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Molecular characterization of *Coxiella burnetii* isolates

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SUMMARY

Restriction fragment length polymorphism (RFLP) was used for the differentiation of 80 *Coxiella burnetii* isolates derived from animals and humans in Europe, USA, Africa and Asia. After *Not*I restriction of total *C. burnetii* DNA and pulsed field gel electrophoresis (PFGE) 20 different restriction patterns were distinguished. The index of discrimination for this typing system was 0·86. Comparison and phylogenetic analysis of the different RFLP patterns revealed evolutionary relationships among groups that corresponded to the geographical origin of the isolates. This finding was confirmed by genetic mapping. No correlation between restriction group and virulence of isolates was detected.

INTRODUCTION

Coxiella burnetii, an obligate intracellular parasite, is the causative agent of the zoonosis Q fever. Infections with this rickettsia occur worldwide in animals and man [1]. In humans, acute pneumonic Q fever and chronic forms associated with endocarditis or granulomatous hepatitis are found [2] whereas in animals inapparent infections are predominant [3]. However, *C. burnetii* may cause abortions in ruminants. Particularly sheep but also goats [4] and cats [5] are known as the source of human infections with *C. burnetii*. Infections are mainly diagnosed by serology, staining smears [6], detection of the antigen by capture ELISA [7] or direct isolation of *C. burnetii* using cell culture techniques. However, such techniques have failed to discriminate *C. burnetii* isolates.

Several attempts have been made to differentiate *C. burnetii* isolates and to determine relatedness among these isolates. Differentiation based on protein profiles using SDS-PAGE and immunoblot was unsatisfactory [8, 9]. Hackstadt [10] demonstrated variations in the lipopolysaccharide (LPS) pattern of *C. burnetii* isolates after SDS-PAGE and silver staining. More promising differentiation techniques are based on manipulation of *C. burnetii* total DNA or plasmids [13, 14]. After *Hae*III digestion of *C. burnetii* total DNA and separation of the resulting fragments by conventional electrophoresis *C. burnetii* isolates could be classified into four different groups [13]. This classification was extended to six groups applying other restriction enzymes [14–16]. Heinzen and colleagues [17] confirmed four of these six groups by PFGE after *Not*I or *Sfi*I digest of total *C. burnetii* DNA. Ten further patterns were recognized by Thiele and colleagues [18, 19] after restriction of total DNA of European and one African isolate with *Not*I.

For the present study 80 *C. burnetii* isolates have been characterized by PFGE and clear similarities of patterns were found indicating relationship among different groups.

Precise identification of the pathogen is supposed to be an important epidemiological marker. Furthermore isolates possess different virulence potential. The genetic basis for these differences is not yet known. Differentiation of *C. burnetii* isolates would probably help to identify the source of Q fever infections and to estimate the virulence of new isolates. Beyond that, characterization of the possible genetic heterogeneity among *C. burnetii* isolates is important

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Table 1. C. burnetii *isolates, their geographical origin and host species*

