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Restriction Endonuclease Analysis of DNA from *Chlamydia trachomatis* Biovars

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DNA from a total of 60 *Chlamydia trachomatis* isolates was examined by restriction endonuclease analysis. Strains from all established biovars and serovars were tested. There was great diversity between the mouse biovar and the lymphogranuloma venereum (LGV) and trachoma biovars. The LGV and trachoma biovar isolates generated similar fragment patterns; however, distinct fragments appeared to be unique to both biovars, thus allowing differentiation of these two major groups. In most cases, strains of the same serovar could be differentiated from one another when a battery of restriction enzymes was used. In addition, in some cases, certain restriction fragments appeared to be characteristic of strains from a particular geographical location. The DNA patterns generated by all *C. trachomatis* isolates differed greatly from the DNA patterns generated from the *Chlamydia psittaci* isolates tested, including TWAR, a human *C. psittaci* strain.

The genus *Chlamydia* is composed of two species, *Chlamydia psittaci* and *Chlamydia trachomatis*. These two species share only about a 10% DNA homology, as shown by DNA-DNA hybridization experiments (10, 21). From hybridization results, it is not clear whether the two species should be classified within the same genus (13). In addition, restriction endonuclease analysis (REA) of the DNA from the two species also supports this lack of DNA relatedness (14).

The species *C. trachomatis* is further divided into three biovars, mouse, lymphogranuloma venereum (LGV), and trachoma (13). In DNA-DNA hybridization experiments, the latter two show over 95% homology between themselves and only approximately 30 to 60% homology with the mouse biovar (10, 21). This lack of homology between the mouse biovar and the other two biovars raises doubt as to whether all three biovars belong to the same species (8).

The LGV and trachoma strains of *C. trachomatis* can be grouped serologically into 15 main categories with some minor subgroupings (18–20). In other systems, such as herpes simplex viruses, further subdivision within serotypes has been possible by restriction endonuclease techniques (5, 6). Here, REA was used with strains to establish the feasibility of using this method with *C. trachomatis*.

In this investigation, we compared the restriction endonuclease DNA fragments of *C. trachomatis* mouse, LGV, and trachoma biovars, as well as *C. psittaci*, including the newly described TWAR strain (4). In addition, DNA from the 15 serovars of the LGV and trachoma biovars was analyzed with restriction endonucleases to compare patterns within the biovars and strains within serovars. We also examined a representative of all 15 *C. trachomatis* serovars, as well as the mouse biovar and the *C. psittaci* strains, for the presence of plasmids. By using REA, we anticipate, as has been seen with other genera, that we will generate data with *C. trachomatis* that will prove this approach to be a useful tool in addressing epidemiological issues.

MATERIALS AND METHODS

Organisms. *C. trachomatis* and *C. psittaci* strains were obtained from San-Pin Wang and Cho-Chou Kuo (University of Washington, Seattle), Julius Schachter (Hooper Foundation, San Francisco, Calif.), S. Darougar (University of London, England), S. Benes (State University of New York, Brooklyn), and the American Type Culture Collection (Rockville, Md.). To prepare stocks, organisms were inoculated onto monolayers of HeLa 229 or McCoy cells by centrifugation (1,000 × *g*, 1 h, 30°C), after which Eagle minimal essential medium with 5% fetal bovine serum, gentamicin (50 μg/ml), and cycloheximide (1 μg/ml) were added (15). After 48 to 72 h of incubation, cells were scraped into 2-SPG (0.2 M sucrose, 0.02 M sodium phosphate [pH 7.2], 5 mM glutamic acid) and frozen at −70°C.

Microimmunofluorescence. All serotyping was performed by microimmunofluorescence (20) by S.-P. Wang. Aliquots from all samples from which DNA was extracted were sent with a blind code for microimmunofluorescence typing to assure that the samples tested contained only the assigned serovar.

