



Serological detection of circulating *Angiostrongylus vasorum* antigen and specific antibodies in dogs from central and northern Italy

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ABSTRACT

The most frequently employed method for the diagnosis of *Angiostrongylus vasorum* in dogs is the detection of first stage larvae (L1) in faeces. The sensitivity of coproscopy, however, is limited in case of low parasite load, intermittent larval excretion, and during pre-patency. An epidemiological survey on dogs was conducted applying serological methods in two Italian regions where angiostrongylosis is endemic in foxes. 265 dog serum samples from Tuscany (central Italy – site A) and 447 from Liguria (north-western Italy – site B) were tested with a sandwich-ELISA for detection of circulating antigen, and with an ELISA using *A. vasorum* adult somatic antigen purified by monoclonal antibodies for specific antibody detection. During previous examinations dogs naturally infected with *Leishmania infantum* ($n=149$), *Dirofilaria immitis* ($n=40$), *Dirofilaria repens* ($n=30$), *Acanthocheilonema reconditum* ($n=27$), *Crenosoma vulpis* ($n=1$), *A. vasorum* ($n=2$), *Capillaria aerophila* ($n=35$), *Capillaria boehmi* ($n=3$), *Toxocara canis* ($n=68$), *Toxascaris leonina* ($n=5$), hookworms ($n=37$) and *Trichuris vulpis* ($n=39$) were detected. Sera of these dogs were used to evaluate cross reactions. In site A, 2 dogs (0.8%) were seropositive for antibody and antigen detection and 4 (1.5%) for antibody detection only. From site B, 4 dogs (0.9%) were seropositive for both tests, while other 4 dogs (0.9%) for antigen detection only and 9 dogs (2%) for antibody detection only. Considering a subgroup of 347 dogs from site B which had also been tested with the Baermann technique, 2 (0.6%) were positive for both tests, 4 (1.2%) for antigen detection only and 9 (2.6%) for antibody detection only. The two dogs which were positive for both serological tests were also positive for *A. vasorum* L1 in the faeces. No significant difference in seropositivities was observed in the group of dogs with other proven parasitic infections. *A. vasorum* serology presents significant advantages (diagnosis before patency, single serum sample instead of repeated faecal samples, rapidity and affordability particularly in case of large number of samples) and it can be considered a valid alternative for diagnosis in individuals and in epidemiological studies.

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1. Introduction

Angiostrongylus vasorum is a metastrongylid nematode of dogs, foxes and other wild carnivores (wolves, coyotes,

and rarely mustelids and felids), living in the right side of the heart and in the pulmonary arteries. The parasite can be at the origin of respiratory and circulatory distress, coagulopathies and neurological signs in dogs, potentially leading to the host's death if the infection is left untreated (Staebler et al., 2005; Koch and Willeßen, 2009). Its life cycle is indirect: definitive hosts shed first-stage larvae (L1) in the faeces (Guilhon and Bressou, 1960; Guilhon, 1963), which, when ingested by an intermediate host (snails and

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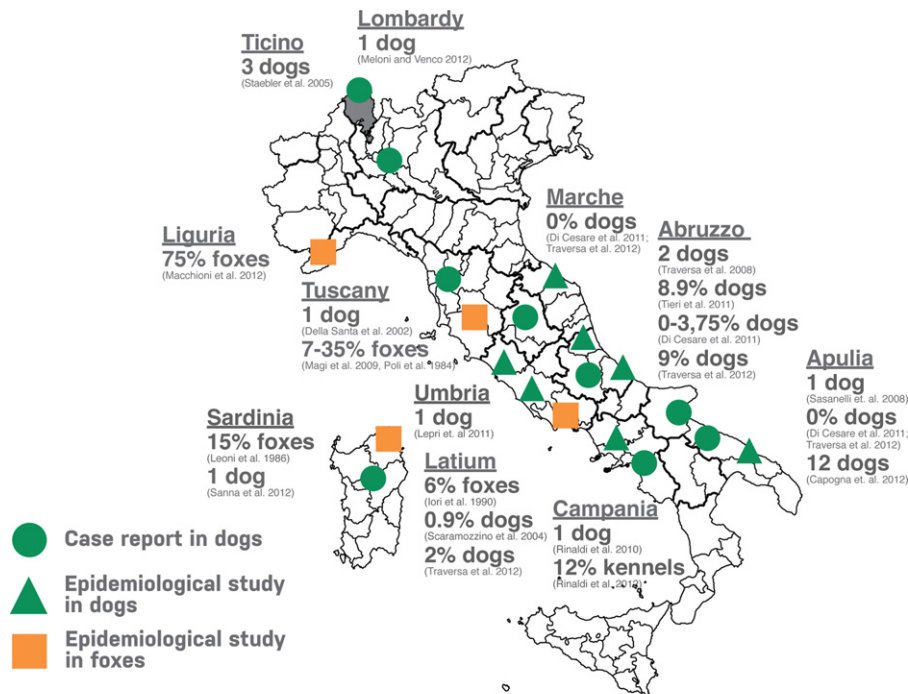


