

Comparison of virulence of *Coxiella burnetii* isolates from bovine milk and from ticks

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Abstract. Laboratory animals (mice and guinea pigs) were infected with the isolates of *Coxiella burnetii* (Derrick, 1939) obtained from bovine milk (M18 and M35) and the ticks *Ixodes ricinus* (Linnaeus, 1758) and *Dermacentor marginatus* (Sulzer, 1776) (Kl₃ and Kl₆, respectively), and with the reference strain Nine Mile. Neither mortality nor lethality occurred with the mice. Antibody response in mice infected with isolates from milk was lower (1 : 16-512) than that from ticks (1 : 32-4096). Onset of seropositivity also occurred later – on the 10th day post-infection (p.i.) for M18 and M35 in comparison with the 7th day for Kl₃ and Kl₆. In guinea pigs, infection manifested by fever. The fever was less evident in guinea pigs infected with isolates from milk (39.5-40.1°C) than in guinea pigs infected with isolates from ticks (39.5-40.6°C). Partially engorged females of *Dermacentor reticulatus* (Fabricius, 1794) were inoculated with isolates M18 and Kl₃. No differences in the multiplication of *C. burnetii* in haemocytes between these two isolates were ascertained.

The etiological agent of Q fever, *Coxiella burnetii* (Derrick, 1939), has been isolated in recent years from two sources in Slovakia and in the Czech Republic from bovine milk (Kocianová et al. 1993) on one hand, and from ticks *Ixodes ricinus* (Linnaeus, 1758), *Dermacentor reticulatus* (Fabricius, 1794), *Dermacentor marginatus* (Sulzer, 1776), *Haemaphysalis inermis* Birula, 1895 and *Haemaphysalis concinna* Koch, 1844 (Řeháček et al. 1991) on the other.

Although *C. burnetii* has been isolated on many occasions and the prevalence of antibodies in herds of cows ranges from 0.3% to 40% (Literák 1995, Literák and Řeháček 1996, Řeháček et al. 1998), in recent years we have not recorded human cases of Q fever caused by autochthonous strains of *C. burnetii*.

In order to assess the health importance of autochthonous strains of *C. burnetii*, it is necessary to know their virulence. As there is lack of information on the virulence of recent isolates of *C. burnetii* from Slovakia and from the Czech Republic, we have tried to characterise it by use of experimental infections.

MATERIALS AND METHODS

For the purpose of experimental infections, two isolates of *C. burnetii* obtained from the milk of seropositive cows were used: M18 from Pliešovce, Slovakia and M35 from Kunín, Czech Republic (Kocianová et al. 1993). These agents were isolated in the spleens of outbred laboratory mice (Swiss strain, Velaz, Prague, Czech Republic) and subsequently transferred and propagated in the yolk sacs of embryonated hen eggs (Brezina 1977). Agents of *C. burnetii* isolated from

ticks (Řeháček et al. 1991) were also used: Kl₃ from *Ixodes ricinus* and Kl₆ from *Dermacentor marginatus*. Tick isolates were propagated in yolk sacs of embryonated hen eggs. To compare the virulence of these isolates, the virulent reference strain Nine Mile of *C. burnetii* phase I, which causes acute Q fever, was used. This strain was originally isolated from *Dermacentor andersoni* ticks (Davis and Cox 1938).

For the purpose of experimental infections, laboratory mice, guinea pigs and *Dermacentor reticulatus* ticks were used. Outbred laboratory mice (Swiss strain, colony of the Slovak Academy of Sciences, Dobrá Voda, Slovakia and Velaz, Prague, Czech Republic) and guinea pigs (colony of the Institute of Virology, Bratislava, Slovakia) were infected intraperitoneally (i.p.) with the isolates M18 and M35 in the third yolk passage (EP₃) suspension and with isolates Kl₃ and Kl₆ in the fourth yolk passage (EP₄) suspension with a dose of 0.5 ml per animal. Stock suspensions of both milk and tick isolates were adjusted to contain the same amount of infection particles 10⁴ EID₅₀. A total of 105 infected mice and 15 as controls and 24 infected guinea pigs and 5 as controls were used.