DNA labeling and isolation. For DNA labeling, 20 h after infection of a monolayer, $^{32}PO_4$ was added to phosphate-free Eagle minimal essential medium with 5% dialyzed fetal bovine serum and cycloheximide (2 μg/ml). At 48 h after infection, the monolayers exhibiting at least 70% infection were treated with sodium dodecyl sulfate (0.5% [wt/vol]) and proteinase K (200 μg/ml) in 10 mM Tris hydrochloride (pH 7.5), containing 5 mM EDTA and 150 mM NaCl, and were incubated for 2 h at 37°C. This was followed by two extractions with phenol and chloroform-isoamyl alcohol (24:1), treatment with RNase (50 μg/ml), and repeated phenol and chloroform extractions. The DNA was precipitated and rinsed with cold ethanol.

Plasmid analysis. Plasmid DNA was performed as previously described (14) and also by an alkaline lysis method (11). Samples were analyzed by electrophoresis on 0.8% agarose gels that were dried, exposed to Kodak X-Omat AR film and developed (14).

Preparation of HSV-2 DNA. Herpes simplex virus type 2 (HSV-2) (strain G) was obtained from the American Type Culture Collection and was grown in McCoy cells; $^{32}PO_4$ was used for labeling. The DNA was extracted and purified by the method described by Hirt (7). The molecular weight values used were those reported by Harland and Brown (5).

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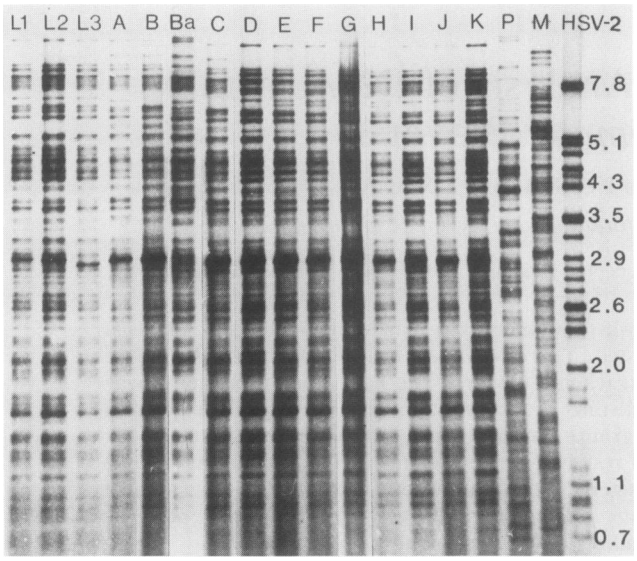

FIG. 1. Autoradiogram of a 0.8% agarose gel containing DNA from the *Eco*RI digests of the *C. trachomatis* mouse biovar (mouse pneumonitis; lane M), *C. psittaci* (lane P), and the 15 *C. trachomatis* serovars L1 (440), L2 (434), L3 (404), A (G-17), B (Har-36), B^a (Apache 2), C (TW-3), D (ICCal-8), E (Bour), F (UW-6), G (UW-57), H (UW-4), I (UW-12), J (UW-36), and K (UW-31). HSV-2 was cleaved with *Bam*HI. The molecular weights (10^6) of the HSV-2 (strain G) bands are indicated to the right of the gel.

The HSV-2 DNA was used as a marker to calculate the molecular weights of specific *Chlamydia* DNA fragments.

Restriction endonuclease enzyme treatment and electrophoresis. DNA samples were divided and digested with the optimum buffer and salt concentration for each particular restriction enzyme (New England BioLabs, Beverly, Mass.). Enzymes were added to achieve complete digestion, and reactions were incubated overnight (18 to 24 h) at 37°C. A solution of bromophenol blue and glycerol was added to each reaction, and samples were loaded onto 0.7 to 1% agarose gels, dried, exposed, and developed as described above.

Restriction enzyme profile data analysis. Data generated from restriction enzyme patterns were analyzed by selecting a few well-resolved fragments that showed differences among the isolates and scoring their presence or absence among the strains. On the basis of these fragments, different patterns were assigned an identification number. Subsequently, a profile was generated by compiling numbers given to each enzyme fragment pattern combination.

