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PHENOTYPIC AND GENOTYPIC HETEROGENEITY OF 8 NEW HUMAN *COXIELLA BURNETII* ISOLATES

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Summary. - Eight new strains of *Coxiella burnetii* were isolated from chronic Q fever patients using centrifugationshell vial technique. Seven patients had endocarditis (including one patient with an immunodeficiency syndrome), and one had a vascular prosthesis infection. Three prototype strains, Nine Mile phase II, Q212 and Priscilla and eight new isolates were cultured in L 929 cells. Heterogeneity of their cytopathic effect was observed. DNAs of the eleven strains have been isolated and purified by standard procedures. Plasmid DNA was separated from chromosomal DNA by a low melting point gel. Electrophoresis in agarose gel showed that seven of the eight new strains had plasmids which were about 40 kb (plasmid V517 was used as size marker). Endonuclease-restriction analysis of the 8 human isolates is currently under investigation.

Key words: Coxiella burnetii; chronic Q fever; cytopathic effect; plasmid DNA

Introduction

Q fever, caused by *Coxiella burnetii (C.b.),* may be acute or chronic (Marrie, 1990). Strains isolated from acute and chronic cases of Q fever differ with respect to lipopolysaccharides, surface protein profiles (Hackstadt *et al.,* 1985), and plasmid type (Samuel *et al.,* 1985). Because *C.b.* are difficult to isolate and to grow, their genetic studies have been limited to few strains. Different plasmids have been found in human and animal isolates of *C.b.* The first plasmid, named QpH_1, was found in the Nine Mile phase I isolate. It is 36 kb in size, has a density of 1.61 g/mm^3 in cesium chloride-ethidium bromide (CsCl-Et Br) gradient and is present at approximately three copies per cell. The second plasmid, designated QpRS (Mallavia *et al.,* 1985; Michael *et al.,* 1990), has been obtained from an animal isolate called Priscilla (placenta of a goat that had aborted), but also from 4 human isolates causing chronic endocarditis. It has considerable homology with QpH_1 but is 39 kb in size. The third plasmid is approximately 51 kb in size and has been named QpDG. It comes from feral rodent isolates obtained near Dugway, Utah, and was designated Dugway.

Stoenner and Lackman, 1960). In a few isolates obtained from humans with endocarditis no plasmid was recovered, and it was shown that plasmid DNA was integrated into the genome of these isolates.

A correlation between disease state and plasmid type has been established (Samuel *et al.*, 1985). All *C.b.* isolates from acute diseases have been found to contain the plasmid QpH_1. Isolates from cases of chronic Q fever possess the plasmid QpRS (Savinelli and Mallavia, 1990) or QpRS sequences that have been integrated into the chromosome. Isolates containing QpDG are currently defined as infectious but avirulent and have not been isolated from humans (Stoenner and Lackman, 1960). We report here the isolation of 8 new strains of *C.b.* from patients suffering of chronic Q fever. Out of seven patients suffering of endocarditis one was immunocompromised; one had a vascular prosthesis infection. These strains, Nine Mile phase II, Q212 and Priscilla strains were cultured in L929 cells. The DNA of the 11 strains has been isolated and purified by standard procedures (Maniatis *et al.*, 1982). Plasmid DNA was separated from chromosomal DNA by electrophoresis in low melting point gels. Electrophoresis in agarose gel showed that plasmids of Nine Mile, Priscilla and 7 of the new humans strains were localized between 34 and 41 kb.

Materials and Methods

Rickettsiae. C. burnetii Nine Mile strain was obtained from O. Baca, Priscilla and Q212 strains were obtained from T. Hackstadt. The eight fresh isolates were coming from patients suffering of chronic Q fever; they were isolated using a centrifugation shell vial technique. Samples (heart valves, arterial prosthesis) were inoculated onto human embryonic lung fibroblasts (HEL), cell monolayers growing in shell vials. *C. burnetii* was detected 6 days later using immunofluorescence (Raoult *et al.*, 1990).

