

Original Article

Molecular Epidemiological Study of Cutaneous Leishmaniasis in the Focus of Bushehr City, Southwestern Iran

***Mohammad Reza Yaghoobi-Ershadi**¹, **Farideh Shahbazi**¹, **Mohammad Darvishi**², **Amir Ahmad Akhavan**¹, **Reza Jafari**³, **Mohammad Khajeian**², **Yavar Rassi**¹, **Hassan Soleimani**⁴, **Mohammad Reza Shirzadi**⁵, **Ahmad Ali Hanafi-Bojd**¹, **Hossein Darabi**⁶, **Mohammad Hossein Arandian**³, **Alireza Sanei-Dehkordi**¹, **Mansour Heidari**⁷

¹School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

²Deputy of Health Services, Bushehr University of Medical Sciences, Bushehr, Iran

³Esfahan Health Research Station, National Institute of Health Research, Esfahan, Iran

⁴Yazd Health Research Station, National Institute of Health Research, Yazd, Iran

⁵Communicable Diseases Management Center, Ministry of Health and Medical Education, Tehran, Iran

⁶The Persian Gulf Tropical Medicine Research, Bushehr University of Medical Sciences, Bushehr, Iran

⁷Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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Abstract

Background: Cutaneous leishmaniasis (CL) represents the most frequent vector borne parasitoses in Iran. The objective of this study was to determine the epidemiological features of CL including human infection and the reservoir host in the city of Bushehr, Bushehr Province, Iran during 2010–2011.

Methods: Studies on human infection was carried out on 2962 school children aged 7–14 years old from 60 primary schools and among 400 households with a total population of 1568 in four infected districts of the city in December 2010. Serosity materials from patients on glass slides were collected for molecular identification of causative agent. Rodents were caught by Sherman traps and examined for identification of the parasite.

Results: Prevalence of scars and ulcers among the inhabitants were 5.86% and 0.12% respectively. Molecular study indicated the presence of two coexisting species: *Leishmania major* and *L. tropica* among patients. The scar rate was 1.24% but no ulcers were seen among the students. Nineteen rodents were caught and identified as *Tatera indica* (47.4%) and *Rattus norvegicus* (52.6%). Specimens from 7 *T. indica* and 9 *R. norvegicus* were examined by two techniques, microscopic examination and nested-PCR. Out of 7 *T. indica*, 14.3% were infected with *L. major* and 42.9% with *L. turanica* by nested-PCR. Out of 9 *R. norvegicus* 22.2% were infected with *L. turanica* and 11.1% with *L. gerbilli*.

Conclusion: Based on this survey *L. major* and *L. tropica* are the causative agents of the disease among patients and *T. indica* plays a predominant role in the dissemination of *L. major* in the city.

Keywords: Molecular Epidemiology, Cutaneous Leishmaniasis, *L. major*, *L. tropica*, *Tatera indica*

Introduction

Cutaneous leishmaniasis (CL) is the first most important vector borne disease at present in Iran (Yaghoobi-Ershadi 2010, 2012). It has been neglected as a major public health problem because it is not a fatal disease. It is still the cause for considerable morbidity of a vast number of people in the endemic foci

and characterized by chronic skin lesions followed by permanent scars and deformation of the infected area (Yaghoobi-Ershadi 2002). For only this reason in urban and rural areas it is politicized and health authorities are usually questioned on the matter in the Islamic Parliament of Iran.

Cutaneous leishmaniasis has been epidemic during the years 1988, 1997 and 2008 in the city of Bushehr (Health Center of Bushehr Province, unpublished data). It is one of the free trade-industrial zones of the country and the Bushehr nuclear power plant which is unique in terms of its technology in the Middle East is located 12 km, southeast of the city along the Persian Gulf, so lots of people travel around and some make several trips in a year for business. If the disease does not receive considerable attention by the health authorities, it may spread into other parts of the country which are free from CL. However the epidemiological aspects of CL have not been examined in the city yet and there is no accurate data on the prevalence, reservoir (s) and vector(s) of the disease.

The objective of this study was to determine for the first time the epidemiological features of CL including human infection and the reservoir hosts in the city during 2010–2011.

Materials and Methods

Study area

The city of Bushehr located in a plain running along the coastal region on the Persian Gulf coast of southwestern Iran and is the administrative center of its province.

