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Diagnosis of Imported Canine Filarial Infections in Germany 2008–2010

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Abstract

Filarial infections of dogs are attracting attention across Europe because of the risk of spread into previously non-endemic areas (e.g. *Dirofilaria repens* with Culicidae as vectors) and as emerging zoonotic agents. The occurrence of filarial infections in German dogs has been analysed based on 8,545 samples collected either from imported animals or following travel into endemic regions. All samples were tested by means of modified Knott's test and heartworm antigen assay within the period 2008–2010. Heartworm antigen was detected in 127 samples (1.49 %; 95 % CI: 1.25–1.77 %), but only 38 dogs also had microfilariae in their blood samples. On the other hand, 125 animals (1.46 %; 95 % CI: 1.23–1.74 %) were only positive in the Knott's test. For discrimination by means of PCR and sequencing a total of 73 blood samples as well as two samples of adult worms were included, which have been sent by veterinarians during 2008–2010. A mono-infection caused by *D. repens* was detected in 35 cases, while *D. immitis* was proven in 15 samples, with 6 of these showing a combination of *D. immitis* and *D. repens*. Imported *Dipetalonema dracunculoides* (transmitted by *Rhipicephalus sanguineus* or *Hippobosca longipennis*) or *Acanthocheilonema reconditum* (fleas and lice serve as intermediate hosts) infections were diagnosed in 24 cases and in a single sample a co-infection of *A. reconditum* and *D. repens* was evident. *D. repens* was the most common filarial infection imported and it was introduced into Germany from eleven European countries. Slovenia and Hungary are reported for the first time as endemic for *D. repens* and *A. reconditum*, respectively. Furthermore this study reports, to the best of our knowledge, for the first time import of *D. dracunculoides*

from the Canary Islands, *A. reconditum* from Majorca, *D. immitis* from Corfu and a co-infection of *D. repens* and *A. reconditum* from Spain as well as mixed infections of *D. repens* and *D. immitis* from Corfu, Sardinia and Bulgaria. Co-infections with other arthropod-borne infections as well as therapeutical follow-up were also considered. Selamectin (as spot-on formulation) was not able to clear microfilaraemia in dogs infected with either *D. repens*, *A. reconditum* or *D. dracunculoides,* whereas a topical moxidectin/imidacloprid formulation was able to eliminate microfilariae in one dog infected with *A. reconditum*.

Introduction

Filarial nematodes in dogs are gaining importance in Germany not only as imported but also as possibly emerging endemic parasites. The first autochthonous case of canine cutaneous dirofilariasis in Germany (hunting dog with ocular infection) was documented in the Central Upper-Rhine valley in July 2004 (Hermosilla et al. 2006). In the same region, the Knott's test revealed unsheathed microfilariae identified as *Dirofilaria repens* by PCR in further 3 (6.8 %; 95% CI: 2.4–18.2 %) of 44 hunting dogs without history of travelling (Pantchev et al. 2009a). In a kennel of 29 sled dogs living in the proximity of Berlin, five animals were found to be positive for *D. repens* microfilariae in 2007. Considering the seasonal travel pattern they presumably were infected in Germany (Sassnau et al. 2009). Heartworm antigen was not detected in any of the samples. While no proven reports of autochthonous cases of *Dirofilaria immitis* infection in Germany exist, this parasitosis is of concern as a disease in dogs that accompany their owners on holidays into or dogs that are imported from endemic areas. For example, 1.2 % of 5,483 dogs with a history of travelling were tested positive for heartworm antigen in 2005–2006 within a travel disease profile using a heartworm antigen assay (Hirsch and Pantchev 2008). Zahler et al. (1997) examined imported filarial infections in Germany by means of heartworm antigen assay, Knott's test and activity pattern of acid phosphatase in unsheathed microfilaria for species differentiation during the period 1993–1996. Of 80 positive dogs 5 were infected with *D. repens* (from Italy, Greece, former Yugoslavia and Hungary) and 3 with *Acanthocheilonema reconditum* (from Spain, 1 dog with additional *D. immitis* infection, and Corsica). Of the remaining 72 samples 45 were tested positive only for *D. immitis* and 27 samples contained microfilariae but delivered a negative heartworm antigen signal. However, microfilariae could not be differentiated further despite of repeated investigation of freshly collected blood samples. This clearly shows the limitation of acid phosphatase staining for species diagnosis and the need for more reliable diagnostic methods. Furthermore, in a recent study three out of five morphological classifications had to be corrected after PCR, demonstrating the risk of inaccurate morphological diagnosis (Rishniw et al. 2006).

With regard to diagnostic procedures for imported or travel-accompanying dogs within Europe, other filarial species have to be considered in addition to *D. immitis* (Tab. 1). However, one of the pitfalls is that filarial screenings within "travel disease profiles" in large diagnostic laboratories are frequently based on heartworm antigen assays alone (Hirsch and Pantchev 2008). Moreover, the awareness of veterinary surgeons in Germany of the use of differentiation of microfilariae based on concentration assays is low so that other filarial species than *D. immitis* are probably underdiagnosed. For instance, a private diagnostic laboratory reported that during the period 2004–2006 only 440 Knott's tests were asked for by the veterinarians and 4.5 % of them were tested positive (Globokar et al. 2010). The aim of this study was to evaluate the occurrence of imported filarial infections by an extended "travel disease profile" including the Knott's test under the conditions of routine diagnosis in a private professional laboratory (IDEXX Vet Med Laboratory Ludwigsburg, Germany). This started

in 2008 (part one of the study). Thereafter further differentiation of positive samples by means of PCR was performed (Institute of Parasitology, University of Leipzig, Germany, part 2 of the study).

Material and methods

Sample collection (part 1 of the study)

In order to investigate the current occurrence of imported filarial infections in dogs living in Germany, the results of a "travel disease profile" were evaluated within the period 2008–2010. This profile includes the option to examine the sample of an imported or a travel-accompanying dog for potential travel infections. Since 2008 it contains – additionally to screening for heartworm infection by means of an antigen ELISA – also a concentration test for microfilariae, besides the detection of antibodies to *Leishmania* spp., *Ehrlichia canis* and *Babesia canis*. In total, this retrospective study included 8,545 dogs. The dogs' blood samples were sent from veterinary surgeons across Germany to the IDEXX Vet Med Laboratory in Ludwigsburg for analysis.

