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PCR Follow-up Examination After Treatment of Canine Leishmaniosis (CaL)

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A study has been performed to investigate the usefulness of the polymerase chain reaction (PCR) for both the diagnosis and the follow-up after treatment of canine leishmaniosis (CaL).

Blood samples (PBL) and/or bone marrow aspirates (BM) could be examined in a total of 18 confirmed cases of primary CaL. PBL was PCR-positive in 87%, whereas the BM was found to be positive in all cases (n=14) tested.

PBL and BM from a total of 13 patients were submitted to PCR examinations after meglumine antimoniate (Glucantime®) treatment. Only one dog showed a negative PCR after 2 treatment cycles (days 1-2: 50 mg/kg bw; days 3-10: 100 mg/kg bw). Examination of the PBL and BM after 15 months remained further PCR negative. All other dogs, from which four were pretreated with allopurinol up to 5 weeks, continued to be positive (92%) at least in the BM.

Ten dogs could be monitored by means of the PCR after allopurinol treatment (2×10 mg/kg/bw/day/) either as a monodrug therapy in seven or as a successive combination with Glucantime® in three cases. Two out of three dogs which showed good clinical improvement after daily administration for five weeks were likewise PCR negative in both the PBL and the BM. The four other dogs remained positive after the single therapy with allopurinol up to 5 weeks. A further three dogs were treated with allopurinol for 5 weeks, 6 and 20 month, respectively, after being clinically cured with Glucantime®. Two of them were PCR negative in the PBL and BM after 5 weeks and 20 month, respectively. The results presented suggest that longterm treatment with allopurinol has some therapeutic benefit in CaL especially when administered following treatment with the pentavalent antimonial Glucantime®.

Keywords : *Leishmania infantum*, Dog, Therapy, Polymerase chain reaction, Glucantime®, Allopurinol

INTRODUCTION

In Europe, the zooanthroponotic visceral leishmaniosis caused by *L. infantum* is endemic in the entire Mediterranean area with its preferentially subtropical climate [5]. The infectious agent whose reservoir is in particular in the dog but also in the fox and the jackal is transmitted by the bite of sandflies of the genus *Phlebotomus*. Recent studies using the PCR have documented that in certain endemic areas, more than 50 % of all dogs may be considered as asymptomatic infected and thus constitute a potential reservoir [4]. However, the dog is not only an asymptomatic carrier. Rather, it may experience life-threatening disease itself after a variable prepatent period of months and even years in some cases.

In the recent decades numerous cases of canine leishmaniosis have been documented also by veterinarians and veterinary hospitals in Germany [12, 13, 20, 26], a country where for climatic reasons, the vector cannot spread. This is attributed to the vehement increase of tourist traffic to and from the

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Mediterranean countries and the love of animals shown by tourists which has resulted in an increasing number of stray dogs being imported from these countries. Beyond this, it is not a rare event that the family dog which cannot be accommodated elsewhere accompanies tourists going south and is thus exposed to a quite considerable risk of infection. Thus, there is reason to regard canine leishmaniosis as one of the diseases which are most frequently imported into Germany [13].

For more than eighty years antimony preparations have been used as a first choice drug to control the different forms of leishmaniosis, initially as the highly toxic trivalent tartar emetic for the treatment of South American muco-cutaneous leishmaniosis in humans [28] but later in the mid of the century as the less toxic pentavalent (SB^{5+}) antimoniates [4]. Antimony compounds inhibit a single step in the parasite glycolysis, the phosphorylation of fructose-6 phosphatase into fructose 1,6-diphosphatase, which finally leads to a fatal energy depletion of the protozoan parasites [11]. There are also some indications, that only the reduced trivalent SB^{3+} metabolite will become active intracellularly against the parasite.

