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Serological survey of domestic animals for tick-borne encephalitis and Bhanja viruses in northeastern Hungary

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ABSTRACT

Blood sera collected from 400 domestic animals (260 cattle, 100 Merino sheep, and 40 Hutzul horses) in northeastern Hungary in 2005 were examined for antibodies against two tick-borne viruses, tick-borne encephalitis flavivirus (TBEV) and Bhanja bunyavirus (BHAV). Using ELISA as screening test and plaque-reduction neutralization as confirmatory test, seropositivity to TBEV was found to be 26.5% in cattle, 7.0% in sheep, and 0.0% in horses. Among cattle, the animals up to 3 years old had significantly lower seroprevalence rate than those in older age groups. Natural foci of tick-borne encephalitis are obviously present in northeastern Hungary. On the other hand, no antibodies neutralizing BHAV were detected in the domestic animals.

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1. Introduction

Tick-borne encephalitis flavivirus (TBEV), family Flaviviridae, is the agent of tick-borne encephalitis (TBE), endemic in many European countries including Hungary. Three antigenically distinct subtypes cause TBE (Theiler and Downs, 1973; Randolph, 2008): the Western or Central European encephalitis subtype (W-TBE or CEE) has been isolated from most European countries and the European part of Russia, while the Siberian (S-TBE) and Far-Eastern (FE-TBE) subtype strains extend from European and Asian Russia (and Japan) to Finland and the Baltic countries. Thus all three subtypes circulate within Latvia, Estonia and Finland (Lundkvist et al., 2001; Golovljova et al., 2004;

Jääskeläinen et al., 2006), but only W-TBE has been recorded in Lithuania (Mickiene et al., 2001). This pattern corresponds to the distributions of the competent tick vectors of TBE: the principal vector of the W-TBE subtype is the hard tick *Ixodes ricinus*, while the main vector of the S-TBE and FE-TBE subtype is *Ixodes persulcatus* (Randolph, 2008). Central European encephalitis (W-TBE) infection is usually subclinical in adult ruminants. Epidemiologically important, goat, sheep and cow excrete the virus in the milk (Van Tongeren, 1955; Grešíková, 1958a,b). Meningoencephalitis with ataxia, jumping, tremor and convulsions can affect lambs (Papadopoulos, 1980).

A very similar disease, caused by louping ill virus (LIV), occurs in sheep of the British Isles—the agent is very closely related to TBEV, in fact it is another (the westernmost) subtype of TBE, because antigenic and genomic similarity between LIV and W-TBE is higher than that between W-TBE and S-TBE (Shiu et al., 1991; Hubálek et al., 1995).

Bhanja virus (BHAV), family Bunyaviridae, causes meningoencephalitis in lambs and leucopaenia in cattle (Theiler and Downs, 1973) and is distributed in southern