Sample collection (part 2 of the study)

In order to determine the probable origin of imported filarial species, the laboratory records of the PCR examinations in the period 2008–2010 were evaluated. PCR is recommended in case of detection of microfilariae in blood samples. Most of the samples were selected according to a positive result in the Knott's test in the "travel disease profile" (part 1), or the PCR was performed based on the suspicion of a filarial infection. PCR was also performed on two isolated adult worms. Data on co-infections with other vector-borne infections were also considered for samples examined with the "travel disease profile". The veterinarian or owner of the dog was asked for information regarding the dog's country of origin, travel activities and age.

Tests performed

Modified Knott's test

EDTA blood samples were screened for the presence of microfilariae using a modified Knott's test. For the modified Knott's test, 1 ml EDTA blood was mixed with 9 ml of 2% formaldehyde solution

Tab. 1 List of diagnostically relevant filarial species in imported/travelling dogs (modified according to Eckert et al. 2008, Anderson 2000)

Species	Occurrence	FH and IH	ML adults	ML microfilariae	Pathology
D. immitis	America, Africa, Asia, Australia, Europe	FH: dog, cat, ferret, wild carnivores IH: Culicidae	Pulmonary arteries, right heart, vena cava	Blood	Cardio-pulmonary disease
D. repens	Europe, Africa, Asia	FH: dog, cat, wild carnivores IH: Culicidae	Subcutis	Blood, skin	Low virulence: pruritus, dermatitis
Ar	South Europe, America, Asia, Australia	FH: dog, wild canids IH: fleas (Cf, Cc, Pi, Eg), lice (Hs)	Subcutis, body cavities, internal organs	Blood	Apathogenic
D	Europe, Asia, Africa	FH: dog, fox, hyaena IH: Rs, Hl	Peritoneal cavity	Blood	Apathogenic
C	South Europe, Africa, South America	FH: dog IH: Rs	Connecting tissue of subcutis/muscle	Skin	Apathogenic

FH: final host, IH intermediate host, ML: main location, Ar: *Acanthocheilonema reconditum*, D: *Dipetalonema dracunculoides*, C: *Cercopithifilaria grassi*, Cf: *Ctenocephalides felis*, Cc: *Ctenocephalides canis*, Pi: *Pulex irritans*, Eg: *Echidnophaga gallinacea*, Hs: *Heterodoxus spiniger*, Rs: *Rhipicephalus sanguineus*, Hl: *Hippobosca longipennis*

in a 15-ml centrifuge tube and spun for 5 min at 300 x g. The supernatant was discarded, leaving 1 ml of solution to which a few drops of methylene-blue solution were added. The sediment was transferred to glass slides, covered with coverslips and examined by light microscopy at x100 and x400 magnifications.

Heartworm antigen test

Serological testing for *D. immitis* antigen was performed using a rapid enzyme immunoassay test system containing specific antibodies (IDEXX SNAP® 3Dx®) following the manufacturer's directions for use.

Serology for co-infections

Most of the dogs from part 2 of the study were also serologically tested by commercial tests for vector-borne co-infections as follows:

Antibodies to *Leishmania* species were examined according to manufacturer's directions by means of indirect immunofluorescence antibody test (Mega Screen FLUOLEISH®, MegaCor, Hörbranz/Austria) or a 96-well microtitre plate ELISA (*Leishmania*-ELISA Dog for detection of IgG antibodies against *L. infantum,* Afosa GmbH, Dahlewitz b. Berlin/Germany).

Antibodies to *Ehrlichia canis* were examined according to manufacturer's directions by means of indirect immunofluorescence antibody test (Mega Screen FLUOEHRLICHIA canis®, MegaCor, Hörbranz/Austria).

Antibodies to *Babesia canis* were examined according to manufacturer's directions by means of indirect immunofluorescence antibody test (Mega Screen FLUOBABESIA®, MegaCor, Hörbranz/Austria).

PCR (Polymerase chain reaction)

Preparation of DNA

Total DNA was extracted from blood samples using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for examination of blood samples. The following modifications were used: 400 µl of blood sample were mixed with 400 µl AL buffer and 40 µl Proteinase K and incubated at 56 °C for 10 min. 400 µl of absolute ethanol (Carl Roth GmbH, Karlsruhe, Germany) were added and after vortexing the mixture was loaded onto the spin column twice, separated by centrifugation each time. Finally, the DNA was eluted using 50 µl AE buffer and stored at –20 °C. For each amplification reaction a 2.5-µl DNA aliquot was used.

PCR to detect and discriminate between canine microfilariae

Three different PCRs were performed simultaneously for each sample: in the first reaction, common filarial primers DIDR-F1 and DIDR-R1 were used, which amplify ribosomal DNA spacer sequences as described elsewhere (Rishniw et al. 2006); the second and third reactions were performed with specific primer sets for *D. immitis* (I1 and I2) and for *D. repens* (R1 and R2), respectively, as described earlier (Favia et al. 1996).

All amplification reactions were prepared in total volumes of 25 µl, consisting of 1x Colorless GoTaq Flexi Buffer (Promega, Mannheim, Germany), 1.5 mM $MgCl_2$, 10 µM of each primer, 0.2 mM of each dNTP and 0.75 units GoTaq Flexi DNA Polymerase (Promega). The PCR cycling conditions were 95 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, at 60 °C for 30 s, at 72 °C for 45 s and, finally, 1 cycle at 72 °C for 7 min using a thermal cycler (iCycler, BioRad, Munich, Germany). All amplification reactions were separated on 1.5 % agarose gel by electrophoresis and photographed under UV light after staining with ethidium bromide.

The banding patterns were used to identify *D. immitis*, *D. repens* and other canine filarial species as single or mixed infections. If reactions with *D. immitis*- and *D. repens*-specific primers were negative, the amplicons generated by using DIRD-F1 and DIDR-R1 primers were purified by polyethylene glycol precipitation (Rosenthal et al. 1993)

Tab. 2 Imported or travel-accompanying dogs from Germany tested by means of modified Knott's test and heartworm antigen assay within the period 2008–2010

	Knott	Antigen	Knott + Antigen	Knott (alone)	Antigen (alone)
Samples tested	8,545	8,545	8,545	8,545	8,545
Positive samples	163	127	38	125	89
Percentage	1.91 %	1.49 %	0.44 %	1.46 %	1.04 %
95 % CI	1.64–2.22 %	1.25–1.77 %	0.32–0.6 %	1.23–1.74 %	0.85–1.28 %

and subjected to the sequencing in both directions. The obtained sequences were used to search for homologies using BLASTn in GenBank™.

