PREVALENCE OF *BORRELIA BURGDORFERI* SENSU LATO GENOSPECIES IN HOST-SEEKING *IXODES RICINUS* TICKS IN SELECTED SOUTH BOHEMIAN LOCATIONS (CZECH REPUBLIC)

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SUMMARY

In selected localities of České Budějovice and Český Krumlov districts, well known by stable high incidence of tick-borne encephalitis (TBE) human cases but with low incidence of Lyme borreliosis, monitoring of *Borrelia burgdorferi* sensu lato (s.l.) in *Ixodes ricinus* ticks was performed. Research was also aimed at the spread of *I. ricinus* to mountain areas of this region (National Park Šumava), as well as at investigating this tick for *B. burgdorferi* s. l. genospecies and TBE virus infection. Altogether 498 nymphs, 88 females and 11 males of *I. ricinus* from lower locations and 58 nymphs from mountain locations (760 – 1080 m above sea level) were tested by polymerase chain reaction. In lower locations total prevalence of *Borrelia burgdorferi* s.l. in *Ixodes ricinus* ticks was 35 %.

Single infection of *Borrelia afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto (s.s.) was found in 59, 50 and 63 ticks, respectively (i.e. in 12.8, 11.2 and 14.1 %). Double infection was found in 42 ticks (6.0 %) and triple infection in three ticks (0.4 %). The high frequency of *B. burgdorferi* s.s. exceeds the as yet reported occurrence in Central Europe. These circumstances are discussed. In mountain locations *B. afzelii* was found in five ticks, that including two co-infection with *B. garinii*, in elevations of 762 m and 1 024 m above sea level, respectively. This fact signals a real danger of human infections in a region that was previously deemed to be without risk. Moreover, this region is more and more the target destination of tourist activities. The results also suggest that the penetration of infection can be rapid and formation and establishment of natural focus of Lyme borreliosis might be rather quick.

Key words: *Ixodes ricinus, Borrelia afzelii*, *Borrelia garinii*, *Borrelia burgdorferi* sensu stricto, high altitude, Czech Republic

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INTRODUCTION

The sharp increase in the incidence of tick-borne encephalitis (TBE) observed in the entire distribution area in Europe in the last decade has also occurred in the Czech Republic (CR), not only in terms of increased occurrence of this infection in areas where it has already been known to be distributed, but also in the form of spreading to new areas, including higher altitudes above sea level. As part of a study of factors contributing to this phenomenon, research was carried out concerning *Ixodes ricinus* ticks and their spontaneous infection with the TBE virus in the region of South Bohemia, in locations with permanently increased occurrence of human infection with this virus, which considerably exceeds the nation-wide average. This research was conducted in 2000 – 2002 (1). By comparison with TBE, the incidence of Lyme borreliosis (LB) in humans in this area is significantly lower. The objective of this work was therefore to examine the infection rate in *I. ricinus* ticks collected in the above high-activity foci of TBE with borreliae, as well as the representation of individual *B. burgdorferi* sensu lato (s.l.) genospecies pathogenic for humans. Genospecies representation in ticks is not known in the Czech Republic as yet.

Part of this research was also concerned with the issue of penetration of *I. ricinus* ticks into the mountainous area of this region (Šumava National Park) that has found an expression in the TBE disease (2), and examination of the ticks to establish infection both with the TBE virus and individual *B. burgdorferi* s.l. genospecies

MATERIAL AND METHODS

Ticks

I. ricinus ticks were collected using the method of flagging on 7 habitats in 5 selected localities in České Budějovice and Český Krumlov districts, South Bohemian region (48° 44′ 16.91″ – 48°

Table 1. *Population density of Ixodes ricinus ticks in single localities and habitats – South Bohemia Region, Czech Republic – May 2001*

Location	Number of ticks collected				Average per 100 m²		
	Female	Male	Nymphs	Total	Female	Male	Nymphs
Borovany habitat 1	37	36	350	423	3.l	3.0	29.2
Borovany habitat 2	26	30	600	656	1.7	2.0	40.0
Borovany habitat 3	62	62	285	409	5.2	5.2	23.8
Římov	105	122	372	599	8.4	9.8	29.8
Nové Hrady	27	24	417	468	1.9	1.5	26.1
Kaplice	11	17	418	446	0.9	1.4	34.8
Trhové Sviny	41	47	387	475	5.1	5.9	48.4
Total	309	338	2829	3476			

