

# Comparison of Avian *Chlamydia psittaci* Isolates by Restriction Endonuclease Analysis and Serovar-Specific Monoclonal Antibodies

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Avian *Chlamydia psittaci* isolates were examined by restriction endonuclease analysis and serovar-specific monoclonal antibodies and compared with ovine abortion and polyarthritides isolates. The avian isolates were divided into four serovars (turkey, psittacine, pigeon, and duck) based on their reactivity to the monoclonal antibodies. The DNA digest patterns were similar across the four avian serovars; most bands were identical when the isolates were tested with *Pst*I, *Bam*HI, and *Eco*RI restriction endonuclease enzymes. The turkey group restriction endonuclease analysis patterns were distinguished from those of the other avian strains by three to four band differences with all enzymes. The duck and pigeon isolates showed only minor DNA pattern differences when compared with the psittacine isolates. Four psittacine isolates from various locations in Texas had an extra band with the *Eco*RI restriction enzyme, suggesting that they were from a common source; however, they were indistinguishable from the other psittacine isolates when examined with the monoclonal antibodies. The avian isolates were distinctly different from either abortion or polyarthritides isolates by both restriction endonuclease analysis and monoclonal antibody analysis. The data demonstrate that the avian isolates form a distinct group or separate biovar with at least four serovars.

The genus *Chlamydia* is divided into three species, *Chlamydia trachomatis*, *C. psittaci*, and *C. pneumoniae* (6, 12). *C. trachomatis* is divided into three biovars based on natural host, disease signs, and biological properties (11, 12). These biovars are further subdivided into 15 serovars on the basis of strain-specific antigens. The third species, *C. pneumoniae*, is a human respiratory pathogen. At this time, the TWAR strain is the only serovar which has been identified (6).

The remaining chlamydiae are grouped into the species *C. psittaci* because of the lack of a satisfactory method to subdivide them. Consequently, *C. psittaci* is a heterologous group which includes isolates from most avian and mammalian species. These isolates produce numerous disease syndromes including pneumonitis, enteritis, conjunctivitis, abortion, arthritis, and encephalomyelitis. Until the relationships among the *C. psittaci* isolates are known, control of disease caused by them will be difficult. At the present time, the significance of isolation of *C. psittaci* in the absence of specific disease syndromes is unknown.

Attempts have been made to subdivide the group by using growth characteristics and serological methods (2-4, 20-23, 30), but success has been limited. In a recent study, Perez-Martinez and Storz (17), using the microimmunofluorescence technique, identified nine immunotypes within the mammalian isolates. These results correlated with the earlier success of Spears and Storz (22) in biotyping *C. psittaci* isolates by using inclusion morphology and response to DEAE-dextran and cycloheximide in cell culture. Recently, attempts have been made to serotype *C. psittaci* isolates of avian origin by using monoclonal antibodies (MAbs) (5, 26, 28). Many of the MAbs have been of genus or subspecies specificity; few have been serovar specific. The results from these studies indicate that there are at least three immunotypes among the avian isolates. We previously reported the production of serovar-specific MAbs that were used to distinguish two avian serovars and one mammalian abortion serovar (1). This paper reports the comparison of avian

isolates by DNA restriction endonuclease analysis (REA) and by typing with serovar-specific MAbs.

## MATERIALS AND METHODS

The origins of the *C. psittaci* isolates are reported in Table 1. The VS1, VS2, and 6BC avian isolates and the mammalian isolates have been maintained in our laboratory by passage in embryonated eggs. The remaining 12 avian isolates were obtained from J. E. Grimes, Texas A&M, College Station, and were received as homogenized yolk sac suspensions. The chlamydiae were adapted for growth in Vero cells by centrifuging the inoculum onto 24-h-old confluent Vero cell monolayers in 25-cm<sup>2</sup> tissue culture flasks. Chlamydial stocks for DNA extraction were produced in 850-cm<sup>2</sup> roller bottles following adaptation to tissue culture. The DNA used in this study was extracted from the third to sixth tissue culture passage.