Since treatment of diseased dogs with these so-called "first line drugs" is not infrequently associated with life-threatening adverse reactions, often relapses and even induction of resistant strains [14, 25], there has been research in recent times to find new strategies for treatment and medicines. The purine analogue allopurinol is one of these medicines [13, 16-18, 27] Originally a common cure for gout, it has been used for some time in human medicine to treat antimoniat resistant cases of visceral leishmaniosis [15, 10]. Unlike antimoniates, it can be bought at a relatively low price and is reported to be comparatively free from adverse drug reactions. Allopurinol will become active against *Leishmania* only after metabolization to allopurinol-ribonucleoside-5-monophosphate which in turn, after having become converted to the corresponding adenosine nucleotide analogue, caused the arrest of the biosynthesis of parasites nucleic acids. Neither allopurinol nor ist metabolites are incorporated into the nucleic acids of the host [11].

Whether treatment with allopurinol given either singly or in combination with pentavalent antimoniates, can result in permanent curing in a parasitological sense is still a subject of controversial discussion since neither dosage nor duration of allopurinol treatment have been clearly established so far. One of the reasons for this shortcoming certainly consists in the fact that neither the classical direct parsitological methods such as the bone-marrow smear nor the indirect serological methods (ELISA, IFAT) have provided well-founded evidence of successful treatment because of their missing sensitivity and lack of interpretability.

Since about 10 years, the polymerase chain reaction (PCR) has provided a highly sensitive method of detection, which recently has also been used for therapy control of protozoan diseases as e.g. babesiosis [24], visceral leishmaniosis [8, 21, 25] and african trypanosomosis, a protozoan disease with some veterinary importance worldwide in the tropics [8].

It has been the objective of the present study to examine the worth of PCR for the diagnosis as well as parasitological follow-up after treatment with n-methyl glucamine antimoniate and allopurinol.

MATERIALS AND METHODS

Samples: The samples originated from patients with a confirmed serological/cytological diagnosis of the cutaneous or cutanovisceral leishmaniosis who had presented themselves at Gerichtliche Veterinärklinik 1 (forensic veterinary department) of Justus Liebig University. Whole blood and/or bone marrow specimens had been collected from dogs before, during and after therapy with Glucantime® or allopurinol. Before transportation, samples were either frozen at −20 °C or transferred to culture medium. The culture medium consisted of RPMI containing 20% FCS, 100 E./mL penicillin, 100 µg /mL streptomycin, 1 mmol L-glutamine)

Glucantime® treatment: One therapeutic cycle (TC) consisted of 10 injections of 50 mg meglumine antimoniate/kg b.w. each on days 1 and 2 and of 100 mg/kg b.w. on days 3 - 10 administered slowly by the i.m. route. If a second TC was performed, an interval of 10 - 14 days between TCs was kept. The course of therapy could be observed in a total of 13 dogs. Four of these patients had been premedicated with allop-

urinol over three to five weeks but with minor clinical recovery. Three dogs were presented again for a third/fourth TC due to recidivation after repeated treatment with Glucantime® using the regimen described.

Allopurinol treatment: Allopurinol treatment consisted in a daily oral administration of 2×10 mg/kg allopurinol. The respective owners treated seven dogs over three to five weeks. Further three patients had been treated before with Glucantime®. These dogs have been additionally treated with allopurinol for 5 weeks, 6 or 20 months, respectively, using the regimen described.

Bone marrow biopsy: The bone marrow sampling has been described elsewhere [26].

Controls: Regularly, whole-blood or bone-marrow specimens from healthy dogs were processed in parallel as negative controls. However, for purposes of internal quality control in the laboratory, specimens were identified only after evaluation of the respective PCR.

***Leishmania* species and strains used:** For specificity and sensitivity testing, cultured forms of *L. infantum* sensu stricto (strain C/ITA/79/ISSB-Ricky, isolated from a dog); *L. donovani* (strain LV 9), *L. tropica* (strain 11/2gb/91, isolated from humans); *L. major* (strain LV 39) and *L. enriettii* (origin Brazil) were used.

