Serologic survey of wild boars for mosquito-borne viruses in South Moravia (Czech Republic)

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ABSTRACT: A serosurvey for mosquito-borne viruses was carried out in 93 wild boars (*Sus scrofa*), using a plaque-reduction neutralization microtest with Vero cells. The boars were sampled on 24 hunting grounds of the Breclav district (South Moravia) from 2000 to 2002. Specific antibodies to *Flavivirus* West Nile (WNV) were detected in six (6.5%) animals, and only in Lanzhot and Kostice, i.e., in the area of the "Soutok" game reserve where WNV was previously isolated from mosquitoes in South Moravia. However, the antibody titres were comparatively low (1:20–1:40). A substantially higher seroprevalence was revealed against *Orthobunyavirus* Tahyna (TAHV): 18 (19.4%) wild boars were positive, and the titres ranged from 1:20 up to 1:640. Only one animal (1.1%) seroreacted with *Orthobunyavirus* Batai (Calovo), at a low titre of 1:20. The sera were additionally examined by a haemagglutination-inhibition test against *Alphavirus* Sindbis: two boars (2.2%) revealed antibodies, the titres were 1:20 and 1:80. The serosurvey indicates that the activity of mosquito-borne viruses in South Moravia has decreased compared with the past decades, but that surveillance for these viruses is still necessary.

Keywords: antibodies; West Nile virus; Tahyna virus; Batai virus; Sindbis virus; Czechland; swine

Mosquito-borne viruses circulate in natural foci between endotherm (homeotherm) vertebrates and mosquitoes. Six vertebrate pathogenic mosquito-borne viruses have been reported in Central Europe (Malkova et al., 1986; Hubalek and Halouzka, 1996; Weissenbock et al., 2002): Sindbis (SINV), West Nile (WNV), Usutu (USUV), Tahyna (TAHV), Batai (BATV; synonym = Calovo), and Lednice (LEDV), and at least two are regarded as being of veterinary importance (WNV, USUV).

The aim of this study was to evaluate the present activity of WNV and other mosquito-borne viruses in South Moravia (Czech Republic), using an indirect method of serological survey of wild mammals (the wild boar, *Sus scrofa*) potentially exposed to these viruses in the field. West Nile virus was already isolated in this region in 1997 and 1999 (Hubalek et al., 1998, 2000) as well as in neighbouring Slovakia (Labuda et al., 1974), and five human cases of WNV fever were described after floods in South Moravia in 1997 (Hubalek et al., 2000). An extensive natural focus of TAHV infections ("Valtice fever") has been well documented in South Moravia since the 1960s, and the virus has been isolated repeatedly from vector mosquitoes (e.g., Danielova et al., 1976; Rosicky and Malkova, 1980). One isolation of BATV was reported from *Anopheles maculipennis* complex mosquitoes in Rakvice (Smetana et al., 1967), while SINV has not yet been isolated in South Moravia, in contrast to neighbouring western Slovakia (Ernek et al., 1973).

MATERIAL AND METHODS

Study sites

All 24 visited hunting grounds belonged to localities in the district of Breclav (number of animals ex-

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amined): Boleradice (3), Charvatska Nova Ves (6), Divaky (9), Hlohovec (4), Hrusky (1), Hustopece (1), Kostice (4), Lanzhot (17), Lednice (4), Moravska Nova Ves (3), Morkuvky (3), Nikolcice (1), Novy Dvur (2), Podivin (2), Postorna (5), Rakvice (1), Tisnov (BV, 8),Tvrdonice (2), Tynec (1), Valtice (8), Velke Bilovice (2), Velke Hosteradky (1), Vranovice (1), and Zajeci (3). Most sampling sites were situated in habitats with abundant mosquito populations.

Blood sampling

The wild boars, which were killed by shooting, and throughout all seasons of the year, were weighed and their age determined. Blood samples were collected from the heart or from the thoracic cavity. After clotting, the blood samples were centrifuged in the laboratory and the sera were then stored at –20°C until use.

