Additional Material: Longlasting insecticidal bed nets for prevention of *Leishmania donovani* infection in India and Nepal: paired cluster randomized trial [posted as supplied by author]

Content

1. Interim analyses

The report is available on request.
The resolution from Prof. Lengeler, chair of the Scientific Advisory Board (SAB) is attached below.

From: lengeler <Christian.Lengeler@xxxxx.xx>
To:
Date: 6/10/2008 3:01 pm
Subject: Interim analysis and report

Dear Kalanet SAB and Dear Kalanet team.

In the absence of any further correspondence and inputs in the matter of the interim analysis and report (submitted by the Kalanet team in April of this year) I would like to confirm that the Kalanet Scientific Advisory Board accepts the reports and recommends continuation of the trial along the lines agreed previously.

Kind regards

Christian Lengeler
Chair, Scientific Advisory Board Kalanet

Prof. Christian Lengeler
Project Leader
Swiss Tropical Institute, P.O. Box, 4002 Basel, Switzerland
Tel: (+41 61) 284 xxxx Christian.Lengeler@xxxx.xx

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2. Direct Agglutination Test (DAT) protocol

The Direct Agglutination Test (DAT) was performed using freeze dried antigen suspension of trypsin-treated, fixed and stained promastigote of *L. donovani* from ITM-Antwerp. The filter papers Whatman #3 with the blood samples, kept at –20^{o} C until the test was conducted, were warmed up to room temperature.

To obtain a 1:400 dilution, a standard 5 mm filter paper disc fully covered with blood was punched out from the filter paper and eluted in 1000 µL DAT buffer (PBS-PH 7.2 supplemented with protein).

After 12h incubation at 4°C, 100 µl were transferred to the first well of a V-shaped microtiter plate. To obtain serial dilutions from 1:400 to 1:25600, 50 µl of the dilution were mixed with 50 µl of DAT-diluent including 2-Mercapto-Ethanol (preparation: 0.24 ml 2-ME per vial of 30ml DAT-diluent) in the subsequent wells using a multi-channel pipette. Serum was diluted from 1:400 to 1:25600.

Then, (1) 50 µL of DAT antigen were added in each well; (2) the plate was sealed, (3) shaken gently, and (4) incubated overnight at ambient temperature. One positive and 1 negative controls were run every 5th plate.

Reading: the tests were read against a white background by two independent readers. A third reader was called as a referee if no consensus was found in the reading.

