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Epidemiological analysis of *Trichinella spiralis* infections of foxes in Brandenburg, Germany

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SUMMARY

In a cross-sectional study conducted between March 1993 and February 1995, 7103 indiscriminately collected foxes were examined for *Trichinella* larvae. A total of 3295 serum samples were serologically investigated with an ELISA based on excretory-secretory antigen. The proportion of serologically positive animals ranged between 3·3% and 17·6% in random samples from individual counties or towns and resulted in an estimated overall prevalence of 7·7% (95% CI: 6·9–8·7%). *Trichinella* larvae were detected in the muscles of five foxes, corresponding to an estimated prevalence of 0·07% in the total sample (95% CI: 0·02–0·16%). The analysis of DNA of the *Trichinella* isolates by random amplification of polymorphic DNA (RAPD) lead to the identification of the isolates as *Trichinella spiralis*. The differences between serological and parasitological findings are discussed.

INTRODUCTION

Trichinella monitoring as part of the meat inspection protocols lead to the almost complete disappearance of autochthonously acquired human infections in many European countries [1, 2]. Industrial pig farming has contributed to the interruption of the domestic cycle, although trichinellosis still occurs in some rural areas in association with traditional swine-rearing practices [3, 4]. By contrast, the silvatic cycle persists even in countries which are considered *Trichinella*-free with respect to the domesticated pig population. Among several other susceptible wildlife animals such as wild boar, bears, racoons, badgers, ferrets, wolves, rodents as well as stray dogs and cats, the fox plays a major role as a host and reservoir for the parasite [1, 4–7]. As foxes are adopting an increasingly synanthropic behaviour, vulpine trichinellosis may return as a threat to domesticated animals, in particular pigs, and eventually also to humans. Therefore, the infection needs to be monitored in foxes.

Previous studies on *Trichinella* spp. infections conducted in eastern Germany failed to detect the parasite in foxes [8–12]. In 1994, a first report has appeared on vulpine trichinellosis in this region [13]. The limited sensitivity of the available techniques of direct parasite detection (trichinoscopy, digestion method) and the numbers of animals investigated could have impaired the results of previous studies and may have lead to an underestimation of the true prevalence [14].

We chose the Federal State of Brandenburg as the study area because of its high population densities of various wildlife animals including foxes and examined a large regionalised random sample of foxes for both *Trichinella* larvae and for antibodies directed against

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larval excretory–secretory (E–S) antigens of the parasite.

MATERIALS AND METHODS

Study area and sampling

Between March 1993 and February 1995, samples from 7103 foxes shot in Federal State of Brandenburg (Germany) were investigated. The study area is situated between 53° 23′–51° 22′ N and 11° 45′–14° 42′ E. The age of each fox was determined, whenever possible, as juvenile (born in the current year) or adult (born in a previous year) according to Wagenknecht [15]. Sampling strategies were planned according to Cannon and Roe [16], assuming infinite sizes of the fox populations per geographical unit (county, 'Landkreis', or town, 'kreisfreie Stadt') and allowing the detection of a 1% prevalence of *Trichinella*-infections and 2% seroprevalence with 99% statistical confidence. Specimens from the diaphragm and forelegs were taken and blood or transudate recovered from the heart, the pleural or peritoneal cavity.

Experimental infections

Two farm foxes were infected by oral application of 150 *T. spiralis* muscle larvae per kg body weight and one fox with a total of 5000 larvae. Another four animals remained uninfected and served as negative controls. All animals were kept individually in kennels and received canned dog feed and water ad libitum. All animals were serologically monitored by serial bleedings.

