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# Copromicroscopic and molecular assays for the detection of cancer-causing parasitic nematode *Spirocerca lupi*

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**Abstract**

*Spirocerca lupi* (Nematoda, Spirurida) is a life-threatening parasitic nematode of dogs that is presently emerging in several countries. Nonetheless, canine spirocercosis is neglected and underestimated, mainly due to diagnostic limitations inherent to clinico-pathologic, diagnostic imaging and laboratory methodologies. Given the significant benefit of improved diagnosis, the present work evaluated the reliability of a recently described copromicroscopic approach, the FLOTAC technique, as well as a PCR-based assay with that of traditional coproscopic techniques to diagnose *S. lupi* infection. Ninety-four faecal field samples were collected from two endemic areas (i.e. 29 and 65 from Kenya and Israel, respectively) and processed using different coproscopic examination techniques. In particular, set I (Kenyan samples) comprised the modified flotation with Sheather's sugar solution and merthiolate–iodine–formalin technique, while set II (Israeli samples) comprised a flotation technique with zinc sulphate solution, a modified sugar flotation procedure and the FLOTAC method. All samples were also subjected to a semi-nested PCR protocol specific for a region internal to the mitochondrial cytochrome *c* oxidase subunit 1 gene of *S. lupi*. The coproscopic examinations showed low sensitivity and high variability, demonstrating the unreliability of the conventional methods for detecting *S. lupi* eggs. Nonetheless, the FLOTAC technique scored the highest number of positives and significantly higher number of *S. lupi* eggs per microscopic field compared to the other coproscopic methods. Additionally, of the coproscopically negative samples, 9 (45%) Kenyan and 21 (38.2%) Israeli samples scored molecularly positive using the PCR-based approach. The potential implications and perspectives for canine spirocercosis of these coproscopic and molecular diagnostic methodologies evaluated herein are discussed.


*Keywords:* Spirocercosis; Dog; Coproscopy; FLOTAC; Mitochondrial DNA; *cox1* gene; Diagnosis

## 1. Introduction

*Spirocerca lupi* (Nematoda, Spirurida) is a parasitic nematode mostly affecting dogs and other canid carnivores, e.g. coyotes and foxes, which become

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infected by ingesting intermediate (i.e. coprophagous beetles) or paratenic (e.g. birds, lizards or rodents) hosts (Bailey, 1972; Anderson, 2000; van der Merwe et al., 2008). The adult stages live within fibrous nodules in the oesophageal wall. The presence of larvae and adult worms in tissues induces lesions and lead to a disease (i.e. canine spirocercosis) characterized by variable clinical signs such as regurgitation/vomiting, depression, weight loss, anaemia, melena, vertebral spondylitis, hypertrophic osteopathy, dyspnoea, aspiration pneumonia, pyothorax and neurological disorders and other abnormalities due to aberrant spinal migration (Mazaki-Tovi et al., 2002; Ranen et al., 2004; Du Plessis et al., 2007; Dvir et al., 2001, 2007). Additionally, the larval migration may lead to aortic aneurisms that may rupture and result in haemothorax and acute death (van der Merwe et al., 2008). Often, the oesophageal nodules go through malignant transformation to fibrosarcoma and osteosarcoma that can metastasize to the lungs (Meléndez and Suárez-Pellín, 2001; Ranen et al., 2004; van der Merwe et al., 2008). This potentially life-threatening parasitosis has been reported worldwide, with higher prevalence in subtropical and tropical areas, and is presently emerging in several countries (Mazaki-Tovi et al., 2002; Traversa et al., 2007; van der Merwe et al., 2008).

