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Comparison of *Chlamydia psittaci* Isolates by DNA Restriction Endonuclease Analysis

MARGARET McCLENAGHAN,* ALAN J. HERRING, AND IAN D. AITKEN
Moredun Research Institute, Edinburgh EH17 7JH, Scotland

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Preparations of DNA from 12 *Chlamydia psittaci* isolates and one *Chlamydia trachomatis* strain were compared by restriction endonuclease analysis. Polyacrylamide gel electrophoresis, followed by silver staining, resulted in optimal resolution of fragments generated by digestion. By this technique, four distinct electropherotypes were demonstrated when ovine abortion, ovine arthritis, and avian and Cal10 strains of *C. psittaci* were examined. Minor profile differences allowed the discrimination of avian isolates derived from psittacine and columbiforme species, and the Cal10 DNA electropherotype was shown to have features in common with these profiles. However, there were no detectable differences in the DNA patterns of eight ovine abortion isolates.

In recent years, interest in the family *Chlamydiaceae* has increased. This family of unique microorganisms consists of a single genus with two species, *Chlamydia trachomatis* and *Chlamydia psittaci*. Because of their pathogenic role in trachoma, sexually transmitted disease, and other human clinical conditions (31), members of *C. trachomatis* have been extensively studied. The development of a microimmunofluorescence typing procedure (40) has allowed the classification of this species, and 15 serovars are currently recognized (39).

In contrast, the relationships among members of the other species are less well understood. *C. psittaci* commonly infects a wide variety of mammals and birds and has been implicated in a range of disease conditions (38). Studies of biological (34, 35), biochemical (2, 13), and serological (3, 5, 6, 29, 30) properties have afforded some measure of differentiation between isolates, but not to the extent achieved with *C. trachomatis*.

One of the most common chlamydial diseases of animals is ovine abortion resulting from placental infection with *C. psittaci* (1). In Great Britain, the disease has been controlled by use of an inactivated vaccine introduced in the 1950s (7), but in the last few years apparent breakdowns in protection have occurred (17). Cross-infection studies and vaccine-challenge experiments in pregnant ewes have suggested differences in virulence, antigenicity, or both among isolates of *C. psittaci* from recent outbreaks of sheep abortion (I. D. Aitken, G. W. Robinson, and I. E. Anderson, unpublished data). Conventional serological methods have failed to discriminate between these "strains." Other workers have detected only minor differences between ovine abortion isolates by immunofluorescent techniques (6, 27) and have emphasized the close relationship of such isolates.

Restriction endonuclease analysis (REA) has proved valuable for strain discrimination among the DNA viruses (33). This report describes the application of REA to several ovine abortion *C. psittaci* isolates and an ovine arthritis strain; other members of the species and one serovar of *C. trachomatis* were also included in this study.

MATERIALS AND METHODS

Chlamydiae. Eight chlamydial isolates associated with ovine abortion were examined, including the vaccine strain A22 and two isolates from vaccinated flocks which had recently experienced abortion. A joint isolate from a lamb with arthritis was also tested. Details of these "strains" are given in Table 1. Avian isolates 725 and 741, recovered from a cockatiel and a wood pigeon, respectively, were supplied by B. J. Bevan (Ministry of Agriculture, Fisheries, and Food, Weybridge, United Kingdom), and the meningopneumonitis strain Cal10 was obtained from J. H. Pearce (University of Birmingham, United Kingdom). An isolate of *C. trachomatis*, LGV serovar L1, was provided by P. Mackie (Belvidere Hospital, Glasgow, United Kingdom). These cultures were passaged in BHK-21 cells on arrival at the laboratory, as described below.

