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Comparison of *Chlamydia psittaci* Isolates by Restriction Endonuclease and DNA Probe Analyses

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DNAs from eight *Chlamydia psittaci* isolates (koala conjunctivitis, avian psittacosis, avian ornithosis, ovine abortion, ovine polyarthritis, sporadic bovine encephalomyelitis, and feline conjunctivitis) and one *Chlamydia trachomatis* isolate (lymphogranuloma venereum) were compared by restriction endonuclease and DNA probe analyses. Digestion with *Hin*dIII yielded a series of discrete fragments which allowed the differentiation of most isolates. A gene probe, pFEN207, which encodes the chlamydia-specific component of the lipopolysaccharide group antigen was used in Southern hybridizations. The probe was chlamydia specific and hybridized to a single *Bam*HI fragment and multiple *Hin*dIII fragments in each isolate. The variation in size of the hybridizing fragments allowed easy differentiation of the isolates and may eventually lead to a meaningful subgrouping of the diverse group of disease agents presently included in the species *C. psittaci*.

The genus *Chlamydia* is divided into two species, *Chlamydia trachomatis*, which is a specific pathogen of humans, and *Chlamydia psittaci*, which causes a range of disease conditions in a wide variety of mammals and birds (17). Despite a number of common biological properties, the two species share less than 10% DNA homology (8) in their genomes (660×10^6 daltons) and are antigenically heterologous (2). Restriction endonuclease analysis with a single enzyme has been used to show a difference between the two species (14, 15); however, the separation of *C. trachomatis* into three groups (lymphogranuloma venereum [LGV] 1 to 3; A, B, Ba, and C; and D to K) required the use of several enzymes and more detailed restriction fragment comparisons. In one of the few studies on *C. psittaci*, McClenaghan et al. (9) demonstrated four distinct electrophoretic groups when ovine abortion, ovine arthritis, avian, and Cal-10 (mouse meningopneumonitis) strains were compared. However, they were unable to differentiate among eight individual ovine abortion isolates.

To date, no attempts to differentiate *C. psittaci* isolates by using chromosomal DNA probes have been described. DNA probes have been used, however, with *C. trachomatis*, in spot hybridization (6, 7, 12), sandwich hybridization (13), and in situ hybridization (5) analyses. Both species share a heat-stable lipopolysaccharide molecule termed the chlamydial group antigen. Nano and Caldwell (10) recently cloned a gene from *C. trachomatis* which directs the expression of this chlamydia-specific lipopolysaccharide epitope in *Escherichia coli*. We have used this probe (pFEN207) in the present study to look for polymorphisms in the corresponding gene in several *C. psittaci* isolates.

The eight *C. psittaci* isolates and one *C. trachomatis* isolate used in this study are listed in Table 1. All isolates were grown in buffalo-green monkey kidney cells (BGM cells; no. 03-242-83; Flow Laboratories, North Ryde, Sydney, Australia) without centrifugation. Tissue culture flasks (75 cm^2) were seeded with 2.6×10^5 BGM cells per ml in 45 ml of growth medium (GM; Dulbecco modified Eagle medium containing 10% fetal calf serum, 100 μg of gentamicin per ml, 100 μg of vancomycin per ml, and 2 μg of amphotericin B per ml [pH 7.2]) and incubated in 5% CO_2 at 37°C overnight. The overlying medium was decanted and replaced with the appropriate dilution of chlamydiae in 9 ml of warm GM. The flasks were incubated in 5% CO_2 at 37°C for 5 h to allow the chlamydiae to attach before the inoculum was decanted and replaced with 45 ml of warm GM plus 0.5 μg of cycloheximide per ml. After incubation for 24 to 48 h, the cycloheximide medium was replaced with normal GM, and incubation was continued. These conditions were used for the growth of KC, AP^1, AP^2, AO, OA, OP, and LGV isolates but were modified for the growth of SBE and FC isolates. For the latter isolates, the BGM monolayer was treated with 20 μg of DEAE-dextran per ml for 30 min at 37°C before the addition of the chlamydial inoculum. In addition, cycloheximide was not included in GM. For all isolates, the flasks were incubated for 5 to 8 days until >80% of the cells contained chlamydial inclusions, as detected by Giemsa staining. An equal volume of SPG (220 mM sucrose, 7 mM K_2HPO_4, 5 mM glutamic acid, 10% fetal calf serum, 100 μg of gentamicin per ml, 100 μg of vancomycin per ml, 2 μg of amphotericin B per ml [pH 7.2]) was added, and the flasks were frozen at −70°C.

