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Rapid Identification of Some *Leptospira* Isolates from Cattle by Random Amplified Polymorphic DNA Fingerprinting

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We compared random amplified polymorphic DNA (RAPD) fingerprinting with cross-absorption agglutination and restriction enzyme analysis for typing bovine leptospires. Using RAPD fingerprinting, we examined a number of *Leptospira* serovars, namely, hardjo genotypes bovis and prajitno, pomona, balcanica, tarassovi, swajizak, kremastos, australis, and zanoni, which are likely to be isolated from Australian cattle. Each serovar and genotype had a unique RAPD profile. Of 26 field isolates of *Leptospira*, 23 were identified as hardjo genotype bovis subtype A, 2 were identified as zanoni, and 1 was identified as pomona by RAPD fingerprinting, and their types were confirmed by cross-absorption agglutination and restriction enzyme analysis.

Pathogenic leptospires comprise over 200 serovars, many of which are assigned to one of the following species by DNA hybridization or ribotyping: *Leptospira interrogans*, *L. weilii*, *L. borgpetersenii*, *L. noguchii*, *L. santarosai*, *L. inadai*, and *L. kirschneri* (11, 12, 26). Serological surveys of Australian cattle found reactions to a number of serovars which may contribute to reproductive failure. The more prevalent were serovars hardjo, pomona, tarassovi, swajizak, medanensis, and kremastos (10, 15, 19, 25). Serovars hardjo, pomona, australis, and zanoni have been isolated from Australian cattle (1, 3, 9, 20).

Serovar hardjo is divided into genotypes prajitno and bovis by restriction enzyme analysis (REA) (14, 21). Genotype bovis is further divided into subtypes A, B, and C with the restriction endonuclease *Hha*I (21). Furthermore, Thiermann et al. (21) reported that bovine isolates of serovar balcanica were identical to hardjo genotype bovis in REA. All Australian serovar hardjo isolates so far examined by REA are genotype bovis (4, 14, 17).

Isolates are identified to the serovar level by cross-absorption agglutination (CAA) and to the genotype level by REA, but each test has disadvantages. CAA is laborious, requires live animals, takes several months to complete, and does not distinguish between genotypes. REA depends on high-resolution electrophoresis, generates complex restriction profiles, and requires relatively large amounts of highly purified DNA, necessitating large-scale culture which may take several weeks to grow.

A rapid and simple typing method which can distinguish between genotypes without the disadvantages of CAA and REA is needed. Rapid identification of isolates would allow farmers to start appropriate vaccination regimens with minimal delays. Also, epidemiological data would be rapidly gathered and assessed to allow the institution of other appropriate control measures. Welsh and McClelland (22) and Williams et al. (24) described a DNA fingerprinting technique based on the random amplification of genomic sequences by using a single primer at low stringency in a polymerase chain reaction (PCR). They called the technique arbitrarily primed PCR or random amplified polymorphic DNA (RAPD). RAPD fingerprinting has been used to type isolates of *Bacillus thuringiensis* (2), *Streptococcus uberis* (5), *Campylobacter jejuni* (8), and *Borrelia burgdorferi* (23).

In this paper, we show that RAPD fingerprinting allows the rapid identification of leptospiral isolates starting with a small-scale broth culture, as would be used for routine in vitro passage of an isolate.

MATERIALS AND METHODS

Strains. The sources of the leptospiral reference strains and field isolates and the *Brucella abortus* reference strain are shown in Table 1. The 11 isolates from the Elizabeth Macarthur Agricultural Institute (EMAI) were identified by CAA and/or REA as reported by Djordjevic et al. (4). All other field isolates were identified by CAA and REA as described below.

Preparation of leptospiral DNA. Leptospires were grown at 30°C to $>10^7$ cells per ml in 30 to 60 ml of EMJH broth (Difco Laboratories, Detroit, Mich.) with 0.09 g of sodium pyruvate per liter (6). DNA was prepared for REA and RAPD fingerprinting as described by Marshall et al. (7) with the following modification. Prior to dialysis, RNA was removed by adding RNase to 100 μg/ml and incubating the mix for 3 h at 37°C, after which the RNase was removed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1). DNA was concentrated in Centricon-30 Microconcentrators (Amicon Division, W. R. Grace and Co., Danvers, Mass.) before analysis by REA. DNA was assayed by comparing its fluorescence with that of DNA standards when spotted on agarose gels and stained with ethidium bromide (16).