Sera

Sera/transudate samples of 3295 foxes shot in the study area were tested for antibodies to larval E–S antigens of *T. spiralis*. *Trichinella*-positive sera ($n = 47$) from experimentally infected foxes and from parasitologically as well as serologically (immunoblot on larval E–S antigen) confirmed vulpine trichinellosis cases were employed in the evaluation of the ELISA used in the serological study. Sera ($n = 40$) from uninfected farm foxes and a pool of 40 *Trichinella*-negative farm foxes served as negative reference samples. Sera from parasitologically and serologically (immunoblot) *Trichinella*-negative animals with proven ancylostomatid ($n = 10$), *Toxocara canis*/*Toxascaris leonina* ($n = 37$), *Mesocestoides* spp. ($n = 23$), *Echinococcus multilocularis* ($n = 6$), taeniid ($n = 10$) or trematode ($n = 5$) monoinfections were included as specificity controls.

Serology

Pork containing *T. spiralis* larvae was kindly supplied by Dr W. P. Voigt, Federal Institute for Consumer Protection and Veterinary Medicine, Berlin. E–S antigen was prepared from larvae recovered by hydrochloride-pepsin digestion and cultured according to Gamble and colleagues [17, 18] with the following modifications: larvae were kept in Dulbecco's modified Eagle Medium (BioWhittaker, Heidelberg, Germany) supplemented with 10 mM Hepes (Serva, Heidelberg, Germany), 2 mM L-glutamine (Biochrom, Berlin, Germany), 50 IU ml^{-1} penicillin (BioWhittaker) and 50 μg ml^{-1} streptomycin (BioWhittaker) for 20 h at 37 °C and 10% CO_2 in the air. Antigen preparations were stored at −20 °C. Microtitre plates (NUNC polysorb C bottom, Roskilde, Denmark) were coated with 0·4 μg E–S antigen per well in a volume of 100 μl at 4 °C overnight and blocked with 2% (w/v) fish gelatine (Serva) in 100 mM sodium bicarbonate buffer, pH 9·6, overnight. The plates were washed three times with phosphate-buffered saline (PBS), pH 7·2 containing 0·05% (v/v) Tween 20 (PBS-T). Fox sera diluted 1/100 in PBS-T supplemented with 2% (w/v) fish gelatine were added to the plate and incubated for 2 h at 37 °C. Each serum was tested in quadruplicate. After three washes, horse radish peroxidase-labelled anti-dog-IgG H + L chains (Dianova GmbH, Hamburg, Germany) was added at a dilution of 1/10000 for 1 h at 37 °C. After five further washes in PBS-T, 0·04% (w/v) 3,3′,5′-tetramethylbenzidine (TMB) dissolved in dimethylsulphoxide was diluted in a buffer consisting of 200 mM sodium acetate and 200 mM citric acid, supplemented with 0·12 μl ml^{-1} hydrogen peroxide (10%) was added as substrate (100 μl). After 10 min, the reaction was stopped by the addition of 50 μl 4 N sulphuric acid and optical densities (OD) at 450 nm determined in a microplate reader (SLT Spectra, SLT, Crailsheim, Germany). Antibody levels are expressed as ELISA indices ([sample OD average − OD average of negative controls]/[OD average of positive controls − OD average of negative controls]). A result was considered valid if the ratio of the mean OD of the positive and negative control on the microtitre plate was $\geqslant 10$ and if the mean OD of the positive control on the plate ranged between 0·95 and 2·20. Sensitivity, specificity

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and optimized cut-off of the ELISA were determined with sera from parasitologically and serologically (tested by immunoblotting on larval E–S antigen) *T. spiralis*-negative or -positive foxes. Cut off-optimization was performed using a two-graph receiver-operating-characteristic (TG-ROC) analysis [19].

Detection of parasites

Trichinella larvae were detected using 10 g muscle tissue from each fox (5 g diaphragm, 5 g foreleg muscles) by the hydrochloride-pepsin digestion method according to the standard procedure described in Annex 1 of Directive 77/96/EEC of 21 December 1976 in the version of Directive 89/321/EEC. If samples from diaphragm or forelegs were not available, tail [M. sacrocaudalis], tongue, laryngeal muscles or M. masseter were used.