**Cell cultures.** Vero cells were cultured in Eagle minimum essential medium with Earle balanced salts, 20 mmol of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-5% fetal bovine serum per liter, 5.4 ml of glucose per liter, 292 mg of glutamine per liter, 2 µg of amphotericin B per ml, and 10 µg of gentamicin sulfate per ml. The same medium with the addition of 0.5 µg of cycloheximide per ml was used following inoculation of the Vero cells with chlamydiae.

**Preparation of chlamydial DNA.** Chlamydiae were harvested between 2 and 4 days following inoculation of the monolayer, depending on the isolate. The cell culture fluid was collected and cellular debris was removed by centrifugation at 500 × *g* for 10 min. Chlamydiae were concentrated by pelleting by centrifugation at 10,000 × *g* for 60 min. Chlamydiae were purified by centrifugation into a 30 to 50% step sucrose gradient at 10,000 × *g* for 60 min. The layer containing the chlamydial elementary bodies was collected, diluted threefold in distilled water, and pelleted by centrifugation at 10,000 × *g* for 60 min. Chlamydiae were resus-

TABLE 1. Isolates of *C. psittaci*

Chlamydial isolate	Host	Yr	Location	Disease	Reference or source
GD	Duck	1960	Europe	Psittacosis	9
80022	Amazon parrot	1980	Texas	Psittacosis	30
WWD	White wing dove	1959	Texas	Psittacosis	7
79068	Cockatiel	1979	Texas	Psittacosis	30
80032	Amazon parrot	1980	Texas	Psittacosis	30
78001	Cockatiel	1978	Texas	Psittacosis	30
VS1	Amazon parrot	1985	Georgia	Psittacosis	1
VS2	Lovebird	1985	Minnesota	Psittacosis	1
80026	Amazon parrot	1980	Texas	Psittacosis	30
80033	Cockatiel	1980	Texas	Psittacosis	30
SE45	Snowy egret	1990	Louisiana	Psittacosis	19
6BC	Parakeet	1941	California	Psittacosis	ATCC <sup>a</sup>
SG125	Sea gull	1957	Oregon	Normal	30
FAL TEX	Turkey	1975	Texas	Ornithosis	30
78105	Budgerigar	1978	Texas	Psittacosis	30
TT3	Turkey	1975	Texas	Ornithosis	Page <sup>b</sup>
NJ1	Turkey	1959	New Jersey	Ornithosis	13
B577	Sheep	1962	Utah	Abortion	24
OSP	Sheep	1985	Oregon	Abortion	1
EBA	Cattle	1959	California	Abortion	25
IPA	Sheep	1968	Iowa	Polyarthrits	15

<sup>a</sup> American Type Culture Collection, VR-125. No reference given.

<sup>b</sup> Isolated by L. Page from the 1974 to 1976 Texas outbreak (16).

pended in 0.5 ml of 25% sucrose in 50 mM Tris–5 mM EDTA (pH 8). High-molecular-weight chlamydial chromosomal DNA was prepared by the techniques of Hull et al. (8) and Thiermann et al. (27). The DNA concentration of each preparation was determined by spectrophotometry (10).

**Restriction endonuclease digestion of DNA.** DNA was digested in 1- to 2- $\mu$ g amounts in a 40- $\mu$ l volume with 5 to 10 U of enzyme per  $\mu$ g of DNA for 3 to 4 h under conditions recommended by the manufacturer (New England BioLabs, Beverly, Mass.). After the addition of 7  $\mu$ l of a 60% sucrose solution as a gel-loading buffer, the samples were electrophoresed on an agarose gel (16 by 30 cm) at 60 V for 16 h on a horizontal electrophoresis box. The electrophoresis gel was made up of 0.7% agarose in Tris-borate buffer (10.8 g of Tris, 0.93 g of disodium EDTA, 5.5 g of boric acid per liter of distilled water). The gels were stained in ethidium bromide (0.25  $\mu$ g/ml) for 60 min and photographed under short-wave UV light through a Kodak 23A red filter (Eastman Kodak, Rochester, N.Y.). Four restriction enzymes (*Bam*HI, *Pst*I, *Eco*RI, and *Sal*I) were screened for use for cleavage of chlamydial DNA. The best results were obtained with *Bam*HI, *Eco*RI, and *Pst*I and are reported in this study.

