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Serological Detection of Circulating *Angiostrongylus vasorum* Antigen- and Parasite-Specific Antibodies in Dogs from Poland

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Abstract

Dogs infected with *Angiostrongylus vasorum*, a potentially lethal parasite living in the heart and pulmonary arteries, may present severe respiratory and neurological sings and coagulopathies. Its occurrence is increasingly reported from various European countries, but little is known about its presence in Poland. In this first large-scale survey, 3,345 sera from polish dogs attending veterinary clinics in different parts of Poland for various reasons were collected and tested by an ELISA for the detection of circulating antigen of *A. vasorum* and by a separate ELISA detecting specific antibodies. A total of 0.51 % (n = 17, 95% Confidence Intervals, CI: 0.30–0.81 %) of the animals were positive in both ELISAs, while 0.78 % (n = 26, CI: 0.51–1.14 %) of the tested dogs were antigen-positive only and 1.29 % (n = 43, CI: 0.93–1.73 %) were positive for specific antibodies only. Regions with antigen- and antibody-positive animals were overlapping and distributed over the whole area of the country, with approximately one

third of positives close to the Baltic Sea, and a limited number of cases close to the German border. These results confirm the occurrence of *A. vasorum* in dogs originating from different parts of Poland. *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.

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 potentially leading to the host's death if the infection is left untreated (Staebler et al. 2005; Koch and Willesen 2009). Definitive hosts of *A. vasorum* shed first-stage larvae (L1) in the faeces (Guilhon and Bressou1960; Guilhon 1963), and in the intermediate host (snails and slugs) they 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 (Bourdeau 1993; Guilhon and Cens 1969), Denmark (Bolt et al. 1992; Rosenlund et al. 1991), Ireland (Dodd 1973) and in Southeast England and Wales (Jacobs and Prole 1975; Simpson and Neal 1982). However, in the last two decades, the number of reports from previously considered non-endemic areas in Europe sharply increased, with further detection of *A. vasorum* in dogs from Italy (Della Santa et al. 2002; Di Cesare et al. 2011), Switzerland (Staebler et al. 2005), Greece (Papazahariadou et al. 2007), The Netherlands (van Doorn et al. 2009) and Hungary (Majoros et al. 2010), and in foxes from some further countries such as the Iberian Peninsula (Segovia et al. 2004; Manas et al. 2005) as well as in Croatia (Rajkovic-Janje et al. 2002). The epidemiology of the parasite in further eastern European countries is poorly known.

Infected dogs often present with severe respiratory symptoms but atypical clinical signs indicating coagulopathies and/or neurological dysfunctions (Mason 1989; Garosi et al. 2005; Gredal et al. 2011; Whitley et al. 2005; Wessmann et al. 2006) make the diagnosis of *A. vasorum* infections particularly challenging. Severe symptoms with frequently fatal outcome (Staebler et al. 2005; Traversa et al. 2008; Denk et al. 2009) may therefore occur, because animal owners often become aware only late during the infection, due to the chronic and subtle course of the pathological processes. Therefore, information about the epidemiological situation concerning the presence of *A. vasorum* is fundamental for both animal owners and practising veterinarians.

Currently, diagnosis of *A. vasorum* infections in dogs is mainly based on the detection of L1 in faecal samples by larval migration techniques. Patency is described to start after 38–57 days post infection (d.p.i.) (Bolt et al. 1994; Schnyder et al. 2010), when damage to the lung parenchyma is already present (Guilhon and Cens 1969; Neff 1971; Dennler et al. 2011). The sensitivity of copromicroscopic methods is furthermore limited in case of intermittent excretion of larvae (Oliveira-Junior et al. 2006; Taubert et al. 2008), low parasite load, and during prepatency. In addition, morphological differentiation of the larvae from other lungworm larvae such as *Crenosoma vulpis* and *Filaroides* spp. requires expertise (McGarry and Morgan 2009). Alternative diagnostic tools such as molecular methods (Jefferies et al. 2009; Al-Sabi et al. 2010) or highly specific serological tests have been developed (Schnyder et al. 2011b; Schucan et al. 2012) to overcome these problems. ELISAs represent a valid and affordable tool for diagnosis in both, individual and population studies, and have been very recently validated in a field study (Guardone et al. 2013).

