MONOCLONAL ANTIBODY BASED DIFFERENTIATION OF *COXIELLA BURNETII* ISOLATES

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Summary. – Isolates of *Coxiella burnetii* from different geographic regions in Europe, USA, Japan and Africa were compared in their binding properties to the monoclonal antibody (MoAb) 1/4/H directed against the lipopolysaccharide (LPS) of *C. burnetii* strain Priscilla. Immunoblot analysis and enzyme-linked immunosorbent assay (ELISA) revealed different binding patterns of *C. burnetii* isolates under study. Most of the isolates tested did react with MoAb 1/4/H. Only four of 20 groups of isolates and one isolate of an otherwise positively reacting group did not react with MoAb 1/4/H. The results indicate a significant variation of LPS structure of the *C. burnetii* isolates studied.

Key words: *Coxiella burnetii*; monoclonal antibody; lipopolysaccharides; immunoblot analysis; ELISA

Introduction

C. burnetii, the etiological agent of Q fever in humans, affects also a large variety of animals, mainly sheep, goats and cattle (Baca and Paretsky, 1983). Its wildlife cycle involves a wide range of mammals, birds, and tick species (Thomas and Palmer, 1994). In humans, most infections are contracted by inhalation of contaminated dust or aerosols. The course of the illness is usually characterized by a self-limiting influenza-like illness or pneumonia (Reimer, 1993), but a severe complication of chronic endocarditis may occur (Raoult *et al.*, 1990). It has been reported that there may be geographical variation in clinical presentation and immune response of reported cases (Thomas and Palmer, 1994) or even strain-dependent variation in virulence (Baca, 1991).

Coxiella LPS is implicated as virulence factor (Baca, 1991). The so-called phase variation of *C. burnetii* is accompanied by a shift in virulence as well as by changes of other biological and physico-chemical properties (Kazár *et al.*, 1974). This phenomenon involves an alteration of the surface LPS (Hackstadt, 1986). This component is known to vary antigenically and biochemically among different *C. burnetii* strains (Hackstadt *et al.*, 1985; Hackstadt, 1986; Novák and Brezina, 1988).

Therefore, we investigated in detail the potential of a monoclonal antibody MoAb 1/4/H, directed mainly against LPS of *C. burnetii* strain Priscilla, which had been described previously (Sekeyová *et al.*, 1995). Our aim was to find out whether this particular MoAb (1) would be able to recognize or distinguish *C. burnetii* isolates which differ genetically as described by Heinzen (Heinzen *et al.*, 1990) and Thiele (Thiele *et al.*, 1993), and (2) could be used for routine antigen identification.

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Abbreviations: BCIP = 5-bromo-4-chloro-indoxyl-phosphate; BGM = buffalo green monkey; ELISA = enzyme-linked immunosorbent assay; LPS = lipopolysaccharide; MoAb = monoclonal antibody; PAGE = polyacrylamide gel electrophoresis; PBS = phosphate buffered saline; PBST = PBS with 0.5% Tween 20; SDS = sodium dodecyl sulphate

Materials and Methods

Antigens. The *C. burnetii* isolates tested (Tables 1, 2 and 3), were propagated in buffalo green monkey (BGM) cell cultures and separated from the cells by repeated centrifugation at 800 x g for 10 mins (Beckman centrifuge Model J2-21, rotor JA-20). *C. burnetii* cells were pelleted from the supernatant by high-speed centrifugation (Arens, 1983). After washing with phosphate buffered saline (PBS) the cells were resuspended in the same buffer.

MoAb 1/4/H was prepared using *C. burnetii* strain Priscilla as antigen (Sekeyová *et al.*, 1995).

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was performed according to Laemmli in Mini Protean II vertical electrophoresis unit (Bio-Rad). Stacking and separating gels contained 5 and 10% acrylamide, respectively. Four µl containing 2.5 x 10^7 cells were applied per slot. The gels were subjected to electrophoresis at 100 mA until the Bromophenol Blue reached the separation gel, and subsequently at 150 mA till the end of run. Separation time was 90 mins.