54′ 0.355″N; 14° 27′ 17.20″ – 14° 46′ 48.43″ E) in spring, in the period from 9 May to 26 May 2001. A total of 3,476 *I. ricinus* specimens were collected. The design of tick collection made it possible to describe the tick population status in this spring period and express it as a number/100 m², and compare it with previous period (1) (Table 1). Most of the ticks were examined for the presence of the TBE virus, and *I. ricinus* specimens (nymphs and adult ticks), 100 from each individual location and habitat, respectively, were examined individually for the presence of borreliae.

In the territory of the Šumava National Park, the collection of ticks was conducted in the altitude range of 762–1,080 metres above sea level (48° 52′ 207″ – 48° 56′ 814″; 13° 38′ 663″ – 13° 51′ 883″) in the period from May 20 to May 29 2002. Most of the ticks collected were examined in isolation experiments for the TBE virus, and a small part, particularly from the highest altitude locations, for the presence of individual *B. burgdorferi* s.l. genospecies.

Habitat Characteristics

Borovany – habitat 1: Border zone between tall mixed woods (oak, pine, spruce) and stands of young oak trees characterized by thick canopy.

- habitat 2: Margin of mixed woods. Three layers with two sub-layers: the upper consists of adult tall oak, pine and singe spruce trees; the lower, of dense young oak trees
- habitat 3: Narrow strip of deciduous woods along a rivulet (oak, hornbeam, alder) with abundant herb undergrowth (mainly *Calamagrostis epigeios*)

Kaplice – small isolated deciduous wood stands (oak, birch, lime trees and shrubs of *Corylus avellana* surrounded by cornfields.

Římov – ravine: bottom covered by abundant herb layer; slopes covered by mixed woods.

Nové Hrady – margin of mixed woods with oak, lime and single spruce trees

Trhové Sviny – margin of small mixed wood stands (oak, pine) forming an "island" surrounded and isolated by wet meadows.

Šumava localities and habitats:

Stodůlky – (48° 55.579′ N – 13°38.663′ E; 1024 m a.s.l.). Sites under solitary mighty spruce trees with branches reaching down to the ground in grassland.

Dobrá – (48° 52.207′ N – 13° 51.833′ E; 762 m a.s.l.). Margin of the mixed woods.

The Detection and Determination of *B. burgdorferi* s.l. genospecies was conducted using a polymerase chain reaction (PCR).

Samples preparation with Chelex® 100 Resin. The sample preparation involved no DNA or RNA purification. The most sufficient way appeared to be the treatment of the ticks by Chelex® 100 resin (Bio-Rad Laboratories, USA). The resin binds all possible inhibitors of the PCR reaction, which may be present in the samples. The 5% Chelex suspension in TE buffer (10 mM Tris-Cl pH 7.2; 1 mM EDTA pH 8.0) for the routine samples treatment was used. Ticks were frozen and then placed one per tube with 5% Chelex 100 suspension (200 µl for nymphs and 500 µl for adult tick). One tick at a time was cut inside the tube using sharp scalpel. The cutting was performed under the liquid level and the scalpel was flamed each time before the next tick cutting was processed. Adult ticks were cut into 4–6 pieces and nymphs into 2–4 pieces. Tube was vortexed vigorously and incubated overnight (or at least 12 hours) at 56 °C. The samples were then incubated at 98–100 °C for 15 minutes, vortexed for 10 seconds and chilled on ice. Supernatant was used directly in PCR or RT-PCR. Samples are stable at 4 °C.

Primer selection. Seventeen primers for PCR designated by different authors and directed on different regions of the borrelia genome were checked (3, 4). The best results were obtained with the primers based on sequences of *B. burgdorferi* s.l. ospA gene. Primers were designed for use in the PCR to type all (SL – for *B. burgdorferi* s.l.) or each (GI – for *B. burgdorferi* s.s., GII – for *B. garinii* and GIII – for *B. afzelii*) of the spirochete genospecies involved in Lyme disease (5). Primers were synthesized by Generi-Biotech (Czech Republic).

