Epidemiol. Infect. (1993), **111**. 297–306

The epidemiology and surveillance of visceral leishmaniasis in the Campania region of Italy. The value of zymodeme typing

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(*Accepted 24 March 1993*)

SUMMARY

Although human visceral leishmaniasis (VL) is a notifiable disease in Italy, there is evidence that the actual number of cases is far higher than that notified. A programme for active surveillance of VL in the 14 Italian endemic regions was launched by the Istituto Superiore di Sanità. We report data collected during a 3-year period of active surveillance in Campania, a south Tyrrhenian region covering 4·5% of the Italian territory. Out of 120 clinically suspected cases referred to medical and diagnostic references centres, there were 52 confirmed VL cases (17·3/year), i.e. 10-fold more than previously notified. Most of the infection sites were in rural areas or peripheral districts of towns in hilly parts of Naples province. An epidemic cluster of 10 cases was identified in a microfocus of Caserta province. The biochemical analysis of 23 *Leishmania* stocks showed a zymodeme composition indicating Campania as an old and well-established focus of VL. The data obtained emphasize that the present notification system for VL in Italy is inadequate.

INTRODUCTION

Visceral leishmaniasis (VL) is an endemic disease in continental and insular regions of central–south Italy (Fig. 1). About 40–70 cases are notified annually, over half of which are from Sicily (data from the Italian Institute of Statistics, ISTAT). In Italy major advances have been made in the last decade on the identification of leishmanial parasites [1, 2], the reservoirs [3, 4] and vectors involved [5, 6], and the nature of man–parasite contacts [7, 8]. Although human VL has been a notifiable disease in Italy since 1956, there is evidence that the actual numbers of cases are far higher than those notified [9]. Therefore, a long-

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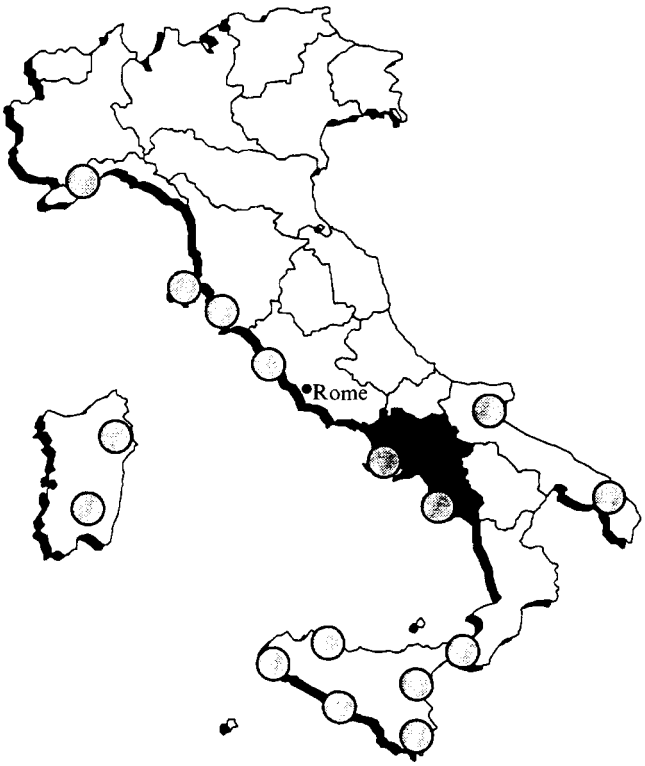

Fig. 1. Present distribution of major macrofoci of visceral leishmaniasis in Italy. Campania region is shown in black.

term programme was launched by the Istituto Superiore di Sanità, Rome, for active surveillance of VL in each of the 14 Italian endemic regions, in order to provide realistic figures and temporal trends of human VL, as well as to improve local diagnostic facilities and patient management.

We report here data collected during a 3-year period of active surveillance in one of these regions, Campania, where human VL is known to have been endemic since 1890, when cases of 'infectious splenic pseudoleukaemia', subsequently identified as infantile VL, were described [10]. Old reports indicate several foci of the disease in the area [11], both on the Tyrrhenian coast and in the inland territories surrounding Vesuvius. The data we obtained show a stable endemicity of VL in the region and emphasize that the present notification system for VL in Italy is inadequate.

