Evaluation of the Efficacy of Olyset® Plus in a Village-Based Cohort Study in the Cukurova Plain, Turkey, in an Area of Hyperendemic Cutaneous Leishmaniasis

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Source: Journal of Vector Ecology, 39(2):395-405. 2014.
Published By: Society for Vector Ecology
URL: http://www.bioone.org/doi/full/10.3376/i1081-1710-39-395

Evaluation of the efficacy of Olyset® Plus in a village-based cohort study in the Cukurova Plain, Turkey, in an area of hyperendemic cutaneous leishmaniasis

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Received 20 June 2014; Accepted 21 August 2014

ABSTRACT: The aim of this study was to measure the protective efficacy of Olyset® Plus, a new long-lasting factory-treated insecticidal net (LLIN) incorporated with 2% permethrin and 1% of the synergist piperonyl butoxide (PBO), against cutaneous leishmaniasis (CL) transmission under field conditions. A village-scale trial, promoting the use of LLIN by the local inhabitants of the study area was conducted as a pilot study in a new hyperendemic focus of CL caused by a *Leishmania infantum*/*L. donovan*i hybrid parasite transmitted by proven vector species *Phlebotomus tobbi* in Cukurova Plain, Adana, Turkey, between May, 2013 and May, 2014. The study area comprised eight villages; two of them were selected as an intervention village with Olyset® Plus net (Kizillar) and a control village without net application (Malihidirli). Six villages with surrounding allopatric barriers were utilized as a buffer zone cluster between intervention and control villages. Monthly entomological surveys were performed in the intervention and control villages and Damyeri, representing the other six villages, to collect adults of *Phlebotomus tobbi*. Results showed a significant reduction in cutaneous leishmaniasis incidence in the intervention village from 4.78% to 0.37%. The protective efficacy rate of LLIN was 92.2%. In contrast, incidence rates increased in the control village from 3.67% to 4.69%. We also evaluated residual insecticide levels of used nets after six and 12 months of usage. It was determined that the nets had retained full insecticidal strength. These results highlight the value of real-world data on bed net effectiveness and longevity to guide decisions regarding sand fly control strategies. To the best of our knowledge, this is the first field study to evaluate Olyset® Plus efficacy in a hyperendemic cutaneous leishmaniasis area. ***Journal of Vector Ecology* 39 (2): 395-405. 2014**.

Keyword Index: Cutaneous leishmaniasis, *Phlebotomus tobbi*, long-lasting insecticidal bed net, permethrin, piperonyl butoxide, Olyset Plus.

INTRODUCTION

Turkey is one of the few countries in the temperate climatic zone on the edge of the European continent in which certain vector-borne diseases are prevalent at endemic and occasionally epidemic proportions. Apart from malaria, cutaneous leishmaniasis (CL) is also endemic in several regions, particularly in southeastern Anatolia. Two *Leishmania* species, *L. tropica* and *L. infantum/donovani* hybrids, are endemic in Turkey (Ok et al. 2002, Svobodova et al. 2009). The third species, *L. major*, has been sporadically reported (Akman et al. 2000, Ok et al. 2002), and there is no evidence for transmission of zoonotic CL by this species. In the recent past, the biggest outbreak with over 11,000 reported cases occurred in Sanliurfa located in southeastern Turkey (Ok et al. 2002) and the parasite was typed as *L. tropica* (Waki et al. 2007). Since 1985, thousands of new CL cases have emerged in the Cukurova Plain and it has also been suggested that the causative agent is *L. tropica* (Uzun et al. 1999, Ok et al. 2002).

Svobodova et al. (2009) reported a new focus of CL in the Cukurova region (South Anatolia, Turkey), where hundreds of human cases continue to occur every year, most of them suffering from relatively small, non-ulcerating lesions. Surprisingly, no cases caused by *L. tropica*, the typical causative agent of CL in the nearby Sanliurfa region (Volf et al. 2000), were found. The causative agent in the Cukurova region was identified as a new *L. donovani/infantum* zymodeme MON-309 using multilocus enzyme electrophoresis analyses, which is similar to MON-188 in multilocus sequence typing (MLST) analyses (Svobodova et al. 2009). Recent whole genome sequencing of parasites isolated from sand flies and humans in the Cukurova region revealed that all of them are derived from a single cross of two diverse strains of *L. infantum* and *L. donovani* with subsequent recombination within the population (Rogers et al. 2014). According to local health authorities, no visceral leishmaniases (VL) have been reported from the study area, and dipstick tests did not reveal VL cases (Svobodova et al. 2009). *Phlebotomus tobbi*, the dominant sand fly species in the study area (Kasap et al. 2009), was identified as a proven vector since late-stage infections were repeatedly found in this sand fly and with dozens of isolates (Svobodova et al. 2009).