Western blot analysis was carried out according to Willems *et al.* (1992). Following SDS-PAGE the gel was electroblotted to Immobilon P membrane (Millipore), which was washed with methanol and distilled water prior to use. The blotting buffer consisted of 192 mmol/l glycin, 25 mmol/l Tris and 20% methanol. The procedure was completed at 4°C and a constant current of 100 mA within 1 hr. After blotting the membrane was washed thoroughly in PBS containing 0.5% Tween 20 (PBST). Free binding sites of the membrane were saturated with blocking solution (3% fish gelatine in PBST). The membrane was then washed in PBS and incubated with MoAb 1/4/H diluted to 1/2000 in PBS. After the addition of anti-mouse IgG-alkaline phosphatase conjugate diluted to 1/20,000, the membrane was shaken for another 45 mins. After washing the membrane 3 times with PBS the bands were visualized using 5-bromo-4-chloro-3-indoxyl-phosphate (BCIP) and Nitroblue tetrazolium salt. In each run a mixture of prestained standards was included. Chemicals from Bio-Rad, Merck, Serva and Sigma were used.

ELISA was performed as described by Jaspers *et al.* (1994). Briefly, microtiter plates Immulon M 129 A (Greiner) were coated with 100 µl per well of serial twofold dilutions (1/500 – 1/512,000) of heat-inactivated cells of *C. burnetii* isolates in the coating buffer in duplicate (the concentrated stock solution of *C.burnetii* cells contained 3 x 10^8 cells/µl). For coating, plates were incubated overnight at 37°C, decanted, dried and stored at 4°C. Remaining free binding sites were blocked with blocking buffer for 15 mins at room temperature. After washing, MoAb 1/4/H diluted to 1/2000 was added. After 1 hr incubation the plates were decanted and washed three more times. Then protein A-peroxidase conjugate diluted to 1/20,000 was added and the incubation continued for 45 mins. Washing was repeated 3 times and tetramethyl

Table 1. *C. burnetii* isolates used and grouped according to *Not*I restriction analysis. Groups I, IV, V, and VI

Genotype group	Reference isolate	Tested isolate	Isolation source	Isolation country	Dot blot test
I	Nine Mile RSA493	Nine Mile RSA493	[1] tick	USA	+
		Bernard	human	France	+
		CS 9[2]	tick	Slovakia	+
		CS 27[2]	tick	Slovakia	+
		J 1[3]	milk	Japan	+
		J 3[3]	milk	Japan	+
		J 27[3]	milk	Japan	–
		Hardthof	bovine	Germany	+
IV	Priscilla Q177	Priscilla Q177[1]	goat	USA	+
		Priscilla 20*	goat	USA	+
		Priscilla 15*	goat	USA	+
		Deborne	human	France	+
V	S Q217	Scurry[1]	human	USA	+
VI	Dugway 5J108-111	Dugway	rodents	USA	–
		Z 3205 b	tick	Germany	–

Dot blot test positive (+), negative (–).

[1] Obtained from L.P. Mallavia, Washington State University, Pullman, Washington, USA.

[2] Obtained from J. Kazár, Institute of Virology, Bratislava, Slovakia.

[3] Obtainned from K. Hirai, Faculty of Agriculture, Gifu University, Gifu, Japan. Unnumbered isolates are from the collection of the Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität Giessen, FRG.

* Number of cell culture passages.

benzidine (Fluka) was added. After 30 mins, the reaction was stopped with H_2SO_4 and the absorbance at 405 nm (A_{405}) was measured in a Titertek Multiskan photometer (Flow).

Dot blot analysis was performed using Immobilon P membranes as desribed by Cowley *et al.* (1992). Briefly, membranes cut into a circle (1.5 cm in diameter) were prewetted with methanol, air dried and the *C. burnetii* antigen (3 x 10^8 cells per 1 µl per dot) was applied. The membrane was air dried overnight. After saturation in blocking buffer for 15 mins, it was washed (5 mins) and incubated with MoAb 1/4/H diluted to 1/2000 in PBS. After addional washing, the alkaline phosphatase-conjugate diluted to 1/20,000 in PBS was added. Following incubation for 45 mins and washing (3x) with PBS the positive reaction was visualized as described above.