Random amplification of polymorphic DNA (RAPD)

DNA isolation from individual *Trichinella* isolates and RAPD were performed essentially as described [20]. Amplification were performed with the primers M-1 (5′-AGGTCACTGA-3′) and M-are (5′-ATCT-GGCAAG-3′) in a Techne PHC-3 thermal cycler (45 cycles at 94 °C, 1 min; 36 °C, 1 min; 72 °C, 1 min). The *Trichinella* reference isolates included in the RAPD analysis were *T. spiralis* ISS116, *T. nativa* ISS10, *T. britovi* ISS89, *T. nelsoni* ISS29, T5 ISS35, T6 ISS34 and T8 ISS149.

Computing

A program written in CLIPPER (Computer Associates International Inc, New York, USA) was used for the documentation of the data in a dBASE file (5.0 for Windows, Borland International Inc., Scotts Valley, CA, USA). Epi-Info 6.03 (Centers for Disease Control and Prevention, Atlanta, GA, USA, and World Health Organisation, Geneva, Switzerland), STATISTICA for Windows (StatSoft Inc., Tulsa, OK, USA), Harvard Graphics 4.0 (Software Publishing Corporation, Santa Clara, CA, USA), RegioGraph 2.0 (MACON GmbH, Waghäusel, Germany) and WinEpiscope 1a (Veterinary Faculty, University of Zaragoza, Spain, and Agricultural University, Wageningen, The Netherlands) were used for statistical analysis, geographical and graphical documentation of the data. 95% confidence intervals (CI) were calculated according to Willer [22]. Prevalence differences were compared by the χ^2 and/or Fisher's exact test as appropriate.

RESULTS

ELISA evaluation

To evaluate an ELISA which was originally designed to test human and porcine sera for its applicability with fox sera, the antibody response of experimentally infected foxes to larval *T. spiralis* antigens was determined (Fig. 1). A massive increase of antibody levels was recorded in all infected animals 3–7 weeks post-infection. The humoral immune responses of these animals persisted at high levels throughout the observation period. All defined *Trichinella*-positive ($n = 47$) and -negative sera ($n = 40$) reacted in the ELISA as expected from their infection status. The results of ELISA and immunoblot showed good agreement ($\kappa = 0{\cdot}977$).

Serological data were obtained for 3 out of 5 animals with proven *Trichinella*-infections as detected by the digestion technique (see later). These animals showed a clear antibody response (indices: 0·590; 0·800; 0·470) to larval E–S antigen of *T. spiralis*. Serum samples of the remaining two foxes with proven trichinellosis were not available.

To check the specificity of the ELISA, sera from *Trichinella*-negative foxes with ancylostomatid ($n = 10$), *Toxocara canis*/*Toxascaris leonina* ($n = 37$), *Mesocestoides* spp. ($n = 23$), *Echinococcus multilocularis* ($n = 6$), taeniid ($n = 10$) or trematode ($n = 5$) mono-infections were examined in the ELISA. The indices of the sera ranged between −0.040 and 0.160. Sera of uninfected foxes kept under experimental conditions ($n = 40$) and a pool of 40 *Trichinella*-uninfected animals obtained from a farm did not react with larval E–S antigen.

A TG-ROC analysis was performed with 40 sera from parasitologically (digestion method) and serologically (immunoblotting) *T. spiralis*-negative foxes and 47 sera obtained from foxes with parasitologically and serologically (immunoblot) proven *Trichinella*-infections. With this panel of sera, the test had a combined optimized specificity and sensitivity (theta 0) of 1·000 when an index of 0·298 was used as the cut-off. Graphic analysis of the frequency distribution of all investigated sera confirmed the validity of this cut-off for the entire collection of sera tested in the ELISA (graph not shown).

