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Characterization of the New *Chlamydia* Agent, TWAR, as a Unique Organism by Restriction Endonuclease Analysis and DNA-DNA Hybridization

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Several molecular techniques were used for comparison of the novel *Chlamydia* agent, TWAR, with *Chlamydia trachomatis* and *Chlamydia psittaci*. Unlike all serotypes of *C. trachomatis* and most strains of *C. psittaci*, the eight TWAR isolates examined did not contain extrachromosomal DNA. TWAR was readily distinguished from *C. trachomatis* or *C. psittaci* by restriction endonuclease analysis, whereas identical or nearly identical restriction patterns were observed among the TWAR isolates. Southern blot analysis with a gene encoding a portion of the *C. trachomatis* serovar L2 major outer membrane protein as the probe showed that TWAR, like *C. psittaci*, contained sequences homologous to this gene. However, while the hybridization patterns were identical for all TWAR isolates, they differed from those of any of the other *Chlamydia* species tested. A *Pst*I gene bank containing TWAR DNA was constructed in pUC19. Random fragments were purified and used for probing *Chlamydia* chromosomal digests. All of the five probes tested were TWAR specific, with the TWAR isolates showing identical patterns of homology. Qualitative studies of the DNA homology revealed that TWAR did not have significant homology to any of the *Chlamydia* strains assayed. Collectively, these results demonstrate that the TWAR isolates represent a single strain or closely allied genotypes and are clearly distinct from any of the other chlamydiae tested.

Chlamydiae are gram-negative, obligate intracellular bacteria that undergo a unique developmental cycle and share a genus-specific antigen. The genus *Chlamydia* is subdivided into two species, *Chlamydia psittaci* and *Chlamydia trachomatis*, with further differentiation of *C. trachomatis* into three biovars: mouse, lymphogranuloma venereum, and trachoma. Only the lymphogranuloma venereum and trachoma biovars are important in human infections, with the trachoma biovar resulting in oculogenital infection and the lymphogranuloma venereum biovar causing sexually transmitted diseases with lymph node involvement (22, 28). Recently, a novel *Chlamydia* agent designated TWAR has been shown to be an important respiratory pathogen (8). It has been isolated from individuals with acute respiratory disease including pneumonia, bronchitis, and pharyngitis (8, 9). The two species of *Chlamydia* are differentiated by several criteria that include inclusion morphology, presence of glycogen in inclusions, and susceptibility to sulfa drugs (22). With respect to these characteristics, TWAR inclusions are more closely related to *C. psittaci* based on their oval and dense appearance and absence of glycogen, in contrast to the vacuolar nature of *C. trachomatis* inclusions which contain glycogen (18). TWAR organisms share genus-specific antigens but are otherwise serologically distinct. Species-specific monoclonal antibodies against *C. trachomatis* do not react with TWAR nor do TWAR-specific monoclonal antibodies react with *C. trachomatis* or *C. psittaci* (18). Although *C. psittaci* and *C. trachomatis* share common antigens (4), certain rRNA gene sequences (24), and a unique developmental cycle (12), they share only 10% DNA homology (17, 33). Not surprisingly, they are easily differentiated on the basis of DNA restriction endonuclease analysis (26). The purpose of this investigation was to compare TWAR isolates with each other and with *C. trachomatis* and *C. psittaci* by restriction endonuclease analysis, Southern hybridizations with random cloned TWAR fragments as DNA probes, and DNA homology studies.

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MATERIALS AND METHODS

Chemicals and enzymes. *Bst*EII, *Dde*I, and *Msp*I were obtained from Pharmacia, Inc. (Piscataway, N.J.). All other restriction enzymes, DNA polymerase I, bacterial alkaline phosphatase, and T4 DNA ligase were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Ultrapure phenol was obtained from Bethesda Research Laboratories, and formamide was obtained from EM Science (Cherry Hill, N.J.). All other chemicals were reagent grade and were obtained from Sigma Chemical Co. (St. Louis, Mo.) or J. T. Baker Chemical Co. (Phillipsburg, N.J.).

