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MOLECULAR CHARACTERIZATION OF CLONED VARIANTS OF *COXIELLA BURNETII* ISOLATED IN CHINA

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Summary. - To study the molecular properties of *Coxiella burnetii* phase variants we cloned the phase variants of *C. burnetii* Qiyi (CBQY) strain by the red plaque technique. Three cloned strains, CBQYIC3 (phase I), CBQYIIC7 (phase II) and CBQYIIC5 (semi-rough-phase) were analysed by SDS-PAGE, immunoblot assay, plasmid isolation and agarose gel electrophoresis of DNA restriction fragments. The results suggest that the unique phase-dependent substance is a lipopolysaccharide and that most protein components of phase I and phase II cells are shared. No significant differences of DNA restriction fragments were found between clonal isolates of phase I and phase II *C. burnetii* CBQY strains. A plasmid of approximately 56 Kb was isolated from both phase I and phase II variants indicating that phase variation probably could not be attributed to its presence or absence.

Key words: Coxiella burnetii; phase variation; SDS-PAGE; immunoblot assay; restriction fragments

Introduction

The obligate intracellular rickettsia *C. burnetii,* the aetiological agent of acute and chronic Q-fever and several zoonozes (Burton *et al.,* 1978; Hackstadt *et al.,* 1981; 1984) is unique from the view point phase variation phenomenon. In nature it exists as phase I, but during repeated passages in chicken embryos, it converts into phase II. This phase variation resemble in many aspects of the well-known smooth to rough (S-to-R) variation occured in gram-negative bacteria. Cells of phase I are analogous to smooth bacteria and are more virulent than cells of phase II (Stoker *et al.,* 1956; Fiset *et al.,* 1957; Kazár *et al.,* 1974). Although studies about substance base of phase variation have been made by many investigators, both of phase I and phase II with different lipopolysaccharides (LPS) have been demonstrated, but up to date the relationship between the phase variation and the proteins and DNA has not been clearly demonstrated. Recent studies of phase variation of *C. burnetii* produced results

which were somewhat contradictory, because that some of the strains used in analysis were not cloned and purified (Baca *et al.*, 1980; Schramek *et al.*, 1982). Among the isolates in China, differences were reported in LPS, proteins and DNA (Yang *et al.*, 1989, Wen *et al.*, 1990), no final conclusions have been made. Here we report, molecular properties of *C. burnetii* phase variants using, we cloned on phase variants of *C. burentii* strain CBQY obtained by the red plaque techniqe and analysed in SDS-PAGE, immunobloting assay, by plasmid isolation and DNA restriction analysis in agarose gel electrophoresis. The result suggests that the unique phase-dependent antigenic substance is LPS and that protein components of phase I and phase II are shared for the most part. No significant differences of DNA restriction fragments between cloned isolates of phase I and phase II *C. burnetii* (CBQY)strains were demonstrated, and the same plasmid was isolated from the both cloned strains.

Materials and Methods

Propagation and purification. The *C. burnetii* strain QIYI (CBQY) was originally isolated from a patient with chronic Q feved in 1962 (Yu *et al.*, 1964). The strains were propagated in mice and chicken embryo yolk sacs. Phase I (CBQYI): EP20/MP52; phase II (CBQYII): EP20/MP3/Ep51. The cloning procedure of the isogenic phase variants of strain CBQY was as previosly described (Cheng *et al.*, 1989; Yu *et al.*, in press). Purified rickettsial suspension was obtained by density gradient centrifugation as described by Zhang *et al.* (1986).

