Eur. J. Epidemiol. 0392-2990
July 1993, p. 419-425

EUROPEAN JOURNAL OF EPIDEMIOLOGY

Vol. 9, No. 4

POLYMORPHISM IN DNA RESTRICTION PATTERNS OF *COXIELLA BURNETII* ISOLATES INVESTIGATED BY PULSED FIELD GEL ELECTROPHORESIS AND IMAGE ANALYSIS

D. THIELE[1], H. WILLEMS, G. KÖPF and H. KRAUSS

Institut für Hygiene und Infektionskrankheiten der Tiere - Justus-Liebig-Universität Frankfurter Str. 89 - 6300 Giessen, Germany.

Key words: *Coxiella burnetii* - Q fever - Pulsed field gel electrophoresis - Image analysis

Pulsed field gel electrophoresis was used for differentiation of *Coxiella burnetii* isolates derived from animals in Europe and one from Namibia. Previously published data on isolates from North America demonstrated four groups with distinct banding patterns. Fingerprints of prototype isolates as references confirmed four different cleavage patterns upon NotI restriction of total DNA as described. With isolates available at our institute five additional DNA banding patterns were obtained from European and one Namibian isolate after endonuclease restriction with NotI. To compare patterns from different electrophoretic runs more precisely an image analysis system was used.

INTRODUCTION

Q fever in man and animals caused by *Coxiella (C.) burnetii* is found worldwide. In man it causes a variety of diseases, e.g. acute flu-like illness, pneumonia, hepatitis or chronic endocarditis. In animals *C. burnetii* is found in the female reproductive system, both uterus and mammary gland, and may cause abortion or infertility. Chronic shedding from these localizations into the environment is a major source for human infection. Disease is mainly diagnosed by serology or direct isolation of *C. burnetii*, using cell culture technique or embryonated chicken eggs.

Such techniques have failed to discriminate *C. burnetii* isolates, but precise identification of the pathogen is an important epidemiological marker. Strains of different virulence do occur. Investigations to differentiate isolates were described by Samuel *et al.* (7). Attempts using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting techniques - based on protein profiles - for differentiation of isolates were unsatisfactory. A more recent report described differences in the lipopolysaccharide (LPS) pattern of different isolates after SDS-PAGE and silver staining (2).

Recently, a new technique for the differentiation of *C. burnetii* isolates was introduced by Heinzen *et al.* in 1990 (3). These authors used pulsed field gel electrophoresis (PFGE) after NotI (GC/GGCCGC) or SfiI (GGCCNNNN/NGGCC) restriction of total *C. burnetii* DNA. Their data partially supported the genomic groupings already derived (genomic groups I-VI representing strains "Hamilton", "Vacca", "Rasche", Biotzere", Corazon" and "Dod") based on plasmid profiles and polymerase chain reaction (PCR) results or restriction-endonuclease-digested DNA separated by SDS-PAGE (1, 4, 5). At least isolates from genomic groups I, IV, V and VI were easily distinguishable with PFGE. These four DNA patterns - each after NotI and SfiI restriction - were derived from eight isolates, all from the North American continent.

[1] Corresponding author.

We have investigated the patterns of reference isolates from the USA and a number of isolates from Europe (Austria, CSFR, Germany, Greece, Italy, Romania, Sweden, Switzerland) and one isolate from Namibia. Differences on the DNA level among isolates of *C. burnetii* from America and Europe after HaeIII restriction have already been described by Vodkin *et al.* (8). The PFGE procedure described by Heinzen *et al.* (3) needed a time consuming running of two or more separate gels with different switch times to get a full characterization of an isolate's restriction enzyme DNA pattern. We investigated the possibility of obtaining a full characterization after only one run with optimized pulse times. In addition, image analysis was tested as a tool to compare more precisely different patterns and to standardize results from different runs with the same parameters against an internal standard.