The objective of this research was to perform a seroepidemiological survey for the detection of circulating antigens of *A. vasorum* and of specific antibodies in sera of dogs originating from Poland in order to increase cognition and, accordingly, disease awareness about the potential occurrence of *A. vasorum* in dogs of this country.

Materials and methods

Sera of 3,345 dogs from Poland were collected from dogs attending veterinary clinics for different reasons. All samples were submitted to a private veterinary diagnostic laboratory (IDEXX Vet Med Lab, Ludwigsburg, Germany) and were tested for a number of different canine vector-borne diseases. Subsamples were sent to the Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Switzerland, and were analysed for the presence of circulating *A. vasorum* antigens using monoclonal and polyclonal antibodies in a sandwich ELISA with a sensitivity of 95.7 % and a specificity of 94.0 %, as previously described (Schnyder et al. 2011b). Additionally, a sandwich ELISA (sensitivity 81.0 %, specificity 98.8 %) using *A. vasorum* adult somatic antigen purified by monoclonal antibodies (mAb Av 5/5) was used for specific antibody detection (Schucan et al. 2012). Test thresholds were regionally determined with 300 randomly selected samples based on the mean value of optical density ($A_{405\ nm}$) plus 3 standard deviations. 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 collected data were analysed by a geographic information system (GIS) using the programme RegioGraph 10 (GfK GeoMarketing, Bruchsal, Germany) to visualise the regional distribution of collected and analysed serum samples and *A. vasorum* antigen- and/or antibody-positive samples. Based on the three digits as points of reference, the locations of positive samples were displayed on maps with administrative and postcode boundaries.

Excel 2007 for Windows (Microsoft Corporation, Redmond, USA) was used to calculate the prevalence values and the 95 % confidence intervals (CI) of prevalence values.

Results

The seropositivity of all tested samples is resumed in Table 1. A total of 0.51 % (n = 17, Confidence Intervals, CI: 0.30–0.81 %) of the animals were positive in both ELISAs, while 0.78 % of the tested dogs were only antigen-positive and 1.29 % were positive for specific antibodies only. The locations of positive sera are shown in Figs. 1–3. Regions with antigen- and antibody-positive animals were overlapping and distributed over the whole area of the country, with approximately one third of positives close to the Baltic Sea and a limited number of cases close to the German border.

Table 1 Serological results of 3,345 dogs serum samples from Poland tested for the presence of circulating antigens of *A. vasorum* (Schnyder et al. 2011b) and of specific antibodies against *A. vasorum* (Schucan et al. 2012)

	Positive samples (n)	%	95 % Confidence Intervals
Antibody-positive	60	1.79	1.37–2.30
Antibody-positive only	43	1.29	0.93–1.73
Antigen-positive	43	1.29	0.93–1.73
Antigen-positive only	26	0.78	0.51–1.14
Antibody- and antigen-positive	17	0.51	0.30–0.81