As a rapid alternative to the above method, rapid-lysis preparations (22) were also used for RAPD PCR. They were obtained by boiling the cells from 1 ml of broth culture (containing $>10^7$ cells per ml) for 5 min in 100 μl of 0.1 mM Tris (pH 7.0). Debris was removed by centrifugation at 16,000 × *g* for 5 min.

REA. DNA (1 to 4 μg) was digested with the enzymes

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TABLE 1. Origins of the strains used in assessing RAPD PCR for the rapid typing of bovine leptospires

Strain	Source
Leptospira serovars	
balcanica 1627 Burgas	WHO/FAO Leptospira Reference Laboratory, Brisbane, Australia
pomona Pomona	WHO/FAO Leptospira Reference Laboratory
hardjo prajitno	WHO/FAO Leptospira Reference Laboratory
hardjo bovis 11135	Commonwealth Serum Laboratories, Parkville, Australia
tarassovi Perepelicin	WHO/FAO Leptospira Reference Laboratory
swajizak Swajizak	WHO/FAO Leptospira Reference Laboratory
kremastos Kremastos	WHO/FAO Leptospira Reference Laboratory
australis Ballico	WHO/FAO Leptospira Reference Laboratory
zanoni Zanoni	WHO/FAO Leptospira Reference Laboratory
hardjo genotype bovis subtype A strains	
83-561	W. Ellis, Department of Agriculture, Belfast, Northern Ireland
83-468	W. Ellis
84-2894	W. Ellis
Kidney 34	W. Ellis
84-2793	W. Ellis
No. 33	W. Ellis
No. 34	W. Ellis
N51	W. Ellis
N233	W. Ellis
6B	W. Ellis
7B	W. Ellis
LT371[a]	W. Ellis
S751	W. Ellis
S731	W. Ellis
hardjo genotype bovis subtype B strain	
Hb-15b-067	C. Bolin, National Animal Disease Center, Ames, Iowa
B. abortus biotype 1[b]	Isolated at ARI, Yeerongpilly, Australia, from a bovine brucellosis reactor
Field isolates	
U2	Bovine urine, isolated at ARI
ARI306	Bovine urine, isolated at ARI
91-117123	Bovine urine, isolated at ARI
91-133451.5	Bovine urine, isolated at ARI
91-147702.5	Bovine urine, isolated at ARI
91-147702.6	Bovine urine, isolated at ARI
92-107821.3	Bovine urine, isolated at ARI
92-112300.2	Bovine urine, isolated at ARI
92-112300.3	Bovine urine, isolated at ARI
92-136291.2	Bovine urine, isolated at ARI
92-1649	Isolated from bovine kidney at Toowoomba Veterinary Laboratory, Toowoomba, Australia
LT500	WHO/FAO Leptospira Reference Laboratory
LT576	WHO/FAO Leptospira Reference Laboratory
LT577	WHO/FAO Leptospira Reference Laboratory
1808	D. Cousins, Western Australia Department of Agriculture, South Perth, Australia
GN88/2700-2	Bovine urine, isolated at EMAI, Camden, Australia
GN88/2825/4	Bovine urine, isolated at EMAI
GN88/4464-1	Bovine urine, isolated at EMAI
GN88/4464-4	Bovine urine, isolated at EMAI
GN88/4464-5	Bovine urine, isolated at EMAI
GN88/4464-6	Bovine urine, isolated at EMAI
GN88/5037-3	Bovine urine, isolated at EMAI
GN88/5235-1	Bovine urine, isolated at EMAI
GN88/5235-3	Bovine urine, isolated at EMAI
GN88/5235/5	Bovine urine, isolated at EMAI
RN85/1282A	Bovine urine, isolated at EMAI

[a] Human isolate.
[b] Typed at the National Brucellosis Reference Center, Canberra, Australia.

