Restriction Endonuclease Analysis as a Taxonomic Tool in the Study of Pig Isolates Belonging to the Australis Serogroup of *Leptospira interrogans*

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Restriction endonuclease analysis was performed on DNAs from the type strains of the Australis serogroup of *Leptospira interrogans* by using 20 restriction enzymes, and the electrophoretic patterns obtained were compared with patterns obtained from 162 Australis serogroup isolates from pigs. It proved to be a quick and reliable method for typing such strains. All of the pig isolates were identified as either serovar bratislava or munchen. It also showed differences at the subserosvar level which may be important in (i) understanding the epidemiology of the Australis serogroup, (ii) the development of suitable vaccines, and (iii) pathogenesis and pathogenicity studies. Two genotypes (B2b and M2) accounted for 92% of isolates from aborted or stillborn piglets, while a third genotype (B2a) was the only one recovered from the brains of piglets with meningitis.

The current classification of *Leptospira interrogans* recognizes 212 serovars arranged in 23 serogroups (19). Differentiation between serovars belonging to a particular serogroup is by cross-agglutination absorption tests (1, 27). Two strains are considered different if, after cross-absorption with adequate amounts of heterologous antigen, 10% or more of the heterologous titer regularly remains in either of the two antisera. A typing system which depends on an arbitrary numerical limit to differentiate between the serovars of a particular serogroup is subjective. This leads to problems of reproducibility both within and between reference laboratories. It is also a very time-consuming method which lacks the sensitivity required to identify intraserovar differences which may be very important in epidemiological or pathogenicity studies.

Marshall et al. (21) proposed the use of restriction endonuclease analysis (REA) of leptospiral DNA as a taxonomic tool. This method is much less time- and labor-consuming than cross-agglutination absorption and has given highly reproducible results. With this method, it has been possible to observe differences between strains of the same serovar which have correlated with differences in the epidemiology of the strains and, possibly, the pathogenicity of the strains (10, 18, 26). It has also shown instances in which strains have been incorrectly identified by conventional typing methods (24, 25).

The Australis serogroup of leptospires has become of increasing interest to veterinarians in the last few years because of (i) an increasing awareness that antibodies to serovar bratislava organisms are widespread in the pig populations of many countries (4), (ii) the recovery of serovar lora (12), munchen (15), and bratislava (6, 7, 9) organisms from pigs, and (iii) the involvement of serovar bratislava and munchen organisms (5, 13) in pig reproductive problems.

The purpose of this study was to examine the serovar type strains of the Australis serogroup by REA and compare their electrophoretic patterns with those of strains belonging to this serogroup which have been isolated from pigs.

**MATERIALS AND METHODS**

Reference strains examined. The following reference serovars (strains) were examined: australis (Ballico), bajaran (Toad 60), bratislava (Jez-Bratislava), fugis (Fudge) (Leptospirosis Reference Laboratory, National Animal Disease Laboratory, Ames, Iowa [LRL]), hawaii (LT 62-68), jahna (Jahna), lora (Lora), munchen (Munchen C90), nicaragua (1011), peruiviana (V42), pina (LT932) (LRL), ramisi (Musa), rusan (507), and soteropolitana (R93) from the current list of *Leptospira* serovars. In addition, a strain which had previously been included in the serogroup—serovar bangkok (Bangkok D-92) (LRL)—was also examined. Except for those marked LRL, all of these strains were obtained from the Leptospira Reference Laboratory, Royal Tropical Institute, Amsterdam, The Netherlands.

Pig isolates. All isolates had previously identified to the serogroup level by cross-agglutination, and selected strains (indicated below) had been identified to the serovar level by cross-agglutination absorption (2).

**Northern Ireland.** We obtained 51 strains from aborted fetuses (5), 48 strains from various organs of 20 sows that had aborted culture-positive fetuses (7), 39 strains from a variety of organs of 10 boars which came from farms where culture-positive fetuses had been obtained (6), six isolates recovered from material from six sows collected at an abattoir, and 11 strains isolated from the brains of piglets with meningitis (4a). Strain 81/1650 had previously been identified as serovar bratislava, while strains 82/568, 82/764, 82/763, 81/2042, and 81/2166 had been identified as serovar munchen.