Identifying the risk of leishmania transmission is essential in designing an appropriate public health response to epidemics and for preventing future cases. Even though CL cases had been reported earlier in the Cukurova region, a rapid accumulation of patients in villages there within the last two decades demonstrates that the outbreak likely began in the foothills of the Taurus and Anti-Taurus Mountains (Votypka et al. 2012). Our previous studies suggest that the transmission cycle could be anthroponotic (Svobodova et al. 2009) and led us to hypothesize that villagers might be at higher risk from sleeping outdoors without bed nets. The moderate manifestation of CL has been overlooked in this rural area, and disease control strategies that target possible reservoirs and phlebotomine vectors have never been put into effect. Furthermore, an understanding of leishmaniasis transmission by local people, including the role of sand flies and the correct use of insecticides, is not widespread (Votypka et al. 2012). Due to all the factors mentioned above, as a second step, we studied the same hyperendemic area to identify the real risk factors associated with *L. infantum/donovani* hybrid infections. We used a case-control study designed to improve the knowledge of villagers about leishmaniasis and to understand the determinants of human outbreak-related CL acquisition in this rural area of the Cukurova region (Votypka et al. 2012). Results of the study revealed that three major risk factors for acquiring CL by *L. donovani* complex exist in the Cukurova region: dog ownership, raising cattle and sleeping without a bed net. Sleeping outside may place people at risk of sand fly exposure and the use of bed nets is usually very important in protecting against leishmaniasis transmission (Bashaye et al. 2009). Ownership of a mosquito net appeared to lower the risk, but it is known that classical mosquito bed nets do not have a fine enough mesh to provide sufficient protection against biting sand flies (Alten et al. 2003a). However, the effect of sleeping outside with or without bed net protection is not necessarily reflected in our results. Another previous study has demonstrated that insecticide-impregnated bed nets are effective in reducing the outdoor human-biting rate of *P. papatasi* and *P. sergenti* in the Sanliurfa province, southeast of Anatolia (Alten et al. 2003a). Those results indicate that field trials in an epidemiologically similar area of Turkey such as the Cukurova region could be fruitful. As in the Sanliurfa region, local people are highly exposed to sand flies while sleeping outside during the summer months in the Cukurova study area. A similar result was obtained in a study performed in two different Iranian cities, Sedeh and Shiraz, by Emami et al. (2009) that showed a statistically significant reduction in the incidence of new cases in intervention areas that received bed nets compared with control areas. The authors suggested that local people should use their bed nets during the entire summer when they sleep outside. We suggest that the use of insecticide-impregnated bed nets would improve personal protection against *P. tobbi* and may reduce the transmission of CL in this region of South Anatolia. Therefore, as a third step, a village-scale field trial with long-lasting insecticidal bed nets (LLIN) was planned in two villages in the area.

We evaluated the performance of a new generation LLIN, Olyset® Plus, in field conditions against both cutaneous leishmaniasis caused by *L. donovani* complex and its proven vector, *P. tobbi*, in the Cukurova region, Adana, Turkey. To the best of our knowledge, this is first field study carried out with Olyset® Plus in an area with hyperendemic cutaneous leishmaniasis.

MATERIALS AND METHODS

Study area

LLIN intervention was performed in the northwest part of the Cukurova region, South Anatolia, in 2013-2014 (Figure 1). South Anatolia is bound by the West Taurus Mountains, the mountain range of Taurus and Anti-Taurus to the north, and the Amanos Mountains to the east. The study area, in the northern part of Cukurova region (South Anatolia, Turkey) (Svobodova et al. 2009), is in the western part of the focus and comprises eight villages approximately 55 km northeast of Adana City at altitudes from 150–280 m a.s.l.: Kizillar, Zerdali, Camili, Damyeri, Otluk, Koyunevi, Malihidirli, and Tepecikoren. Tepecikören (37° 21′ 46′′ N, 35° 37′ 40′′ E) is the only village with a part-time health office (one day per week). The Kozan City Governmental hospital is the only health care facility in the area with anti-leishmania drugs and a leishmania diagnostic capability.

Most of the study area is fertile ('mollisol' soil) and used for agricultural activities, but pine (Pinus) and fir (Abies) forests are also cultivated. Citrus orchards and cotton fields are more common in the study area. The mean annual precipitation is 636.8 mm with 66% relative humidity, and the mean annual temperature is 18.7° C. During the sand fly season, from May to October, the mean relative humidity is 66% and the mean temperature is 27.9° C (min 13.7° and max 45.7° C). Residents live in single-family houses consisting of one or more rooms including a living room, small kitchen, and bathroom constructed of concrete, stone, briquette, or adobe, surrounded by gardens with henhouses and sheep or cattle sheds. Compounds and houses usually consist of an entrance leading to a courtyard. Living rooms serve as a bedroom in the winter. Animal quarters are sometimes built at the furthest end of the same compound opposite the living area, but generally they are built under the living room separated with a cement wall. Sometimes households keep their animals outside the compound but near enough to be watched closely. In some houses, animals live in the living room together with householders. The inhabitants perform various activities in the courtyards in the daytime during summer. They also watch television and sleep in the courtyards on summer nights using a Taht, a traditional sleeping sheet.

Study population and case-control study

Examining population and case-control studies have been well described in two our previous field studies carried out between 2005 and 2007 (Svobodova et al. 2009, Votypka et al. 2012). Inhabitants are basically subsistence farmers, with many of the men working as manual laborers in large cultivated fields. Some people work as laborers in nearby small factories, but agricultural production is the primary income source. The main activities of the women consist of household duties, along with work on the farms during spring and summer.

Patients were diagnosed by physicians based on clinical grounds at the Kozan City Governmental Hospital or by local doctors visiting villages. A case was defined as a person having at least one active leishmania lesion and/or a