***Chlamydia* strains.** *Chlamydia* strains studied included the following: (i) TWAR (TW-183, AR-39, AR-231, AR-277, AR-388, AR-427, AR-458, and LR-65) (8, 18); (ii) *C. psittaci* (6BC [7], meningopneumonitis [Mn] [5], feline pneumonitis [FP] [2], guinea pig inclusion conjunctivitis [GPIC] [23], and sheep abortion [OA] [a local isolate obtained from P. Dilbeck, Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman]); and (iii) *C. trachomatis* (B/TW-5/OT, D/UW-3/Cx, and L2/434/Bu) (29, 32). The TWAR strains have been described previously (8, 18). All TWAR strains were pharyngeal isolates, except TW-183, which was isolated from the conjunctiva. 6BC is an avian psittacosis strain. The remaining *C. psittaci* strains are of mammalian origin.

Growth and purification of organisms. All chlamydia strains were adapted to grow in HeLa 229 cell cultures (19). The organisms were purified with a linear gradient of meglumine diatrizoate (Hypaque-76; Winthrop-Breon Laboratories, New York, N.Y.) (14). Briefly, infected cells were harvested after 3 days of incubation. Inoculated cells were

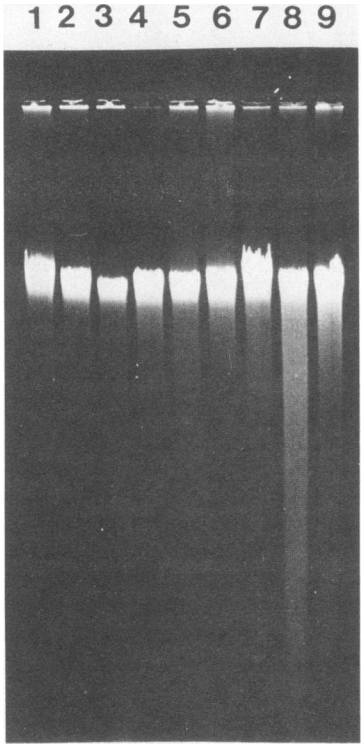

FIG. 1. Electrophoresis of undigested DNA on a 0.8% agarose gel. Lanes: 1, *C. trachomatis* serovar D; 2, *C. psittaci* Mn; 3 to 9, TWAR isolates AR-388, AR-231, LR-65, TW-183, AR-39, AR-458, and AR-277, respectively.

disrupted by sonication. Cell debris was removed by centrifugation at 500 × *g* for 10 min. The organisms were recovered by centrifugation at 30,000 × *g* for 20 min and were purified by first being pelleted through a 30% meglumine diatrizoate cushion at 20,000 × *g* for 40 min and then being banded in a 30 to 65% linear gradient of meglumine diatrizoate at 45,000 × *g* for 90 min. The organisms were collected, washed with Hanks balanced salt solution, suspended in sucrose-phosphate-glutamate buffer (19), aliquoted, and frozen at −75°C until used. The final products usually contained 1.0×10^8 to 5.0×10^8 inclusion-forming units of organisms per ml.

DNA purification. Chlamydia DNA was isolated as previously described for *C. trachomatis* (31). Basically, purified elementary bodies were treated with proteinase K (64 μg/ml) for 1 h at 37°C and solubilized in 1% sodium dodecyl sulfate. After phenol extraction, the aqueous layer was treated with RNase (50 μg/ml) for 30 min at 37°C, extracted with phenol-chloroform, and precipitated with ethanol.

Restriction endonuclease digestions. Restriction digestions were done by following the directions of the manufacturer. Restriction fragments were separated by electrophoresis on 0.8% agarose gels or on 7.5% polyacrylamide gels since no single gel concentration can permit resolution of all fragments. Subsequently, gels were stained with ethidium bromide and photographed.

Detection of methylated bases. To detect the presence of 5-methyl adenine, we used the isoschizomers *Dpn*I and *Mbo*I. While *Dpn*I digests GATC only if the adenine is methylated, *Mbo*I digests only unmethylated DNA (6). *Msp*I and *Hpa*II were used for detection of 5-methyl cytosine. *Hpa*II cuts at the sequence CCGG only if the internal cytosine is methylated, while *Msp*I cuts regardless of methylation at this site (6).