RESULTS

DNA isolated from *C. trachomatis* and *C. psittaci* was analyzed by agarose gel electrophoresis. All 60 *C. trachomatis* isolates, which included members of all 15 serovars and the mouse biovar, as well as the Texas turkey strain of *C. psittaci*, in addition to the main chlamydial chromosomal DNA, contained a plasmid (data not shown). However, the human *C. psittaci* strain, TWAR-183, did not appear to contain the plasmid shared by the other *Chlamydia* isolates examined. The molecular weight of the *C. trachomatis* plasmid appeared to be the same, ca. 4.5×10^6, in all isolates, except for strain 404 (serogroup L3), which had a lower molecular weight, ca. 4.4×10^6.

Chlamydial DNA isolated from elementary bodies was digested with restriction enzymes, and the fragments were analyzed by agarose gels. The two *C. psittaci* isolates, TWAR and Texas turkey, differed greatly from one another (data not shown). Members of the LGV and trachoma biovars generated similar *Eco*RI restriction enzyme patterns while differing greatly from the mouse biovar and the Texas turkey strain of *C. psittaci* (Fig. 1). In addition to the 15 *C. trachomatis* serovar representatives (Fig. 1), 45 other *C. trachomatis* strains were examined and, in contrast to the differences apparent between the mouse and LGV and trachoma biovars, all generated similar patterns; however, differences did exist that afforded differentiation of the LGV and trachoma biovars and also, in some cases, differentiation of members within the same serovar.

By using *Eco*RI fragments, the 15 serovars tested could be placed in three main groups (Fig. 2). All 18 strains belonging to the LGV biovar had a 3.3×10^6 fragment not present in the 42 trachoma biovar isolates. Within the trachoma biovar, all 42 strains examined, representing all 12 serovars, possessed a single fragment or a doublet in the 5.6×10^6- to 5.7×10^6-dalton region not present in any members of the LGV biovar. All seven isolates from serovars A, B, B^a, and C had a doublet consisting of a 5.6×10^6 and a 5.7×10^6 fragment in this region, whereas the 35 isolates from serovars D through K had a single 5.6×10^6 band. In addition, six of seven strains tested from serovars, A, B, B^a, and C possessed a 9.3×10^6 fragment not present in any of the 35 isolates from serovars D through K or the 18 LGV isolates tested. Therefore, with an *Eco*RI cleavage based on the absence or presence of a 9.3×10^6-, 5.6×10^6-, 5.7×10^6-, or 3.3×10^6-dalton fragment, 15 *C. trachomatis* serovars could be placed into three main groups (LGV 1 through 3; A through C and B^a; and D through K). The differentiation of the LGV and trachoma biovars was also seen with *Pst*I when the 18 LGV isolates tested had a fragment pattern not present in any of the trachoma isolates examined (data not shown).

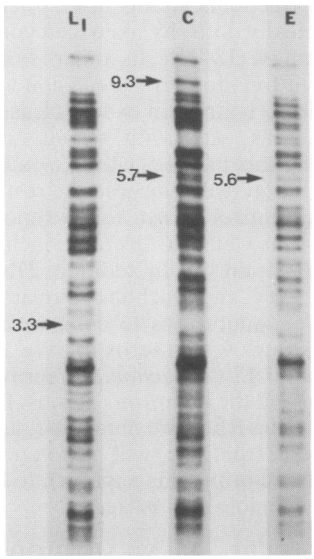

FIG. 2. *Eco*RI digests of the three serovars of *C. trachomatis* L1 (440), C (TW-3), and E (Bour). Each serovar represents one of three groups, L:1 through 3; A, B, B^a, and C; and D through K. Fragments characteristic of each group are indicated by the arrows. Molecular weights (10^6) are indicated.