Elementary bodies were purified by a modification of the method of McClenaghan et al. (9). Infected flasks were thawed at 37°C, and the inclusions were disrupted by being shaken with glass beads for 1 min before being centrifuged at 2,000 × *g* for 10 min to remove cell debris. The supernatant was then pelleted at 45,000 × *g* for 45 min, and the pellet was resuspended in approximately 1 ml of SPG per 100 ml of original cell culture. This suspension was layered onto a discontinuous (32, 44, 54, and 60%) Urografin-76 (Schering AG, Berlin, Federal Republic of Germany) gradient in 20 mM Tris hydrochloride–150 mM KCl (pH 7.5) and centrifuged at 50,000 × *g* for 60 min at 8°C. Two diffuse bands, present at the 32/44 and 44/54 interfaces, were pooled and washed in 20 ml of SPG. The washed pellet of purified elementary bodies was resuspended in 1 ml of SPG per 100 ml of the original cell culture and stored at −70°C.

Genomic DNA was extracted from purified elementary bodies by a modification of the method of Wenman and Lovett (19). The suspension of purified elementary bodies was centrifuged at 45,000 × *g* for 45 min at 4°C, and the

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TABLE 1. Chlamydial isolates used

Species and isolates	Description	Source
C. psittaci		
KC	Koala conjunctivitis isolate from infected *Phascolarctos cinereus*	R. G. Grice[a]
AP[1]	Avian psittacosis isolate from livers of infected native birds	Our isolate
AP[2]	Avian psittacosis isolate from spleens of infected parrots	Our isolate
AO	Avian ornithosis isolate from imported birds	R. L. Doherty[b] (3)
OA	Ovine abortion isolate (ATCC VR-656)	ATCC[c] (18)
OP	Ovine polyarthritis isolate (ATCC VR-629)	ATCC (11)
SBE	Sporadic bovine encephalomyelitis isolate from bovine lung tissue	K. Quinn[d]
FC	Feline conjunctivitis isolate	M. J. Studdert[e]
C. trachomatis LGV	Laboratory-adapted LGV strain L2	D. M. Graham[e]

[a] State Health Department, Brisbane, Queensland Australia.
[b] University of Queensland, Brisbane, Queensland, Australia.
[c] ATCC, American Type Culture Collection, Rockville, Md.
[d] Colin Blumer Regional Veterinary Laboratory, Armidale, New South Wales, Australia.
[e] University of Melbourne, Victoria, Australia.

pellet was suspended in 2.3 ml of DNA lysis buffer (40 mM Tris hydrochloride, 100 mM NaCl, 0.5% sodium dodecyl sulfate, 20 mM EDTA [pH 7.2]). Proteinase K was added to 1.4 mg/ml, and the mixture was incubated at 55°C for 20 min and then at 37°C for 40 min. The resulting viscous solution was extracted once with phenol, twice with phenol-chloroform, once with chloroform, and twice with ether before precipitation of the DNA with 2 volumes of ethanol at −20°C overnight. RNA was removed by RNase A (50 μg/ml) digestion (30 min, 37°C) followed by phenol-chloroform extraction and ethanol precipitation. The precipitated DNA was dissolved in TE (10 mM Tris hydrochloride, 1 mM EDTA [pH 7.2]) to a concentration of 100 to 500 μg/ml.

Samples of chlamydial DNA (1 to 2 μg) were digested with 10 U of either *Hin*dIII or *Bam*HI (Boehringer GmbH, Mannheim, Federal Republic of Germany) for 2 h at 37°C. The reaction mixtures were electrophoresed for 18 h in 0.6% agarose in TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA [pH 8.0]) containing 0.5 μg of ethidium bromide per ml, photographed for comparison of digest profiles, and then transferred to Hybond-N membranes (Amersham Corp., Arlington Heights, Ill.) by Southern blotting (16). *Hin*dIII-digested lambda DNAs were used as size markers. Recombinant plasmid pFEN207, kindly provided by F. E. Nano, Rocky Mountain Laboratories, Hamilton, Mont., was used to probe the Southern blots. This plasmid consists of a 6.5-kilobase (kb) fragment of *C. trachomatis* LGV-434 DNA inserted into the *Bam*HI site of pUC8 and has been shown to direct the expression of the chlamydia-specific lipopolysaccharide epitope in *E. coli* (10). pFEN207 DNA was nick translated with [α-^{32}P]dCTP (Amersham) to ca. 10^7 cpm/μg of DNA. DNA hybridization reactions were performed for 24 h at 65°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10× Denhardt solution–20 mM Na_2HPO_4–100 μg of denatured salmon sperm DNA per ml–0.1% sodium dodecyl sulfate–10% dextran sulfate containing ca. 10^6 cpm of probe DNA per ml. The filters were washed twice at room temperature for 10 min in 2× SSC–0.1% sodium dodecyl sulfate and then twice at 65°C for 1 h in 0.1× SSC–0.1% sodium dodecyl sulfate before autoradiography.