Table 2. *C. burnetii* isolates used and grouped according to *Not*I restriction analysis. Groups 1-16

Group	Tested isolate	Source	Country	Dot blot test
1	CS Florian[2]	human	Slovakia	+
	München	sheep	Germany	+
	CS Henzerling	human	Italy	+
2	Stanica	human	Romania	+
	Z 104	sheep	Germany	+
	München Kl. 5*	human	Germany	+
	Henzerling Kl. 5*	human	Italy	+
	Z 4313	sheep	Germany	+
3	CS Henzerling R	human	Slovakia	–
4	Z 3464	goat	Germany	+
5	Z 3567	sheep	Germany	+
6	Brustel	human	France	–
	Z 2534	goat	Austria	–
	Z 3055	sheep	Germany	–
7	Z 2775	bovine	Germany	+
8	Brasov	human	Romania	–
9	Namibia	goat	Namibia	+
10	R 1140	human	Russia	+
11	Schperling	human	Russia	+
12	Ouaret	human	France	+
13	Campoy	human	France	+
	Jaquemot	human	France	+
14	Pallier	human	France	+
15	Lombardi	human	France	+
16	Butin	human	France	+
	Raphael	human	France	+

* Cloned in phase II. For the rest of legend see Table 1.

Table 3. *C. burnetii* isolates not yet differentiated by restriction analysis

Tested isolate	Isolation		Dot blot test
	source	country	
J 60[3]	milk	Japan	+
J 82[3]	milk	Japan	–
Z 349	?	Germany	+
Z 4488	?	Germany	+

(?) = unknown. For the rest of legend see Table 1.

Results

Dot blot analysis

Referring to the grouping originally proposed and meanwhile extended by Thiele *et al.* (1993; Thiele, D., personal communication) on the basis of *Not*I restriction analysis, all available isolates (from USA, Europe, Japan and Africa) were initially analyzed by dot blot to demonstrate their binding to MoAb 1/4/H. Most isolates from Table 1, except the reference strain Dugway and the isolate Z 3205 b (group No. IV), and the isolate J27 (group No. I) reacted with this antibody. Isolates from groups No. 3, 6 and 8 (Table 2), as well as one isolate from Japan not yet classified genetically (Table 3) did not react with MoAb 1/4/H. The reaction of all other isolates (listed in Tables 2 and 3) was positive.

Western blot analysis

Isolates from different regions of the world revealed significant differences in Western blot analysis. As expected, the analysis of the reference *C. burnetii* strains (Nine Mile RSA 493, Priscilla Q 177, S Q217 and Dugway 5J108-11) with MoAb 1/4/H clearly resulted in four different patterns (Fig. 1). Eight bands within the range of 24 –100 K were

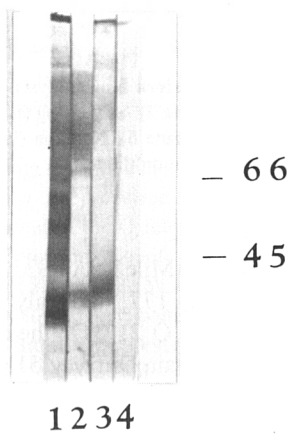

Fig. 1
Western blot analysis
Nine Mile RSA 493 (lane 1), Priscilla Q 177 (lane 2), Scurry S Q 217 (lane 3), Dugway 5J108-111 (lane 4). K values of size standards (lane M) are shown on the right.

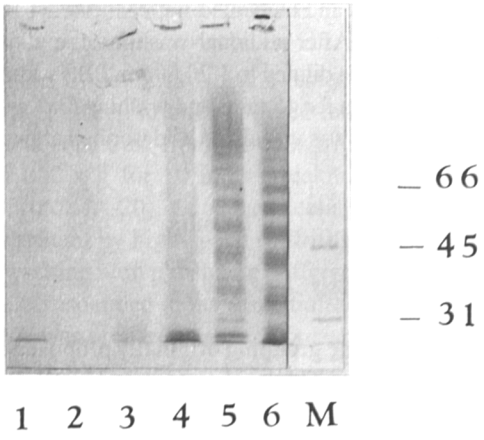

Fig. 2
Western blot analysis
Z 3205 b (lane 1), München (lane 2), Henzerling Kl. 5 (lane 3), Z 3567 (lane 4), CS Florian (lane 5), Raphael (lane 6). K values of size standards (lane M) are shown on the right.