DNA extraction and polymerase chain reaction (PCR): DNA was standardized using the QIAamp Blood Kit® (Qiagen, Germany) for cleanup.

For the detection of genomic *Leishmania* DNA, the PCR procedure described by Piarroux et al. [23] was used. This procedure permitted the identification of a highly repetitive 100 bp sequence of *Leishmania infantum*.

For each run, a total volume of 50 μL was used consisting of 10 μL of extracted specimen DNA, 0.2 mM each of dNTP (dUTP, dATP, dCTP, dGTP, Boehringer, Germany), PCR buffer containing 1.5 mM $MgCl_2$ (Perkin Elmer, Germany), *Taq* polymerase Gold (Perkin Elmer, Germany) and 1 μM of each primer. The material used was overlayered with 50 μL mineral oil in each case. Amplification was performed over 34 cycles using a TRIO-Thermocycler® (Biometra, Germany), denaturing at 94℃ (first cycle 3 min, all further cycles 30 sec), annealing at 59 ℃ (30 sec) and extension at 70 ℃ (30 sec, in the last cycle 10 min). If the PCR was negative in the first run a second PCR-run has been performed.

Detection of amplificates: Amplification products were visualized by submarine minigel electrophoresis in a 4% composite gel (Biozym, Germany) stained with ethidium bromide. Subsequent gel evaluation was performed with the aid of Phoretix 1D Gel-Analysis Software (SLG, Germany).

After *Southern* Blot hybridization with the respective peroxidase-labelled probes, the specificity of the amplification products was confirmed using the ECL® direct nucleic acid labelling and detection system in accordance with the manufacturer's data (Amersham, Germany). The DNA probes needed for this purpose were obtained by sequential amplification of the respective PCR amplificates from *L. infantum* (100 bp products) with subsequent cleanup (Qiagen, Germany).

Possible cross contamination was prevented by the use of aerosol-protected filter tips and disposable gloves and strict separation of areas for sample preparation, PCR runs (UV-irradiated laminar flow bench), amplification and gel electrophoresis.

RESULTS

Analytical sensitivity and specificity of PCR: For the determination of specificity, a dilution series of cleaned *L. infantum* DNA was prepared. A minimum of 0.1 pg (1 parasite) could be detected after adding the DNA from 10^5 dog leukocytes (approx. 0.7 μg DNA). Specificity testing revealed for *L. infantum* and *L. donovani* (100 pg each) the expected amplification product of 100 bp. The *L. major* strain used reacted only weakly and became detectable only after *Southern* Blot hybridization. In contrast *L. tropica* und *L. enriettii* did not produce a corresponding amplification product (data not shown).

Diagnosis of the agent in clinical material with the aid of PCR: For the evaluation of diagnostic PCR, 15 blood samples and 14 bone marrow samples from a total of 18 confirmed and untreated cases of CaL was available. The blood was *Leishmania*-positive in 13 out of 15 cases and the bone marrow in all cases (14 of 14). Compared to the immediate deep-freezing of specimens (PBL^{df}, Bm^{df}), precultivation (PBL^{pk}, BM^{pk}) had no statistically significant influence on PCR diagnosis of primary CaL (Bm^{pc+}12/12;

Bm^{df+}: 7/8; Wb^{pc+}: 9/11; Wb^{df+}:8/10). For a synoptic view, see Table 1.

Controls: All whole-blood and bone-marrow specimens (n=30) of the control animals which had been regularly run in parallel proved to be negative in gel analysis as well as in *Southern* blotting (specificity 100%).

Therapy follow-up using the PCR

Totally, after treatment with (Glucantime®, 12/13 (92.3%) of the patients continued to be *Leishmania* PCR-positive. In the blood, 78 % (7/9) were still found to be positive after a single therapeutic cycle while after 2 and more cycles, a mere 37.5 % (3/8) were found to be positive on testing. In contrast,

Table 1 Results of PCR examinations of blood and bone marrow specimens from symptomatic canine leishmaniosis (CaL) patients (n=18) before treatment.