Restriction group	Isolate	Geographical origin	Host species
I	Nine Mile RSA493	USA	Tick
	Balaceanu	Romania	Human
	Bernard	France	Human
	CS1	Slovak Republic	?
	CS3	Slovak Republic	?
	CS4	Slovak Republic	?
	CS5	Slovak Republic	?
	CS6	Slovak Republic	?
	CS7	Slovak Republic	Tick
	CS8	Slovak Republic	Tick
	CS9	Slovak Republic	Tick
	CS10	Slovak Republic	Tick
	CS11	Slovak Republic	?
	CS18	Slovak Republic	?
	CS Dayer	Slovak Republic	Tick
	CS L 35	Slovak Republic	?
	CS Poland	Poland	Tick
	Hardthof	Germany	Cattle
	J1	Japan	Cattle
	J3	Japan	Cattle
	J27	Japan	Cattle
IV	Priscilla Q177	USA	Goat
	Deborne	France	Human
V	Scurry Q217	USA	Human
	CS S	USA	Peacock
VI	Dugway 5J108-111	USA	Rodent
	Z257	Germany	Cattle
	Z3027	Germany	Cattle
	Z3205a	Germany	Cattle
	Z3205b	Germany	Cattle
	Z3351	Germany	Cattle
	Z3568	Germany	Cattle
	Z3749	Germany	Cattle
1	Boren	USA	Cattle
	CS 48	Slovak Republic	Tick
	CS F	Slovak Republic	?
	CS II/Ia	Slovak Republic	Tick
	CS Ixodes	Russia	Tick
	CS S1	Russia	Cattle
	Florian	Slovak Republic	?
	Frankfurt	Germany	Cattle
	Gbud	Slovak Republic	Cattle
	Geier	Romania	Human
	Henzerling	Italy	Human
	München	Germany	Sheep
	RT 1	North Western Russia	Mouse
	RT 3	North Western Russia	Tick
2	Andelfingen	Switzerland	Cattle
	CS Z 57	Slovak Republic	?
	Henzerling K1.5	Italy	Human
	Herzberg	Greece	Human
	München K1.5	Germany	Human
	S 1	Sweden	Cattle
	S 4	Sweden	Cattle
	Soyta	Switzerland	Cattle

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Table 1. (*cont.*)

Restriction group	Isolate	Geographical origin	Host species
	Stanica	Romania	Human
	Utvinis	Romania	Human
	Z104	Germany	Sheep
	Z3478	Germany	Sheep
	Z3574	Germany	Sheep
	Z4313	Germany	Sheep
	Z4485	Germany	Sheep
3	CS R Henzerling*	Italy	Human
4	Z3464	Germany	Goat
5	Z3567	Germany	Sheep
6	Brustel	France	Human
	Z2534	Austria	Goat
	Z3055	Germany	Sheep
7	Z2775	Germany	Cattle
8	Brasov	Romania	Human
9	Namibia	Namibia	Goat
10	R1140	Southern Russia	Human
11	Schperling	Kirgisia	Human
12	Ouaret	France	Human
13	Campoy	France	Human
	Jaquemot	France	Human
14	Pallier	France	Human
15	Lombardi	France	Human
16	Butin	France	Human
	Raphael	France	Human

* Chlortetracycline resistant isolate derived from isolate 'Henzerling' [33].

for the development of vaccines since they must induce protection against an array of strains.

MATERIALS AND METHODS

Rickettsial isolates: Isolates of *C. burnetii*, their origin and their history are given in Table 1. All isolates were propagated in buffalo green monkey (BGM) cell cultures (Flow, Bonn, Germany) [20]. BGM monolayers in synthetic material cell culture flasks and Roux flasks respectively were inoculated with the infective agent. For the first passage the bacterial suspension was centrifuged onto the cell culture. Eighteen hours later the cell culture medium (Eagle's Minimal Essential Medium with Earl's salt, glutamin, vitamins and FCS) was exchanged. After 6–9 days the infected cell culture was homogenized ultrasonically and inoculated on BGM monolayers in larger flasks. While changing the cell culture medium weekly, the supernatants containing coxiellae were collected and centrifuged. The resulting pellet was rehomogenized ultrasonically in PBS. Further centrifugation steps and washing in PBS were performed to separate BGM cells and coxiellae.

The isolates all belong to different Q fever outbreaks, individual cases or animal infections without geographical or chronological relation. The French, German and Slovakian isolates were collected nationwide.