METHODS

Area

Campania lies along the Tyrrhenian coast of south Italy, covering an area of 13600 km^2 (4·5% of Italy) (Fig. 1). The population is 5600000, one-fifth of whom live in Naples. The region is divided into five provinces: Naples, Salerno and Caserta, which include coastal and hilly territories, and Avellino and Benevento, which include mountainous areas (Apennines). It was recently estimated that about 900000 people of Campania are at risk of leishmania infection [12].

Surveillance

Local medical reference centres (MRCs) were established in two paediatric divisions and two infectious diseases departments of Naples, where all clinically suspected patients were referred and proven VL cases treated. A diagnostic reference centre (DRC) was established at the Istituto Superiore di Sanità, Rome, where biological samples were sent for diagnosis, confirmation and patient follow-up. The Regional Epidemiology Unit (REU) was in charge of disseminating information among medical personnel of the Local Health Units and of departments of paediatrics, infectious diseases and haematology of universities and hospitals of the region. The REU also collected all available data on confirmed VL cases.

A VL case was identified according to the following parameters, obtained in sequence.

(1) Presence of the following signs and symptoms: irregular fever resistant to antibiotics; splenomegaly; pancytopenia; alteration of albumin/globulin ratio.

(2) Detection of serum anti-leishmanial antibodies showing an IFAT titre equal to or higher than 80 [13]. This did not apply to patients infected with human immunodeficiency virus (HIV), which may cause depletion of anti-leishmanial antibodies in VL patients [13]. In order to increase test sensitivity, the antigen was developed in the laboratory from the WHO reference strain of *L. infantum* and freshly prepared every 30–50 days.

(3) Demonstration of leishmania from a bone-marrow aspirate, partly smeared and stained with Giemsa's stain, and partly seeded into Evans's modified Tobie's medium [14].

In the follow-up of drug-treated patients, serological tests alone, or serological plus parasitological tests, were performed according to the patient's clinical status.

Parasite characterization

Leishmanial stocks isolated from VL patients were characterized by electrophoretic analysis of 13 isoenzymes (15 enzymic loci):[1] PGM, GPI, GOT1, GOT2, ME, 6PGD, G6PD, MDH, NH1, NH2, MPI, ICD, DIA, GLUD and FH. The techniques employed are those reported by Gramiccia and colleagues [2].

Treatment

During the first 2 years of surveillance most of the VL patients were treated with a combination of meglumine autimoniate (Glucantime) and allopurinol, according to the regimen described by di Martino and colleagues [15]. Only a few

[1] PGM, phosphoglucomutase (E.C. 2.7.5.1); GPI, glucose-phosphate isomerase (E.C. 5.3.1.9); GOT, glutamate-oxaloacetate transaminase (E.C. 2.6.1.1); ME, malic enzyme (E.C. 1.1.1.40); 6PGD, 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44); G6PD, glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49); MDH, malate dehydrogenase (E.C. 1.1.1.37); NH, nucleoside purine hydrolase (E.C. 3.2.2.1); MPI, mannose phosphate isomerase (E.C. 5.3.1.8); ICD, isocitrate dehydrogenase (E.C. 1.1.1.42); DIA, diaphorase nicotinamide adenine dinucleotide (reduced form) (E.C. 1.6.2.2); GLUD, glutamate dehydrogenase (E.C. 1.4.1.3); FH, fumarate hydratase (E.C. 4.2.1.2).

patients received the standard regimen of meglumine antimoniate (25 mg SbV/kg daily for a minimum of 20 days). In the third year, most of the patients were enrolled in a clinical trial on liposomal amphotericin B treatment [16].

Data recording

Leishmaniasis transmission in Italy is seasonal, coinciding with the activity period of the phlebotomine vectors from June to October [17]. Hence the annual incidence of VL cases referred to an 'epidemiological year', corresponding to new infections occurring after one 'transmission season (TS)' [18, 19]. Since a few clinical cases may appear as early as the end of each TS, and in some subjects the incubation period may last over 1 year [20], the overall annual recording consisted of a 12-month period from October to September.

Leishmanin skin test

Leishmanin skin testing (LST) was carried out in a VL focus of Caserta province. The antigen, derived from promastigotes of the WHO reference strain of *L. infantum*, was prepared according to Gramiccia and colleagues [8]; 0·1 ml of leishmanin was injected intradermally and skin reaction was read 48 h after inoculation by the ball-point method [21]. All reactions with an induration size of 5 mm or more were recorded as positive.