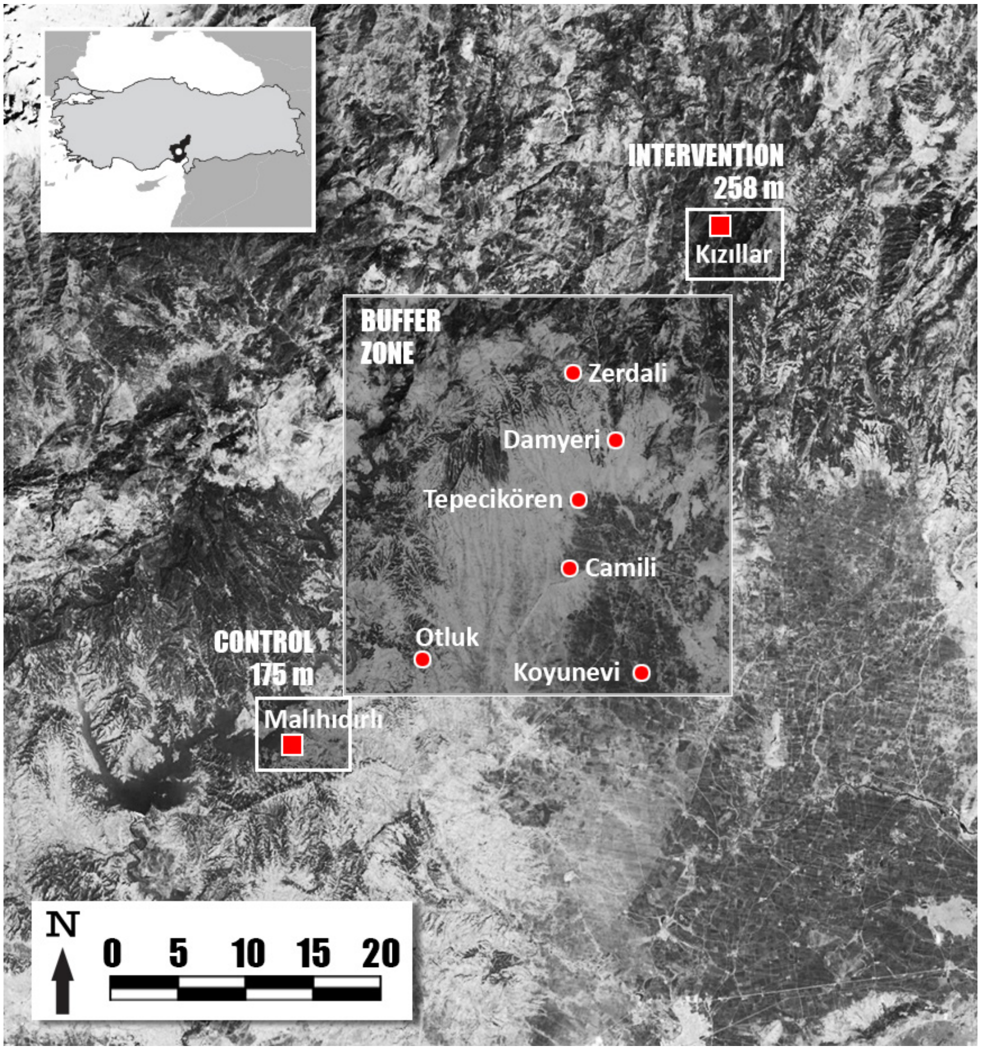

Figure 1. Map of the study area.

typical scar. Most case patients had been treated before the case-control study began, following standard practice and treatment of patients with CL, i.e., multiple weekly injections of Glucantime® (meglumine antimoniate) for at least two months. Case patients were sought in their homes, based on name lists from health centers and the familiarity of local health doctors. The controls were chosen preferentially according to the site where the cases occurred (i.e., from the neighboring houses), with the age and gender of cases and controls also taken into account.

Data collected by a risk factor questionnaire included the presence of domestic animals and where they were kept at night, storage of dried dung, house construction materials, toilet location, job travel to other areas, and socioeconomic indicators (age, gender, occupation, education, time living in the village, and family size). The remaining questions focused on epidemiological and clinical characteristics, such as sleeping outdoors, the number of lesions and/or scars and their bodily locations, and the time that lesions appeared. Participants were asked about methods they used to prevent sand fly bites (use of bed nets and insecticides) and their knowledge of leishmania transmission. For young children, parents provided consent and responded to the questionnaire (see Votypka et al. 2012 for detailed information).

Selection of study villages

On the basis of available epidemiological data with the Public Health Institution of Turkey and our previous data bank (Votypka et al. 2012), some villages were short-listed. Ongoing (EU FP 7. Edenext project) and preliminary entomological surveys were carried out to get some idea of the sand fly productivity (Belen and Alten 2011). Along with this information, three pre-intervention and bed net usage surveys were conducted in the operation area between October, 2012 and May, 2013, to determine the annual leishmaniasis incidence rate between. During this time, three questionnaires were completed with 8,203 inhabitants (4,103 male, 4,100 female) in eight villages (Figure 1). Statistical procedures were conducted on physical characteristics of settlements, socio-economic levels of the population living in experimental areas, migration rates of the areas, and the ratio of cutaneous leishmaniasis cases to compare settlements with each other and to help determine the selection of intervention and control villages (Votypka et al. 2012). On the basis of the average cutaneous leishmaniasis incidence rate, the sample size of the population for both intervention and control villages was calculated as minimum 800 and maximum 1,000. Accordingly, eight villages were divided into three operational zones and assigned either 1) LLIN- Olyset® Plus- (Kizillar; population 1,066), 2) control (Malihidirli; population 789) or 3) a cluster of six villages with surrounding allopatric barriers between the above two villages (=buffer zone).

Olyset® Plus distribution and study design

Olyset® Plus nets were supplied by the manufacturer (Sumitomo Chemical Co. Ltd). The nets were factory treated, containing 2% w/w permethrin corresponding to 20 g AI/kg (about 800 mg of AI/m^2) and 1% w/w piperonyl butoxide, as a pyrethroid synergist, corresponding to 10 g PBO/kg (about 400 mg of PBO/m^2) and were made of 150 denier white polyethylene monofilament fiber with 80 holes/ in^2 knitted in a raschel pattern. All the nets were of extra family/high size (width 180 cm, length 190 cm, height 210 cm).