TABLE 1. REA profile number of 18 strains of the LGV biovar

Serogroup and strain	Source[a]	Origin	Profile no.[b]								
			P	B	Bn	E	N	PI	SI	SII	X
L1											
440	BU	Vietnam	1	1	1	1	4	1	1	1	1
IOL 1962	PE	Great Britain	1	2	1	1	3	1	2	2	1
EA-1	BU	Ethiopia	1	2	2	1	1	1	2	2	1
L2											
434	BU	San Francisco-New York	1	1	1	1	3	1	3	ND[c]	1
UW-297	BU	Seattle	1	1	1	1	3	1	1	ND	1
UW-329	BU	Seattle	1	1	1	1	4	1	1	1	1
UW-330	R	Seattle	1	1	1	1	3	1	1	1	1
UW-396	R	Seattle	1	1	1	1	3	1	1	1	1
UW-447	R	Seattle	1	1	1	1	4	1	1	1	1
EA-2	BU	Ethiopia	1	1	2	1	2	1	1	1	1
EA-5	CX	Ethiopia	1	3	2	1	5	1	1	1	1
FER	BU	Australia	1	1	1	1	2	1	1	1	1
CAR	BU	Australia	1	1	1	1	1	1	3	3	1
MU-17	BU	Australia	1	1	3	1	2	1	1	1	1
L3											
404	BU	Philippines	2	1	1	1	2	1	1	1	1
IOL 253	BU	Great Britain	1	1	1	1	3	1	3	3	1
EA-3	BU	Ethiopia	1	2	2	1	1	1	2	2	1
EA-4	MU	Ethiopia	1	2	1	1	3	1	2	2	1

[a] Abbreviations: BU, bubo; PE, penile erosion; CX, cervix; R, rectum; MU, male urethra.
[b] Profile generated by plasmid size (P) and fragment patterns generated by *Bam*HI (B), *Ban*II (Bn), *Eco*RI (E), *Nco*I (N), *Pst*I (PI), *Sac*I (SI), *Sac*II (SII), and *Xba*I (X).
[c] ND, Not determined.

TABLE 2. REA profile of 19 clinical isolates

Isolate no.	Source[a]	Serogroup	Profile no.[b]					
			P	B	Bn	E	N	PI
7	CX	D	1	5	4	2	7	4
10	CX	D	1	9	4	2	7	4
17	CX	D	1	9	4	2	9	4
3	CX	E	1	7	3	3	11	3
6	CX	E	1	8	3	5	11	3
15	CX	E	1	8	3	6	11	5
20	CX	E	1	8	ND[c]	6	ND	ND
18	CX	F	1	4	ND	7	ND	ND
9	CX	H	1	9	4	4	7	2
12	CX	H	1	6	4	5	7	2
14	CX	H	1	4	4	2	8	2
1	CX	I′	1	9	4	2	7	2
2	MU	I′	1	9	4	2	7	2
11	CX	I′	1	9	4	2	7	2
13	MU	I′	1	9	4	2	8	2
16	MU	J	1	9	4	2	8	4
4	CX	K	3	5	4	4	6	4
8	CX	K	1	7	5	2	7	4
19	CX	K	3	6	4	2	10	4

[a] Abbreviations: CX, cervix; MU, male urethra.
[b] Profile number represents plasmid difference by *Eco*RI cleavage (P) and the following restriction enzyme profile patterns: *Bam*HI (B), *Ban*II (Bn), *Eco*RI (E), *Nco*I (N), and *Pst*I (PI).
[c] ND, Not determined.

Fragment patterns generated with each restriction enzyme were assigned a number, depending on the pattern generated. An example of this can be seen in Table 1. With *Bam*HI there were only three different patterns generated and each pattern was arbitrarily assigned a number. This is in contrast to the enzyme *Pst*I, with which no differences were found among patterns and thus only one pattern number was assigned. These pattern numbers were compiled for each strain to generate an overall profile number. Within the LGV and trachoma biovars, it was evident that the members of the LGV biovar showed the least amount of interstrain differences. This can be seen by comparing the variety of profile numbers generated by the two biovars. There was less variability in the profile numbers seen with the LGV isolates (Table 1) compared with those generated by trachoma isolates (Table 2). To assure that the patterns seen were stable, DNA from LGV strains and selected trachoma strains were digested with restriction enzymes after 20 in vitro passages and patterns remained the same. In addition, every isolate was repeated at least twice, and no changes in REA were seen.