Field studies were carried out over a period of 22 months (from September 2009 to end of June 2011) in the city of Bushehr (Latitude: 28° 55' 30" N, Longitude 50° 50' 17" E, Altitude: 5 m above sea level) (Fig. 1). The city had a population of 221016 in 2011, while this was 133753 in 1991 with an increase about two folds in the last two decades. The area has a hot desert climate though it does receive more rainfall than most cities on the Persian Gulf. The rain is confined to the period from November to May, when temperature is pleasantly mild and is extremely erratic. The long summer from April to October is brutally hot, humid and completely

rainless. In 2010, the maximum and minimum mean monthly temperature was 39 and 12.1 °C in August and February respectively, and the total annual rainfall was 4.29 mm with a minimum of 0.1 mm in May and 2.45 mm in February. The minimum mean monthly relative humidity was 58 % (December) and the maximum was 74 % in January (Bushehr Meteorological Organization, unpublished data).

Population studies

The study was carried out in two population groups: (1) school children and (2) the population of the infected parts of the city (to obtain data on human infection rate in all age groups). For the first group, a list of all the elementary schools was obtained from the Department of Education. One hundred and twenty classes were selected by cluster sampling technique. Each class was visited and in each class, a list was prepared from all the school children and they were questioned and examined for the presence of ulcer(s) or scar(s).

For each case having ulcers or scars a form was completed to record the necessary information such as name, address, age, sex, number of ulcers or scars, site of ulcer(s) or scar(s), date and place of acquiring the disease, etc. Smears were prepared from scraping of the edge of the ulcer fixed in methanol, stained with Giemsa and examined under a light microscope for the presence of amastigotes. Serocity materials from some CL patients on glass slides were used for molecular identification of causative agent. All the school children were visited in December 2010.

For the second group, four infected districts of the city were selected, called Tangak in the south, Sangi, in the city center, Chaharmahalleh in the north and Imamzadeh in the southwest of the city. One hundred households from each district whose buildings were located near each other were visited and all members of the households

examined by coincidence of visiting schools in the selected parts of the city.

The χ^2 test was used to determine any statistical significant difference in disease prevalence between males and females of school children and inhabitants of the infected districts.

Collection and examination of rodents

Colonies of rodents were identified and caught using 35 Sherman traps baited with cucumber and dates monthly. In the laboratory they were identified by morphological characters (Etemad 1978) then regardless of the presence of lesions, impression smears were prepared from the ear lobes of the animals (Edrissian et al. 1982, Mohebbali et al. 2004) fixed in methanol and stained by the standard Giemsa method, and examined carefully under the light microscope (1000 X) for detection of *Leishmania* amastigotes during September and December of 2011.

Sample Collection

Twenty specimens were collected from suspected CL patients who were referred to the health center of Bushehr. The tissue samples from patients and rodents were placed in labeled micro tubes, stored in 70% ethanol (PBS) at -20 °C until further examination.

DNA Extraction

The smears from suspected cases of leishmaniasis were used, all the slides were washed with absolute ethanol and after drying washed three times in cold sterile PBS (pH 7.2). The smear on the slides was scrapped off and collected in the 1.5 ml microtubes. Genomic DNA was extracted and purified using Qiagen extraction Kit (Qiagen, Germany, Cat.no.69504) according to the manufacturer's manual with the minor modification of increasing incubation time to increase the yield of DNA in the final step. DNA was stored at -20 °C until analysis. Before submitting the tissue from rodents to the DNA extraction procedure described for the

slides, the tissue was subjected to 13 freeze/thaw cycles, using liquid nitrogen and boiling water, to disrupt the tissues, and treated as described above. The concentration of extracted DNA was measured by NanoDrop (Thermo Fisher Scientific, USA).

Molecular Assays

Primer design for amplification of ITS2

Primers designed previously and used to amplify a 230 bp product in *L. major*, a 215 bp product in *L. tropica*, a 206 bp in *L. gerbili* and a 141 bp in *L. turanica* across the internal transcribed spacer 2. The external primers, Leish out F (5'-AAA CTC CTC TCT GGT GCT TGC-3') and Leish out R (5'-AAA CAA AGG TTG TCG GGG G-3'), and internal primers, Leish in F (5'-AAT TCA ACT TCG CGT TGG CC-3') and Leish in R (5'-CCT CTC TTT TTT CTC TGT GC-3') were selected to distinguish among the parasite species in a nested PCR system (Akhavan et al. 2010a).