In the first week of May, 2013, the last questionnaire was administered in selected villages with a total of 2,000 inhabitants to determine the incidence of CL cases between December, 2012 and May, 2013, and to get detailed information about the sleeping patterns of families. For this, both intervention and control villages were divided into six sub-areas on the map according to their official quarter names, and again, each house was numbered on the map to facilitate follow-up during intervention and post-intervention periods. The requirement of the nets was ascertained through a sleeping pattern survey in the villages. Before the start of the trial, community group meetings were organized in the study villages, and inhabitants were educated on the proper and regular use of nets and the importance of the study with health education messages. The distribution of nets according to the sleeping pattern survey was carried out the second fortnight of May, 2013, the number of nets distributed to each household was recorded in the register, and signatures of the recipients were obtained. Each member of the family was also recorded with his/her personal information such as name, age, sex, education level, etc. Six hundred and sixty-five nets were distributed in the intervention village (Kizillar) to cover the entire population of this village. Thus, we obtained an average of one bed net per two persons (min. one person, max. three persons under each net). Additionally 100 nets were provided to the manager of the primary school and "Imam" (prayer leader) for distributing to new families coming from outside the study area for their summer holidays or to replace those nets that had been torn or lost. During the intervention period, the supervisory project staff, in consultation with the community, formed a village committee consisting of governing body members and other opinion leaders to monitor the proper use and maintenance of the nets. After the intervention, to determine accurate CL incidence trends and to compare villages to each other, four follow-up questionnaires were conducted every 90 days between June, 2013 and May, 2014.

To measure insecticide retention, samples of net pieces from top, bottom, right and left sides, and front and back of whole nets (each side was marked during distribution) were cut from additionally distributed bed nets that were provided to 15 selected families at the beginning of the intervention. These selected families set up the bed nets in the same courtyards with the other nets being used. Each of these additional bed nets was washed at least three times using natural soap by inhabitants during the six-month period. Chemical analyses of these additional nets was carried out ten days after starting the intervention, and again three and six months after the start of the intervention, to determine the content of permethrin and piperonyl butoxide (PBO). A total of 90 pieces (25 cm x 25 cm) were cut from each of the 15 additional nets according to the WHO sampling method for LLINs and pooled for chemical analysis. The average permethrin and PBO contents were determined using the CIPAC method 331/LN/M/3 (www. cipac.org). This method involved extraction of permethrin and PBO from the net samples in a water bath (85–90° C) for 45 min with heptane in the presence of triphenyl phosphate as an internal standard and determination by gas chromatography with flame ionization detection (Pennetier et al. 2013).

Entomological sampling

Adult sand fly densities were measured in five randomly selected houses in both the trial and control villages and in five houses of a village (Damyeri) belonging to the buffer zone cluster from May to late October (Figure 1). The randomly selected houses were changed every month during the pre-intervention period. However, during the intervention phase, sand fly monitoring was restricted to only sampling fixed houses. This was to determine the seasonal population fluctuations of these vectors and to assess possible impact of impregnated bed nets on sand fly populations. The total number of sand flies in a house was measured every month in the night between 18:00 and 07:00 by the following methods. Twenty-five miniature CDC light traps (John W. Hock, U.S.A.) and 500 sticky papers were used for sampling in each collecting night. Papers, 20x20 cm, were prepared in the laboratory, numbered, and left in castor oil overnight. Twenty sticky papers were placed in each household as sand fly baits for 24 h. One was nailed to the top of each corner of the bedroom (each house had at least two bedrooms), one was placed on the front wall of each bedroom, two in the kitchen, if present, two in the stable or animal shelter, and six in each courtyard. Sand fly density was then estimated by checking both sides of the paper and the data were recorded. For the sampling of exophilic species, light traps were maintained according to the standard used by Southwood (1966). A total of 25 light traps (five light traps per village) were used for sampling in each collecting day. Light traps were operated between the hours 18:00-07:00 in each sampling station. Light traps were run every month from May to late October.

Specimens were stored in 96% alcohol for morphological identification. Identifications were based on the morphology of male genitalia and female spermathecae and pharynges using the keys of Theodor (1958), Lewis (1982), and Killick-Kendrick et al. (1991). Both the preparation and identification of specimens were made individually.

Statistical analysis

Significance of possible sources of error (treatment and date) was evaluated by Kruskal-Wallis one-way analysis of variance (ANOVA) (Zar 1996). Significance between treatments was set at 5% level.

Ethical considerations

The proposal was approved by the Ethical Review Board of Ministry of Health, Public Health Institution of Turkey and officially declared as number B.10.1.HSK.14.05. The households in both intervention and control villages were informed about the purposes of the study and educated by study team and medical doctors from local health centers. A medical doctor was on hand during the trial to respond to any side effects of the nets or to treat any cases of fever.

RESULTS

Annual changes in CL incidence: Impact of Olyset® Plus on reduction of CL

Approximately 2,000 inhabitants were interviewed just before the intervention, and 1,855 of them were included in the study. It was subsequently found that 145 of them had been infected before May, 2012 (7.25%), and were consequently potentially immune to reinfection. A total of 80 persons were newly infected for the entire study area between May, 2012 and May, 2013, a yearly incidence of 4.31% in one year. In the same period, yearly incidence was determined according to both intervention and control areas as follows: Kizillar (intervention village) 4.78% (51/1066) and Malihidirli (control village) 3.67% (29/789).

Table 1 and Figure 2 show the CL cases in each village during the pre-intervention period (May 2012-May 2013) and the post-intervention study period (May, 2013 to May, 2014). During the pre-intervention period, CL prevalence was remarkably high in both the intervention village where treated bed nets were distributed and in the control village. Incidence declined greatly in the intervention village after the introduction of Olyset® Plus, reaching an incidence rate of 0.37% by the end of the post-intervention year. The reduction rate of yearly CL cases was 92.2%. The differences in CL incidence were statistically compared for the year 2013-2014 for the intervention village, and it was found that the declining differences at the end of one-year period were significant (P<0.05). During the post-intervention period, the incidence of CL fluctuated but never declined greatly from its initial level in the control village. CL incidence remained at almost initial levels until December and a partial increase in the annual incidence rate was seen in the control village (4.69%; 37 new patients in the study year). This difference was also found to be statistically significant (P<0.05) between the intervention and control villages (Table 1).