Pulsed field gel electrophoresis (*PFGE*): Preparation of gel plugs containing 5×10^7 *C. burnetii* particles for restriction endonuclease digestion was a modification of the procedure described by Heinzen [17] and followed the previously published protocol [16]. Embedded *C. burnetii* DNA was digested at 37 °C for 12 h in sterile cups containing 400 μl reaction buffer and 10 U/ml restriction enzyme *Not*I (Amersham, Bad Homburg; Germany). Prepared samples were stored at 4 °C. Running gels consisted of 1% MBC agarose (Bio-Rad, München, Germany, Cat. No. 162-0133) in 1 × TBE buffer (0·1 M Tris, 0·1 M boric acid, 2 mM EDTA). Electrophoresis was performed using the contour-clamped homogenous electric field (CHEF) apparatus from Bio-Rad. Lambda/*Hin*dIII (Pharmacia, Freiburg, Germany), 5 kb ladder (4·9 kb concatemers, Bio-Rad) or *C. burnetii* isolate Nine Mile DNA digested with *Not*I served as molecular weight standards. Running conditions for the counter-

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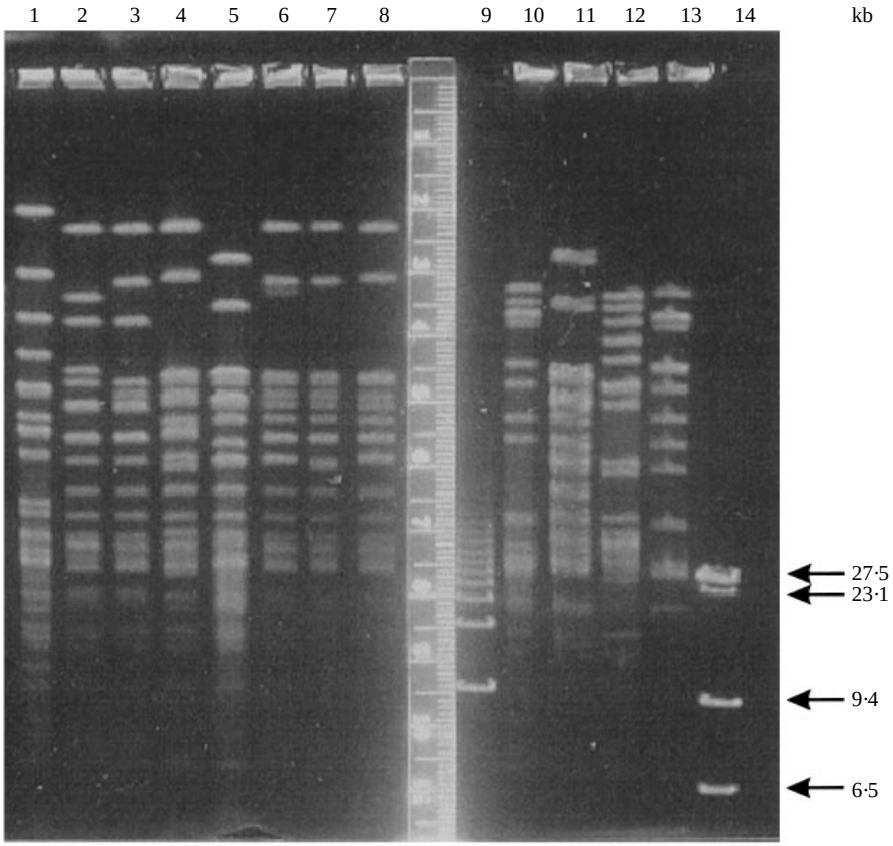

Fig. 1. CHEF-PFGE patterns after *Not*I restriction of total DNA from the following *C. burnetii* isolates as examples: lane 1, Namibia; lane 2, R1140; lane 3, Schperling; lane 4, Ouaret; lane 5, Campoy; lane 6, Pallier; lane 7, Lombardi; lane 8, Butin; lane 9, '5 kb ladder' (molecular weight marker); lane 10, Nine Mile; lane 11, Priscilla; lane 12, Scurry Q217; lane 13, Dugway; lane 14, Lambda-DNA/*Hin*dIII (molecular weight marker).

clamped homogenous electric field (CHEF-) PFGE were as follows: pulse time 0·1–11 s during 8 h, linear gradient, subsequently pulse time 9·0–34 s during following 12 h, linear gradient and continuous voltage of 6 V/cm. Gels were stained for 30 min with ethidium bromide (f.c. 0·5 μg/ml) and photographed under UV light. Fragment sizes of resulting restriction patterns were analysed by image analysis program Bioprofil (Fröbel, Lindau, Germany).