MATERIALS AND METHODS

C. burnetii isolates (Table 1) were propagated in buffalo green monkey (BGM) cell cultures. Preparation of gel plugs for restriction endonuclease digestion was a modification of the procedure described by Heinzen *et al.* (3). Briefly, purified heat-treated *C. burnetii* particles (15 min, 85°C) were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) at a concentration of 1.25 x 10^9/ml and diluted with one volume of 1% InCert low gelling ($\leq$ 30°C) and low melting ($\leq$ 65°C) agarose (FMC BioProducts, Rockland, ME, USA) in TE buffer at 50°C melted with the microwave procedure. The InCert agarose used was a new brand with improved gel strenght ($\geq$ 400 g/cm^2 at 1% agarose concentration). The suspension was transferred to 250 µl sample holders, each filled with 125 µl and solidified on ice for a minimum of 10 min. According to the supplier's instructions, this length of time is criticial for the formation of stronger gel plugs. Plug-embedded *C. burnetii* particles were lysed in two volumes of ES buffer (0.5 M EDTA pH 7.5 and 1% N-lauroylsarcosine) for 30 min at room temperature and deproteinized with proteinase K (f.c. 500 µg/ml) (Boehringer, Mannheim, F.R.G.), overnight at 56°C. The blocks were washed twice in 10 volumes of TE for 30 min and twice with the same volume or 1 h at 50°C in TE buffer supplemented with phenylmethyl-sulfonylfluoride (PMSF, f.c. 1.0 mM) to inactivate proteinase K. Care has to be taken at this step because PMSF is extremely destructive to mucuous membranes and is fatal if inhaled, swallowed or absorbed through the skin. PMSF aqueous solutions (pH > 8.6) can be safely discarded after they have been stored for several hours at room temperature (6). After two finale TE washes (10 volumes) the plugs were stored in TE at 4°C for no longer than 3 days, until used for restriction digest.

Restriction enzymes (NotI, SfiI) were purchased from USB (Cleveland, Ohio, USA). The embedded *C.*

TABLE 1. - Isolates of *C. burnetii* used for pulsed field gel electrophoresis.

Isolate	Source
Nine Mile RSA493[1]	USA, tick
Priscilla Q177[1]	USA, goat
S Q217[1]	USA, human
Dugway 5J108-111[1]	USA, rodents
Z 2534	Austria, goat
CS II/Ia[2]	CSFR
CS 27[2]	CSFR
CS 48[2]	CSFR
Nine Mile Brat.[2] 10 MP	CSFR, orig. USA, tick
Nine Mile Brat.[2] 15 MP	CSFR, orig. USA, tick
FFM	Germany, bovine
Hardthof	Germany, bovine
München	Germany, sheep
Rostock Z 3205 a	Germany, bovine
Z 2775	Germany, bovine
Z 3027	Germany, bovine
Z 3055	Germany, sheep
Herzberg	Greece, human
Henzerling	Italy, human
Balaceanu	Romania, human
Boţen	Romania, bovine
Brasov	Romania, human
Geier	Romania, human
Stanica	Romania, human
Utvinis	Romania, human
S1	Sweden, sheep
S4	Sweden, sheep
Andelfingen[3]	Swiss, bovine
Soyta[3]	Swiss, bovine
Namibia	Namibia, goat

[1] obtained from L.P. Mallavia, Washington State Univ., Pullman, Washington, USA;
[2] obtained from J. Kazár, Inst. of Virology, Bratislava, CSFR;
[3] obtained from H. Becht, Inst. of Virology, J.L.U. Giessen, F.R.G.

burnetii particles were digested for 48 h in sterile Eppendorf cups containing 400 µl reaction buffer and the appropriate restriction enzyme (70 U/ml for NotI and 62.5 U/ml for SfiI). Multiple enzyme digestion was performed in two steps, using first the enzyme requiring the lowest salt concentration (SfiI was incubated at 50°C in low salt buffer and NotI was incubated at 37°C in high salt buffer). Digestion was stopped by adding EDTA (f.c. 5 mM). After incubation at room temperature for 1 h the plugs were ready for pulsed field gel electrophoresis.