Phlebotomine fauna

Over the 24-month period including the pre-intervention phase (May, 2012 to May, 2013) and the intervention phase (May, 2013 to May, 2014), the total number of sand flies collected in intervention (Kizillar) and control (Malihidirli) villages, and also in a selected village from the buffer zone (Damyeri) was 11,287. These belonged to the following seven species: *Phlebotomus tobbi* (8,141-72.12%), *P. perfiliewi* (1,177-10.42%), *P. papatasi* (980-8.68%), *Sergentomyia dentata* (674-5.97%), *P. (Larroussius)* sp. (unidentified) (253-2.24%), *P. major s.l* (39-0.34%), and *P. sergenti* (23-0.20%). *P. tobbi* was the most abundant species in both phases. Interestingly, *P. sergenti*, the proven vector of *Leishmania tropica* in southeastern Anatolia, was found to be only 0.20% of the total number of sand flies caught (Table 2).

Figure 3 shows the seasonal population fluctuations of adult *P. tobbi* captured in three villages during both the pre-intervention and intervention phases. Among the species caught, only the seasonal population dynamics of *P. tobbi* were evaluated as this species was shown to be the only vector of the *Leishmania infantum/donovani* hybrid in the entire study area. In both phases, a total of 6,749 *P. tobbi* were captured from three villages; 42.15% (n=2,845) were collected in the pre-intervention phase and 57.84% (n=3,904) during the intervention phase. Even though the population density of *P. tobbi* increased during the intervention phase, there was no significant difference in terms of the density of *P. tobbi* population between the two phases. The numbers and ratio of *P. tobbi* collected in each village according to different phases were determined to be as follows: 32.61% (n=928) in Kizillar (intervention village), 37.82% (n=1,076) in Malihidirli (control village), and 29.56% (n=841) in Damyeri (buffer zone) in the pre-intervention phase, and 30.07 % (n=1,174), 26.43% (n=1,032), and 43.49% (n=1,698) in the intervention phase, respectively. There was also no significant difference in sand fly population density between villages in either pre- or post-intervention phases (P>0.05). However, we observed a slight but not significant increase (11.74%) in sand fly population density for the years 2013 and 2014 in the intervention village.

Sand fly activity within the region started at the beginning of May and lasted until the month of November, coinciding with the increase and decrease of the average temperature and humidity (Figure 3). Overall, *P. tobbi* was the predominant species during the active seasons in both the pre-intervention and intervention phases. The population of *P. tobbi* reached its peak values in September while two peaks were defined, one during August and another in September for the control village in the pre-intervention year and for the buffer zone village in the intervention year. Like other species identified in the study area, the *P. tobbi* population also showed "uni-model" (one peak per year) seasonal dynamics. In general, there was no significant difference in the population size each month between intervention and control villages in both years. But the population size in Kizillar (intervention) was approximately two-fold larger than that of Malihidirli (control) in the month of September in the intervention year.

Insecticide retention in Olyset® Plus nets

The chemical analysis of the nets used for the field trial showed that the permethrin content of Olyset® Plus nets that were washed 3 times by homeowners for six months remained close to the initial levels of 20±5 g AI/kg. With

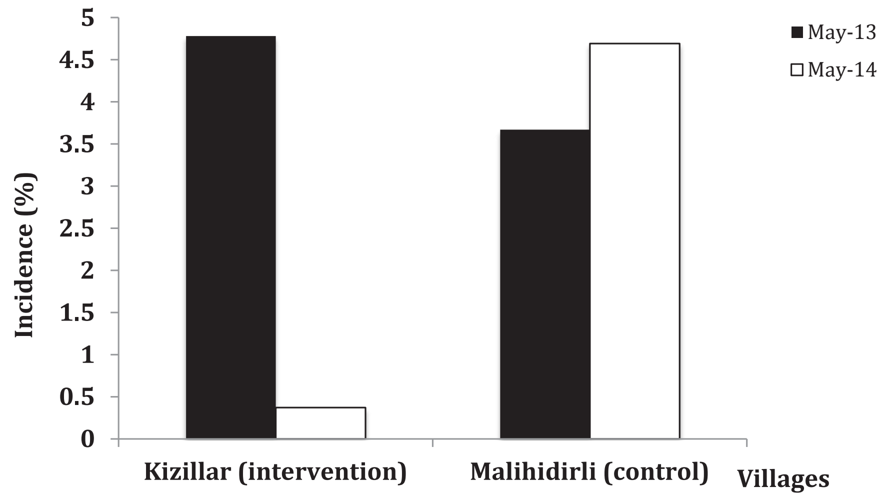

Figure 2. The annual changes of cutaneous leishmaniasis incidence (%) in intervention and control villages between May, 2013 and May, 2014.