SDS-polyacrylamide gel electrophoresis (PAGE). Whole cell samples were analysed by modified SDS-PAGE (Wen *et al.*, 1990; Laemmli *et al.*, 1970). Slab gel was composed of 5 % polyacrylamide spacer gel and 12.5 % or 17.5 % separating gel (1.5 mm thick). Each rickettsial sample (3–6 μg protein) was boiled for 5 min in equal vol sample buffer (0.25 mol/l, pH 6.8 glycine.HCl buffer, containing 2 % SDS, 10 % glycerol and 5 % 2-mercaptoethanol). The proteinase K digests of whole-cell lysates (treated at final enzyme concentration 0.5 μg/μl for 2 hrs at 37°C) were separated by electrophoresis and stained with Coomassie brilliant blue or silver nitrate as described Tsai and Frasch (1982).

Immunoblot. The components separated by PAGE were transferred from gel to the nitrocellulose membrane (NCM) by electrotransfer at 200 mA (Towbin *et al.*, 1979). Antigens were detected after blocking with 10 % bovine serum albumine and 0.05 % Tween-20 in phosphate-buffered saline (PBS, pH 7.2, overnight at 37°C). After incubation with the primary antibody overnight at 4°C, the NCM sheet was washed three times in PBS (containing 0.05 % Tween-20) incubated for 1 hr at 37°C with goat anti-mouse IgG/Px, washed as before and reacted with the substrate (0.2 mol/l, pH 7.4 Tris-HCl buffer containing 0.05 % 3,3-diaminobenzidine tetrahydrochloride and 0.03 % hydrogen peroxide).

Electrophoresis of restriction endonuclease fragments. The DNA was extracted and purified as described (Marmur *et al.*, 1961; Maniatis *et al.*, 1982; Vodkin *et al.*, 1986). The restriction enzymes (*Hae*II, *Pst*I, *Bam*HI, *Hidn*III, *Hap*I, *Xho*I, *Sa*II) were used according to instructions (provided by Sino-American Biotechnology CO). DNA fragments were analysed on 0.8 % agarose gel in 89 mmol/l Tris-HCl (pH 8.3) containing 89 mmol/l boric acid and 2.5 mmol/l EDTA as described by Southern (1979).

Plasmid isolation. Purified *C. burnetii* cells mixed with 5 ml sample buffer (0.5 mol/l EDTA, 1 % SDS, 2 mg/ml proteinase K and 1 % low gelling agarose) were incubated overnight at 50°C at final concentration for *C. burnetii* of 5 μg/ml. Next day the mixture was poured into the insert hold at 4°C for 2 hrs and then the inserts were placed into gel slots and electrophoresed under following conditions: 15 V/cm, 1.5 % agarose, 60 sec pulse time, buffer temperature 15°C (Schwartz *et al.*,

1984; Clark *et al.*, 1988). The plasmid was isolated and purified as described by Hansen *et al.* (1978). The molecular weight of plasmid is determided as described by Meyer *et al.* (1976).

Results

Results of SDS-PAGE

The whole cell components of three cloned phase variants of CBQY variants were analysed by SDS-PAGE stained with Coomassie briliant blue (Fig. 1). The gel patterns of all the variants showed similarity: each clone revealed about 35 bands, in which 7 major bands Mr 79.8, 56.2, 41.6, 39.8, 30.0, 20.1 and 17.8 kD were identified except that CBQYIIC7 had a 95.5 kD band which was absent in two others. After being proteinase K treated there was no difference among the three variants. The result of silver staining is shown in Fig. 2. The patterns which did not digest with proteinase K showed no significant difference among the variants except for two bands (36.3 and 39.8 kD) in CBQYIC3, which were not observed in CBQYIIC7 and CBQYIIC5. But after being digested with proteinase K apparent differences were seen among the variants. Most bands are not observed in CBQYIIC5 and CBQYIIC7 within the range of 30–94 kD, and three additional major bands, 44.7, 39.9 and 35.5 kD were found in the CBQYIC3. The major bands of both CBQYIC3 and CBQYIIC5 have M_r 31.6 kD, but in CBQYIIC7 the largest band had M_r 17.5 kD. In the range of 17.5 to 30 kD CBQYIIC5 and CBQYIC3 had 3–4 bands more than CBQYIIC7, and the bands of CBQYIC3 were stained more deeply than CBQYIIC5. In order to show clearly the strain-specific differences beyond the 30 kD, we used