Plaque-reduction neutralization microtest (PRNμT)

The seroneutralization method was originally proposed by Madrid and Porterfield (1969, 1974), and later adopted to a microtechnique on 96-well (flat-bottomed) sterile microplates (Sarstedt) for cell culture (Hubalek et al., 1979). Vero E6 cells were serially propagated in Leibovitz L-15 medium (Sigma) supplemented with 10% of foetal calf serum (FCS, Gibco Bio-Cult) and antibiotics. Tested sera were inactivated at 56°C for 30 m, and diluted 1:10 in L-15 medium for screening; 30 μl of the diluted serum was mixed in a microplate well with 30-μl test dose of the virus (containing 20–30 PFU of individual viruses) in L-15 supplemented with 3% of inactivated FCS, and incubated at 37°C for 60 min; 60 μl of Vero cell suspension (in L-15 with 3% FCS) was then added to each test well (20 000 to 30 000 cells), and 120 μl of carboxymethylcellulose sodium salt overlay (1.5% CMC of medium viscosity – BDH in PBS mixed with the same volume of L-15 with 3% of inactivated FCS) was added after an incubation at 37°C for 4 h in each well. Sera were tested in duplicate, and controls included the virus test dose and its titration, immune WNV reference serum; control negative serum; and cells without virus. The microplates were incubated at 37°C for 5 days, and the microplate cultures were then stained with 0.1% acidic solution of naphthalene blue black (Fluka).

The virus strains used in PRNμT were: (1) West Nile virus (WNV) Egyptian topotype strain Eg-101 (Melnick et al., 1951), passaged 17 times in suckling mouse brain, homogenized in PBS with 0.4% of bovine serum albumin fraction V, Sigma (PBS-BSA), and centrifuged; (2) Tahyna virus (TAHV) strain T16 (Bardos et al., 1975), passaged six times in suckling mouse brain, homogenized in PBS-PBS, and centrifuged; (3) Batai virus (BATV) strain 184 (Bardos and Cupkova, 1962), passaged 10 times in suckling mouse brain, homogenized in PBS-BSA, and centrifuged. Unfortunately, we were unable to use Sindbis *Alphavirus* strains Eg339 or P3328 because they did not produce good plaques in Vero E6 cells.

Sera reactive with a virus, revealing 90% or greater reduction in the number of plaques at the 1:10 dilution during the screening (corresponding to the 1:20 final dilution of the serum – after mixing with the virus test dose), were titrated by twofold dilutions, and those dilutions corresponding to a 90% reduction of plaque numbers were regarded as the serum titres ($PRN\mu T_{90}$). Reciprocal titres ≥ 20 were considered positive. Boar sera reacting with WNV were also tested in PRNμT on SPEV cells with *Flavivirus* of tick-borne encephalitis (TBEV) strain Hypr (Pospisil et al., 1954), passaged 55 times in HeLa cells followed by 11 passages in mouse brain, in order to exclude cross reactions with this antigenically partially related virus occurring in Moravia.

Haemagglutination-inhibition test (HIT)

We encountered problems with the plaquing of Sindbis virus in microcultures of Vero E6 cells. Therefore, we used the haemagglutination-inhibition test (HIT) in microplates for this virus (Eg-339; Taylor et al., 1955). All serum samples for this assay were acetone-extracted and tested with saccharose- and acetone-processed antigen by using eight haemagglutinin units (Clarke and Casals, 1958); titres > 20 were considered positive.

Statistical methods

The statistical significance of differences between proportions was evaluated by using cross-tables 2 × 2 or 2 × 3 and the χ^2 test (SOLO 4.0 package, BMDP Statistical Software, Los Angeles).

RESULTS

The serosurvey was carried out in 93 wild boars sampled on 24 hunting grounds. Specific neutralizing antibodies to WNV were detected in six (6.5%) animals and only from the Lanzhot and Kostice hunting grounds (Table 1). However, the antibody titres were comparatively low (1:20–1:40). Some additional animals seroreacted with WNV (A35, A36, A48, A60 – see Table 1), but at the same time they also reacted with TBEV at similar or higher titres. Those results were, therefore, regarded as flavivirus cross reactions and the corresponding animals were not taken in consideration as specific reactors.