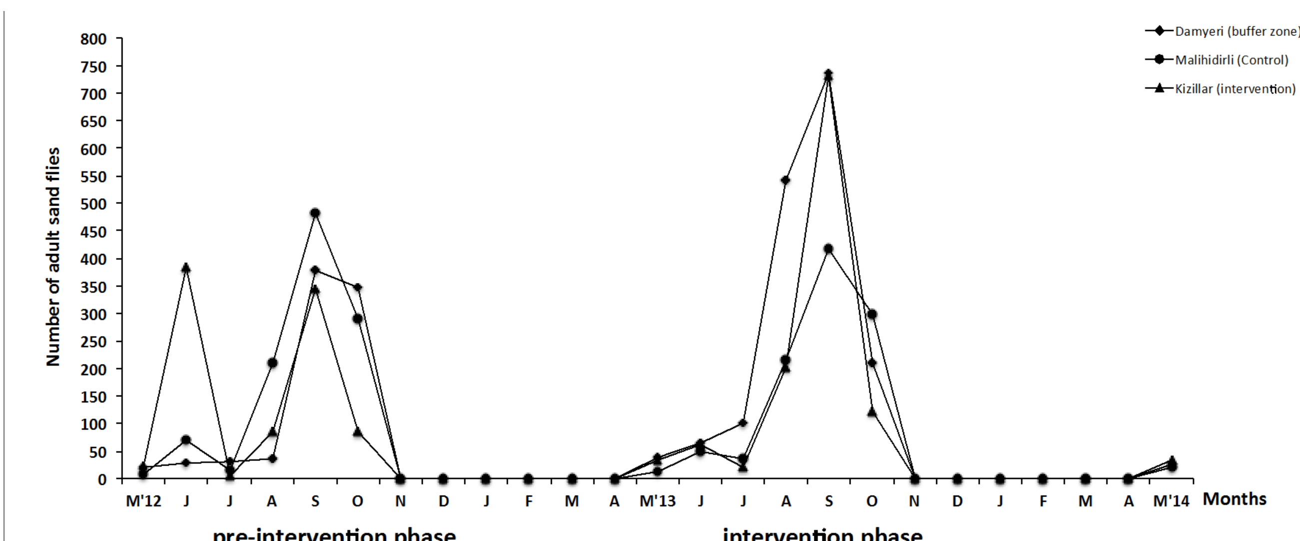

Figure 3. Seasonal population fluctuations of *Phlebotomus tobbi* distributing in Kizillar (intervention), Malihidirli (control) and Damyeri villages in the study area.

Table 1. Settlements, population numbers, number of females and males, and differences of incidence rates between years and villages.

						cutaneous leishmaniasis incidence							
						Pre-intervention year May 2012 to May 2013				Intervention year May 2013 to May2014			
City	District	Village	C/I	Population	Sex (F/M)	2012	2013	total	incidence %	2013	2014	total	incidence %
Adana	Imamoglu	Malihidirli	control	789	385/404	13	16	29	3.67	17	27	37	4.69
Adana	Kozan	Kizillar	intervention	1066	519/547	24	27	51	4.78	0	4	4	0.37

Table 2. Phlebotomine sand flies and number of individuals collected in pre-intervention and intervention phases in the study area.

Species	pre-intervention phase			intervention phase			TOTAL
	n (%) females	n (%) males	n (%) total	n (%) females	n (%) males	n (%) total	
Phlebotomus (Larroussius) tobbi	814 (23.4)	2,665 (76.6)	3,479 (75.35)	2,000 (42.90)	2,662 (57.10)	4,662 (69.89)	8,141 (72.12)
Phlebotomus (Phlebotomus) perfiliewi	286 (66.35)	145 (33.65)	431 (9.33)	333 (44.63)	413 (55.37)	746 (11.18)	1,177 (10.42)
Phlebotomus (Larroussius) papatasi	149 (51.37)	150 (48.63)	290 (6.28)	370 (53.62)	320 (46.38)	690 (10.34)	980 (8.68)
Sergentomyia dentata	95 (47.5)	105 (52.5)	200 (4.33)	274 (57.80)	200 (42.20)	474 (7.10)	674 (5.97)
P. (Larroussius) sp. (unidentified)	101 (57.7)	74 (42.3)	175 (3.79)	30 (38.46)	48 61.54)	78 (1.16)	253 (2.24)
Phlebotomus (Larroussius) major s.l	17 (73.91)	6 (26.09)	23 (0.49)	8 (50)	8 (50)	16 (0.23)	39 (0.34)
Phlebotomus (Paraphlebotomus) sergenti	9 (47.36)	10 (52.64)	19 (0.41)	0 (0)	4 (100)	4 (0.05)	23 (0.20)
			4,617 (100)			6,670 (100)	11,287 (100)

three- and six-month Olyset® Plus nets, permethrin contents of 19.02±0.77 g AI/kg and 18.63±0.11 g AI/kg, respectively, were detected, corresponding to an overall retention of 95.1% and 93.1%. The PBO content in Olyset® Plus was also close to the initial dose of 10±2.5 g AI/kg, nets containing 9.01±0.11 g AI/kg at three months and 8.01 g AI/kg at six months, corresponding to an overall retention of 90.1% and 80.1% g AI/kg, respectively. Both the permethrin and PBO content in the used Olyset® Plus nets did not decrease significantly (Table 3). There was also no observable difference in insecticide contents of different sides of the nets in terms of consecutive months. It was determined that Olyset® Plus had long-lasting activity under natural conditions. This would indicate that under the use conditions in the study area, these nets should continue working for at least three years, which is the minimum WHOPES requirement for a long-lasting net. Further detailed tests performed in the laboratory and under field conditions showed similar results (unpublished data).

DISCUSSION

Results of this field trial are based on comparative data collected from two different villages using Olyset® Plus and no nets during one year of use in an area where the primary cutaneous leishmaniasis vector *Phlebotomus tobbi* (Svobodova et al. 2009) were fully susceptible to deltamethrin and permethrin (unpublished data). In Turkey, synthetic pyrethroids have long been used in rotation with other insecticides for mostly indoor residual spraying in high-risk malarious and leishmaniosis areas, but the susceptibility status of vectors to pyrethroids has remained nearly stable. This study is also based on use in a rural area of Turkey, where net distribution and the instructions regarding bed net use are not systematically done. In this sense, this study can be regarded as a good "surveillance program" to monitor both ongoing leishmaniasis and also malaria control efforts in Turkey under ordinary conditions.