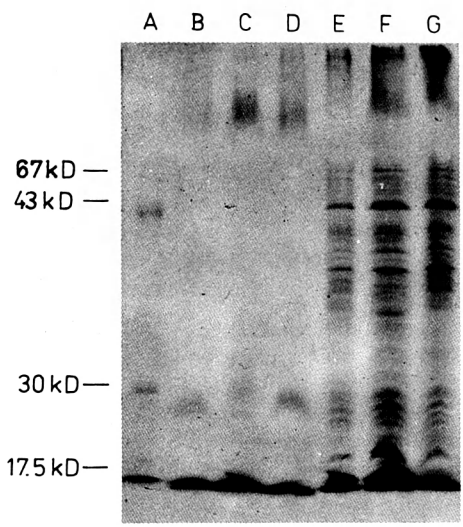

Fig. 1
The protein profile of three strains in SDS-PAGE stained with Coomassie briliant blue
A (markers); B - D CBQYIC3, CBQYIIC7, CBQYIIC5 treated with proteinase K; E - G CBQYIC3, CBQYIIC7, CBQYIIC5.

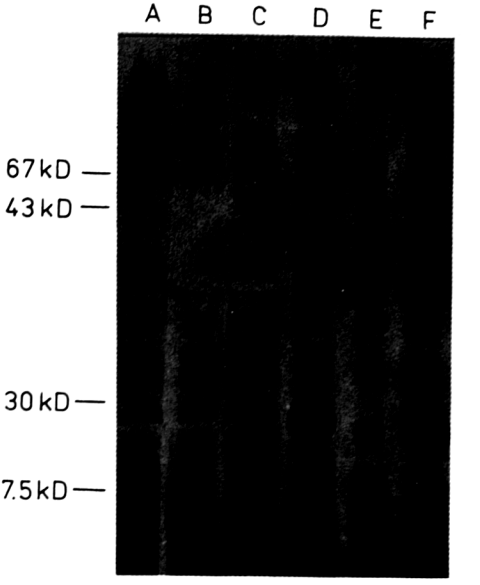

Fig. 2
The profile of the three strains in SDS-PAGE stained with LPS-staining
A - C CBQYIC3, CBQYIIC7, CBQYIIC5 treated with proteinase K; D - F CBQYIC3, CBQYIIC7, CBQYIIC5.

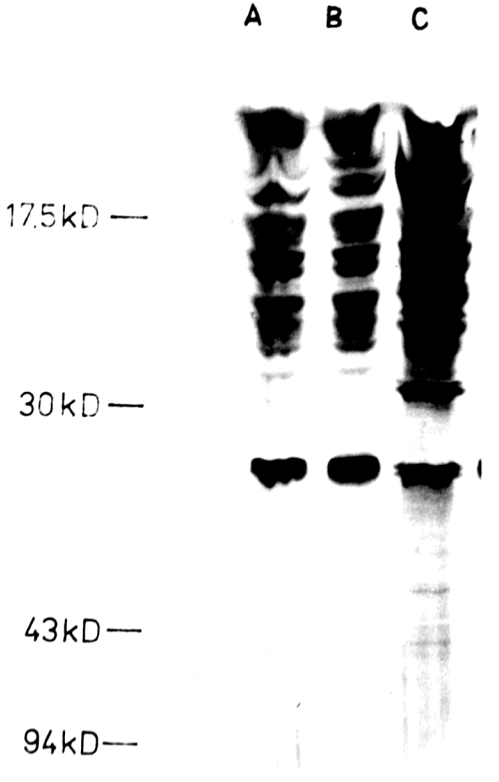

Fig. 3
The profile of three strains on SDS-PAGE stained with LPS-staining
A (CBQYIIC5); B (CBQYIIC7); C (CBQYIC3).
*17.5 % separation gel

a 17.5 % separation gel (Fig. 3). Within the 30–94 kD range the three variants had a 31.6 kD common band apart from this there were 18 bands common in CBQYIC3, but CBQYIIC5 and CBQYIIC7 differed from CBQYIC3 by loss of the majority of bands in this range. Compared with CBQYIIC5, the CBQYIIC7 strain lacks at least a 43 kD band. Within the range 17.5–30 kD a 29 kD band is present in CBQYIIC5 but not in CBQYIIC7.