Despite its diffusion and importance in canine medicine, *S. lupi* is an underestimated and neglected parasite, mainly due to diagnostic limitations in living animals. In fact, diagnosis at early infection stages is unreliable and difficult, with exception of aortic aneurism rupture, when it is too late. The vast majority of infected dogs are diagnosed only when the disease is advanced. In particular, clinical diagnosis of canine spirocercosis is challenging, because symptoms vary considerably with disease stage, parasite burden, larval migration, aberrant localization and potential complications, thus overlapping a range of other diseases (Dvir et al., 2001, 2007; Mazaki-Tovi et al., 2002; Ranen et al., 2004). Although oesophagoscopy and radiography play a pivotal role in diagnosing infection, both modalities mostly do not allow an aetiological diagnosis, are reliable only at advanced disease stages and are invasive and insensitive, respectively (van der Merwe et al., 2008). In addition, routine coproscopy has inherent limitations in terms of sensitivity and specificity and the reliability of faecal flotation is influenced by several factors as maturation stage of the oesophageal nodule and of the nematodes, number of adult female worms within the nodule, number of eggs shed by adult females and the eggs' relatively small size and higher specific gravity compared to other nematodes' eggs (Evans, 1983; Fox et al., 1988; van der Merwe et al., 2008). Modified sugar flotation techniques and flotation with sodium nitrate or zinc sulphate solutions have been reported to be the most effective methods in detecting *S. lupi* eggs in faecal flotation (Cabrera and Bailey, 1964; Markovics and Medinski, 1996), although sensitivity of coproscopy has been reported from 3% to 70–80%, and in suspected animals with negative faecal samples, faecal examination should be repeated to improve sensitivity (Mazaki-Tovi et al., 2002).

Recently, both coproscopic and innovative molecular approaches have been developed to overcome the constraints of the classical diagnostic methodologies used in parasitology. Regarding copromicroscopical diagnosis, a novel multivalent faecal egg count method, i.e. the FLOTAC technique, has been recently demonstrated to be powerful for diagnosing a range of parasitic infections of human and veterinary importance (Rinaldi et al., 2007; Utzinger et al., 2008). This method relies on the use of the FLOTAC® apparatus, which allows detection and quantification of helminth eggs and/or larvae in up to 1 g of faeces, and has been reported to improve the sensitivity of other conventional coproscopic approaches (Cringoli, 2006). This method has never been assessed in canine spirocercosis.

In addition, regions within the mitochondrial (mtDNA) and ribosomal DNA (rDNA) contain useful genetic markers in molecular diagnostic tools specific for human and veterinary nematode infections, which are able to overcome inherent limits of the classical approaches (Chilton, 2004; Gasser, 2006; Traversa, 2007). An informative region within the mtDNA gene encoding for the cytochrome *c* oxidase subunit 1 (*cox1*) of *S. lupi* has been recently showed to be potentially useful in developing tools for an *ante mortem* diagnosis of canine spirocercosis (Traversa et al., 2007). Hence, the present work aimed to compare the diagnostic reliability of the FLOTAC and a *cox1*-based molecular assays with that of traditional coproscopic approaches in the diagnosis of *S. lupi* infection using faecal field samples from two geographical areas endemic for canine spirocercosis.

## 2. Materials and methods

### 2.1. Sample collection

Overall, 94 canine faecal samples were randomly collected in highly *S. lupi*-endemic areas (Brodey et al., 1977; Mazaki-Tovi et al., 2002). Specifically, 29 and 65 stool samples were collected from dogs owned by

Samburu people in villages of Northern Kenya during a rabies vaccination campaign and from two public parks in towns in central Israel, respectively. All samples were stored in ethanol and comparatively processed with two sets of coproscopic and molecular examination, scheduled according to geographic provenance and amount of faeces available.

### 2.2. Coproscopic techniques

Kenyan samples (nos. 1–29) were processed with modified Sheather's sugar solution (specific gravity [SG] 1.300) flotation and merthiolate–iodine–formalin (MIF) technique, while Israeli samples (nos. 30–94) were examined with zinc sulphate flotation ($ZnSO_4$, SG 1.350), a modified sugar flotation procedure (Markovics and Medinski, 1996) (MM) and the FLOTAC flotation method.

An aliquot of each faecal sample was stored at −20 °C pending molecular analysis (Section 2.3.2).