Our results can be summarized as follows: 1) Olyset® Plus can provide significant personal protection with a 92% reduction rate from the bites of sand flies and subsequently reduce the risk of CL infection; 2) There was no strong evidence that the use of pyrethroid- impregnated bed nets had an impact on reducing the mean total density of *P. tobbi* in the intervention areas compared with the control areas; 3) There was no significant decrease of both permethrin and PBO content of Olyset® Plus under normal conditions of use over a one-year period; 4) Considering that leishmaniasis is still endemic in the study area (Votypka et al. 2012), development of a net incorporating a pyrethroid with a synergist is promising against cutaneous leishmaniasis vectors and it revealed that Olyset® Plus has high level efficacy under field conditions. This study demonstrated the benefit of incorporating permethrin and PBO together in a long-lasting insecticidal net for leishmaniasis control.

The longitudinal design of the study allowed all individuals to be followed up during the study period (May, 2013 to May, 2014) every three months after the distribution of the bed nets (intervention period) according to the study design of previous large-scale trials performed in Sanliurfa City where cutaneous leishmaniasis is hyperendemic in southeast of Anatolia (Alten et al. 2003a). This helped reduce the margin of error and gradually made the estimate of previously infected individuals more accurate.

The efficacy and community-effectiveness of insecticide-impregnated bed nets in preventing morbidity and mortality from several vector-borne diseases, such as malaria and leishmaniasis in several disease endemic countries, has been frequently demonstrated in the past. The studies reported by Jalouk et al. (2007) was carried out from 1997 to 1999 and from 2001 to 2003 in ten and four villages, respectively, in Aleppo, Syria. Both studies not only confirmed the high efficacy of ITNs in preventing CL during the one-year post intervention (about 85% in the 1997-1999 trial), but also suggested that the interruption of this control measure might restore the pre-intervention disease incidence within one to two years. The other study, also performed in the Middle East in Bam City, Iran, showed a significant reduction in the cumulative CL incidence in the intervention area and a reversal of the relative risk (Noazin et al. 2013). Sharma et al. (2009) noted that the Olyset Net, which is a first generation product using Olyset®

Table 3. Active ingredient and synergist contents of Olyset® Plus net samples used in the study area.

	Position of piece*					
	top	bottom	right side	left side	front	behind
Just after intervention						
Permethrin content (g/kg ±SE)	19.90±0.18**	20.03±0.08	22.12±0.10	20.01±0.21	20.98±0.17	21.84±028
PBO content (g/kg ±SE)	9.97±0.11	9.94±0.17	11.02±0.09	10.07±0.07	10.01±0.13	9.29±0.16
3 months						
Permethrin content (g/kg ±SE)	19.02±0.77	19.99±0.11	20.33±0.07	19.76±0.18	20.00±0.02	20.17±0.29
PBO content (g/kg ±SE)	9.02±0.64	9.66±0.23	10.01±0.06	9.96±0.09	9.44±0.44	9.01±0.11
6 months						
Permethrin content (g/kg ±SE)	18.63±0.11	19.03±0.44	19.95±0.04	19.07±0.09	19.33±0.16	19.37±0.22
PBO content (g/kg ±SE)	8.01±0.49	8.89±0.17	9.92±0.14	9.03±0.10	8.97±0.33	8.88±0.61

*Unwashed pieces.

**Average value of all pieces cut from fifteen bed nets.

technology, maintained high bioefficacy (>80%) against *An. culicifacies* and *An. fluviatilis* in India, even after repeated washings. One of the interesting results on the protective efficacy of LLINs (PermaNet) obtained from a comparative study of kala-azar vector control measures in eastern Nepal demonstrated that, even though indoor residual spraying was an effective control measure to decrease vector density, LLINs were also found to be effective and can be considered as a promising alternative vector control tool in visceral leishmaniasis elimination initiatives (Das et al. 2010). One of the most important results of protective efficacy of LLINs published by Komazawa et al. (2012) showed that LLIN coverage level (about 35%) could induce a community effect to protect children sleeping without bed nets even in malaria endemic areas.

To the best of our knowledge, this is the first field study carried with Olyset® Plus in a hyperendemic cutaneous leishmaniasis area. We found a considerable and consistent reduction in cutaneous leishmaniasis incidence in the intervention areas following the introduction of Olyset® Plus compared with the control areas. Although *P. tobbi* were abundant in the intervention area, the results of the study suggest that their ability to transmit *L. infantum/donovani* was greatly reduced because of the treated bed nets. In the intervention year, the reduction in incidence rate was found to be considerable (12.91-fold) and very high (4.78% to 0.37%; $P<0.05$).

Despite the success in reducing parasite transmission, this study has some limitations. There were difficulties in determining three-month reduction rates between intervention and control areas due to various factors. The main factor was to calculate human movements and migration rates, particularly those moving from outside the study area, during the hot summer and part of the autumn because temperature and relative humidity are more moderate than at lower altitudes. We also distributed more than 100 extra bed nets to inhabitants based on previous questionnaires to cover for possible migrants into the area.

Though medical treatment is one of the major tools for decreasing parasite transmission, it is interesting to note that it has not been a factor in preventing transmission of the disease in our study population because of the following problems. Almost all patients sought treatment in 2012, and the survey team thought that the presence of hospitals in Adana and Kozan Cities would bias the results by reducing the incidence in the control areas. The technicians in these hospitals were actively involved in the treatment of CL lesions. However, the high prevalence of the disease throughout the post-intervention period in the control areas showed that treatment did not play a major role, although the fact that many patients interrupted treatment before the lesions healed completely may have mitigated the effects. We assumed that this situation is common in parts of Anatolia. Our previous study conducted in southeastern Anatolia between 1999 and 2002 (Alten et al. 2003a,b) showed very similar results concerning treatment activities in the study area.