Culture. Chlamydiae were cultured in mycoplasma-free BHK-21 cell monolayers grown in plastic flasks (75 cm^2). The cells were treated for 3 days with 80 μg of 5-iodo-2′-deoxyuridine per ml before inoculation with a 10^{-3} dilution of a heavily infected yolk sac or cell culture in maintenance medium (BHK-21 Glasgow medium supplemented with 2% newborn calf serum, 2.95 mg of tryptose phosphate broth per ml, 200 μg of streptomycin per ml, and 25 U of mycostatin per ml). The cultures were then centrifuged for 1 h at 200 × *g* and room temperature and incubated at 37°C. Giemsa-stained coverslip monolayers were used to monitor the progress of infection. Seven days after inoculation, cells were gently detached from the plastic, harvested along with the medium, and stored at −70°C until purification procedures.

Chlamydiae were also cultured in embryonated chicken eggs. Organisms were inoculated into the yolk sac at 7 days of age; on embryo death, yolk sacs were harvested and stored at −70°C. Degree of infection was established by modified Ziehl-Neelsen staining (36) of smear preparations, and only heavily infected yolk sac cultures were selected for subsequent use.

Purification. Centrifugations and homogenizations were performed at 4°C during the following procedure.

Chlamydial cell culture harvests of 100 ml were disrupted with a glass-Teflon homogenizer and centrifuged at 1,400 × *g* for 5 min to remove cell debris. The supernatant was layered onto 30% (vol/vol) Renografin-76 diluted with 20 mM Tris–150 mM KCl (TKCl) (pH 7.5); Renografin-76, a solution of diatrizoate meglumine and diatrizoate sodium in citrate buffer, was kindly donated by E. R. Squibb & Sons, Princeton, N.J. After centrifugation at 50,000 × *g* for 45 min, the pellets were resuspended in 1 ml of TKCl, layered onto 30 to 60% (vol/vol) Renografin-76 gradients in TKCl, and centri-

* Corresponding author.

TABLE 1. Details of ovine *C. psittaci* isolates

Isolate designation	Associated disease condition	Flock history
A22 (Scotland)	Abortion	High-passage (>40) vaccine strain, originally isolated from an outbreak of abortion in the 1950s (7)
H574 (Scotland)	Abortion	From an individual case of abortion in a small unvaccinated flock
S26/3 (Scotland)	Abortion	Regular vaccination practiced, severe outbreak of abortion in year of isolation
S507 (Scotland)	Abortion	Regular vaccination practiced, severe outbreak of abortion 2 years previously, few cases only in year of isolation
ZC-19 (England)[a]	Abortion	Abortion outbreak in vaccinated flock
ZC-25 (England)[a]	Abortion	Unvaccinated flock with no previous history of abortion, serious outbreak in year of isolation
ZC-26 (England)[a]	Abortion	Unvaccinated flock with no previous history of abortion, serious outbreak in year of isolation
ZC-31 (Wales)[a]	Abortion	Unvaccinated flock with no previous history of abortion, serious outbreak in year of isolation
P787 (Scotland)	Arthritis	Isolated from the synovial fluid of an affected lamb, no previous history of abortion in flock

[a] These isolates were kindly supplied by F. W. A. Johnson, Veterinary Field Station, University of Liverpool, Wirral, United Kingdom.

fuged at 50,000 × *g* for 2 h. Two diffuse bands were visible in the middle region of the gradient; these fractions were collected, diluted with TKCl, and centrifuged at 50,000 × *g* for 45 min. The pellets were resuspended in 0.5 ml of TKCl.

An alternative method of purification was introduced to facilitate the handling of avian isolates considered to be virulent for man. The chlamydial suspension obtained after centrifugation through 30% Renografin-76 was mixed with an equal volume of 2% (wt/vol) *N*-lauroylsarcosine in 20 mM Tris (pH 7.5) and incubated at 4°C for 10 min. This was followed by centrifugation through 15% sucrose containing 1% (wt/vol) sarcosine in 20 mM Tris. The resulting pellets were used for DNA extraction.