*Eco*RI (Boehringer Mannheim GmbH, Mannheim, Germany) and *Hha*I (GIBCO BRL, Gaithersburg, Md.) by following the manufacturers' instructions. Digests were examined by electrophoresis in horizontal 0.7% agarose gels (20 by 20 cm) (Ultrapure; GIBCO BRL) in half-strength TBE (single-strength TBE is 0.1 M Tris–0.089 M boric acid–0.002 M EDTA [pH 8.3]). Gels were stained with ethidium bromide and photographed over a UV light source.

Serotyping. Leptospiral isolates were serotyped by CAA at the WHO/FAO Leptospira Reference Laboratory, Laboratory of Microbiology and Pathology, Brisbane, Australia.

RAPD. One 19-mer primer (L10; 5′GTAGAGCTCGCG GCACTTG3′) and one 17-mer primer (M13 universal sequencing primer, US; 5′GTAAAACGACGGCCAGT3′) were used. These primers were selected from a collection of one 17-mer, three 19-mers, and two 20-mers (GC content, 53 to 84%) on the basis of RAPD fingerprint clarity and their ability to differentiate between serovars pomona and hardjo

genotype prajitno and between serovars balcanica and hardjo genotype bovis. Primers were supplied by the Center for Molecular Biology and Biotechnology, University of Queensland, St. Lucia, Australia.

PCR mixtures contained 50 ng of purified DNA or 1 μl of rapid-lysis preparation and one of the above primers at 2 μM, 4 mM magnesium chloride, 200 μM (each) deoxynucleoside triphosphate, 2.5 U of Amplitaq polymerase, and PCR Buffer II (Perkin-Elmer Cetus, Norwalk, Conn.) in a 100-μl reaction volume and were overlaid with 100 μl of paraffin oil. The temperature program consisted of 1 cycle with 3 min at 97°C, 1 min at 40°C, and 1 min at 72°C; 4 cycles with 1 min at 97°C, 1 min at 40°C, and 1 min at 72°C; 24 cycles with 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C; and 1 cycle with 1 min at 95°C, 1 min at 55°C, and 7 min at 72°C. The temperature program was run in an FTS-1 Fast Thermal Sequencer (Corbett Research, Sydney, Australia). Reaction products were analyzed on 2.5% agarose gels, stained with ethidium bromide, and photographed.

Alkali blotting and DNA hybridization. RAPD reaction products were fractionated by electrophoresis on 1.5% agarose gels and were transferred to Hybond-N+ nylon (Amersham International plc, Amersham, United Kingdom) by capillary blotting in 0.4 M NaOH (13, 18).

After digestion with *Eco*RI, hardjo genotype bovis strain 11135 DNA was labelled with digoxigenin-11-dUTP (Boehringer Mannheim) by random priming and hybridized to the nylon membranes at 60°C. Labelling and hybridization were performed according to the instructions supplied by the respective manufacturers.

RESULTS

To establish a rapid identification system based on RAPD fingerprinting, we prepared RAPD profiles for a number of standard strains. Profiles generated from field isolates were compared with these profiles to enable identification of the isolates. The isolates were also typed by REA and CAA to confirm the RAPD identifications.

RAPD profiles of standard strains. Purified DNA from two *Leptospira* serovars likely to be isolated from Australian cattle (pomona and hardjo genotypes prajitno and bovis strain 11135) and serovar balcanica was amplified with primers L10 and US (Figs. 1 and 2). Profiles containing up to 10 intense bands and a number of fainter bands were generated when the reaction products were run on agarose gels. Although the serovar pomona and hardjo genotype prajitno profiles had a number of common bands and the serovar balcanica and hardjo genotype bovis profiles were similar, there were sufficient differences to enable differentiation of the serovars and genotypes. These differences involved mainly major bands with primer L10 and fainter bands with the US primer and are indicated with arrowheads on Fig. 1 and 2.

To minimize the time required to prepare sample DNA, we amplified rapid-lysis preparations of serovars pomona, balcanica, and hardjo (genotypes prajitno and bovis strain 11135). Some bands differed in relative intensity when rapid-lysis preparations were used, and more high-molecular-weight products were produced when purified DNA was used (Fig. 1 and 2). However, the profiles generated from rapid-lysis preparations and purified DNA were comparable and characteristic for each serovar and genotype.