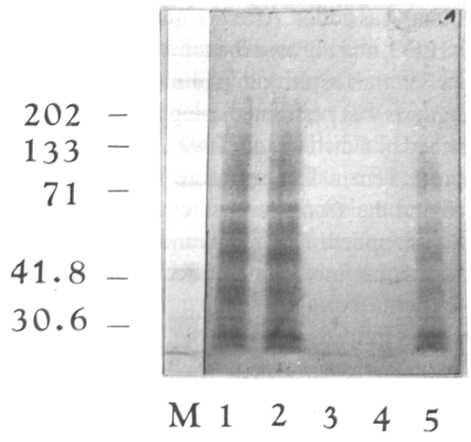

Fig.4
Western blot analysis
J 1 (lane 1), J 3 (lane 2), J 27 (lane 3), J 82 (lane 4), J 60 (lane 5). K values of size standards (lane M) are shown of the left.

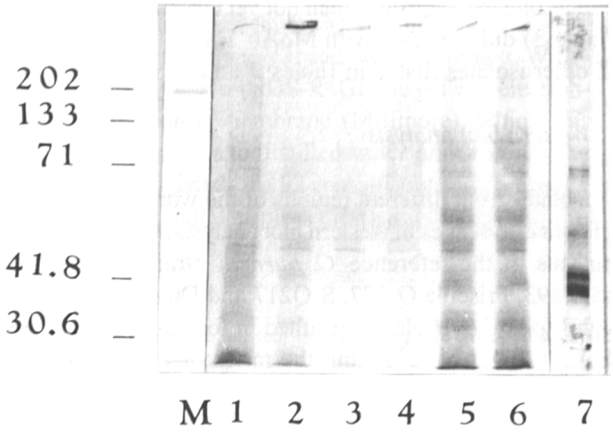

Fig. 3
Western blot analysis
Z 2775 (lane 1), R 1140 (lane 2), Schperling (lane 3), Campoy (lane 4), Pallier (lane 5), Lombardi (lane 6), Namibia (lane 7). K values of size standards (lane M) are shown on the left.

detected with strain Nine Mile RSA 493, five bands (29-80 K) with strain Priscilla Q 177, and only three major bands (29-80 K) with strain S Q 217. On the other hand, the MoAb did not recognize strain Dugway 5J108-111 at all.

Results of screening of *C. burnetii* isolates with MoAb 1/4/H are presented in Figs. 2-4.

The MoAb did not react with isolate Z 3205 b and only a weak reaction could be seen with the isolate Henzerling Kl. 5. There was a slight smear in the range of 100 K with isolate München. A ladder-like pattern in the range from 29 K to more than 100 K was obtained with isolates Z 3567, CS Florian and Raphael (Fig. 2).

There was one clear common band in the region around 50 K with isolates Z 2775, R 1140, Schperling and Campoy, while with isolates Pallier and Lombardi a ladder-like pattern was identified. Two dominating bands in the region around 40 K were detected with isolate Namibia (Fig. 3).

Fig. 4. presents the results obtained with the Japanese isolates. Whereas the isolates J 1, J 3, and J 60 were recognized by MoAb 1/4/H yielding a ladder-like pattern, two isolates, namely J 27 and J 82, were not identified at all. Isolates Z 3464 and Ouaret produced patterns comparable to those obtained with strain Nine Mile RSA 493 (data not shown). In accordance with dot blot analysis, there was a negative reaction when the isolates of the groups 3, 6 and 8 were used (data not shown). The isolate Z 4313 was not tested.

ELISA

ELISA carried out with MoAb 1/4/H and various isolates of *C. burnetii* revealed different affinities. The highest A_{405} was obtained with the standard reference strains Priscilla (1200) and Scurry (800). The reactivity with strain Nine Mile was lower (300), while there was no reaction at all with strain Dugway. The strain Z 3464 from another group of tested isolates was the only one responding in a higher A_{405} range (490). Reactions similar to that of Nine Mile were observed with the isolates München 5, Campoy, Lombardi, and Pallier (around 200).