DNA hybridization: Recombinant *omp* DNA [21] was biotinylated as a probe using a commercial nick translation biotinylation kit (Serva, Heidelberg, Germany).

After depurination of the large DNA fragments with 0·25 M HCl (2 × 15 min) and denaturation of DNA strands by 0·5 M NaOH/1·5 M NaCl (2 × 30 min) DNA fragments were downward blotted [22] on Biodyne B (Pall, Dreieich, Germany) nylon membranes with 20 × SSC (3 M NaCl, 0·3 M sodium citrate) as transfer buffer. Hybridization was carried out at 60 °C overnight using the 'Southern Light' hybridization kit (Serva).

Calculation of the coefficient of similarity: Similarity among isolates was determined by using Dice coefficient (F), also known as the coefficient of similarity [23]. The Dice coefficient expresses the portion of shared DNA fragments in two isolates and was calculated from the formula $F = 2n_{xy}/(n_x + n_y)$, where n_x is the total number of DNA fragments from isolate X, n_y is the total number from isolate Y and n_{xy} is the number of fragments identical in the two isolates. An F value of 1·0 indicates that the two isolates have identical restriction patterns and a value of 0 complete dissimilarity.

Index of discrimination: The discriminatory power of a typing method is its ability to distinguish between unrelated isolates. It is determined by the number of types defined by the test method and the relative frequencies of the types. The index of discrimination is based on the probability that two unrelated isolates will be placed into different typing groups. To calculate the index for the present *C. burnetii* discriminating method the equation of Hunter and Gaston was applied [24].

Phylogenetic analysis: Phylogenetic analysis of restriction patterns and construction of the phylo-

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Table 2. *Hybridization patterns* (omp *groups*) *of* C. burnetii *restriction groups and* C. burnetii *isolates*, *respectively*, *according to genetic mapping of the* omp *gene*

Hybridization pattern (*omp* group)	*omp*-bearing *Not*I fragment	*Not*I restriction group	*C. burnetii* isolate	Geographical origin
1	27·5 kb	1	Henzerling	Italy
		2	Z4485	Germany
		3	CS-R	Italy
		4	Z3464	Germany
		5	Z3567	Germany
		6	Z3055	Germany
		7	Z2775	Germany
		8	Brasov	Romania
		I	Nine Mile	USA
		VI	Dugway	USA
2	180 kb	9	Namibia	Namibia
3	135 kb	10	R1140	Southern Russia
		11	Schperling	Kirgisia
4	160 kb	12	Ouaret	France
		13	Campoy	France
		14	Pallier	France
		15	Lombardi	France
		16	Butin	France
		IV	Priscilla	USA
5	150 kb	V	Scurry Q217	USA

genetic dendrogram was performed utilizing UPGMA (unweighted pair group method with arithmetic averages) [25].

RESULTS

Analysis of 80 different *C. burnetii* isolates by CHEF-PFGE (Fig. 1) after digestion of the total DNA with the restriction endonuclease *Not*I led to 20 different restriction fragment patterns (Fig. 1, Tables 1, 2). Separating *Not*I digested total DNA of *C. burnetii* isolates revealed fragments in the range 2·1–390 kb. Reproducibility was extensively tested using aliquoted samples in parallel on the same gel and comparing identical samples from independent experiments. Obtained patterns were always consistent and reproducible. Four distinguished patterns (I, IV, V and VI) for reference isolates [17] were reproduced (Fig. 2*a*). The new profiles were designated as restriction groups 1–16 (Fig. 2*b*).