Chlamydiae grown in chicken embryo yolk sacs required a lengthier purification procedure. Six heavily infected yolk sacs were washed in Hanks salts and thoroughly disrupted with sterile sand and TKCl, by using a pestle and mortar. The resulting suspension was centrifuged at 1,400 × *g* for 5 min, the supernatant was further centrifuged through 30% Renografin-76 in TKCl, and the pellets were dispersed in TKCl. This suspension was cleared at 2,000 × *g* for 5 min, and the supernatant was processed by two cycles of Renografin-76 density gradient centrifugation.

Chlamydiae were also purified from infected ovine placental tissue by essentially the method developed for yolk sac extraction, except that the material was further treated with sarcosine as described for the avian isolates.

At the various stages of purification, samples were taken for electron microscopic examination. These samples were negatively stained with phosphotungstic acid (pH 7.4) and provided a useful measure of suspension purity.

DNA extraction. The following procedure was based on the method described by Wenman and Lovett (42). The chlamydial suspension was homogenized before the addition of 50 mM Tris containing 20 mM Na_2EDTA, 20% (wt/vol) sucrose, 0.7% (wt/vol) *N*-lauroylsarcosine, and 200 μg of proteinase K. This mixture was incubated at 55°C for 15 min and then at 37°C for 45 min. An equal volume of 'phenol'-chloroform (11) was added, and the mixture was vortexed and centrifuged at 2,000 × *g* for 10 min. Nucleic acid was precipitated from the aqueous phase by addition of a 1/20-volume of 5 M NaCl and 2 volumes of ethanol. After two further cycles of centrifugation and precipitation, the DNA was measured spectrophotometrically, precipitated once more, and redissolved in 10 mM Tris with 1 mM EDTA (pH 7.5) at a concentration of 200 μg/ml.

Restriction endonuclease treatment. DNA was digested in 4- to 10-μg amounts at a concentration of 80 μg/ml with 4 U of enzyme per μg of DNA for 2 to 3 h, under the conditions recommended by the manufacturer. The enzymes were obtained from Bethesda Research Laboratories Ltd., United Kingdom, New England Biolabs Inc., Beverly, Mass., and Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Electrophoresis. Agarose electrophoresis was carried out in horizontal gels of 0.6 or 0.75% agarose in Tris-phosphate buffer (18) at ca. 1 V/cm for 16 h. For DNA digests, ethidium bromide was incorporated into the gel at 0.5 μg/ml, whereas for plasmid detection gels were stained after electrophoresis. Restriction enzyme digests were loaded at 2 μg per track, and results were visualized by illumination with longwave UV light and photographed with a Wratten 2A filter. An *Eco*RI digest of bacteriophage λ DNA and an *Hae*III digest of bacteriophage ΦX174 replicative form DNA (Bethesda Research Laboratories) were used as size markers.

Polyacrylamide gel electrophoresis (PAGE) was conducted under either the conditions described by Laemmli (16) or those described by Loening (18). Gels were run under constant-current conditions at a current density of 9.5 mA/cm for 16 h. Two gel formats were employed, 14 by 20 cm or 16 by 32 cm; in both gel types the thickness was 0.15 cm. The discontinuous gels were run with a 1.5-cm-deep stacking gel of 3% polyacrylamide, and 1 μg of restriction enzyme digest was loaded in each well.

Gels were stained with silver by the method described by Herring et al. (11) except that the gels were washed for 4 h before being soaked in silver nitrate; this involved five changes of 10% ethanol with 0.5% acetic acid to remove sodium dodecyl sulfate and glycine.

RESULTS

In preliminary studies, samples of A22 DNA were cleaved by using a panel of 12 restriction endonucleases, and the resulting digests were analyzed in agarose gels. The total number of bands observed in this system was consistent with the estimated genome size of chlamydiae (14, 28) when compared with the fragment number generated by herpesviruses (33). Restriction endonucleases with four-base recognition sequences produced fragments too small for effective resolution by low-concentration agarose gels, whereas en-

zymes with six-base recognition sites yielded a low number of discrete fragments in the higher-molecular-weight range (10 to 20 kilobases), together with many poorly resolved smaller fragments. The resolution of these larger fragments in 0.75% agarose gels is demonstrated in Fig. 1 which shows *Eco*RI digests of chlamydial DNA preparations alongside a digest of uninfected BHK-21 DNA as a control and phage DNA size markers. No differences were detected in the DNA profiles of four abortion isolates compared in this manner.