Less reactive were the isolates R 1140 and Butin (120), while Z 2775, Namibia, München, Z 3568 and Schperling proved the lowest reactivity with MoAb 1/4/H (< 100). Only three of the Japanese isolates, namely J 1, J 3 and J 60, gave a weak response (< 100). Again, we were not able to identify the presence of a reactive epitope with MoAb 1/4/H

in ELISA in the isolates previously listed as negative in Western blot and dot blot analyses.

Discussion

Intracellular parasites have developed evolutionarily different mechanisms enabling them to survive in an intracellular environment and to escape from attack by the host immune system (Moulder, 1985). *C. burnetii* has adapted to such an extent that it is able to thrive under harsh conditions of acidic pH in the phagolysosome of the host cell (Hackstadt and Williams, 1981; Baca and Paretsky, 1983). Whether the biochemical structure of the LPS determines the chance of this organism to survive within the phagolysosome of the infected cell or not, thus leading to different grades of virulence among strains, is unknown (Baca, 1994). A possible role of phase I *C. burnetii* LPS may be a masking of underlying antigens from the immune system, allowing the organism to escape immune surveillance (Hackstadt, 1986). Thus, it is of great interest to investigate the LPS of *C. burnetii* in detail with the aim to determine immunodominant sites on the LPS molecule, which can be detected by MoAbs (Thiele *et al.*, 1992; Wen Bo-Hai *et al.*, 1991; Williams *et al.*, 1984; Zhi Ning *et al.*, 1991). In this study, we have used MoAb 1/4/H directed mainly against LPS of *C. burnetii* strain Priscilla (Sekeyová *et al.*, 1995). ELISA and immunoblot techniques were applied to examine binding properties of this MoAb to different *C. burnetii* isolates. The dot blot analysis was employed mainly for screening of positively reacting isolates. More precise data about the binding properties of MoAb 1/4/H were obtained by Western blot analysis, which allows to differentiate the humoral response to a number of different antigenic components. As a matter of fact, it revealed irregularities in binding properties of MoAb 1/4/H to *C. burnetii* isolates under study, thus proving the idea of possible differences in its surface LPS structure, as it has been described for *C. burnetii* reference strains (Hackstadt *et al.*, 1985; Hackstadt, 1986). Interestingly, 4 of the 20 tested groups, namely No. VI (reference strain Dugway 5J108-111, isolate Z 3205 b), No. 3 (isolate CS Henzerling R), No. 6 (isolates Brustel, Z 2534 and Z 3055) and No. 8 (isolate Brasov) did not react at all. Only one isolate of group No. I was negative as well. This indicates the absence of any binding site for MoAb 1/4/H on LPS molecule of the isolates. Therefore it seems that these isolates posess not only different genotypes as found by Thiele *et al.* (1993), but also distinct phenotypic features. Apart from non-reaction slight differences in the LPS structure have been observed with these isolates by their varying Western blot patterns. Unusual was the pattern of isolate Namibia, the only African isolate used in this study.

Similarly, a different reactivity of the *C. burnetii* isolates with MoAb 1/4/H was revealed by ELISA. Of interest was the observation that none of the tested isolates was able to reach the reaction level obtained with the prototype strain Priscilla. This observation is not in accordance with the lower antibody-binding capacity of Priscilla strain described in another study (Kováčová *et al.*, 1994), in which, however, polyclonal rabbit sera were used. More important was the finding that the *C. burnetii* isolates reacted in ELISA with MoAb 1/4/H in a different manner, and some of them did not react at all.

Although the MoAb used in our study is suitable for detection of isolates belonging to group IV Priscilla Q 177, there are isolates which cannot be detected with this MoAb. The use of MoAbs in general is undoubtly of a great help when utilized in antigen-capture tests for diagnostic purposes. However, it seems that a cocktail of several MoAbs directed against various binding sites and strains is needed to identify all possible strains. On the other hand, further study of the LPS structure of *C. burnetii*, including the isolates belonging to the non-reacting groups may contribute to our better knowledge of the surface structures of *C. burnetii*.

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