**Other pig isolates.** We obtained two strains of serovar munchen that were isolated from pigs in England (pigs 344 and 84/14) (15, 22a), four strains of serovar bratislava isolated from two sows from the United States (sow 26 oviduct and kidney and sow 30 uterus and kidney) (9), and one strain of serovar lora from a pig in The Netherlands (pig 133) (12).

**Preparation of whole-cell DNA.** Leptospires were har-
vested from 300 ml of an exponentially growing culture in liquid E medium (3) containing no rabbit serum, 5-fluorouracil, or nalidixic acid. Leptospires were harvested by centrifugation and washed twice in 0.01 M phosphate-buffered saline (pH 8.0); the resulting pellet of leptospires was suspended in 2 ml of phosphate-buffered saline divided equally into two microcentrifuge tubes and centrifuged; and the supernatant was discarded. One of the pellets was stored at −70°C until required, and the other was processed further. It was suspended in 0.5 ml of Tris-EDTA buffer (1.21 g of Tris and 0.38 g of Na₂-EDTA per liter of distilled water) (pH 8.0) and placed in an ice bath for 3 to 5 min, and then 100 μl of lysozyme (0.01 g of lysozyme per ml of distilled water; Sigma L-2879) was added and the contents were mixed gently by inversion of the tube several times. This mixture was allowed to stand on ice for at least 45 min or until total lysis occurred. To this was then added 30 μl of sodium dodecyl sulfate (SDS) solution (0.2 g of SDS per ml of distilled water), and the combination was mixed and kept on ice for 45 min. Then 4 μl of RNase (0.1 g/ml of distilled water; Sigma R-4875) was added, and the mixture was incubated at 37°C for 90 min, after which time 40 μl of proteinase K (0.02 g/ml of distilled water; Sigma P-0390) was added and the mixture was incubated at 37°C for a further 90 min. The mixture was then extracted successively with equal volumes of phenol, phenol-chloroform-isooamyl alcohol (25:24:1), and chloroform-isooamyl alcohol (24:1:20). It was then extracted twice with equal volumes of water-saturated ether (20). The DNA solution was then added to a fresh microcentrifuge tube containing 50 μl of 5 M sodium acetate solution (0.408 g of sodium acetate trihydrate per ml, pH 5.2) and mixed well, double the volume of 95% ethanol was added, and the mixture was kept overnight at −20°C. The resulting pellet of DNA was resuspended in 70% ethanol for 5 min, and then the ethanol was decanted and the DNA pellet was dried by incubation at 37°C in a stream of nitrogen for 30 min. The DNA pellet was then dissolved in 100 μl of distilled water and kept at −70°C until required.

Restriction endonuclease digestion of DNA. Purified leptospiral DNA (3 μg) was mixed with 10 U of restriction enzyme in a 20-μl reaction mixture containing the buffer recommended by the manufacturer for each restriction enzyme. All restriction enzymes were purchased from GIBCO Ltd., Paisley, Scotland. The mixture was incubated for 3 h at 37°C. After addition of 5 μl of a tracking dye (0.1% bromophenol blue, 20% Ficoll 400 in distilled water), electrophoresis was performed on a horizontal electrophoresis box (20 by 25 cm). A variety of electrophoresis conditions were used, depending on which restriction enzyme was used; run times varied between 18 and 24 h, and the voltages used varied between 60 and 75 V. Different gel concentrations were also used; these ranged from 0.4 to 0.8% agarose (A-9539; Sigma Chemical Co., Poole, Dorset, England) in Tris-borate buffer (10.8 g of Tris, 0.99 g of Na₂-EDTA, 5.5 g of boric acid per liter of distilled water). The gels were stained in ethidium bromide (0.05 μl/ml) for 45 min and photographed under short-wavelength UV light through a Kodak 23A red filter.

Twenty restriction enzymes (AluI, BglIII, Clai, DdeI, EcoRI, HhaI, HinclII, HindIII, HpaII, KpnI, MboII, MspI, NciI, NdeI, PstI, PvuII, SacI, SalI, SstI, Thul, and XbaI) were initially used to examine the serovar type strains. Complete digestion and best results were obtained with Clai, HhaI, HpaII, and combined digestion with BglII and HhaI; consequently, all strains were examined by using these enzymes.