#### 2.2.1. Flotation procedures

For the flotation procedures with $ZnSO_4$ and Sheather's sugar solutions, 2–3 g of faeces were added to 20 ml of each solution, centrifuged at 1500 rpm for 5 min, and a supernatant aliquot (100 μl) was aspirated with a Pasteur pipette. The supernatant was then transferred to a glass slide, covered with a 18 mm × 18 mm coverslip and examined using a light microscope at 200× and 400× magnifications (Euzeby, 1981; Foreyt, 2001).

The MM technique was performed as previously reported (Markovics and Medinski, 1996). Briefly, 1 g of faeces was mixed in 15 ml of tap water and strained through a wire screen. Then, the suspension was centrifuged at 2000 rpm for 10 min and the sediment re-suspended in a sugar solution (SG 1.250), filled to the rim, covered with a 18 mm × 18 mm coverslip and centrifuged at 2000 rpm for 20 min. The coverslip was transferred on a slide and examined using a light microscope at 200× and 400× magnifications.

#### 2.2.2. Merthiolate–iodine–formalin (MIF) technique

Two to three grams of faeces were dissolved in 10% formalin and filtrated through tea strainer or gauze. Then, 5 ml of MIF solution (5 ml of 40% formaldehyde, 40 ml of 0.1% sodium merthiolate, 1 ml of glycerine, 50 ml of water), 6 ml of ether and 10 drops of Lugol's solution (1 g of crystalline iodine and 2 g of potassium-iodide in 100 ml water) were added. The tube was sealed, thoroughly mixed and centrifuged for 2 min at 1500 rpm. A small sediment drop was transferred to a slide, covered with a 18 mm × 18 mm coverslip and examined using a light microscope at 200× and 400× magnifications (Blagg et al., 1955).

#### 2.2.3. FLOTAC

Two grams from each Israeli faecal sample were suspended in 18 ml of tap water. The suspension was then homogenized using a mixer and filtered through a wire mesh. After discarding the debris, 12 ml of suspension were placed in a tube and centrifuged at 1500 rpm for 2 min. The supernatant was discarded and the tube was then filled with a $ZnSO_4$ solution (SG 1.350) to up to a total volume of 12 ml and slowly agitated. This type of solution was selected as optimal from a panel of fourteen solutions with SGs ranging from 1.200 to 1.450 (Cringoli et al., 2004) used in a preliminary study performed on all samples that have resulted positive for *S. lupi* eggs in the flotation procedures described above (Section 2.2.1). The suspension was aspirated using a Pasteur pipette and used to fill the chamber with a 18 mm × 18 mm grid of the FLOTAC® apparatus (Veterinary Parasitology, Faculty of Veterinary Medicine, University of Naples Federico II, Naples, Italy). The apparatus was then centrifuged at 1000 rpm for 5 min and translated (Cringoli, 2006). The egg count was performed under both grids (10 ml total volume) with no multiplication factor.

#### 2.2.4. Statistical analysis

The egg number/microscopic field faeces results of three coproscopic methods (conventional $ZnSO_4$ flotation, MM technique and FLOTAC method) in Israeli faecal samples were compared using one-way analysis of variance (ANOVA) with Bonferroni's correction of alpha for multiple comparisons using statistical software (SPSS 13.0 for Microsoft Windows, SPSS Inc.).

### 2.3. Semi-nested PCR

The optimal conditions of the molecular protocol (Section 2.3.1) were determined by preliminary analysis of five *S. lupi* egg samples recovered from naturally infected dogs which were definitely diagnosed (i.e. thoracic radiography, oesophagoscopy and faecal flotation) and hospitalized at the Hebrew University Veterinary Teaching Hospital (Israel).

Later on, an aliquot of each of the 94 field Kenyan and Israeli samples were examined using molecular methods (Section 2.3.2).