3. Map with the location of the 26 KALANET trial clusters in India and Nepal.

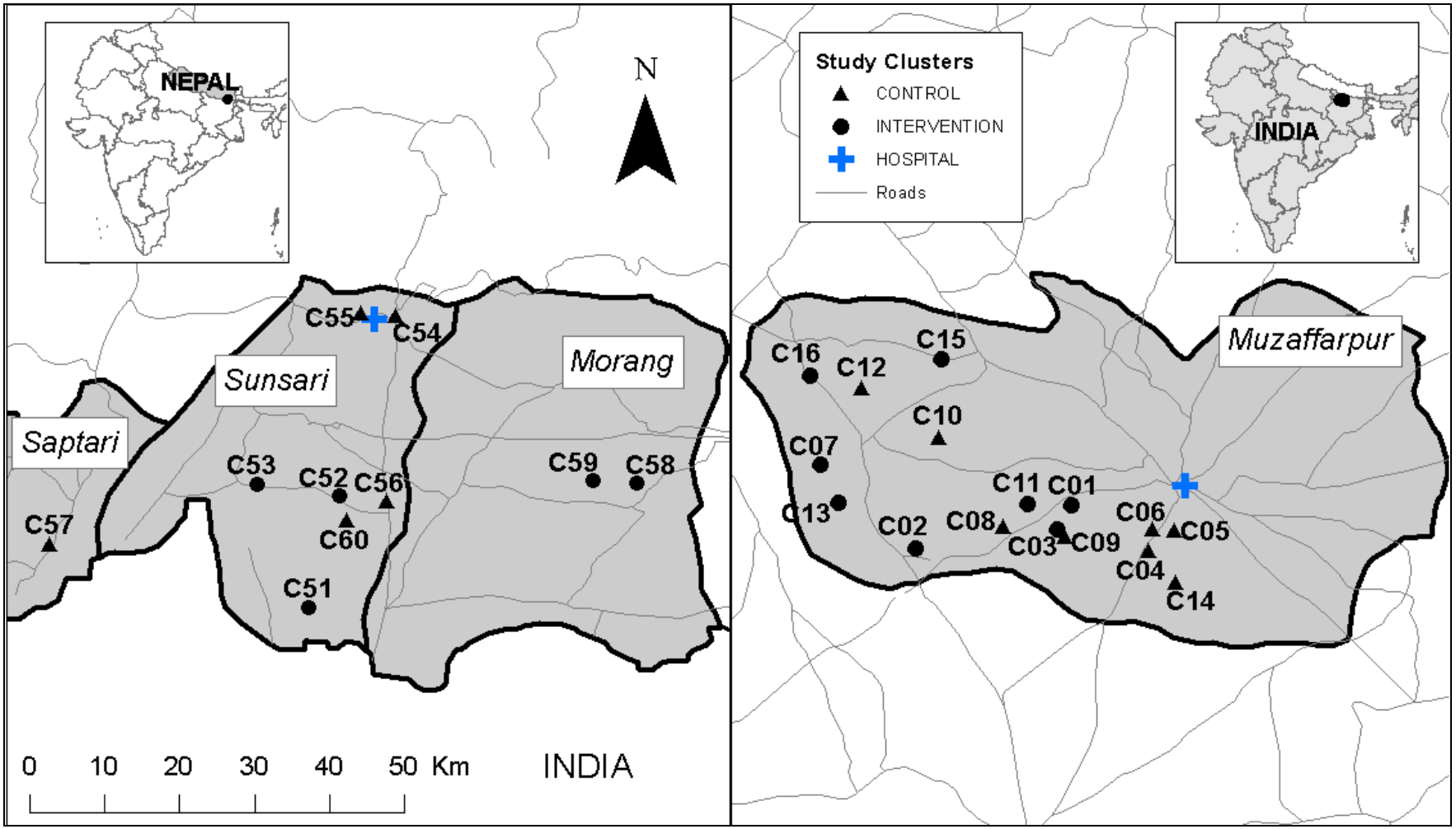

Figure Add-3.1: Map with the location of the KALANET study clusters, 10 in Nepal (left) and 16 in India (right). Intervention and control clusters are identified with a circle and a triangle respectively. The location of the reference hospitals: B.P. Koirala Institute of Health Sciences in Dharan, Nepal and Kala-Azar Medical Research Centre in Muzaffarpur, India are also indicated.

4. Visceral Leishmaniasis case definitions.

Case-definitions

Visceral leishmaniasis certain:

1. Clinical suspect patient with a positive bone marrow or spleen aspirate at KALANET reference hospitals[1].
2. Clinical suspect patient with (a) a positive rK39 dipstick (performed by the KALANET team) and (b) good clinical and haematological response to anti leishmanial treatment given at KALANET reference hospitals.
3. Clinical suspect patient with (a) a positive rK39 dipstick (performed by the KALANET team) and (b) good clinical response to anti-leishmanial treatment given outside KALANET reference hospitals (e.g. other hospital, private physician) and (c) retrospective confirmation of treatment from the care-provider (see below).
4. Clinical suspect patient with (a) diagnosis (rK39 dipstick, parasitology) not performed by the KALANET team and (b) good clinical response to anti-leishmanial treatment given outside KALANET reference hospitals and (c) retrospective confirmation of diagnosis and treatment from the care-provider(s)
5. Patient who died of parasitologicaly proven (certain) visceral leishmaniasis.

Remarks:

- A clinical suspect patient is an individual with history of fever for > 2 weeks and splenomegaly
- Retrospective confirmation of diagnosis and treatment is obtained by the KALANET team, usually the medical coordinator, from verification of proper medical records of the patient , if they are available from the patient or the care-provider
- Definition 4: if information on diagnosis can not be collected by the KALANET team, a DAT seroconversion observed at the following serological survey will retrospectively confirm the diagnosis.

Visceral leishmaniasis probable:

1. Clinical suspect patient with (a) a positive rK39 dipstick (performed by the KALANET team) and with (b) good clinical response to anti-leishmanial treatment given outside KALANET reference hospitals and (c) with no retrospective confirmation of treatment from the care-provider
2. Clinical suspect patient with (a) diagnosis (rK39 dipstick, parasitology) not performed by the KALANET team and (b) good clinical response to anti-leishmanial treatment given outside KALANET reference hospitals and (c) no retrospective confirmation of diagnosis and treatment from the care-provider(s) and (d) with DAT seroconversion at the following serological survey.
3. Patient who died of probable visceral leishmaniasis.

Remark:

- If diagnosis and treatment performed outside KALANET settings can not be confirmed from the care-provider and if DAT seroconversion is not demonstrated, the patient will not be included as a VL case in the analysis.

[1]Kala-Azar Medical Research Centre (KAMRC), Muzaffarpur in India and B.P. Koirala Institute of Health Sciences, Dharan in Nepal.

5. Number of VL cases and people at risk per pair and cluster

Table Add-5.1: Visceral Leishmaniasis (VL) cases by pair (2 months' incubation period)

Pair (Clusters)	Intervention		Control	
	VL Cases	Subjects	VL Cases	Subjects
INDIA				
A (C16-C12)	1	979	6	1121
B (C02-C14)	7	899	4	727
C (C01-C09)	6	813	8	815
D (C07-C10)	9	639	2	505
E (C15-C08)	2	527	0	561
F (C13-C06)	0	485	4	459
G (C11-C04)	2	580	3	1308
H (C03-C05)	4	1065	4	711
NEPAL				
I (C58-C54)	1	771	4	995
J (C51-C57)	2	1204	5	1060
K (C52-C56)	1	599	0	584
L (C53-C55)	0	739	0	512
M (C59-C60)	2	529	0	623
Total	37	9829	40	9981
Total India	31	5988	31	6207
Total Nepal	6	3842	9	3774

6. Incident seroconversions in each cluster by pair and intervention group

Table Add-6.1: Number and incidence of seroconversions in each cluster by pair and intervention group. Unadjusted and adjusted risk ratio (RR) per pair. The adjusted analysis includes the following covariates: age group, gender, country, times sprayed and socio-economic status (SES).

