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Roe deer as sentinels for endemicity of tick-borne encephalitis virus

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SUMMARY

The suitability of serological surveys of roe deer (*Capreolus capreolus*) in determining the spread of tick-borne encephalitis virus (TBEV) was tested in a south German area with a low risk of TBEV infection to humans. Sera obtained from 192 hunted roe were screened by an haemagglutination–inhibition test (HAI) and in an ELISA developed in our laboratory. Those found positive were tested in a neutralization test (NT). Fifty (26·0%) sera reacted positive by ELISA and 43 (86·0%) of these were confirmed by HAI or NT. Forty-seven (24·5%) samples were positive by HAI, 44 (93·6%) of which were also positive in NT or ELISA. Only insignificant increase of the antibody prevalence with age ($P = 0{\cdot}17$ for HAI antibodies) suggests that most infections occur at an early age in scattered natural foci. The antibody prevalence in females was lower than in males (OR = 0·63; $P = 0{\cdot}02$ for HAI antibodies). In determining the distribution of seropositive roe we increased the sample size to 235 sera. No antibodies were detected in 56 (23·8%) sera collected in the eastern third of the county. The areas of high antibody prevalence in roe match those in which humans have been infected. We conclude that serosurveys of roe deer are useful in marking out areas in which humans face the risk of infection, provided that an adequate number of sera, preferably from males, is available.

INTRODUCTION

Tick-borne encephalitis (TBE) is known to be endemic in some parts of Central Europe. The prevalence in these endemic areas, however, varies greatly [1]. TBE is caused by the tick-borne encephalitis virus (TBEV), the only arthropod-borne flavivirus known to occur in Central Europe. In at least two-thirds of humans the infection is asymptomatic, but, particularly in adults, the disease may take a severe course. The case fatality rate is 1–2%, while residual neurological symptoms lasting for months or even lifelong can be observed in 10–30% of patients with either meningoencephalitis or encephalomyelitis [1]. There is no specific treatment and, in endemic areas, the question of prophylaxis arises.

Inactivated vaccines as well as specific immunoglobulin preparations are available [1, 2]. In the highly endemic areas the indication for prophylaxis is fairly clear-cut. In other regions, in which just a few human cases are known, measures to define the endemic areas are desirable before immunoprophylaxis is recommended. Seroepidemiological studies in risk groups, i.e. foresters, hunters and farmers, have proved useful [3–5]. However, because these risk groups have been widely vaccinated in recent years [1, 2], it is now difficult to obtain an adequate number of serum samples from unvaccinated persons so as to perform a meaningful surveillance.

The roe deer (*Capreolus capreolus*) is a common game species in almost all TBE endemic areas, and it is an important host of the tick *Ixodes ricinus*, the main vector of TBEV. Like other large mammals such as sheep, goats and cattle, the roe deer does not play a role in the maintenance of TBEV, because generally the level of viraemia after natural infection is too low to infect ticks. However, because they develop antibodies [1, 6, 7] they are likely to be useful sentinel animals to determine the proportions of TBEV endemic areas. Antibodies in roe deer and other large mammals have been used before to support the search for natural TBEV foci, and roe deer have been proposed to be particularly suited for this purpose [8]. However, to our knowledge, no systematic survey on a large roe population, including age, sex and shooting sites of the tested animals, has been reported in an area with focal TBEV endemicity. This report deals with such a TBEV antibody surveillance in roe deer. In addition to the traditional haemagglutination–inhibition test, an ELISA, developed in our laboratory, was used for antibody detection. Finally, we tried to confirm positive reactions obtained with either of these tests by a neutralization test.

MATERIALS AND METHODS

Description of the surveyed area

The County of Tuebingen, located in southwest Germany and drained by the Neckar river, comprises an area of 519 km². About 36% of the county is covered by forest, while about 50% is farmland. The average annual temperature in the Neckar valley is 8·2 °C and the average annual precipitation is 730 l/m². The altitude varies between mountains of up to 845 m above sea level in the east and as relatively low as 300 m in the Neckar valley, thus resulting in considerable climate variations [9]. Roe deer are abundant in all parts of the county while the density of *I. ricinus* varies widely (unpublished observations). The human population of the county is about 183000 with approx. two-thirds concentrated in three towns: Tuebingen (77000), Rottenburg (34000) and Moessingen (16000) [9].