At the beginning of the implementation, extensive health training took place in the villages to explain the properties and correct use of Olyset® Plus bed nets. While being trained, the villagers received information about cutaneous leishmaniasis and sand flies as well as how to use bed nets. However, this training could not be repeated for the immigrants because of geographical conditions and long distances. As a result, after a short period, even some of the people who received the training were influenced by the behavior of immigrant relatives and stopped using bed nets during dinner time, TV watching, and even sleeping, when the *P. tobbi* population was high (20:00-22:00).

An objection to using nets has been raised among some inhabitants that bed nets restrict ventilation, especially during the summer when it is hot and the humidity is higher. Some additional parameters such as a large number of persons living in the same house (up to ten), lower sanitary levels, or the feeding of domestic animals in courtyards or under houses, could contribute to relatively higher incidence levels in intervention villages than we expected at the beginning.

We have no strong evidence that the use of Olyset® Plus nets had an impact on reducing the mean total density of *P. tobbi* in the intervention areas compared with the control areas. The mean density was quite similar in the intervention and control areas during the 2013-2014 transmission season with seasonal fluctuations. One would expect that the insecticide absorbed by the bed nets would have resulted in a lower mean sand fly density in the intervention areas compared with the control areas. Dead insects, including sand flies, were found in the mornings on and around the treated bed nets during survey team visits to households in the intervention settlements. This indicates a possible impact on the reduction of the overall density of the insect population, including sand flies.

We emphasize that this study was not designed to measure insecticidal efficacy of permethrin and PBO against sand flies. It is also known that *P. tobbi* is an opportunistic feeder that feeds mainly on cattle and is both endophilic and exophilic. Svobodova et al. (2009) proved that *P. tobbi* prefers cattle blood (159/225 females; 70.7%) more than human blood (23/225 females; 10.2%) in the study area. However, some studies performed under laboratory and also field conditions in recent years have shown very promising results on the insecticidal effectiveness of LLINs. Pennetier et al. (2013) compared the bioefficacy of two types of LLINs (Olyset® Plus and Olyset Net) under laboratory conditions and found 100% knockdown effect and 100% mortality rate of Olyset® Plus against female *An. gambiae* under laboratory conditions. The authors also determined high and significant blood-feeding inhibition rates with all LLIN treatments compared to control ($p<0.05$) in the experimental huts of Malanville in Benin. In the study conducted in a hyperendemic tribal area of Orissa, India with Olyset Nets, a significant reduction was found at the rate of 80.6, 94.1, and 76.7% in the entry rate of *An. culcifacies*, *An. fluviatilis*, and other Anopheline species, respectively, with an overall reduction of 63.5% in total mosquitoes. The floor sheet collections in houses with Olyset Nets indicated 39% immediate mortality in total mosquitoes. The overall feeding success rate of mosquitoes in the trial village was 18% in comparison to 44.2 and 79.1% in villages with untreated nets and no nets, respectively (Sharma et al. 2009).

Regarding insecticide retention rates, no significant differences were found in terms of both permethrin and PBO level between fresh and six months used three times-washed bed nets (Table 3). In fact, according to previous publications (Penneiter et al. 2005, 2009, Van Bortel et al. 2009, Corbel et al. 2010, Yewhalaw et al. 2012) this result was expected. Pennetier et al. (2013) showed very strong evidence for a benefit of incorporating the synergist PBO with a pyrethroid insecticide (Olyset® Plus) into mosquito netting compared with Olyset Net without synergist against susceptible and multi-resistance malaria vectors with different experiments under laboratory conditions and in field conditions. They found the knock-down effect was 100% after three washes, and the mortality rate was 64% for three consecutive washes on the same day. The authors also found that the overall permethrin and PBO retention in Olyset® Plus even after 20 washes was 64.1% and 44.2%, respectively. After the experimental hut study, the permethrin and PBO content in the tested Olyset® Plus did not decrease significantly.

The present study showed Olyset® Plus nets are an effective long-lasting personal protection tool that is operationally feasible to prevent sand fly bites in high leishmania transmission areas where other interventions have a limited role. In addition, considering the highly endophilic and endophagic behavior of *P. tobbi* in Turkey (Svobodova et al. 2009), it is also suggested that the use of impregnated bed nets in an endemic area with a cooler climate in summer or autumn where people sleep in rooms at night would provide an invaluable control measure against these vectors of cutaneous leishmaniasis and may have better results with regard to the interruption of transmission of CL. These encouraging results need to be complemented by a large-scale field trial to assess the durability and acceptability of this new tool for cutaneous leishmaniasis vector control.

Acknowledgments

Our appreciation and thanks go to all who participated so generously and actively in this project. The cooperation and patience of the population of the settlements during this long project was of great help. The hard work of the staff from the Directorate of Health in Kozan and Imamoglu/Adana, as well as that of the administrators contributed in no mean measure in fulfilling the aims of the project. Equally important was the constant support received from the Republic of Turkey Ministry of Health, Public Health Institute, Ankara and Adana, as well as the interest shown by the Hacettepe University and Ege University. Olyset® Plus nets used in this study were obtained by Sumitomo Chemical Company, Ltd. We especially thank Dr. John Lucas from Sumitomo Chemical Company, Ltd. for his help supplying procedures and his technical input and comment. We also thank Dr. Mehmet Ali Torunoglu, Dr. Ali Tanju Altınsu, Dr. Fuat Gunes, and Technician Salim Calis for their administrative, technical, and scientific support. This study was funded by EU grant FP7-261504 EDENext and is catalogued by the EDENext Steering Committee as EDENext234 (http://www.edenext.eu). The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

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