The laboratory mice and guinea pigs were tested microscopically and serologically using microagglutination reaction (MAR) and enzyme-linked immunoadsorbent assay (ELISA). The temperature of the guinea pigs was monitored (daily during 14 days) and a temperature over 39.5°C (i.e., fever) was considered to be a clinical sign of infection. Microscopic examination (magnification: ×1000) of *C. burnetii* was done using impressions of spleens, which were stained as described by Giménez (1964). Blood and spleen samples were taken from laboratory mice on the 7th, 10th and 14th days p.i. and after that, every 7th day until the 35th day of infection (end of the experiment). Blood samples were taken from guinea pigs in the 4th, 8th and 12th weeks p.i.

Coxiella burnetii was also inoculated into 60 partially engorged adult female *Dermacentor reticulatus* ticks, after being confirmed as negative for *C. burnetii* using the haemocytometer test (Řeháček et al. 1971). The ticks were infected intracoelomally (i.coel.) with M18 and Kl₃ isolates, each in a dose of 10⁴ EID₅₀ / 25 µl. Multiplication of *C. burnetii* was detected microscopically in haemocytetes, stained as described by Giménez (1964).

The serological investigations MAR and ELISA were carried out with the antigen derived from the standard strain of *C. burnetii*, Nine Mile (Institute of Virology, Bratislava, Slovakia). In MAR (Fiset et al. 1969) the titre of sera ≥ 16 was considered positive. In ELISA test (Kováčová et al. 1987) titre was calculated from the mean value of negative sera plus 2×SD. Each value higher or equal was considered positive.

The fever in the guinea pigs was statistically evaluated by two-way analysis of variation (Zar 1996) on the statistical software package Programme STATGRAPHICS Plus, Version 7.

RESULTS

Laboratory mice. No mortalities were recorded and no visible clinical signs of illness were observed in mice infected with isolates from either milk or ticks. *C. burnetii* was detected in spleen on the fourth day after infection with milk isolates M18 and M35. On days 7, 10 and 14, the number of *C. burnetii* particles increased. On the 21st day only single particles were found and on the 28th and 35th days p.i., *C. burnetii* was not detected. In isolates from ticks (namely Kl₆), tens to hundreds of particles were observed as early as the 4th day p.i. On the 7th, 10th, 14th and 21st days the amount of *C. burnetii* particles rose in the vacuole of cytoplasm. On the 28th day the number of particles decreased and on the 35th day only a single particle could be found.

Antibodies against *C. burnetii* were detected (by MAR) in mice infected with the isolates M35 and M18 from the 10th and 14th days p.i., respectively, and during the course of monitoring, a maximum titre of 1 : 512 was reached (M18 on the 35th day). For isolates Kl₃ and Kl₆ and the Nine Mile strain, antibodies were detected from the 7th day. During the course of monitoring, a higher level of antibodies was reached for Nine Mile strain (1 : 4096) and the Kl₆ (1 : 1024) and Kl₃ (1 : 4096) isolates (Fig. 1). As detected by ELISA, an antibody response after inoculation of isolate M18 occurred on the 10th day, and on the 7th day p.i. after Kl₃ infection. We can conclude that isolates of *C. burnetii* from ticks (Kl₃) produce antibodies earlier and in higher titres than those from milk (M18) (Table 1).

Three weeks after inoculation of laboratory mice with isolates from milk, the amount of coxiellae was approximately 2-4 log lower (10^{5.5}-10⁶ ID₅₀/ml) than after infections with isolates from ticks (10⁸-10¹⁰ ID₅₀/ml). Similar differences were also found during titration of *C. burnetii* in yolk sacs of hen eggs.

Guinea pigs. In reaction to being infected with *C. burnetii*, guinea pigs developed a fever. After infection with M18 (n = 6) and M35 (n = 6) the fever appeared on the 5th day p.i. (lasting an average of three days), with an average of 39.7°C in the case of M18 (range 39.5-40.1°C) and 39.6°C in M35 (range 39.5-39.8°C). In guinea pigs infected with isolates Kl₃ (n = 6) and Kl₆ (n = 6), the fever appeared on the 2nd-3rd days p.i. (lasting an average of six and a half days), with an average of 40.0°C for both isolates (range 39.5-40.3°C for Kl₃ and 39.5-40.6°C for Kl₆). The fever in guinea pigs infected with milk isolate M35 was lower than in guinea pigs infected with isolates from ticks. Differences in temperatures between all *C. burnetii* isolates were statistically significant according to two-way analysis of variation (F = 95.438, df₁ = 3, df₂ = 160, P ≤ 10⁻⁵). MAR-detected antibodies were found for both types of isolates during the set sampling period, and the level of titres achieved was different. Two months p.i. with M18 and M35 the antibodies had fallen to titres 1 : 16-32. During this time, titres rose to 1 : 64-512 for tick isolates. Three months p.i. the titre levels still differed (Table 2).