	Incidence seroconversions % (n/N)			
Pair (Clusters)	Intervention	Control	Unadjusted RR	Adjusted RR[a]
INDIA				
A (C16-C12)	8.5 (52/613)	7.7 (51/660)	1.10	1.10
B (C02-C14)	8.3 (46/552)	3.7 (15/408)	2.27	2.45
C (C01-C09)	2.3 (12/533)	8.6 (44/509)	0.26	0.26
D (C07-C10)	11.2 (41/366)	13.3 (36/271)	0.84	0.95
E (C15-C08)	7.5 (22/294)	9.0 (31/345)	0.83	0.72
F (C13-C06)	4.5 (11/244)	3.6 (11/308)	1.26	1.12
G (C11-C04)	16.2 (60/371)	5.0 (42/847)	3.26	3.31
H (C03-C05)	5.4 (32/595)	5.1 (23/452)	1.06	1.10
NEPAL				
I (C58-C54)	1.4 (8/565)	3.0 (20/668)	0.47	0.46
J (C51-C57)	4.7 (44/936)	3.2 (21/648)	1.45	1.42
K (C52-C56)	2.1 (8/390)	1.3 (5/392)	1.61	1.58
L (C53-C55)	0.9 (5/539)	13.4 (41/307)	0.07	0.07
M (C59-C60)	1.6 (6/374)	1.0 (5/504)	1.62	1.59

a) Adjusted for age group, gender, times sprayed and socio-economic status.

7. Subgroup analyses

Material and Methods

Subgroup analyses were carried out for the effect of longlasting insecticidal bed nets (LNs) on *L. donovani* infection by country, age (<14, 14 to 38 and >38 years old) and socioeconomic status (SES) groups. To test for effect modification, the effect of intervention was compared in the youngest age group (<14) with the older age group (≥ 14) and the lowest SES group compared to the rest. The effect of LNs was analyzed using higher DAT cut offs.

Results

The analysis of the effect of LNs by increasing age showed a trend, with the RR ranging from 0.69 in the youngest age group to 1.15 in the oldest, but none of the RRs were statistically significant (table 4). The effect modification was borderline significant with RRs (<14y/+14y) of 0.52 (95% CI 0.37-0.73) in intervention and 0.77 (95% CI 0.56-1.07) in control group (p=0.06). An analysis of LN effect by SES group showed no major differences (data not shown).

When higher cut-offs were used in DAT for defining seroconversion, the protective effect of LNs seemed to progressively increase up to RR=0.80 when a titre cut-off 1:12800 was used, the effect was however non-statistically significant and this trend disappeared in the adjusted model (table 7.1).

Table Add-7.1: Subgroup analyses results: effect of long lasting insecticidal bed nets (LN) on (1) different age groups, and (2) using different cut offs for seroconversion in Direct Agglutination Test (DAT). Unadjusted and adjusted risk ratios for the intervention compared to control from the cluster analysis. The adjusted analysis includes the following covariates: gender, country, times sprayed, socio-economic status (SES) and age group (when appropriate).

	Intervention % Serocon. (n/N)	Control % Serocon. (n/N)	Unadjusted RR (95% CI)	No interv. effect (p)	Adjusted RR (95% CI)	No interv. effect (p)
Age group						
< 14	4.45 (121/2718)	5.02 (137/2730)	0.69 (0.33-1.45)	0.30	0.70 (0.33-1.47)[a]	0.31
14 to 38	5.57 (119/2137)	5.64 (121/2144)	0.98 (0.46-2.10)	0.95	0.93 (0.44-1.98)[a]	0.84
38+	7.05 (107/1517)	6.02 (87/1445)	1.15 (0.60-2.20)	0.65	1.20 (0.63-2.30)[a]	0.55
Titre cut off						
3200	3.04 (194/6372)	3.23 (204/6319)	0.86 (0.50-1.49)	0.57	0.87 (0.51-1.50)[b]	0.60
6400	1.63 (104/6372)	1.99 (126/6319)	0.84 (0.51-1.39)	0.48	0.88 (0.55-1.42)[b]	0.58
12800	1.02 (65/6372)	1.25 (79/6319)	0.80 (0.41-1.55)	0.47	1.00 (0.67-1.51)[b]	0.98

a) Simultaneously adjusted for gender, times sprayed and socio-economic status. b) Simultaneously adjusted for age group, gender, times sprayed and socio-economic status.

8. Effect of LNs when seroconverters from 800 to 1600 (1 titre difference) are included in the analysis.

Table Add-8.1: Risk of seroconverting during the 2 years of the study (a person was DAT positive if the titre ≥ 1600 – no minimum 2 titres difference required).