Collection of samples

Sera were collected by private hunters and rangers of the state forest department. The hunters filled in a form for each animal, recording date, sex and estimated age. The shooting site was marked on a map.

Serum samples were obtained from the bullet wound, a dissected blood vessel, or the peritoneal or thoracic cavity, transferred into 50 ml screw-capped plastic

tubes and refrigerated at 4–8 °C. In a minority of cases the blood was frozen in the deep freeze. The hunters notified the Department of Medical Virology and Epidemiology in Tuebingen by telephone, so that the samples could be collected within 24–48 h, centrifuged and kept frozen in aliquots at −20 °C until further processing. The majority of the sera were highly haemolysed and 10–20% had to be discarded due to gross contamination.

During the 1986–7 hunting season 192 blood specimens from 70 female and 122 male animals were collected. During the seasons that followed (1987–92) we collected an additional 43 sera from regions poorly covered in 1986–7 which were tested by HAI and NT and used only for mapping the spatial distribution of the seropositive animals.

Haemagglutination–inhibition test (HAI)

The HAI was done essentially as described by Clarke and Casals [10], modified for the microtitre system [11]. Briefly, we used a haemagglutinating antigen produced by sucrose–acetone extraction from suckling mouse brains which were infected with the TBEV strain Vie 415B [12]. Twofold serum dilutions in 0·64-M KCl-borate buffer pH 9, in saline ranging from 1/10 to 1/1280 were incubated with 4 haemagglutinating units of antigen for 3 h. After adjustment of the pH to 6·4, goose erythrocytes were added. The sedimentation pattern was read after 45 min incubation at room temperature. The serum dilution which completely inhibited haemagglutination was considered as the titre. Incomplete haemagglutination inhibition at a dilution of 1/10 was considered as equivocal and recorded as 1/5.

Neutralization test (NT)

The NT used has been described before [12]. Briefly, aliquots (50 μl) of twofold serum dilutions 1/10–1/1280 were incubated with 100 $TCID_{50}$ virus (TBEV strain Vie 415B) in flat bottomed microtitre plates for 90 min at 37 °C. A cell suspension of a porcine cell line (PS clone D) was added and the cytopathic effect (CPE) was determined after 7 days incubation at 37 °C. The serum dilution which completely inhibited the CPE was considered as the titre.

Enzyme-immunoassay (ELISA)

The development of the ELISA has been described in detail [13]. Briefly, anti-roe deer IgG was prepared by immunizing rabbits with IgG prepared by ammonium sulphate precipitation of serum from a captive roe buck. The rabbit IgG was purified by affinity chromatography with protein A-sepharose [14]. Plastic test strips coated with TBEV antigen were obtained from Immuno (Heidelberg, Germany). They were treated with 10% v/v calf serum in phosphate buffered saline pH 7·2, (PBS-C) before use. 200 μl of serum diluted 1/50 in PBS-C were added to the coated plastic test strips and kept overnight in a moist chamber in the refrigerator. After three washes with PBS-C, 200 μl of rabbit-anti-roe IgG was added and, after incubation for 2 h at 37 °C in the moist chamber, the strips were washed thoroughly with PBS-C and incubated further for 2 h with 200 μl peroxidase-labelled anti-rabbit IgG (Behringwerke AG, Marburg, Germany). 200 μl substrate was added, consisting of 10 mg *O*-phenylene-diamine (Dakopatts,

DAKO GmbH, Hamburg, Germany), dissolved in 10 ml 0·1 M phosphate buffer, pH 6·0, containing 0·005% H_2O_2. The enzyme-substrate reaction was stopped by adding 2 M sulphuric acid (50 μl per well) after 8 min. Absorbance was read photometrically at 492 nm (Microelisa Autoreader, MR 580, Dynatech, Denkendorf, Germany). The average $OD_{492\,nm}$ value of HAI- and NT-negative plus HAI-equivocal sera ($n = 144$) was calculated and 1·5 standard deviations were added. Sera with $OD_{492\,nm}$ values above this limit were considered positive.