Estimation of similarity (Dice coefficient) among 20 restriction patterns revealed high similarities for several restriction groups. As compared with group 1, groups 2–5 showed similarity of 93·3% ($F = 0{\cdot}933$), 93·6%, 89% and 83%, respectively. Although groups 6 and 7 differed from all other groups the degree of mutual similarity was 93%. The restriction pattern of the isolate 'Namibia' in group 9 was unique among the isolates tested. Among restriction groups 10–16, similarity of 92·6% has been calculated for groups 10 and 11, 96·4% for 14 and 15 and 98·2% for 15 and 16. The index of discrimination for the present RFLP based *C. burnetii* differentiation system was calculated for 0·86.

Applying the UPGMA [25] method for analysing similarity and relationship of the 20 different *C. burnetii Not*I restriction patterns led to the dendrogram shown in Figure 3, representing the genetic distances and by this means the phylogenetic relationship of *C. burnetii* restriction groups.

Genetic mapping of the *omp* gene (EMBL database Acc. No. M88613) [26, 21] to *Not*I digested CHEF-PFGE separated total DNA of *C. burnetii* isolates revealed a singular *omp*-bearing *Not*I fragment for every isolate and five *omp*-bearing *Not*I fragments different in size altogether. After hybridization five groups of isolates corresponding to the size of the *omp*-bearing *Not*I fragment were distinguishable (Table 2). These groups will be designated as *omp* groups. The first *omp* group (Table 2) consists mainly of isolates from central Europe (Slovak Republic, Germany, Italy) whilst the only African isolate

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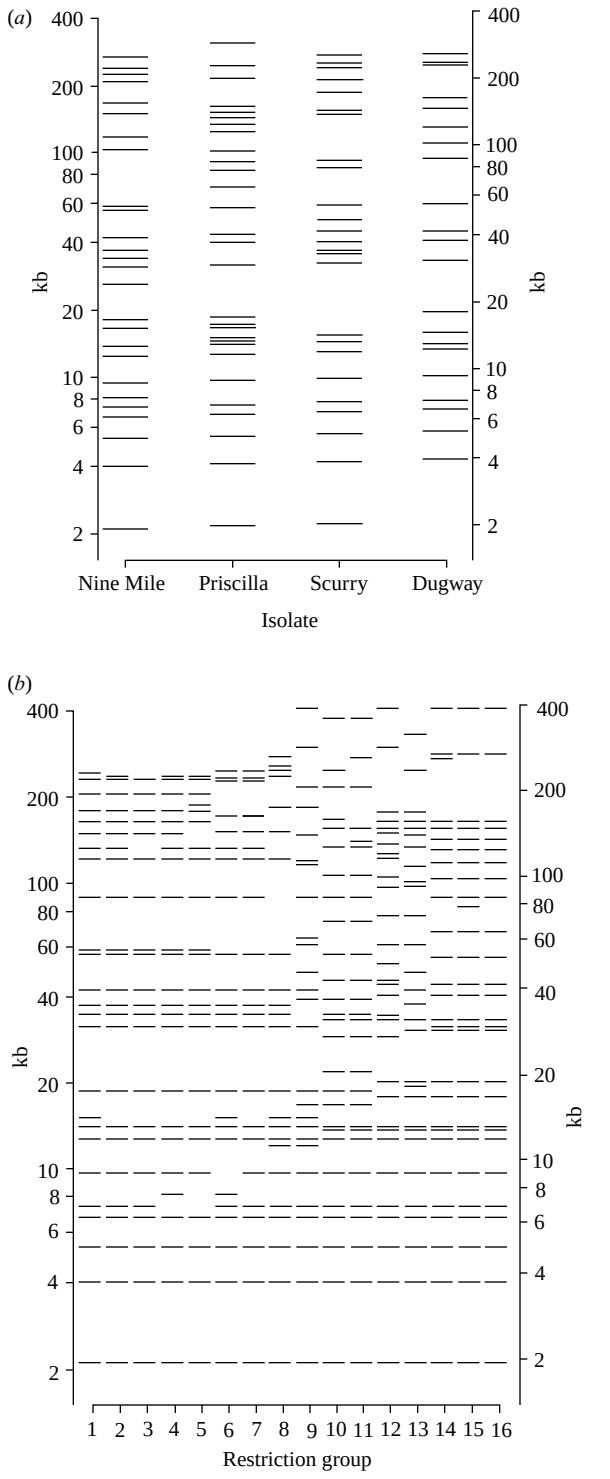