Propagation and purification of rickettsiae. The isolates were subcultured in L929 fibroblasts. The medium (MEM, glutamine 1 %, foetal calf serum 4 %) was changed every 4 to 10 days. The cytopathic effect of *C.b.* in L929 cells was observed. When cells were heavily infected (more than 70 % of infected cells), they were harvested and propagated in other flasks. Cell lysis was performed with trypsin at 5 % (0.1 vol), 30 min at 37 °C. Rickettsiae were purified by Renografin density gradient centrifugation (Renografin 7 % in SPG buffer pH 7.2). First, the cell fragments were pelleted by centrifugation at 4 °C for 15 min at 1000 rev/min, then *C.b.* was pelleted by centrifugation at 4 °C for 15 min at 8000 rev/min. The pellets were washed 10 min twice with Rinaldini solution, checked by Gimenez staining and frozen at -20 °C.

DNA isolation and purification. The quantity of microorganisms obtained from four 150 cm^2 cell flasks infected at 70 to 80 % were suspended in 300 μl Tris-HCl pH 7.5 buffer. This suspension was incubated 4 hr at 37 °C with 50 μl of proteinase K at the concentration of 20 mg/ml. Lysis was completed by adding sodium dodecyl sulphate (SDS) to a 1 % final concentration. The DNA was extracted by double centrifugation for 5 min at 15 000 rev/min in phenol and phenolchloroform. The DNA was precipitated with 3 vol of absolute ethanol overnight at -20 °C. After a centrifugation at 15 000 rev/min for 10 min, the supernatant was removed by aspiration and the pellet was washed with 70 % ethanol. The repelleted DNA was lyophylized and suspended in a small volume of 10 mmol/l Tris-HCl (pH 8), 1 mmol/l EDTA which was exposed for 30 min at 37 °C to RNase (1 μl of 1 mg/ml RNase).

Electrophoresis. DNA samples were analysed electrophoretically in 0.8 % agarose low melting point gels (BIO-RAD) prepared with Tris-borate buffer. Samples were run with 4 vol of DNA plus 1 vol of tracking-dye mix. Plasmids HB 101 (41 kb) and V517 (34 kb) were obtained from *Ph.*

Sansonetti were used as size markers. After electrophoresis, the plasmids were cut under UV-light in the low melting point gel removing chromosomal DNA. After fluidification (5 min at 50 °C) the plasmid DNA was extracted and precipitated as described above, than suspended in a small vol of 10 mmol/l Tris-HCl (pH 8), 1 mmol/l EDTA.

Results

Rickettsial isolation and culture

C. burnetii was isolated from 8 patients suffering of chronic Q fever. The samples (7 heart valves, 1 vascular prosthesis) were inoculated onto human embryonic lung fibroblasts (HEL) growing in shell vials. These strains have been established and still successfully subcultured in L929 cells. The cytopathic effect of *C.b.* in L929 cells was observed for all the strains, but was quite different when comparing the 8 isolates, Nine Mile phase II, Priscilla and Q212 strain. In fact, some subcultures were heavily (70 to 80 %) infected within 10 to 15 days, others needed about 3 months (Priscilla strain). We noticed also heterogeneity concerning the size and the aspect of phagolysosomes and the whole infected cells. Q212 and two of the new strains had an impressing cytopathic effect (enormous phagolysosome as big as the cell), Priscilla and one of the new isolates formed small round cells (Figs 1 and 2).