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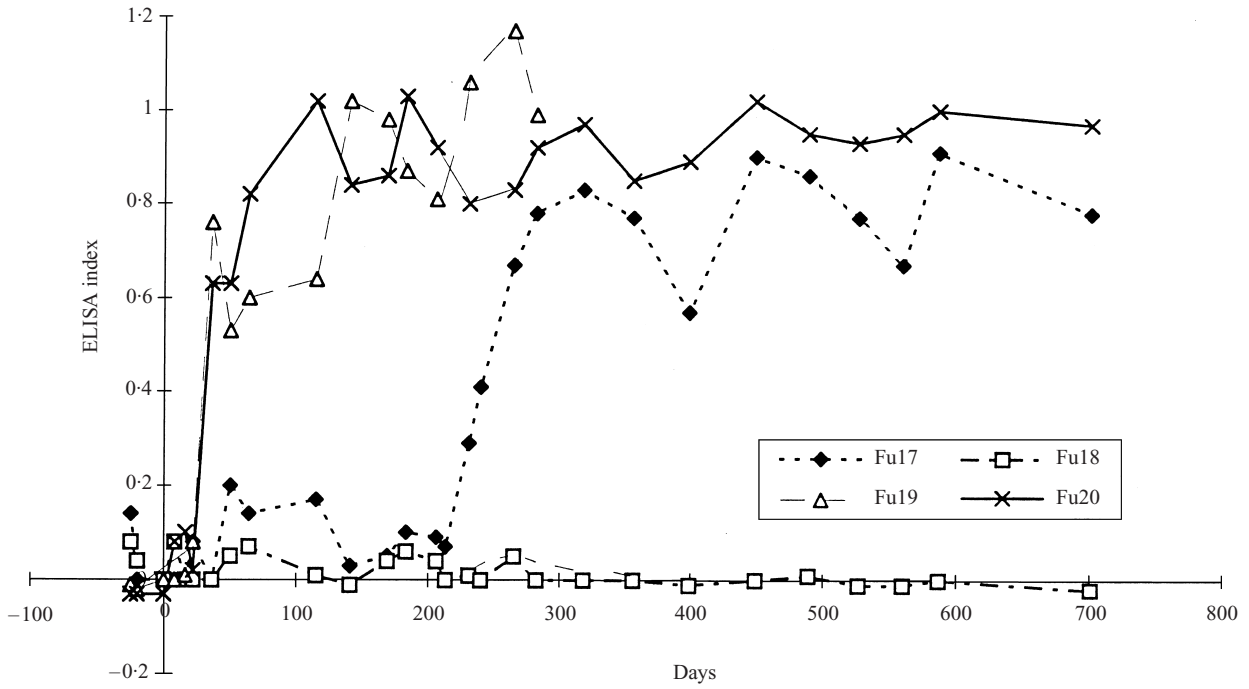

Fig. 1. Detection of antibodies to *T. spiralis* in experimentally infected foxes. Foxes were infected by oral uptake of 150 *T. spiralis* muscle larvae per kg body weight on day 0 (Fu19, Fu20) or with 5000 muscle larvae on day 206 (Fu17), or were left uninfected (Fu18). All animals were serologically monitored using an ELISA with larval *T. spiralis* E–S antigen.

Seroprevalence

Two hundred and fifty-five of 3295 sera tested contained antibodies reactive with larval *T. spiralis* E–S antigen. This corresponds to an overall estimated prevalence of 7·7% (95% CI: 6·9–8·7%). Calculated on the basis of individual counties or towns, between 3·3 and 17·6% of the serum samples contained antibodies reacting with larval E–S antigen of *T. spiralis* (Fig. 2). When the data set was split to perform multiple pairwise comparisons between a counties, the type I error α should be corrected to α': $\alpha = \alpha/J$ with $J = (a(a-1)/2)$ [23]. With this correction, significant differences between counties or towns could not be detected. Without the correction, a few pairwise comparisons (Prignitz and Oberhavel, Dahme-Spreewald, Elbe-Elster, Oberspreewald; Oberspreewald and Uckermark, Barnim and Cottbus) were significant ($0{\cdot}01 < P < 0{\cdot}05$) by the χ^2 or Fisher's exact test.