Fig. 2. (*a*) CHEF-PFGE patterns of *C. burnetii* reference isolates representing *Not*I restriction groups I, IV, V and VI (schematically, fragment size in kb). (*b*) CHEF-PFGE patterns of *C. burnetii* *Not*I restriction groups 1–16 (schematically, fragment size in kb).

(Namibia, restriction group 9) represents the second group. The third *omp* group consists of two isolates from South East Russia and Kirgisia respectively.

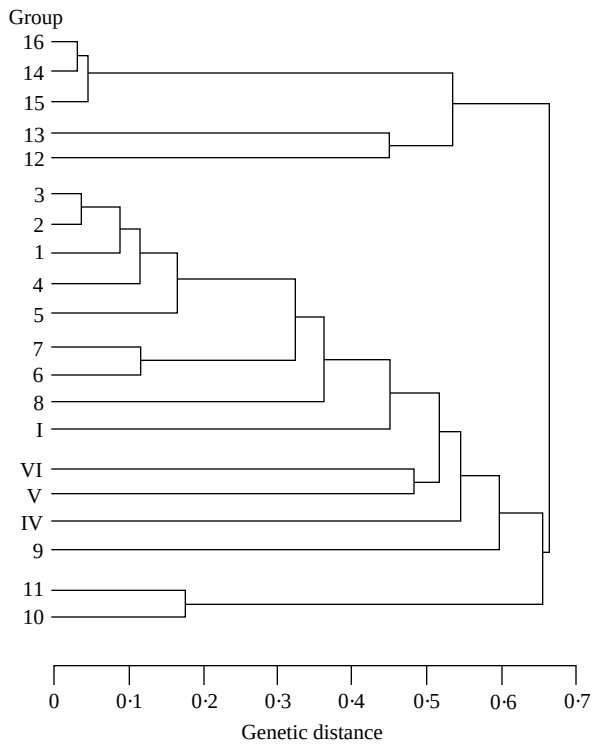

Fig. 3. Dendrogram of DNA divergence generated from banding patterns of *Not*I restriction groups of *C. burnetii* isolates by the unweighted pair group method with arithmetic averages (UPGMA).

Other Russian coxiellae isolated in North Western Russia belong to the first group. The fourth group represents most of the French isolates and contains only one American isolate. Finally, the plasmidless isolates [27] form a fifth *omp* group.

DISCUSSION

Although *C. burnetii* is considered as homogenous species by serological methods and sequence analysis of 16S rRNA gene [28], data presented in this study underline the genetic diversity among isolates. In addition to four *Not*I restriction patterns for reference isolates [17] and 10 *Not*I patterns detected by Thiele [18, 19] 6 further *Not*I patterns were distinguished and thus 42 additional *C. burnetii* isolates were classified by RFLP. The restriction enzyme *Not*I was chosen with regard to comparison of new and elder results.

Analysis of RFLP after PFGE must be considered as a powerful tool for typing isolates. If more than 10 distinct DNA fragments are resolved by PFGE, this method is considered to be robust and of high discriminatory ability [29]. Once analysis of RFLP is established many isolates can be tested within a short

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time and with a high level of reproducibility. The discriminatory ability of the present RFLP analysis (index of discrimination) is far better than the ability of many traditional typing systems e.g. some serotyping systems with indices of 0·5–0·7, and in the present study almost reaches the recommended value (0·9) [24].

