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New Method for Classification of Leptospiral Isolates Belonging to Serogroup Pomona by Restriction Endonuclease Analysis: Serovar *kennewicki*

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The genomes of leptospiral field isolates belonging to serogroup Pomona were analyzed and compared with those of type strains by cleavage with restriction endonucleases. This new classification method shows differences among these organisms not indicated by the conventional serological typing method. No differences were observed among isolates from the United States, Canada, and New Zealand. Although all isolates selected for this study had been serologically typed as belonging to serovar *pomona*, the restriction endonuclease analysis indicates that they belong to serovar *kennewicki*. *kennewicki*, a serovar of North American origin, has recently been eliminated from the official serovar list because it was found to be indistinguishable from serovar *pomona* by the serological method.

The classification of the genus *Leptospira* has been confusing and complicated. Two species are recognized; *L. interrogans*, representing the pathogens; and *L. biflexa*, representing the free-living saprophytes. Among *L. interrogans* there are at least 180 serovars, representing 19 serogroups based on antigenic relatedness. Classification is based on the microscopic agglutination test with group- and serovar-specific hyperimmune sera. This method continues to be the conventional typing tool, despite numerous attempts to supersede it: in 1965 Bakoss and Chorvath (1) and in 1967 Green et al. (5) studied the properties of lipases; in 1969 Haapala et al. (6) compared DNA base compositions, as did Brendle et al. in 1974 (2). In 1974 Chang et al. (3) examined axial filament antigens by immunodiffusion analysis. In 1967 Kmety (10) proposed an improvement of the microscopic agglutination test with a factor analysis method. This method has proven complicated and difficult to reproduce in different laboratories.

Recently, examination of genomic DNA by restriction endonuclease analysis has gained recognition in the classification of certain viruses (7, 12, 14). The application of restriction endonuclease analysis (REA) to the classification of leptospires was first proposed by Marshall et al. (11). The technique has proven to be sensitive enough to differentiate among the different leptospiral serovars while showing consistent results among the various field isolates of the same serovar. Serogroup Pomona, which includes only six serovars, was chosen for this study because of its economic importance in livestock in the United States.

MATERIALS AND METHODS

Leptospiral strains. The six Pomona serogroup reference strains (*pomona*, Pomona; *proechimys*, LT 796; *monjakov*, Monjakov; *mozdok*, 5621; *tropica*, CA299U; and *kennewicki*, LT 1026) were obtained from the Centers for Disease Control, Atlanta, Ga. A total of 25 field isolates were typed by conventional serological methods as belonging to serovar *pomona*. The origin and animal source of these isolates is described in Table 1.

Preparation of whole-cell DNA. High-molecular-weight chromosomal DNA was prepared by the technique of Hull et al. (8) with modifications. Leptospires were harvested from 500 ml of an exponentially growing culture in bovine polysorbate 80 medium (4). Cells were harvested by centrifugation, washed twice in 0.01 M phosphate-buffered saline (pH 7.2), and suspended in 1.6 ml of 25% sucrose in 50 mM Tris–1 mM EDTA (pH 8) on ice. The suspension was transferred to ultracentrifuge tubes, and lysozyme was added (0.4 ml at 5 mg/ml), followed in 15 min by the addition of 0.01 ml of proteinase K at 20 mg/ml. After the proteinase K was thoroughly mixed in, 0.4 ml of 0.5 M EDTA was added and mixed in gently. Cells were lysed by the addition of 0.25 ml of Sarkosyl (10%). The tubes were then covered with aluminum foil and incubated overnight at 65°C. A 35-ml amount of CsCl solution (75.65 g of CsCl in TE buffer [60 ml of 10 mM Tris (pH 8)–1 mM EDTA] containing 50 μg of the proteinase K inhibitor phenylmethylsulfonyl fluoride per ml) was added, and the lysate was centrifuged at 50,000 rpm (242,000 × *g*) overnight in a Beckman VTi 50 rotor. The diffuse DNA band was collected from the side through a 16-gauge needle and dialyzed against TE buffer for 24 h. The final DNA concentration of each preparation was determined by spectrophotometry.

For field isolates, the technique was reduced to one-third its volume with 150 ml of culture. The lysate was centrifuged at 65,000 rpm (404,000 × *g*) for 3 h in a VTi 65 Beckman rotor.