	Bone marrow*		Peripheral blood*	
	deep-frozen (−20℃)	precultured (7 days)	deep-frozen (−20℃)	precultured (7 days)
PCR-positive	7	12	8	9
PCR-negative	1	0	2	2
PCR-positive/total dogs	14/14		13/15	
(compiled PCR data)	(100%)		(87%)	

*The detection rate was not significantly different (chi-square test=2.41, df=3, p=.49) when the results received from the native samples were compared with the results received from the precultured samples.

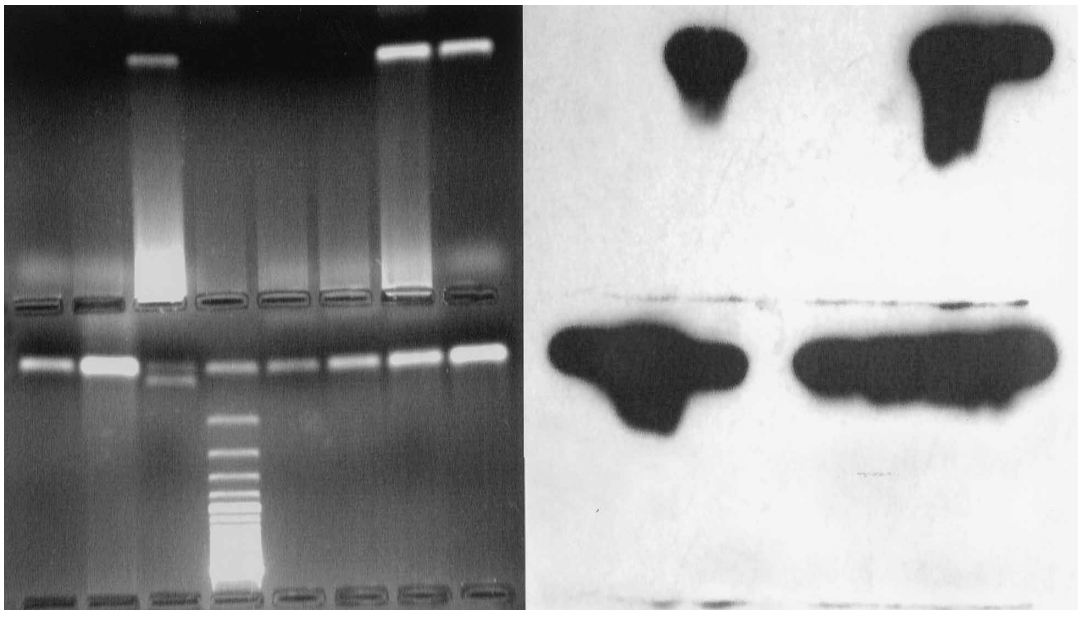

Fig. 1 PCR-amplification and *Southern*-blot hybridization of a 100 bp-sequence of *L. infantum* (PIARROUX et al.; 1993) in clinical blood (PBL) and bone marrow samples (BM) before, during and after 2 treatment cycles using Glucantime®.
Bottom row: lane 1: PBL (day 0); lane 2: BM (day 0); lane 3: PBL (day 4); lane 4: 100 bp-Ladder; lane 5: PBL (day 7); lane 6: PBL (day 10); lane 7: BM (day 10); lane 8: *L. infantum* (10 pg).
Top row: lane 1: Control (0.7 μg Dog-DNA); lane 2: PBL (day 25); lane 3: BM (day 25); lane 4: PBL (day 28); lane 5: PBL (day 31); lane 6: PBL (day 35); lane 7: BM (day 35); lane 8: *L. infantum* (10 pg).

the rate of positivity in the bone marrow was 88.9 % (8/9) after a single cycle and still 87.5 % (7/8) after 2 and more cycles. The negative results in the bone marrow were based on the examination of a single dog only. Clinically, all dogs have been shown good to excellent improvement. An example of the PCR follow-up is shown in Fig. 1.