RESULTS

From October 1989 to September 1992, 120 clinically suspected cases were referred to the MRCs. Samples for diagnosis confirmation usually reached the DRC in Rome within 1–2 days from patient's hospitalization and, on the same day, the medical personnel in charge were informed of the diagnosis. Only a few suspected cases were referred to other health centres. In confirmed cases, the DRC was informed retrospectively.

There were 52 confirmed VL cases (average 17·3/year), of which 11 occurred after TS 1989, 13 after TS 1990 and 28 after TS 1991 (Fig. 2). All but one of the cases were HIV-negative. The VL cases recorded in Campania through passive notification during the 10 years before surveillance are also shown for comparison. From 1980 to September 1989 15 VL cases were notified (average 1·5/year), the highest number of cases/year being 3, in 1983, 1984 and 1989.

Age and sex

Approximately the same proportion was found between paediatric (below 16 years) and adult cases (ratio 1·08) and between males and females (ratio 1·2) (Table 1). However, in 1990–92 the frequency of infantile VL showed an increasing trend. from 36 to 57 % of cases, which was not statistically significant.

Seasonal distribution

The monthly distribution of cases referred to the date of hospitalization is shown in the graph of Figure 3. No cases were reported in October of any year, and only a few in November and December. From January onward the graph shows an irregular trend, with peaks in March and September.

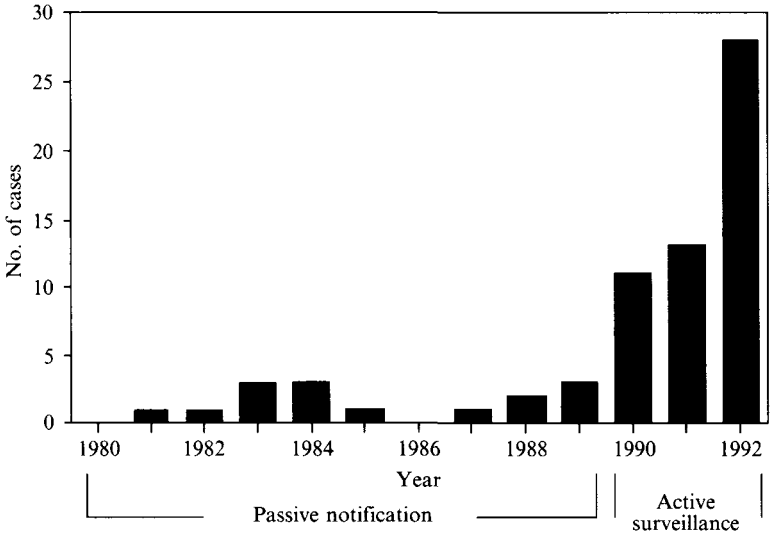

Fig. 2. Visceral leishmaniasis cases recorded in Campania from 1980 to September 1992 through passive notification and active surveillance.

Table 1. *Age and sex distribution of 52 VL cases recorded in Campania from October 1989 to September 1992*

Age (years)	October 1989 to September 1990			October 1990 to September 1991			October 1991 to September 1992			Totals		
	M	F	Total	M	F	Total	M	F	Total	M	F	total
< 16	3	1	4	2	5	7	9	7	16	14	13	27
⩾ 16	2	5	7	4	2	6	8	4	12	14	11	25
Total	5	6	11	6	7	13	17	11	28	28	24	52

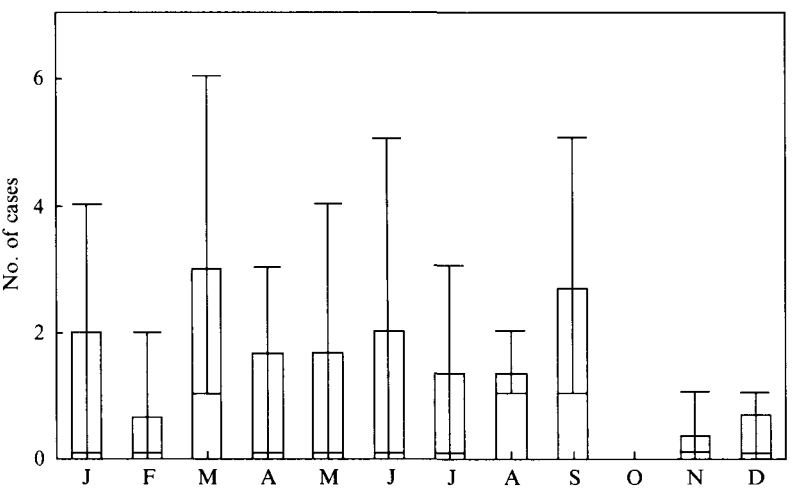

Fig. 3. Seasonal distribution of visceral leishmaniasis cases recorded in Campania from October 1989 to September 1992. Bars represent the mean number of cases of the 3 years plus minimum and maximum.

