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Rapid and Simplified Protocol for Isolation and Characterization of Leptospiral Chromosomal DNA for Taxonomy and Diagnosis

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We have developed a rapid method for the isolation of leptospiral chromosomal DNA which yields DNA of a purity suitable for restriction endonuclease analysis. A small volume (15 to 20 ml) of an exponentially growing culture of leptospires yielded 2 to 4 μg of chromosomal DNA. In a 1-day protocol, the DNA was isolated, restricted with endonucleases, and fractionated on an agarose gel. Chromosomal DNA from dinger zones (visible subsurface zones of leptospiral growth) of first semisolid subcultures of field isolates was also isolated and characterized, thus greatly speeding up the diagnostic process.

Restriction enzyme analysis of leptospiral chromosomal DNA as a means of characterization and diagnosis has already been shown to be a valuable tool (6–8, 12; A. B. Thiermann and W. A. Ellis, Proc. Comm. Eur. Commun. Spec. Meet., in press). Nevertheless, improvements are needed to isolate chromosomal DNA of a suitable purity and concentration for more rapid diagnosis. Although several isolation procedures for bacterial chromosomal DNA are available, many of them are either too time-consuming and expensive for many diagnostic laboratories, or if the protocol is facile and economic, the DNA is generally not of a suitable purity for complete digestion by restriction endonuclease.

The experimental procedure reported here was designed to purify, digest to completion by restriction enzymes, and fractionate leptospiral DNA on an agarose gel by rapid and cost-efficient means. Problems of dilute or impure DNA samples were also resolved, so that a minimum of time is required from first field isolation to restriction enzyme analysis characterization.

MATERIALS AND METHODS

Leptospiral serovars. As a control, all leptospiral strains tested were first characterized to serovar by conventional serologic methods (3). Reference strains and field isolates of *Leptospira interrogans* belonging to various serogroups were assayed in this study. Strains Wint 5'80, and Wint 5'84, serovar hardjo, serotype sejroe, were isolated from bovine urine in Ontario, Canada; strain Hond Utrech IV, serovar canicola, serotype canicola, was the typing strain. These were maintained in bovine serum albumin–polysorbate-80 liquid medium (4) at 29°C. Cell density was monitored by a Coleman photonephelocolorimeter.

Isolation of leptospiral chromosomal DNA. Approximately 20 ml of an exponentially growing leptospire liquid culture was pelleted at 31,000 × *g* for 20 min at 4°C, and the pellet was suspended in 1 ml of 10 mM Tris–1 mM EDTA (TE; pH 8.0). The cells were then transferred to a 1.5-ml Microfuge tube and repelleted. The pellet was suspended in 0.5 ml of 50 mM glucose–10 mM EDTA–25 mM Tris hydrochloride (pH 8.0) containing freshly added lysozyme at 10 mg/ml. This solution was incubated at 37°C until viscous (20 to 30 min). Subsequently, 40 μl of 10% sodium dodecyl sulfate solution was added, and the solution was again incubated at 37°C for 15 to 20 min, followed by the addition of 20 μl of a cold potassium-acetate solution (3 M potassium–5 M acetate). After mixing, the tubes were placed in an ice bath for 5 to 10 min. The solution was centrifuged for 10 min, and the supernatant containing the chromosomal DNA was transferred to another tube. The solution was phenol extracted (50% phenol [pH 7.0] and 50% chloroform-isoamyl alcohol [25:1, vol/vol]) by inverting the tube 20 times, followed by a chloroform-isoamyl alcohol (25:1, vol/vol) extraction, also inverted 20 times. To the upper phase, 60 μl of a 3 M sodium acetate solution was added, followed by the addition of 750 μl of isopropyl alcohol. This solution was held at −20°C for 30 min. The precipitated DNA was pelleted in a Microfuge for 10 min. The pellet was washed with 70% ethanol and vacuum dried. The dried pellet was resuspended in 20 to 30 μl of TE buffer. Approximately 2 to 4 μg of DNA was obtained by this procedure, which was enough for characterization by two to three different restriction enzymes. Dilute solutions of DNA were concentrated by extraction with unsaturated 2-butanol. However, the butanol-concentrated DNA was vacuum dessicated for 15 to 20 min, rather than ethanol precipitated as reported elsewhere (10, 11). The DNA was then ready for digestion with endonucleases.

Isolation of chromosomal DNA from leptospires growing in semisolid medium. Slow-growing or new isolates of leptospires not yet fully adapted to liquid medium were isolated from semisolid dinger zones (visible subsurface zones) of growth by this same protocol. After the first centrifugation, the agar pellet surrounding the cells was aspirated.

