Restriction Endonuclease DNA Analysis of *Leptospira interrogans* Serovars *Icterohaemorrhagiae* and *Copenhageni*

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Strain Ictero No. I showed endonuclease cleavage patterns which differed from those of the other four strains only when it was digested with enzymes *Kpn*I and *Hind*III. When digested with *Kpn*I, an extra band of about 5.4 kb was clearly produced, and when digested with *Hind*III, an extra band of about 25 kb was produced. When the other 13 enzymes were used, no differences were found between the endonuclease cleavage patterns among the five strains. Moreover, strains RGA, M20, Shiromizu and Shibaura could not be distinguished by the restriction endonuclease DNA analysis using all 15 endonucleases. In addition, six newly isolated leptospires from patients with leptospirosis and from *Rattus norvegicus* were compared with the Ictero No. I and M20 strains, by restriction endonuclease DNA analysis using enzymes *Kpn*I and *Hind*III. Three leptospires belonging to serovar *icterohaemorrhagiae* showed the same endonuclease cleavage patterns as the M20 strain. The other three strains, which belong to serovar *copenhageni*, showed almost the same endonuclease cleavage patterns as the M20 strain; only the Kai ima 702 strain produced an extra band which was not identical to the Ictero No. I-specific extra band when digested with *Hind*III. The leptospiral restriction endonuclease DNA analysis has revealed taxonomic structures that are unrecognized by serology alone.

The etiological agent of Weil's disease was first isolated by Inada and Ido in 1915 (6). Now there are about 180 serovars of pathogenic leptospires. The microscopic agglutination test and the cross-absorption test are still standard reference procedures for serological classification of leptospires (2). However, interactions between serovars inevitably sometimes result in incorrect identification and these procedures are rather troublesome and time consuming. Recently, newly developed methods such as the monoclonal antibody technique (9) and restriction endonuclease DNA analysis (4) have been introduced in taxonomy of leptospires. There have been some attempts at reclassification of leptospires using these new techniques (7, 8, 13, 15, 16).

In order to clarify the genetic relationship between serovars *icterohaemorrhagiae*...
and copenhageni, 11 strains of these two serovars were analyzed by restriction endonuclease DNA analysis.

MATERIALS AND METHODS

Leptospiral strains. Serovar icterohaemorrhagiae strains Ictero No. I, RGA, RN 7421, RN 7426 and Mori and serovar copenhageni strains M20, Shiromizu, Shibaura, Kaijima 702, RN 7964 and RN 7977 were used (Table 1). The Ictero No. I strain was the first isolate of the causative agent of Weil’s disease (6), and was provided by Dr. S. Yamamoto. Strains RGA and M20 were provided by the National Institute of Health, Tokyo. Strain Shibaura, also provided by the National Institute of Health, has been used for the preparation of biological products in Japan (1). The Shiromizu strain isolated from a patient with Weil’s disease in 1948 is a representative strain in our laboratory. The six other strains listed were isolated in Fukuoka Prefecture. Three of these strains, Kaijima 702, RN 7964, and RN 7977 belong to serovar copenhageni; the other three RN 7421, RN 7426, and Mori, belong to serovar icterohaemorrhagiae (7).

Preparation of DNAs. High molecular weight chromosomal DNA was prepared according to the method described by Maniatis et al (10). Briefly, leptospires of an exponentially growing culture (600 ml) were centrifuged at 13,000 × g for 5 min and the pellet was suspended in phosphate buffer saline (PBS), pH 7.5 and centrifuged at 13,000 × g for 5 min. The pellet was resuspended in a 3 ml solution of 100 mM Tris (pH 8.0), 10 mM EDTA, 200 mM NaCl, 100 μg of proteinase K per ml and 0.5% sodium dodecylsulfate (SDS) and allowed to stand at 37°C overnight. DNA was extracted twice with TE (10 mM Tris pH 8.0, 1 mM EDTA)-saturated phenol and phenol/chloroform/isoamyl alcohol (25: 24: 1) and finally extracted with chloroform/isoamyl alcohol (24: 1). After precipitation by 100% ethanol and washing with 70% ethanol, the DNA was dried and suspended in TE medium (10 mM Tris pH 8.0, 1 mM EDTA). In order to remove the contaminating RNA, RNase A (20 μg/ml) was added and the solution was allowed to stand at 37°C for 2 hr. Proteinase K (100 μg/ml), 0.2 M NaCl and 0.1% SDS were then added and