Immunoblotting results

The whole cell components of both phase I and phase II, which are transfered to NCM, were analysed with the pooled mouse anti-*C. burnetii* antisera as first antibody. About 10 bands were precipitated in the patterns (Fig. 4) of CBQYIC3 and CBQYIIC7, but in CBQYIIC7 at least a 89.0 kD band was absent. When the cell components were analysed on NCM with McAbI-2 B8, the patterns from CBQYIC3 consisted of 20.0, 17.8, and 15.4 kD proteins, but that of CBQYIIC7 only from 17.8 kD and 15.4 kD protein bands. After digestion with proteinase K the 17.8 and 15.4 kD bands appeared in both of CBQYIIC7 and CBQYIC3. The gel patterns of CBQYIC3 which reacted with

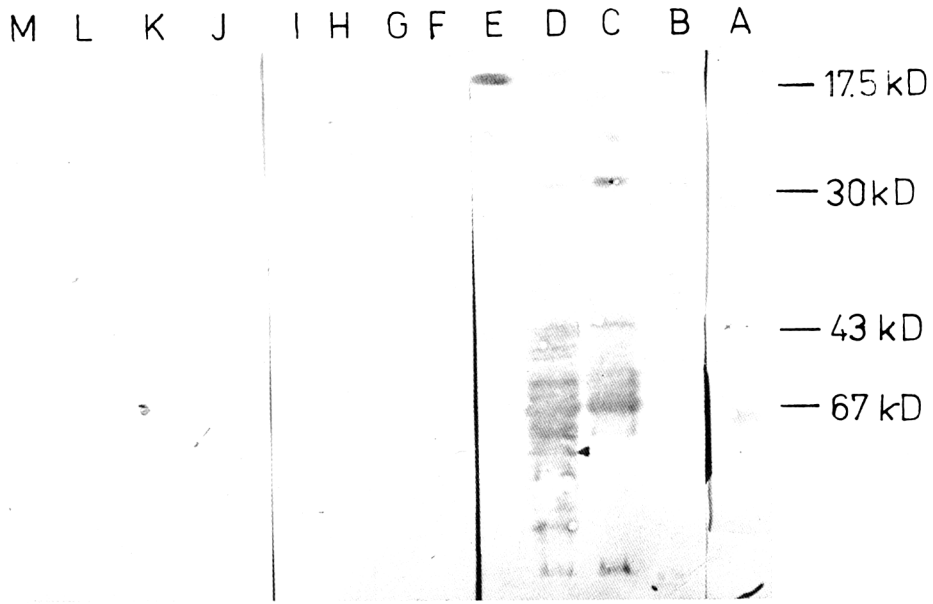

Fig. 4
Immunoblotting assay
A (marker); B - E antigens with PcAb, B (CBQYIIC7 treated with proteinase K), C (CBQYIIC7), D (CBQYIC3), E (CBQYIC3 treated with proteinase K); F - I antigens with McAb II, F (CBQYIC3 treated with proteinase K), G (CBQYIC3), H (CBQYIIC7), I (CBQYIIC7 treated with proteinase K); J - K antigens with McAb I, J (CBQYIC3 treated with proteinase K), K (CBQYIC3); L (CBQYIIC7); M (CBQYIIC7 treated with proteinase K).

McAbII 2-2 F8 were similar to each other and contained 19.5, 17.8, and 15.4 kD bands, but after digestion with proteinase K both of the variants were negative.