PCR for co-infections

The molecular investigation on *Babesia* spp. DNA was performed by means of a conventional PCR according to Carret et al. (1999). No further differentiation was performed on samples with positive detection of *Babesia* DNA.

Results

Part 1 of the study

The results of part 1 of the study, which included a total of 8,545 samples, namely the results of heartworm antigen and Knott's test, are presented in Tab. 2. Heartworm antigen was detected in 127 samples (1.49 %; 95 % CI: 1.25–1.77 %), but only 38 dogs also revealed microfilariae in their blood samples. On the other hand, 125 animals (1.46 %; 95 % CI: 1.23–1.74 %) were only positive in the Knott's test.

Part 2 of the study

Species detected and countries of origin

For discrimination by means of PCR and sequencing, a total of 73 blood samples and two samples of adult worms were sent in by veterinary surgeons during 2008–2010. *D. repens* alone was detected in 35 cases (Tab. 3). *D. immitis* was proven in 15 samples, in 6 of them together with *D. repens* (Tab. 4). Tab. 5 shows the results for *Acanthocheilonema* and *Dipetalonema*: *D. dracunculoides* was found in 10 cases, *A. reconditum* in 12 cases, and in one sample an infestation with both *A. reconditum* and *D. repens* was evident. In two dogs (no. 23 and 25) no sequence analysis could be accomplished, but the size of the band obtained in the PCR reaction with common filarial primers DIDR-F1 and DIDR-R1 corresponded to *A. reconditum* or *D. dracunculoides* (Rishniw et al. 2006). Further two samples were positive for *D. immitis* based on the result of the heartworm antigen assay, however, molecular analysis revealed infection with *D. repens* (Tab. 3, dog no. 1) or *D. dracunculoides* (Tab. 5, dog no. 16) in these cases.

D. repens was the most common imported filarial infection and was found in a total of 42 samples. Furthermore, it was obviously imported to Germany from particularly many (11) European countries (Hungary: n = 14, Greece: n = 7, Italy: n = 5; Spain and Romania: each n = 4; Poland and Croatia: each n = 2; Bulgaria, Slovenia, Austria and France: each n = 1). *D. immitis* was the second most commonly detected species with 15 positive PCR results and two samples which only demonstrated heartworm antigen. Those dogs were brought to Germany from five different countries: Greece (n = 6), Spain (n = 6), Italy (n = 3), Hungary (n = 1) and Bulgaria (n = 1). Finally, 25 cases with either *A. reconditum* or *D. dracunculoides* could be diagnosed for 23 dogs imported from Spain (n = 6 from the Canaries and one from Majorca) and one dog from Portugal and Hungary each, respectively.

Tab. 3 Canine *Dirofilaria repens* infections imported to Germany during 2008–2010

Dog	Age (years)/ gender	Origin of infection	Knott	Heartworm antigen	PCR/ sequencing	Co-infections/ comments
1	2/f	Spain	+	+	*D. repens*	Ec 1:2,560; L/B –
2	6/f	Spain	+	–	*D. repens*	Bc 1:320; B +; Ec –
3	6/m	Spain	nc	nc	*D. repens*	L –
4	5/f	France (Mazeres)	nc	nc	*D. repens*	
5[a]	6/m	Italy	+	–	*D. repens*	Ec >1:2,560; L 1:50; B –
6	9/m	Italy (Adria)	+	–	*D. repens*	
7	5–6/f	Italy (Sardinia)	+	–	*D. repens*	Ec/L/Bc –
8[b]	6/f	Austria (Gablitz)	nc	nc	*D. repens*	PCR performed on a worm
9	3/m	Poland (near Warsaw)	+	nc	*D. repens*	Ec/L/B –
10	8/m	Poland (Baltic coast)	+	–	*D. repens*	Bc/B –
11	4/f	Slovenia	+	–	*D. repens*	Bc 1:40; Ec/L –
12	2/m	Croatia	+	nc	*D. repens*	L 1:50
13	2/m	Croatia (near Zagreb)	+	–	*D. repens*	Ec/L/Bc –
14	11/m	Romania	+	–	*D. repens*	Ec/L/Bc –
15	10/f	Romania	+	–	*D. repens*	Ec/L/Bc –
16	4/f	Romania	+	–	*D. repens*	Bc 1:160
17	6/f	Romania	+	–	*D. repens*	Ec/L/Bc –
18	8/f	Hungary	+	nc	*D. repens*	
19	10/f	Hungary	nc	–	*D. repens*	Ec/L/B –
20	unknown	Hungary (Pecs)	nc	nc	*D. repens*	
21	5/m	Hungary	+	–	*D. repens*	Ec/L/Bc –
22	3/f	Hungary	+	–	*D. repens*	Ec/L/Bc –
23	unknown	Hungary	nc	–	*D. repens*	
24	1/m	Hungary	+	–	*D. repens*	Ec/L/Bc –
25	4/f	Hungary	+	–	*D. repens*	Bc 1:160; Ec/L –
26	2/m	Hungary	nc	nc	*D. repens*	Ec –
27	4/m	Hungary	+	–	*D. repens*	Ec/L/Bc –
28	8/m	Hungary	+	–	*D. repens*	
29	6/f	Hungary	+	–	*D. repens*	Ec/L/Bc –
30	4/m	Hungary	+	–	*D. repens*	Ec/L/Bc –
31[c]	5/m	Greece	+	–	*D. repens*	Ec/L/Bc –
32	unknown/m	Greece	+	–	*D. repens*	
33	1/m	Greece	+	–	*D. repens*	Ec/L/Bc –
34	2/f	Greece	+	–	*D. repens*	Ec/L/Bc –
35	2/f	Greece	+	–	*D. repens*	Ec/L/Bc –

B: *Babesia* spp. PCR, Bc: *Babesia canis* antibodies (IFA), Ec: *Ehrlichia canis* antibodies (IFA), L: *Leishmania* antibodies (IFA or ELISA), nc: not conducted, m: male, f: female, +: positive, –: negative