Fig. 1. Occurrence of *Angiostrongylus vasorum* in dogs and foxes in Italy and close to the national borders (Ticino, Switzerland).

slugs), develop to infective third-stage larvae (L3). Since its first description in the south of France (Serres, 1854), *A. vasorum* has been reported as a common parasite of foxes and less frequently in dogs in several countries in Europe, Africa, North and South America. Traditionally, *A. vasorum* has been considered endemic in distinct isolated foci in southern France (Guilhon, 1969; Bourdeau, 1993), Denmark (Willingham et al., 1996; Taubert et al., 2009) and southern Britain (Martin and Neal, 1992; Chapman et al., 2004), but in the last two decades the number of reports from previously considered non endemic areas sharply increased (Staebler et al., 2005; Papazahariadou et al., 2007; Helm et al., 2009; van Doorn et al., 2009; Yamakawa et al., 2009; Majoros et al., 2010), clearly indicating that the parasite is widely present in Europe.

The epidemiology of *A. vasorum* in Italy is poorly known, although the attention of parasitologists and clinicians to this parasite is currently increasing. Its presence has been reported in foxes in Tuscany (Poli et al., 1984; Magi et al., 2009), in Sardinia (Leoni et al., 1986), in Latium (Iori et al., 1990) and in Liguria (Macchioni et al., 2012), while the first dog infected with *A. vasorum* was described with respiratory distress and originated from Tuscany (Della Santa et al., 2002), followed by two dogs with severe respiratory signs in Abruzzo (Traversa et al., 2008), by one dog with clinical signs in Apulia (Sasanelli et al., 2008) and by a fatal case in Campania (Rinaldi et al., 2010). Although very recently new cases have been described (Lepri et al., 2011; Capogna et al., 2012; Meloni and Venco, 2012; Sanna et al., 2012), and epidemiological surveys have been conducted (Scaramozzino

et al., 2008; Di Cesare et al., 2011; Tieri et al., 2011; Rinaldi et al., 2012; Traversa et al., 2012), data are mainly limited to central Italy (see also Fig. 1).

The most frequently employed diagnostic method in dogs is currently the detection of L1 in faeces, applying larval migration techniques such as the Baermann–Wetzel technique (Eckert et al., 2008). The sensitivity of copromicroscopic methods, however, is limited in case of intermittent excretion of larvae (Oliveira-Junior et al., 2006; Taubert et al., 2009), low parasite load, and during prepatency. In addition, morphological differentiation of the larvae from other lungworm larvae such as *Crenosoma vulpis* and *Filaroides* spp. is challenging (McGarry and Morgan, 2009). Repeated testing of faecal samples is recommended to increase the diagnostic sensitivity (Barutzki and Schaper, 2009; Taubert et al., 2009). However, there has been agreement within the veterinary community that, due to i.e. difficulties to collect multiple faecal samples and to guarantee proper storage of faecal samples before analysis, alternative, new sensitive diagnostic techniques were needed (Traversa and Guglielmini, 2008; Schnyder et al., 2011a).

Serological methods have been developed to overcome these problems (Cury et al., 1996; Verzberger-Epshtein et al., 2008; Jefferies et al., 2011), but currently no immunological tests for the diagnosis of *A. vasorum* infections are commercially available. Newly presented serological methods for the detection of circulating antigen of *A. vasorum* (Schnyder et al., 2011a) and of specific antibodies (Schucan et al., 2012) may represent a valid alternative

for epidemiological surveys of dog populations and for individual diagnosis of canine angiostrongylosis. The aim of our work was to determine the seroprevalences of *A. vasorum* in dogs of two areas of central and northern Italy known to be endemic for angiostrongylosis in foxes applying these newly developed serological detection methods, and to validate the ELISAs on a field study.