A substantially higher seroprevalence was revealed against TAHV: 18 (19.4%) wild boars were seropositive, the titres ranged from 1:20 up to 1:640

Table 1. Survey of wild boars seroreacting with Tahyna or West Nile virus in PRNμT

No.	Age (years)	Weight (kg)	Locality	Date	TAH titre	WNV titre	TBE titre
A7	< 1	20	Lanzhot	July 2002	< 20	**20**	< 20
A13	< 1	25	Lanzhot	July 2002	< 20	**40**	20
A16	< 1	12	Kostice	July 2002	< 20	**40**	< 20
A28	< 1	8	Lanzhot	July 2002	**160**	< 20	n.t.
A32	<1	10	Lednice	July 2002	**640**	< 20	n.t.
A41	<1	30	Lednice	July 2002	**20**	< 20	n.t.
B19	< 1	30	Zajeci	Nov.2000	**160**	≤ 20	< 20
A6	1	47	Kostice	July 2002	< 20	**20**	< 20
A20	1	31	Vel. Bilovice	July 2002	**160**	< 20	n.t.
A24	1	55	Lanzhot	July 2002	< 20	**20**	< 20
A29	1	44	Lanžhot	July 2002	< 20	**20**	< 20
A35	1	38	Charv. N. Ves	July 2002	< 20	20	20
A36	1	40	Charv. N. Ves	July 2002	< 20	20	**160**
A43	1	50	Lanzhot	July 2002	**20**	< 20	n.t.
A45	1	60	Lanzhot	July 2002	**320**	< 20	n.t.
A50	1	64	Postorna	July 2002	**160**	≤ 20	< 20
A54	1	55	Podivin	July 2002	**320**	< 20	n.t.
A56	1	45	Postorna	July 2002	**640**	≤ 20	20
A58	1	40	Charv. N. Ves	July 2002	**20**	< 20	n.t.
B2	1	38	Novy Dvur	July 2002	**160**	< 20	n.t.
B23	1–2	70	Divaky	Nov. 2000	**320**	< 20	n.t.
B13	> 2	70	Tisnov (BV)	Nov. 2000	**80**	< 20	n.t.
A42	3	80	Lanzhot	July 2002	**80**	< 20	n.t.
A48	3	70	Lanzhot	July 2002	< 20	20	**40**
A51	3	80	Tvrdonice	July 2002	**320**	< 20	n.t.
A60	4	80	Lanzhot	July 2002	< 20	40	**80**
B27	4	80	Boleradice	Jan. 2001	**640**	< 20	n.t.
A59	7	150	Lanzhot	July 2002	**640**	< 20	n.t.

n.t. = not tested; titres in bold are regarded as specific for particular virus

(Table 1). The three age groups of the examined boars were: (1) < 1 year (37 animals); (2) 1 to 2 years (46 animals); and (3) > 2 years (10 animals). There were four boars (10.8%) seropositive for TAHV in the first age group, nine (19.6%) seropositives in the second group, while five (50.0%) animals in the oldest group had specific antibodies to TAHV. The differences in the seropositivity rate between the three age groups of the wild boar are statistically significant: $\chi^2 = 7.748$ ($P = 0.021$; *d.f.* = 2). The significant pair-wise differences are those between the age groups 1 and 3 ($\chi^2 = 7.809$; $P = 0.005$; *d.f.* = 1), and between the age groups 2 and 3 ($\chi^2 = 4.058$; $P = 0.044$), the difference between the age groups 1 and 2 ($\chi^2 = 1.190$; $P = 0.275$) is not significant. The oldest wild boars, therefore, showed a significantly higher proportion of seropositive animals. This statistical comparison could not be applied to the other viruses due to low numbers of seropositive individuals.

Only one animal (a 7-year old boar A59, shot on Lanzhot hunting grounds in July 2002) seroreacted with BATV (1.1%), at a low titre of 1:20.

The sera, when additionally examined by HIT against SINV, revealed two boars (2.2%) with antibodies: a young animal B4, shot on Boleradice hunting grounds in October 2000, and a > 2-year old boar B13 shot on Tisnov (BV) hunting grounds in November 2000; the titres of antibodies were 1:20 and 1:80, respectively.