Nested-PCR

We used nested-PCR to identify the *Leishmania* species. Conditions and parameters for PCR were as previously described with the minor modification (Akhavan et al. 2010). All samples were tested in 25 µl amplification reaction mixtures with 12.5 µl of the master mix (Taq DNA polymerase, 2X Master Mix Red, Amplicon, Germany), 1.8 µl of primers (each primer: 10 pmol), 10.7 µl H₂O, and 1 µl of template DNA (20 ng). The first-round PCR was performed based on the following conditions: initial denaturation at 95 °C for 5 min; followed by 35 cycles including denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. The second-round (nested) PCR was performed as the same first round exception for annealing at 58 °C for 30 s. At the end, 10 µl of the reaction mix was analyzed by 2.5% agarose gel electrophoresis.

Additionally, for all PCR reactions one negative control without DNA and one positive control with standard DNA were included to confirm the results of two rounds of nested-PCR. The PCR products of the negative and positive controls of the first-round PCR were used as negative and positive controls in the second round (respectively). Finally, 10 µl of the PCR products were loaded on 2.5% (W/V) agarose gels, and stained with ethidium bromide to visualize by electrophoresis. Initially, ITS-PCR was confirmed with standard DNA of reference strains *L. major* (MRHO/IR/75/ER), *L. gerbilli* (MRHO/CN/60/GERBILLI) and *L. turanica* (MRHO/SU/1983/MARZ-051) *L. tropica* (MHOM/IR/o4/Mash10) as positive controls and distilled water were used as negative controls (Akhavan et al. 2010a, 2010b).

RFLP-PCR Analysis

The results of Nested-PCR were confirmed by enzymatic analysis using the MNII enzyme. PCR products (20 µl) were digested with MNII 2 µl at 37 °C for 4 h without prior purification using conditions recommended by the supplier (Fermentas Life Sciences, Germany). The restriction fragments were subjected to electrophoresis in 3% agarose gel containing ethidium bromide for 3 h at 65v and visualized on a UV transilluminator.

Results

Altogether, 60 primary schools with 2962 school children (1533 boys and 1429 girls) from 7 to 14 years of age were visited in December 2010. The overall scar rate was 1.24% but no ulcers were seen among them (Table 1). This means a medium endemicity of the disease which has recently become endemic, otherwise a higher scar rate should be observed.

In children with scar, 81.9% were recorded with only one and 18.1% with two scars. Hands, legs, and face were the most

affected parts of the body with 34.9, 34.9 and 27.9% of scars respectively.

A study of prevalence among 400 households with a total population of 1568 in four infected districts of the city showed 5.86% for scars and 0.12% for ulcers. The scar rate was higher than what was seen for school children. The scar rate was 3.92% for individuals under 10 years of age and 6.16% for those above 10 years old. Males and females were equally infected. Out of 38 individuals with scar cases, 2 had contracted the disease in Shiraz and one in Khuzestan (south of the country), one in Gilan-e-Gharb (western Iran) and the rest in the city of Bushehr. Prevalence of ulcers among the inhabitants was 0.12 % and the infected age group was 25⁺ with a rate of 0.24%. No active case was seen less than 24 years of age. A 45 year old man presented with approximately 3 month history of ulcer on the right lower leg. He was staying in the district of Imamzadeh, south of the city. The other case was also 45 year old with 6 month history of ulcer on his right hand and he was staying at the same district of the city. Both of them had contracted the disease in 2010 in Bushehr City. Out of 13 specimens, 2 specimens were positive by microscopic examination.

Leishmania DNA was found in 8 specimens collected from 13 (61.5%) suspected CL patients. The visualized obtained bands in 2 infected specimens of human indigenous cases were similar to the standard strain of *L. major*, which was equal to 231 bp and in 6 specimens were similar to the standard strain of *L. tropica*, which was equal to 215 bp (Fig. 2). Treatment was provided for the 8 subjects with molecular diagnosis of leishmaniasis.