Data analysis

To test the significance of the differences in age- and sex-specific antibody prevalences binary logistic regression analysis was performed by using the commercial program IMP Statistics and Graphics Guide Version 3 (SAS Institute Inc.). Fisher's exact test was used as indicated.

RESULTS

Relationship between the results obtained by HAI and in ELISA

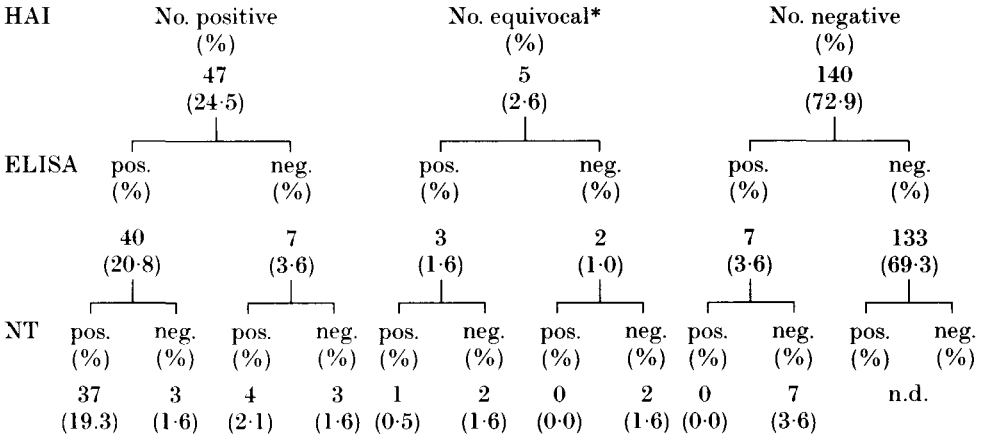
Initially, the sera were screened by HAI and in ELISA. Positively and equivocally reacting sera were tested in NT. The results are shown in Table 1. Forty-seven of 192 (24·5%) sera were positive by HAI tests with titres $\geqslant 10$. Forty-one (87·2%) of these showed neutralizing activity. Of the remaining 6 sera, 3 (6·4%) were positive in the ELISA. Therefore, the specificity of 93·6% of the sera positive by HAI was confirmed in NT, ELISA or both. Fifty out of 192 (26·0%) sera were positive in ELISA. Forty-one (82·0%) were confirmed by HAI, NT or both. Including 2 sera which reacted equivocally by HAI, 86% of all sera positive in ELISA were confirmed by HAI or in NT. Because 3 out of 5 sera which reacted equivocally by HAI were positive in ELISA, it may be assumed that the ELISA is somewhat more sensitive than the HAI. Some or all of the 7 (3·6%) sera positive in ELISA but negative by HAI and in NT could be true-positive. However, 4 out of 142 (2·8%) ELISA-negative sera were positive by HAI and in NT. Therefore, they must be considered as false-negative results. A good linear correlation between the HAI and the NT titres was observed (Table 2); however, 3 out of 12 sera with HAI titres of 20 were negative in ELISA.

Age- and sex-specific prevalence of TBEV-antibodies in roe sera

The distribution of the HAI- and ELISA-positive sera according to age and sex of the donors is given in Table 3. The results are remarkable in two respects. First, there is a comparatively high antibody prevalence in the first year of life which increases only slowly with age. This increase is statistically not significant ($P = 0·176$ for HAI antibodies by logistic regression analysis). Secondly, the antibody prevalence is lower in females than in males (OR = 0·63; $P = 0·018$ for HAI antibodies by logistic regression analysis). A sex difference which almost reaches statistical significance can be observed in animals in their first year of life ($P = 0·057$ by Fisher's exact test).

The age distribution of the HAI titres in male and female animals is shown in Fig. 1. Thirteen of 41 (31·7%) and 8 of 11 (72·7%) sera of male and female animals, respectively, showed antibody titres of $\leqslant 10$ ($P = 0·02$ by Fisher's exact test).

Table 1. *TBEV-antibodies in sera of roe deer. Comparison of results obtained in HAI, ELISA, and NT*



(%) Percentage of all sera tested ($n = 192$); * partial haemagglutination at serum dilution 1/10.