2. Materials and methods

2.1. Dog sera

Serum samples of 712 dogs from two different areas of Italy were tested (Fig. 2): 265 from Tuscany (central Italy – site A) and 447 from Liguria (north-western Italy – site B). In site A dogs were mainly sampled in kennels (240), at the moment of their arrival. A smaller amount of samples (25) was sent to the University of Pisa by veterinary clinicians. In site B 304 samples were taken from privately owned hunting dogs, 130 were sampled in kennels and 13 from pet dogs. Individual data about sex, age, living habitat and geographical origin were collected. Sera from site A (106 female and 159 male dogs) derived from a previously reported survey on filarial infections and were tested with Knott's test and Dirocheck® ELISA (Magi et al., 2011). Sera from site B (212 female and 235 male dogs) derived from an on-going epidemiological survey on helminths of wild and domestic canids; for 347 dogs coprological examinations were performed (flotation and Baermann–Wetzel technique), together with Knott's test and Dirocheck® ELISA. Furthermore all sera included in the study were tested for antibodies against *Leishmania infantum* with ELISA (following the procedure described in Mettler et al., 2005). All dogs came from rural or semirural environments and had not received any anthelmintic treatment for at least 3 months before the sampling. Their age ranged from 6 months to 15 years.

2.2. ELISA for the detection of *Angiostrongylus vasorum* circulating antigen and of specific antibodies against *A. vasorum*

Procedures for the production of polyclonal antibodies against *A. vasorum* adult E/S-antigen and of monoclonal antibodies (mAb) against *A. vasorum* adult antigen are

described in detail in Schnyder et al. (2011a). *A. vasorum* adult somatic antigen was produced from specimens isolated during cardiopulmonary examination of naturally infected foxes from Italy, following the procedure previously described (Schucan et al., 2012).

A sandwich-ELISA was performed for antigen detection as described in Schnyder et al. (2011a), and a sandwich-ELISA using *A. vasorum* adult somatic antigen purified by monoclonal antibodies (mAb Av 5/5) was used for specific antibody detection (Schucan et al., 2012). All test runs included a background control, a conjugate control, three positive control sera from three experimentally infected dogs and two negative control sera from uninfected dogs.

The cut-off value of each test was determined using the mean value of optical density ($A_{405\text{nm}}$) plus 4 standard deviations (SD) of thirty sera from dogs of site B which tested negative for Baermann–Wetzel technique performed over three days. Four SD were applied for a better discrimination between positive and negative sera, based on the results with predefined sera.

2.3. Statistical analysis

Excel 2007 for Windows (Microsoft Corporation, Redmond, USA) was used to calculate the prevalences values, the confidence interval (CI) of the sensitivities and the specificities and for the calculation of means and standard deviations (SD).

3. Results

3.1. Parasitological surveys

During the above mentioned parasitological surveys dogs were found to be naturally infected with *L. infantum* ($n=31$ in site A and $n=118$ in site B) diagnosed by ELISA as previously described (Mettler et al., 2005), *Dirofilaria immitis*, diagnosed by the presence of circulating antigen (DiroCHEK® Synbiotics, San Diego, USA; $n=34$ in site A and $n=6$ in site B) and/or microfilariae (identified with the Knott's modified test and the acid phosphatase staining; $n=27$ in site A and $n=2$ in site B); *Dirofilaria repens* ($n=26$ in site A and $n=4$ in site B) and *Acanthocheilonema reconditum* ($n=4$ in site A and $n=23$ in site B) diagnosed by the presence of microfilariae (identified with the Knott's modified test and the acid phosphatase stain); *Crenosoma vulpis* ($n=1$, site B) and *A. vasorum* ($n=2$, site B) diagnosed by the presence of L1 in faeces detected by Baermann–Wetzel technique (Eckert et al., 2008); *Capillaria aerophila* ($n=35$, site B), *Toxocara canis* ($n=68$, site B), *Toxascaris leonina* ($n=5$, site B), hookworms ($n=37$, site B) and *Trichuris vulpis* ($n=39$, site B) detected by coproscopy after flotation in centrifuge (Dryden et al., 2005); *Capillaria boehmi* ($n=3$, site B) detected by coproscopy after flotation and confirmed by scanning electron microscopy and PCR-coupled sequencing (Guardone et al., 2012; Magi et al., 2012).