[a] died of canine monocytic ehrlichiosis

[b] adult non-fertile female surgically removed from a pea-sized subcutaneous mandibular cyst

[c] six treatments with selamectin (Stronghold®) spot-on at monthly intervals did not completely eliminate the microfilariae in this dog

Tab. 4. *Dirofilaria immitis* infections and co-infections of *D. immitis* and *D. repens* diagnosed in imported/travelling German dogs during 2008–2010

Dog	Age (years)/ gender	Origin of infection	Knott	Heartworm antigen	PCR/ sequencing	Co-infections/ comments
1	6/f	Spain	+	+	Di	L –
2	5/m	Spain	nc	nc	Di	
3	3/m	Spain	+	+	Di	
4	2/m	Spain	nc	+	Di	Ec/L –; Bc 1:40
5	unknown/m	Italy	+	+	Di	Ec/L/Bc –
6	7/m	Italy	+	+	Di and Dr	Ec >1:2,560; L 1:100; B –
7[a]	unknown/f	Italy (Sardinia)	+	–	Di and Dr	Ec/L –; Bc 1:40
8	2/m	Hungary	nc	–	Di and Dr	Ec 1:640
9	1/m	Bulgaria (Burgas)	+	+	Di and Dr	
10	4/m	Greece	nc	+	Di	L –
11	7/m	Greece	+	+	Di	L 1:50; Ec/Bc –
12	2/f	Greece	nc	+	Di	Ec >1:2,560; L 16 TU; Bc –
13	2/f	Greece	+	–	Di	Ec/L/Bc –
14[b]	2/f	Greece	+	+	Di and Dr	L 1:1,600; Ec/B –
15	2/f	Greece (Corfu)	nc	+	Di and Dr	Ec >1:2,560; L 1:100; B –

B: *Babesia* spp. PCR, Bc: *Babesia canis* antibodies (IFA), Di: *Dirofilaria immitis*, Dr: *Dirofilaria repens*, Ec: *Ehrlichia canis* antibodies (IFA), L: *Leishmania* antibodies (IFA or ELISA), TU: test units (ELISA), nc: not conducted, m: male, f: female, +: positive, –: negative

[a] presumable adulticidal treatment prior importation to Germany

[b] heartworms detected also on ultrasound examination

Age of dogs and filarial infections

For dogs infected with *D. repens* (Tab. 3) the median age was 5 years (ranging from 1 to 11 years); for dogs positive for *D. immitis* (Tab. 4), the median age was 2 years (ranging from 1 to 7 years). Dogs tested positive for *A. reconditum* or *D. dracunculoides* (Tab. 5) showed a median age of 2.5 years, with a range of 9 months to 10 years.

Co-infections with other arthropod-borne pathogens

Four dogs imported from Greece showed also antibodies against *Leishmania infantum* and two of them additionally antibodies to *Ehrlichia canis*. In samples obtained from dogs imported from Spain, antibodies to *Babesia canis* were detected in two cases (one of them with a positive PCR result for *Babesia* spp.), antibodies to *L. infantum* in two cases (one of them also with a positive serology for *E. canis*) and one dog with antibodies only to *E. canis*. Two dogs introduced from Italy showed antibodies to *L. infantum* and *E. canis*, and in one case *B. canis* serology was also positive. Antibodies to *B. canis* were found in one case from Slovenia, Poland and Hungary, respectively, and one dog from Hungary showed antibodies to *E. canis*. One dog imported from Croatia displayed a positive serologic reaction to *L. infantum* at a low titre of 1:50 (Tabs. 3–5).

Tab. 5 Canine *Acanthocheilonema reconditum* and *Dipetalonema dracunculoides* infections diagnosed in imported/travelling German dogs during 2008–2010

Dog	Age (years)/ gender	Origin of infection	Knott	Heartworm antigen	PCR/ sequencing	Co-infections/ comments
1	unknown/m	Spain	nc	nc	Ar	
2[a]	unknown/m	Spain	+	–	Ar	Ec/L/Bc –
3	6/m	Spain	+	–	Ar	Ec 1:2,560; L 1:400; Bc –
4[b]	9 months/m	Spain	+	–	Ar	Ec/L/Bc –
5[c]	1/f	Spain	nc	–	Ar	PCR performed on worms; L 95,6 TU
6	4/f	Spain (mainland)	+	nc	Ar	
7[d]	unknown/m	Spain (Almeria)	+	–	Ar	
8	1/f	Spain (Malaga)	+	–	Ar	Ec/L/Bc –
9	4/m	Spain (Majorca, Palma)	+	–	Ar	Ec/L/Bc –
10	7/m	Spain (CI–Tenerife)	+	–	Ar	Ec/L/Bc –
11	5/f	Spain (CI)	+	–	Ar	Ec/L/Bc –
12	3/f	Spain (CI)	+	–	Ar and *D. repens*	Ec/L/Bc –
13	10/m	Spain (CI–F)	+	–	D	Ec/L/Bc –
14	1/f	Spain (CI–F)	+	–	D	Ec/L/Bc–
15	2/f	Spain (CI)	+	–	D	Ec/L/Bc –
16	1/f	Spain (Alicante)	+	+	D	Ec/L –
17	4/f	Spain (La Albuera)	+	–	D	Ec/L/Bc –
18	2/f	Spain (Gava)	nc	–	D	
19	1/f	Spain	+	–	D	Ec/L/Bc –
20	2/m	Spain[e]	+	–	D	
21[f]	1/m	Spain	+	–	D	Ec/L/Bc –
22	3/w	Spain (Malaga)	+	–	D	Ec/L/Bc –
23	4/m	Spain	+	–	Ar or D[g]	Ec/L/Bc –
24	6/m	Hungary	nc	–	Ar	
25	2/f	Portugal	nc	–	Ar or D[g]	Ec/L/B –

B: *Babesia* spp. PCR, Bc: *Babesia canis* antibodies (IFA), Ar: *Acanthocheilonema reconditum*, D: *Dipetalonema dracunculoides*, Ec: *Ehrlichia canis* antibodies (IFA), L: *Leishmania* antibodies (IFA or ELISA), TU: test units (ELISA), CI: Canary Islands, F: Fuerteventura, nc: not conducted, m: male, f: female, +: positive, –: negative

[a] tested microfilariae-free 2 months later after a single spot-on therapy with moxidectin/imidacloprid (Advocate®)