Gene bank construction. TWAR isolate AR-39 chromosomal DNA was digested with *Pst*I and ligated to similarly digested pUC19 by a standard protocol (20). The vector had been treated with bacterial alkaline phosphatase to prevent self-ligation. Recombinant plasmids were transformed into *Escherichia coli* TB1 by the method of Hanahan (10). The transformation mixture was plated onto L agar containing 50 μg of ampicillin per ml and 40 μg of 5-bromo-4-chloro-3-indoyl-β-D-galactoside per ml. Plasmids were isolated by the method of Holmes and Quigley (13) and digested with *Pst*I for evaluation of insert size. Cloned fragments were purified by electroelution from SeaKem GTG agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) followed by passage through an Elutip column (Schleicher & Schuell, Inc., Keene, N.H.) according to the directions of the supplier.

Hybridizations. After electrophoresis, restriction fragments were transferred to BioTrace nitrocellulose sheets (Gelman Sciences, Inc., Ann Arbor, Mich.) by the method of Southern (30). Probe DNA was nick translated by standard methods with [^{32}P]dATP and [^{32}P]dGTP to a specific activity of 10^8 cpm/μg. Generally, 3×10^6 cpm were added per hybridization. The hybridization reaction buffer included 50% formamide, 5× Denhardt reagent, 5× SSPE (1× SSPE is composed of 180 mM NaCl, 10 mM NaH_2PO_4 [pH 7.4], and 1 mM EDTA, pH 7.4), 10 μg of salmon sperm DNA per ml, and 0.1% sodium dodecyl sulfate. Filters were prehybridized at 42°C for 4 h. Hybridization mixtures were incubated at 42°C for 18 h. Washings were done at high stringency with three washes in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 15 min each at 52°C followed by three washes with 0.1× SSC. Filters were exposed to X-Omat film (Eastman Kodak Co., Rochester, N.Y.) for 4 to 18 h before developing.

Dot blots. Depending on the particular experiment, 0.2 to 5 μg of purified elementary body DNA was boiled for 10 min in 0.3 M NaCl. Subsequently, 2.0 M ammonium acetate was added to a final volume of 100 μl. Samples were applied to nitrocellulose (Trans-Blot; Bio-Rad Laboratories, Richmond, Calif.) with a Bio-Dot microfiltration apparatus (Bio-Rad). Before filtration, the nitrocellulose was washed in 1 M ammonium acetate to increase DNA adherence. The nitrocellulose was baked at 80°C for 2 h, and subsequently it was prehybridized as described previously. Nick-translated whole chromosomal chlamydia DNA was denatured by boiling with subsequent quick cooling and used as the probe in these hybridization experiments. To attempt to roughly quantitate the percent homology, we cut the nitrocellulose into individual dots which were suspended in Omnifluor (New England Nuclear Corp., Boston, Mass.) for scintillation counting.

RESULTS

Plasmid screening. Plasmids have been described for both *C. trachomatis* and *C. psittaci*. While plasmids from both species are similar in molecular weight, they share little, if any, DNA homology (16). None of the seven TWAR isolates examined contained any visible plasmid DNA, as determined by agarose gel electrophoresis (Fig. 1), in contrast to the *C. trachomatis* and *C. psittaci* controls which contain a 7.3 and 6.2-kilobase-pair (kbp) plasmid, respectively (16). TWAR isolate AR-427 was screened separately and also found to lack plasmid DNA.

Restriction endonuclease analysis. Restriction endonuclease analysis was used for comparison of TWAR isolates with each other and with strains of the two *Chlamydia* species. Ten different restriction enzymes including *Bam*HI, *Bst*EII, *Dde*I, *Dpn*I, *Eco*RI, *Hin*dIII, *Mbo*I, *Msp*I, *Pst*I, *Sau*3A, and *Sst*I were used on eight TWAR isolates, three *C. trachomatis* serovars (B, D, and L2), and five different strains of *C. psittaci* including both avian and mammalian isolates. When digested with *Msp*I, TWAR isolates were readily differentiated from the trachoma and lymphogranuloma venereum biovars of *C. trachomatis* and the four different strains of *C. psittaci* tested (Fig. 2). In separate experiments, *C. psittaci* FP gave different restriction patterns from TWAR isolates. Moreover, restriction patterns that distinguished TWAR from the other *Chlamydia* species were observed with all other enzymes tested (data not shown).