After allopurinol treatment over 3-5 weeks, testing revealed 71 % (5/7) of whole-blood specimens and 66 % (4/6) of bone-marrow specimens to be positive. Two dogs were completely PCR-negative in both the bone marrow and the blood. Four dogs were further treated with Glucantime® due to minor clinical improvement, but continued to be PCR- positive.

One out of three dogs which were subsequently treated with allopurinol after previous Glucantime-courses continued to be positive in blood and bone marrow after 6 months of treatment. Two patients were PCR-negative after 5 weeks and 20 month, respectively.

Details of treatment parameters are shown in Table 2 and 3.

DISCUSSION

The results demonstrate that the polymerase chain reaction (PCR) has made available a supplementary method of high sensitivity and specificity for the diagnosis of canine leishmaniosis, in addition to the classical serological methods such as IFAT and ELISA. PCR is particularly useful where serological methods do not provide clear evidence, e.g. because of the presence of a fresh infection or immunosuppression. Moreover,

Table 2 Comparative PCR-results of blood and bone marrow specimens after several treatment cycles using Glucantime® in dogs (n=13).

Material	Before treatment*		One treatment cycle		Two treatment cycles		≥Three treatment cycles	
	PCR results							
	positive	negative	positive	negative	positive	negative	positive	negative
Blood (PBL)	8	4	7	2	2	4	1	1
Bone marrow (BM)	10	0	8	1	4	1	3	0
PCR-positive/total dogs	12/12**		9/10		5/6		3/3	
(compiled PCR data)	(100%)		(90%)		(83%)		(100%)	

* Four dogs had been pretreated with allopurinol for 3 to 5 weeks without success. Further three dogs had to be presented to the veterinary hospital due to relapses after previous Glucantime® courses. Each two were PCR negative in the blood but positive in the BM before the following treatment course.

**No pre-treatment samples were available in one dog.

Table 3 Comparative PCR-results of blood and bone marrow specimens after therapy with allopurinol in dogs given either singly (n=7) or after initial Glucantime® course (n=3).

Material	Allopurinol therapy for 3-5 weeks (2×10 mg/kg b.w. daily)		Allopurinol therapy for up to 20 month (2×10 mg/kg b.w. daily) after an initial Glucantime® course	
	PCR Results			
	positive	negative	positive	negative
Blood (PBL)	5	2	1	2
Bone marrow (BM)	4	2	1	2
PCR-positive/total dogs	5/7		1/3	
(compiled PCR data)	(71%)		(33%)	

PCR appears to be an excellent tool for epidemiological surveys [4], because the classical serological tests seems to to be regularly underestimate the prevalence of leishmaniosis. [2, 9].

In our study, a specific detection of *Leishmania* DNA was already possible in the peripheral blood of 87 % of untreated animals. In two patients, however, a second PCR run of the blood has been necessary for positive results, indicating the low number of blood-circulating parasites in some cases concerned. In a recent published PCR-study on dogs with primary leishmaniosis only 5 out of 13 whole blood samples gave positive reactions [19]. Such differences may be related to lack of PCR standardization especially the use of different DNA extraction methods, primer sets or cycling programs. Our PCR diagnosis of CaL could then be confirmed by examination of the bone marrow in all patients. But, under the common conditions of practice, biopsies can be taken only in rare cases. As an alternative, lymph node biopsy might offer itself [19]. Further alternatives for the PCR diagnosis may be material from modified skin or conjunctiva of the eye. Diagnosis from the mentioned material has been demonstrated in approximately 89 % of all symptomatic dogs [4].

When compared with PCR diagnosis from frozen material, sampling in a culture medium for one week did not result in a significant improvement of the detection rate in our studies, neither for whole blood nor for bone marrow. Obviously, this has also been due to the mostly high detection rate in the deep-frozen specimens ($\geq 80\%$). However, we always prefer dispatch in culture medium where the cold chain during transportation cannot be reliably maintained, or where there are only limited possibilities for deep-freezing of specimens.