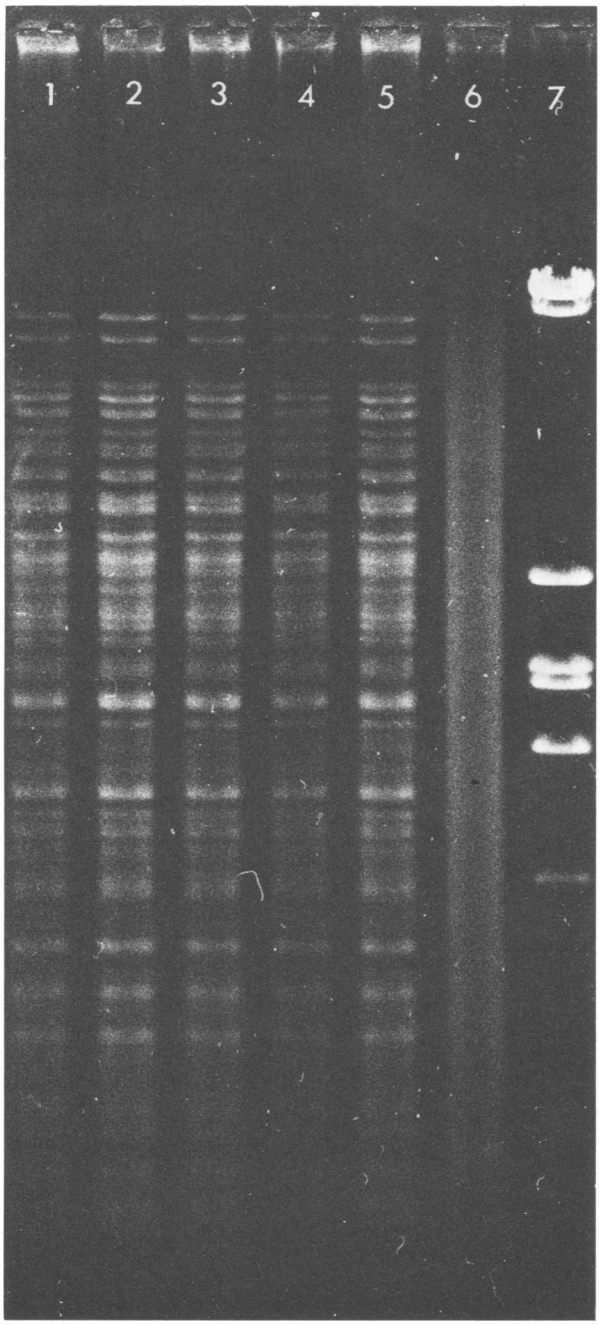

FIG. 1. Agarose gel (0.6%) showing *Eco*RI digests of DNA extracted from four ovine abortion isolates of *C. psittaci* cultured in BHK-21 cells. Lanes: 1, A22; 2 and 3, H574; 4, S26/3; and 5, S507. *Eco*RI digests of uninfected BHK-21 DNA (lane 6) and phage λ DNA (lane 7) are also included.