Electrophoresis was performed using the countour-clamped homogeneous electric field (CHEF-DR) electrophoresis system from Bio-Rad (Richmond, CA, USA). Running gels were poured in the format 13 cm x 14 cm x 0.55 cm using molecular biology certified agarose, f.c. 1% (Bio-Rad) in 1 x TBE buffer. Non-rectangular edges of gel plugs (125 µl) were cut with a glass cover slip. A sterilized glass rod was used to gently push the gel slice into the well, until it touched the bottom of the well and keeping the slice in close contact to the front of the slot, thereby strictly avoiding air trapping. Finally the gel slice was overlaid with liquid agarose of the same concentration as the running gel. Lambda HindIII (Pharmacia, Freiburg, F.R.G.), "5 kb ladder" (concatemers of 4.9 kb Bio-Rad), *Saccharomyces cerevesiae* chromosomes (Bio-Rad) or *C. burnetii* Nine Mile digested with NotI served as standards.

The gel was loaded into the electrophoresis unit filled with 1 x TBE buffer that had been precooled to 14°C. Running conditions were as follows: initial switch time ramping 0.1-11 s during 8 h, linear gradient, final switch time ramping 9.0-34 s during 12 h, linear gradient. To monitor separation at different time intervals ethidium bromide was always included in the running gel (1.6 mg/100 ml gel), although the electrophoretic mobility of linear double-stranded DNA is reduced by approximately 15% in the presence of the dye. For final documentation and detection of small bands gels were restained with ethidium bromide.

The resulting restriction patterns were analyzed with the video system Bio-Profil from Fröbel (Lindau, F.R.G.), allowing densitometry as well as direct "on screen" comparison of DNA banding pattens from non-adjacent slots from one gel as well as between different electrophoretic runs. Mobility shifts of runs with identical conditions could be corrected against a standard as reference.

RESULTS

CHEF-PFGE is an excellent tool for the separation of total *C. burnetii* DNA fragments after NotI or SfiI restriction. Fragments in the molecular size range of approximately 500 kb to 6 kb could be separated. This could be achieved after optimizing conditions using only one gel run with two switch time rampings. Reproducibility was extensively tested using aliquoted samples in parallel on the same gel run and comparing identical samples from independent experiments. Obtained patterns were always consistent and reproducible. Attempts to prepare DNA without the use of gel plugs always yielded sheared fragments and distinct bands visible only in the lower molecular weight range (Fig. 1a, 1b).

Initial PFGE rsults with reference isolates (Nine Mile RSA493, Priscilla Q177, S Q217 and Dugway 5J108-111) clearly demonstrated four different banding patterns as expected after NotI (Fig. 2a-2d) or SfiI (results not shown) restriction. Using the Nine Mile isolate received from Bratislava after 10 and 15 mouse passages, NotI restriction banding patterns were identical to those obtained with Nine Mile RSA 493 (Fig. 3).

NotI restriction of the European isolates and one from Namibia revealed five additional profiles. The resulting nine profiles are shown schematically as "bar codes" in figure 4. In the range from 6 kb to more than 300 kb 17 to 26 bands were detected. In all isolates two additional NotI fragments of 4 kb and 5 kb could be demonstrated with conventional agarose gel