**RESULTS**

With Clai, HhaI, HpaII, and a combination of BglIII and HhaI, the type strains of serovars australis, bahan, fugis, hawain, nicaragua, peruviana, pina, ramisi, rashan, soteropolitan, and bangkok showed markedly different electrophoresis patterns (Fig. 1). Serovars bratislava, jalna, lora, and muenchen formed a group which showed similar electrophoretic patterns but could be consistently distinguished by differences in the high-molecular-weight regions (Fig. 2).

All of the pig-derives isolates examined in this study gave patterns which closely resembled either serovar bratislava or muenchen. Identification by RFLP concurred with the cross-agglutination serotyping results for the 12 strains from the United Kingdom and the United States which had been serotyped previously. The one divergence between serotyping and the RFLP analysis was in the pig-derived strain from The Netherlands which had previously been serotyped as serovar lora but had RFLP profiles identical to those of serovar bratislava.

With HhaI, HpaII, and BglII-HhaI, the bratislava strains could be divided into two genotypes—B1 and B2 (Fig. 2)—on the basis of their high-molecular-weight fragments. Within the bratislava genotype 2 strains, two distinct patterns could be distinguished by using Clai (Fig. 3), and these were designated B2a and B2b.

The muenchen isolates (designated genotype M2) from pigs were all identical; however, when cut with HhaI, in one high-molecular-weight region of the profile they showed a difference in band spacing from that of the type strain, designated genotype M1 (Fig. 4).

On the basis of these criteria, strains belonging to two serovars, bratislava and muenchen, are found in pigs in the United Kingdom (Table 1). Serovar bratislava genotype B2a

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**FIG. 1.** REA patterns of chromosomal DNAs from the reference serovars of the Australis serogroup following digestion with *HhaI* and subsequent electrophoresis for 24 h at 70 V in a 0.6% agarose gel. Lanes: a, fragment size markers (sizes are given in kilobase pairs); b, australis; c, bahan; d, bangkok; e, fugis; f, hawain; g, nicaragua; h, peruviana; i, pina; k, ramisi; l, rashan; m, soteropolitan; n, bratislava; o, jalna; p, lora; q, muenchen.
accounted for 23% of the United Kingdom isolates, bratislava genotype B2b accounted for 41%, and muenchen genotype M2 accounted for the remaining 36%. B2a has also been identified in The Netherlands and in the United States.

Analysis of genotypes with respect to the clinical history, age, and sex of the pigs from which isolates had been recovered (Table 1) and the organs from which they were isolated produced a number of points of note. Strains identified as bratislava genotype B2b accounted for 63% of the isolates from aborted or stillborn piglets, and muenchen genotype M2 made up a further 29% of such isolates, whereas bratislava genotype B2a was found in only 8% of such cases. Also, genotype B2b strains were found only in material with a history of reproductive wastage; i.e., they were isolated only from aborted or stillborn pigs, sows which had aborted, or boars from farms from which the organisms had also been recovered from aborted piglets. Only B2a strains were recovered from piglets with meningitis.

Analysis of isolates with respect to the organs from which they came revealed no genotype-organ predisposition, apart from the isolation of only B2a from brain tissue. Analysis of the 48 strains from different organs of 20 aborted sows indicated that 3 of the sows had mixed-serovar infections, a further 7 had mixed-genotype infections of serovar bratislava, 9 were infected with serovar muenchen only, and 1 was infected with serovar bratislava only. Analysis of 39 isolates from 10 boars revealed a similar pattern; 1 boar was