Electrophoresis of the restriction fragments endonuclease and plasmid isolation

The DNA of the CBQYIC3 and CBQYIIC7 was digested with 8 restriction enzymes (Figs 5, 6), but no significant differences were seen.

Using pulsed field gel electrophoresis (PFGE) apparatus D4-4A (China), the gel patterns of CBQYIC3, CBQYIIC5 and CBQYIIC7 had two bands. One of them has the size of about 56 kb and the another is nearly as large as the chromosomal of V517 strain (Fig. 7). The plasmid was purified and then analysed by electrophoresis. The result shown in Fig. 8 depicts a single band in all the three cloned variants respectively, which is as large as the largest plasmid of V517 (about 56 kb).

Discussion

It has been asserted that phase variation of *C. burnetii* occurs only under the laboratory conditions, but the current results show that there are variations

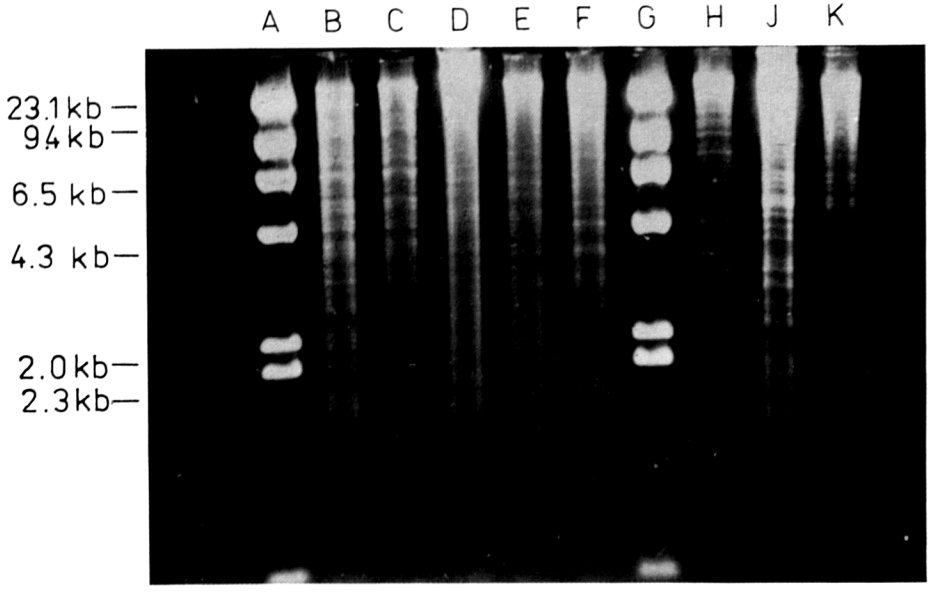

Fig. 5

Gel electrophoregram of 4 kinds of restriction endonuclease digestion of the DNA of *C. burnetii* (0.8 % agrose gel)

A and G (λDNA-*Hind*III); B (CBQYIC3 *Eco*RI); C (CBQYIIC7 *Eco*RI); D (CBQYIC3 *Hind*III); E (CBQYIIC7 *Hind*III); F (CBQYIC3 *Bam*HI); H (CBQYIIC7 *Bam*HI); I (CBQYIC3 *Pst*I); J (CBQYIIC7 *Pst*I).

among the variants in nature. Clearly delineated differences in plasmid DNA and LPS structure were seen between the human endocarditis isolates and the acute Q-fever isolates. So it is important to use the cloned pure isogenic strain to obtain the correct data. In this study, the CBQYIIC7 and CBQYIIC5 isolates were cloned in primary chicken embryo cells after over 51 serial egg passages and the CBQYIC3 was cloned after over 52 mice passages. Recent studies of the structure of LPSI and LPSII of *C. burnetii* produced results which were somewhat contraditory. Williams (1984) reported that many different proteins exists in phase I and phase II cells in addition to several common protein components. The unique phase dependent substance is LPS and that protein components of phase I and phase II cells are shared at most part (Hackstadt *et al.*, 1988). The purified whole cell antigens of three cloned CBQY variants were subjected to SDS-PAGE, the migrating profile of each variant was shared,