DNA amplification. 25 µl of supernatant from Chelex 100 treated sample were used as a template in PCR under the following conditions: 95 °C – 5 min, 30 cycles of (95 °C –30 s, 55 °C – 30 s, 72 °C – 1 min). Due to high A/T contents in *B. burgdorferi* genome, the dNTPs mixture was prepared as follows: 26 µl 10 mM ATP, 26 µl 10 mM dTTP, 14 µl 10 mM dCTP and 14 µl 10 mM dGTP were mixed and used in PCR reactions in final concentration 100 µM. Twenty pmol of each primer was used in 50 µl reaction (according recommendation of Promega, USA). PCR products were resolved on 1% agarose electrophoresis gel with the following visualization under UV light.

Table 2. *Prevalence of Borrelia burgdorferi s.l. in Ixodes ricinus in single localities and habitats*

Locality /Habitat	Nymphs			Females			Males			Total		
	Number		% infected	Number		% infected	Number		% infected	Number		% infected
	tested	infected		tested	infected		tested	infected		tested	infected	
Borovany 1	69	39	57	19	7	37	12	3	25	100	49	49
Borovany 2	74	27	37	12	5	42	14	0	0	100	32	32
Borovany 3	61	28	46	18	4	22	21	1	5	100	33	33
Římov	66	22	33	10	8	80	24	1	4	100	31	31
Nové Hrady	75	27	36	3	0	0	19	6	32	97	33	34
Kaplice	81	30	37	12	8	67	7	0	0	100	38	38
Trhové Sviny	72	17	24	14	3	21	14	5	36	100	25	25
Total	498	190	38	88	35	40	111	16	14	697	241	35

Table 3. *Frequency of occurrence of Borrelia burgdorferi s.l. genospecies in Ixodes ricinus ticks (n = 697) – South Bohemia Region, Czech Republic – May 2001*

Total			Single infection			Co-infection		
Genospecies	Number	%	Genospecies	Number	%	Genospecies	Number	%
B. afzelii	89	12.8	*B. afzelii*	59	8.5	*B. afz.+ gar.*	10	1.4
B. garinii	78	11.2	*B. garinii*	50	7.2	*B. afz.*+ s.s.	17	2.4
B.b. s.s.	98	14.1	*B.b.* s.s.	63	9.0	*B. gar.*+ s.s.	15	2.2
						B. afz. + gar.+ s.s.	3	0.4

The detection of the TBE virus in ticks was conducted using an isolation test on pork kidney cell line followed by three blind passages (20 N, 4–6 F, and 7–13 M) and has been described in more detail by Danielová et al. (1).

RESULTS

Region of the České Budějovice and Český Krumlov Districts

The density of *I. ricinus* tick populations varied significantly with the individual locations and habitats. It is expressed as a part of the total number of ticks per 100 m^2 collected in individual locations and habitats (Table 1).

Out of the total number 3,479 ticks, 498 nymphs, 88 females and 111 males have been examined for the presence of borreliae, targeting the following genospecies of interest: *B. afzelii*, *B. garinii*, and *B. burgdorferi* s.s. The prevalence of *B. burgdorferi* s.l. by individual *I. ricinus* stages, various locations, and habitats is given in Table 2. It is shown from the numbers given that the proportions of the total number of positive ticks were as follows: nymphs were represented at 79%, females at 14%, and males at 7%. The total prevalence of *B. burgdorferi* s.l. in ticks reached 35 %. Surprising finding is that the incidence in nymphs exceeds that in adults (38 versus 26 %).

The ticks examined were found to contain *B. afzelii*, *B. garinii*, and *B. burgdorferi* s.s. The representation of individual genospecies is given in Table 3. The genospecies in several positive samples remained undetermined – these include different genospecies not defined for the purpose of the research, or technical reasons.