When chlamydial DNA was digested with *Hin*dIII and the fragments were analyzed in agarose gels, *C. psittaci* and *C. trachomatis* could be clearly differentiated from one another by their fragment patterns (Fig. 1). Of the diverse *C. psittaci* isolates studied, five of eight could be differentiated from one another. Only the three avian isolates, AP[1], AP[2], and AO, gave similar profiles, and cleavage of AP[1] DNA with a battery of nine other restriction endonucleases (*Bam*HI, *Eco*RI, *Pst*I, *Sma*I, *Sac*I, *Sal*I, *Bgl*II, *Bst*EII, and *Xho*I) failed to generate profiles more suited to strain differentiation (data not shown). While the two ovine isolates (OA and OP) shared a number of common *Hin*dIII bands, it was apparent that isolate OP had, in fact, more bands in common with isolate SBE than with isolate OA. Isolate KC was easily differentiated from all the others by the presence of a doublet at around 10 kb and the absence of larger bands.

While restriction endonuclease analysis has been used by other workers to dissect the chlamydial genome, their efforts have been mainly applied to *C. trachomatis* (14, 15) or the avian and ovine strains of *C. psittaci* (4, 9). Our results have extended these studies to include bovine, feline, and koala strains, which all appear to have quite distinct patterns. This technique has obvious applications in epidemiological studies and is presently being used to determine if single or

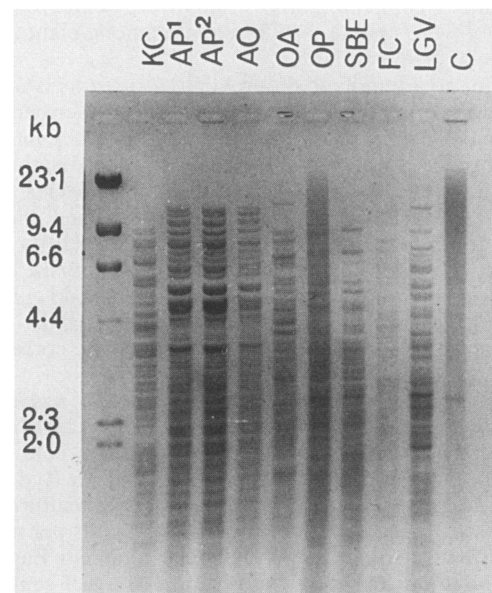

FIG. 1. Agarose gel (0.6%) showing *Hin*dIII digests of genomic DNAs extracted from eight *C. psittaci* isolates, from one *C. trachomatis* isolate, or from uninfected BGM cells (C).

multiple strains of *C. psittaci* are responsible for the range of chlamydial diseases seen in koalas in Australia (1).

While restriction endonuclease analysis is a useful technique for strain comparison, the banding patterns are reasonably complex, exhibit a degree of variability between laboratories, and are not well suited for routine studies. A DNA probe would be much better suited to these requirements. Our present results showed that DNAs from all nine chlamydial isolates tested but not from control BGM cells hybridized to the pFEN207 probe (Fig. 2 and 3). Hybridization was, as expected, much stronger with LGV DNA than with any of the *C. psittaci* DNAs tested. This was because the pFEN207 probe was prepared from LGV DNA, so it indicates a much greater degree of homology with LGV DNA than with *C. psittaci* DNA. Probing of *Bam*HI-digested DNAs resulted in a single band which varied in size among the isolates (Fig. 2). The sizes (in kilobases) of the hybridizing fragments for the different isolates were as follows: KC, 6.4; AP[1], 10.0; AP[2], 10.0; AO, 10.0; OA, 9.0; OP, 7.5; SBE, 7.6; FC, 18.5; and LGV, 6.5. The small difference in the sizes of the hybridizing bands between OP and SBE and between KC and LGV has been found by us on several occasions. The hybridization patterns obtained with *Hin*dIII-digested DNAs were more complicated (Fig. 3). Dominant bands of 1.8 and 3.4 kb for KC, 2.0 kb for AP[1], AP[2], and AO, 2.0 and 2.4 kb for OA, 1.5 kb for OP and SBE, 2.3 kb for FC, and 1.4, 1.6, 1.8, and 2.8 kb for LGV were present. Other faintly hybridizing bands could be seen in all lanes after longer exposures.