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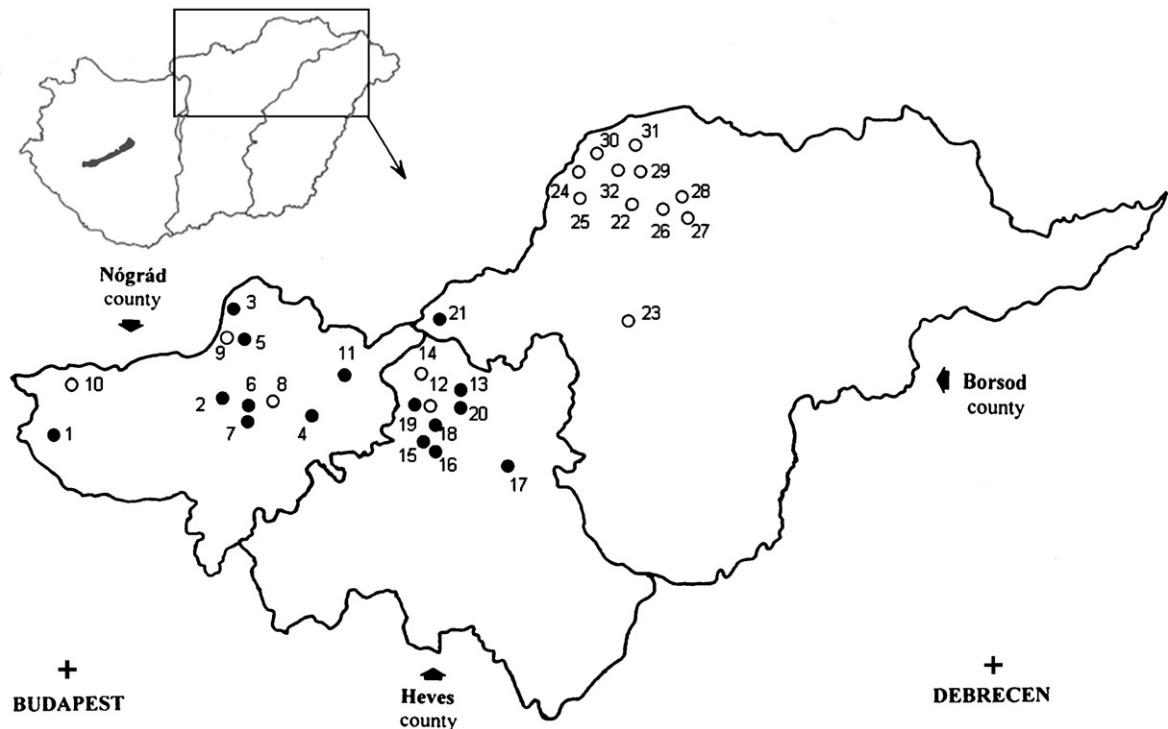


Fig. 1. Geographical distribution of animal sera investigated. Cattle sera were from the following locations: (1) Diósjenő; (2) Nógrádsipek; (3) Nógrádszakál; (4) Sámsonháza; (5) Piliny; (6) Zsunypuszta; (7) Felsőtold; (8) Kisbárkány; (9) Ludányhalászi; (10) Patak; (11) Kazár; (12) Pétervására; (13) Tarnaleesz; (14) Istenmezeje; (15) Mátraderecske; (16) Recsk; (17) Egerszólát; (18) Kisfüzes; (19) Ivád; (20) Fedémes; (21) Domaháza; (22) Alsótelkes; (23) Sajólászlófalva; (24) Aggtelek; (25) Trizs; (26) Szendrő; (27) Abod; (28) Galvács; (29) Perkupa; (30) Jósavafő; (31) Szögliget; (32) Szinpetri. Places where seropositivity was detected are marked by solid circles, lack of seropositivity is indicated by open circles. Horse sera came from location 30 and sheep sera came from location 21.

Asia, Africa and southeastern Europe (Hubálek, 1987). In Europe, BHAV is transmitted by metastriate ixodid ticks *Haemaphysalis punctata* and *Dermacentor marginatus*. Veterinary monitoring of sheep and goats in natural foci has been recommended (Hubálek and Halouzka, 1996).

2. Materials and methods

2.1. Serum samples

Blood samples were collected during the year 2005 from 400 grazed domestic animals in northern Hungary: 260 cattle sera from 32 localities (11 in Nógrád county, 9 in Heves county and 12 in Borsod-Abaúj-Zemplén county; Fig. 1), 100 Merino sheep sera from 5 flocks at Domaháza, and 40 horse (historical Hutzul breed) sera from Jósavafő.

2.2. Serological tests

2.2.1. TBEV

2.2.1.1. ELISA. Serum samples were tested in commercially available EIA TBEV-Ig kit (Test-Line, Ltd., Czech Republic) according to the instructions of the manufacturer. Native serum samples were inactivated at 56 °C for 30 min. The optical density was measured at 450 nm.