3.2. ELISA results

Serological results of the ELISAs for antigen and antibody detection are shown in detail in Table 1. Briefly, in site



Fig. 2. Study areas in Italy and sample size.

Table 1

Serological results of 712 dogs from Italy tested for the presence of circulating *Angiostrongylus vasorum* antigen and/or for specific antibodies against *A. vasorum* by ELISA. Tests performed as described in Schnyder et al. (2011a) and Schucan et al. (2012). CI: confidence interval.

Serological results for <i>A. vasorum</i> serology	Site A (Tuscany, N = 265)		Site B (Liguria, N = 447)		Dogs tested with the Baermann technique (site B, N = 347) ^a	
	N pos.	Prevalence % (95% CI)	N pos.	Prevalence % (95% CI)	N pos.	Prevalence % (95% CI)
Dogs seropositive for antigen and antibody detection	2	0.8 (0.1–2.7)	4	0.9 (0.2–2.3)	2 ^a	0.6 (0.1–2.1)
Dogs seropositive for antigen detection only	0	0(0.0–1.1)	4	0.9 (0.2–2.3)	4	1.2 (0.3–2.9)
Dogs seropositive for antibody detection only	4	1.5 (0.4–3.8)	9	2.0 (0.9–3.8)	9	2.6 (1.2–4.9)
Total seropositive samples for at least one ELISA test	6	2.3 (0.9–5.0)	17	3.8 (2.2–6.0)	15	4.3 (2.4–7.0)

^a The only two dogs which were positive for *Angiostrongylus vasorum* L1 with the Baermann–Wetzel technique were contemporaneously seropositive in both ELISA tests.

A 2 dogs (0.8%) out of the 265 examined were seropositive for both antibody and antigen detection and from site B 4 dogs (0.9%) out of the 447 examined were seropositive for both tests. Considering the subgroup of 347 dogs from site B, which was also tested with the Baermann–Wetzel technique, 2 dogs (0.6%) were positive in both tests: these two dogs were also the only ones positive for *A. vasorum* L1 detection in the faeces.

3.3. Cross reactions

The results of the ELISA tests of dogs with other proven parasitic infections are shown in detail in Table 2. On the whole, no significant difference in seropositivity was observed between the group of dogs with other proven parasitic infections and the group of dogs negative for other parasites.

4. Discussion

This is the first study on the seroprevalence of *A. vasorum* in Italy, therefore the prevalence values found cannot be directly compared with equivalent surveys in this country. However, our results in both study areas are similar to the values found in Latium with faecal analysis and post-mortem examinations (Scaramozzino et al., 2008) and in Abruzzo with the examination of individual faecal samples (Di Cesare et al., 2011). In a further Italian study (Tieri et al., 2011), interestingly, the prevalence found was much higher (8.9%) and comparable to the values found in symptomatic dogs from known endemic areas of England (Morgan et al., 2010), suggesting the possible presence of hyperendemic foci also in Italy. Regarding Europe, it is noteworthy to compare the results of the present study with the only other seroepidemiological survey performed with the same ELISA tests in Europe: our values are close to those found in the United Kingdom (0.97%) and higher than those found in Germany (0.3%) (Schnyder et al., 2011b). The prevalence values reported in Europe in surveys conducted by copromicroscopic examinations show high variations depending if dogs with or without clinical signs compatible

with *A. vasorum* infection have been tested: in the United Kingdom Morgan et al. (2010) found a prevalence of 15% in dogs with clinical signs and of 2% in apparently healthy dogs. In Germany the reported prevalence in symptomatic dogs varies from 1.2% to 7.4% (Taubert et al., 2009; Barutzki and Schaper, 2009), while only 0.1% of non-symptomatic dogs was found positive for *A. vasorum* in another study (Barutzki and Schaper, 2003). In Denmark a prevalence of 2.2% was found in dogs with clinical signs (Taubert et al., 2009), while in randomly sampled dogs a prevalence of 0.8% was found in The Netherlands (van Doorn et al., 2009) and of 1.1% in hunting and shepherd dogs in Greece (Papazahariadou et al., 2007). The dogs of our study were randomly sampled: this because we aimed to investigate the prevalence in a population at risk, considering the endemicity of the parasite in foxes in the study areas and the rural or semirural origin of the dogs, and because *A. vasorum* infection can be asymptomatic, especially in the early stages of the infection.