The sex of 2466 serologically tested foxes was known: 1417 (57·5%) animals were male and 1049 (42·5%) were female (Fig. 3). When males and females in this fraction of the tested sample were analysed with respect to the detection of antibodies directed against *T. spiralis*, no statistically significant differences became evident between the genders (Fig. 3). The age (juvenile/adult) was determined for 2137 animals of the serologically tested sample: 342 animals (16·0%) were classified as juvenile and 1795 (84·0%) as adult. For 1769 foxes, both sex and age were known. The seroprevalence was higher in adult male foxes (7·4%) as compared with juvenile male animals (2·3%), but this association was weak [uncorrected $P = 0{\cdot}0323$; Mantel–Haenszel corrected $P = 0{\cdot}0324$; Yates-corrected $P = 0{\cdot}510$; Odds ratio 3·35 ($1{\cdot}07 <$ OR $< 16{\cdot}91$); Relative risk 3·18 ($1{\cdot}01 <$ RR $< 9{\cdot}96$); Fig. 3]. No other tested strata revealed statistically significant differences.

Direct parasite detection

Trichinella larvae were found in 5 out of 7103 examined red foxes (Fig. 2). Thus, the estimated prevalence of *Trichinella*-infections of foxes in Brandenburg as detected by isolation of muscle larvae amounted to 0·07% (95% CI: 0·02–0·16%). All foxes which were found infected with *Trichinella* larvae were shot in the counties Barnim, Spree-Neiße and Uckermark in the East of Brandenburg. When the results obtained in counties where infected foxes were found were compared to those of the counties and towns where no infected were detected by the digestion

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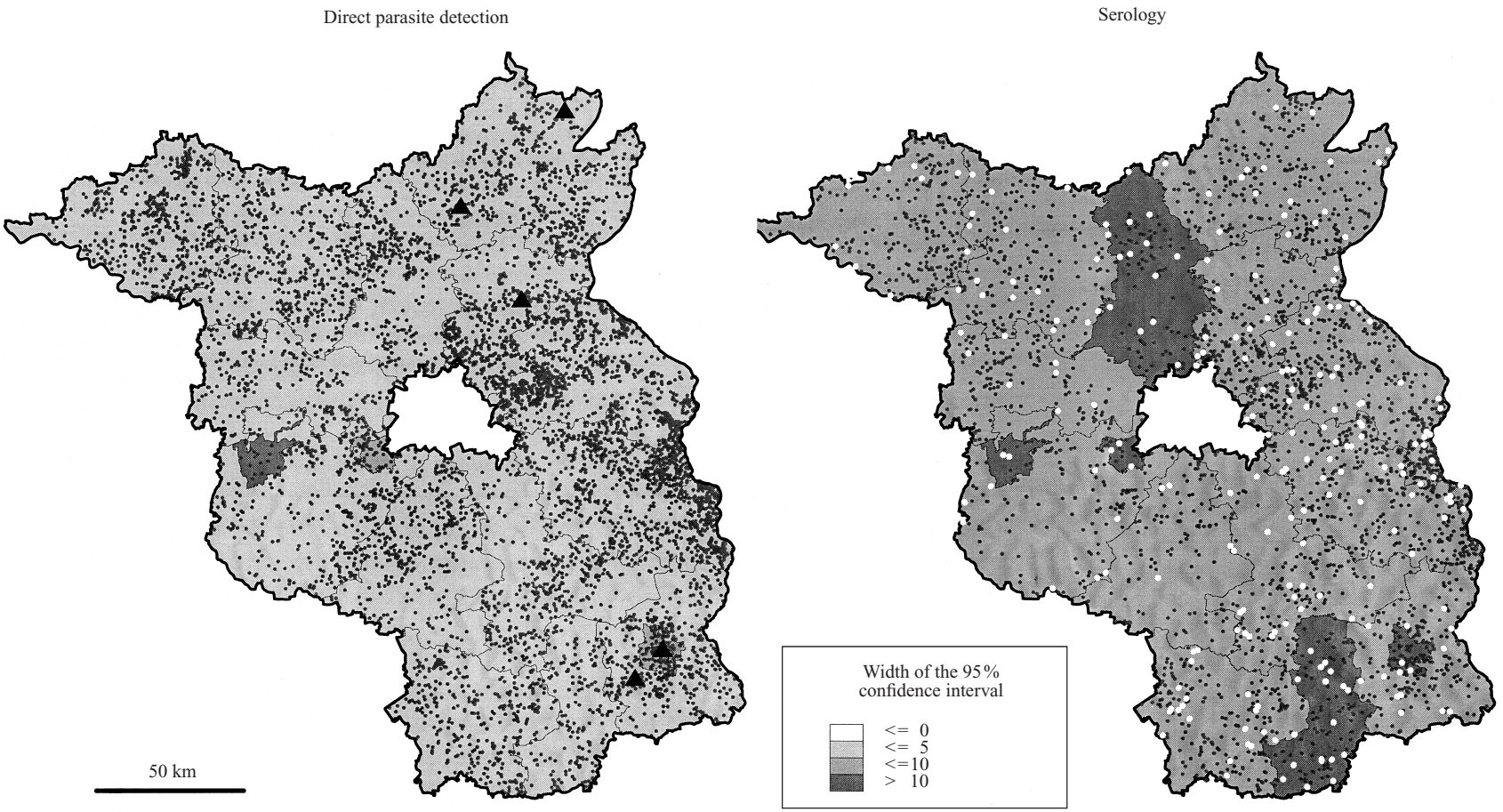