#### 2.3.1. Protocol validation

Egg samples were subjected to genomic DNA extraction with a commercial kit (QIAgen Stool Mini Kit, QIAgen Gmbh, Germany) and all extracts were undertaken to a semi-nested PCR protocol, amplifying an internal region of the *S. lupi cox1* gene. In the first PCR round, a 689-bp-long region of the *cox1* gene was amplified using the primers NTF (5′-TGATTGGTGGTTTTGGTAA-3′) and NTR (5′-ATAAGTACGAGTATCAATATC-3′) as previously described for Spirurida (Casiraghi et al., 2001). Following the criteria of Sharrocks (1994), the forward primer SlInt (5′-TGACTTTGGATCAGATAAG-3′) was designed in a region of the *S. lupi cox1* gene without sequence polymorphisms (Traversa et al., 2007) and used in the second PCR round together with primer NTR to achieve specific amplification of a product of ∼400 bp. The protocol of the semi-nested PCR was optimized by consequent trials based on different primer concentration titrations, various annealing times, temperatures and cycles number. Mixtures were prepared in 50 μl reaction containing 100 pmol of each primer in both steps, 4 μl (DNA extract in the first step) or 5 μl (1:40 dilution of the primary PCR product in the second step) of template, 3 mM $MgCl_2$, 200 μm of each dNTP, 1× reaction buffer (100 mM Tris–HCl pH 8.3 and 500 mM KCl), 0.5 μg bovine serum albumin and 5 U of *Taq* Gold polymerase (Applied Biosystems, Foster City, CA, USA).

PCRs were performed in a thermal cycler (2700, Applied Biosystems, Foster City, CA, USA) using the following cycling protocol: 12 min at 94 °C, 40 cycles at 94 °C for 1 min (first step) or 45 s (second step), 54 °C (first step) or 60 °C (second step) for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

The successful PCR reactions were purified using Ultrafree-DA columns (Millipore, Billerica, MA) and sequenced (MWG Biotech/M-Medical, Milan, Italy). All generated sequences were aligned using MEGA 4.0 (Tamura et al., 2007), compared with one another and with those of the *cox1* of all nematodes (including *S. lupi*) available in the GenBank™ using the Nucleotide-Nucleotide Basic Local Alignment Search Tool (nblast) (Altschul et al., 1997).

DNA from other common canine endoparasites collected by the authors or provided by colleagues, from canine blood and intestinal samples (Table 1) was extracted using the QIAgen DNEasy Tissue Kit (QIAgen, Gmbh, Germany) and subjected to the above PCR protocol.

Table 1
DNA samples used to validate the semi-nested PCR assay specificity for *Spirocerca lupi cox1* gene

| Samples | Stage |
|---|---|
| *Cystoisospora* spp. | O |
| *Giardia* spp. | C |
| *Echinococcus granulosus* | Sa |
| *Taenia multiceps* | Ssa |
| *Taenia hydatigena* | Ssa |
| *Taenia pisiformis* | Ssa |
| *Taenia ovis* | Ssa |
| *Dipylidium caninum* | Ssa |
| *Ancylostoma caninum* | Sa |
| *Uncinaria stenocephala* | Sa |
| *Strongyloides stercoralis* | Sa |
| *Crenosoma vulpis* | Sa |
| *Toxocara canis* | Sa |
| *Toxascaris leonina* | Sa |
| *Trichuris vulpis* | Sa |
| *Eucoleus aerophilus* | Sa |
| Canine blood | – |
| Canine intestine tissue | – |

O: concentrated oocysts of faecal origin; C: concentrated cysts of faecal origin; Sa: single adult specimen; Ssa: single segment from adult specimen.

#### 2.3.2. Sample analysis

Genomic DNA was extracted with the QIAgen Stool Mini Kit using ∼0.5 g of faecal material from each of the 94 field samples. All extracts were subjected to the above molecular protocol (Section 2.3.1). Each second round product was electrophoresed and visualised by ethidium bromide under UV transillumination. Amplicons were further purified, sequenced and analysed as described above (Section 2.3.1). The presence of inhibition in the PCR reactions that did not produce any amplicon detectable on agarose gels was verified by *S. lupi* DNA-spiking into DNA extracts. The smallest amount of *S. lupi* DNA yielding detectable amplicons by the semi-nested PCR was estimated by multiple serial titrations into faecal samples collected from non-parasitized dog, as previously described (Traversa et al., 2004).