Of the *Chlamydia* isolates that have been examined, only *C. psittaci* OA isolates (11), one ovine arthritis strain (21), and TWAR isolates lack plasmid DNA. Using AR-388 as the representative TWAR isolate for comparison with the OA isolate, we observed distinct restriction patterns with all enzymes used (Fig. 3).

Within the limits of resolution of this method, banding patterns of the TWAR isolates were identical with eight of the enzymes used. When the DNA was digested with *Msp*I (Fig. 2) or *Bst*EII (data not shown), one extra DNA fragment that was found with both AR-39 and TW-183 and that was absent from the other six TWAR isolates could be identified by agarose gel electrophoresis. No further differences were resolved by polyacrylamide gel electrophoresis (data not shown).

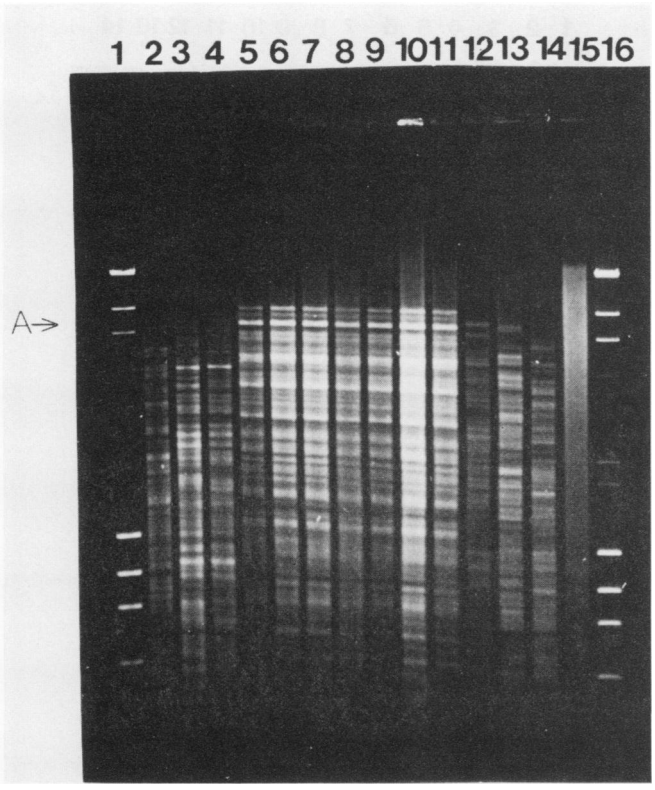

FIG. 2. *Msp*I digests of two serovars of *C. trachomatis*, seven TWAR isolates, and four *C. psittaci* strains. Lanes: 1, lambda *Hin*dIII and φX174 molecular weight markers; 2, *C. psittaci* Mn; 3 and 4, *C. trachomatis* serovars D and L2, respectively; 5 to 11, TWAR isolates AR-231, AR-39, AR-277, AR-458, LR-65, AR-388, and TW183, respectively; 12 to 14, *C. psittaci* GPIC, 6BC, and OA, respectively; 15, HeLa; 16, lambda *Hin*dIII and φX174 *Hae*III molecular weight markers. The letter A marks the position of the faint extra band seen in TW-183 (lane 11) and AR-39 (lane 6).