	Intervention		Control	
	Events	**Subjects**	**Events**	**Subjects**
INDIA				
A (C16-C12)	53	613	55	660
B (C02-C14)	48	552	15	408
C (C01-C09)	12	533	44	509
D (C07-C10)	41	366	37	271
E (C15-C08)	23	294	32	345
F (C13-C06)	12	244	11	308
G (C11-C04)	60	371	43	847
H (C03-C05)	33	595	23	452
NEPAL				
I (C58-C54)	8	565	20	668
J (C51-C57)	45	936	22	648
K (C52-C56)	8	390	5	392
L (C53-C55)	5	539	41	307
M (C59-C60)	6	374	5	504
Total	354	6372	353	6319
Total India	282	3568	260	3800
Total Nepal	72	2804	93	2519

Table Add-8.2: Unadjusted and adjusted risk ratios for seroconversion using the definition DAT ≥1600 (no minimum 2 titres difference required). The adjusted analysis includes age group, gender, times sprayed and socio-economic status.

Seroconversion (DAT ≥ 1600)	**Intervention n (%)**	**Control n (%)**	**Unadjusted Intervention effect Risk Ratio (95% CI)**	**p**	**Adjusted Intervention effect Risk Ratio (95% CI)**	**p**
Overall	354 (5.6)	353 (5.6)	0.90 (0.49; 1.66)	0.7140	0.89 (0.48; 1.63)	0.6732
India	282 (7.9)	260 (6.8)	1.10 (0.58; 2.07)	0.7365	1.09 (0.58; 2.06)	0.7469
Nepal	72 (2.6)	93 (3.7)	0.66 (0.12; 3.53)	0.5245	0.57 (0.11; 2.93)	0.3935

9. Effect of LNs on Visceral Leishmaniasis (VL) when all suspected VL cases are considered.

Table Add-9.1: All suspected Visceral Leishmaniasis (VL) cases reported from individuals living at least 6 months in the study clusters from IS1 to IS3. Number of cases detailed per pair and cluster

Pair	Intervention		Control	
	VL Cases	Subjects	VL Cases	Subjects
INDIA				
A (C16-C12)	6	979	14	1121
B (C02-C14)	8	899	5	727
C (C01-C09)	6	813	21	815
D (C07-C10)	14	639	5	505
E (C15-C08)	2	527	1	561
F (C13-C06)	1	485	7	459
G (C11-C04)	3	580	7	1308
H (C03-C05)	9	1065	5	711
NEPAL				
I (C58-C54)	6	771	8	995
J (C51-C57)	7	1204	7	1060
K (C52-C56)	3	599	2	584
L (C53-C55)	0	739	0	512
M (C59-C60)	3	529	0	623
Total	68	9829	82	9981
Total India	49	5987	65	6207
Total Nepal	19	3842	17	3774

Table Add-9.2: Unadjusted and adjusted risk ratios (RR) for visceral leishmaniasis (VL) using all suspected cases detected between IS1 and IS3. The adjusted analysis includes age group, gender, times sprayed and socio-economic status.

All suspected VL cases	Intervention n (%)	Control n (%)	Unadjusted Intervention effect Risk Ratio (95% CI)	p	Adjusted Intervention effect Risk Ratio (95% CI)	p
Overall	68 (0.7)	82 (0.8)	0.86 (0.44; 1.68)	0.6277	1.03 (0.69; 1.52)	0.8846
India	49 (0.8)	65 (1.0)	0.76 (0.32; 1.80)	0.4701	0.81 (0.50; 1.33)	0.3491
Nepal	12 (0.3)	17 (0.5)	1.34 (0.37; 4.92)	0.5214	1.44 (0.48; 4.33)	0.3665

10. Effect of LNs on Visceral Leishmaniasis (VL) taking 6 months incubation period

Table Add-10.1: Visceral Leishmaniasis (VL) cases by pair (6 months' incubation period)

Pair	Intervention		Control	
	VL cases	Subjects	VL cases	Subjects
INDIA				
A (C16-C12)	0	979	4	1121
B (C02-C14)	6	899	3	727
C (C01-C09)	5	813	5	815
D (C07-C10)	10	639	1	505
E (C15-C08)	2	527	0	561
F (C13-C06)	0	485	3	459
G (C11-C04)	2	580	3	1308
H (C03-C05)	2	1065	2	711
NEPAL				
I (C58-C54)	1	771	4	995
J (C51-C57)	1	1204	5	1060
K (C52-C56)	1	599	0	584
L (C53-C55)	0	739	0	512
M (C59-C60)	2	529	0	623
Total	32	9829	30	9981
Total India	27	5987	21	6207
Total Nepal	5	3842	9	3774

Table Add-10.2: Unadjusted and adjusted risk ratios and risk differences for visceral leishmaniasis (6 month incubation period). The adjusted analysis includes age group, gender, times sprayed and socio-economic status.