So far classification of *C. burnetii* isolates was established particularly to determine the virulence of new isolates. Understanding of relatedness among *C. burnetii* isolates was not of major interest, possibly because the differentiation methods did not reveal sufficient diversity and comparatively few isolates had been analysed. Vodkin [13] mentioned RFLP differences between Northern American and European *C. burnetii* isolates, whereby European isolates showed similar restriction patterns. Up to now diversity among *C. burnetii* isolates has never been used to quantify relatedness.

The potential to detect even slight differences among bacterial isolates by PFGE supports phylogenetic analysis by UPGMA. Phylogenetic analysis of the RFLP patterns by UPGMA is well established but it should always be kept in mind that a dendrogram is a statistical result that is affected strongly by the stochastic error of nucleotide substitution [30]. The phylogenetic relatedness of *C. burnetii* restriction groups is presented in Fig. 3. Formation of groups was confirmed by mapping of the *omp* gene (Table 2).

In this study we demonstrated that genetic similarity i.e. relatedness of *C. burnetii* isolates determined by RFLP and hybridization patterns corresponded to the geographical origin (Tables 1, 2). Classification of *C. burnetii* isolates by RFLP corresponding to the geographical origin may help to detect the source of infection in case of Q fever outbreaks (e.g. imported animals) and how the pathogen is spread.

The present results also underline that virulence may be a host dependant function, independent of plasmid type [11] as has been shown by Stein and Raoult [31]. Closely related isolates like the French isolates may cause either acute ('Butin', 'Pallier', 'Lombardi') or chronic ('Ouaret', 'Jaquemot', 'Raphael', 'Bernard', 'Deborne', 'Campoy', 'Brustel') Q fever. Even if isolates ('Butin', 'Raphael') are identical in restriction pattern and plasmid type [19], infections may exhibit acute hepatitis or endocarditis. The French *C. burnetii* isolate 'Bernard' (restriciton group I) also demonstrated that plasmid type and disease do not correlate. This isolate contains the 'acute' QpH1 plasmid although it is an endocarditis-causing isolate. Presence of the QpH1 plasmid in the isolate 'Bernard' was demonstrated by PCR [19] and can be deduced from restriction group I since a different plasmid would lead to another restriction pattern.

Beyond that, analysis of phase I and phase II organisms [32] of the same isolate led to identical restriction patterns, indicating that phase variation is determined by minor genetic differences. This finding confirms the results of Mallavia and colleagues [15] and Hendrix and colleagues [16].

Although geographically disparate isolates can be distinguished by RFLP, no distinct serological or obvious biological differences have been demonstrated [13]. Genetic diversity in coxiellae may be mainly the result of DNA rearrangements rather than extensive transitions or transversions since cross hybridization experiments revealed a high degree of homology [14, 16]. Vodkin and colleagues [13] described that singular bands of RFLP patterns in one isolate regularly correspond to other bands of different isolates indicating the loss of restricition sites without deletion of the whole DNA fragment or genes.

These findings are supported in the present study since the *omp* gene could be detected in every isolate. This may be due to the crucial importance of this gene of which the function is still cryptic or due to the overall genetic homogeneity of *C. burnetii* apart from varying restriction sites.

Stein and colleagues [28] demonstrated upon 16S rRNA studies that *C. burnetii* isolates are closely related. They suggest that the species *C. burnetii* within the phylogenetic homogenous genus Coxiella should not be divided. Although our findings indicate some genetic differences among *C. burnetii* isolates we designated the presented 20 groups still as 'restriction groups'. However, following Tenover and colleagues [29] a group of isolates that can be distinguished from other isolates by genetic characteristics represents a strain. This means that *C. burnetii Not*I restriction groups may be considered as strains. Nevertheless and despite RFLP, restriction groups should not be considered as subspecies due to the high degree of DNA homology [14, 15].

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