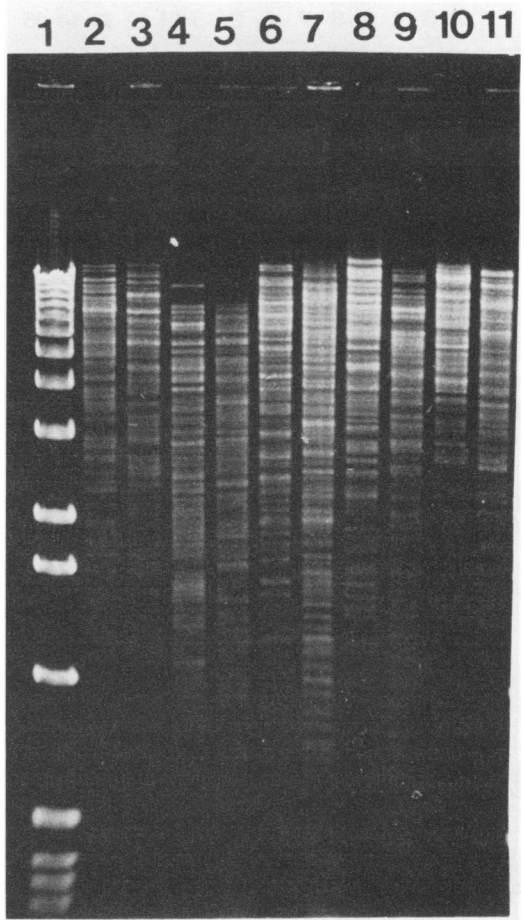

FIG. 3. Restriction digests comparing TWAR isolate AR-388 with a *C. psittaci* OA isolate. Lanes: 1, 1-kilobase ladder; 2, AR-388, *Pst*I; 3, OA, *Pst*I; 4, AR-388, *Hin*dIII; 5, OA, *Hin*dIII; 6, AR-388, *Eco*RI; 7, OA, *Eco*RI; 8, AR-388, *Sst*I; 9, OA, *Sst*I; 10, AR-388, *Bam*HI; 11, OA, *Bam*HI.

In studies of methylation of chlamydia DNA, all *Chlamydia* isolates tested were cut by the enzyme *Mbo*I but not by *Dpn*I, and all were cut equally well with both *Msp*I and *Hpa*II. These results suggest that chlamydia DNA does not contain 6-methyl adenine or 5-methyl cytosine.

To further investigate the relationship between the TWAR isolates and the other *Chlamydia* strains, five random TWAR DNA fragments having approximate sizes of 500, 900, 1,200, 3,200, and 3,800 base pairs were purified from a gene bank of AR-39 DNA and used for probing chromosomal digests. When the 3.2-kbp TWAR fragment was used as a probe of chlamydia DNA digested with *Hin*dIII, the hybridizing fragment was the same for all TWAR isolates (Fig. 4). Identical results were observed when two other restriction enzymes, *Pst*I and *Hin*dIII, were used for digestions and genomic DNA was probed with the 3.2-kbp fragment. Additionally, this probe was TWAR specific, as no reactivity was observed with the *C. trachomatis* or *C. psittaci* strains tested. The other four different probes tested were also TWAR specific (data not shown). Each probe hybridized to a distinct restriction fragment(s). In each case, all TWAR

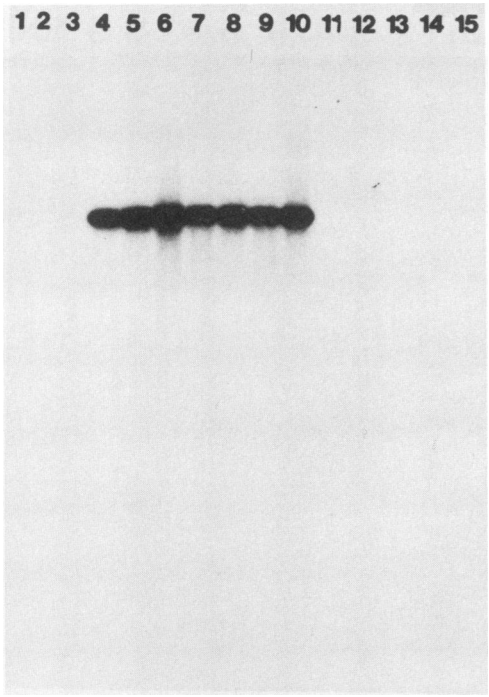

FIG. 4. Autoradiograph of Southern hybridizations of two *C. trachomatis* strains, seven TWAR isolates, and four *C. psittaci* strains digested with *Hin*dIII and probed with a 3.2-kbp *Pst*I TWAR fragment. Lanes: 1, *C. psittaci* Mn; 2 and 3, *C. trachomatis* serovars D and L2, respectively; 4 to 10, TWAR isolates AR-231, AR-39, AR-277, AR-458, LR-65, AR-388, and TW-183, respectively; 11 to 13, *C. psittaci* GPIC, 6BC, and OA, respectively; 14, HeLa; 15, lambda *Hin*dIII and φX174 *Hae*III molecular weight markers.

isolates had the same pattern of hybridization. These cumulative results suggest that the first TWAR isolate, which was isolated 20 years ago from the conjunctiva of a child in Taiwan, is the same strain as or a strain closely related to those isolated from individuals with acute respiratory disease in Seattle in 1985 (8).