Fig. 2. Spatial distribution of *Trichinella*-infected foxes. The places of origin of all examined foxes were plotted on maps of the study area. Each triangle represents a *Trichinella*-positive fox (direct parasite detection, left map), white circles (serology, right map) stand for individual seropositive foxes and black dots represent animals tested negative. The background colour of the maps shows the width of the 95% CI for the prevalence estimation at the county or town level.

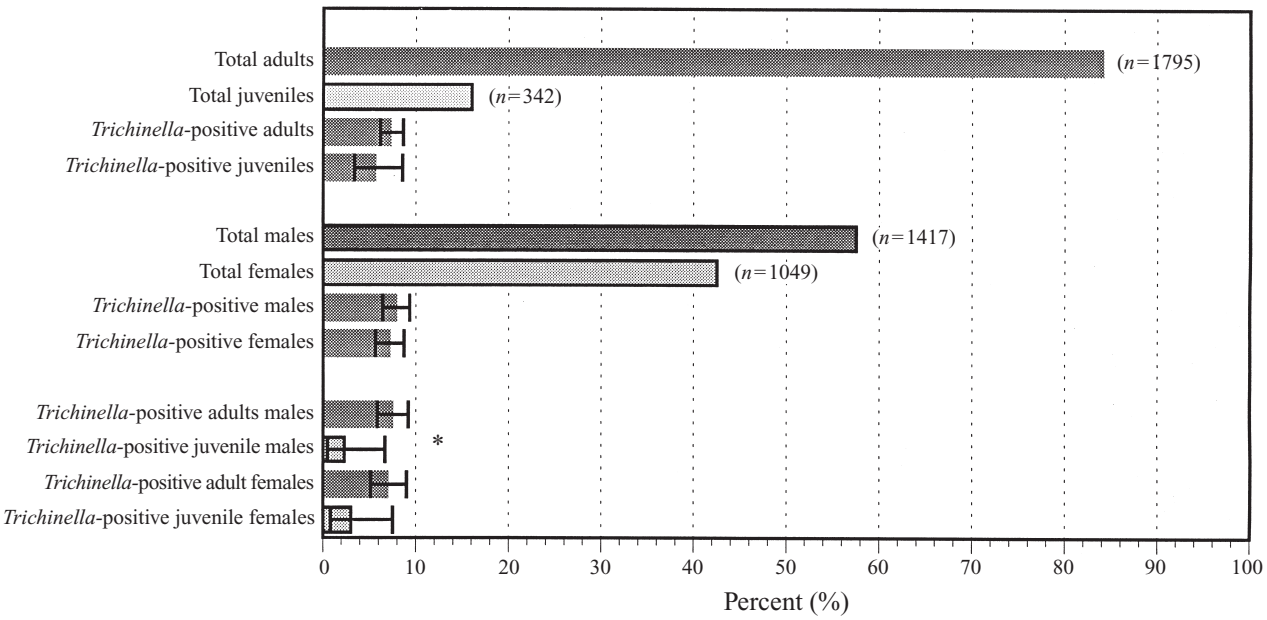

Fig. 3. Prevalence of *Trichinella*-infections in different age groups, male and female foxes. The serologically tested sample was stratified according to sex and age (juvenile/adult) of the foxes. Percentages or estimated percentages (columns) and 95% CIs (bars) are shown. A statistically significant difference is indicated by an asterisk.

method, the differences were statistically significant ($P = 0{\cdot}002$).

Parasite typing

DNA from three *Trichinella* isolates was amplified with two different random primers. All patterns were compared to reference isolates of *T. spiralis*, and also to *T. nativa*, *T. britovi*, *T. nelsoni*, T5, T6 and T8. The patterns obtained with the Brandenburg isolates were similar or identical with the *T. spiralis* reference, but clearly differed from the patterns of the other reference isolates. These findings allowed the unambiguous classification of the parasites from Brandenburg as *T. spiralis*. The two remaining isolates could not be analysed by RAPD due to lack of larval DNA.

DISCUSSION

Anecdotal reports and a few cross-sectional studies suggest that *Trichinella* infections occur in foxes in several if not all European countries [1, 4, 5, 7, 24]. The detected prevalences were often low, ranging between estimated prevalences of 0·1% in Denmark and 1·3% in Switzerland [7, 25, 26]. However, the limited sensitivity of the techniques frequently used to detect muscle larvae may lead to an underestimation of the true prevalence. Previous studies in Germany considered the parasite spectrum of foxes [8, 12, 28–30], *Trichinella* spp. infections in wildlife animals including foxes [9, 31] or specifically vulpine *Trichinella* infections [10, 32]. All these studies failed to detect the parasite. Wagner and colleagues (1988) investigated 3889 animals sampled in West Germany and detected a single *Trichinella*-infected fox [26]. Only a few years ago vulpine trichinellosis was discovered in East Germany [13].