A. vasorum is common in wildlife, mainly in foxes, which represent the wild host reservoir of this parasite (Bolt et al., 1994). In this host *A. vasorum* is generally found in higher prevalence values (5%, Sréter et al., 2003; 92.7%, Tønsgberg et al., 2004; 22.7%, Mañas et al., 2005; 48.6%, Saeed et al., 2006; 7.3%, Morgan et al., 2008). As already mentioned, in Italy its presence in foxes has been reported in several regions and the parasite was known to be endemic in wildlife in the two areas sampled in this study, Tuscany – site A (Poli et al., 1984; Magi et al., 2009) and Liguria – site B (Macchioni et al., 2012). Interestingly, the presence of *A. vasorum* in these areas matches the prediction of the potential parasite diffusion hypothesized by Morgan et al. (2009): site B and the northern part of site A were identified to be highly suitable for parasite transmission. The higher presence of *A. vasorum* in foxes can be connected to the fact that canine angiostrongylosis appears as an epiphenomenon of the cycle “gasteropoda-fox” (Bolt et al., 1994), suggesting that *A. vasorum* is a common parasite among wild canids, from which it is transmitted only occasionally to the dog population (Eckert and Lämmler, 1972). High fox population densities in areas of human settlements are likely to

Table 2

Dogs from Italy ($n=612$) tested for presence of circulating *Angiostrongylus vasorum* antigen and for the presence of specific antibodies against *A. vasorum*. n.a.: not available. Percentages in brackets represent the overall prevalence values of each parasite in the respective study areas.

Diagnosed parasitic infections ^a	Site A (Tuscany) $N=265$				Site B (Liguria) $N=347$			
	Dogs N (%)	<i>A. vasorum</i> serology: positive antigen detection (N)	<i>A. vasorum</i> serology: positive antibody detection (N)	Seronegative in both tests (N)	Dogs N (%)	<i>A. vasorum</i> serology: positive antigen detection (N)	<i>A. vasorum</i> serology: positive antibody detection (N)	Seronegative in both tests (N)
<i>Leishmania infantum</i> (ELISA)	31 (12%)	1	1	30	66 (19%)	1 ^b	4 ^c	61
<i>Dirofilaria immitis</i> (DiroCHECK [®])	34 (13%)	1	1	33	6 (1.7%)	1 ^b	1 ^c	4
<i>Dirofilaria immitis</i> (Knott's test)	27 (10%)	0	0	27	2 (0.6%)	0	1 ^c	1
<i>Dirofilaria repens</i>	26 (10%)	0	1	25	4 (1.1%)	1	0	3
<i>Acantocheilonema reconditum</i>	4 (1.5%)	0	0	4	23 (6.6%)	1 ^d	1 ^d	22
<i>Crenosoma vulpis</i>	n.a.	n.a.	n.a.	n.a.	1 (0.3%)	0	0	1
<i>Capillaria aerophila</i>	n.a.	n.a.	n.a.	n.a.	35 (11%)	2 ^{e,f}	2 ^e	32
<i>Capillaria boehmi</i>	n.a.	n.a.	n.a.	n.a.	3 (0.8%)	0	0	3
Hookworms	n.a.	n.a.	n.a.	n.a.	37 (10%)	1 ^f	2	34
<i>Toxocara canis</i>	n.a.	n.a.	n.a.	n.a.	68 (20%)	1 ^f	0	67
<i>Toxascaris leonina</i>	n.a.	n.a.	n.a.	n.a.	5 (1.4%)	0	0	5
<i>Trichuris vulpis</i>	n.a.	n.a.	n.a.	n.a.	39 (11%)	2 ^d	1 ^d	37

^a For methods see Section 2.