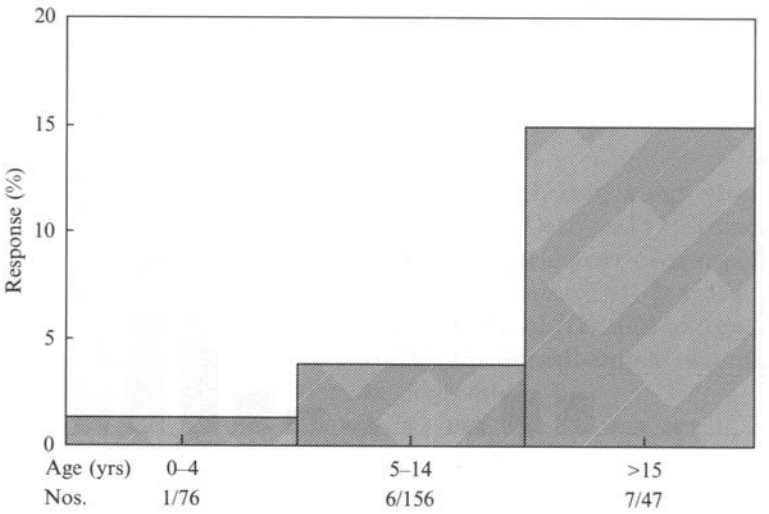

Fig. 4. Response to the leishmanin skin test according to age of 279 subjects in Maddaloni, Campania.

Geographical distribution and environment

Fifty-one cases (98%) were infected in Campania. Thirteen infection sites were clearly identified by the occurrence of infantile VL in subjects who had never left their place of birth. These foci consisted of rural areas or peripheral districts of small towns in hilly territories (100–300 m above sea level). Over half of the cases (28, 54%) were from towns surrounding Vesuvius (Naples province). Here, the annual incidence had been constant and the patients lived in seven different foci on the slopes of the volcano. Eight cases were from the coastal territories of the Salerno province and from the island of Ischia. Five cases had lived in more than one of the above foci, but the site of infection remains undetermined.

Epidemic cluster

After TS 1991, six VL cases were diagnosed at the beginning of 1992 in Maddaloni, a small town in Caserta province. Four more cases were detected during the following months. All of the 10 patients acquired the disease in an area located on the slope of a hill, where about 5000 people live. Hence, in this microfocus the VL incidence after TS 1991 was 2/1000. In Maddaloni, a few VL cases had already been diagnosed in the past, but apparently none in the previous 4–5 years. This suggests the occurrence of an outbreak of VL in this area.

In April 1992 an LST survey was carried out in Maddaloni to determine the size of the outbreak. The tests were carried out in kindergartens and schools in districts of, or close to, the area where the VL cases lived. Both children and their parents were examined. A total of 279 subjects were tested. As shown in Figure 4, positivity rates in the age groups of 0–4 and 5–14 years were 1·3% and 3·8%, respectively. The mean positivity rate of subjects over 15 years was 14·9%. By comparison with other surveys previously carried out in Italian VL foci [7, 8, 22], this positivity-rate pattern suggests the presence in Maddaloni of a mild and