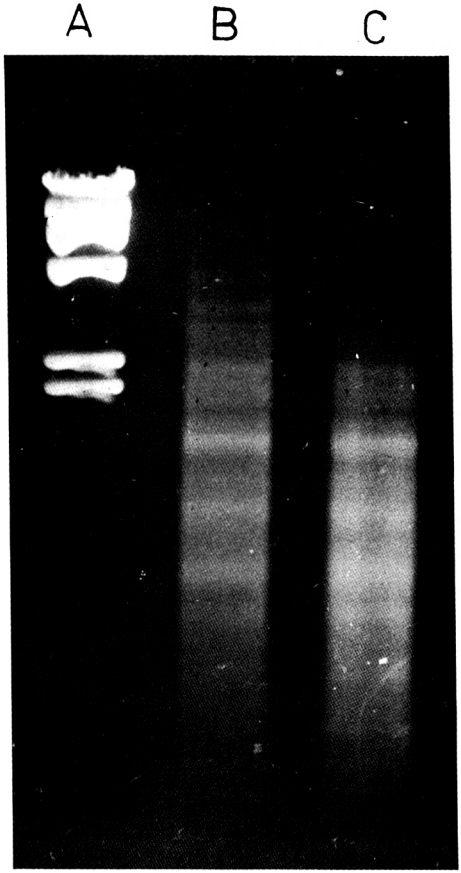

Fig. 6
Gel electrophoregram of *Hae*III digestion of DNA from *C. burnetii* (1 % agarose gel) A (λ DNA-*Hind*III); B (CBQYIC3); C (CBQYIIC7).

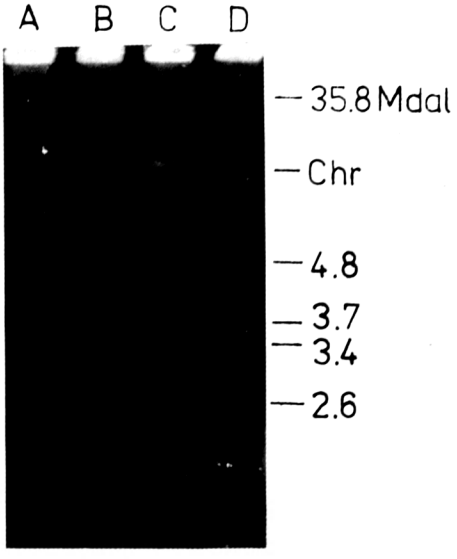

Fig. 7
Separation of the plasmid of *C. burnetii* by DY-4A electrophoresis apparatus
A (CBQYIC3); B (CBQYIIC7); C (CBQYIIC5); D (V517).

except that of a 95.5 kD band present in CBQYIIC7 and absent in CBQYIC3 and CBQYIIC7. After LPS-silver staining, the result indicates significant differences among the strains. It seems plausible that during phase variation of *C. burnetii* great changes take place in the LPS. When compared phase I with phase II isolates, these differed in the range of 30 to 94 kD in phase II. The semi-rough-phase I (CBQYIIC5) also lacks the components in this region, but there were 3-4 bands within 17.5-30 kD region which were absent in CBQYIIC7. Amino (26) suggested that a unique disaccharide, galactosaminuronyl-α(1,6)-glucosamine(GalNU-α(1,6)-GlcN, $C_{12}H_{22}N_{20}O_{10}$) and two sugars, virenose and dihydrohydroxystreptose, could be used as biochemical markers of truncated LPS. Smooth-phase I LPS contained all three compounds, semi-rough phase I LPS only virenose and rough phase II LPS contained none of the three compounds. In our studies it is uncertain whether the patterns of LPS silver staining reflected this difference of the three compounds among CBQYIC3, CBQYIIC5 and CBQYIIC7. The immunoblots of whole cell digests with polyclonal antibody (the serum of mice infected CBQY strain for 30 days) showed the absence of at least a 89.1 kD major band for CBQYIIC7. The result of immunoblotting assay with monoclonal antibody (phase I 3-2 B8) showed three positive bands (20, 17.8 and 15.4 kD) in CBQYIC3 and in CBQYIIC7 two bands (17.8 and 15.4 kD). After digestion with proteinase K the 17.8 and 15.4 kD band appear in both of CBQYIIC7 and CBQYIC3. We think, therefore, that the McAb I determined a LPS between 15.4-20.0 kD. When we used McAb II 2-2 F8 for immunoblotting assay, three bands, 19.5, 17.8 and 15.4 kD were observed in the migrating profile of both CBQYIC3 and CBQYIIC7. But after