**Identification of serovars.** The MABs to the VS1, NJ1, B577, IPA, and GD isolates were used in the indirect fluorescent-antibody test as described previously (1) to determine the serotypes of the isolates. Briefly, 24-h-old monolayers of Vero cells grown in 96-well multiwell dishes were inoculated with each isolate. The inoculum was then centrifuged onto the monolayer and the cultures were incubated for 24 to 72 h at 37°C, depending on the time of optimal inclusion development by the specific isolate. After incubation, the infected monolayers were fixed with 50% acetone–50% methyl alcohol for 5 min. The isolates were serotyped by testing with serovar-specific MABs at dilutions of 1:16 and 1:512. All positive cultures were retested by using a twofold dilution series.

The specificity and titers of the MABs to the VS1, NJ1, and B577 isolates were described previously. The MABs to

the duck and ovine polyarthrits isolates (GD and IPA) were produced and tested for specificity by the same procedures and methods of selection as described previously (1). All MABs had titers of 1:2,048 or higher to the homologous isolates and no reaction at 1:16 with heterologous isolates (1).

## RESULTS

Comparison of the *Bam*HI restriction endonuclease digest patterns of the avian isolates is shown in Fig. 1. The bands produced by all of the isolates are similar; only a limited number of differences are seen. Lane 2 and lanes 4 through 12 are of the psittacine type. These isolates are indistinguishable with the *Bam*HI digest pattern. Lanes 13 through 16 are of the virulent turkey type and are similar. Three characteristic band differences distinguish the virulent turkey isolates from the psittacine isolates (designated by arrowheads). Lane 1 is a duck isolate (German duck [GD]) and lane 3 is a pigeon isolate (white wing dove [WWD]). Minor band differences separating them from the psittacine isolates are marked with arrows.

The *Eco*RI digest patterns are similar (Fig. 2), with only minor differences noted among the psittacine, turkey, duck, and pigeon isolates. The inclusion of an extra band (brackets) was noted in four psittacine isolates (lanes 2, 5, 9, and 10). These were isolated in 1980 from psittacine birds from Texas. The results with *Pst*I were similar, with only minor band differences among turkey, psittacine, duck, and pigeon isolates (data not shown).

Psittacine isolates, turkey isolates, abortion isolates, and an ovine polyarthrits isolate were compared following digestion with *Pst*I enzyme (Fig. 3). Multiple band differences are seen when the avian isolates are compared with the mammalian isolates and also when the ovine polyarthrits isolate is compared with the abortion isolates.

Avian isolates were compared with one another by using serovar-specific MABs in the indirect fluorescent-antibody