<table>
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<th>Serovar</th>
<th>Strain</th>
<th>Origin</th>
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<tr>
<td>icterohaemorrhagiae</td>
<td>Ictero No. I</td>
<td>Man Japan</td>
<td>1914</td>
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<tr>
<td></td>
<td>RGA</td>
<td>Man Germany</td>
<td>1915</td>
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<tr>
<td></td>
<td>RN 7421</td>
<td>Rat Japan</td>
<td>1974</td>
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<tr>
<td></td>
<td>RN 7426</td>
<td>Rat Japan</td>
<td>1974</td>
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<td></td>
<td>Mori</td>
<td>Man Japan</td>
<td>1980</td>
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<td></td>
<td>M20</td>
<td>Man Denmark</td>
<td>1935</td>
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<tr>
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<td>Shiromizu</td>
<td>Man Japan</td>
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<td></td>
<td>Shibaura</td>
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<tr>
<td></td>
<td>Kaijima 702</td>
<td>Rat Japan</td>
<td>1970</td>
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<td></td>
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<td>Rat Japan</td>
<td>1979</td>
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<td></td>
<td>RN 7977</td>
<td>Rat Japan</td>
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the solution was allowed to stand at 37 C for another 2 hr. Then, DNA was extracted with TE-saturated phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), then precipitated by ethanol and washed with 70% ethanol. After being dried, it was suspended in TE medium (500 µl). These DNA preparations were stored at 4 C.

Restriction endonuclease digestion of DNAs. Two micrograms of leptospiral DNA was digested at 37 C for 1.5 hr with 10 to 15 units of restriction endonucleases (AluI, BglII, EcoRI, HaeIII, HhaI, HindIII, KpnI, PstI, SacI, SalI, SmaI, StyI, and XbaI) and digested at 30 C with the enzyme BamHI (TAKARA SHUZO Co., Ltd., Kyoto, Japan) in the appropriate enzyme buffer made according to the manufacturer's specifications.

Gel electrophoresis and photography. After addition of a tracking dye (0.1% bromophenol blue, 0.1% xylene cyanol, 50% glycerol in distilled water), each sample was electrophoresed at 70 mA for about 6 hr until the tracking dye had travelled a distance of 12 cm. Gels consisted of 0.7% agarose (Sigma type II; Sigma Chemical Co., St. Louis, Mo., U.S.A.) in electrophoresis buffer (40 mM Tris, 20 mM sodium-acetate, and 2 mM EDTA, pH 8.0). The gels were stained with ethidium bromide (1 µg/ml) for 30 min and photographed under short wave UV light.

RESULTS

Chromosomal DNAs of two strains of serovar icterohaemorrhagiae, Ictero No. I and RGA, and three strains of serovar copenhageni, M20, Shiromizu and Shibaura, were digested with 15 endonucleases and their endonuclease cleavage patterns were examined.

The endonuclease cleavage patterns produced with these enzymes differed from each other. Complete digestion and good resolution were obtained with nine endonucleases (AluI, BamHI, BglII, EcoRI, HaeIII, HhaI, HindIII, PstI, and StyI) (Fig. 1). When the other six enzymes (KpnI, SacI, SalI, SmaI, XbaI, and XhoI) were used, a large portion of the DNA was electrophoresed at a high-molecular-weight position in bulk; a relatively small number of fragments were produced (Fig. 2).

These five strains could not be distinguished from each other by restriction endonuclease DNA analysis using the 13 endonucleases AluI, BamHI, BglII, EcoRI, HaeIII, HhaI, PstI, SacI, SalI, SmaI, StyI, XbaI, and XhoI. On the other hand, when enzymes KpnI and HindIII were used, strain Ictero No. I showed different endonuclease cleavage patterns from those of the other four strains. An extra band of about 5.4 kb was produced by KpnI digestion, and an extra band of about 25 kb was produced by HindIII digestion (Fig. 3). This difference was consistently present with a range of various concentrations of enzymes.

Six newly isolated strains were examined by restriction endonuclease DNA analysis with the enzymes KpnI and HindIII. RN 7421, RN 7426, and Mori, which belong to serovar icterohaemorrhagiae, showed the same restriction patterns as those of the M20 strain. (Fig. 4). Kaijima 702, RN 7964, and RN 7977, which belong to serovar copenhageni, showed almost identical endonuclease cleavage patterns.
Fig. 1. Restriction endonuclease DNA analysis. DNAs of five strains were digested with enzymes EcoRI (A) and HaeIII (B). Lanes: a, iDNA digested with StyI; b, RGA; c, Ictero No. 1; d, Shiromizu; e, M20; f, Shibaura.

Fig. 2. Restriction endonuclease DNA analysis. DNAs of five strains were digested with enzymes SacI (A) and SalI (B). Lanes: a, iDNA digested with StyI; b, RGA; c, Ictero No. 1; d, Shiromizu; e, M20; f, Shibaura.
Fig. 3. Restriction endonuclease DNA analysis. DNAs of five strains were digested with enzymes KpnI (A) and HindIII (B). Lanes: a, 2DNA digested with SphI; b, RGA; c, Ictero No. 1; d, Shiromizu; e, M20; f, Shibaura.