Experience with strain comparisons of herpesvirus has shown that restriction fragment distributions in the size range 0.5 to 3.0 kilobases which appear identical in agarose gels, may be readily discriminated with PAGE techniques (P. F. Nettleton, J. M. Sharp, A. J. Herring, and J. A. Herring, *in* C. Wittman, R. A. Gaskell, and H. J. Rziha (ed.), *Proceedings of the European Economic Community Workshop on Latent Persistent Herpesvirus Infection in Veterinary Medicine, Tübingen, Federal Republic of Germany, September 1982*, in press). Different gel concentrations and buffer formulations, including gradient gels, were compared by using chlamydial DNA digests, and it was found that optimum resolution over the highest-molecular-weight range was achieved with a discontinuous buffer system and a 7.5% acrylamide concentration. Digestion with four-base recognition sequence enzymes generated too many bands for satisfactory PAGE analysis; however, the six-base recognition enzyme *Eco*RI gave a fragment distribution with ca. 90 bands clearly resolved. Therefore, in subsequent experiments, *Eco*RI and occasionally *Hin*dIII were employed.

The DNA profiles of two abortion isolates (A22 and S26/3) extracted from different cultural conditions were compared to investigate possible host cell contamination and to ensure that enzymatic digestion had proceeded to completion. Identical profiles were obtained from material derived from cell culture, yolk sac, and placental tissue; similarly, uniform profiles were observed irrespective of the purification procedure employed. In contrast, LGV serovar L1 DNA from BHK-21 cell cultures gave a distinctly different profile.

When DNA samples from eight abortion isolates were examined, no differences could be detected despite the substantial numbers of clearly resolved bands which could be compared. This finding was consistent for both *Eco*RI and *Hin*dIII digests. The *Eco*RI profiles of four isolates are shown in Fig. 2, together with those of an ovine arthritis isolate, Cal10 and two avian isolates. The profiles allowed ready differentiation of the various types of *C. psittaci*. The two avian isolates gave very similar profiles, but minor variations in a few bands enabled the strains to be distinguished (Fig. 3). Such differences were seen consistently when the two avian DNA samples were examined by electrophoresis. Moreover, whereas the Cal10 strain could be clearly discriminated from the avian isolates, there were many similar features in these three profiles which did not occur in the ovine abortion or arthritis patterns (Fig. 2).

Recently, other workers have reported a plasmid-like fraction in chlamydial DNA (20, 25) with a size that allows simple detection in agarose gels. Electrophoresis of undigested avian chlamydial DNA resulted in the resolution of faint bands separate from the main genomic DNA which were resistant to digestion with 20 μg of ribonuclease A per ml; such bands were not seen with ovine abortion, arthritis, or Cal10 undigested DNA. When 1-μg amounts of DNA were examined, the plasmid-like bands were at the limits of detection with our illumination system, indicating ca. 10-ng quantities to be present. The two bands had sizes of 8.2 and 4.0 kilobases, which indicates a copy number of about three per cell for the smaller fragment.

DISCUSSION

The use of direct nucleic acid analysis has been particularly valuable for strain differentiation of certain RNA and DNA viruses, but application of the technique to more complex microorganisms has been limited. However, this method of analysis has successfully identified *Mycoplasma hyorhinis* contamination in stocks of human herpesvirus (4) and has proven a useful taxonomic tool in the study of