^b One dog was seropositive for circulating *A. vasorum* antigen, for *Dirofilaria immitis* antigen and had antibodies against *L. infantum*.

^c Two dogs positive for *D. immitis* (one for antigen and one for microfilariae) and with antibodies against *L. infantum* also had antibodies against *A. vasorum*.

^d In a dog positive for *Acantocheilonema reconditum* and *Trichuris vulpis* and seropositive in both *A. vasorum* ELISAs, the co-infection with *A. vasorum* was confirmed by Baermann analysis.

^e In a dog positive for *Capillaria aerophila* seropositive in both *A. vasorum* ELISAs, the co-infection with *A. vasorum* was confirmed by Baermann analysis.

^f One dog positive for hookworms and *Toxocara canis* was also positive for *A. vasorum* circulating antigens.

increase opportunities for *A. vasorum* transmission from foxes to dogs, as described for *Echinococcus multilocularis* (Deplazes et al., 2004). In a further previously performed study the prevalence appears to be higher in hunting dog breeds (Conboy, 2004).

The here adopted sandwich ELISAs proved to be reliable tools for field studies and clinical diagnosis. The presence of dogs seropositive only to one test may be due to different reasons: for example, the presence of dogs positive only for the antibody detection test may be due to an early infection stage where no antigen and/or L1 are being produced yet, or to undeclared treatment that sterilized or eliminated the adult parasites. In fact, while dogs become positive 35–77 days post infection (p.i.) in the antigen test and again negative within 16–34 days after efficacious treatment (Schnyder et al., 2011a), antibodies can be detected already after 21 days p.i. and persist up to 9 weeks after treatment. Also, cross reactions and false positive results have to be considered, even if the specificity has been shown to be as high as 94.0% for the antigen test (Schnyder et al., 2011a) and 98.8% for the antibody test (Schucan et al., 2012). This has been confirmed with the results of the ELISAs in dogs with other parasitic infections of this study: cross reactions are very limited or absent. In fact, a high number of the examined animals which were negative for all performed test for *A. vasorum* had other proven parasitic infections. Therefore, the few positive reactions observed with sera of dogs with other proven helminthic infections are more likely caused by occult *A. vasorum* coinfections, than by cross-reactions. All parasitic species found in the dogs are widely distributed in Europe and would therefore represent a diagnostic challenge for *A. vasorum* serology. *D. immitis* is currently still limited to southern Europe, while *D. repens* has been recently shown to be spreading from eastern and southern to central Europe (Genchi et al., 2011). In particular, *A. vasorum* and *D. immitis* share common epitopes and can induce strong cross-reactions (Schnyder and Deplazes, 2012), as seen using crude adult somatic antigen for antibody detection. The mAbs selected for the sandwich ELISAs were evaluated with E/S antigen of both *A. vasorum* and *D. immitis* and only clones that did not react with *D. immitis* adult E/S antigens were selected (Schnyder et al., 2011a).

Also leishmaniosis, associated with a polyclonal B-cell activation and hypergammaglobulinaemia, a very frequent canine infection in Southern Europe currently spreading northwards (Maroli et al., 2008; Otranto et al., 2009), seems not to interfere with the results of the tests, as well as the presence of a wide range of cardiopulmonary (*C. vulpis*, *C. aerophila*, *C. boehmi*) and gastrointestinal nematodes (*T. canis*, *T. leonina*, hookworms, *T. vulpis*).

In conclusion, the detection of circulating antigens and specific antibodies against *A. vasorum* by ELISA represents a valid alternative not only for a reliable diagnosis and follow-up investigation after anthelmintic treatment in individuals, but also for population studies. Diagnostic testing with ELISA represents significant advantages: it enables to diagnose infection with *A. vasorum* before patency, it requires a single serum sample instead of repeated faecal samples and it has the potential for a rapid diagnostic test, particularly in the case of a large number of samples.

The serological approach may increase the knowledge concerning the epidemiological situation and therefore disease awareness of this potentially fatal parasite for dogs. An early diagnosis is essential to ensure anthelmintic treatment before the onset of pathological changes, which can be present before clinical signs, as previously shown (Schnyder et al., 2009, 2010). This is particularly true considering the confirmed establishment and potential expansion of *A. vasorum* in various European countries.

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