[b] 2 spot-on treatments with selamectin (Stronghold®) 1 month apart did not eliminate microfilariae

[c] several vital worms isolated from ascites (abdominal cavity) at spaying (ovariohysterectomy) (Fig. 1 and 2)

[d] 3 weeks prior to the positive Knott's test, dog was treated with selamectin (Stronghold®) spot-on

[e] locality Central/southern Spain, close to border with Portugal

[f] 3 spot-on treatments with selamectin (Stronghold®) 1 month apart did not eliminate microfilariae

[g] sequencing for species diagnosis was not possible

Discussion

Our results demonstrate that filarial infections are relevant in terms of their occurrence in dogs in Germany with a history of import from or travel to countries in eastern or southern Europe. Out of 8,545 respective dogs tested for filarial infections with the "travel disease profile", 127 (1.49 %; 95 % CI: 1.25–1.77 %) were positive for heartworm antigen. These are supposed to be *D. immitis* infections because other filarial species such as *Acanthocheilonema* (previously *Dipetalonema*) *reconditum* (Weil et al. 1984) or *Dirofilaria repens* (Pantchev et al. 2009a; Sassnau et al. 2009) do not react in the applied heartworm antigen assay. Only 38 out of 127 heartworm antigen-positive samples also contained visible microfilariae (Tab. 2). The remaining 89 samples (70 %) might represent occult infections (infection with adult *D. immitis* in the absence of circulating microfilariae), a condition observed in 10 to 67 % of dogs infected with heartworms (Rawlings et al. 1982). Rawlings et al (1982) report on four different types of occult infection: prepatency (up to 6 months after infection), unisexual infections, drug-induced sterility of adult *D. immitis* (a condition which could be due to macrocyclic lactone or doxycycline treatment in the current study) or finally an immune-mediated clearance of microfilariae by means of ADCC (antibody-dependent cellular cytotoxicity) through IgM/IgG and neutrophils as described by Rzepczyk and Bishop (1984). In experimentally infected dogs, heartworm antigen was first detectable 6.5–8.5 months after infection and was produced exclusively by adult females (Weil et al. 1984; Weil 1987).

On the other hand, in 125 blood samples (1.46 %; 95 % CI: 1.23–1.74 %) only microfilariae were detected. The high proportion of microfilariae-positive but antigen-negative dogs emphasises the importance of microfilariae-based screening tests for dogs travelling across Europe or moved between countries. Courtney and Zeng (2001a) found, in a group of 963 dogs with necropsy-confirmed heartworm infections, 834 (86.6 %) to be positive by a heartworm antigen test, while 504 (52.3 %) were microfilaraemic in the modified Knott's test. Only two (0.4 %) of the microfilaraemic dogs were negative for heartworm antigen and another 18 (3.6 %) showed a very weak positive signal. We conclude that most of the 125 microfilaraemic dogs in the present study were infected with other filarial species than *D. immitis*.

Considering all diagnostic results, PCR and sequencing corresponded well to the results of detection of microfilariae and heartworm antigen. Interestingly, three animals (Tab. 4, dogs no. 7, 8 and 13) were found to be positive for *D. immitis* by means of PCR, but were negative in the heartworm antigen assay. This discrepancy may be due to possible persistence of microfilariae after the death of adults, e.g. following adulticidal therapy as has been performed prior to importation in one dog, or due to low sensitivity in cases of a low burden of adult worms. A study by Courtney and Zeng (2001b) suggests that sensitivity is crucially dependant on the number of adult female worms (0, 1–2 or > 2). Furthermore, in case of low worm burdens or after chemoprophylaxis with macrocyclic lactones, antigenaemia may be delayed to approximately 9 months post infection (Nelson et al. 2005).

Moreover, in the present study two samples were positive for *D. immitis* based on the result of the heartworm antigen assay alone, despite the fact that the molecular detection only found *D. repens* (Tab. 3, dog no. 1) or *D. dracunculoides* (Tab. 5, dog no. 16). In these cases, the number of circulating microfilariae of *D. immitis* was probably lower than the detection limit (5 microfilariae per 1 ml blood), and the DNA could not be amplified by PCR. Because low microfilaraemia may thus lead to false negative results, blood should be collected at the appropriate time considering the periodicity of microfilaraemia. Webber and Hawking (1955) showed that the maximum number of microfilariae in the case of *D. repens* (Sardinian strain) occurs

Fig. 1 *Acanthocheilonema reconditum* adults isolated from the abdominal cavity of a female dog from Spain (Tab. 5, Dog no. 5)

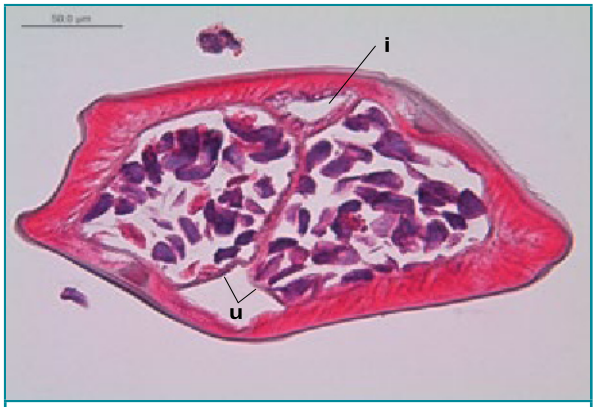

Fig. 2 *Acanthocheilonema reconditum* female from Fig. 1 in cross section. Note the smaller size of the worm (compared to Fig. 3), the small intestine (i) and the uteri with developing microfilariae (u)

at midnight (between 10 p.m. and 3 a.m.) and the minimum number about noon (between 11 a.m. and 2 p.m.), whereas in the case of *D. immitis* (Chinese strain), the highest count of microfilariae in the peripheral circulation occurs about 6 p.m. and the lowest about 6 a.m. Depending on the time when samples are collected, the number of microfilariae can be reduced to 20–40 % (*D. repens*) and 5–20 % (*D. immitis*) of the counts obtained at the optimal time of examination. This should be considered for filarial diagnosis in dogs. There is currently no data available regarding periodicity of microfilariae of *A. reconditum* or *D. dracunculoides*, but the activity pattern of known vectors involved, e.g. *Hippobosca longipennis* (Tab. 1), favour the hypothesis of diurnal accumulation in peripheral blood. Moreover, it should be considered that the prepatent period in other filarial species differs from that of *Dirofilaria* spp. Due to the much shorter prepatency of *D. dracunculoides* (69–76 days; Olmeda-Garcia et al. 1993) and *A. reconditum* (61–68 days; Farnell and Faulkner 1978), diagnostic tests for microfilariae may be conducted as early as 3 months post infection (pi) in dogs suspected to be infected with these worms.