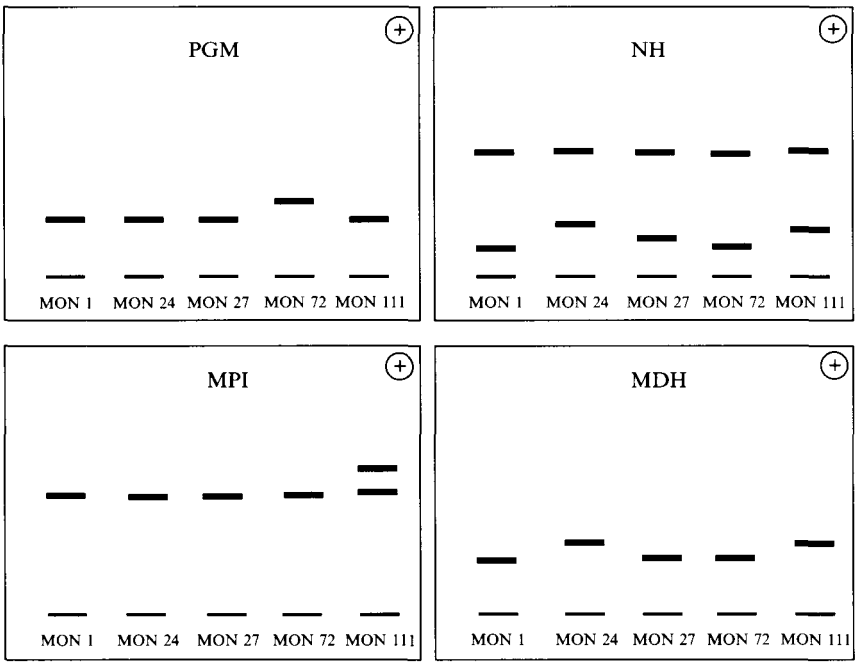

Fig. 5. Diagram showing the relative electrophoretic mobility of four isoenzyme markers for the main *Leishmania infantum* zymodemes found in Italy (PGM, phosphoglucomulatase; NH, nucleoside purine hydrolase; MPI, mannose phosphate isomerase; MDH, malate dehydrogenase). The zymodemes show identical mobility in the remaining nine enzymes examined. Montpellier (MON) 1, MON 27 and MON 72 are the main cause of visceral leishmaniasis, while MON 24 and MON 111 are common agents of cutaneous leishmaniasis in HIV-negative subjects [2]. Note that MON 72 differs from MON 1, the commonest *L. infantum* zymodeme, only in the mobility of PGM.

constant level of leishmania transmission to man, but not however the occurrence of a widespread epidemic.

Lethality

Three deaths (6%) occurred in the course of or after drug treatment. An infantile case developed a fulminant hepatitis after 2 weeks of antimonial plus allopurinol treatment [23]. An adult case not referred to an MRC received a course of pentamidine and died a few weeks after the end of treatment. A second adult case with undiagnosed VL, who had a long hospitalization, died a few days after the correct diagnosis was made and antimonial drug treatment had been instituted.

Characterization of leishmania

Twenty-three *Leishmania* strains (i.e. from 44% of the cases recorded) were isolated and typed by isoenzyme electrophoretic analysis. All were identified as *L. infantum*, belonging to two zymodemes: Montpellier (MON) 72 (12 strains), so far identified only in Italy in the Naples area [24], and MON 1 (11 strains), the commonest *L. infantum* zymodeme of the Mediterranean basin [1] (Fig. 5). Zymodeme MON 72 was apparently responsible for the outbreak in Maddaloni (4

strains typed out of 10 cases). It was also isolated from several cases which had occurred in the Vesuvius area. Zymodeme MON 1 was isolated from different foci in the provinces of Naples (including Vesuvius) and Salerno. No difference was observed between the cases due to MON 72 and those due to MON 1 as regards clinical signs and symptoms, and their response to drug treatment.

DISCUSSION

Active surveillance found 10-fold more VL cases than previously notified. The poor efficacy of the notification system for VL in Italy, however, is not confined to the region surveyed. In Tuscany, Bettini and colleagues [9] found that the number of cases reported in literature was twice as high as those officially recorded. The same situation seems to be common in other Mediterranean countries [25].

Although surveillance was preceded by disseminating specific information among different health services, it is highly probable that in the course of the 3 years the efficiency of surveillance has increased. Thus, excluding the epidemic cluster of Maddaloni, the progression from 11 to 18 cases during the 3 years may simply reflect surveillance improvement.

The degree of endemicity of VL in Campania (annual incidence of 2/100000 population estimated at risk of leishmaniasis) was higher than that in Sicily, the most traditional VL 'macrofocus' in Italy, where active monitoring during 1990–1 showed an average of 25 VL cases/year, i.e. 1/100000 population exposed [12, 26].

Prompt diagnosis and treatment led to a low mortality (6%). Drug toxicity (antimonials and pentamidine) or late diagnosis were the causes of death. During the third year, however, drug toxicity problems were overcome by the use of a regimen based on liposomal amphotericin B, much less toxic than antimony [16].

