin examination	Culture of skin specimens for LD	*PCR Result	Referred for treatment	Treatment status (number of injections)	Reason of partial treatment or not treated	Status after one year
T-085	26.00	Male	2	Negative	Negative	2	Yes	Partial (18)	Loss of wages	Not cured
T-013	30.00	Female	2	Negative	Negative	3	Yes	Complete (120)	-	Cured
T-073	7.00	Female	2	Negative	Negative	3	Yes	Complete (120)	-	Cured
T-034	34.33	Female	1	Negative	Negative	2	Yes	Complete (120)	-	Cured
T-035	25.00	Female	3	Negative	Negative	3	Yes	Complete (120)	-	Cured
T-015	7.00	Female	2	Negative	Negative	1	Yes	Partial (54)	Insolvency	Not cured
T-019	26.00	Male	2	Negative	Negative	3	Yes	Complete (120)	-	Cured
T-028	45.00	Male	2	Negative	Negative	3	Yes	Partial (59)	Insolvency	Not cured
T-084	13.00	Female	1	Negative	Negative	3	Yes	Complete (120)	-	Cured
T-040	22.00	Male	1	Negative	Negative	2	Yes	Partial (62)	Insolvency	Cured
T-041	17.00	Female	2	Negative	Negative	2	Yes	Partial (40)	Loss of school days	Cured
T-042	10.00	Female	2	Negative	Negative	1	Yes	Partial (40)	Loss of school days	Cured
T-057	7.00	Female	2	Negative	Negative	1	Yes	Complete (120)	-	Cured
T-022	70.00	Male	2	Negative	Negative	0	No	Not referred	-	Cured
T-072	25.00	Male	1	Negative	Negative	0	No	Not referred	-	Not cured
T-074	2.25	Male	2	Negative	Negative	0	No	Not referred	-	Not cured
T-087	20.00	Male	2	Negative	Negative	0	No	Not referred	-	Not cured
T-088	30.00	Female	1	Negative	Negative	0	No	Not referred	-	Not cured
T-120	13.00	Male	1	Negative	Negative	0	No	Not referred	-	Not cured
T-121	10.00	Male	2	Negative	Negative	0	No	Not referred	-	Not cured
T-123	25.00	Female	2	Negative	Negative	0	No	Not referred	-	Rash increased
T-124	2.50	Female	2	Negative	Negative	1	Yes	Not taken	Does not feel sick	Not cured
T-126	19.00	Male	1	Negative	Negative	0	No	Not referred	-	Not cured
T-141	21.00	Male	1	Negative	Negative	0	No	Not referred	-	Not cured
T-029	25.00	Male	2	Negative	Negative	1	Yes	Not taken	Loss of wages	Not cured
T-063	30.00	Female	2	Negative	Negative	1	Yes	Not taken	Does not feel sick	Not cured
T-086	38.00	Female	2	Negative	Negative	3	Yes	Not taken	Does not feel sick	Not cured
T-110	20.00	Male	2	Positive	Negative	3	Yes	Not taken	Loss of wages	Not cured

*0 = Negative both by skin specimen PCR and peripheral blood buffy coat PCR, 1 = positive by only peripheral buffy coat PCR, 2 = positive only by skin specimen PCR, 3 = Positive by both PCR.
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detection of VL and PKDL cases by the existing health facilities in rural Bangladesh may not be possible due to lack of human resources. We found that the TVVs were useful for finding suspected PKDL cases. Thus, the TVVs should be used by the NKEP for an annual active case search. We feel that it is sufficient to conduct an annual search, particularly for PKDL, since no new cases of suspected PKDL were found in our study after the initial active case search by the TVVs.

The proportion of PKDL cases among the self-reported suspects was higher (76.9%) compared to the referred PKDL cases (34.6%). The majority of the self-reported cases were directed to our study clinic from nearby public union health posts that lacked facilities for rK39 testing. This suggests that many people are concerned about signs and symptoms of possible PKDL but remain undiagnosed due to lack of diagnostic facilities, particularly rK39-based rapid tests, in the union health posts. The NKEP should thus, strengthen the existing public union health posts in the VL-endemic areas by equipping them with tools to diagnose PKDL.