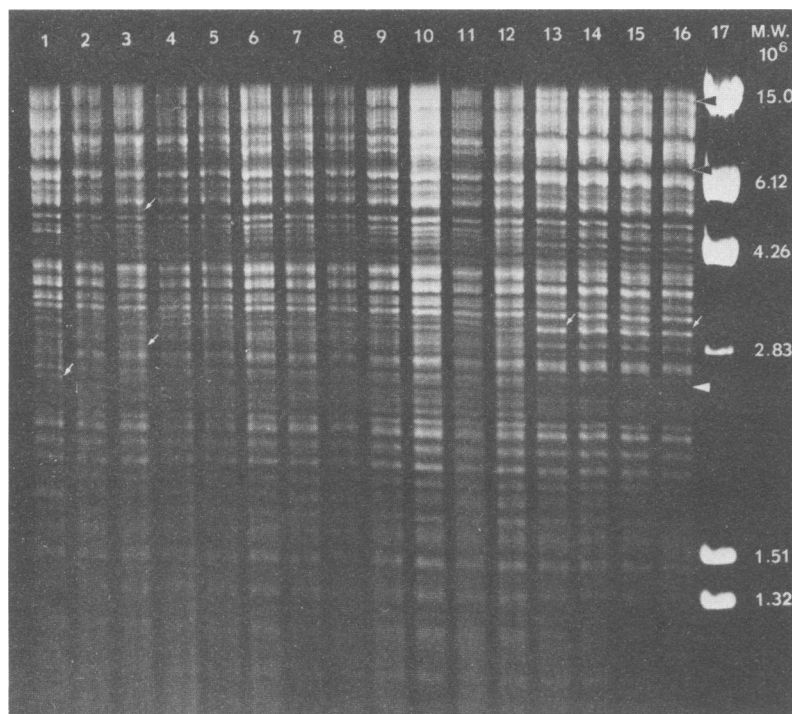


FIG. 1. REA digest patterns of chlamydial chromosomal DNA digested with *Bam*HI. Isolates include the following: lane 1, GD (duck); lane 2, 80022 (Amazon parrot); lane 3, WWD (white wing dove); lane 4, 79068 (cockatiel); lane 5, 80032 (Amazon parrot); lane 6, 78001 (cockatiel); lane 7, VS1 (Amazon parrot); lane 8, VS2 (lovebird); lane 9, 80026 (Amazon parrot); lane 10, 80033 (cockatiel); lane 11, SE45 (snowy egret); lane 12, 6BC (parakeet); lane 13, SG125 (sea gull); lane 14, FAL TEX (turkey); lane 15, 78105 (budgerigar); lane 16, TT3 (turkey); lane 17, *Hind*III digest of lambda DNA. Arrows indicate extra or missing bands. Arrowheads indicate difference between turkey and psittacine isolates. M.W., Molecular weight.

test. Reactions to selected MABs are given in Table 2. Either the reactions were over 1:2,048 or there was no reaction at 1:16. The avian isolates formed four separate serovars. Three of the serovars were distinct, with none of the MABs cross-reacting over 1:16; these included the psittacine, duck, and turkey serovars. The fourth serovar is the pigeon serovar, which is represented by the WWD isolate. It was differentiated from the psittacine group by the failure of the VS1/E8 MAB to react with it.

The mammalian isolates (abortion and polyarthritis) were serologically distinct when tested with the serovar-specific MABs (Table 2). The serovar-specific MABs to these isolates did not cross-react with any of the avian isolates or with one another.

## DISCUSSION

The results show that these avian isolates represent four serological groups or serovars. Three of the serovars (psittacine, duck, and turkey) showed no cross-reactions with serovar-specific MABs. The fourth serovar, designated pigeon and represented by the WWD isolate, reacted with two of the three psittacine serovar-specific MABs. The third MAB produced no cross-reaction with the WWD isolate at a dilution of 1:16, indicating that the pigeon serovar is lacking an epitope present in the psittacine strain. Similar results have been obtained with a number of other pigeon isolates (data not shown). The pigeon and psittacine serovars are closely related; however, in addition to their cross-reactivity, their REA digest patterns are very similar. No cross-reactions between the avian serovars and the two mamma-

lian serovars were seen with any of the serovar-specific MABs.

The REA digest patterns clearly show that all of the avian isolates are closely related. Over 95% of the bands are the same. The psittacine, duck, and pigeon strains showed similar digest patterns with the restriction enzymes used; in contrast, the turkey strains had three to four band differences with each of the enzymes. The mammalian serovars (abortion and polyarthritis) were distinctly different from each other and from the avian isolates. Major REA pattern differences were found among the avian group, the abortion group, and the polyarthritis isolate. This is in contrast to findings within the *C. trachomatis* species in which only minor band differences and minor DNA homology differences were detected between the lymphogranuloma venereum and trachomatis biovars (18, 29).