In total, a group of 13 dogs was subjected to follow-up with the aid of PCR after therapy with Glucantime®. 92% of these dogs continued to be PCR-positive although the dogs clinically improved, so that these animals could be regarded as potential candidates for relapse. It should be noted that in cases having undergone treatment, the agent can be regularly detected only in the bone marrow while in the blood, the agent can be detected sporadically (approx. 30%) after termination of the standard therapy (2 treatment cycles). This would also seem to be the reason why Berrahal and co-workers [4] detected persistent infection after Glucantime® treatment in only 63 % of cases even though immunoblot studies had suggested the presence of persistent infection. The group mentioned used only skin biopsies for the control of therapy. For this reason, we principally recommend PCR examination of the bone marrow for therapy control.

A single patient proved to be still PCR-negative even 1 ½ years after Glucantime® treatment both in the blood and in the bone marrow. In the animal concerned, however, clinical symptomatology was little pronounced and beyond this, there was only a low ELISA titre (1:40) at the beginning of therapy. We thus assume that an early stage of disease was present where therapy and elimination of the parasites with Glucantime® still appeared to be possible. It is, however, impossible to make a definitive statement on this at present on account of the comparatively short post-therapeutic phase. Generally speaking, the opinion should be maintained that only in very rare cases, parasitological curing with Glucantime® as a single drug will be possible.

Also on account of our negative experience with pentavalent antimoniates which likewise can exhibit a considerable hepatotoxic and nephrotoxic potential [25], we investigated the question whether in the dog, allopurinol would constitute a suitable alternative or additive to Glucantime®. The leishmanicidal effect of the gout cure, allopurinol, has been known since the seventies [22]. However, not before the eighties, it has been used in the treatment of antimoniate-resistant human cases of leishmaniosis [15] Moreover, the additive efficacy of a combination of antimoniates/allopurinol has been already described for human patients with visceral leishmaniosis who were unresponsive to or had relapsed after treatment with antimoniates [7,10]. Treatment of dogs with a successive application of meglumine antimoniate and allopurinol has been reported for the first time in 1994 [1]. Since that time several reports with a noteworthy clinically success of treatment were even published using oral doses of 10 to 30 mg allopurinol/kg b.w. daily for up to 9 months as a

monodrug therapy. [13, 16-18, 27].

Based on our parasitological follow-up, we are now in a position to corroborate in part these results. After daily treatment with allopurinol alone for up to 5 weeks 2 out of 7 dogs has become PCR negative in both the blood and the bone marrow. Likewise, the PCR proved also to be negative when the specimens were precultured before examination.The PCR-results correlate good with the clinical course since both patients exhibit excellent clinical improvement after the mondrug therapy. Only one clinically cured dog continued to be PCR-positive in the bone marrow. Therefore, we decide to maintain the therapy in this patient. On the other hand 4 patients did not respond to the monodrug therapy and all blood and bone marrow samples continued to be PCR-positive as well so that a supplemental treatment with Glucantime® has become necessary. Whether in part a lack of compliance by animal owners in connexion with the daily dosage of allopurinol has been responsible for these failures has to be elucidated. It should also be noted that the subsequent Glucantime® course after allopurinol therapy did not lead to negative PCR-results, although the dog according to the treatment developed some clinical improvement.

Three other patients were treated daily with 20 mg/kg b.w. for up to 20 months, after they had been treated with Glucantime®. Two of them were PCR negative in both the blood and the bone marrow and only one dog showed a *L. infantum* specific product after having treated continuously for 6 month using allopurinol.