With certain restriction enzymes, such as *Xba*I and *Pst*I, there were no detectable differences in the 18 LGV strains examined (3 L1 strains, 11 L2 strains, and 4 L3 strains). However, a few differences could be detected within LGV serovars with *Eco*RI, *Bam*HI, *Ban*II, *Nco*I, *Sac*I, and *Sac*II. Within the L1 serovar, the three strains examined each had a different profile and, thus, could be distinguished from one another. This was also true of the four L3 strains. Among the 11 L2 strains, there were eight different profiles generated. There were five strains, all of which were isolated in the Seattle area, which generated one of two profiles, which varied only in the *Nco*I pattern, whereas the other strains from common geographic origins showed more variation. In addition, four of the five strains isolated from Ethiopia had a unique *Ban*II pattern not seen with the other LGV strains. These four isolates, while having the same *Ban*II pattern, were of the L1, L2, and L3 serogroups.

Within the trachoma biovar, differences were also present in restriction enzyme digests that allowed differentiation among some strains of the same serovar. For example, with *Eco*RI, two strains each of serovars A and C could be differentiated from one another (Fig. 3). The ability to distinguish among strains within the same serovar was not always possible with every enzyme used. For example, the two C strains (Fig. 3), while exhibiting fragment differences with *Eco*RI, had identical patterns in our gel system with *Bam*HI.

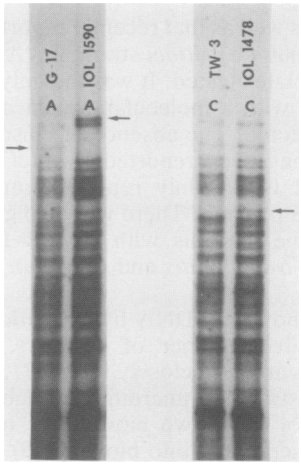

FIG. 3. *Eco*RI digests of two isolates each of serovars A and C run on a 0.8% agarose gel. Arrows mark the fragment differences in isolates.

A total of 19 random clinical isolates from the New York area, 16 from cervical cultures, and three from male urethral cultures, were serotyped and examined by restriction enzymes. Table 2 shows the serotype and enzyme profiles for these isolates. All were members of the trachoma biovar. Of these 19 clinical isolates, 2 (isolate numbers 4 and 19) had a plasmid that differed in *Eco*RI restriction sites from the other strains tested. This was seen with *Eco*RI digests of plasmid and chromosomal preparations in which the plasmid from these two isolates was cleaved only once by this enzyme (data not shown). These were the only 2 isolates of the 60 *C. trachomatis* isolates tested that were missing an *Eco*RI cleavage site, with the plasmid from the other isolates being cleaved twice. However, with all other enzymes tested, the plasmid in these two isolates generated patterns identical to the plasmids present in the other isolates (data not shown). While these two isolates appeared to have the same plasmid and both were of serotype K, they were found to have different restriction site polymorphism.

Of these 19 random clinical isolates, there were six serovars represented and, with six restriction enzymes, 17 restriction enzyme profiles were generated (Table 2). The only isolates not able to be differentiated with the enzyme battery used were three of the four I′ isolates (isolate numbers 1, 2, and 11) which shared a common profile number. Although the four E isolates had different overall composite profiles, three of the strains possessed the same fragment pattern with *Ban*II and *Nco*I, which was not shared by isolates of any of the other serovars.

DISCUSSION

In this report, we have shown that REA of *Chlamydia* DNA allows differentiation among species, biovars, serovars, and strains within a serovar. Up to this point, DNA hybridization, plasmid analysis, and previous restriction enzyme data showed a marked difference among the two *Chlamydia* species, *C. trachomatis* and *C. psittaci* (9, 10, 14). DNA hybridization studies showed only a 40 to 60% homology between the mouse biovar and the LGV and trachoma biovars, thus leading to questions of whether these biovars should be included in the same species (8, 21). In addition, our results show a marked difference in REA between the mouse and the other two *C. trachomatis* biovars.