Table 2. *TBEV antibodies in roe deer. Results of NT and ELISA as related to HAI titres*

HAI		NT positive			ELISA positive	
Titre	*n*	*n*	%	GM†	*n*	%
< 5	140	n.d.‡	—	—	7	5·0
5*	5	1	20·0	10·0	3	60·0
10	17	11	64·7	17·6	13	76·5
20	12	12	100·0	27·4	9	75·0
40	8	8	100·0	33·6	8	100·0
⩾ 80	10	10	100·0	85·7	10	100·0

* Equivocal HAI at 1/10.
† GM, reciprocal geometrical mean titre.
‡ n.d., not done.

Moreover, while in HAI-seropositive males there were 2 of 41 (4·8%) which could not be confirmed in NT or ELISA, 3 of 11 (27·3%) such sera were found in the female group ($P = 0·057$ by Fisher's exact test; n.s.).

Geographical distribution of roe deer positive for TBEV-antibodies

Fig. 2 shows a map of the County of Tuebingen in which the shooting sites and the antibody status of the roe (as detected by HAI and NT) are shown. In addition, results of 43 sera, collected between 1987 and 1992, are included. The antibody-positive animals are not equally distributed but show the highest density in the southwest while along the eastern border of the county no antibody-positive roe was found. However, even in the south-western part of the county many sera lacked antibody. Further, there is a suggestion that seropositive deer are not equally distributed but are clustered in certain areas. The best dividing

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Table 3. *Prevalence of HAI and ELISA TBEV-antibodies in roe deer according to age and sex*

Age group (yrs)	Male			Female		
	n	HAI pos.* (%)	ELISA pos. (%)	*n*	HAI pos.* (%)	ELISA pos. (%)
1	38	11 (28·9)	11 (28·9)	31	3 (9·7)	3 (9·7)
2	18	3 (16·7)	4 (22·2)	12	2 (16·6)	2 (16·6)
3+4	44	18 (40·9)	18 (40·9)	20	5 (25·0)	2 (10·0)
5–8	22	9 (40·0)	8 (40·9)	7	1 (14·3)	2 (28·6)
Total	122	41 (33·6)	41 (33·6)	70	11 (15·7)	9 (12·9)

Age group (yrs)	Total		
	n	HAI pos.* (%)	ELISA pos. (%)
1	69	14 (20·3)	14 (20·3)
2	30	5 (12·5)	6 (15·0)
3+4	64	23 (35·9)	20 (34·5)
5–8	29	10 (34·5)	10 (34·5)
Total	192	52 (27·1)	50 (26·0)

* Includes all sera with HAI titres ⩾ 10 or partial haemagglutination at serum dilution 1/10.