Rapid-lysis preparations of five other serovars likely to be found in Australian cattle (tarassovi, swajizak, kremastos, australis, and zanoni) were amplified with primers L10 and

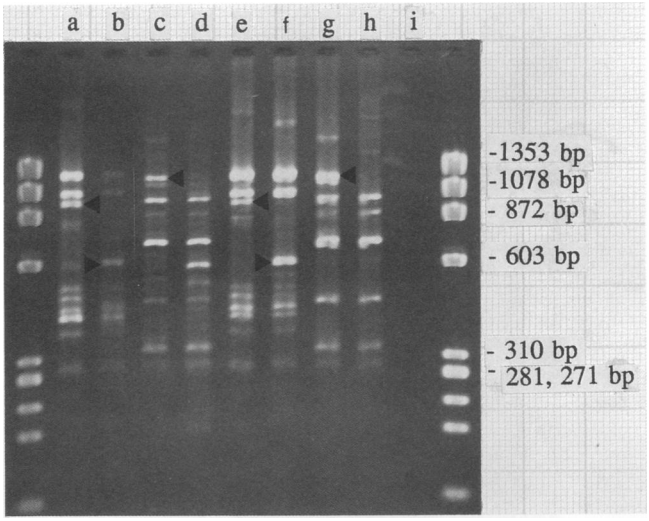

FIG. 1. RAPD profiles of *Leptospira* serovars with the L10 primer. Lanes a to d, 1 μl of rapid lysate per reaction; lanes e to h, 50 ng of purified DNA per reaction. Lanes: a and e, serovar pomona; b and f, hardjo genotype prajitno; c and g, hardjo genotype bovis strain 11135; d and h, balcanica; i, control reaction without template DNA. Arrowheads indicate features useful for differentiating pomona from hardjo genotype prajitno and hardjo genotype bovis from balcanica.

US. Serovar medanensis, also found in serological surveys of Australian cattle, was omitted because of the unavailability of a reference culture. The profiles are shown in Fig. 3 and 4. Again, characteristic profiles were generated for each serovar. However, the profiles of serovars kremastos, australis, zanoni, and swajizak were similar and resembled those of serovars pomona and hardjo genotype prajitno. The

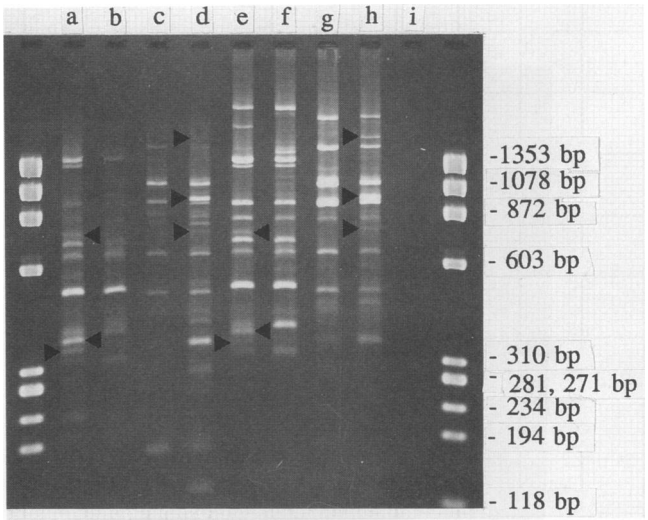

FIG. 2. RAPD profiles of *Leptospira* serovars with the US primer. Lanes a to d, 1 μl of rapid lysate per reaction; lanes e to h, 50 ng of purified DNA per reaction. Lanes: a and e, serovar pomona; b and f, hardjo genotype prajitno; c and g, hardjo genotype bovis strain 11135; d and h, balcanica; i, control reaction without template DNA. Arrowheads indicate features useful for differentiating pomona from hardjo genotype prajitno and hardjo genotype bovis from balcanica.