Clinical manifestations of the PKDL cases did not differ from those reported in the literature [1–6,18]. We found that the median duration of onset of PKDL was slightly longer compared to that reported recently [4]. This difference might be explained by the differences in the study design, and recall bias in our study population also might contribute to this difference. Itching was reported in 25% of the cases. This symptom among the PKDL patients was also reported by others, and inflammatory reactions in the dermis observed by Ismail *et al* might explain this symptom [19].

The prevalence of PKDL in our study was 6.2 per 10,000 people which was lower than that found by a recent study in another VL-endemic area of Bangladesh [3]. The limitation of our study was that the TVVs looked for the suspected PKDL cases based on the past history of VL and skin rash. Since PKDL may develop in individuals without a past history of VL, it was possible that the TVVs could not track all suspected PKDL cases. However, we believe that this risk was minimal because the percentage of PKDL without past history of VL was low (about

10% of all PKDL cases) in Bangladesh [4]. Additionally, no self-reported PKDL case from Kanthal union was presented to the study clinic or the Upazila Health Complex in the months following the study.

Slit skin examination and culture methods were not very much useful for the diagnosis of PKDL since the LD parasite was found in only one by slit skin examination, and all the 28 cases were negative by culture method. Similar results were reported by others [18]. PCR of skin specimens and peripheral blood buffy coat was more useful for the detection of LD compared to slit skin examination and culture. PCR helped confirm the diagnosis of PKDL in additional 17 patients who were negative for LD by conventional methods. Seven of the 17 patients who received complete treatment were cured. Without this useful diagnostic tool, these 7 patients would not be considered PKDL patients because they were negative by SSE and culture and would continue to serve as a reservoir of anthroponotic VL, thus threatening the community's health. In Bangladesh, many tertiary hospitals are now equipped with PCR technology. Thus, the NKEP should consider the use of PCR for the diagnosis of PKDL. However, the feasibility and cost-effectiveness of such a diagnostic algorithm may be a concern for the NKEP and needs to be explored first.

We observed a significantly-positive relationship between PKDL grade and PCR positivity, particularly with peripheral blood buffy coat PCR. The circulation of the LD parasites in peripheral blood increases with the severity of disease and indirectly indicated that the origin of LD DNA in blood was skin. So far, this is the first documentation of such a relationship between PKDL grade and PCR method. It also supports an earlier experiment that patients with PKDL may serve as a reservoir for the transmission of the LD parasite which circulates in their blood [20]. Although the severity of PKDL was equally distributed among the males and females, the positive PCR results were more common in the females, especially for peripheral buffy coat PCR. We have no explanation for this phenomenon.

Ten of the 28 probable PKDL cases were not referred for treatment because we failed to demonstrate LD or LD DNA among them. At follow-up, only one had improvement in his skin rashes, which occurred spontaneously. Our current diagnostic capabilities limited our ability to determine whether these 10 cases were false-positive probable PKDL cases or if they were false-negative confirmed PKDL cases. The former explanation is less likely to be true because PKDL is typically not self-limited in the Indian subcontinent and less likely to be cured without treatment

[1–6]. All except one patient had persistent skin lesions after a one year in this study. Thus, the development of more sensitive tests for the diagnosis of PKDL is urgently needed and should be encouraged by national and international researchers and funding agencies.

The poor treatment-seeking behavior of and treatment compliance by patients with PKDL patients present a challenge for the NKEP. Only seven of the 18 cases (38.9%) completed treatment after referral to the hospital. The reason behind not being treated and partially treated was respectively feeling otherwise healthy and concern about loss of daily wage or loss of school day. Intensive motivation and some financial support from the NKEP may improve this situation.

In conclusion, the prevalence of PKDL is high in a VL-endemic area of Bangladesh. The use of TVVs feasible to actively detect PKDL suspects, and in conjunction with PCR techniques, this holds promise as an effective strategy for the NKEP to help meet goals for the elimination of VL, if the ways for improving treatment-seeking behavior and treatment compliance are found.

Supporting Information

Checklist S1 STROBE Checklist
Found at: doi:10.1371/journal.pntd.0000832.s001 (0.23 MB DOC)

Author Contributions

Conceived and designed the experiments: DM KNN MMH MK MSH AK RH. Performed the experiments: DM KNN MMH MK MSH RH. Analyzed the data: DM KNN MMH MSH AK TT RH. Contributed reagents/materials/analysis tools: DM KNN MMH MK AK RH. Wrote the paper: DM KNN MMH MK MSH AK TT RH.

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