The test was regarded valid when the optical density (OD) value of the positive control was $\leq 0.5 \times$ average OD

of the negative control, and when OD value of the negative control was ≥ 0.200 . Results were expressed as a ratio of average OD value of the negative control/OD value of the sample. The cut-off value for positive sera was ≥ 1.9 . All these positive sera were then tested in confirmatory assay, the plaque-reduction neutralization microtest (PRN μ T).

2.2.1.2. PRNT. Plaque-reduction neutralization microtest (PRN μ T) (Hubálek et al., 1979) on SPEV (porcine embryo kidney) cells, which is based on PRNT assay suggested by Madrid and Porterfield (1969), was used with the TBEV strain Hypr—a prototype of Central European encephalitis subtype of TBEV, isolated by Pospíšil et al. (1954) from a human patient. Sera were inactivated at 56 °C for 30 min, and diluted 1:5 in Leibovitz L-15 medium (Sigma, USA). Thirty microliter of diluted sera (in duplicate) were mixed with 30 μ l of the TBEV suspension (containing about 30 plaque-forming units, PFU) in flat-bottomed microtiter plates (Sarstedt, USA), and incubated at 37 °C for 60 min. Then 60 μ l of cell suspension (about 20,000 cells) in Leibovitz L-15 medium (Sigma, USA) with 2% foetal calf serum (Sigma, USA) and antibiotics were added to each well and incubated at 37 °C for 4 h. Thereafter 120 μ l of a carboxy-methyl cellulose overlay was added to each well and incubated at 37 °C for 4 days. The cells were stained by naphthol blue-black solution for 50 min at room temperature. Sera reducing the number of PFU by 90%

(PRN μ T₉₀) at the screening dilution 1:10 were considered positive.

2.2.2. Bhanja virus

The serological assay was performed in analogy to PRN μ T used for TBEV. The BHAV strain applied was Bg 326 which was isolated from *Haemaphysalis* ticks in Bulgaria (Pavlov et al., 1978). The test was conducted on Vero E6 cells in flat-bottomed microtiter plates, and evaluated after an incubation at 37 °C for 3 days.

2.3. Statistical analysis

A SOLO statistical program (BMDP Statistical Software, Los Angeles, California, USA) with 2 × *n* tables and χ^2 -test was used to compare prevalence data between individual

counties and among age categories. Differences in proportions were considered as significant when $p \leq 0.05$.

3. Results

3.1. Tick-borne encephalitis virus

Of the 260 cattle sera tested, 69 (i.e., 26.5%) were positive for TBEV in both ELISA and PRN μ T₉₀: 29/105 (27.6%) in Nógrád county, 22/70 (31.4%) in Heves county, and 18/85 (21.2%) in Borsod-Abaúj-Zemplén county (Table 1). The difference in seroprevalence rate among counties was not significant ($\chi^2 = 2.175$; $p = 0.337$). Nevertheless, all positive bovine samples of Borsod-Abaúj-Zemplén county were collected in one place (Domaháza), where 69.2% of cattle were positive. No

Table 1
Antibodies against tick-borne encephalitis virus in cattle sera in northern Hungary.