Nineteen rodents were caught and identified as *Tatera indica* (47.4%) and *Rattus norvegicus* (52.6%). Specimens from 7 *T. indica* and 9 *R. norvegicus*, captured from the city were examined by two diagnostic techniques, direct (microscopic) examination and nested-PCR. All specimens were negative by

direct examination but out of 7 *T. indica*, 14.3% were infected with *L. major* and 42.9% with *L. turanica* by nested-PCR. Out of 9 *R. norvegicus* 22.2% were infected with *L. turanica* and 11.1% with *L. gerbilli*. We also

found mixed natural infections with *L. gerbilli* and *L. turanica* in 11.1% of *R. norvegicus* by the same molecular method (Table 2, Fig. 3–5). The study showed that *T. indica* acts as the animal reservoir host in the city of Bushehr.

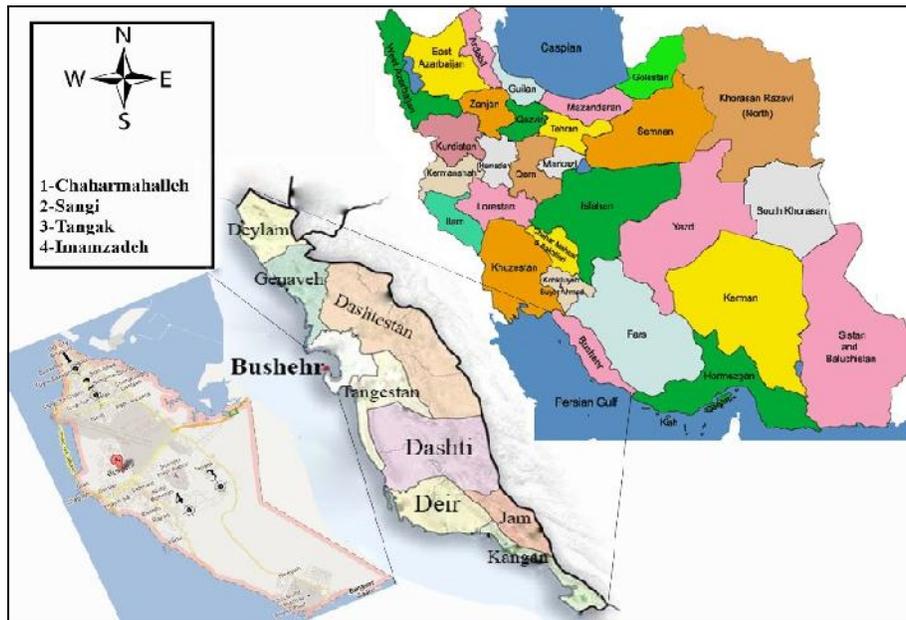


Fig. 1. Map of the city of Bushehr, showing the geographical location and study sites

Table 1. The prevalence of scar rate among the school children (both sexes) of primary schools in the city of Bushehr, December 2010

Age (yr)	No. observed	No. of scars	%	No. of ulcers	%
7	665	8	1.2	0	0
8	520	2	0.38	0	0
9	622	4	0.64	0	0
10	618	16	2.58	0	0
11	519	3	0.57	0	0
12	15	4	26.6	0	0
13	2	0	0	0	0
14	1	0	0	0	0
Total	2962	37	1.24		

Table 2. Natural *Leishmania* infection rates of rodents by Nested-PCR in the city of Bushehr, Iran, Sept. 2010–Jan. 2011

Rodent species	No. of examined	<i>Leishmania</i> species			
		<i>L. turanica</i>	<i>L. major</i>	<i>L. gerbilli</i>	<i>L. gerbilli</i> + <i>L. turanica</i>
<i>R. norvegicus</i>	9	22.2 (2/9)	–	11.1 (1/9)	11.1 (1/9)
<i>T. indica</i>	7	42.9 (3/7)	14.3 (1/7)	–	–

Figures in parentheses are numbers of positive/no of examined rodents and figures at the top of them are percent of positive`

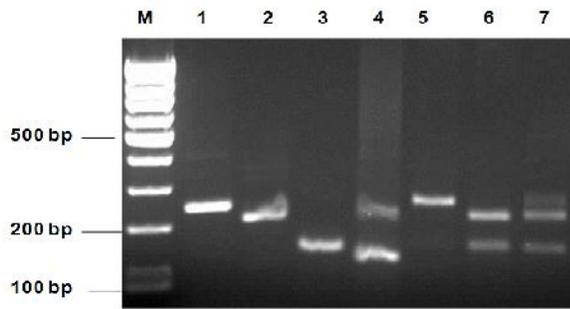


Fig. 2. Nested-PCR amplification of DNA extracted from reference strains and Giemsa-stained smears

Lane M, 100 bp DNA ladder (Fermentas), lane 1–4, reference strains, *Leishmania major*, *Leishmania gerbilli*, *Leishmania turanica*, *Leishmania tropica*, respectively, lane 5, *Leishmania major* isolated from Giemsa-stained smears of human, lane 6, *Leishmania tropica* isolated from Giemsa-stained smears of human, lane 7, mixed infection of *Leishmania major* and *Leishmania tropica* isolated from Giemsa-stained smears of human.