Ascribing cases to well-defined TSs is a common practice in studies on zoonotic cutaneous leishmaniasis by *L. major* [18]. In this type of leishmaniasis, however, almost all new cases appear within a few months after the end of the TS. In the calculation of VL incidence, due to the high variability of the incubation period, this procedure should be applied only to infants exposed to a single TS. However, by careful analysis of case histories, it was possible to ascribe most cases to a definite TS. Doubts may arise for cases which occurred at the end of each TS (see Fig. 3), representing either very short or very long incubation periods.

Outbreaks of VL caused by *L. infantum* are very rare, as compared with the frequency and extent of those caused by *L. donovani* [20]. In Italy an outbreak occurred in Emilia Romagna in 1971–2 with 60 cases [27]. Since inter-human transmission of *L. infantum* is thought to be impossible, the causes of these outbreaks should be found in changes in phlebotomine vector and/or canine reservoir populations. The surveillance allowed us to monitor the extent of the outbreak in Maddaloni from its beginning. The LST survey clearly indicated that the phenomenon was limited and circumscribed. In Emilia Romagna, Pampiglione and colleagues [22] found about 50% of LST positives in 64 healthy subjects of the age groups 0–4 and 5–14 years in the area involved by the outbreak. The rates found by us (1·3% and 3·8%, respectively) were considerably lower.

The multi-focality of the disease can be explained by the large pool of infected

dogs in the area. From annual surveys carried out by the Zooprophylactic Institute of the region, it was shown that from 1987 to 1992 about 3300 dogs were found infected out of 14600 examined. Dogs were also found to harbour the same *L. infantum* zymodemes affecting man in the area, MON 1 and MON 72 [24].

Several *L. infantum* zymodemes cause leishmaniases in Italy, although MON 1 is the commonest [1]. However, among approximately 400 *Leishmania* strains from man and dogs examined so far in Italy, MON 72 has been found only in Campania (Naples province and Maddaloni). This suggests that this area represents an old and well-established focus of VL.

ACKNOWLEDGEMENTS

This study received financial support by Regione Campania.

REFERENCES

1. Gramiccia M, Gradoni L, Angelici MC. Epidemiology of Mediterranean leishmaniasis caused by *Leishmania infantum*: isoenzyme and kDNA analysis for the identification of parasites from man, vectors and reservoirs. In: Hart DT, ed. Leishmaniases. The current status and new strategies for control. NATO ASI ser. A. New York: Plenum Press, 1989; 21–37.
2. Gramiccia M, Gradoni L, Troiani M. HIV-*Leishmania* co-infections in Italy. Isoenzyme characterization of *Leishmania* causing visceral leishmaniasis in HIV patients. Trans R Soc Trop Med Hyg 1992; **86**: 161–3.
3. Pozio E, Gradoni L, Gramiccia M. La leishmaniose canine en Italie de 1910 à 1983. Ann Parasitol Hum Comp 1985; **60**: 543–53.
4. Gradoni L, Pozio E, Gramiccia M, Maroli M, Bettini S. Leishmaniasis in Tuscany (Italy). VII. Studies on the role of the black rat, *Rattus rattus*, in the epidemiology of visceral leishmaniasis. Trans R Soc Trop Med Hyg 1983; **77**: 427–31.
5. Bettini S, Gramiccia M, Gradoni L, Atzeni MC. Leishmaniasis in Sardinia. II. Natural infection of *Phlebotomus perniciosus*, Newstead, 1911, by *Leishmania infantum* Nicolle, 1908, in the province of Cagliari. Trans R Soc Trop Med Hyg 1986; **80**: 458–9.
6. Maroli M, Gramiccia M, Gradoni L. Natural infection of *Phlebotomus perfiliewi* with *Leishmania infantum* in a cutaneous leishmaniasis focus of the Abruzzi region, Italy. Trans R Soc Trop Med Hyg 1987; **81**: 596–8.
7. Bettini S, Gramiccia M, Gradoni L, Pozio E, Mugnai S, Maroli M. Leishmaniasis in Tuscany (Italy). VIII. Human population response to leishmanin in the focus of Monte Argentario (Grosseto) and epidemiological evaluation. Ann Parasitol Hum Comp 1983; **58**: 539–47.
8. Gramiccia M, Bettini S, Gradoni L et al. Leishmaniasis in Sardinia. 5. Leishmanin reaction in the human population of a focus of low endemicity of canine leishmaniasis. Trans R Soc Trop Med Hyg 1990; **84**: 371–4.
9. Bettini S, Maroli M, Gradoni L. Leishmaniasis in Tuscany (Italy). IV. An analysis of all recorded human cases. Trans R Soc Trop Hyg 1981; **75**: 338–44.
10. Cardarelli A. Nosografia della pseudoleucemia splenica (infettiva) dei bambini. Boll R Accad Med Chir Napoli (Naples) 1890; 2: 17–44.
11. Pampiglione S, Bettini S. Bibliografia italiana delle leishmaniosi dalle origini al 1980. Ann Ist Super Sanità (Rome) 1981; **17**: 11–150.
12. Gradoni L, Scalone A, Gramiccia M. Estimation of population risk of leishmanial infections in Italy. Trans R Soc Trop Med Hyg. Submitted.
13. Gradoni L, Scalone A, Gramiccia M. HIV-*Leishmania* co-infections in Italy: serological data as an indication of the sequence of acquisition of the two infections. Trans R Soc Trop Med Hyg 1993; **87**: 94–6.
14. Evans DA. *Leishmania*. In: Taylor AER, Baker, JR, eds. *In vitro* methods for parasite cultivation. London: Academic Press, 1987: 52–75.