All the molecular procedures were performed in separate rooms (i.e. DNA preparation and pre-PCR steps in a different room to post-PCR manipulation) to avoid PCR contamination and were validated twice.

## 3. Results

Overall, 9 (31%) and 10 (15.4%) of 29 and 65 faecal Kenyan and Israeli samples, respectively, were positive for *S. lupi* eggs in at least one coproscopic test (Tables 2 and 3). Of the 9 positive Kenyan samples, 4 were

Table 2
Kenyan samples (S) scored positive (+) or negative (−) for *Spirocerca lupi* (Sl), protozoa (P), tapeworms (T) and intestinal nematodes (IN) in modified Sheather's sugar solution (Fl), merthiolate–iodine–formalin (MIF) flotation techniques and *S. lupi cox1* molecular assay (PCR)

| S | Fl | | | | MIF | | | | PCR |
|---|---|---|---|---|---|---|---|---|---|
| | Sl | P | T | IN | Sl | P | T | IN | |
| 1 | − | − | − | + | − | − | − | + | − |
| 2 | + | + | + | − | + | − | + | − | + |
| 3 | − | − | − | − | − | − | − | − | − |
| 4 | − | − | − | − | − | − | − | − | − |
| 5 | − | + | + | + | − | + | − | + | + |
| 6 | − | − | + | − | − | − | + | − | + |
| 7 | − | − | + | − | − | − | − | − | + |
| 8 | − | + | − | − | − | + | − | − | + |
| 9 | − | − | − | − | − | − | − | − | − |
| 10 | − | + | − | − | + | − | − | − | + |
| 11 | − | − | + | − | + | − | − | − | + |
| 12 | + | + | − | − | − | − | − | − | + |
| 13 | − | − | − | − | − | − | − | − | + |
| 14 | − | − | + | − | − | − | − | − | − |
| 15 | − | − | − | − | − | − | − | − | − |
| 16 | − | − | − | − | − | − | − | − | − |
| 17 | + | − | − | − | + | − | − | − | + |
| 18 | + | + | − | − | + | − | − | + | + |
| 19 | − | − | − | − | − | − | − | − | − |
| 20 | + | + | − | − | − | − | − | − | + |
| 21 | − | + | + | − | − | + | − | − | + |
| 22 | − | − | − | − | − | − | − | − | + |
| 23 | − | − | − | − | − | − | − | − | − |
| 24 | − | − | − | − | + | + | − | − | + |
| 25 | − | − | − | − | − | − | − | − | − |
| 26 | − | − | − | − | − | − | − | − | − |
| 27 | + | − | − | − | + | − | − | − | + |
| 28 | − | − | − | − | − | − | − | − | + |
| 29 | − | − | − | − | − | − | − | − | + |

positive in both Sheather's flotation and MIF technique, 2 only in the former and 3 only in the latter tests. These 7 samples and 7 additional samples of the 20 *S. lupi*-negative samples were also positive for other endoparasites (e.g. protozoa, cestodes and intestinal nematodes) (Table 2). Only 1 of the 10 coproscopically *S. lupi*-positive Israeli samples was positive in all three diagnostic tests, while all scored positive in the FLOTAC apparatus (Table 3). Seven samples were positive in $ZnSO_4$ flotation but only 1 of these was positive also in the MM technique, and was also positive in the FLOTAC method. The 2 $ZnSO_4$ flotation-negative samples were positive using the MM method (Table 3). Four *S. lupi*-positive and 9 *S. lupi*-negative samples were also positive for other protozoa, cestodes and/or intestinal nematodes (Table 3).

The number of *S. lupi* eggs detected in all coproscopically positive Israeli samples in the 18 mm × 18 mm microscopic field in each of the three coproscopic methods used is reported in Fig. 1. In particular, the number of microscopically detected eggs was markedly higher when samples were examined using the FLOTAC apparatus. Results of the ANOVA analysis showed that the FLOTAC method yielded a statistically ($p < 0.017$) higher egg number per 18 mm × 18 mm microscopic field in Israeli faecal samples compared with conventional $ZnSO_4$ flotation and the MM technique. There was no statistical difference between the results of the two latter methods.