Restriction endonuclease digestion of DNA. Purified bacterial DNA (2 μg) was mixed with 4 to 5 U of restriction enzyme in a 20-μl reaction mixture. All enzymes were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and reaction conditions were those recommended by the manufacturer. After the addition of 5 μl of a tracking dye (0.1% bromphenol blue, 20% Ficoll type 400 in distilled water), the samples were electrophoresed at 60 V for 16 h on a horizontal electrophoresis box (20 by 25 cm; model H-4; Bethesda Research Laboratories). The gel was

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TABLE 1. Source and origin of leptospiral isolates

Isolate	Animal source	Origin[a]
T-0361	Cow kidney	Iowa
T-5289	Cow kidney	Iowa
50K	Cow kidney	NADC
31K	Cow kidney	NADC
27U	Cow urine	NADC
RM-39	Steer kidney	Iowa
RM-34	Swine fetus	Iowa
RM-40	Swine fetus	Iowa
RM-70	Swine fetus	Iowa
RM-200	Swine fetus	Iowa
RM-211	Swine fetus	Iowa
DM-2H	Human blood	NADC
HCE	Human blood	NADC
ColE	Human blood	NADC
J. Minn	Swine	Minnesota (R. Johnson)
Po-X	Calf kidney	Indiana (C. Armstrong)
NZ-465	Unknown	New Zealand (R. Marshall)
NISKU-2	New Zealand sheep kidney	Alberta (ADRI, Alberta)
Huber 13	Cow urine	Ontario (ADRI, Ontario)
LC 81-03	Calf kidney	Alberta (ADRI, Alberta)
LC 81-19	Skunk kidney	Alberta (ADRI, Alberta)
LC 82-05	Swine fetus	Alberta (ADRI, Alberta)
LC 82-09	Sow urine	Alberta (ADRI, Alberta)
W 1801-73	Fox kidney	Ontario (ADRI, Ontario)
Po-sk	Skunk kidney	Ontario (ADRI, Ontario)

[a] NADC, National Animal Disease Center; ADRI, Animal Diseases Research Institute.

made up of 0.7% agarose (ME; Marine Colloids, Rockland, Maine) in Tris-borate buffer (10.8 g of Tris, 0.93 g of Na_2 EDTA, and 5.5 g of boric acid per liter of distilled water). The gels were stained in ethidium bromide (0.25 μg/ml) for 45 min and photographed under short-wave UV light through a Kodak 23A red filter.

Twelve restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Hha*I, *Hin*dIII, *Kpn*I, *Pvu*II, *Sal*I, *Sma*I, *Sst*I, *Xba*I, and *Xho*I) were screened for cleavage of leptospiral DNA. Complete digestion and best results were obtained with *Bgl*II, *Eco*RI, *Hha*I, and *Hin*dIII. Therefore, all comparative preparations were digested with these enzymes.

RESULTS

Comparison of *Eco*RI restriction patterns of serogroup Pomona reference strains, and isolates from the United States, Canada, and New Zealand are shown in Fig. 1. The restriction patterns of all 25 field isolates (serovar *pomona*) and of the serovar *kennewicki* reference strain were identical. Differences in restriction pattern were observed between all reference strains of serogroup Pomona.

After digestion with enzyme *Hha*I (Fig. 2), all field isolates had a pattern identical to that of reference strain *kennewicki*. Differences between the various reference strains were more apparent with *Hha*I than with *Eco*RI. Similar results were observed when the other restriction enzymes were used, but the best separations were obtained with *Hha*I and *Eco*RI.

DISCUSSION

The results of this study show that REA can be used as a reliable tool to classify leptospires of serogroup Pomona. Our study shows there was no difference in restriction pattern among the 25 field isolates examined. When analyzing the restriction patterns of the six reference strains in serogroup Pomona, differences in patterns could be observed with the various restriction enzymes tested. Serovars *proechimys*, *mozdok*, and *tropica* each had a distinctly different restriction pattern. However, only minor differences could be observed among REA patterns of *pomona*, *monjakov*, and *kennewicki*.

The minor differences in the restriction pattern between the reference strains of *pomona*, *monjakov*, and *kennewicki* are consistent with the high degree of serological cross-reaction between these strains, which are often considered serologically identical. Although the differences in the REA patterns are minor, they are consistent when different restriction endonuclease enzymes are used. All field isolates tested, regardless of the animal species they were isolated from, their country of origin, and degree of laboratory adaptation, show identical REA patterns. This is suggestive of the stability of the genome of the organisms.

In our study, the REA patterns of all field isolates matched that of the serovar *kennewicki* reference strain and not that of *pomona*, as would be expected from serological typing. Although serovars *pomona* and *kennewicki* show subtle serological differences, serovar *kennewicki* was deleted from the serovar list in the latest edition of *Bergey's Manual* (9). It is now considered identical to serovar *pomona*. On the basis of our results, we propose restoring serovar *kennewicki* as a different serovar in the Pomona serogroup.

Since in the United States, Canada, and New Zealand, vaccines are formulated with serovar *pomona* and not *kennewicki*, the characterization of all isolates tested as belonging to serovar *kennewicki* may have significant implications.