	Intervention n (%)	Control n (%)	Unadjusted Intervention effect	p	Adjusted Intervention effect	p
Risk Ratio (95% CI)						
Overall	32 (0.3)	30 (0.3)	1.25 (0.52; 2.99)	0.5875	1.07 (0.54; 2.14)	0.8261
India	27 (0.5)	21 (0.3)	1.37 (0.48; 3.90)	0.5005	0.89 (0.38; 2.12)	0.7658
Nepal	5 (0.1)	9 (0.2)	0.87 (0.10; 7.44)	0.8465	1.39 (0.14; 14.32)	0.6818

11. The effect of bed net (LN and untreated) use.

Methods

The effect of bed net use on the risk of seroconversion was examined by comparing the number of seroconverters in three groups: LN group (intervention clusters) and people with and without an untreated net in control clusters. The statistical methods described in the manuscript for the primary analyses were applied.

Results

Table Add-11.1: The use of bed nets in the study population

	Use of Nets, N (%)	Seroconversion n(%)
Overall		
Treated net	6372 (50.2)	347 (5.5)
Untreated net	4761 (37.5)	228 (4.8)
No use of nets	1557 (12.3)	117 (7.5)
India		
Treated net	3568 (48.4)	276 (7.7)
Untreated net	2501 (34.0)	155 (6.2)
No use of nets	1298 (17.6)	98 (7.6)
Nepal		
Treated net	2804 (52.7)	71 (2.5)
Untreated net	2260 (42.5)	73 (3.2)
No use of nets	259 (4.9)	19 (7.3)

Table 11.1 presents the number of individuals and proportion of seroconversions per group. As expected the group using treated nets is the largest (50%, 6372/12690) as this corresponds to the intervention group. We do, however, have 12% (1557/12690) of the subjects, who report not using nets. This proportion differs between India and Nepal as 18% (1298/7367) of the Indian study population do not use nets compared to 5% (259/5323) of the study population from Nepal. The overall proportion of seroconverters was highest in the group of individuals not using bed nets (although this was perhaps not the case in India).

Table Add-11.2: The effect of treated net compared to no net; treated net compared to untreated net and untreated net to no net. The adjusted analysis includes age group, gender, times sprayed and socio-economic status.

Seroconversion	n (%)	n (%)	Unadjusted RR (95% CI)	Test for no intervention effect	Adjusted RR (95% CI)	Test for no intervention effect
Treated net vs no net	**Treated nets**	**No nets**				
Overall	347 (5.5)	117 (7.5)	0.71 (0.36; 1.39)	0.2834	0.45 (0.19; 1.03)	0.0579
India	276 (7.7)	98 (7.6)	1.03 (0.47; 2.24)	0.9367	0.91 (0.42; 1.96)	0.7788
Nepal	71 (2.5)	19 (7.3)	0.25 (0.06; 1.03)	0.0530	0.27 (0.06; 1.26)	0.0779
Treated vs untreated net	**Treated**	**Untreated**				
Overall	347 (5.5)	228 (4.8)	1.08 (0.57; 2.04)	0.8061	1.06 (0.56; 2.04)	0.8374
India	276 (7.7)	155 (6.2)	1.27 (0.67; 2.40)	0.3993	1.31 (0.67; 2.56)	0.1956
Nepal	71 (2.5)	73 (3.2)	0.82 (0.13; 5.23)	0.7851	0.62 (0.12; 3.12)	0.4540
Untreated vs no net	**Untreated**	**No nets**				
Overall	228 (4.8)	117 (7.5)	0·52 (0·27; 1·00)	0·0487	0·57 (0·30; 1·10)	0·0870
India	155 (6.2)	98 (7.6)	0·75 (0·42; 1·33)	0·2656	0·83 (0·49; 1·38)	0·3974
Nepal	73 (3.2)	19 (7.3)	0·28 (0·04; 1·90)	0·1241	0·38 (0·06; 2·64)	0·2122

Table 11.2 shows the effect of bed net use on the risk of seroconversion. Overall the risk of seroconversion decreased by 29% for treated nets compared to no net use. This decrease is, however, not statistically significant as the confidence interval includes 1 (p=0.2834). Most of this effect is due to the effect in Nepal, where the treated nets reduce the risk by 75% compared to no nets (this effect is borderline statistically significant, p=0.0530). The overall risk ratio is borderline statistically significant after adjusting for age group, gender, times sprayed and socio-economic status (p=0.0579). The adjusted risk ratio is 0.45 (95% CI 0.19 to 1.03) this change is mainly due to the change when adjusting in India where using treated nets now becomes beneficial. In the control clusters, the overall risk of seroconversion decreased by 48% for people using nets compared to people without nets, the effect is borderline statistically significant (p=0.049) in the unadjusted model but not in the adjusted model (p=0.087).

In general the two groups using nets have very similar risks of seroconverting and the main difference lies in the group not using nets (Table 11.2). There are no statistically significant differences between the two groups using nets.

12. Primary analysis not accounting for pairing

Some studies suggest that in community trials with a small number of pairs the pairing can be ignored in the analyses.[1] Table 12.1 shows the results from the primary analysis on the cluster level, but ignoring the pairing. The results and confidence intervals obtained are similar to those obtained in the analysis taking into account the pairing (see manuscript for further details).