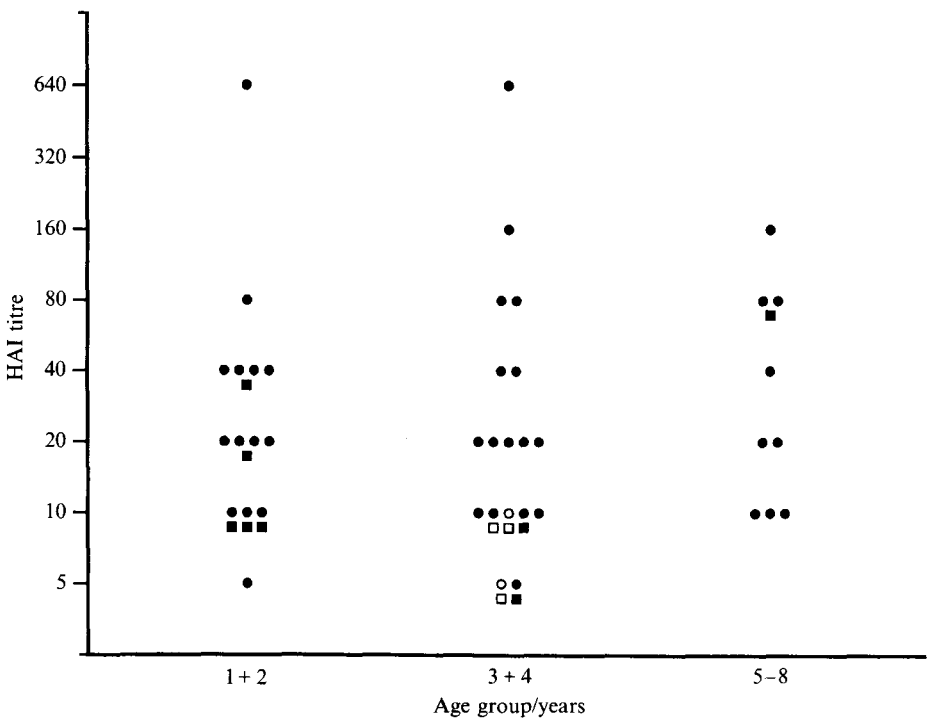

Fig. 1. Distribution of titres of sera reacting positive in HAI according to age and sex. ● Male, seropositive in NT or ELISA; ○ male, seronegative in NT and ELISA: ■ female, seropositive in NT or ELISA; □ female, seronegative in NT and ELISA.

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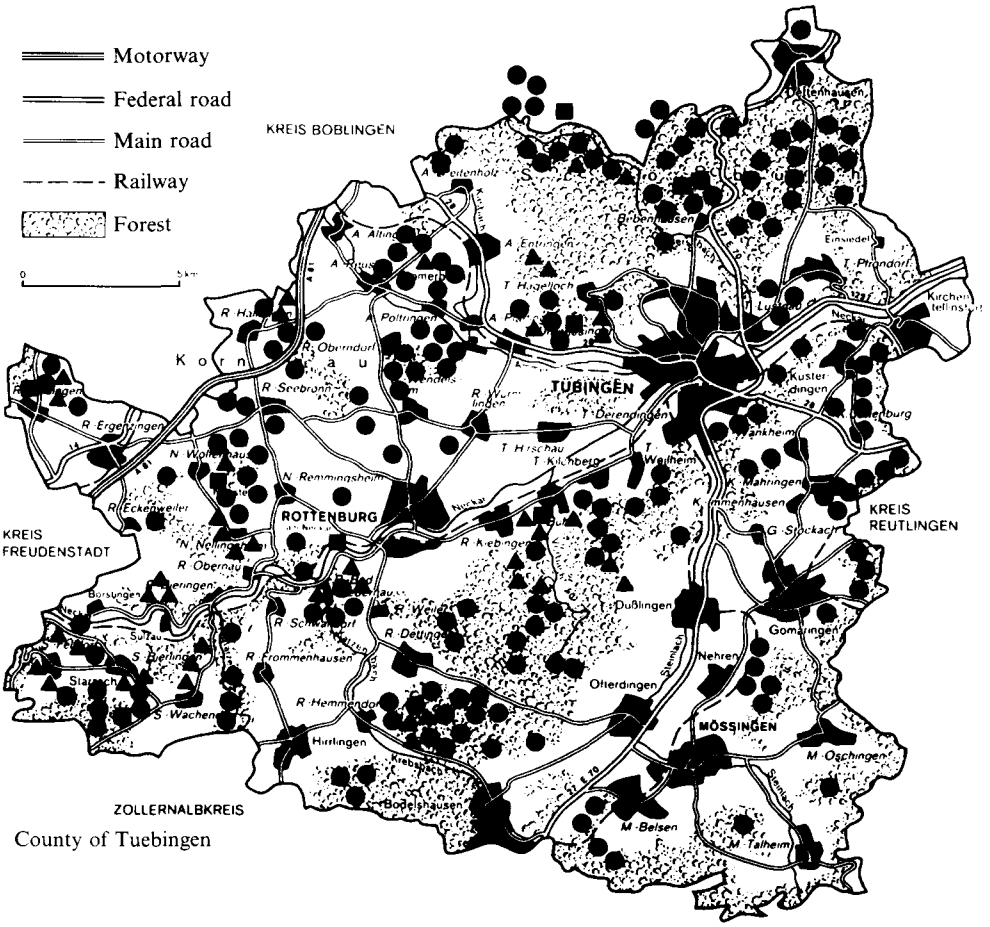

Fig. 2. Geographical distribution of shooting sites of roe deer tested for TBEV-antibodies in the County of Tuebingen. ● Roe without TBEV-antibodies; ▲ roe TBEV-seropositive in NT; ■ roe TBEV-seropositive by HAI but not in NT.

line between the TBEV-positive and the TBEV-negative areas was the highly used B27 road. In most forested areas this road is lined by fences forming an effective barrier against exchanges between the eastern and western roe subpopulations. The sample of the eastern subpopulation comprised sera from 26 males and 30 females. The age distribution was similar to that of the total population. All 56 sera, collected east of the B27, showed negative results by HAI and none of 41 sera tested in ELISA proved to be positive, while the prevalence of HAI-antibodies in the western part was 41 in 105 (39·0%) males and 13 in 74 (17·6%) females.