The proportions of the individual genospecies represented as well as their co-infection are given in Fig. 1. It is apparent from this overall result that the frequency of occurrence of *B. burg-*

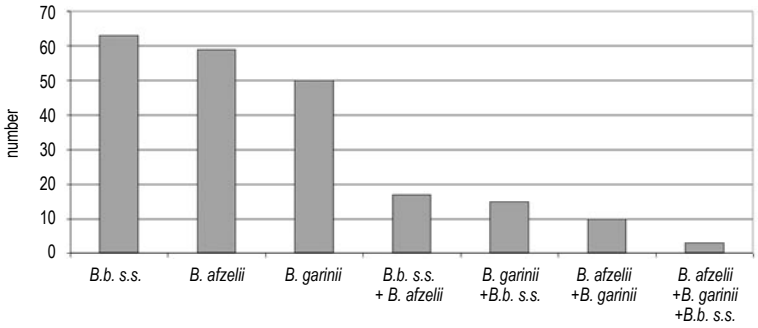

Fig. 1. *Genospecies Borrelia burgdorferi s.l. found in Ixodes ricinus ticks (n= 697) in the South Bohemian Region, Czech Republic – May 2001.*

Table 4. *Infection and co-infection of Borrelia burgdorferi s.l. genospecies in infected Ixodes ricinus ticks by localities and habitats – South Bohemian Region, Czech Republic – May 2001*

Locality/Habitat	Single	Number	%	Co-infection	Number	%
Borovany 1 n = 49	*B. afzelii*	8	16	B.afz.+gar.	4	8
	B. garinii	12	25	B.afz.+s.s.	1	2
	B.b. s.s.	19	39	B.gar.+s.s.	2	4
	B.b. s.l.	3	6			
Borovany 2 n = 32	*B. afzelii*	4	13	B.afz.+gar.	3	9
	B. garinii	6	19	B.afz.+s.s.	1	3
	B.b. s.s.	11	34	B.gar.+s.s.	4	13
	B.b. s.l..	3	9			
Borovany 3 n = 33	*B. afzelii*	6	18	B.afz.+s.s.	1	3
	B. garinii	11	33	B.gar.+s.s.	4	12
	B.b. s.s.	8	24			
	B.b.s..l.	3	9			
Římov n = 31	*B. afzelii*	11	36	B.afz.+gar.	1	3
	B. garinii	4	13	B.afz.+s.s.	4	13
	B.b. s.s.	6	19	B.afz.+gar.+s.s.	2	6
	B.b..s.l.	3	10			
Nové Hrady n = 33	*B. afzelii*	14	43	B.afz.+s.s.	2	6
	B. garinii	6	18	B.gar.+s.s.	3	9
	B.b. .ss.	3	9			
	B.b. s.l.	5	15			
Kaplice n = 38	*B. afzelii*	8	21	B.afz.+s.s.	7	19
	B. garinii	9	24	B.gar.+s.s.	1	2
	B.b. s.s.	9	24			
	B.b. s.l.	4	10			
Trhové Sviny n = 25	*B. afzelii*	8	32	B.afz.+gar.	2	8
	B. garinii	2	8	B.afz.+s.s.	1	4
	B.b. s.s.	7	28	B.gar.+s.s.	1	4
	B.b. s.l.	3	12	B.afz.+gar.+s.s.	1	4

dorferi s.s. was distinctly high, and its proportion exceeds the findings reported from Central Europe thus far.

The ratios of the genospecies found varied with the individual locations and habitats (Table 4). These habitats constitute 5 groups based on similar individual genospecies ratios (Fig. 2). A commentary to this fact is included in the discussion.

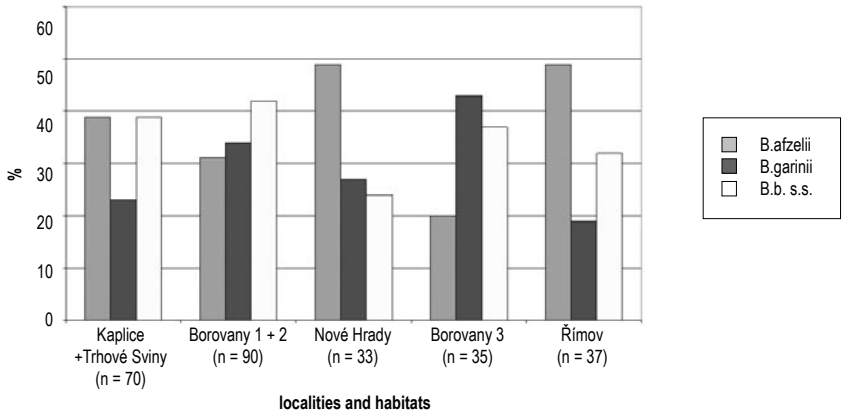

Fig. 2. *Differences in prevalence of Borrelia burgdorferi s.l. genospecies in Ixodes ricinus ticks in different types of habitats – South Bohemian Region, Czech Republic – May 2001.*