Table Add-12.1: Primary analysis repeated ignoring the pairing. The adjusted analysis includes age group, gender, times sprayed and socio-economic status. (RR=Risk Ratio)

Seroconversion	Unadjusted RR (95% CI)	Test for no intervention effect	Adjusted RR (95% CI)	Test for no intervention effect
Overall	0.90 (0.45; 1.78)	0.7481	0.95 (0.50; 1.80)	0.8696
India	1.09 (0.61; 1.94)	0.7564	1.08 (0.60; 1.93)	0.7881
Nepal	0.66 (0.19; 2.23)	0.4531	0.51 (0.17; 1.51)	0.1878

Reference:
1. Diehr P, Martin DC, Koepsell T, Cheadle A. Breaking the matches in a paired t-test for community interventions when the number of pairs is small. *Stat Med* 1995;**14**(13):1491-504.

13. Coefficient of intraclass correlation (ICC).

Assumed ICC:
The coefficients of intraclass correlation (ICC) were estimated from the coefficient of variation (*k*) using the formula for binary outcomes described in Hayes & Moulton (2009 - page 18; section 2.3.4)[1].
For the sample size calculation the coefficient of variation was assumed to be 0.25 corresponding to the intracluster correlation coefficient of 0.003 (ignoring pairing and setting the overall risk of seroconversion to 0.04).

Observed ICC:
It is impossible to estimate the intracluster correlation coefficient of the coefficient of variation based on data from a matched trial because the between-cluster variability is confounded with variations in the intervention effect. However, ignoring the pairing, the observed ICC could be estimated as 0.03.

References:
[1]Hayes R, Moulton L. Cluster Randomised Trials. Chapman & Hall/crc, London 2009.

14. Quality control Direct Agglutination Test

The Direct Agglutination Test (DAT) was done by survey batches in two laboratories: B.P Koirala Institute of Health Sciences (BPKIHS) in Dharan, Nepal for the samples taken in Nepal and Banaras Hindu University (BHU) in Varanasi, India, for the Indian samples. The laboratory of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium was used as a reference laboratory.

Before the trial:
A standardisation workshop was organised at BPKIHS in Dharan before starting the trial. To confirm that the protocol was well standardised in both countries, 100 samples (non trial-related) were exchanged between laboratories and the DAT results compared.

During the trial:
During the serosurveys three blood spots were collected from each consenting participant. The third spot, identified as "QC", was for quality control purposes exclusively (Figure 14.1).

For each one of the three immunological surveys, 10 % of samples in each country were selected on the following basis: a random sample of 10% of all DAT-negative samples and a random sample of 10% of the DAT-positive samples. These samples were exchanged between country laboratories, re-tested and the results compared. Discordance was defined as a titer difference of 2 or more between the results from the two laboratories in a given sample. The discordant samples were sent to the reference laboratory in ITM-Antwerp for definitive classification.

Results were plotted in Bland Altman and scatter plots to check for systematic errors. The Kappa test was used to compare the results on the QC samples. Discordance rate was calculated by cluster, by day and by batch of antigen to examine clustering.

Conclusion:
Between laboratory reproducibility was good and comparable with previous studies.[1] No systematic errors were found. Errors were randomly spread in the total set of samples.

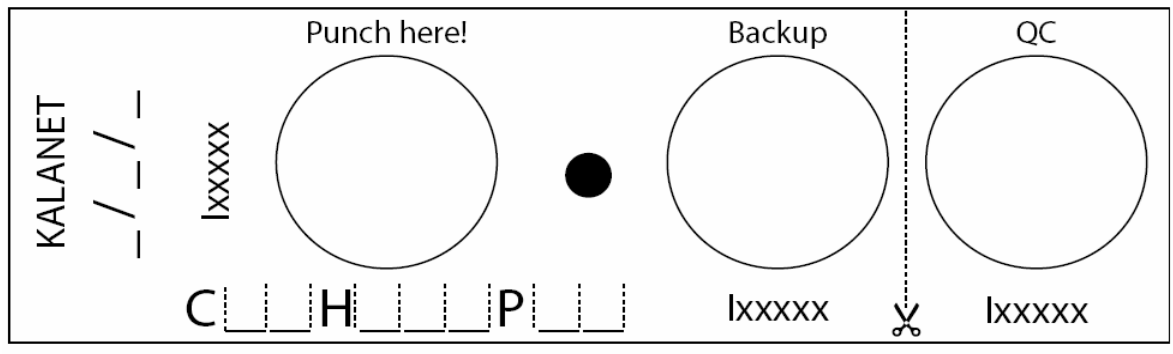

Figure Add-14.1: Design of the filter paper used to collect blood samples for Direct Agglutination Test (DAT). Three blood spots were taken in each immunological survey, the last spot (QC) was used for Quality Control.

Reference:
1. Boelaert M, El-Safi S, Hailu A, Mukhtar M, Rijal S, Sundar S, Wasunna M, Aseffa A, Mbui J, Menten J, Desjeux P, Peeling RW. Diagnostic tests for kala-azar: a multi-centre study of the freeze-dried DAT, rK39 strip test and KAtex in East Africa and the Indian subcontinent. *Trans R Soc Trop Med Hyg* 2008;**102**(1):32-40

15. Trial outcomes analysed using a random effects logistic regression model

Materials and Methods:

Cluster-specific odds ratios (OR) for the trial outcomes (DAT seroconversion, VL, malaria and all causes of death) were obtained applying a logistic regression model using the individual level data and including a fixed effect of pair and a random effect of cluster to take the matching and the clustering into account respectively.