Fig. 3 shows the presumed site of exposure and in those cases in which this was unknown, the place of residence of 14 TBE patients admitted between 1986 and 1992 to the University Hospitals of Tuebingen. It can be assumed that all serious TBE cases, which occurred during this time in the county of Tuebingen, are included. All the places of residence or the presumed sites of exposure are located in areas of high TBEV-antibody prevalence in roe.

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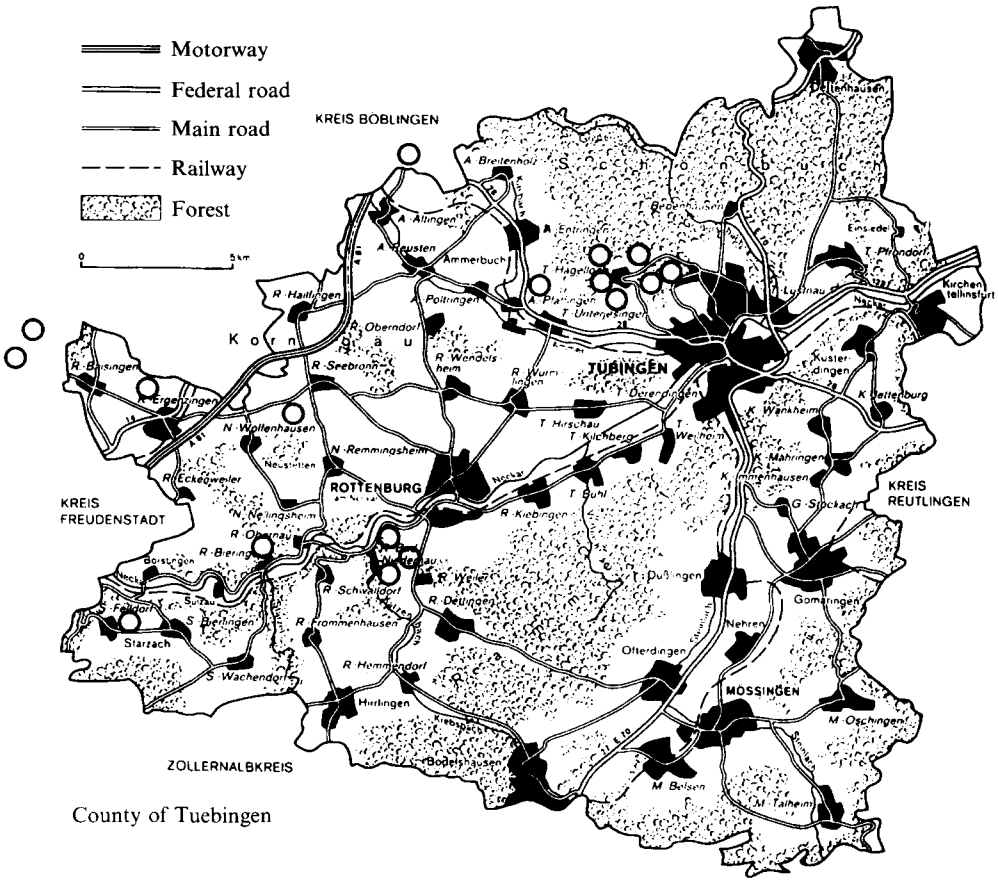

Fig. 3. Geographical distribution of the places of exposure, or, respectively, of residence of TBE-patients treated at the University Hospitals of Tuebingen between 1986 and 1992. ○ *Place of patient's exposure or residence.*

DISCUSSION

The ideal sentinel animal for TBEV surveillance should have an adequately limited home range, be available in large numbers, be well dispersed in the surveillance area, and should show a long-lasting antibody response after natural infection. As the roe deer fulfils some of these requirements, its surveillance offers a basis for targeting immunoprophylaxis to human beings facing the risk of TBEV-infection. The home range of the female roe is c. 5 km in diameter, while bucks tend to be more mobile [15]. After experimental infection via infected ticks [6] or virus injection [7] roe deer develop a low-grade viraemia followed by an antibody response [6, 7]. However, nothing is known about the minimum infectious dose or the duration of the antibody response.