The four psittacine isolates with an extra band in the  $12 \times 10^6$ -molecular-weight area in the *Eco*RI digest pattern are of interest epidemiologically. Three of the isolates were from Amazon parrots and one was from a cockatiel. The birds had been recently purchased from two pet stores in different areas of Texas during 1980. It appears that the birds had been infected from a common source because of the distinct REA pattern in comparison with other psittacine isolates. However, the reaction with the MABs was the same as with other psittacine isolates.

The results are in agreement with earlier studies of avian isolates. An early study with antisera produced in chickens to various isolates demonstrated that there are serological differences among isolates from turkeys, psittacine birds, and pigeons (2). Because of problems encountered in pro-

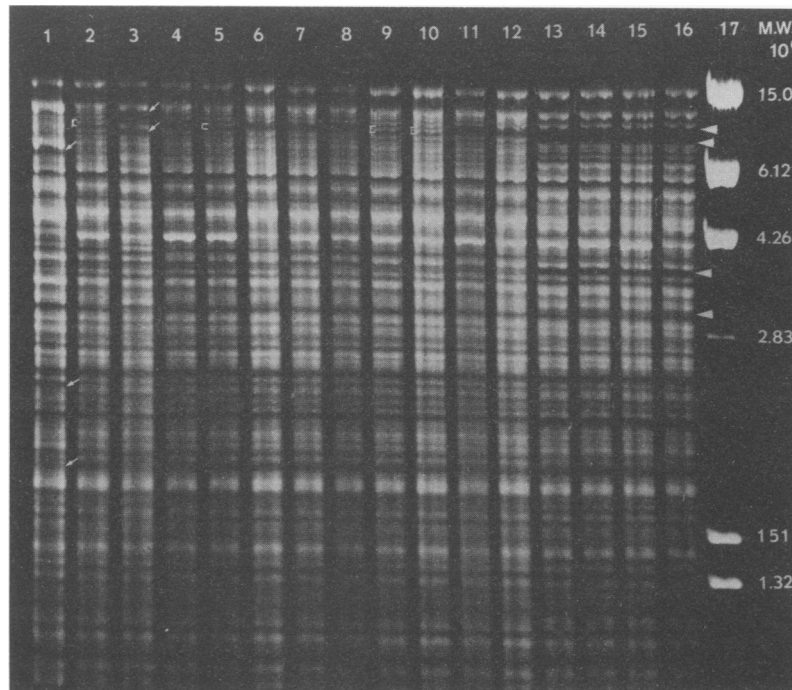


FIG. 2. REA digest patterns of chlamydial chromosomal DNA digested with *EcoRI*. Isolates include the following: lane 1, GD (duck); lane 2, 80022 (Amazon parrot); lane 3, WWD (white wing dove); lane 4, 79068 (cockatiel); lane 5, 80032 (Amazon parrot); lane 6, 78001 (cockatiel); lane 7, VS1 (Amazon parrot); lane 8, VS2 (lovebird); lane 9, 80026 (Amazon parrot); lane 10, 80033 (cockatiel); lane 11, SE45 (snowy egret); lane 12, 6BC (parakeet); lane 13, SG125 (sea gull); lane 14, FAL TEX (turkey); lane 15, 78105 (budgerigar); lane 16, TT3 (turkey); lane 17, *HindIII* digest of lambda DNA. Arrows indicate extra or missing bands in the duck and pigeon isolates. Arrowheads indicate difference between turkey and psittacine isolates. Brackets indicate extra band present in four psittacine isolates. M.W., Molecular weight.