Fig. 4. Restriction endonuclease DNA analysis. DNAs of five strains were digested with enzymes KpnI (A) and HindIII (B). Lanes: a, 2DNA digested with SphI; b, Ictero No. 1; c, M20; d, Mori; e, RN 7421; f, RN 7426.
to those of the M20 strain (Fig. 5). Only the Kaijima 702 strain produced a high-molecular-weight extra band when digested with HindIII; however it was not identical to the Ictero No. I-specific extra band which was produced when the Ictero No. I strain was digested with HindIII.

DISCUSSION

When digested with nine of the enzymes (AluI, BamHI, BglII, EcoRI, HaeIII, HhaI, HindIII, PstI, and StyI), the DNA of five classic strains, Ictero No. I, RGA, M20, Shiromizu, and Shibaura, was completely digested and good resolution was obtained. On the other hand, a large portion of the DNA was not completely digested and a small number of fragments were produced when the DNA was digested with six of the enzymes: KpnI, SacI, SalI, SmaI, XbaI, and XhoI. This difference may reflect the fact that leptospiral DNA has a large number of recognition sequences for the nine enzymes AluI, BamHI, BglII, EcoRI, HaeIII, HhaI, HindIII, PstI, and StyI. Conversely, the leptospiral DNA has a relatively small number of recognition sequences for the six enzymes KpnI, SacI, SalI, SmaI, XbaI, and XhoI.

An analysis of the leptospiral DNA base composition (5) showed that the guanine + cytosine (GC) content of leptospiral DNA is about 36–39%. According to these results, it may be expected that enzymes with GC-rich restriction sequences
would digest leptospiral DNA incompletely and, conversely, enzymes with adenine + thymine (AT)-rich restriction sequences would digest leptospiral DNA completely. In this study, the enzymes which incompletely digested the leptospiral DNA tended to have GC-rich restriction sequences and the enzymes which completely digested the DNA tended to have AT-rich restriction sequences.

Only the Ictero No. I strain was differentiated by restriction endonuclease DNA analysis using the enzymes KpnI and HindIII. The four other strains were not differentiated by any of the enzymes used. Moreover, the Ictero No. I strain produced the same endonuclease cleavage patterns as the four other strains by restriction endonuclease DNA analysis with all of the other 15 enzymes except KpnI and HindIII.

Originally, restriction endonuclease DNA analysis was developed for viral DNA analysis (3, 4). The application of restriction endonuclease DNA analysis to the classification of leptospires was first proposed by Marshall et al (11) in 1981. There have been some studies which show that restriction endonuclease DNA analysis could be useful in the taxonomy of leptospires (13, 15, 16). Marshall et al (12) showed that serovar icterohaemorrhagiae strain RGA and serovar copenhageni strain M20 are easily distinguishable by restriction endonuclease DNA analysis with the enzyme EcoRI; high molecular-weight bands in the RGA strain were not seen in the M20 strain.

In this study, however, no differences were found between these two serovars, not only with enzyme EcoRI but also with the 14 other enzymes used. The reason for this difference is unclear.

Taxonomically, serovar icterohaemorrhagiae and serovar copenhageni were differentiated by the conventional serological methods, and in our previous study using monoclonal antibodies (7, 14) the serovar icterohaemorrhagiae RGA and serovar copenhageni M20 strains were easily distinguished from each other. In this study, however, these two serovars were not differentiated by endonuclease DNA analysis with the 15 endonucleases used.

In this study, the genetic relationship between serovar icterohaemorrhagiae and serovar copenhageni was analysed by using restriction endonuclease DNA analysis. Only the Ictero No. I strain could be differentiated from the four other classic strains: serovar copenhageni strains M20, Shiromizu and Shibaura, and serovar icterohaemorrhagiae strain RGA. The Ictero No. I strain also differed from the six newly isolated strains: serovar icterohaemorrhagiae RN 7421, RN 7426, Mori strains, and serovar copenhageni Kaijima 702, RN 7964 and RN 7977 strains. Although the Ictero No. I strain belongs to serovar icterohaemorrhagiae, these findings indicate that this strain has some unique characteristics.

However, there are differences between the results based on serological and genetic grounds. This new method has revealed taxonomic structures that have hitherto been unrecognized on the basis of serology alone. Thus, this new classification method is useful for revealing the differences among these organisms not found by the conventional serological typing methods.

It remains to be established whether these genetic differences are directly
related to the phenotypic differences. To solve this interesting problem, a more detailed analysis is required.

In our laboratory, we produced an Ictero No. I-specific monoclonal antibody which can distinguish the Ictero No. I strain from the RGA strain. It will be reported in a separate paper.

REFERENCES


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