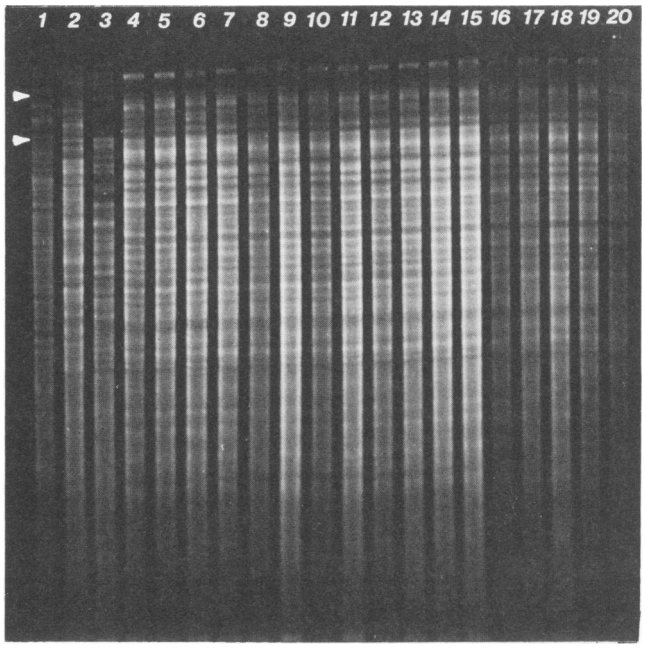

FIG. 1. REA patterns of chromosomal DNA from serovars in serogroup Pomona reference strains and Pomona field isolates digested with *Eco*RI. Reference strains: *proechimys* (lane 1), *mozdok* (lane 2), *tropica* (lane 3), *pomona* (National Animal Disease Center) (lane 4), *pomona* (lane 5), *monjakov* (lane 6), *kennewicki* (lane 7); field isolates: RM-34 (lane 8), RM-39 (lane 9), RM-40 (lane 10), RM-70 (lane 11), RM-200 (lane 12), T-0361 (lane 13), T-5289 (lane 14), Po-sk (lane 15), Nisku-2 (lane 16), 50K (lane 17), 31K (lane 18), 27U (lane 19), and Po-X (lane 20). Area of difference between *pomona*, *monjakov*, and *kennewicki* is indicated between arrows.

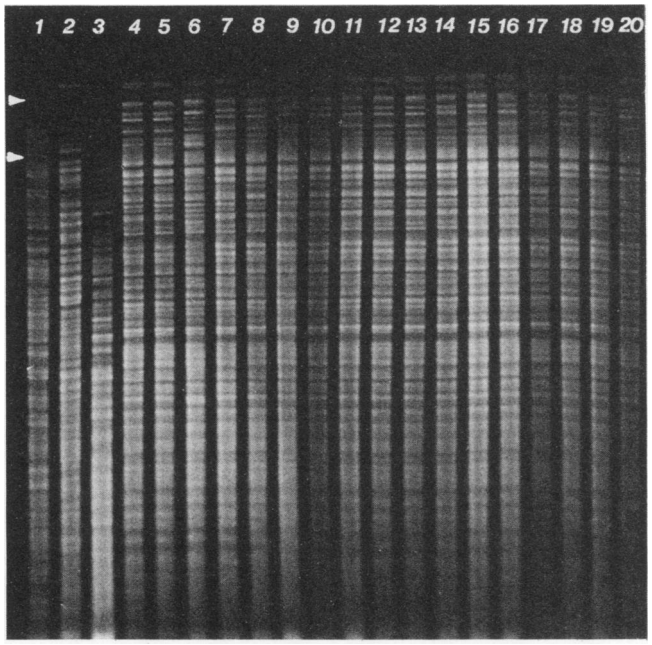

FIG. 2. REA patterns of chromosomal DNA from serovars in serogroup Pomona reference strains and Pomona field isolates digested with *Hha*I. Reference strains: *proechimys* (lane 1), *mozdok* (lane 2), *tropica* (lane 3), *pomona* (National Animal Disease Center) (lane 4), *pomona* (lane 5), *monjakov* (lane 6), *kennewicki* (lane 7); field isolates: RM-34 (lane 8), RM-39 (lane 9), RM-40 (lane 10), RM-70 (lane 11), RM-200 (lane 12), RM-211 (lane 13), T-0361 (lane 14), T-5289 (lane 15), Po-sk (lane 16), Nisku-2 (lane 17), 50K (lane 18), 31K (lane 19), 27U (lane 20). Area of difference between *pomona*, *monjakov*, and *kennewicki* is indicated between arrows.

This could account for some cases of clinical leptospirosis and leptospiral isolations (strains RM-70, RM-200, RM-211) from vaccinated herds. Further investigations are needed to determine whether serovar *pomona* is present in these countries.

The restriction endonuclease analysis of chromosomal DNA has shown to be an accurate and reliable method to compare and classify pathogenic leptospires (11, 13). Isolates belonging to other serogroups are being investigated by REA to determine their exact identities. This technique should shed some light on existing classification problems with serovar *hardjo* in cattle worldwide. Robinson et al. (13) have already demonstrated differences between certain *hardjo* isolates and their reference strain.

We believe that the extraction procedures and electrophoretic techniques employed in our study are rapid and reproducible and result in patterns with good resolution (Fig. 1 and 2). Restriction enzymes *Eco*RI and *Hha*I continue to be the enzymes of choice because they produce complete digestions with good definition of the high-molecular-weight fragments.

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