***Dermacentor reticulatus* ticks.** In ticks inoculated with isolate M18 the prevalence of positive specimen was high (30 positive/30 tested) and the proportion of infected haemocytetes was almost 100% after three weeks, as was the case in ticks infected with isolate Kl₃ (30 positive/30 tested). In both cases, the haemocytetes contained hundreds of cells of *C. burnetii*. Differences between the proportion of infected haemocytetes were not found.

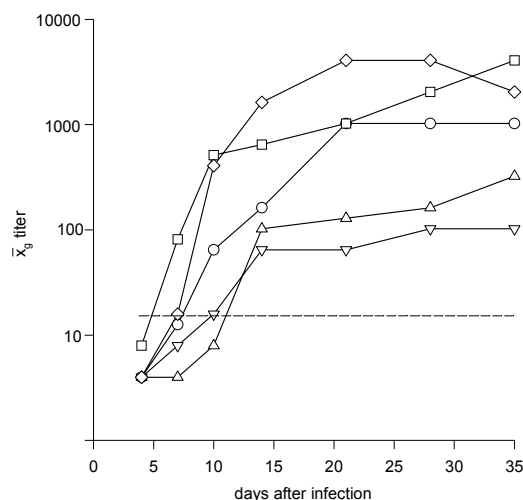


Fig. 1. Antibody response (MAR) in laboratory mice experimentally infected with *C. burnetii*. —○— Kl₆; —□— Kl₃; —△— M18; —▽— M35; —◇— Nine Mile; ----- level of positive titres

Table 1. Antibodies to *Coxiella burnetii* in laboratory mice experimentally infected with isolates M18 from milk and Kl₃ from *Ixodes ricinus* ticks (as revealed by ELISA).

Days post infection	Isolate M18		Isolate Kl ₃	
	seroprevalence	titres	seroprevalence	titres
7	0/3*	(100) ⁺ 100 ⁺⁺	1/3	(100-500) 170.99
10	1/3	(100-1000) 215.44	3/3	(500) 500
14	3/3	(1000) 1000	3/3	(1000) 1000
21	3/3	(1000-2000) 1587.40	3/3	(2000) 2000
28	3/3	(1000) 1000	4/4	(2000) 2000
35	3/3	(1000) 1000	1/1	(2000) 2000

* positive/tested animals; ⁺ titres of sera (minimum-maximum); ⁺⁺ mean of geometric titres

Table 2. Agglutination antibodies to *Coxiella burnetii* in guinea pigs experimentally infected with isolates from milk (M18, M35) and isolates from ticks (Kl₃, Kl₆).

<i>C. burnetii</i> isolates	4 weeks p.i.		8 weeks p.i.		12 weeks p.i.	
	seroprevalence	titres	seroprevalence	titres	seroprevalence	titres
M18	4/4*	(32-256) ⁺ 101.59 ⁺⁺	4/4	(16-32) 20.15	3/4	(16-32) 20.15
M35	4/4	(32-256) 101.59	4/4	(16-32) 25.39	3/4	(8-16) 12.69
Kl ₃	4/4	(128-256) 203.18	4/4	(64-512) 256	4/4	(32-256) 128
Kl ₆	4/4	(128) 128	4/4	(128-512) 203.18	4/4	(64-256) 161.26
Nine Mile	4/4	(128-512) 203.18	4/4	(128-512) 256	4/4	(64-256) 161.26

* positive/tested animals; ⁺ titres of sera (minimum-maximum); ⁺⁺ mean of geometric titres

DISCUSSION

In Slovakia and in the Czech Republic, autochthonous strains of *C. burnetii* are present in many herds of cattle and in various natural foci (Literák and Řeháček 1996). The lack of clinical signs of illness in cattle herds can be explained by a low level of virulence of the strains circulating in the environment (Literák and Calvo Rodriguez 1994, Literák and Kroupa 1998). Experimental infections with isolates from milk should contribute to clarifying the question of *C. burnetii* circulation in the environment.

Testing of isolates of *C. burnetii* (EP₃) from bovine milk (M18 and M35) on pregnant sheep confirmed their low virulence (Kocianová et al. 1998). The animals did not fall ill and healthy lambs were born, although clinical Q fever did manifest itself in the sheep, mainly during the course of pregnancy. Agglutination antibodies were found 5-10 days p.i. with titres of 1 : 16-128 in those sheep that had been infected intranasally or

intravenously (i.v.). Complement-fixing (CF) antibodies were detected at the same time with titres of 1 : 8-16, and in the period around birth (8-11 weeks p.i.), they were found in all sheep in higher levels (1 : 16-32) and persisted until the end of the experiment, i.e., five months.