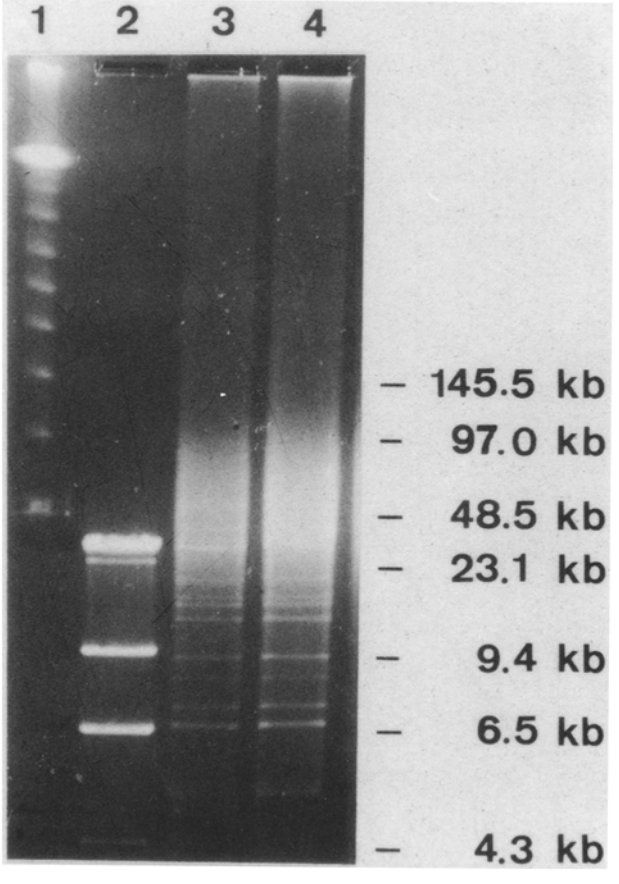

Figure 1a. - CHEF-PFGE of NotI restricted total DNA prepared by conventional methods. lane 1: lambda concatemers, lane 2: lambda HindIII, lane 3: Namibia isolate, lane 4: Namibia isolate.

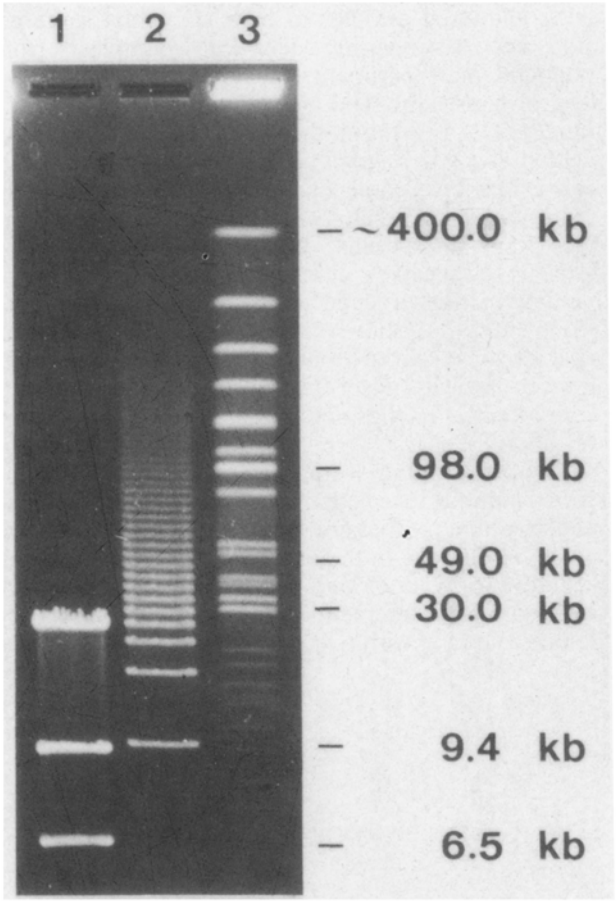

Figure 1b. - Same Namibia total DNA NotI restriction as in Fig. 1a but prepared in gel plugs and electrophoresed on a different gel. lane 1: lambda HindIII, lane 2: 4.9 kb concatemers, lane 3: Namibia isolate.

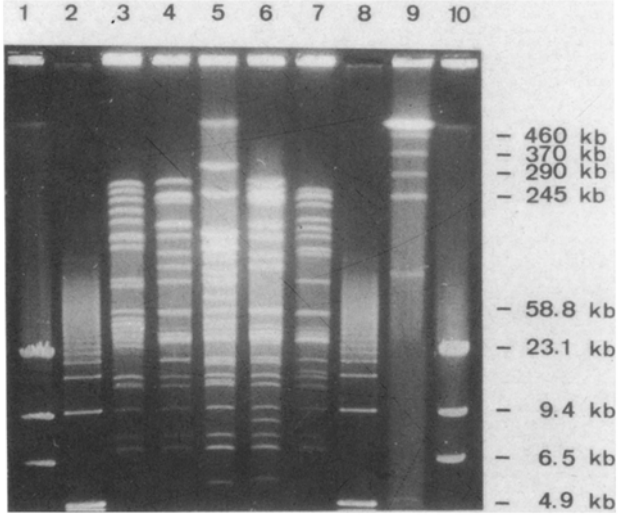

Figure 2. - CHEF-PFGE patterns after NotI restriction of total DNA of the following isolates: lane 3: S Q217, lane 4: Dugway 5J108-111, lane 5: Priscilla Q177, lane 6: Nine Mile RSA493, lane 7: München. Lane 1, 10: markers lambda HindIII and uncut Nine Mile RSA493, lanes 2, 8: 4.9 kb concatemers, lane 9: *S. cerevisiae* strain YNN295 chromosomes.