County locality	Age (months)				Total
	≤36	37–60	61–96	97–212	
Nógrád					
(1) Diósjenő		1/9^a	1/1		2/10
(2) Nógrádsipek	0/7	1/3			1/10
(3) Nógrádszakál	1/2	0/1	0/3	1/4	2/10
(4) Sámsonháza	0/5		0/2	1/3	1/10
(5) Piliny	0/1	5/5	4/4		9/10
(6) Zsunypuszta		2/2	0/3	1/5	3/10
(7) Felsőtold		1/1	9/9		10/10
(8) Kisbárkány	0/3		0/1	0/1	0/5
(9) Ludányhalászi		0/10			0/10
(10) Patak	0/1	0/7	0/2		0/10
(11) Kazár	0/6	1/3		0/1	1/10
Total	1/25	11/41	14/25	3/14	29/105 (27.6%)
Heves					
(12) Pétervására	0/10				0/10
(13) Tarnalelesz		2/3			2/3
(14) Istenmezeje	0/2				0/2
(15) Mátraderecske			1/4	1/6	2/10
(16) Recsk		0/1	2/5	3/4	5/10
(17) Egerszólát		0/2	2/6	1/2	3/10
(18) Kisfüzes	1/5	0/2	0/3		1/10
(19) Ivád	0/2	2/4	2/3	0/1	4/10
(20) Fedémes		1/1		4/4	5/5
Total	1/19	5/13	7/21	9/17	22/70 (31.4%)
Borsod-Abaúj-Zemplén					
(21) Domaháza		6/6	2/4	10/16	18/26
(22) Alsótelkes		0/1	0/9	0/2	0/12
(23) Sajólászlófalva	0/1	0/4	0/5		0/10
(24) Aggtelek		0/2	0/1	0/2	0/5
(25) Trizs			0/2	0/2	0/4
(26) Szendrő		0/2	0/1	0/4	0/7
(27) Abod				0/2	0/2
(28) Galvács	0/1		0/1	0/1	0/3
(29) Perkupa			0/2		0/2
(30) Jósvafő				0/1	0/1
(31) Szögliget			0/1	0/1	0/2
(32) Szinpetri	0/2	0/2	0/4	0/3	0/11
Total	0/4	6/17	2/30	10/34	18/85 (21.2%)
Total, northern Hungary	2/48 (4.2%)	22/71 (31.0%)	23/76 (30.3%)	22/65 (33.8%)	69/260 (26.5%)

Bold values indicate seropositive animals.

^a No. positive/no.examined.

seropositivity was detected in the eastern part of the evaluated region (Fig. 1).

Distribution of positive cattle sera was also analyzed according to the age groups. The animals up to 36 months (3 years) old (A: young cattle) had significantly lower seroprevalence rate than those of the older age groups (B: 37–60; C: 61–96; D: 97–212-month-old age group) (A vs. B: $p = 0.0003$; A vs. C: $p = 0.0004$; A vs. D: $p = 0.0001$). No significant difference was found among the three older age categories (B vs. C: $p = 0.924$; B vs. D: $p = 0.722$; C vs. D: $p = 0.649$). Within particular counties, the seroprevalence in age categories was 4.0% (young cattle group), 26.8% (age group B), 56.0% (age group C), and 21.4% (age group D) in Nógrád county. In Heves county, seroprevalence against TBEV increased from 5.3% in the youngest cattle age group to 52.9% in the age group D. Seroprevalence in the whole Borsod-Abaúj-Zemplén county decreased from 35.3% in the age group B to 6.7% in the group C, and then increased to 29.4% (group D). All results are summarized in Table 1.

Of the 100 sheep samples, 7 were positive in both ELISA and PRN μ T₉₀. All the seropositive sheep were at least 3 years old. There was no significant difference in TBEV seroprevalence between sheep age groups corresponding to those of cattle (A: 5.3%, B: 5.7%, C: 12.5%, and D: 10%). No seropositive horses were detected.

3.2. Bhanja virus

All serum samples (260 cattle, 100 Merino sheep and 40 Hutzul horses) examined in PRN μ T against BHAV were negative.

4. Discussion

4.1. Tick-borne encephalitis virus

This is the first report on the seroprevalence of TBEV in domestic animals of northeast Hungary. The selection criteria of the study area were based on natural foci of TBE that have been described previously, taking into account the incidence of human cases (Molnár, 1982; Ferenczi et al., 2005; Rác et al., 2006). In particular, for TBE risk-assessment a good correlation was demonstrated between the incidence of disease and the level of forestation (Rác et al., 2006). In this way the region evaluated in the present study (northeast Hungary) was estimated to have a similar rate of exposure as the area (southwest Hungary) recognized with the highest risk of TBE. Furthermore, although the overall number of diagnosed human TBE cases in Hungary significantly decreased between 1991 and 2000, this could largely be attributed to a tendency of decline in the southern part of the country, whereas the incidence remained relatively constant in the northern region (Ferenczi et al., 2005).