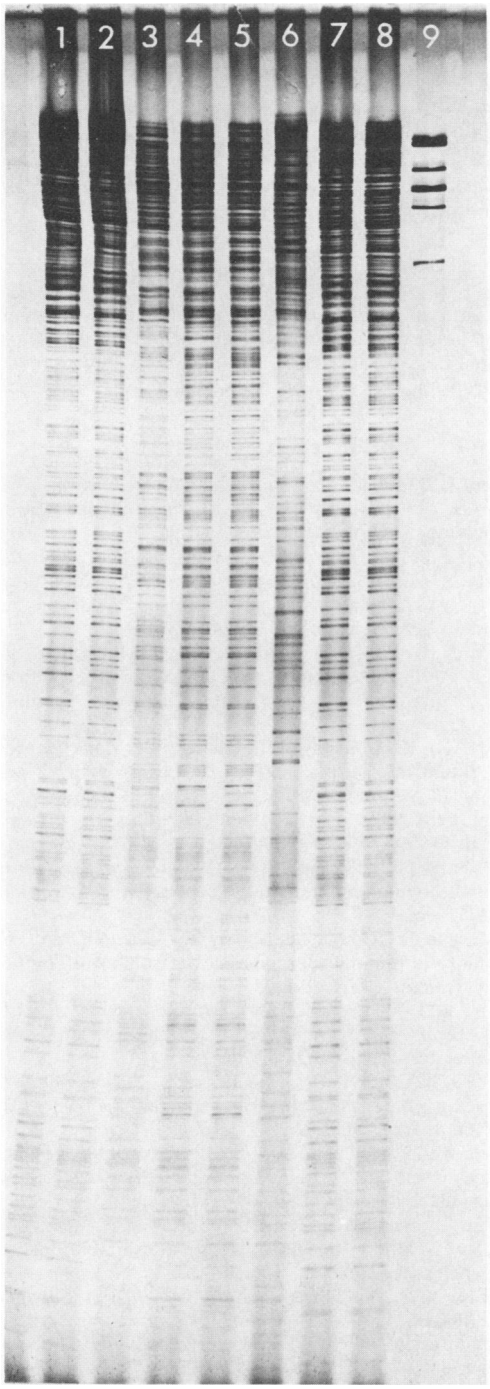

FIG. 2. Silver-stained polyacrylamide gel (7.5%) showing *Eco*RI DNA digests from a range of *C. psittaci* isolates. Lanes: 1 and 2, ovine abortion ZC-19 and A22, respectively; 3, Cal10; 4, avian 725; 5, avian 741; 6, ovine arthritis P787; 7, ovine abortion S26/3; and 8, ZC-25. An *Eco*RI digest of phage λ DNA is also shown (lane 9).

trypanosomes (24). The major problem with the fingerprinting of genomes in the molecular weight range of 5×10^8 to 7×10^8 is that too many fragments are produced for total resolution. Separation of all the digest fragments is not strictly necessary where the goal is simply strain discrimination; as with ribonuclease T1 analysis of RNA, the essential requirement is that as many fragments as possible be well resolved.

Our studies of chlamydial DNA were carried out with PAGE techniques together with sensitive silver staining which obviated the need for radioisotopic labeling of material. The small amounts of DNA required for electrophoresis were economic in use of material and resulted in high-quality resolution of digest fragments. By this method, ca. 90 fragments were well resolved compared with the low number of large fragments separated by agarose gels. Although the highest-molecular-weight fragments were not successfully resolved by PAGE, effectively more profile information was

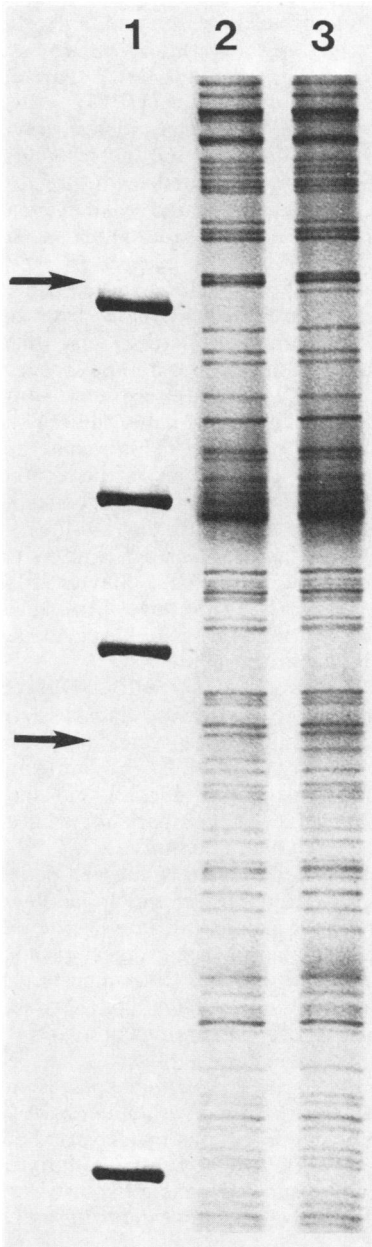

FIG. 3. Silver-stained polyacrylamide gel (7.5%) comparing *Eco*RI DNA digests from two avian *C. psittaci* isolates, 741 columbiforme (lane 2) and 725 psittacine (lane 3). Areas of profile variation are arrowed. Lane 1 shows part of the *Hae*III DNA digest profile of bacteriophage, ΦX174, with fragment sizes of 1,353, 1,078, 872, and 603 base pairs.