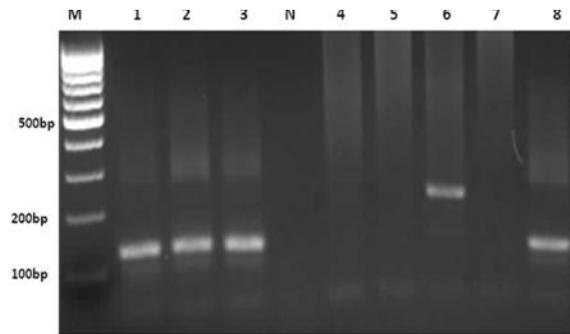


Fig. 3. Nested-PCR amplification of DNA extracted from rodents

Lane M, 100 bp DNA ladder (Fermentas), lane 1, *Leishmania turanica* isolated from *Tatera indica* (skin samples), lane 2–3, *Leishmania turanica* isolated from *Rattus norvegicus* (skin samples), lane 4–5, negative sample, lane 6, *Leishmania major* isolated from *Tatera indica* (skin sample), lane 7, Negative sample, lane 8, *Leishmania turanica* (reference strain), lane N, negative control (distilled water).

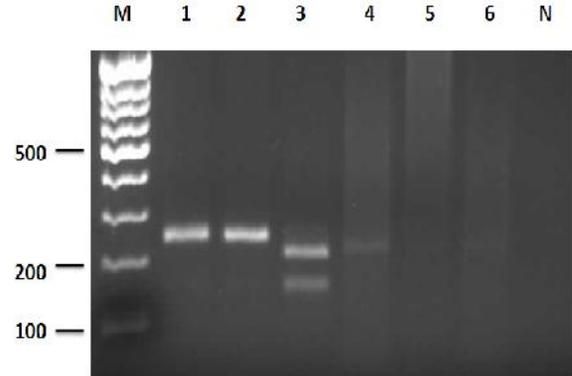


Fig. 4. Nested-PCR amplification of DNA extracted from rodents

Lane M, 100 bp DNA ladder (Fermentas), lane 1, reference strain, *Leishmania major*, lane 2, *Leishmania major* isolated from *Tatera indica* (skin sample), lane 3, mixed infection of *Leishmania gerbilli* and *Leishmania turanica* isolated from *Rattus norvegicus* (skin samples), lane 4, *Leishmania gerbilli* isolated from *Rattus norvegicus* (skin sample), lane 5, negative sample, lane 6, *Leishmania gerbilli* isolated from *Rattus norvegicus* (skin sample), lane N, negative control (distilled water).

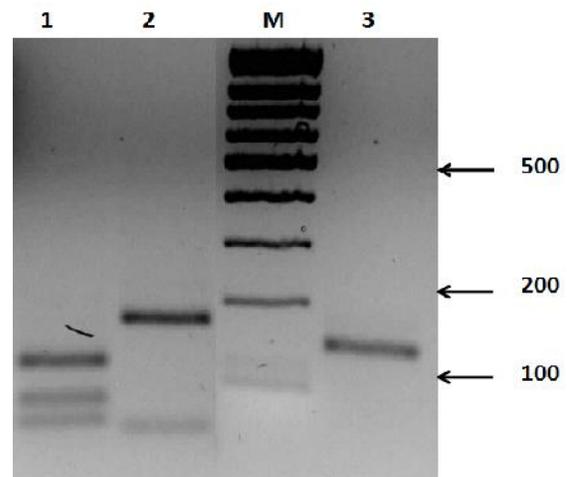


Fig. 5. Restriction products of nested-PCR amplicons after digestion with MnlI

Lane M, 100 bp DNA ladder (Fermentas), lane 1, *Leishmania major*, lane 2, *Leishmania gerbilli*, lane 3, *Leishmania turanica*.