In a second step the basic model was adjusted for a series of covariates: age and gender at individual level, IRS and social status at household level and country at cluster level. First each covariate was added one at a time to the basic model as a fixed effect to investigate their individual impact on the intervention effect. Finally, all the potential confounders were included simultaneously. Only the latter results are presented below.

For the adjusted analyses, the age of the individuals at baseline was grouped into five approximately equally sized groups 0-4, 5-11, 12-23, 24-39 and 40+. The information on number of times each household was sprayed during the trial was grouped as 0, 1 and 2 or more. A score was calculated for each household to reflect the socio-economic position of the family. These scores were grouped into five equal groups (within country) where 0 represents the poorest group and 4 the least poor group.

Results:

Table Add-15.1 summarises the effect of LN on DAT seroconversion, incident VL, malaria and all causes of death using a multilevel model. When the individual data were analysed using the mixed logistic regression model, the overall odds for *L. donovani* infection were reduced in the individuals living in intervention clusters by 9% compared to individuals in control clusters. The effect was, however not statistically significant (OR=0.91; 95% CI: 0.62-1.34). The results were not significantly modified in the adjusted model (OR=0.91; 95% CI 0.61-1.37). When the individual data were analysed per country LNs seem to have an opposite effect on DAT seroconversion in India (OR=1.13) and Nepal (OR=0.62) but the effect was in both cases non-statistically significant in the crude analysis. Similar results were obtained in the adjusted model: OR=1.17 and 0.51 in India and Nepal respectively.

The mixed logistic regression model showed that LNs reduced the odds of VL by 11% (OR=0.89; 95% CI 0.57-1.40), but the effect was, again, not statistically significant and did not change in the adjusted model. Results per country were similar to the overall effect.

The overall effect of LNs on malaria showed a statistically significant reduction of cases in intervention clusters (OR=0.61; 95% CI 0.42-0.89) which was slightly increased post-adjustment (OR=0.56; 95% CI 0.39-0.78). The results observed on malaria cases were mainly based on the effect of LNs in India due to the bias on the number of cases (220/225 malaria cases reported in India). Similarly the total number of deaths was reduced in intervention clusters (OR=0.72; 95% CI 0.56-0.93) and this effect was greater in India than in Nepal.

Discussion:

Table Add-15.2 presents the trial results obtained from the cluster level model (Relative Risk = RR) used in the manuscript and the multilevel model presented above.

The trial results obtained using a multilevel model (mixed logistic regression) were analogous to those obtained using a cluster level model (presented in the manuscript). The effect of LN on *L. donovani* infection (the main outcome) was almost identical (RR=0.90 and OR=0.91) and non-statistically significant in both models. The effect on VL cases was larger in the mixed model (OR=0.89) compared to the cluster level model (RR=0.99) but was non-statistically significant in both cases. LN reduced the number of malaria cases, this effect was similar in both models (RR=0.63 and OR=0.61) but was only statistically significant in the crude multilevel model. The effect of LN on malaria was increased (RR=0.46 and OR=0.56) and was statistically significant in both adjusted models. The main difference was that the mixed model was able to detect a statistically significant effect of LN on all causes of death, the magnitude of the effect was however similar in both cases: RR=0.75 and OR=0.72.

The conclusions of the trial were not modified when an alternative analytical method was used.

Table Add-15-1: Trial outcomes using a random effects logistic regression model

Variable	Intervention	Control	Effect of LN: Unadjusted OR (95% CI)	Effect of LN: Test for no intervention effect (p)	Effect of LN: Adjusted OR[a] (95% CI)	Effect of LN: Test for no intervention effect (p)
OVERALL						
	(N=6372, 13 clusters)	(N=6319, 13 clusters)				
No. Seroconversions (%)	347 (5.4)	345 (5.5)	0.91 (0.62-1.34)	0.64	0.91 (0.61-1.37)	0.65
	(N=9829, 13 clusters)	(N=9981, 13 clusters)				
VL cases (%)[c]	37 (0.38)	40 (0.40)	0.89 (0.57-1.40)	0.61	0.79 (0.46-1.36)	0.39
Malaria (%)[c]	88 (0.90)	137 (1.37)	0.61 (0.42-0.89)	0.01	0.56 (0.39-0.78)	0.00
All causes of death (%)	124 (1.26)	167 (1.67)	0.72 (0.56-0.93)	0.02	0.77 (0.60-1.00)	0.05
INDIA						
	(N=3568, 8 clusters)	(N=3800, 8 clusters)				
No. Seroconversions (%)	276 (7.7)	253 (6.7)	1.13 (0.77-1.64)	0.54	1.17 (0.78-1.74)	0.45
	(N=5988, 8 clusters)	(N=6207, 8 clusters)				
VL cases (%)[c]	31 (0.52)	31 (0.50)	0.95 (0.57-1.57)	0.83	0.78 (0.40-1.51)	0.45
Malaria (%)[c]	87 (1.45)	133 (2.14)	0.63 (0.43-0.92)	0.02	0.57 (0.40-0.80)	0.01
All causes of death (%)	80 (1.34)	121 (1.95)	0.63 (0.44-0.90)	0.02	0.70 (0.51-0.97)	0.03
NEPAL						
	(N=2804, 5 clusters)	(N=2519, 5 clusters)				
No. Seroconversions (%)	71 (2.5)	92 (3.6)	0.62 (0.28-1.37)	0.26	0.51 (0.22-1.17)	0.14
	(N=3842, 5 clusters)	(N=3774, 5 clusters)				
VL cases (%)[c]	6 (0.16)	9 (0.24)	0.69 (0.25-1.96)	0.49	0.71 (0.25-2.03)	0.52
Malaria (%)[c]	1 (0.03)	4 (0.11)	0.26 (0.03-2.33)	0.18	--[b]	
All causes of death (%)	44 (1.15)	46 (1.22)	0.94 (0.62-1.42)	0.75	0.95 (0.61-1.48)	0.82