The animals investigated in the present study were mostly shot between May and October or December and March. This seasonality of the sampling was due to influences of the hunting season and the oral immunization campaign against rabies as most foxes analysed in this study also served as indicator foxes in the monitoring of the efficacy of the current rabies control programme. Whether the seasonality has any impact on the total (sero)prevalence remains to be clarified. The surplus of male foxes (57·5%) in the sample is at least in part due to the fact that male animals are shot during the entire hunting period, while female animals are spared during pregnancy (February–April). However, recent evidence suggests that there can also be a 'natural' surplus of male animals in a fox population [33]. The surplus of adult animals over juvenile foxes in the sample can be explained by the fact that juvenile animals are only shot from April/May onwards. In addition, the distinction between juvenile and adult foxes becomes increasingly difficult from October onwards which

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may lead to an increase of the proportion of unclassified animals. As a consequence of this seasonality in the age structure of the sample, the slightly higher prevalence in adult as compared with juvenile foxes may be caused by confounding. Due to the small size of some relevant strata this possible effect could not be further analysed. One can speculate, however, that the juvenile foxes may be less exposed to the infection with *Trichinella* spp., at least as long as the cubs are not weaned. A similar effect has been observed for infections unweaned cubs with *Echinococcus multilocularis* [34]. On the other hand, adult foxes spend a longer 'time at risk' and are therefore more likely to get infected and to mount an antibody response to *Trichinella* spp.