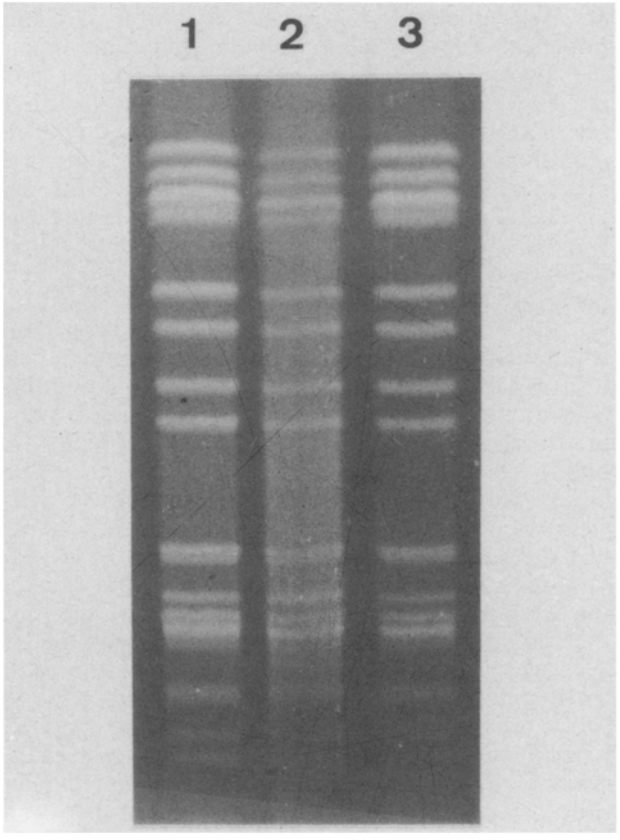

Figure 3. - CHEF-PFGE patterns after NotI restriction of total DNA of the following isolates: lane 1: Nine Mile Brat. 15 MP, lane 2: Nine Mile RSA493, lane 3: Nine Mile Brat. 10 MP.

electrophoresis. Using SfiI restriction instead, consistently fewer bands were found. Results from SfiI and double digest SfiI and NotI are presented in Figures. 5 and 6.

Isolates with identical DNA banding patterns after NotI restriction were put into the same restriction group (Table 2). The majority of the European isolates had a unique pattern clearly distinct from those four obtained with the American isolates (Fig. 2e). Another group of isolates (Balaceanu, Romania; CS 27, CSFR; Hardthof, Germany and the Nine Mile isolate previously obtained from Bratislava after different passage histories) could be grouped together with the reference isolate Nine Mile RSA493 (Fig. 3). Two isolates derived from cows from different parts of Germany (West and East Germany) were identical to the Dugway isolate after NotI or SfiI and combined NotI/SfiI restriction although the Dugway isolates originally were only found in rodents without causing any disease in guinea pigs and unknown virulence for humans (3, 5) (Fig. 6). Four isolates, including the one from Namibia, showed a unique restriction pattern (Table 2, Fig. 4).

Using the image analysis system we determined the molecular weight in the range of 6-400 kb.