ducing high-titer antisera, a classification system was not developed. Pathogenesis studies have indicated differences in virulence of isolates from various sources and differences in host range (14). These studies have indicated that, for most birds, turkey isolates were more virulent than pigeon isolates; however, turkey isolates had little effect on pigeons and sparrows. A recent study, using many of the same isolates (30), groups isolates into high and low infectivity on the basis of chicken embryo lethal doses needed to infect tissue culture monolayers. The high-infectivity group corresponds to the turkey strains in our study. The low-infectivity group includes isolates that are designated pigeon (WWD), duck (GD), and psittacine. In our laboratory, these isolates have similar growth characteristics in tissue culture and their REA patterns are more similar to those of one another than to that of the turkey isolates.

MAb studies also indicate that differences exist among avian isolates. Toyofuku et al. (28) separated 9 feral pigeon isolates and 16 budgerigar isolates into three and four groups, respectively, by reaction to MAbs. The MAbs used were to a pigeon isolate and included three genus-specific and two strain-specific MAbs. In a later study, using the same MAbs and MAbs to a budgerigar isolate, they identified three budgerigar and three pigeon immunotypes (26). Fukushi et al. (5) divided 77 avian and mammalian *C. psittaci* isolates into four groups, using 11 MAbs produced to a psittacine isolate. The above studies indicate that there are a number of strain differences in the avian isolates of *C. psittaci*. It is difficult to compare these results with ours, as these researchers used primarily genus-specific MAbs and serovar-specific MAbs to a limited number of isolates. In

addition, the isolates and the methods of testing are different from those used in our laboratory. The MAbs we use are serovar specific and, when used in the indirect fluorescent-antibody test as performed in our laboratory, provide an all-or-none reaction at a 1:500 dilution.

The species *C. trachomatis* is divided into biovars on the basis of host, disease syndrome, and other biological properties (12). It is further divided into serovars by immunological procedures. *C. psittaci* has never been subdivided into biovars and serovars because of the lack of easily reproducible methods to serotype the isolates and to determine genetic relationships of the organisms.

The REA and the serovar-specific MAbs used in this study have the potential for use in developing a classification procedure for *C. psittaci*. The serovar-specific MAbs are easily produced and can be used at dilutions of 1:500 to 1:1,000 in the indirect fluorescent-antibody test. The results are easily interpreted, as the serovar-specific MAbs give an all-or-none reaction when used at the proper dilution. REA can then be used to provide a rapid test on the genetic relatedness of new serovars identified by the MAbs.

The four avian serovars are members of one biovar when the *C. trachomatis* classification standards (11) are applied to them. The serovars are genetically related as determined by the REA and are endemic to the avian host; all produce a similar type of disease in birds and have similar growth characteristics in tissue culture.

The mammalian isolates represent the two main antigenic groups found in sheep and cattle. They have been designated serovars 1 and 2 (12), which correspond to biotypes 1 and 2 (22). According to *C. trachomatis* classification methods,

TABLE 2. Reaction of MABs in immunofluorescence assay

Chlamydial isolate	Reaction <sup>a</sup> of MAB:									
	GD/B6	VS1/E3	VS1/F9	VS1/E8	NJ1/A4	NJ1/C1	B577/F3	B577/A11	IPA/2F8	IPA/2E9
GD	+++	-	-	-	-	-	-	-	-	-
80022	-	+++	+++	+++	-	-	-	-	-	-
WWD	-	+++	+++	-	-	-	-	-	-	-
79068	-	+++	+++	+++	-	-	-	-	-	-
80032	-	+++	+++	+++	-	-	-	-	-	-
78001	-	+++	+++	+++	-	-	-	-	-	-
VS1	-	+++	+++	+++	-	-	-	-	-	-
VS2	-	+++	+++	+++	-	-	-	-	-	-
80026	-	+++	+++	+++	-	-	-	-	-	-
80033	-	+++	+++	+++	-	-	-	-	-	-
SE45	-	+++	+++	+++	-	-	-	-	-	-
6BC	-	+++	+++	+++	-	-	-	-	-	-
SG125	-	-	-	-	+++	+++	-	-	-	-
FAL TEX	-	-	-	-	+++	+++	-	-	-	-
78105	-	-	-	-	+++	+++	-	-	-	-
TT3	-	-	-	-	+++	+++	-	-	-	-
NJ1	-	-	-	-	+++	+++	-	-	-	-
B577	-	-	-	-	-	-	+++	+++	-	-
OSP	-	-	-	-	-	-	+++	+++	-	-
EBA	-	-	-	-	-	-	+++	+++	-	-
IPA	-	-	-	-	-	-	-	-	+++	+++