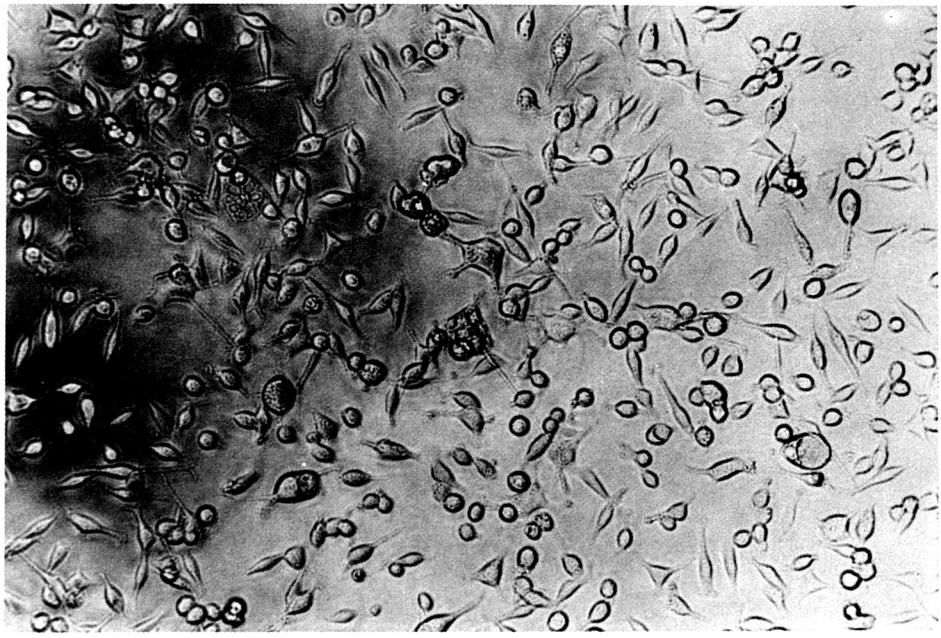

Fig. 1
Cytopathic effect of *C. burnetii* Priscilla strain in L929 fibroblasts

Rickettsial lysis and plasmid isolation

C. burnetii is difficult to lyse and is resistant to the action of lysozymes. We obtained efficient lysis of the microorganisms after treatment with proteinase K and SDS. DNA extraction and precipitation was done by standard procedures (Maniatis *et al.*, 1982). The use of a low melting point gel permitted the separation of plasmid DNA from chromosomal DNA. Plasmid screening by electrophoresis showed that 7 of the new isolates, Nine Mile and Priscilla strain and individualized plasmids (Fig. 3). Q212 and one of the new strains had no individualized plasmid DNA. The plasmid DNA of the 7 new strains, Nine Mile phase II and Priscilla strain were found to migrate in the agarose gel as a single band in the area limited by the two size markers (34 and 41 kb).

Discussion

Shell vial technique is efficient for the isolation of *C. burnetii.* Followed by subculture in L 929 cells it allows to obtain a wide variety of human and animal strains which can be utilized for further physiologic and genetic studies. Lysis