Surprisingly, 23 out of 25 cases of infection with filarial species other than *Dirofilaria* came from Spain (Tab. 5). Our data demonstrate first records of *D. dracunculoides* from the Canary Islands, of filarial infections on Fuerteventura as well as a co-infection of *A. reconditum* and *D. repens* from this country. Furthermore, no previous importation of *A. reconditum* from the island of Majorca has been described. Stenzenberger und Gothe (1999) investigated 700 dogs on Tenerife for vector-borne diseases and found 190 (27.1 %) to be infected with filaria, which represented the most common arthropod-borne infection. Most dogs (n = 158) harboured *D. immitis*, 15 dogs were additionally infected with *A. reconditum* und three with *D. repens*, whereas two dogs were infected with *D. repens* only. 30 samples positive for microfilariae could not be differentiated further by activity pattern of acid phosphatase, which substantiates the limitations of this method compared to PCR and sequencing. Other data obtained for dogs that lived for shorter or longer periods in Spain were in concordance with previous reports from this country (Guerrero et al. 1989; Gomez-Bautista and Rojo-Vazquez 1990; Ortega-Mora et al. 1991; Solano-Galego et al. 2006) with the exception of occurrence of *A. reconditum* in Almeria, however. This parasite has been described in the bordering Murcia area before.

One case of *A. reconditum* was imported from Hungary, which to our knowledge is the first reported case from this country confirmed by sequencing. Most dogs with *D. repens* infection came from Hungary (n = 14). This is in concordance with the high

prevalence (regionally 14–30 %) of this parasite in Hungary (Fok et al. 2010) and with the currently high number of dogs imported to Germany by animal welfare organisations. Thus, it is particularly important to test such dogs and to treat them, if necessary, with an appropriate filaricide (Fok et al. 2010). The dog with co-infection (*D. immitis* and *D. repens*; Tab. 4 , dog no. 8) represents the second reported case of *D. immitis* infection from Hungary. The first one was described by Jacso et al. (2009), who also showed a co-infection of *D. immitis* and *D. repens* confirmed by PCR on a blood sample. Interestingly, both cases were negative for heartworm antigen. This was likely due to the low number of adult nematodes. Only one male and one gravid female worm were found in the right cardiac ventricle of the first case (Jacso et al. 2009).

Slovenia was previously not reported as a source of infection with *D. repens*. So far, only one case of heartworm infection was described for Slovenia (Brglez and Senk 1987). Recently, canine *D. repens* infection was reported from Poland. This was based on morphological identification (Demiaszkiewicz and Polanczyk 2010) and by means of PCR (Sapierzynski et al. 2010). Unfortunately, no information on the geographic location where the infection was probably attracted is available. One of the *D. repens*-infected dogs in the present study travelled with its owner to the Baltic coast, and one dog lived near Warsaw prior to import.

Interestingly, the second most frequent source of *D. repens* after Hungary as well as the origin of most heartworm infections was Greece (Tabs. 3 and 4). Two dogs showed a co-infection with both species. Such co-infections have been described from hunting dogs in northern Greece at a rate of 3 % (Papazahariadou et al. 1994). The finding of *D. immitis* and *D. repens* in a dog originating from the island of Corfu appears of particular interest as the only previously reported case in this location was a *D. repens* infection in a human (Tzanetou et al. 2009).

Five dogs harbouring *D. repens* came from Italy, and two of them were also infected with heartworm. Tarantini et al. (1983) investigated 578 dogs on Sardinia and found *D. repens* (7.4 %), *D. immitis* (3.5 %), *A. reconditum* (0.3 %) as well as 0.2 % dogs co-infected with *D. repens* and *A. reconditum*. Neither these authors nor other studies from Sardinia (reviewed by Weise 2004) could identify a co-infection of *D. immitis* and *D. repens*, as diagnosed in dog no. 7 (Tab. 4) in the present study. However, such co-infections in Italy are conceivable as in the region of Lazio (Central Italy) *D. repens* and *D. immitis* were identified in head and thorax of a single mosquito (*Aedes albopictus*; Cancrini et al. 2007).

Four dogs imported from Romania were positive for *D. repens*. Data on the prevalence of *D. immitis* and *D. repens* in dogs in Romania are summarised by Olteanu (1996). These parasites are present all over the country but in strongly variable levels (up to 35 % prevalence) with many cases in the south. Since 1996, no further studies on the distribution and expansion of *Dirofilaria* spp. in Romania have been performed and our cases are the first confirmed by means of PCR.

Two cases of *D. repens* infection were identified in dogs imported from Croatia. Microfilariae are frequently diagnosed in dogs in this country (average 15.5 %), and microfilariae of *Dirofilaria* spp. have been detected by chance in clinically healthy dogs (Zivicnjak et al. 2007). Dzaja et al. (2008) described the first case of a dog infected with *D. repens* by means of morphological description of microfilariae after necropsy.

In Bulgaria, *D. immitis* has been detected frequently, e.g. in 7.4 % of 258 dogs investigated by means of Knott's test and heartworm antigen (Georgieva et al. 2001), but so far only one report of *D. repens* infection exists, where in 2 out of 192 street dogs adult worms were found during necropsy (Kanev et al. 1996). The present study reports the first case of co-infection with *D. immitis* and *D. repens* in Bulgaria from the area of Burgas.