All coproscopically positive samples were positive also using semi-nested PCR (Tables 2 and 3). Of the coproscopically negative samples, 9 (45%) Kenyan and 21 (38.2%) Israeli samples were molecularly positive for the *S. lupi* ~400 bp amplicon detected on agarose gel (Tables 2 and 3). There was no evidence of PCR inhibition in any of the PCR-negative samples. No amplicons were produced from any of the control DNA samples (Table 1) or from faecal samples positive for other canine endoparasites (Tables 2 and 3).

The smallest amount of parasitic DNA yielding amplicons detectable on an agarose gel by the semi-nested PCR was 0.2 fg. Sequencing of all PCR products (i.e. 386-bp long) generated by the second round with the primer set SlInt-NTR confirmed their identification as *S. lupi*. The DNA sequences obtained were identical and showed a 100% identity homology with the previously described Afro-Asiatic *S. lupi* haplotype (Traversa et al., 2007).

## 4. Discussion

The 94 faecal field samples collected from both endemic areas yielded variable results when subjected to various coproscopic methods. The examination results of Kenyan samples (Table 2) suggest that the MIF technique is a superior method compared to Sheather's flotation in detecting *S. lupi* eggs, however, these also highlight the variability of coproscopic methods used and their apparently low sensitivity for detecting *S. lupi* eggs. The results of the coproscopic examination of Israeli samples (see Table 3) indicate that $ZnSO_4$ flotation and MM method also appear to be insensitive diagnostic tests for canine spirocercosis. Conversely, the FLOTAC® apparatus had a higher detection rate, including in samples that scored negative in both $ZnSO_4$ flotation and the MM method.

The overall coproscopic results demonstrate that the classical approaches appear insensitive and unreliable tests to definitely diagnose *S. lupi* infection. Indeed, the sensitivity of conventional faecal flotations was reported to be as low as 3% in previous studies (Evans,

Table 3
Israeli samples (S) scored positive (+) or negative (−) for *Spirocerca lupi* (Sl), protozoa (P), tapeworms (T) and intestinal nematodes (IN) using conventional $ZnSO_4$ flotation (Fl), Markovics and Medinski technique (MM), FLOTAC method (FLO) and *S. lupi cox1* molecular assay (PCR)

| S | Fl | | | | MM | | | | FLO | | | | PCR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sl | P | T | IN | Sl | P | T | IN | Sl | P | T | IN | |
| 30 | + | − | − | − | − | − | − | − | + | − | − | − | + |
| 31 | + | − | − | − | − | − | − | − | + | − | − | − | + |
| 32 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 33 | − | − | − | − | + | − | − | + | + | − | − | + | + |
| 34 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 35 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 36 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 37 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 38 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 39 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 40 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 41 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 42 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 43 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 44 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 45 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 46 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 47 | − | − | − | − | − | − | + | − | − | − | − | − | + |
| 48 | − | − | − | − | − | − | − | + | − | − | − | + | − |
| 49 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 50 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 51 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 52 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 53 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 54 | + | − | − | − | − | − | − | − | + | − | − | − | + |
| 55 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 56 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 57 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 58 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 59 | − | − | − | − | − | − | − | + | − | − | − | − | + |
| 60 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 61 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 62 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 63 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 64 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 65 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 66 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 67 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 68 | + | − | − | − | − | − | − | + | + | − | − | + | + |
| 69 | − | − | − | + | − | − | + | + | − | − | − | + | − |
| 70 | − | − | + | − | − | − | + | − | − | − | + | − | − |
| 71 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 72 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 73 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 74 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 75 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 76 | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 77 | − | − | − | − | − | − | − | − | + | − | − | − | + |
| 78 | − | − | − | + | + | − | − | + | + | − | − | + | + |
| 79 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 80 | − | − | − | − | − | − | − | − | − | − | − | + | − |
| 81 | − | − | − | − | − | − | − | − | − | − | − | + | − |
| 82 | − | − | − | − | − | − | − | − | − | − | − | + | − |
| 83 | − | − | − | − | − | − | − | − | − | − | − | − | + |
| 84 | − | − | − | − | − | − | − | − | − | − | − | − | − |