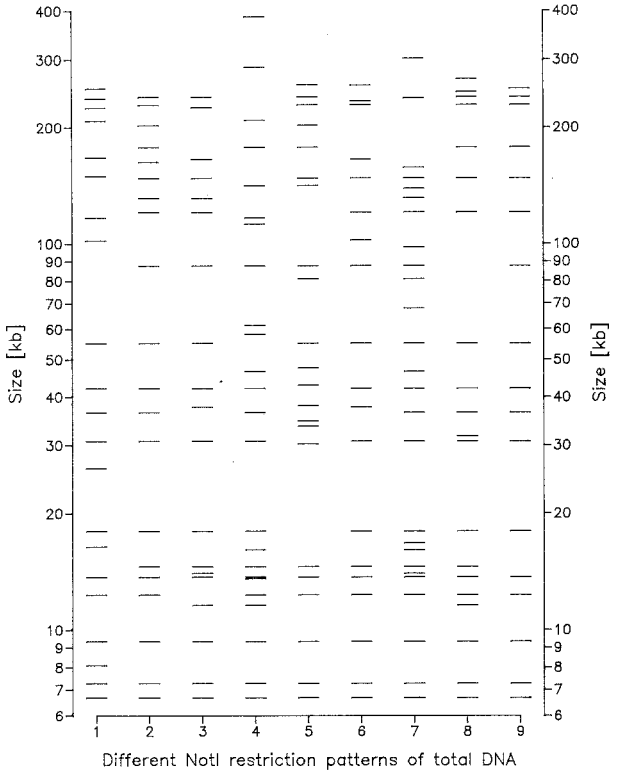

Figure 4. - "Bar codes" representing nine different patterns after NotI restriction of total DNA. An example for each pattern is given. Lane 1: Nine Mile RSA493 (21), lane 2: München (20), lane 3: Z2534 (19), lane 4: Namibia (24), lane 5: S Q217 (22), lane 6: Dugway 5J108-111 (19), lane 7: Priscilla Q177 (26), lane 8: Brasov (20), lane 9: Z2775 (17), number in brackets indicates number of bands.

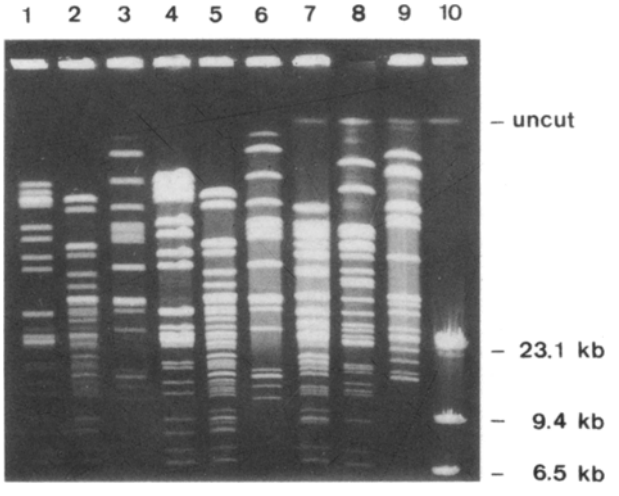

Figure 5. - CHEF-PFGE pattern after NotI, SfiI and NotI/SfiI restriction of total DNA of the following isolates: lane 1: Hardthof NotI, lane 2: Hardthof NotI/SfiI, lane 3: Hardthof SfiI, lane 4: Nine Mile RSA493 NotI, lane 5: Nine Mile RSA493 NotI/SfiI, lane 6: Nine Mile RSA493 SfiI, lane 7: Priscilla Q177 NotI/SfiI, lane 8: Priscilla Q177 NotI, lane 9: Priscilla SfiI, lane 10: lambda HindIII and uncut total DNA of the isolate Nine Mile Brat. 10 MP as marker.

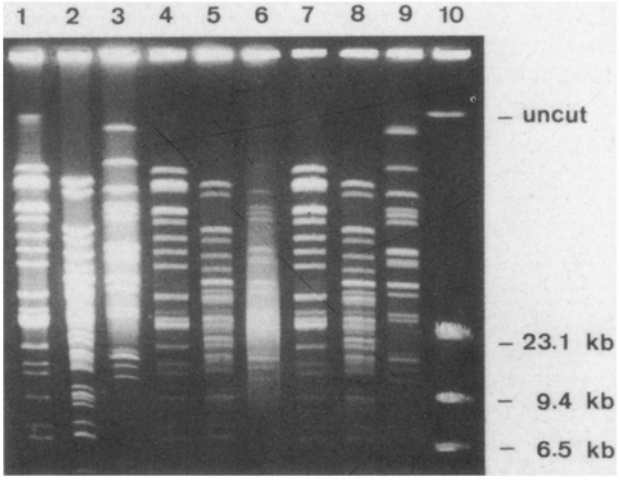

Figure 6. - CHEF-PFGE pattern after NotI, SfiI or NotI and SfiI restriction of the following isolates lane 1: Rostock Z 3205 b NotI, lane 2: Rostock Z 3205 b NotI/SfiI, lane 3: Rostock Z 3205 b SfiI, lane 4: Dugway 5J108-111 NotI, lane 5: Dugway 5J108-111 NotI/SfiI, lane 6: Dugway 5J108-111 SfiI, lane 7: Z 3027 NotI, lane 8: Z 3027 NotI/SfiI, lane 9: Z 3027 SfiI, lane 10: lambda HindIII and uncut Nine Mile Brat. 10 MP as marker. Lanes 1, 4, 7 and 2, 5, 8 as well as 3, 6 and 9 represent identical patterns.