15. di Martino L, Pettoello Mantovani M, Gradoni L, Gramiccia M, Guandalini S. Low dosage combination of meglumine antimoniate plus allopurinol as first choice treatment of infantile visceral leishmaniasis in Italy. Trans R Soc Trop Med Hyg 1990; **84**: 534–5.
16. Davidson RN, Croft SL, Scott A, Maini M, Moody AH, Bryceson ADM. Liposomal amphotericin B in drug-resistant visceral leishmaniasis. Lancet 1991; **337**: 1061–2.
17. Maroli M, Bettini S. Leishmaniasis in Tuscany (Italy): I. An investigation on phlebotomine sandflies in Grosseto Province. Trans R Soc Trop Med Hyg 1977; **71**: 315–21.
18. Lysenko AJ, Beljaev, AE. Quantitative approaches to epidemiology. In: Peters W, Killick-Kendrick R, eds. The leishmaniases in biology and medicine. Vol I. Biology and epidemiology. London: Academic Press, 1987: 263–90.
19. Gradoni L, Gramiccia M, Mancianti F, Pieri S. Effectiveness of control measures against canine leishmaniasis in the isle of Elba, Italy. In: Hart DT, ed. Leishmaniasis. The current status and new strategies for control. NATO ASI ser A, New York: Plenum Press, 1989: 71–6.
20. World Health Organization. Control of the leishmaniases. Tech Rep Ser No. 793, 1990: 158.
21. Sokal JE. Measurement of delayed skin-test responses. N Eng J Med 1975; **293**: 501–2.
22. Pampiglione S, Manson-Bahr PEC, La Placa M, Borgatti MA, Musumeci S. Studies on Mediterranean leishmaniasis. 3. The leishmanin skin test in kala-azar. Trans R Soc Trop Med Hyg 1975; **69**: 60–8.
23. Di Martino L, Vajro P, Nocerino A, Scotti S, Napolitano G, Vegnente A. Fulminant hepatitis in an Italian infant with visceral leishmaniasis. Trans R Soc Trop Med Hyg 1992; **86**: 34.
24. Gramiccia M, Gradoni L, Di Martino L, Romano R, Ercolini D. Two syntopic zymodemes of *Leishmania infantum* cause human and canine visceral leishmaniasis in the Naples area. Italy. Acta Trop 1992; **50**: 357–9.
25. Jeannel D, Tuppin P, Brucker G, Danis M, Gentilini M. Leishmaniasis in France. Lancet 1989; ii: 804.
26. Scarlata F, Infantino D, Giordano S, Cascio A. Monitoraggio della leishmaniosi viscerale in Sicilia nel biennio 1990–1991. Parassitologia 1992; **34** (suppl 1): 158–9.
27. Pampiglione S, La Placa M, Schlick G. Studies on Mediterranean leishmaniasis. I. An outbreak of visceral leishmaniasis in northern Italy. Trans R Soc Trop Med Hyg 1974; **68**: 349–59.