Restriction enzyme digestion and fractionation. Leptospiral chromosomal DNA samples were digested with restriction enzymes purchased from Bethesda Research Laboratories, Inc., and used according to the manufacturer's specifications. In addition, the reaction mixture contained 1 μl of a 10-mg/ml heat-inactivated RNase solution and 1 μl of a 0.1 M spermidine solution (2). Digested DNA fragments were fractionated on a 0.7% agarose gel stained with ethidium bromide and photographed through a Kodak 23A filter with UV irradiation.

RESULTS

Comparison of restriction patterns of DNA isolated by the rapid protocol with that of DNA isolated through cesium chloride and ultracentrifugation. *Eco*RI was used to digest

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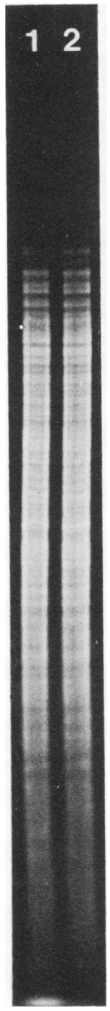

FIG. 1. *Eco*RI digest of serovar canicola DNA isolated by the rapid isolation method (lane 1) and by the CsCl isolation method (11; lane 2).

leptospiral chromosomal DNA isolated either by ultracentrifugation (Thiermann and Ellis, in press) or by the protocol described here. After fractionation on an agarose gel, the restriction patterns of the two samples were identical (Fig. 1). The rapidly isolated DNA was pure enough to allow complete digestion by the restriction enzyme, yielding distinct and reproducible fragments.

Semisolid dinger zone isolate characterization. The pattern of chromosomal DNA isolated from leptospires growing in dinger zones of growth in semisolid isolation medium was also compared with the restriction endonuclease pattern of ultracentrifuge-purified DNA. The *Eco*RI digestion of the DNA is shown in Fig. 2. The digestion patterns of the DNA from the two protocols were identical.

DISCUSSION

We have developed a procedure for the rapid isolation and characterization of chromosomal DNA from *L. interrogans*. Several different protocols were tested, but for speed and purity the method described here was the one of preference. Of particular interest is the fact that this technique more closely resembles plasmid isolation rather than extant bacterial chromosomal isolation. The major difference between this procedure and plasmid isolation (1) is that an alkaline (1) denaturation step was omitted here. Hansen and Olsen (5) noted that when bacterial plasmids are isolated, an alkaline denaturation step is essential to prevent large amounts of chromosomal DNA from contaminating the plasmid DNA. Treatment with alkalai or RNase causes breaks in the DNA, leading to the unfolding and denaturation of the condensed chromosome (8, 13, 14). Upon neutralization of the extract in the presence of a high-salt concentration, the chromosomal DNA is precipitated, presumably owing to interstrand reassociation at multiple sites, which then leads to the formation of an insoluble DNA network (8). The performance of cell lysis at 37°C rather than on ice also facilitates the release of the bacterial chromosomal DNA from cellular membrane attachment sites (8, 14). This, coupled with a salt-precipitation step in the absence of alkalai, effectively precipitates proteins and lipids, while leaving the chromosomal DNA in the supernatant. We used these findings to our advantage in isolating leptospiral chromosomal DNA. The same results were obtained with *Escherichia coli* HB101, *Oxalobacter formigenes*, and *Streptococcus* spp. with minor modifications (unpublished data).

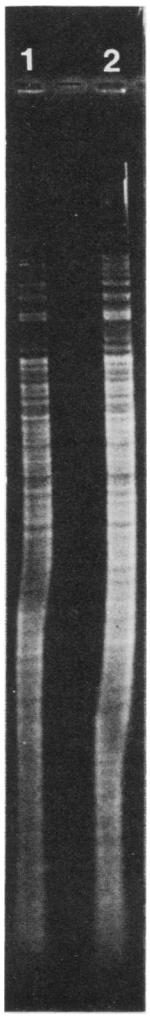

FIG. 2. *Eco*RI digest of serovar hardjo Wint 5'84 isolated by the CsCl method (lane 1) and Wint 5'80 (lane 2) DNA isolated from semisolid culture by the rapid isolation method.

It is also of importance to note that leptospiral DNA could be isolated from semisolid cultures of new or slow-growing organisms, thus allowing rapid and more accurate diagnosis of leptospires at the serovar level by restriction enzyme analysis. This is especially important in the proper characterization of pathogens after their first subculture.

It is apparent from the findings of Thiermann et al. (12; Thiermann and Ellis, in press) and Marshall et al. (6, 7, 9) that restriction enzyme analysis of *L. interrogans* is the most accurate means of proper identification of the pathogen. The isolation protocol described here should enhance diagnosis in that it is a simple, rapid, and cost-efficient means of characterizing the organism at the genetic level. The chromosomal DNA isolated by this technique produced reproducible restriction-banding patterns when digested with various restriction endonucleases.'l2;6q

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