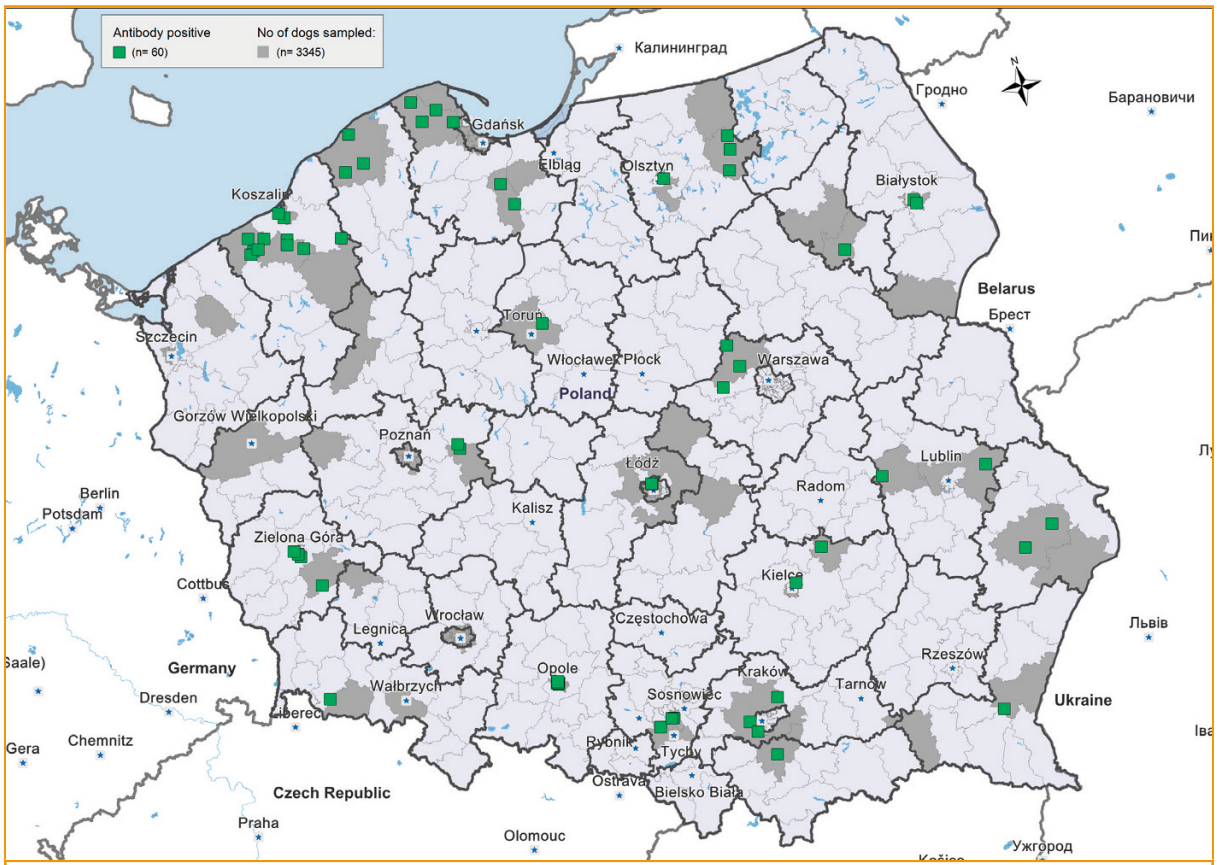

Fig. 1 Occurrence of *Angiostrongylus vasorum* in 3,345 dogs from Poland detected by ELISA. Dark grey areas represent the origin of the tested dog sera. Dogs with specific antibodies against *A. vasorum* (Schucan et al. 2012)

Discussion

To the authors knowledge, this is the first report of *A. vasorum* in Poland. The use of ELISA techniques allowed a large seroepidemiological survey, showing that this parasite is present. Both tests had been previously validated in a field study: ELISAs were shown to confirm samples from Baermann-positive dogs, and additionally detected non-patent infections (Guardone et al. 2013); therefore, they are highly sensitive methods. However, there is a different denotation for the two adopted ELISAs: while specific antibody detection is useful for the determination of parasite exposure, antigen detection represents an actual infection. This explains the higher number of positive dogs for antibody detection compared with antigen detection. The presence of dogs seropositive only to one test may be due to different reasons: for example, the presence of dogs positive only for antibody detection may be due to an early infection stage where no circulating antigens are being produced yet. 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. 2011b), antibodies can be detected already after 21 days p.i. and persist up to 9 weeks after treatment (Schucan et al. 2012). In naturally infected and untreated dogs, adults may persist up to five years (Guilhon and Cens 1969), during which it can be assumed that dogs would also persist positive for antigen and antibody detection. Also cross-reactions and false positive results have to be considered, even if the specificity has been