Table 3 (*Continued*)

| S | Fl | | | | MM | | | | FLO | | | | PCR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sl | P | T | IN | Sl | P | T | IN | Sl | P | T | IN | |
| 85 | – | – | – | – | – | – | – | – | – | – | – | + | – |
| 86 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 87 | – | – | – | – | – | – | – | – | – | – | – | – | + |
| 88 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 89 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 90 | – | – | – | – | – | – | – | – | – | – | – | – | + |
| 91 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 92 | + | – | – | – | + | – | – | – | + | – | – | – | + |
| 93 | + | – | – | – | – | – | – | – | + | – | – | + | + |
| 94 | + | – | – | – | + | – | – | – | + | – | – | – | + |

1983; Fox et al., 1988). This may result from inherent difficulties in *S. lupi* egg detection, e.g. small dimension (∼11 μm × 30 μm), unique shape compared to other canine parasites' eggs and errors in their identification as fat globules, plant seeds and faecal debris (Fox et al., 1988; Markovics and Medinski, 1996; Dvir et al., 2001; Mazaki-Tovi et al., 2002). These difficulties, along with other limitations have led previous investigators to recommend repeated faecal flotations by skilled laboratory technicians to increase their diagnostic sensitivity (Mazaki-Tovi et al., 2002; van der Merwe et al., 2008). In light of the present results, the FLOTAC® technique appears to overcome some of the limits of the classical parasitological methods commonly used for diagnosing canine spirocercosis. It was the most sensitive diagnostic coproscopic method, since it yielded a higher detection rate (Table 3) and a statistically significant higher egg number detected in the same 18 mm × 18 mm surface microscopic fields (Fig. 1) compared to the other microscopic methods used. Thus, the use of this apparatus may be extremely useful for clinical and epidemiological purposes especially when molecular methodologies and/or instruments are unavailable.

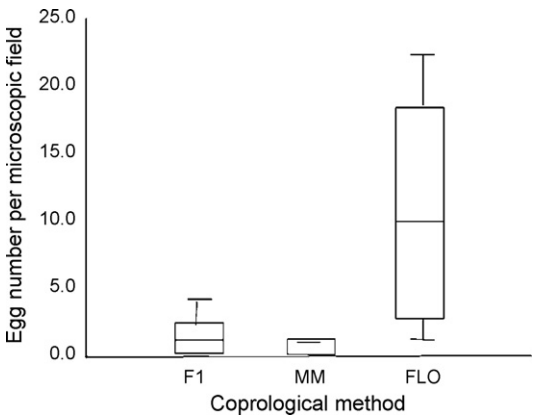


Fig. 1. Number of *Spirocerca lupi* eggs detected in a 18 mm × 18 mm microscopic field in 10 coproscopically positive Israeli faecal samples in each of three coproscopic methods used. Fl: conventional flotation with $ZnSO_4$; MM: Markovics and Medinski technique; FLO: FLOTAC method.

The PCR-based approach validated for the *S. lupi* mitochondrial *cox1* proved to be sensitive in detecting and identifying *S. lupi* infection using canine faecal field samples. The values of sensitivity and specificity of the molecular assay in standardized conditions using concentrated *S. lupi* eggs (Section 2.3.1) were of 100%. The diagnostic sensitivity of the molecular assay in collected field samples cannot be calculated, as the actual parasitological status of sampled dogs was unknown, thus false negative results of the PCR assay cannot be ruled out. However, given the minimum amount of *S. lupi* DNA required for effective amplification, i.e. ∼0.2 fg, which correspond to about 1/4000 of a single nematode immature stage (Verweij et al., 2000), the PCR-negative samples in this study were collected from animals that were most probably not infected by *S. lupi* adult worms and negative samples did not contain larvated eggs of the parasite.