<sup>a</sup> +++, Bright fluorescence of inclusion bodies observed at a dilution of 1:2,048 or higher; -, no visible reaction at 1:16 dilution.

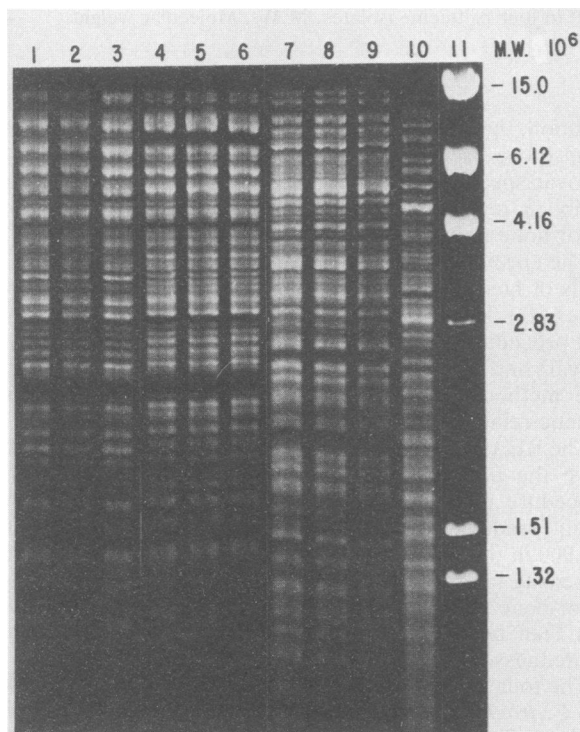


FIG. 3. REA digest patterns of chlamydial chromosomal DNA digested with *Pst*I. Isolates include the following: lane 1, VS1 (Amazon parrot); lane 2, VS2 (lovebird); lane 3, 6BC (parakeet); lane 4, NJ1 (turkey); lane 5, 78105 (budgerigar); lane 6, TT3 (turkey); lane 7, EBA (bovine abortion); lane 8, OSP (ovine abortion); lane 9, B577 (ovine abortion); lane 10, IPA (sheep polyarthritis); lane 11, *Hind*III digest of lambda DNA. M.W., Molecular weight.

these serovars represent two additional biovars in the *C. psittaci* group, each currently having one serovar. These serovars show major differences in REA patterns by all restriction enzymes, indicating major genetic differences. They have different growth characteristics in tissue culture and produce different disease syndromes. Serovar 1 is associated with abortions; serovar 2 is associated with polyarthritis and conjunctivitis. It is expected that other serovars belonging to each biovar will be found when the use of serovar-specific MABs becomes more common.

A number of other isolates of *C. psittaci* are known to be serologically distinct from the avian and mammalian isolates presented in this report (17). Isolates can be rapidly screened with known serovar-specific MABs to determine whether they are serologically related to known serovars. Isolates failing to react with known serovars can easily be analyzed by REA to determine their genetic relationship to the known mammalian and avian groups. The isolates can be grouped at that time with known mammalian or avian groups or be assigned a new designation based on serological and genetic relatedness. Factors such as host specificity, disease syndrome, and growth characteristics must also be considered.

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