In our survey, five *Trichinella*-infected foxes were detected which were all shot in the Eastern half of Brandenburg. However, the low number of infected animals does not allow conclusions on a potential clustering of the infection in this region of the study area. On the contrary, the results of the serological part of the study suggest that vulpine trichinellosis is rather homogeneously distributed in Brandenburg. The fact that a few significant differences were found at the county level, may contradict this notion at first sight. However, the recommended correction of the type I error for pairwise comparisons of split data sets [23] which leads to a more conservative estimate showed that these differences may be spurious. Some higher prevalences were found at the town-level which should not be overinterpreted in view of limited numbers of sample available from these towns.

A serological test based on the use of larval E–S antigens of *T. spiralis* [17, 18] was adopted for foxes, evaluated and optimized for the detection of vulpine antibodies to *Trichinella* spp. Under optimal conditions, the ELISA was 100 % sensitive and specific on a test population consisting of defined sera. A similar test had been successfully used to investigate the seroprevalence among foxes in Switzerland [7]. The seroepidemiological results obtained with this test indicate a hugely higher prevalence of *Trichinella*-infections in foxes than the digestion method. In general, the discrepancy between serology and the digestion technique can be explained as follows: (i) In some cases the muscle samples examined do not consist entirely of tissue from the predilection sites or are of minor quality [35]. This may reduce the sensitivity of the digestion method. As a consequence, the prevalence of *T. spiralis*-infections in foxes may be underestimated by this method. (ii) Infections with few muscle larvae may lead to a reduced sensitivity of the digestion method. (iii) Persisting antibodies may lead to an overestimation of the true prevalence in cases of transient infections. The results of experimental infections reported in this publication indicate that antibodies can persist for 2 years at least. The excess of adult animals over juveniles in the sample may augment the effect of an overestimation the seroprevalence versus the actual prevalence of the infection itself. On the other hand, adult foxes are exposed to the infection for a longer period of time which increases the likelihood of an adult animal to be seropositive. (iv) Potential cross-reactions with other antigens may lead to a lower specificity of the ELISA as compared to the isolation and determination of larvae, although testing with a limited number of sera from animals with *Mesocestoides*, *Toxocara*/*Toxascaris* or ancylostomatid mono-infections cast no doubt on the specificity of the assay used.

A higher sensitivity of the ELISA as compared to direct parasite detection was also recorded in a Swiss study [7]. The authors estimated that the larval E–S antigen ELISA used was ten times more sensitive than the peptic digestion method. It was argued that the intensity of infection might be low in foxes (leading to false-negative results in direct parasite detection) while the prevalence could still be rather high at the same time.

The spatial distribution of seropositive foxes cannot be distinguished from a random distribution (K. Wacker, C. Staubach, unpublished). Thus, at a large scale, the serological results of this survey provide no evidence that vulpine trichinellosis clusters in certain regions of the study area.

Typing of 3 out of 5 isolates by RAPD [20] resulted in the unambiguous classification of the parasites as *T. spiralis*. This finding is in accord with the notion that *T. spiralis* is the predominant species in Central and Northern Europe [27]. Results of a study recently undertaken in France, Italy and the Extremadura region of Spain suggests that *T. spiralis* is present among silvatic animals at lower altitude than *T. britovi* which has more frequently been identified in mountain regions [4]. Similarly, *T. britovi* was detected in foxes in a study conducted in Switzerland [36]. These findings are in accord with our own data which were obtained in the East of the North-German lowlands.

Likewise there is no indication of a possible link between the occurrence of trichinellosis in wild life animals and infections in domesticated pigs [37].

Despite the prevalence of the parasite in foxes, there were no recent cases of trichinellosis in pigs or humans in Brandenburg. Even in wild boars, there was only a single case of trichinellosis recorded in the monitored area in recent years. These data suggest that *T. spiralis*-infections persist in the study area in a silvatic cycle with foxes serving as a reservoir and that the fox does not appear suitable as an indicator for the trichinellosis situation in domesticated animals.

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