Demonstration of shared sequences. In previous studies, a cloned fragment encoding a portion of the L2 major outer membrane protein gene of *C. trachomatis* was shown to hybridize weakly to the Mn strain of *C. psittaci* in dot blots (31). Both the TWAR isolates and the different *C. psittaci* strains contain sequences that demonstrated homology when the hybridization was done under stringent conditions (Fig. 5). Identical patterns of homology were observed among the TWAR isolates that differed from the patterns observed for *C. psittaci* or *C. trachomatis*. The faintly hybridizing band seen for AR-39 (Fig. 5, lane 4) represents a partial digestion and was not observed in other hybridizations.

DNA homology studies. To determine the relatedness of TWAR to *C. trachomatis* and *C. psittaci*, we used dot-blot analyses with whole chromosomal DNA as the probe. In all experiments, HeLa DNA was spotted as a control, as most *Chlamydia* DNA preparations contain a small amount of HeLa DNA contamination. Unfortunately, accurate quantitation of results was precluded because the amount varied in each preparation. Whole chromosomal DNAs of TWAR strain AR-39, *C. trachomatis* serovar D, and *C. psittaci* 6BC, FP, OA, and Mn were nick translated and used as probes of dot blots containing denatured chromosomal DNA of the various isolates. Neither of the two *C. trachomatis* biovars nor the five *C. psittaci* strains demonstrated any significant homology to the AR-39 probe (Fig. 6). On the other hand, all TWAR isolates gave strong hybridization signals. Analogous results were obtained when TW-183 was used as the probe. When the reciprocal experiments were done with DNA from *C. trachomatis* serovar D or *C. psittaci* 6BC, FP, Mn, or OA as the probe, no reactivity above background levels was observed with any of the TWAR isolates (data not shown).

DISCUSSION

We used DNA analysis to define further the novel *Chlamydia* agent, TWAR, an important human respiratory pathogen. Several conclusions can be made from our studies. First, unlike all serovars of *C. trachomatis* and most strains of *C. psittaci*, none of the TWAR isolates contained plasmid DNA. Second, analysis by restriction endonuclease digestion coupled to Southern blots with TWAR fragments as DNA probes suggests that the TWAR isolates are the same strain or have closely allied genotypes. These results confirm our previous serological analyses suggesting that the isolates were the same or closely related strains (18). Third, we showed that TWAR and several strains of *C. psittaci* contain sequences that are homologous to a gene encoding a portion of the L2 major outer membrane protein gene. However, the blotting pattern of TWAR differed from that of either *C. trachomatis* or *C. psittaci*, while all TWAR isolates had the