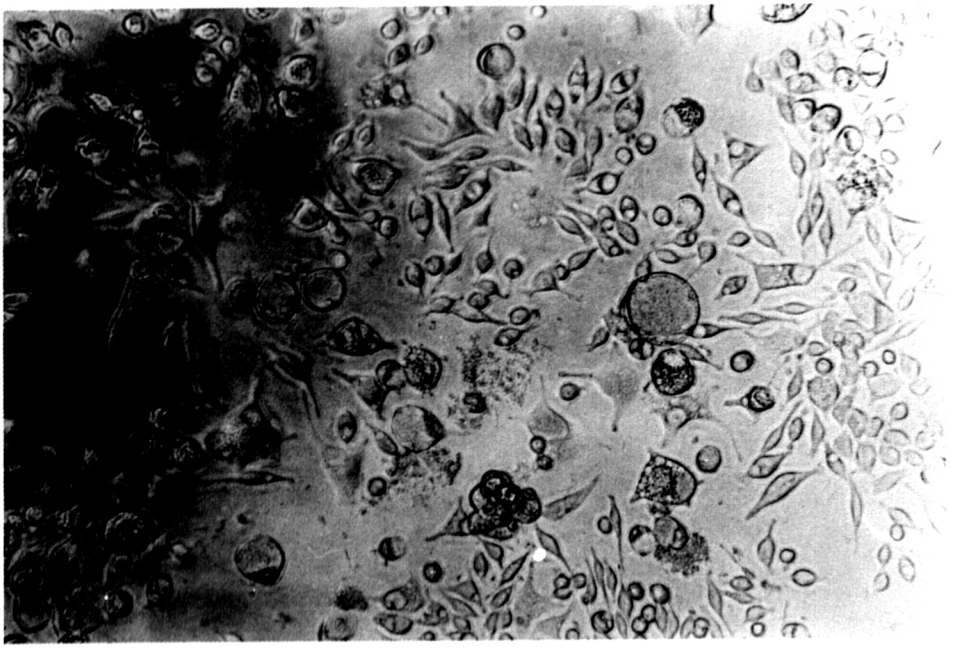

Fig. 2
Cytopathic effect of a new human *C. burnetii* chronic endocarditis isolate in L929 fibroblasts

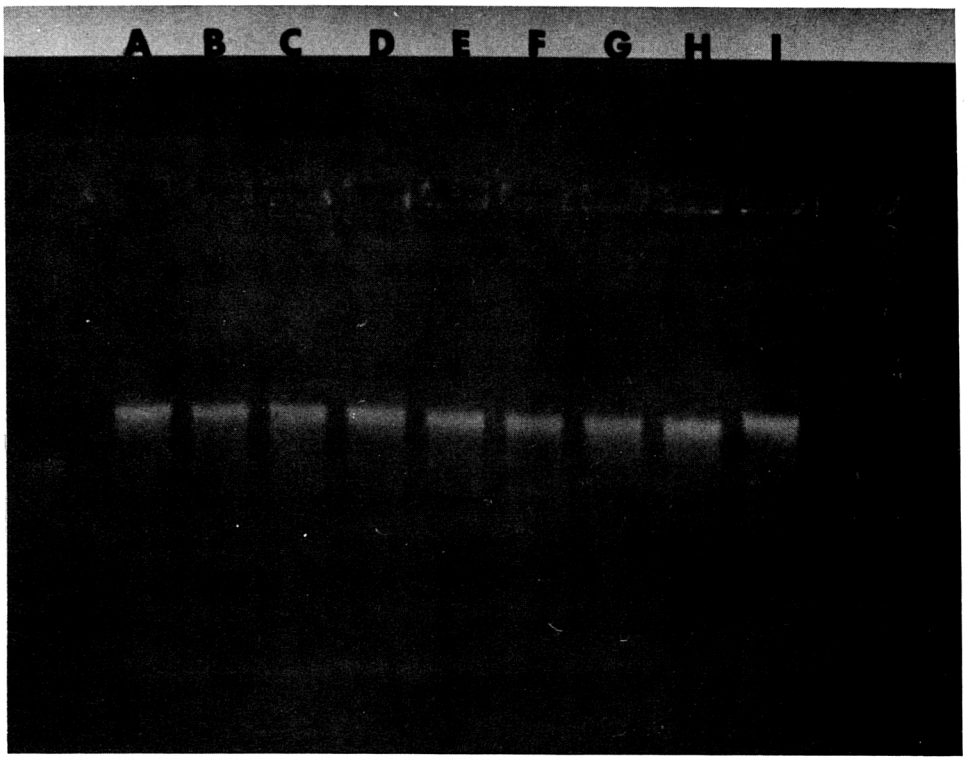

Fig. 3
Plasmid-DNA electrophoresis of 9 strains of *C. burnetii* in 0.8 % agarose gel
Lanes: A to G, 8 new human isolates,
H, Nine Mile strain (36 kb),
I, Priscilla strain (39 kb).

of microorganisms, plasmid isolation and purification are possible with simple standard procedures. The new genetic data which were reported the last few years for the pathogenicity of *C. burnetii* are only based on few strains, especially for chronic Q fever. We were interested to correlate the disease state and plasmid type, to determine if other plasmid types exist and whether there is a genetic support for virulence in the immunocompromised host. Our technique allows the recovery of more new isolates of *C. burnetii* and their plasmid purification. Our rapid plasmid screening should allow to start the restriction analysis in order to characterize the plasmid DNA of all these new strains.

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