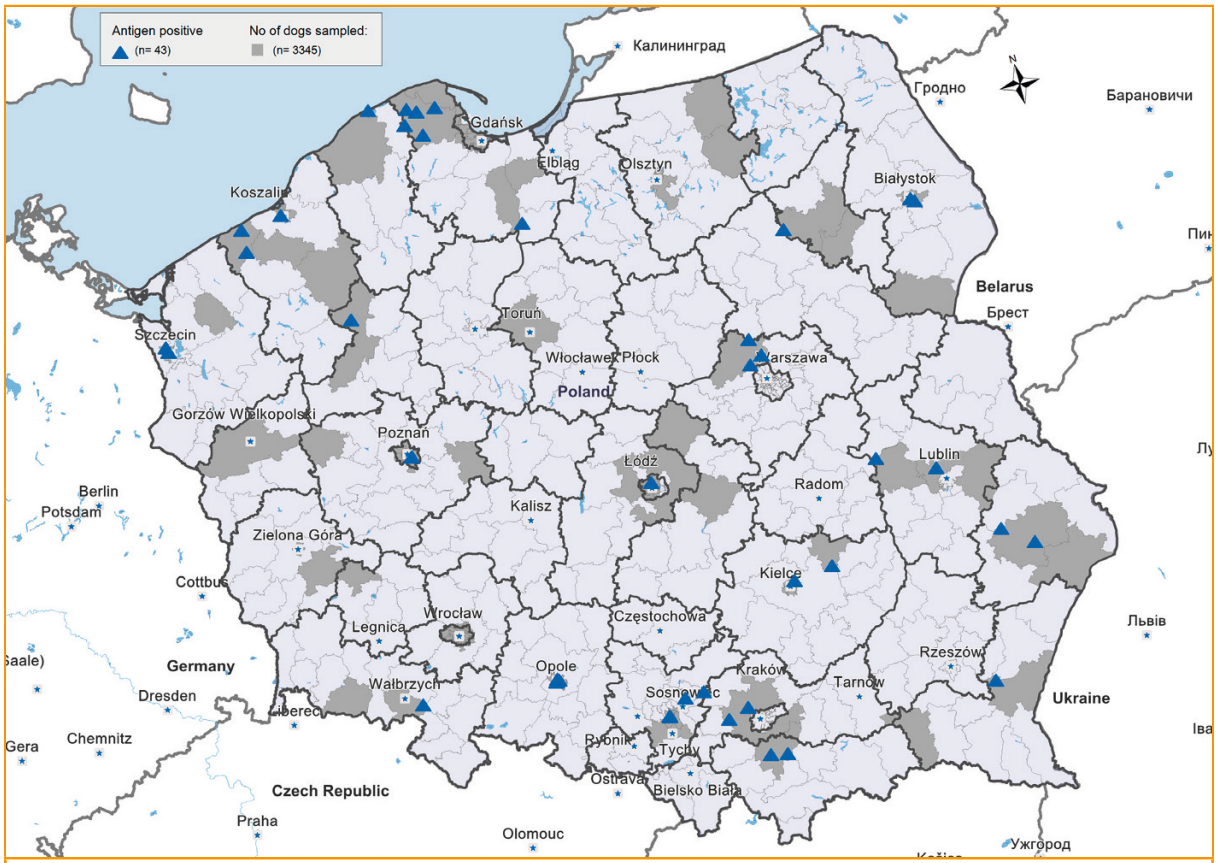

Fig. 2 Dogs positive for circulating antigen of *Angiostrongylus vasorum* (Schnyder et al. 2011b)

shown to be as high as 94.0 % for the antigen test (Schnyder et al. 2011b) and 98.8 % for the antibody test (Schucan et al. 2012). Thus, using them in combination can enhance the performance of the serological tests. Nevertheless, the confirmation of the occurrence of *A. vasorum* in Poland through clinically confirmed positive cases with larval excretion or at necropsy would be advisable.