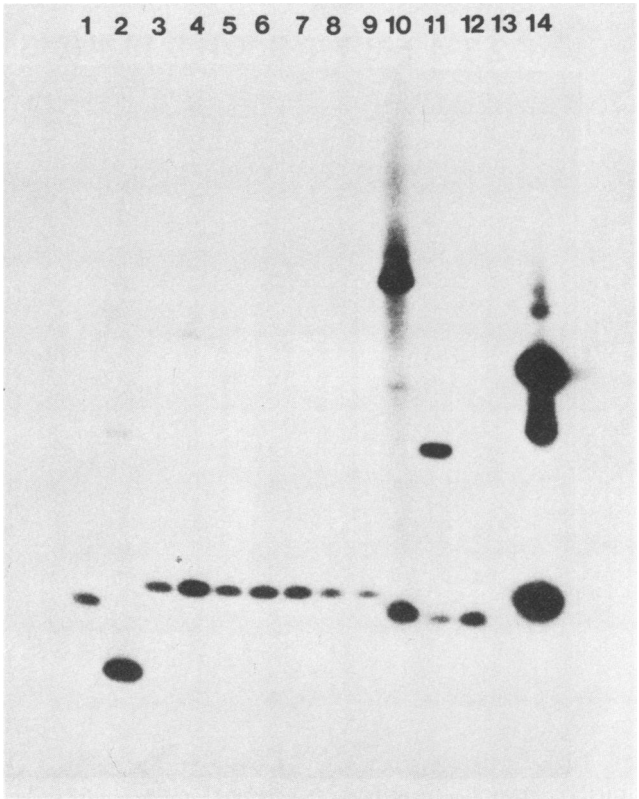

FIG. 5. Autoradiograph of Southern hybridization of *Chlamydia* DNA digested with *Msp*I and hybridized to a 1.1-kbp *C. trachomatis* clone which codes for a part of the L2 major outer membrane protein. Lanes: 1, *C. psittaci* Mn; 2, *C. trachomatis* serovar D; 3 to 9, TWAR isolates AR-231, AR-39, AR-427, AR-458, LR-65, AR-388, and TW-183, respectively; 10 to 12, *C. psittaci* GPIC, FP, and 6BC, respectively; 13, HeLa; 14, molecular size markers of 1.1, 2.6, and 3.7 kbp containing sequences hybridizing to the probe.

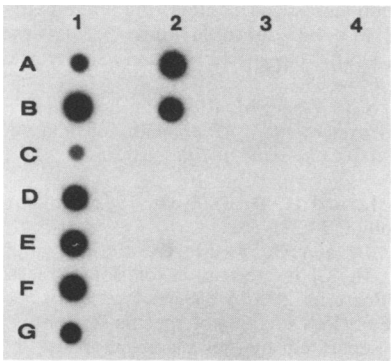

FIG. 6. Autoradiograph of a dot blot probed with TWAR isolate AR-39 chromosomal DNA. Lane 1, A to G: TWAR isolates AR-39 (diluted fivefold), AR-39 (undiluted), AR-39 (diluted 10-fold), AR-388, AR-231, AR-458, and AR-427, respectively. Lane 2, A and B: TWAR isolates LR-65 and TW-183, respectively. Lane 2, C to E: *C. trachomatis* serovars L2, B, and D, respectively. Lane 3, A to C, E, and F: *C. psittaci* 6BC, GPIC, Mn, FP, and OA, respectively. Lane 4, A to D: HeLa DNA diluted 2-fold, 4-fold, 10-fold, and 100-fold, respectively.

same pattern of hybridization. These results suggest that these sequences are highly conserved within the genus *Chlamydia*. We have isolated clones of AR-39 DNA containing sequences which hybridized to this probe (data not shown). Unfortunately, these clones are unstable, a problem that has been encountered frequently in attempts to clone these sequences from *C. trachomatis* (unpublished data from our laboratory). Last, homology studies showed that TWAR does not share any significant homology with the human biovars of *C. trachomatis* or with the mammalian and avian *C. psittaci* strains assayed. The specificity of whole chromosomal TWAR DNA coupled with the isolation of TWAR-specific fragments represent potential diagnostic tools for the identification of TWAR in respiratory infections.

To date, the information obtained from morphological, epidemiological, and antigenic analyses has strongly indicated that TWAR is distinct from *C. trachomatis* and more closely related to *C. psittaci* (8, 18). However, unlike the bird-to-human transmission of *C. psittaci*, no avian or animal host has been shown to be associated with the spread of TWAR (8, 27). The epidemiology of TWAR infections has suggested that transmission is from human to human and that the TWAR organism is a primary human pathogen (8, 9, 27). In addition, recent ultrastructural analysis has shown that TWAR elementary body morphology is unique among the *Chlamydia* species (3). The elementary body is typically pear shaped with a large periplasmic space, in contrast to the other *Chlamydia* elementary bodies which are round with an indistinct periplasmic space. The results from this study show that the organism can be differentiated from *C. trachomatis* or any of the *C. psittaci* strains tested on the basis of restriction endonuclease analysis. The qualitative studies of DNA homology clearly show that the TWAR organism does not share extensive homology with the *C. trachomatis* or the *C. psittaci* strains tested. To quantitate the relatedness of TWAR to *C. trachomatis* and *C. psittaci*, further analysis with solution hybridization is necessary because of the limitations of filter hybridizations in determining DNA complexity and composition (1, 15). The heterogeneity of the different strains making up the *C. psittaci* species (21, 25) and the paucity of information concerning the DNA homologies of these different members prevent extrapolation of these results for comparison of TWAR with *C. psittaci* strains not tested in this study. However, TWAR isolates are clearly distinct from the avian and mammalian isolates that we utilized. The results presented in this paper coupled with our previous studies suggest that the TWAR isolates represent a novel entity.

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