a) Simultaneously adjusted for age group, gender, times sprayed and socio-economic status. b) Too few Malaria cases to adjust for covariates. c) Rare outcomes: some clusters did not record any event during the study period.

Table Add-15.2: Trial outcomes obtained from the cluster level model presented in the manuscript and the random effects logistic regression model described above. RR= Relative Risk and OR= Odds Ratio

Variable	Cluster level model		Multilevel model	
	Unadjusted RR (95% CI)	Adjusted RR[a] (95% CI)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)
OVERALL				
No. Seroconversions (%)	0.90 (0.49-1.65)	0.89 (0.48-1.64)	0.91 (0.62-1.34)	0.91 (0.61-1.37)
VL cases (%)[c]	0.99 (0.46-2.16)	1.15 (0.61-2.16)	0.89 (0.57-1.40)	0.79 (0.46-1.36)
Malaria (%)[c]	0.63 (0.29-1.36)	0.46 (0.28-0.77)	0.61 (0.42-0.89)	0.56 (0.39-0.78)
All causes of death (%)	0.75 (0.50-1.13)	0.78 (0.56-1.10)	0.72 (0.56-0.93)	0.77 (0.60-1.00)
INDIA				
No. Seroconversions (%)	1.09 (0.58-2.04)	1.09 (0.58; 2.05)	1.13 (0.77-1.64)	1.17 (0.78-1.74)
VL cases (%)[c]	1.00 (0.41-2.44)	0.94 (0.44-2.02)	0.95 (0.57-1.57)	0.78 (0.40-1.51)
Malaria (%)[c]	0.64 (0.36-1.13)	0.60 (0.38-0.94)	0.63 (0.43-0.92)	0.57 (0.40-0.80)
All causes of death (%)	0.62 (0.32-1.19)	0.72 (0.44-1.19)	0.63 (0.44-0.90)	0.70 (0.51-0.97)
NEPAL				
No. Seroconversions (%)	0.66 (0.12-3.56)	0.57 (0.11-2.97)	0.62 (0.28-1.37)	0.51 (0.22-1.17)
VL cases (%)[c]	0.96 (0.13-7.39)	1.55 (0.17-14.18)	0.69 (0.25-1.96)	0.71 (0.25-2.03)
Malaria (%)[c]	0.18 (0.00-14.38)	--[b]	0.26 (0.03-2.33)	--[b]
All causes of death (%)	1.02 (0.67-1.55)	1.06 (0.69-1.64)	0.94 (0.62-1.42)	0.95 (0.61-1.48)

a) Simultaneously adjusted for age group, gender, times sprayed and socio-economic status. b) Too few Malaria cases to adjust for covariates. c) Rare outcomes: some clusters did not record any event during the study period.

16. Effect of LN excluding clusters (C11 and C55) with high seroconversion rates (outliers).

Material and Methods:
Clusters C11and C55 had a very high seroconversion rates compared to the rest of clusters. Those clusters could be considered outliers. To asses their impact on the trial outcomes, the effect of LN on *L. donovani* infection was re-analysed excluding those clusters. The analytical method used was the same described in the manuscript. In this new analysis pairing was not taken into account.

Results:
The results of the effect of LN on *L. donovani* infection obtained excluding the outliers (clusters C11 and C55) detailed in Table Add-16.1 are similar to those reported in the manuscript: RR=0.88 and RR=0.89 respectively. Both effects were non-statistically significant.

Table Add-16.1: Primary cluster level analysis repeated excluding cluster C11 and C55 and ignoring the pairing. The adjusted analysis includes age group, gender, times sprayed and socio-economic status.

Seroconversion	**Unadjusted**		**Adjusted**	
	Intervention effect	**p**	**Intervention effect**	**p**
Relative Risk				
Overall	0.88 (0.45-1.72)	0.6872	0.94 (0.50-1.76)	0.8417
India	0.96 (0.55-1.69)	0.8917	0.89 (0.51-1.55)	0.6633
Nepal	0.98 (0.38-2.53)	0.9529	0.74 (0.33-1.69)	0.4222