Although the number of cases is presently low these results suggest that permanent treatment with allopurinol especially subsequent to Glucantime® treatment but in part also as a monodrug therapy for several weeks may result in a considerable reduction of the parasite burden to below the detection limit of the PCR. It remains still unclear in these cases, however, whether a complete elimination of the pathogen can be achieved since *L. infantum* may be present in all organs of an infected dogs. We could demonstrate that in an euthanized dog suffering from CaL more than 80 % of all organs examined are PCR positive (data not shown). From such "cryptic" retreats, recidivation may originate even years later. Additonally, it could be shown in a similiar experiment with a treatment scheme of high dose cycles of meglumine antimoniate base (140 mg of salt/kg/bw daily for 20 days) plus allopurinol (20 mg/kg bw daily for 30 days) that five of six treated dogs still had parasites in the spleen ten month after they had been cured [1]. Further evidence of persistent infections in most of clinically cured Cal patients resulted from a recent PCR investigation [6]. In this study eight out of nine dogs remained PCR- or in vitro culture-positive after allopurinol treatment alone for up to 24 month (10 mg allopurinol/kg bw daily). Therefore, PCR investigations of our negative dogs in regular intervals are advisable to prove the uncertainty whether the parasites are completely eliminated or whether relapse must be expected.

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REFERENCES

1) Alvar J, Molinar R, San Andres M, Nieto J, Vitutia M, Gonzalez F, San Andres MD, Boggio J, Rodirguez F, Sainz A, Escacena C: Canine leishmaniasis: clinical, parasitological and entomological follow-up after chemotherapy. Ann Trop Med Parasitol 88: 371-378, 1994.
2) Ashford DA, Bozza M, Freire M, Miranda JC, Sherlock I, Eulalio C, Lopes U, Fernandes O, Degrave W, Barker RH Jr: Comparison of the polymerase chain reaction and serology for the detection of canine visceral leishmaniasis. Am J Trop Med Hyg 53: 251-255, 1995.
3) Berman JD: Chemotherapy for leishmaniasis: biochemical mechanism, clinical efficacy, and future strategies. Rev Infect Dis 10: 560-586, 1988.
4) Berrahal RF, Roze CMM, Berenger A, Escoffier K, Lamouroux D, Dunan S: Canine leishmaniasis: identification of asymptomatic carriers by polymerase chain reaction and immunoblotting. Am J Trop Med Hyg 53: 251-255, 1996.
5) Bettini S, Gradoni L: Canine leishmaniasis in the mediterranean area and ist implications for human leishmaniasis. Insect Sci Applic 2: 241-245, 1986.
6) Cavaliero T, Arnold P, Mathis A, Glaus T, Deplazes P: Langzeitbehandlung von naturlich mit *L. infantum* infizierten Hunden mit Allopurinol. 18. Conference of the German Society of Parasitology, Dresden, Germany, Abstr 16, 1998.
7) Chunge CN, Gachihi G, Muigai R, Wasunna K, Rashid JR, Chulaj JD, Anabwani G, Oster CN,

Bryceson ADM: Visceral leishmaniasis unresponsive to antimonial drugs. III. Sucessfull treatment using a combination of sodium stibogluconate plus allopurinol. Transact Roy Soc Trop Med Hyg 79: 715-718, 1985.