In our study, as well as that recently reported by Campbell et al. (3), the human *C. psittaci* strain, TWAR, differed from all *Chlamydia* isolates tested. It was the only strain tested in which a plasmid with a molecular weight of ca. 4.5×10^6 could not be detected. The absence of plasmids in other *C. psittaci* strains has been reported by McClenaghan et al. (12), but TWAR is the only reported human chlamydial strain lacking this plasmid. There was also great variation in restriction enzyme patterns with TWAR DNA compared with all other *C. trachomatis* and *C. psittaci* isolates examined.

As has been shown by DNA hybridization and previous REA, on a limited number of serovars, the LGV and trachoma biovars are very closely related (10, 14). Antigenic analysis of these strains by microimmunofluorescence shows that the strains of these two biovars are interrelated and, thus, cannot be separated into biovars (18). However, Batteiger et al. (2) described biochemical differences in two outer membrane proteins of a molecular mass of about 60 and 12 kilodaltons that enabled differentiation of the two biovars from one another. Allan and Pearce (1) described methionine requirement differences between the two biovars. Our findings of unique restriction enzyme fragments between the two biovars is the first time differentiation between the two biovars has been reported that included all established serovars. Thus, by studies such as these with proteins and nucleic acid, we are initiating our understanding at the molecular level of the well-known differences in biological properties and pathogenicity of these two biovars.

Within each serovar, it was not possible with the enzymes and gel systems used here to find unique fragment patterns for each serovar similar to those that allowed biovar distinctions. Although they represent a limited sample, it appeared that members of serovar E from different geographical locations had fragments with *Ban*II that were not present in the other serovars. If this finding holds up, then this could suggest that, with enough REA, serovar distinction is possible.

There was some evidence for geographical clustering in some of the isolates tested. An example of this could be seen with the LGV isolates that were examined from Ethiopia. There were 5 isolates tested which represented the three LGV serovars and, with *Ban*II, 4 of these isolates had a unique banding pattern that was not present in any of the other 14 LGV strains tested. In addition, there were three other enzyme patterns that were present in three of the Ethiopian isolates that were only shared by one other non-Ethiopian isolate.

Besides a geographical clustering of isolates, it may be that the isolates can be grouped by DNA patterns based on their pathogenicity or tropism for particular tissue or cells. There is some evidence for this with *C. psittaci*. McClenaghan et al. (12) compared restriction enzyme fragments from isolates of *C. psittaci* and focused their analysis on the lower-molecular-weight DNA fragments. On the basis of this information, they were able to discern four main patterns, which corresponded to the type of infection from which the strain was isolated. The isolates used in their study from ovine abortion and ovine arthritis differed, whereas eight abortion isolates tested were identical. Thus, there appeared to be a correlation between pathogenicity and DNA patterns. This may be a reflection of clonal dissemination among chlamydial strains as has been described with other bacterial species (16, 17) and may be similar to what we have found with the 15 *C. trachomatis* serovars we have examined. The isolates from clinically diagnosed LGV have fragment patterns distinguishable from the isolates from other genital infections and trachoma. Therefore, although probably all are derived from a common progenitor, LGV and trachoma biovars mutated from one another, which conferred different selective advantages for each group. This would explain the finding that while being very homogenous in the overall REA, these two biovars can be distinguished by specific restriction enzyme sites.

One of the most useful findings revealed by this study was that, in some cases, members of the same serovar could be differentiated. Just as with other systems (i.e., herpes simplex virus) in which REA is used, not all enzymes revealed differences between two strains. In some cases, five to six enzymes were tested before differences were apparent with the methodology used here. In comparison to *C. trachomatis* serotyping, REA was a more specific tool for differentiating strains within a serovar. Of 19 random isolates examined, there were six different serovars but 17 restriction enzyme profiles. Therefore, in conjunction with serotyping, this method of strain differentiation might prove useful in studies

addressing epidemiological questions such as strain transmission and latency.

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