In this paper we describe the results of a TBEV-antibody survey on 192 roe sera collected in an area covering 519 km^2 which is marked by the occurrence of natural TBEV foci. In aiming to control the results of our antibody determinations by HAI in an independent test system, we developed an ELISA and used both tests in examining the 192 roe serum samples. Sera with either positive or equivocal reaction in any of these tests were further tested for neutralizing antibodies.

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The results obtained by HAI, in NT and ELISA correlated well. Forty-seven (24·5%) and 50 (26·0%) sera were positive by HAI (titre 10) and in the ELISA, respectively, but 4 out of 41 (9·8%) sera which proved positive by HAI and in NT were negative in ELISA while 3 out of 47 (6·4%) sera positive by HAI were negative in NT and ELISA. Three out of 5 sera which reacted equivocally by HAI were positive in ELISA. These results suggest that the ELISA was slightly more sensitive than the HAI, but, on the other hand, certainly less specific. It is evident that both tests have some practical disadvantages. Goose erythrocytes and haemagglutinating antigen extracted from infected suckling mouse brain are essential for the HAI, while the ELISA is species-specific and necessitates anti-roe-Ig antibodies which at present are not commercially available.

The age distribution with relatively high antibody prevalence in young animals and a rather slow increase with age can be explained most easily by the occurrence of the virus in scattered natural foci, areas with population densities of small mammals and ticks high enough to allow continuous TBEV transmission [1]. Animals born to herds whose home range includes such a natural focus will be infected early, while others may not be exposed at all during their life. The suggestive clustering of antibody-positive roe deer in certain areas (Fig. 2) supports this proposition. A surprising result of our survey was that TBEV-antibodies were found significantly less often in female than in male roe. A lower prevalence of antibodies in females was observed even in animals in their first year of life ($P = 0{\cdot}057$), an age-group which keeps close to their mothers. Therefore, sex differences in mobility [15] leading to greater exposure to ticks of the males cannot explain our results. To our knowledge nothing is known about a difference in antibody response related to sex in deer. Therefore, we have no explanation for the sex differences in antibody prevalence in our study.

From observations in humans we suspected that several endemic TBEV foci were present in the county, and it was the main practical aim of our study to narrow down the area in which immunoprophylaxis is required after tick bite. No sera positive by HAI or in ELISA were detected in the east of the county in our first study of 192 sera. The sample was increased to 235 sera in the following 5 years, and no positives were detected by HAI in the 56 (23·8%) sera collected east of the highly used B27 road. This area comprises about one third of the county, while the HAI antibody prevalence (titre $\geqslant 5$) in the western part was 39·0% in 105 males and 17·6% in 74 females. It should, however, be emphasized that even in high risk areas a considerable proportion of sera lacked detectable antibodies.

The antibody distribution in roe is consistent with our experience in humans. All the 14 human TBE cases from the County of Tuebingen, diagnosed between 1986 and 1992 at the University Hospitals of Tuebingen, were probably infected in the western part of the county. Therefore, at present we see no indication for post-exposure TBE prophylaxis after tick bites occurring east of the B27 road. A comparison with the distribution of earlier human cases [16] and antibody surveys in humans [17] suggests that the endemic area, as described in this paper, has remained largely unchanged for at least 20 years. This observation compares well with those of other authors [18, 19] which describe long-lasting stability of natural TBEV foci over many years.

We conclude that antibody surveys in roe deer can be useful in tracing TBEV

endemic areas. We found, however, that even in a highly endemic area a high proportion of the animals, particularly the females, were seronegative in our tests. A considerable sample size is therefore necessary to arrive at valid conclusions. The ELISA, though useful in confirming HAI results, did not essentially improve the results obtained using HAI and was less specific.

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