Martinov et al. (1989) tested the virulence of the *C. burnetii* strain Tchilnov (EP₁₂₅), originally isolated from sheep, on pregnant sheep infected i.v. The animals developed a fever, during which other clinical signs emerged. Lambs were stillborn or died within 24 hours post parturition. From the 8th day onwards, adult mothers had elevated CF antibodies (1 : 8-32), and the highest titre (1 : 256-512) appeared between 40-50 days p.i.

Another difference in the virulence between the investigated isolates is the course of the fever in guinea pigs. A lower level and shorter course of fever in the animals is typical of milk isolates. In this instance, it is of course necessary to emphasise that the *C. burnetii*

Priscilla strain isolates from an aborting goat, which may cause chronic Q fever in humans, is also only mildly virulent for guinea pigs (Kazár et al. 1993).

Differences between isolates from milk (M18 and M35) and the reference strain of *C. burnetii* have also been found by Sekeyová et al. (1996). They compared the prototype Nine Mile and Priscilla strains with isolates from ticks and milk in a microimmunofluorescence test, an ELISA, and an immunoblot by the use of monoclonal antibodies against Nine Mile strain and Priscilla strain of an acute case and a chronic case of Q fever, respectively. When comparing monoclonal antibodies with isolates M18 and M35, to isolates from ticks and the prototype homologous strains, a markedly lower reaction was detected in the former, in all of the above mentioned tests.

In our experiments, the same results were obtained when comparing the virulence of *C. burnetii* isolates from ticks and from bovine milk with the reference strain Nine Mile. Seroconversion in the laboratory animals infected with the isolates from ticks and from Nine Mile was evident, and the intensity of the fever in the guinea pigs was different. Mice infected with the milk isolate demonstrated a lower number of *C. burnetii* particles in the spleen as compared with those injected with coxiellae originating from ticks. The species of tick from which the *C. burnetii* was isolated probably did not play a significant role.

The same results were demonstrated in our experiments comparing the virulence of *C. burnetii* isolates from ticks used with the reference strain Nine Mile. Seroconversion in infected laboratory animals was evident and the intensity of the infection was higher.

The spontaneous abatement of a focus that had been initiated by imported infected livestock, and that had spread to animals and people over several years without maintenance in ticks, is believed to have resulted in the disappearance of the disease in areas of Italy (Babudieri 1962). This hypothesis was also employed to explain the recession of acute forms of Q fever during the 1950s in former Czechoslovakia (Syrůček et al. 1958). Likewise, Pautov (1973) concluded that when *C. burnetii* is transferred from sheep and from cattle to

cattle, it loses its virulence and finally becomes incapable of provoking infection. Decline of virulence of *C. burnetii* during the passages in the same species of tick was observed by Daiter and Gromova (1970). Klimchuk and Kushnir (1989) studied characteristics of two strains of *C. burnetii* from the natural foci of Q fever in north-western Ukraine. They monitored dynamics of antibody response and the course of fever in guinea pigs and laboratory mice. Strain Gishin, isolated from ixodid ticks, was characterised by high virulence and the Politsky strain, which was isolated from the common vole, considerably exceeded in the virulent properties.

SDS-PAGE, immunoblotting and serological methods were used to compare protein and lipopolysaccharide (LPS) profiles, and antigenicity of 12 *C. burnetii* strains isolated mostly from ticks, with three reference strains (originating from tick, human and goat). No significant differences in protein and LPS profiles were observed among tick strains from Europe and Mongolia. The LPS profiles of these strains appeared to be more related to those of Nine Mile strain associated with acute Q fever, than to those of strains associated with chronic Q fever. Reference Nine Mile strain gave double bands at approximately 14 and 16 kD, which were absent in the chronic strains (Kováčová et al. 1994).

Our results may support the hypothesis that strains of *C. burnetii*, circulating in cattle herds without a prolonged or extensive tick intervention gradually decrease their virulence. The cattle stock from which the investigated isolate M35 originated was kept in closed stables during the lactation and no ticks were seen on these animals. The cows from another locality (isolates M18) grazed on pastures, however, no massive tick infestation was seen.

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