The hybridization patterns obtained with pFEN207 revealed size polymorphisms between isolates in the *Bam*HI and *Hin*dIII restriction enzyme sites of the lipopolysaccharide gene. These polymorphisms were most evident when *Bam*HI-digested chlamydial DNA was probed, as only one band per isolate was visible. Hybridization of *Bam*HI digests with this probe may therefore prove quite useful in comparing the relatedness of *C. psittaci* isolates, particularly as a good typing scheme for this species does not exist at present. This method of strain characterization offers several advantages over the other methods which have been used to subdivide the chlamydiae. The DNA, once extracted, can be stored virtually indefinitely and, at some later time, digested with enzymes and hybridized with gene probes. Southern blots can be reprobed with a series of DNA probes. The patterns of hybridization tend to be simple, allowing isolates to be characterized by a series of fragment sizes revealed by one or more standard probes. Most importantly, the use of genetic markers eliminates variations due to phenotypic expression which may render conventional serotyping procedures inconclusive.

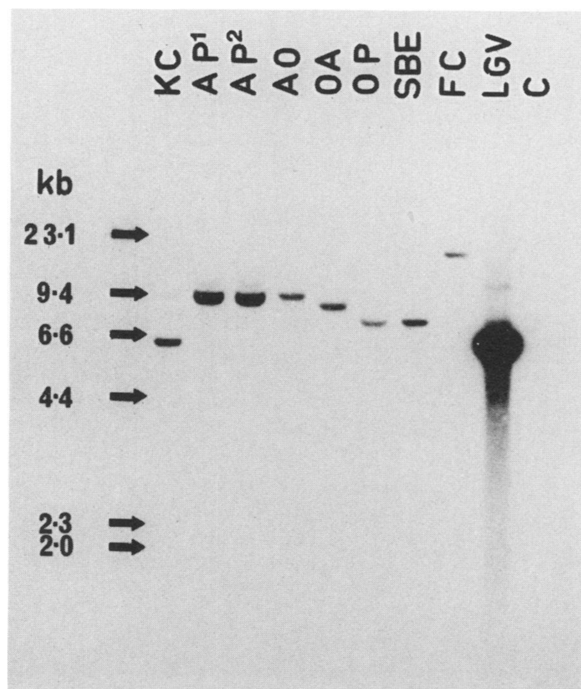

FIG. 2. Southern hybridization of *Bam*HI-digested DNAs from eight *C. psittaci* isolates, from one *C. trachomatis* isolate, or from uninfected BGM cells (C). Hybridization was carried out with ^{32}P-labeled pFEN207.

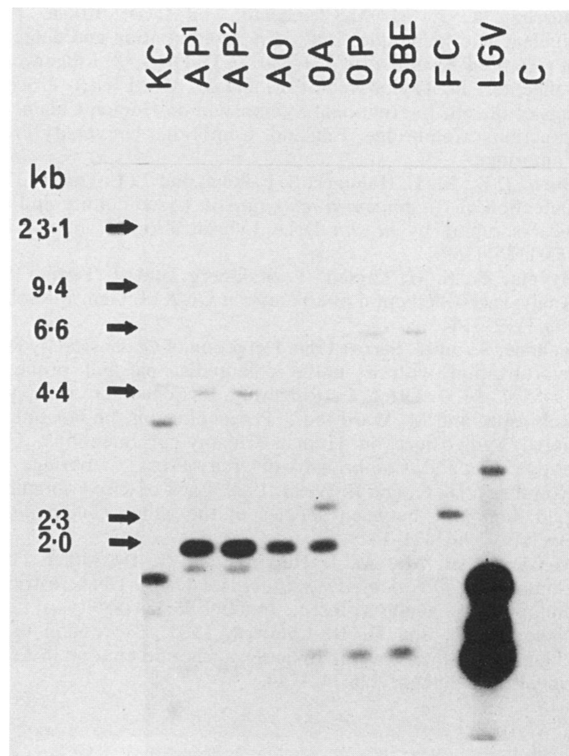

FIG. 3. Southern hybridization of *Hin*dIII-digested DNAs from eight *C. psittaci* isolates, from one *C. trachomatis* isolate, or from uninfected BGM cells (C). Hybridization was carried out with ^{32}P-labeled pFEN207.

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