8) Clausen P-H, Waiswa C, Moritz A, Steuber S: Post treatment detection of cryptic/latent parasitic infections (bovine trypanosomosis, canine leishmaniosis) by DNA amplification. In: Hygiene und Mikrobiologie Methodische Entwicklungen in der mikrobiologischen Nukleinsäure-Diagnostik, 3. und 4. Poster Workshop, Einhorn Presse Verlag, Abstr 24, 1997.
9) Dye C, Vidor E, Dereure J: Serological diagnosis of leishmaniasis- on detecting infection as well as disease. Epidemiol Infect 11: 647-656, 1993.
10) Di Martino L, Mantovani MP, Gradoni L, Gramiccia M, Guandalini S: Low dosage combination of meglumine antimoniate plus allopurinol as first choice treatment of infantile visceral leishmaniasis in Italy. Transact Roy Soc Trop Med Hyg 84: 534-535, 1990.
11) Frayha GJ, Smyth JD, Gobert JG, Savel J: The mechanismus of antiprotozoal and anthelmintic drugs in man. Gen Pharmacol 28: 273-299, 1997.
12) Gothe R: Leishmaniosen des Hundes in Deutschland: Erregerfauna und - biologie, Epidemiologie, Klinik, Pathogenese, Diagnose, Therapie und Prophylaxe. Kleintierpraxis 36: 45-84, 1991.
13) Gothe R: Leishmaniose des Hundes in Deutschland: epidemiologische Fallanalyse und Alternative zur bisherigen kausalen Therapie. Tierärztl Prax 25: 68-73, 1997.
14) Gramiccia M, Gradoni L, Orsini S: Decreased sensitivity to meglumine antimoniate (Glucantime) of *Leishmania infantum* isolated from dogs after several courses of drug treatment. Ann Trop Med Parasit 86: 613-620, 1992.
15) Kager PA, Rees PH, Wellde BT, Hockmeyer WT, Lyerly WH: Allpurinol in the treatment of visceral leishmaniasis. Transact Roy Soc Trop Med Hyg 75: 556-559, 1981.
16) Lester SJ, Kenyon JE: Use of allopurinol to treat visceral leishmaniosis in a dog. JAVMA 209: 615-617, 1996.
17) Liste F, Gascon M: Allopurinol in the treatment of canine visceral leishmaniasis. Vet Rec 137: 23-24, 1995.
18) Lindner T: Aus der Praxis: Kombinationstherapie der Hundeleishmaniose (HL) mit Megluminantimonat und Allopurinol am Beispiel einer Teckelhündin. Tierärztl Umschau 50: 797-798, 1995.
19) Mathis A., Deplazes P: PCR and in vitro cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. J Clin Microbiol 33: 1145-1149, 1995.
20) Opitz M: Hautmanifestation bei der Leishmaniose des Hundes. Tierärztl Prax 24: 284-291, 1996.
21) Osman OF, Kager PA, Zijlstra EE, El-Hassan AM, Oskam L: Use of the PCR on lymph-node samples as test of cure of visceral leishmaniasis. Ann Trop Med Parasit 91: 845-850, 1997.
22) Pfaller MA, Marr JJ: Antileishmanial effect of allopurinol. Antimicrob Agents Chemotherap 5: 496-472, 1974.
23) Piarroux R, Azaiez R, Lossi AM, Reynier P, Muscatelli F, Gambarelli F, Fontes SS, Dumon H, Quilici M: Isolation and characterization of a repetitive DNA sequence from *Leishmania infantum*: development of a visceral leishmaniasis polymerase chain reaction. Am J Trop Med Hyg 49: 364-369, 1993.
24) Shaio MF, Yang KD: Response of babesiosis to a combined regimen of quinine and azithromcin. Transact Roy Soc Trop Med Hyg 91: 214-215, 1997
25) Steuber S, Kroker R: V. Antiprotozoika. In; Pharmakotherapie bei Haus- und Nutztieren, Löscher W, Ungemach FR, Kroker R (Eds.), 3rd Edt., Parey Buchverlag, Berlin und Hamburg, 355-377, 1997.
26) Steuber S, Schirrmann I, Greiner M., Moritz A.: Therapiekontrolle der kaninen Leishmaniose mit Hilfe der Polymerase Kettenreaktion (PCR). Mitt Österr Ges Tropenmed Parasit 19: 97-104, 1997.
27) Vercammen F, De Deken R, Kageruka P: First evaluation of the use of allopurinol as a single drug for the treatment of canine visceral leihmaniosis. Vlaams Dierg Tijds 64: 208-211, 1995
28) Vianna G: Tratamento da leishmaniose tegumentar por injeccoes intravenosas de tartaro emetico. Annaes do 7o Congresso Brasileiro de Medicina e Cirurgia 4: 426-428, 1912.