Discussion

A study based on our detailed observation of school children and also local people showed scars of CL in all age groups with a maximum of 8.9% among 15–19 years old indicated that the disease is prevalent with a low endemicity in the city of Bushehr. The disease agents are *L. major* and *L. tropica* but in order to determine the dominant causative agent, more specimens from patients should be examined in the coming years.

Statistical analysis in the school survey and also community data (patients with scars) showed non-significant differences by sex ($P < 0.005$). It should be mentioned that there are some difficulties in using scars to assess past infection. Scars may be missed, become less detectable through time or may have causes other than leishmaniasis.

Based on this survey at the present time the Indian gerbil (*T. indica*) and the Norway rat (*R. norvegicus*) are well established in the infected districts of the city. *Rattus norvegicus* exists in coastal areas near the sea, in garbage dumps and in sewer systems but *T. indica* whether is essentially a field rodent, occurs in sandy parts where the city of Bushehr has extended its expanse. It is found near human dwellings under bushes and appears to be the main reservoir host of zoonotic cutaneous leishmaniasis in the city. 3.1% of the animals had *L. major* infection. This rodent species has been also reported as the main animal reservoir host in other foci of Bushehr Province such as Dashti and dashtestan counties and also Khuzestan, and Ilam Provinces with leishmanial infection rate between 2.3–9% in Iran and also in sub-saharan Africa (Javadian et al. 1998, Hamzavi et al. 2000). Gerbils infected by *L. major* having a great effect in the transmission cycle of cutaneous leishmaniasis due to *L. major* (CLM) so it is important to accurately assess the rate of *L. major* infection in important reservoirs (Gramiccia and Gradoni 2005).

The results of the current study shows that *L. major* and *L. turanica* are circulating in *T. indica* populations and *L. turanica*, *L. gerbilli* and also mixed natural infections with *L. turanica* and *L. gerbilli* in *R. norvegicus* populations of Bushehr City. In previous studies *L. turanica* has been isolated in *Rhombomys opimus* in Sabzevar northeast of the country (Yaghoobi-Ershadi et al. 2004) and mixed natural infections with 3 species, *L. major*, *L. gerbilli*, and *L. turanica* in *R. opimus* in Esfahan area in central part of Iran (Akhavan et al. 2010a, 2010b). *Leishmania turanica* was also isolated from *Nesokia indica* in an area lies on the border between Iran and Iraq in 2009 (Hajjaran et al. 2009). *Leishmania turanica* was also reported as the dominant species in *R. opimus* populations in hypoendemic, mesoendemic and hyperendemic foci of ZCL in Turkmenistan and Uzbekistan in 2001 (Srelkova 1996, Strelkova et al. 2001). Based on the results *L. major* and *L. tropica* are the causative agents of the disease among patients and *T. indica* plays a predominant role in the dissemination of *L. major* in the city of Bushehr.

The occurrence of CL in the city of Bushehr seems to be the results of expansion of the city and urbanization, constructing of buildings nearby rodent colonies, increase of nonendemic people in south Pars Projects, Bushehr military complex and The Bushehr Nuclear Power Plant. The people take a trip more than one month in summer during the active season of sand flies to other Zoonotic cutaneous leishmaniasis or Anthroponotic cutaneous leishmaniasis foci of the country and all are exposed to the infected bites of sand flies.

Any residual insecticide spraying is not recommended during the next year due to the low prevalence of the disease in the city. If the present epidemiological situation for

breeding of the vector are changed (example setting up big gardens in different parts of the city and so forth) and the disease becomes epidemic in the future, deltamethrin 0.025 (g/m) spraying in the houses with active cases and also their neighboring houses and in districts with high density of *Phlebotomus sergenti* or *P. papatasi* should be applied only once during the active season of sand flies and repeated once again two weeks before the beginning of sand fly activity in the next year. Infected stray dogs should be found and eliminated if any. Destruction of rodent burrows and using 2.5% zinc phosphide baits or Coumavec (a mixture of coumatetralyl 0.5% and etofenprox 0.5%) within 500-meter circle of houses is recommended (Yaghoobi-Ershadi et al. 2000, Yaghoobi-Ershadi et al. 2005, Veysi et al. 2012).

Active and passive case detection and rapid treatment should be provided for the positive subjects. Intensive health education programs have to be strongly supported in order to promote awareness among the exposed population. Regular epidemiological studies to address risk factors and transmission patterns are also necessary in order to integrate information on the current situation into control strategies.

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