Genome sizes obtained by summing up NotI fragment molecular weight sizes from each isolate yielded total DNA sizes between 1.4 Mb and 1.9 Mb. The video system also allowed direct comparison of patterns from different runs after correction against NotI-digested Nine Mile RSA493 DNA as an internal standard (Fig. 7).

DISCUSSION

Only a few methods have been described for the differentiation of *C. burnetii* isolates. Serological methods completely failed in this task, with the exception of classification of phase I or phase II using the complement fixation test (CFT). Differentiation based on protein or LPS profiles was not successful or was inconvenient with low resolution. Improvements with respect to better differentiation were possible when research was carried out on the DNA level. Plasmid profile investigation detected three different plasmids QpH1, QpRS and QpDG and one group of plasmidless isolates. Restriction fragment lenght polymorphisms (RFLPs) of total DNA after restriction with BamHI followed by SDS-PAGE and silver staining placed the isolates tested into one of six groups (4). Due to the high cutting frequency of BamHI, resulting RFLP patterns contain numerous bands difficult to compare. We used a rare cutting restriction enzyme on total DNA from coxiella isolates in combination with the CHEF-PFGE technique and image analysis. A previous report using a similar CHEF-PFGE technique with isolates from

TABLE 2. - *Coxiella burnetii* isolates with identical banding patterns after NotI restriction are grouped together.

Genomic group (Lit. 5)	Reference Isolate (Lit. 5)	Test Isolate
I	**Nine Mile RSA493**	Nine Mile Bra. 10 MP Nine Mile Bra. 15 MP Balanceanu CS 27 Hardthof
IV	**Priscilla Q177**	
V	**S Q217**	
VI	**Dugway 5J108-111**	Rostock Z 3205 a Z 3027
		Andelfingen Boren Cs II/Ia CS 48 Frankfurt Geier Henzerling Herzberg München S1 S4 Soyta Stanica Utvinis
		Z 2534 Z 3055
		Z 2775
		Brasov
		Namibia

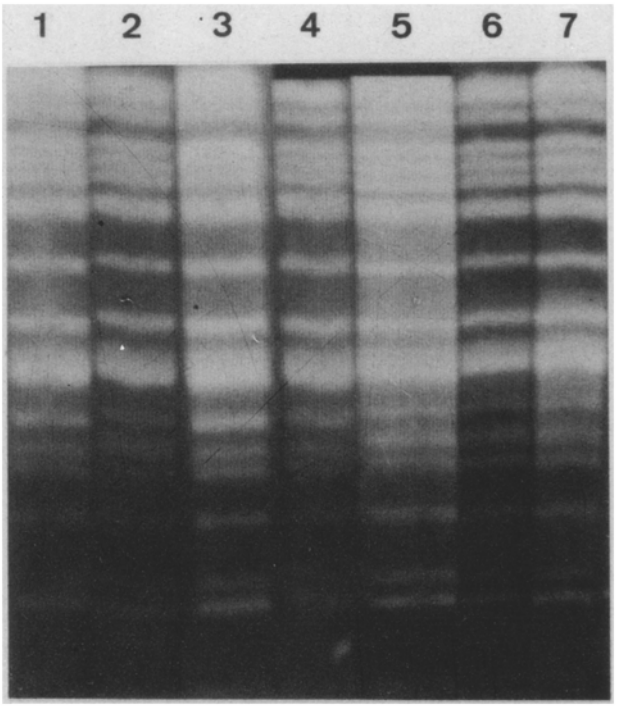

Figure 7. - CHEF-PFGE patterns after NotI restriction of total DNA from the following isolates: lane 1: Utvinis, lane 2: Boren, lane 3: Stanica, lane 4: S4, lane 5: Geier, lane 6: Henzerling, lane 7: München. Patterns were obtained from different runs corrected against a standard and aligned directly on screen using an image analysis system. Picture taken from screen.