Germany confines on its eastern border with Poland, and in a previous study, 1.2 % of 958 examined dogs tested positive by means of Baermann technique during 2003–2007 (Taubert et al. 2009). However, infected animals in Germany mainly originated from southern and western parts of the country, although regional differences were not statistically significant. In another study performed with preselected dogs showing cardiopulmonary signs, 7.4 % of the dogs were positive for larval excretion (Barutzki and Schaper 2009), also concentrated in western and southern parts of the country. When testing sera submitted to private veterinary diagnostic laboratories for various medical reasons, seroprevalences were clearly lower than prevalences obtained through testing faecal samples from clinically suspect dogs: prevalences obtained with the same ELISAs as in the present study performed with 4,000 sera from German dogs confirmed the accumulation of positive cases in southwestern parts of the country, with a total of 0.3 % being positive for antibody and antigen detection (Schnyder et al. 2011a). Dogs positive for both tests, in which circulating antigens as well as specific antibodies against *A. vasorum* are detected, can be assumed to harbour an

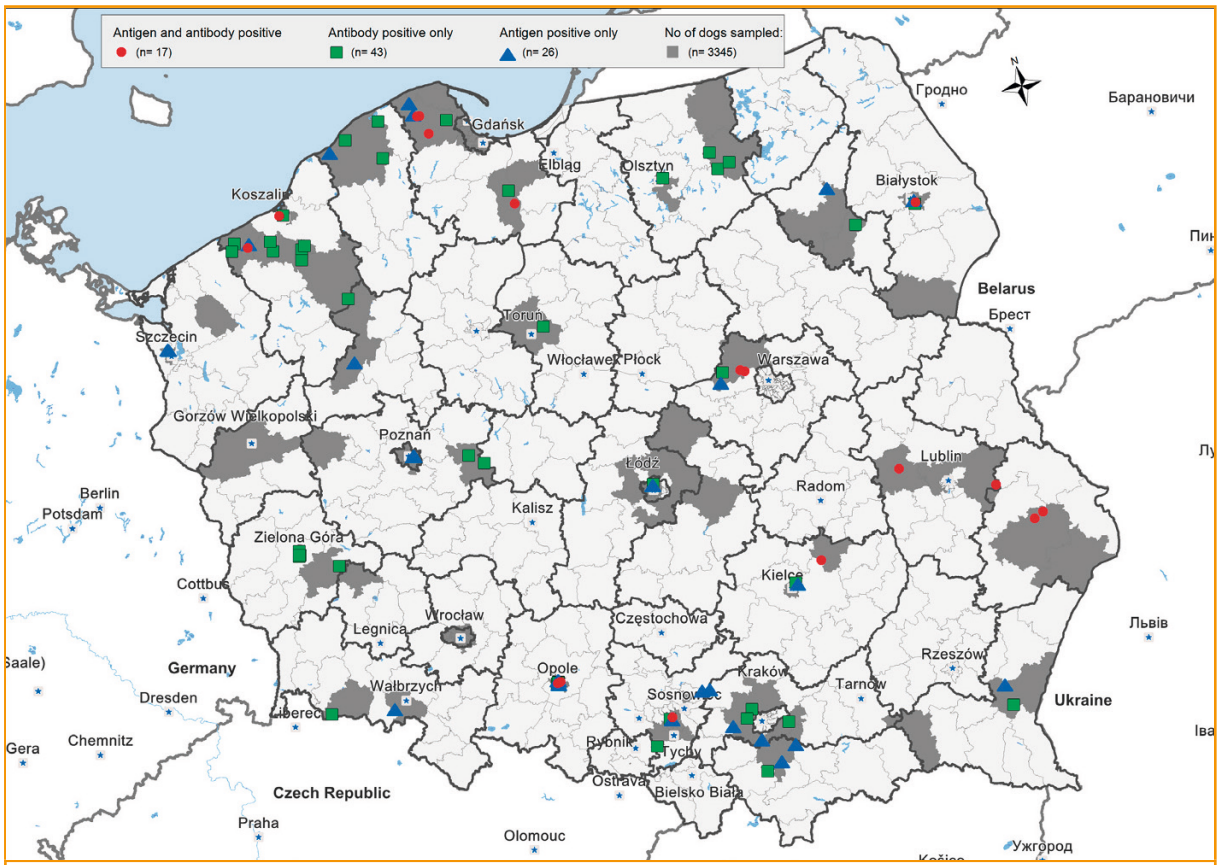

Fig. 3 Combined testing for *Angiostrongylus vasorum* antigen and antibody defection