being digested with proteinase K, all three bands disapeared. Based on these observations, protein antigens are shared in the 15.4–19.5 kD fraction of CBQYIC3 and CBQYIIC7, but in intact phase I cells, the phase I LPS sterically blocks the access of phase II antibodies to proteins. After immunoblotting assay CBQYIC3 reacted positively with McAb II as a result of the surface proteins exposure.

It has been reported that Nine Mile strain phase II variant is absent a 4.4 kb restriction fragment, but in other phase II variants, this deletion do not always appear, for example Grita M44 (Vodkin *et al.*, 1986; O'Rourke *et al.*, 1985). We used eight kinds of restriction enzymes to analyse whole DNA of cloned isogenic strains of *C. burentii* isolated in China, CBQYIC3 and CBQYIIC7. No obvious difference was found. The gel pattern of CBQYIIC7 digested with *Hae*III also contained 4.4 kb fragment. It has been reported in China that the *Hae*III 4.3 kb fragment was present in strain CBQY (isolated from a patient in Sichuan) in strain Xingiao (isolated from tick in Sichuan), but not in strain Ys-8 (isolated from sheep placenta in Yunnan). The result shows that the isolates of *C. burnetii,* show genetic heterogeneity which may be related to the geographical location of given strains (Yang *et al.*, 1989).

We found that both CBQYIC3 and CBQYIIC7 contained a plasmid about 56 kb by PFGE and plasmid purification. This corresponds Samuel's report (1988) indicating that the structure and function of plasmid was similar in both phase I and phase II cells. It has been reported that *C. burnetii* contained three kinds of

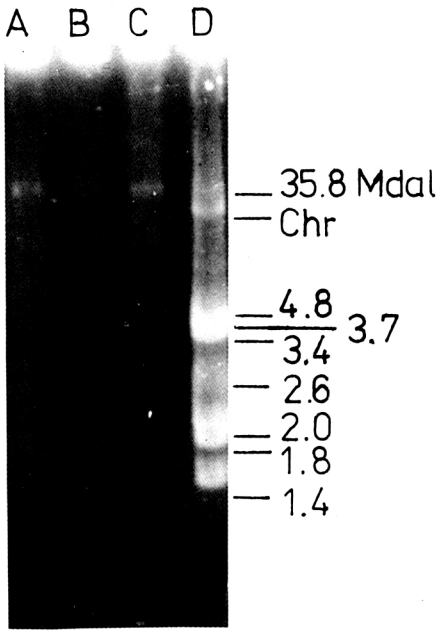

Fig. 8
Gel electrophoregram of the plasmid of *C. burnetii*
A (CBQYIC3); B (CBQYIIC7); C (CBQYIIC5); D (V517).

plasmids: Q_PHI (36 kb), Q_PRS (38–39 kb) and Q_PDG (45 kb). The molecular weight which we reported in this paper is different from Q_PHI, Q_PRS and Q_PDG. The CBQY strain was isolated in the marrow of a chronic Q-fever patient who suffered from multiple body system invasions beside marrow infection. The relationship between molecular structure of plasmid and pathogenicity remains to be defined.

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