At the same time, seropositivity of cattle or sheep to TBEV has only been evaluated in regions other than northeast Hungary (Molnár, 1982), and no similar data have been available on horses. Interestingly, the proportion of cattle showing seropositivity was lowest in the western part of the country (3–15.7%). However, the incidence of antibodies to TBEV in samples of cows (38.8%)

from the southeastern region, and of sheep (19%) in northwestern Hungary (Molnár, 1982) exceeded the prevalence rates in northeast Hungary reported in the present study (i.e., 26.5% in cattle and 7% in sheep).

In neighbouring southeastern Slovakia, several sero-surveys for TBEV were carried out among local domestic animals including sheep and cattle. For instance, Hubálek et al. (1985, 1986) found haemagglutination-inhibiting and neutralizing (PRNT) antibodies to TBEV in 8–25% of sheep, 44–54% of goats, and 2–14% of cattle sampled during 1982 and 1983.

As a plausible explanation for the high overall seroprevalence reported here for TBEV in cattle, all sampled animals were beef producers kept extensively, which usually implies a high level of repeated tick infestation (S. Hornok, personal observation). Thus the chances for TBEV transmission also become greater with the advance of age, as indicated by higher rates of seropositivity in older animals of the present study. Exposure to infected ticks is still regarded as the major risk factor in contracting TBEV, despite the fact that raw milk consumption has been implicated in human TBE epidemiology in several European countries (Grešiková, 1958b; Rieger et al., 1998). Potential of cow-to-calf natural transmission of TBEV should also deserve attention and evaluation, especially if the calving period coincides with the highest spring activity of ticks.

TBEV seroprevalence among sheep at Domaháza was approximately only one-tenth of that detected in local cattle. This suggests that although the age distribution, the annual period spent on pastures and the extent of grazed area was similar for herds and flocks in the relevant region, there still may have been differences in the rate of tick exposure between cattle and sheep. This could be, in part, attributed to their unique grazing habit or feeding preference, influencing contact with ticks which quest at certain heights on the vegetation. Cattle and sheep are also known to have variable predisposition for tick attachment, depending on body surface and predilection sites (Ogore et al., 1999).

Since ixodid ticks were found on horses during the present study (data not shown), their TBEV seronegativity cannot be explained by the lack of vector availability. On the other hand, horse samples were obtained in an area (Jósvafő) where cattle were also found seronegative. This result indicates that the eastern part of the evaluated region of northern Hungary appears to be non-endemic.

4.2. Bhanja virus

The seronegativity of grazed domestic animals (sheep, cattle, horse) for BHAV in northern Hungary has been surprising in that antibodies neutralizing this bunyavirus were detected some 30 years ago in the neighbouring Slovak territory in 63% of 19 examined goats as well as in 7% of 28 sheep (Bárdoš et al., 1977), later in 27% of 120 sheep (Hubálek and Juřicová, 1984; Hubálek et al., 1985, 1986) and then the virus was also isolated from *D. marginatus* ticks (Hubálek et al., 1988) in the Slovak Karst at Kečovo area, ecologically identical to, and the continuation of, the Hungarian Aggtelek Karst at Jósvafő. It is possible that either the BHAV activity decreased in this

region, or the domestic animals tested in Hungary did not have effective contact with the main European tick vector of this virus, *H. punctata* (Hubálek et al., 1985). Disappearance of *H. punctata* from formerly inhabited places of the three evaluated counties was recently reported (Hornok and Farkas, in press). In conclusion, an updated evaluation of the occurrence of BHAV in other endemic parts of eastern Europe is strongly encouraged.

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