active *A. vasorum* infection inducing an immunological reaction with production of antibodies. Higher seroprevalences were obtained from kennel and privately owned dogs from areas of central and northern Italy known to be endemic for angiostrongylosis in foxes (0.8–0.9 % of the dogs were positive for both, antigen and antibody detection, Guardone et al. 2013). Seroprevalences were also higher in a highly endemic area of England, with 0.97 % of the dogs being positive in both ELISAs. Therefore, with 0.51 % of dogs being positive in both ELISAs, seroprevalence in Poland is situated in-between seroprevalences obtained in Germany and highly endemic areas. These data suggest that dogs suffering from canine angiostrongylosis must be present in Poland. The low prevalences may explain why, to our knowledge, no cases are reported as yet. Furthermore, due to the high variability of clinical signs and the often chronic course of the infection, clinical diagnosis is challenging and may be missed, in particular if the infection has never been previously detected by the involved clinicians. The localisation of cases also suggests that *A. vasorum* is not necessarily spreading from Germany, since only a restricted number of cases have been detected in the adjacent areas of Poland and Germany (Barutzki and Schaper 2009). It can be assumed that *A. vasorum* has been present focally since long in Poland. Also movement of dogs from endemic to non-endemic areas have to be considered. Adequate intermediate hosts were expected in large areas of Europe eastern from Germany and Italy, and, based on climatic modelling, potentially suitable areas for *A. vasorum* were hypothesised in Poland, in addition to the Baltic States, Slovakia, Czech Republic, Hungary, Slovenia,

Croatia, Serbia, Bulgaria, parts of Romania and Ucraina (Morgan et al. 2009). For instance, definitive hosts infected with *A. vasorum* have already been confirmed in Hungary (Majoros et al. 2010), Croatia (Rajkovic-Janje et al. 2002) and Greece (Papazahariadou et al. 2007).

During the last decades, reports of lung worm-affected animals have increased and different reasons for the apparent spread of lung worms have been discussed, in particular for the metastrongyloids *Aelurostrongylus abstrusus, A. vasorum* and *Crenosoma vulpis,* but also for *Dirofilaria immitis* (Filariidae) and *Eucoleus aerophilus* (Trichuridae). Regional climate changes in vector epidemiology and movements in animal populations were taken into account (Morgan et al. 2009; Traversa et al. 2010). The evidence of higher prevalences in foxes compared to dogs (Koch and Willesen 2009) accounts for the fact that wildlife, in absence of obvious geographic barriers, plays an important role in expansion and establishment of *A. vasorum*. An increasing fox population prone to human settlements (Deplazes et al. 2004) generally also increases the infectious threat for dogs.

It is therefore of essential importance to increase disease awareness and cognition of the occurrence of *A. vasorum* in previously unreported countries in order to prevent fatal cases of canine angiostrongylosis. As previously shown (Guardone et al. 2013), the detection of circulating antigens and specific antibodies against *A. vasorum* by ELISA represents a useful and reliable tool for both, the population studies and individual clinical diagnosis. Diagnostic testing with ELISAs enables detection of infection with *A. vasorum* before patency, requires a single serum sample instead of repeated faecal samples, and has the potential for a rapid diagnostic test, particularly in the case of a large number of samples. An early diagnosis is essential to ensure anthelminthic treatment before the onset of pathological changes, which can be present before clinical signs, as previously shown (Schnyder et al. 2009, 2010). Adequate anthelmintic treatments (Conboy 2004; Willesen et al. 2007; Schnyder et al. 2009) allow good prognosis for *A. vasorum*-affected dogs. Thus, the monthly use of macrocyclic lactones (Schnyder et al. 2009) or routine screening of dogs for *A. vasorum* infection (Verzberger-Epshtein et al. 2008; Schnyder et al. 2011b) for prevention of clinical angiostrongylosis are already recommended in known endemic areas.

Ethical standards

All institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of interest

N Pantchev was employed by Idexx Germany, A Szwedko was employed by Eskulap/Idexx Poland. R Schaper and D Kowalska were employed by Bayer Animal Health in Germany and Poland. The study was supported by Idexx Poland and Germany and Bayer Animal Health Poland and Germany.

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