The first case of *D. repens* infection in a dog from Austria was documented in Burgenland (Zurndorf) by Löwenstein and Spallinger (2009), and further

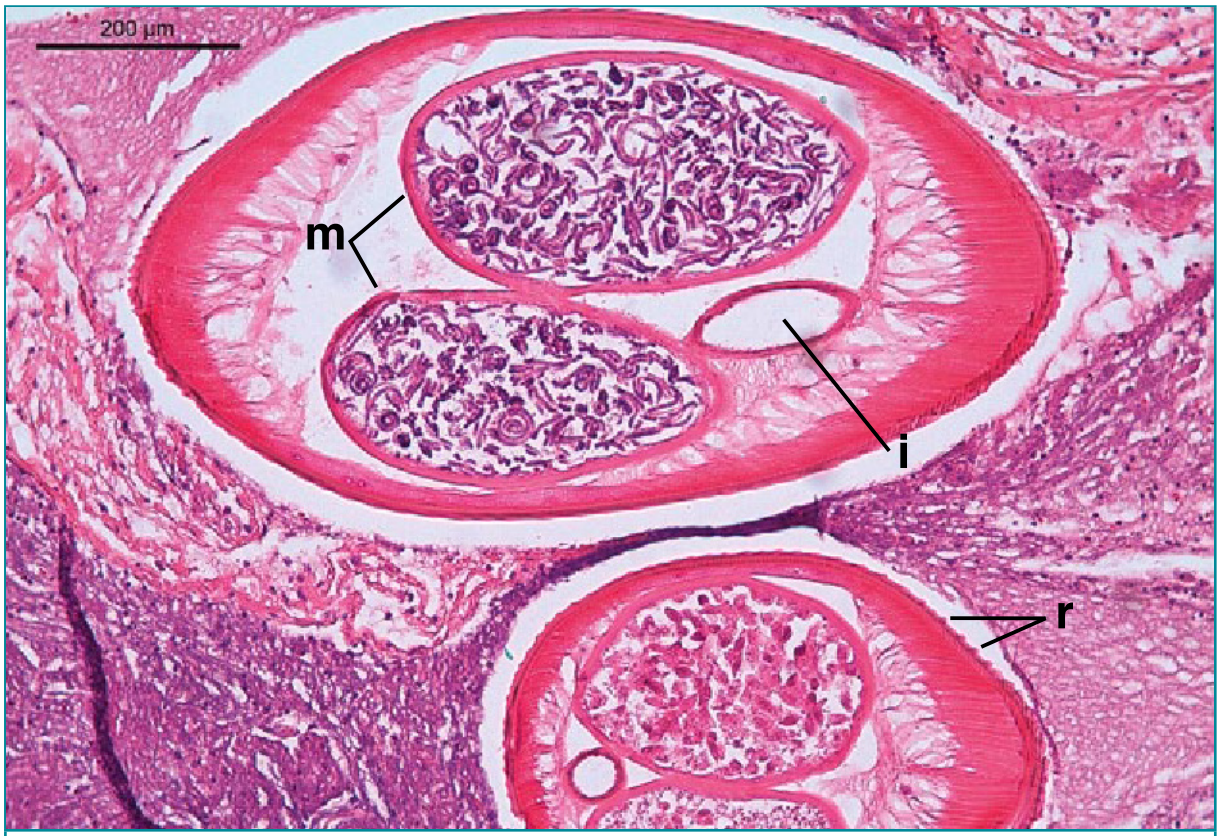

Fig. 3 *Dirofilaria repens* female in cross section in the subcutaneous mammalian tissue (primary neoplasm suspicion) in a dog from Italy (confirmed by PCR). Note the small intestine (i), the uteri filled with microfilariae (m) as well as the longitudinal, evenly spaced ridges on the surface of the cuticle (r)

presumably autochthonous cases were subsequently reported from Lower Austria (Gänserndorf) and Burgenland (Neusiedl; Duscher et al. 2009), and this is in accordance with the findings in the present study from Gablitz (Lower Austria), pointing to the possibility of endemicity in this region.

In France, *D. repens* was described for a dog from Mazeres (department Ariège, region Midi-Pyrénées). *D. repens* is known to occur in the department Aveyron (region Midi-Pyrénées; Cazelles and Montagner 1995) and in the bordering departments Haute-Garonne and Tarn-et-Garonne (Ducos de Lahitte 1990; Chauve 1997) and thus appears to be endemic in these regions.

In the present study, *D. repens* was the most common imported canine filarial infection, and it originated from many European countries. Climate change has been proposed as a possible factor for spreading of *Dirofilaria* infections into the North of Europe (Genchi et al. 2008; Pantchev et al. 2009a). However, *D. immitis* is so far not autochthonous in Germany, in contrast to *D. repens* (Pantchev et al. 2009a), and in northern France (Pantchev et al. 2009b). *D. repens* infections in dogs are mostly asymptomatic or they are occasionally misidentified as subcutaneous tumour (Fig. 3), while heartworm infections may cause severe clinical symptoms. Laboratory screening profiles for travelling or imported dogs in the past were based predominantly on heartworm antigen tests (Hirsch and Pantchev 2008). Thus, dogs harbouring *D. immitis* are generally treated with adulticides and microfilaricides and cured in many cases (Genchi et al. 2009). Dogs infected