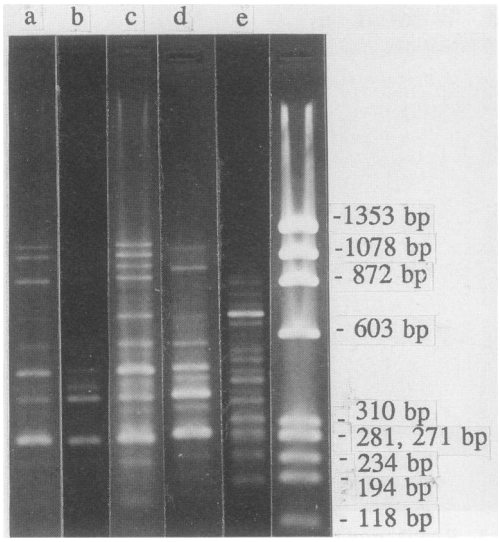

FIG. 3. RAPD profiles of *Leptospira* serovars with the L10 primer. Lanes: a, serovar kremastos; b, zanoni; c, swajizak; d, australis; e, tarassovi.

profiles of serovar tarassovi were similar to those of serovars balcanica and hardjo genotype bovis.

Amplification in RAPD fingerprinting is specific. Alkali blots of RAPD products from hardjo genotype bovis strain 11135, the unrelated bacterium *B. abortus* biotype 1, and control reactions without target DNA were hybridized with digoxigenin-labelled *Eco*RI-digested chromosomal DNA from 11135. The 11135 probe bound to the 11135 profiles but not to the *B. abortus* or the control profiles (results not shown). Thus, the RAPD profiles were due to the amplification of sequences on the *Leptospira* genome rather than interactions between primer molecules.

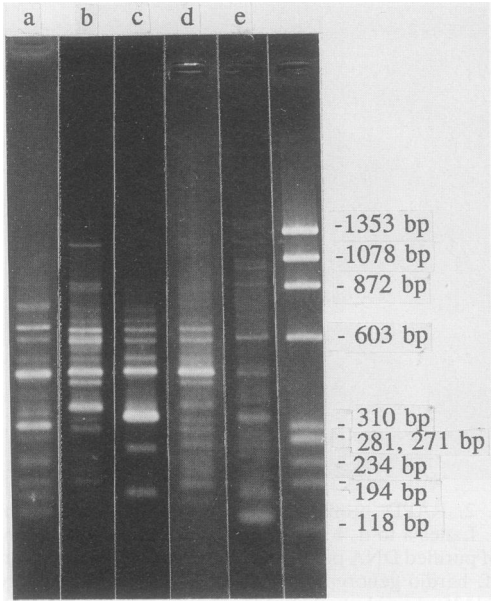

FIG. 4. RAPD profiles of *Leptospira* serovars with the US primer. Lanes: a, serovar kremastos; b, zanoni; c, swajizak; d, australis; e, tarassovi.

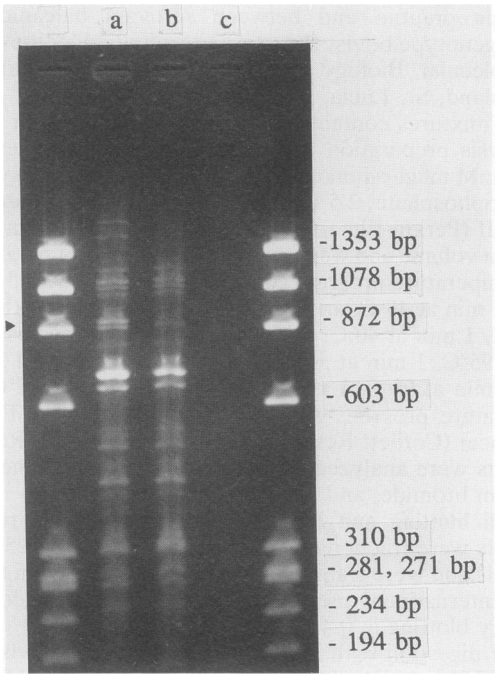

FIG. 5. RAPD profiles of A and B subtypes of serovar hardjo genotype bovis with the L10 primer. Lanes: a, representative of subtype A; b, subtype B; c, control reaction without template DNA. The arrowhead marks the band present in subtype A profiles but not in the subtype B profile.

RAPD PCR resolves differences within the hardjo bovis genotype. Fourteen hardjo genotype bovis subtype A isolates (13 bovine isolates and 1 human isolate) from W. Ellis, Belfast Northern Ireland, and a hardjo genotype bovis subtype B bovine isolate from C. Bolin, Ames, Iowa, were examined by RAPD PCR for subgenotype variation. DNA samples were prepared by rapid lysis. All of the subtype A isolates had identical profiles. The subtype B isolate differed slightly from the subtype A isolates when amplified with L10 primer. The difference is shown in Fig. 5.