North America only detected four different RFLPs (3).

The described "two step" CHEF-PFGE technique with only one run for the differentiation of *C. burnetii* isolates proved to be a reproducible and reliable alternative for the classification of these isolates. Patterns from within runs and from separate runs of the same isolate could be compared after standardization on screen with the image analysis system and were always identical. NotI fragments below 10 kb so far not described have been detected, revealing common fragments of ca. 4, 5, and 6.5 kb. When DNA fingerprinting is done by in gel side-by-side comparison, DNA fragment size determination is not needed. However, this can become expensive when many isolates are involved. Size determination using an isolate as a standard allows the comparison of patterns across time and space (Fig. 7).

With the reference isolates Nine Mile RSA493, Priscilla Q177, S Q217 and Dugway 5J108-111, four different patterns were obtained as previously published (3). In contrast, with the European isolates and the one from Namibia five additional distinct patterns were observed, supporting data previously published demonstrating variation between isolates from different geographical regions after HaeIII restriction (8). Although heterogeneous (in regards to geographic origins), most of the European isolates were identical upon NotI restriction. Two NotI restriction patterns of isolates obtained from bovine samples from Germany were identical to restriction patterns of the Dugway isolate, previously isolated only from rodents.

In summary, we have demonstrated a considerable heterogeneity among *C. burnetii* RFLPs, even using enzymes that have few recognition sites and generate large DNA fragments. Therefore RFLPs obtained after restriction with rare cutting enzymes are useful as epidemiological markers. Common restriction fragments may serve as a basis for vaccine development since protection should be induced against an array of isolates from various geographical

areas. However, it is still necessary to determine if isolates with the same total DNA restriction pattern are likely to represent a single strain. In addition, as proposed by Heinzen *et al.* (3), CHEF-PFGE provides a powerful technique for deriving a physical macrorestriction map of *C. burnetii* chromosomal DNA, thereby improving further genetic studies.

Acknowledgements

Excellent technical assistance by Mrs. R. Frank is gratefully acknowledged.

REFERENCES

1. *Frazier M.E., Mallavia L.P., Samuel J.E.* and *Baca O.G.* (1990): DNA probes for the identification of *Coxiella burnetii* strains. In Rickettsiology: Current Issues and Perspectives, eds. Hechemy K.E., Paretsky D., Walker D.H. and Mallavia L.P. - Ann. N.Y. Acad. Sci. *590:* 445-456.

2. *Hackstadt T.* (1986): Antigenic variation in phase I lipopolysaccharide of *Coxiella burnetii* isolates - Infect. Immun. *52:* 337-340.

3. *Heinzen R., Stiegler G.L., Whiting L.L., Schmitt S.A., Mallavia L.P.* and *Frazier M.E.* (1990): Use of pulsed field gel electrophoresis to differentiate *Coxiella burnetii* strains. In Rickettsiology: Current Issues and Perspectives, eds. Hechemy K.E., Paretsky D., Walker D.H. and Mallavia L.P. - Ann. N.Y. Acad. Sci. *590:* 504-513.

4. *Hendrix L.R., Samuel J.E.* and *Mallavia L.P.* (1991): Differentiation of *Coxiella burnetii* isolates by analysis of restriction-endonuclease-digested DNA separated by SDS-PAGE - J. Gen. Microbiol. *137:* 269-276.

5. *Mallavia L.P.* and *Samuel J.E.* (1989): Genetic diversity of *Coxiella burnetii.* In: Intracellular Parasitism, ed. James Moulder - CRC Press, Boca Raton, Fla., pp. 117-126.

6. *Sambrook J., Fritsch E.F.* and *Maniatis T.* (1989): In Molecular cloning. A laboratory manual, second edition - Cold Spring Harbor Laboratory Press pp. 6-56.

7. *Samuel J.E., Frazier M.E.* and *Mallavia L.P.* (1985): Correlation of plasmid type and disease caused by *Coxiella burnetii* - Infect. Immun. *49:* 775-779.

8. *Vodkin M.H., Williams J.C.* and *Stephenson E.H.* (1986): Genetic heterogeneity among isolates of *Coxiella burnetii* - J. Gen. Microbiol. *132:* 455-463.