DISCUSSION

In this study, we used PRNμT for all viruses except for SINV. The neutralization test is regarded as the 'gold standard' in arbovirus serology and is used for the verification of other serological tests (ELISA, heamagglutination-inhibition test – HIT) because it is generally more specific and discriminatory. However, it is well known that, e.g., flaviviruses are responsible for a high degree of serological cross-reactivity, sometimes even in the neutralization test (Theiler and Downs, 1973; Madrid and Porterfield, 1974; Calisher et al., 1989; Weingartl et al., 2003; Weissenboock et al., 2003; Niedrig et al., 2007). Several antigenically related flaviviruses might co-occur in one area, e.g., TBEV and WNV in Central Europe. It is sometimes very difficult to decide which particular antigen is responsible for the antibody production – controversial results may thus be published. It is always necessary to interpret results of flavivirus (WNV) serology with great care, especially those obtained from serosurveys in wild vertebrates (e.g., shot-killed game animals) where non-specific inhibitors of viruses may occasionally occur (e.g., Holden et al., 1965; Theiler and Downs, 1973). In the PRNμT, we estimated the results conservatively, as a 90% reduction in the number of plaques (not a 50% reduction which is sometimes used), and 1:20 dilution (instead of the usual 1:10) as a titre cut-off point.

In general, the data indicate a limited WNV activity in South Moravia during 2000–2002, restricted to the area of the "Soutok" game reserve at Lanzhot. This is in concordance with previous attempts at isolation, when WNV was detected in *Culex pipiens* mosquitoes in this area previously (Hubalek et al., 1998, 2000). It is possible that antibodies detected in wild boar were formed to the local (enzootic) genomic lineage 3 ("Rabensburg") of WNV, antigenically indistinguishable from the lineages 1 and 2 of WNV (Bakonyi et al., 2005). In nearby western Slovakia (Zahorska lowland), where WNV was also isolated from mosquitoes (Labuda et al., 1974), Kozuch et al. (1976) detected neutralizing antibodies to WNV in 2.0% of examined wild boars.

TAHV antibodies, on the other hand, were detected in wild boar at a much higher frequency (19.4%) and over a wider range, involving the hunting grounds of Lanzhot, Tvrdonice, Postorna, Lednice, Valtice, Podivin, Zajeci, and Boleradice, all characterized by the presence of floodplain forest or wetland/fishpond ecosystems and by abundant mosquito populations. We found that the older the boar, the higher the probability of the presence of TAHV antibodies. Such a pattern is typical for animals living in an enzootic, long-term natural focus of infection. However, in the past, the seroprevalence was even higher. For instance Juricova (1992) and Juricova and Hubalek (1999) found 41.7% and 46.7% of wild boar with TAHV haemagglutination-inhibiting antibodies in the years 1990 and 1993–1997, respectively. In addition, as much as 73.3% (Danielova and Marhoul, 1968; neutralization test) and 54.8% (Kolman, 1973; HIT) of South-Moravian domestic pigs were found seropositive against TAHV in 1963–1964. Kozuch et al. (1976) detected antibodies neutralizing TAHV in 28.9% of examined wild boars in western Slovakia, and Aspock and Kunz (1971) found antibodies against TAHV in one of four tested wild boars in eastern Austria.

BATV antibodies were detected at a very low level in the present study. In the past, Juricova

and Hubalek (1999) found as much as 18.7% of wild boars in South Moravia with BATV haemagglutination-inhibiting antibodies. Kozuch et al. (1976) detected antibodies neutralizing BATV in 5.3% of wild boars in western Slovakia. In addition, Kolman (1973) detected BATV antibodies in 17.2% of South-Moravian domestic pigs in 1964.

SINV antibodies were also detected at a low frequency in this study. Previously, Juricova (1992) did not detect SINV antibodies in wild boar sampled in 1990, while 10.0% of boars were seropositive during 1993–1997 in South Moravia (Juricova and Hubalek, 1999). Kozuch et al. (1976) detected no antibodies neutralizing SINV in wild boars in western Slovakia.

We did not test the wild boars for the presence of antibodies against the two remaining mosquito-borne viruses known to occur sporadically in Central Europe, i.e. USUV and LEDV. These viruses are ornithophilic, and antibodies against them will be mainly found in free-living birds, not in mammals. Bird groups susceptible to LEDV are wetland anseriforms (Malkova et al., 1986) and for USUV largely thrushes (family Turdidae) and birds of prey (Weissenbock et al., 2002).

In conclusion, the serosurvey in wild boars indicates that the activity of mosquito-borne viruses has decreased compared with the past decades in South Moravia. However, long-term precipitations or floods could reverse the situation, and surveillance for these viruses still remains necessary.

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