Identification of bovine isolates by RAPD fingerprinting. Twenty-six Australian bovine leptospiral isolates (1 from Western Australia, 3 from the WHO/FAO Leptospira Reference Laboratory, 10 from the Animal Research Institute [ARI], 1 from the Toowoomba Veterinary Laboratory, and 11 from EMAI) were RAPD fingerprinted with L10 and US primers. Except for the EMAI isolates, which were fingerprinted from purified DNA, DNA samples were prepared by rapid lysis.

Identification of the isolates was the same with both primers, except that the L10 primer allowed the hardjo genotype bovis isolates to be assigned to a subtype. The Western Australia isolate, WHO/FAO Leptospira Reference Laboratory isolates, and Toowoomba isolate all produced profiles identical to that of hardjo genotype bovis subtype A, were all genotyped as hardjo genotype bovis subtype A by REA, and were all serotyped as hardjo at the WHO/FAO Leptospira Reference Laboratory. Of the 11 isolates from EMAI, 10 produced profiles identical to that of hardjo genotype bovis subtype A and 1 (GN88/2700-2) produced profiles identical to that of serovar pomona. The EMAI isolates were identified as serovar hardjo by CAA and genotype bovis by REA or, in the case of GN88/2700-2, as

pomona by CAA (4). Of the 10 ARI isolates, 8 produced profiles identical to that of hardjo genotype bovis subtype A and were identified as hardjo genotype bovis subtype A by REA and hardjo by CAA. The other two ARI isolates produced profiles identical to that of serovar zanoni and were identified as zanoni by CAA. In REA, these isolates produced identical profiles which closely resembled that of the zanoni reference strain Zanoni. One of these (92-112300.2) is the isolate reported by McClintock et al. (9).

DISCUSSION

We have shown that RAPD fingerprinting is a rapid and reliable method for typing bovine leptospires. The technique would probably be useful for typing leptospires from other sources. Using either the L10 or US primer, the technique clearly distinguished between type strains of serovars pomona, hardjo, balcanica, tarassovi, swajizak, kremastos, australis, and zanoni and the prajitno and bovis genotypes of hardjo. However, the RAPD profiles of serovars pomona, hardjo genotype prajitno, kremastos, australis, zanoni, and swajizak were similar, as were the profiles of serovars hardjo genotype bovis, balcanica, and tarassovi. These findings support the grouping of serovars pomona, hardjo genotype prajitno, kremastos, australis, and zanoni in *L. interrogans* and serovars hardjo genotype bovis, balcanica, and tarassovi in *L. borgpetersenii* (11, 12, 26). We suggest that serovar swajizak may also belong to *L. interrogans*. DNA hybridization would confirm this.

Twenty-three bovine field isolates were typed as hardjo genotype bovis subtype A and two were typed as zanoni by RAPD fingerprinting, and their types were confirmed by REA and CAA. One isolate was typed as pomona, and its type was confirmed by CAA. Thus, RAPD fingerprinting identified isolates to the level of serovar, genotype, and subtype.

With both primers, all isolates were correctly identified. Primer L10 appears more discriminatory than US, as it distinguished the hardjo genotype bovis subtype B strain from the subtype A strains. Also, the differences between similar serovars and genotypes with primer L10 involved major bands, whereas mainly fainter bands were used for differentiation of similar serovars and genotypes with the US primer. Thus, L10 appears the more useful of the two primers.

RAPD fingerprinting worked equally well with highly purified DNA and crude culture lysates. Welsh and McClelland (22) reported similar results in this regard, and rapid-lysis preparations have been used in RAPD analysis of *B. thuringiensis* and *C. jejuni* isolates (2, 8). This makes RAPD fingerprinting ideal for the rapid identification of isolates and gives the technique a distinct advantage over REA, which requires relatively large amounts of highly purified DNA. Furthermore, RAPD profiles are less complex and easier to compare than REA profiles, and the technique does not require high-resolution electrophoresis. RAPD fingerprinting is much faster than CAA and does not require live animals. In fact, an isolate could be typed in as little as 1 day starting with a small broth culture as is used for routine in vitro passage.

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