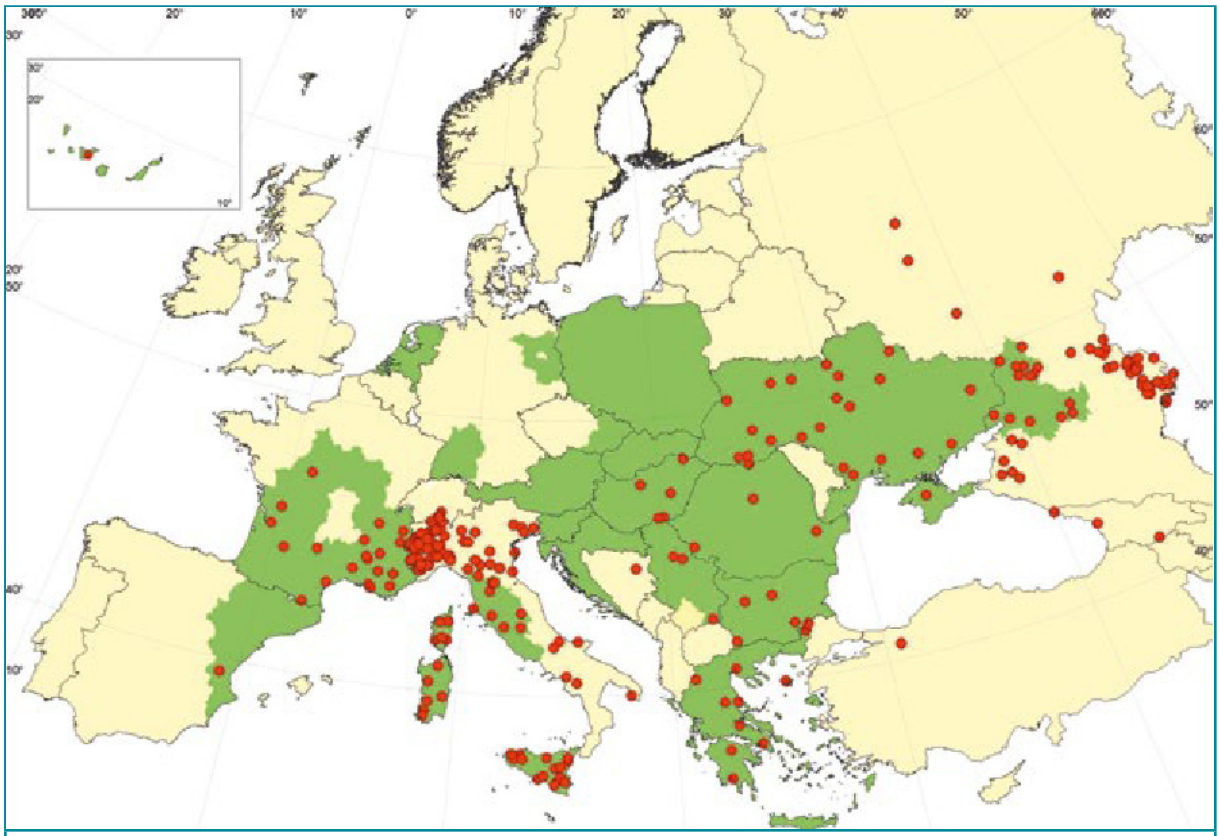

Fig. 4 Endemic areas (at least one confirmed case) of *Dirofilaria repens* in Europe, Canary islands (projected on the map) and Middle East: green areas indicate documented cases in animals (dogs, cats, foxes), whereas red points show documented human cases (based on literature and the current study). The extent of epidemiological assessment of *D. repens* cases varies between countries. The data presented in the map may therefore not be entirely complete, and it cannot be excluded that infections will occur in new areas (map software RegioGraph)

with *D. repens* often originate from other countries, although autochthonous transmission in Germany has been reported (Sassnau et al. 2009), and this parasite remains undiagnosed and untreated in many dogs that thus may serve as reservoir hosts for the local mosquito population for several years. The maximum life span of *D. repens* females in dogs is described to be as long as 43 months, plus additional 280 days when worms are transferred to a second dog, while fertility is maintained (Webber and Hawking 1955). Interestingly, the median age of *D. repens*-infected dogs in the present study was 5 years, while dogs infected with *D. immitis* or *A. reconditum*/*D. dracunculoides* showed a lower median age of 2 and 2.5 years, respectively. The current spread of *D. repens* across Europe (Fig. 4) is of major concern, especially regarding its zoonotic potential, and underlines the importance and necessity of appropriate diagnostic approaches for dogs imported from or travelling to endemic areas by means of microfilariae concentration assays and PCR. Apart from (possibly) Hungary, there is so far no tendency of spreading in Europe for *Acanthocheilonema* or *Dipetalonema*, but the results of the present study indicate that the relatively high numbers of imported dogs in (mainly from Spain), as well as the existence of suitable vectors (Tab. 1), may lead to endemic expansion in Germany.

Some of the dogs (part 2 of the study) have been treated with macrocyclic lactones in order to eliminate the microfilariae. Selamectin was not able to clear microfilaraemia in one dog infected with *D. repens*

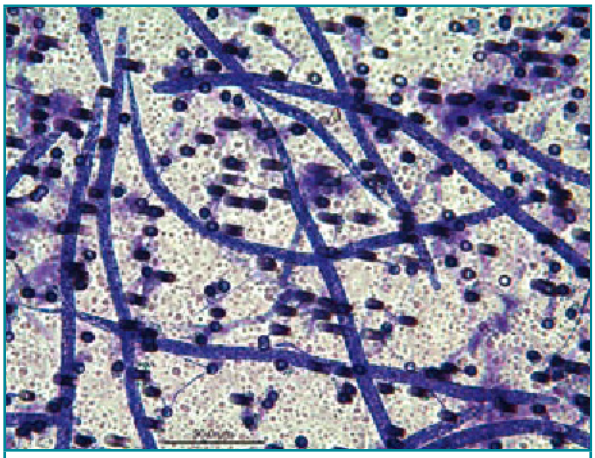

Fig. 5 Microfilariae from a blood sample after concentration with the filtration test (clinical suspicion of heartworm infection in a dog imported from Corsica based on ultrasound/X-ray examination and a positive antigen test; around the microfilariae stained remnants of blood cells are visible)

(up to six-monthly spot-on applications; dog no. 31, Tab. 3), two dogs diagnosed with *A. reconditum* (up to two-monthly spot-on applications; dogs no. 4 and 7, Tab. 5) as well as one dog with *D. dracunculoides* infection (up to three-monthly spot-on applications; dog no. 21, Tab. 5). A current study in Hungary showed that 35 % of dogs remained microfilaraemic (*D. repens*) after selamectin spot-on applications (monthly or biweekly) for a treatment period up to 9 months (Jacso et al. 2010). One spot-on treatment with moxidectin/imidacloprid was able to eliminate microfilariae in one dog infected with *A. reconditum* (dog no. 2, Tab. 5), which confirms the microfilaricidal efficacy of moxidectin, previously demonstrated for *D. immitis* and *D. repens* (Fok et al. 2010).

Conclusion

Filarial infections of dogs are of relevance in Germany, however, often neglected. The current spread of *D. repens* across Europe is of major concern, especially regarding its zoonotic potential. In order to prevent the establishment of new endemic spots as well as the expansion of further filarial species, imported dogs should be tested – additionally to screening for heartworm infection by means of an antigen ELISA – for microfilariae by means of modified Knott's or filtration test (Fig. 5) and molecular methods for their differentiation. Differences regarding the prepatent period or periodicity of microfilaraemia in the circulation should be taken into consideration.

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Compliance statement

All investigations comply with the current laws of the countries in which they were performed.

Disclosure statement

There are no commercial associations for authors M.E., A.D., V.D. or N.P. of IDEXX Laboratories, there is no commercial conflict of interest since the information generated here is solely for scientific dissemination. There are no conflicts of interest to report by any author.

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