Indeed, the molecular approach yielded a higher prevalence of positive results, and thus is more sensitive compared to all the coproscopic methods used in the present study for the faecal diagnosis of canine spirocercosis (Tables 2 and 3). In all the positive samples, the PCR yielded *S. lupi*-specific amplicons regardless of co-infection with other endoparasites (i.e. protozoa, cestodes and intestinal nematodes). In addition, the 100% specificity of the assay was also confirmed by the sequencing of all amplicons produced by the second round of the semi-nested PCR and by the absence of control samples amplification (Table 1).

The coproscopic and molecular methodologies examined herein can now be used for the specific diagnosis of canine spirocercosis, thus having relevant

implications for field epidemiological investigations as well in the clinical evaluation and treatment, and monitoring of the disease.

In fact, dogs suspected with spirocercosis are often examined by collateral diagnostic imaging modalities in addition to coproscopy. However, these modalities show inherent limitations. Thoracic radiography lacks sensitivity (up to 50% false negatives) and specificity, as its findings are often misleading due to the variable range of lesions induced by *S. lupi* adults and migrating larvae in canine patients (Mazaki-Tovi et al., 2002; van der Merwe et al., 2008). Nodules and neoplasms caused by *S. lupi* sometimes are hard to be radiographically differentiated and discriminated from other opaque soft-tissue masses. Conversely, oesophagoscopy is more specific and sensitive and has an advantage in differentiating between a typical *S. lupi* nodule from an oesophageal neoplasm or other lesions, mostly when the nodule is mature (i.e. presenting a patent passage represented by a nipple-shaped protuberance through which the adult females of *S. lupi* protrude to lay eggs). In addition, endoscopically obtained biopsies from oesophageal masses may be sometimes used to diagnose the disease, especially after the neoplastic transformation of the lesions has occurred. However, the histological results are often impaired by the low microscopic quality of examined tissue due to the typical oesophageal structure. They are difficult to interpret and moreover, the differentiation between a tumour and a nodule may be challenging (Berry, 2000; Dvir et al., 2001; Mazaki-Tovi et al., 2002; Ranen et al., 2004; van der Merwe et al., 2008). In addition, radiography often requires patient restraint or sedation, while endoscopy requires general anaesthesia, both of which are expensive, time consuming and can be life-threatening (Joubert et al., 2005). Additionally, in epidemiological field studies focusing on stray dogs or on dogs difficult to handle (e.g. those of Samburu people in Kenya examined in the present study), above-mentioned methods are all unplayable.

Due to the above limitations, the conventional diagnostic methodologies are prone to failure, thus often delaying or preventing effective and timely anthelmintic treatment. Worthy of note is that a timely macrocyclic lactone treatment has been shown to lead to resolution of oesophageal nodules and clinical improvement of infected dogs and, most importantly, it prevents malignant transformation of oesophageal nodules (Berry, 2000; Lavy et al., 2002, 2003). Hence, the molecular tool presented herein is an extremely sensitive and useful method to achieve an early and specific diagnosis that at present is not possible with the classical techniques.

Additionally, future experimental studies investigating the efficacy of anthelminthic drugs in treatment and prophylaxis of canine spirocercosis could use such a sensitive and specific molecular tool for monitoring faecal and other tissue *S. lupi*-specific DNA levels in treated animals, thus circumventing other more complicated tools and/or the need to sacrifice them.

Finally, the detection of *S. lupi* DNA by the molecular tool presented herein may open new avenues in elucidating some epidemiological aspects of canine spirocercosis, such as diffusion and parasitization pattern in different definitive hosts, as well as in investigating the presently unclear identity and population dynamics of intermediate and paratenic hosts of *S. lupi* in different geographic areas. For example, the relationship between the nematode and its vector/s could be investigated using this tool. In fact, DNA-based techniques have been successfully previously applied for spirurid nematode DNA detection in their intermediate hosts (Otranto et al., 2006), thus overcoming the constraints of the traditional tests, i.e. laborious and time-consuming vector dissections and microscopic detection of larval stages, inherently associated with low sensitivity and frequent misidentification.

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