<table>
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<tr>
<th>Source</th>
<th>No. of isolates with the following genotypea:</th>
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<tbody>
<tr>
<td></td>
<td>Bratislava B2a</td>
</tr>
<tr>
<td>Northern Ireland</td>
<td></td>
</tr>
<tr>
<td>Sows that aborted</td>
<td>9</td>
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<tr>
<td>Boars</td>
<td>10</td>
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<tr>
<td>Aborted piglets</td>
<td>4</td>
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<tr>
<td>Piglets with</td>
<td>11</td>
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<tr>
<td>meningitis</td>
<td></td>
</tr>
<tr>
<td>Abattoir sows</td>
<td>2</td>
</tr>
<tr>
<td>Elsewhere</td>
<td></td>
</tr>
<tr>
<td>Sows from England</td>
<td>2</td>
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<tr>
<td>Sow from The</td>
<td>1</td>
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<tr>
<td>Netherlands</td>
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<tr>
<td>Sows from the</td>
<td>4</td>
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<td>United States</td>
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*The bratislava B2a and Muenchen M1 genotypes were not detected.*
infected with serovar muenchen only, 2 were infected with both serovars bratislava and muenchen, 4 were infected with mixed B2a and B2b infections, and 3 were infected with single-genotype bratislava B2b infections.

**DISCUSSION**

In this study, REA proved to be a quick and reliable method for typing strains belonging to the Australis serogroup of *L. interrogans* and gave results which, with one exception, complement previous conventional serological findings. This is consistent with previous work on other *L. interrogans* serogroups (23, 25, 26). The one inconsistency was in the identification of the pig-derived isolate from the Netherlands (pig 133); this had previously been identified as serovar lora (12) but had a REA pattern identical to that of the bratislava B2a strains from Northern Ireland and the United States. The REA findings seem to be more in harmony with epidemiological findings than with the published serological findings, since it would be more reasonable to expect a pig-derived isolate to resemble strains which are widely found in pigs and not a strain which was originally isolated from small rodents in Italy (22). Similar divergences between REA typing and published serotyping results have been observed with North American Serogroup isolates, and in those cases the REA findings tied in with the epidemiological findings much better than did cross-agglutination absorption data. The fact that the REA patterns of serovars lora, jalna, bratislava, and muenchen were broadly similar (Fig. 1) and distinct from the other type strains was also consistent with epidemiological considerations, since these strains were all isolated in Europe while the remaining type strains were not.

This study also showed differences in strains which may have important implications for (i) our understanding of the epidemiology of Australis group infection, (ii) the control of Australis infection in pigs, and (iii) pathogenicity and pathogenesis studies. Genotypes B2b and M2 were associated with abortion and stillbirth in pigs, whereas a third genotype, B2a, was the only one recovered from neonatal meningitis cases.

The epidemiology of Australis group infections in the United Kingdom is unclear, and diverging views have been expressed. Widespread infection of free-living animals has been identified (14, 16, 17), and Hathaway et al. (14) have suggested that free-living species constitute primary sources of infection for domestic species. In contrast, Ellis et al. (5, 8) have suggested that pigs and horses may act as maintenance hosts for strains belonging to this serogroup. Conventional serological typing of isolates has been unable to differentiate between wildlife and domestic animal isolates of serovars bratislava and muenchen and has been unable to resolve this issue. In this study, REA was able to show differences at the subserovar level in both serovars bratislava and muenchen, and it is possible that this technique will enable demonstration of similarities and/or differences between wildlife and domestic species isolates. A knowledge of which species maintain which strains would be important to the construction of control measures.

In the United Kingdom, Australis serogroup infection in pigs is controlled by the feeding of medicated rations to sows on a continuous or semicontinuous basis (4). This has a number of major drawbacks, including recurrence of clinical disease within a few months of the end of treatment and problems with antibiotic residues. Vaccination is a possible alternative control measure, and a commercial bratislava bacterin is available in the United States, where field trials with affected herds have shown significant improvement in reproductive performance in gestation following vaccination (11). In view of the findings of this study, a vaccine for use in pigs in the United Kingdom should ideally have proven efficacy against all three genotypes (B2a, B2b, and M2) identified as occurring in pigs there. Before applying such an argument to the situation in other countries, in particular, the United States, it is necessary to have a more meaningful number of isolates than is currently available.

Pathogenesis and pathogenicity studies are difficult and time-consuming, and since three genotypes were found in this study it is necessary to study the effects of all three on pigs; however, the difference in the distribution of the three genotypes in pigs with different clinical histories indicates that the priorities are to assess the roles of B2b and M2 strains as abortifacient agents in pregnant sows and the pathogenicity of B2a strains for young pigs.

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**REFERENCES**

5. Ellis, W. A. Unpublished data.