***Table 5**. Clinical manifestation of Lyme borreliosis in patiens from the districts České Budějovice and Český Krumlov*

Year	Manifestation			
	Dermal (ECM)	Neurological	Arthritic	Total
1997	12	5	1	18
1998	15	5	1	21
1999	20	10	3	33
2000	30	9	2	41
2001	12	1	2	15
Total	89	30	9	128
%	70	23	7	100

Different genospecies ratios were also found for individual stages and sex status of *I. ricinus*, as it is documented by findings from the habitats Borovany 1 and Římov. *B. burgdorferi* s.s. was markedly predominant in females and conversely rather low positivity in males was found (Fig. 3).

Due to the high proportion of *B. burgdorferi* s.s. (known to cause chiefly the arthritic disorders) found in the ticks, we sought to establish the proportion of Lyme borreliosis with symptoms of arthritis in patients from the České Budějovice and Český Krumlov districts. Table 5 gives excerpted data on 5-year morbidity (including the year of research and the four previous years) obtained from the EPIDAT database, National Institute of Public Health, Prague.

Šumava National Park (NP) Region

In NP Šumava, a total of 430 *I. ricinus* ticks were collected. Of these, 246 nymphs, 61 females, and 65 males were examined in isolation experiments for the presence of the TBE virus with negative results.

Regarding the presence of individual *Borrelia burgdorferi* s.l. genospecies, a total of 58 nymphs *I. ricinus* ticks were examined. Three of 20 *I. ricinus* nymphs tested from the locality Dobrá (762 m above sea level) were infected with *B. afzelii*, that included twice co-infection with *B. garinii*. Likewise two nymphs from 20 tested in the locality Stodůlky (1024 m above sea level) were infected with *B. afzelii*. Eighteen nymphs from other localities (927 – 1080 m above sea level) were negative.

DISCUSSION

In our study *B. burgdorferi* s.l. genospecies were detected in tick samples by polymerase chain reaction. Many different methods of sample treatment for obtaining better results in PCR reactions were already described, but all of them included the DNA purification step for its further use as a template in polymerase chain reaction. However, in spite of all positive features, any purification leads to a loss of DNA, at times considerable. We have used the technique that involves no DNA purification, but includes the removal or limitation of PCR inhibiting substances from the sample. Chelex® 100 resin binds the remaining inhibitors of the PCR reaction and helps with the stabilization of double stranded DNA (6). We have optimized the PCR cycle to minimize the duration of the reaction. The amount of amplified pathogen DNA was sufficient to be visualized on the agarose gel without further re-amplification of the PCR product. The use of SYBR Green I increased the sensitivity of DNA detection on the gel.

It is difficult to draw a precise map of the distribution and frequency of different *Borrelia burgdorferi* s.l. genospecies in continental Europe until more data have been collected (7). This corresponds with the situation in the Czech Republic; an overview published by Hubálek and Halouzka, 1997 (8), states only the presence of *B. afzelii*, *B. garinii* and a single finding of B. lusitaniae. The high frequency of *B. burgdorferi* s.s. is, therefore, a rather serious result, also in comparison with literary data. Beside it, literary sources state that the frequency of *B. burgdorferi* s.s. seems to decrease in Europe from West to East (7, 9).

Contrary to most literary data (10) that the frequency of occurrence of *Borrelia burgdorferi* s.l. is usually higher in adults than in nymphs, our frequency of occurrence in individual genospecies is practically identical for *I. ricinus* nymphs and femals. The decisive point is, therefore, that the ticks must have acquired the infection in the larval stage. Interestingly, there was a difference in the genospecies ratios found in various locations and habitats. There is no concrete comparative data so far for this finding in the literature. This can relate to the different character of the habitats determining a difference in the ecosystems in which borreliae circulate. The character of these habitats influences mainly the composition of hosts for lower developmental tick stages. In our observation, the difference was relevant mainly in three habitat groups:

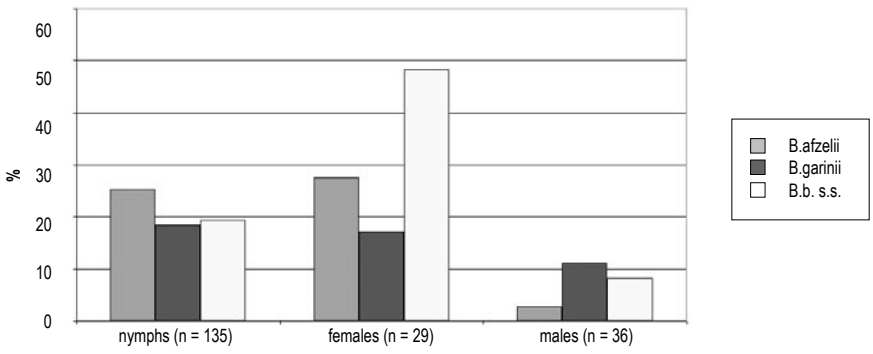

***Fig. 3.** Percentage of Borrelia burgdorferi s.l. occurrence in Ixodes ricinus nymphs, females and males tested. Location Borovany 1 and Římov – South Bohemian Region, Czech Republic – May 2001.*

a) margins of large wood complexes – abundant in songbirds (Borovany 1 and 2);
b) isolated groves – here, small mammals are significant, and there is the periodic presence of small ground mammals from adjacent agricultural areas (Kaplice, Trhové Sviny);
c) narrow penetrating small valleys (ravines) (Římov).

The importance of the relation of different genospecies to the habitat of occurrence is also discussed by Kurtenbach et al. (11)

We have observed a disparity between the relatively high percentage of ticks infected with borreliae and the low numbers of human Lyme borreliosis diseases. At the same time, there was also an obvious disparity between the high incidence of human infection with tick-borne encephalitis virus and the low incidence of Lyme borreliosis (at an identical level of human contact with ticks). The general conclusion made on a nation-wide scale describing the relation of these two infections as being clearly in favour of Lyme borreliosis does not hold true for this situation. So far, this anomaly has not been pointed out and further research is required to provide an adequate explanation.

We attempted to document this circumstance, at least in part, using data from the EPIDAT database. Regarding the high frequency of *B. burgdorferi* s.s., known in the first place to be the cause of arthritic symptoms of the disease (9), we focused on the occurrence of these symptoms in inhabitants from both above-mentioned districts, as in the localities under study there has been reported 14 Lyme borreliosis cases (5 dermal, 6 neurological, 3 arthritic symptoms) only in 1997 – 2001, that means too low amount. In spite of being aware of all factors limiting the validity of the conclusions, in particular the low numbers and pitfalls in the field of diagnostics, we assume that the relatively high number of neurological and arthritic cases compared to skin symptoms of borreliosis might have a factual basis in the spectrum of genospecies found in ticks.

The high infection rate of *I. ricinus* ticks with different *B. burgdorferi* s.l genospecies and their occurrence in recreation mountain areas indicate a high risk of Lyme borreliosis and the necessity to count with it also in these areas, where formerly it was not taken into account, in order to start with antibiotic therapy in time, as later treatment is less effective or ineffective.

It is the only possibility to prevent a severe course of this illness, as no vaccine against European genospecies exists yet. Moreover, neither vaccination against TBE which is available on the Czech market since the beginning of the 90´s has not decreased the incidence of this disease, though it is the only protection against TBE as any specific therapy does not exist so far. Despite a massive public education campaign, the vaccination rate in the Czech Republic is estimated at 10 %, which according to Austrian experience is not sufficient for a decrease in general morbidity (12).

CONCLUSION

There has been found a significantly higher representation of *B. burgdorferi* s.s. genospecies than has been known in Central Europe.

Different representation of *B. burgdorferi* s.l genospecies not only in close localities, but also in the same locality but in different habitats is not given by the geographical position but by the diversity of habitats, primarily by the tick host composition.

The occurrence rate of *B. afzelii*, *B. garinii* in ticks found at the altitude over 1,000 metres above sea level signals a real danger of human infections in a region that was previously deemed to be without risk. Moreover, this region is more and more a target destination of tourist activities. The results also suggest that the penetration and formation of a focus of infection is easier and more rapid in the case of Lyme borreliosis than in the case of tick-borne encephalitis.

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