achieved and the use of longer gels (32 cm) facilitated the expansion of the complex pattern under examination. Traditionally, such a gel system has been used for small fragments of <1.5 kilobases (21), but the present work and other work (10) indicate a role in the analysis of larger DNA molecules. A cautionary comment is necessary, however, regarding the comparison of profiles between gels, because patterns of bands are not reproduced exactly. It is probable that this effect is due to slight differences in voltage gradient, affecting resolution (44). Thus for fine strain comparisons, DNA digests must be compared in the same gel, preferably in adjoining tracks.

Representatives of the established species *C. trachomatis* and *C. psittaci* were easily differentiated by this technique, confirming the findings of Peterson and de la Maza (25) who used $^{32}P_i$-labeled DNA preparations in agarose gels. The results also accord with previous DNA characterization by G+C ratio and homology studies (15, 41).

Comparisons of *C. psittaci* DNA digests revealed a substantial level of variation within the species, and four distinct electropherotypes were obtained with preparations from ovine abortion, ovine arthritis, and avian and Cal10 isolates. These variations were not confined to host species diversity, since ovine abortion isolates gave a markedly different profile than ovine arthritis material. Such a distinction has previously been made between isolates from these disease conditions by growth parameters, cross-neutralization, plaque reduction, and microimmunofluorescence techniques (6, 29, 34, 37). However, despite repeated testing, the DNA profiles of the seven abortion isolates and the vaccine strain A22 were consistently uniform. This result, obtained with isolates from a range of locations in the United Kingdom, suggests that the chlamydial genome has considerable stability and contrasts with the finding that strains of herpesvirus can nearly all be distinguished by REA (19). It also implies that the differences in biological behavior of the abortion isolates reflect variations at the level of a small number of genetic loci, rather than gross differences such as that revealed with the arthritis isolate.

Minor differences were consistently observed in the migration patterns of the two avian digests, derived from a psittacine and a columbiforme species, respectively. These slight variations may represent the antigenic variation known to exist in this group (23, 32), but since only two isolates were available for comparison, a more extensive study of avian chlamydiae is required.

The origin of the Cal10 strain is not well defined since the isolation was made from a ferret which had been inoculated with human throat washings (8); however, by REA, Cal10 gave a unique and characteristic digest profile which had obvious similarities with avian DNA digests. This observation agrees with the work of Hilleman (12) and Manire and Meyer (22) who suggested a serological relationship between Cal10 and ornithosis isolates.

The heterogeneity of *C. psittaci* species members has frequently been demonstrated with a number of parameters, including growth characteristics under varied culture conditions (34), amino acid requirements (2), fatty acid analysis (13), and various serological reactions (5, 6, 9, 29, 30). Our results show that DNA REA studies represent a valuable additional tool for the taxonomy and epidemiology of *C. psittaci*. The fundamental nature of this type of analysis results in precise information since genetic differences determine strain variation. This chlamydial species is ubiquitous in warm-blooded animals and was also recently isolated from a colony of *Xenopus laevis* frogs (43). The compilation of a library of genomic profiles could be useful to elucidate the spread of *C. psittaci* in the field, and such analyses could also identify potential areas of human zoonoses.

The presence of nongenomic DNA in avian isolates was detected, with a molecular weight less than that described for the chlamydial plasmid (20, 25). Since chlamydial-associated virus has been reported in duck isolates (26), the function of this extra-chromosomal DNA remains to be determined.

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