Differentiation of Hard-to-Type Bacterial Strains by RNA Mismatch Cleavage

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ABSTRACT

Many bacteria are difficult to subtype due to high genetic relatedness. In the cases of pathogens of medical or veterinary importance, subtyping is an essential tool of epidemiologists. This report describes a method for molecular subtyping based on the detection of point mutations without DNA sequencing or specialized equipment. The method, known as RNA mismatch cleavage, hybridizes RNA transcripts derived from PCR-amplified DNA, with a control RNA transcript followed by RNase cleavage at point-mutation mismatches. The method was successful in distinguishing all six *Brucella* species tested and was able to distinguish 11 of the 18 biovars studied. Of the remaining seven biovars (all of which are *Brucella abortus* strains), three subgroups were identified. The method should be applicable to all hard-to-subtype bacterial strains.

INTRODUCTION

Subtyping of bacterial strains has been a difficult task with certain genera due to the low levels of DNA sequence divergence (extremely high similarity) among strains and species. One such genus is *Brucella*, a pathogenic Gram-negative bacteria consisting of at least six closely related species (4,11). Members of this genus are important because they cause disease in humans and animals. Currently, subtyping of *Brucella* species for epidemiology is based on cultural characteristics (8). Identification of select species by polymerase chain reaction (PCR) has been accomplished (3,5), but attempts at molecular subtyping within species, based on DNA, has been unsatisfactory. This is partly because the observed DNA sequence divergence is very low, around 4%, based on DNA:DNA hybridization studies (11).

RNA hybrid mismatch cleavage (9, 10,12) is a point-mutation screening procedure, which has been used to find and characterize genetic mutations. In this procedure, a sense transcript of the test DNA is hybridized with the antisense transcript derived from a reference or wild-type strain. Points of mismatch (sequence divergence) are then cleaved with a ribonuclease, resulting in a specific cleavage pattern. This study examines the applicability of this technique to subtype bacterial strains known to have very low DNA sequence diversity. The subtyping of *Brucella* strains utilizes minute DNA sequence differences within the *Brucella* genetic element IS711 (6), a multicopy 842-bp insertion sequence found only in the species *Brucella*.

MATERIALS AND METHODS

Bacterial Strains and Vectors

Eighteen strains of *Brucella* were chosen as representative of the classical

Brucella species and subtypes (biovars) (Table 1). Culturing of the bacteria and DNA isolation methods were done as previously published (1,7). The reference DNA used for hybridization was derived from the plasmid pBO31-I1, which contains the first reported copy of the insertion sequence element IS711 (6) from *Brucella ovis*.

PCR

Figure 1 gives a schematic overview of the process. Target DNA was amplified by nested PCR. The first-stage PCR amplified a 1121-bp fragment containing the entire 842-bp IS711 element by using primers (Table 2) flanking a specific copy of IS711, designated IS711A, which is common to all eighteen *Brucella* strains used in this study. Conditions for PCR of IS711 have been previously described (3). Briefly, 2.5 ng of each genomic DNA were amplified in a master mixture consisting of 60 mM Tris-HCl (pH 9.0), 15 mM (NH₄)₂SO₄, 1.5 mM

Figure 1. Schematic diagram of the RNA hybrid mismatch cleavage procedure. The basic elements of the procedure are depicted with the experimental DNA presented on the left of the diagram and the reference DNA represented on the right side. IS711 DNA is shown as thick, shaded boxes. RNA is depicted by thin, shaded boxes. Small arrows represent oligonucleotide primers; small, shaded boxes labeled SP6 and T7 indicate incorporation of these promoters into the primer sequences. Small, black rectangles within RNA transcripts symbolize sequence mismatch.

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MgCl₂, 250 μM (each) of the four dNTPs [Catalog No. K1220-02(E); Invitrogen, San Diego, CA, USA], 0.2 μM of each primer and 0.5 U of AmpliTaq® DNA Polymerase (PE Biosystems, Foster City, CA, USA) inactivated per manufacturer's instructions with TaqStart™ antibodies (CLONTECH Laboratories, Palo Alto, CA, USA), per 25 μL reaction. Samples were cycled 36× through a program consisting of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. This was followed by a 5-min incubation at 72°C.

In the second-stage PCR, the nested primers (Table 2) contained either the T7 or SP6 promoter sites connected to the borders of the insertion sequence (IS) element, resulting in an 883-bp amplification product. Amplification conditions were the same as those in the first-stage PCR, except that 0.2 μL of the first-stage PCR products was used as targets, and the temperature cycle conditions were the following: 3 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 2 min. This was followed by incubations of 33 cycles of 95°C for 1 min and 72°C for 2 min. Next, the samples were incubated at 72°C for 5 min. Five microliters of product were analyzed by agarose gel electrophoresis to assess the concentration and purity of the products.

Point-Mutation Detection

Reagents and protocols for RNA synthesis, hybridization and cleavage were obtained from the Mutation-Finder™ Kit (Catalog No. 50570; FMC BioProducts, Rockland, ME, USA) optimized for mutation detection. The manufacturer did not disclose the exact formulations.

Briefly, 2 μL of the second-stage amplification product from each strain were transcribed in a final volume of 10 μL using SP6 RNA Polymerase (Ambion, Austin, TX, USA) on the sense strand. The reference DNA was amplified from the originally described IS711 copy (6) of *B. ovis* on plasmid pBO31-I1 (7). Sufficient reference DNA (25 μL of amplified product) was transcribed using T7 RNA Polymerase (Ambion) to produce enough antisense strand for hybridization with each of the 18 test samples and controls. For all

Table 1. Bacterial Strains Used in This Study

Organism	Strain Designations	Source
<i>B. abortus</i> biovar 1	544	NADC ^a
<i>B. abortus</i> biovar 2	2016	NADC
<i>B. abortus</i> biovar 3	Tulya	NADC
<i>B. abortus</i> biovar 4	292	NADC
<i>B. abortus</i> biovar 5	B3196	NADC
<i>B. abortus</i> biovar 6	870	NADC
<i>B. abortus</i> biovar 9	C68	NADC
<i>B. canis</i>	RM-6/66	NADC
<i>B. melitensis</i> biovar 1	16M	NADC
<i>B. melitensis</i> biovar 2	63/9	NADC
<i>B. melitensis</i> biovar 3	Ether	NADC
<i>B. neotomae</i>	SE-1169	NADC
<i>B. ovis</i>	ANH3572	NADC
<i>B. ovis</i>	ATCC 25840	NADC
<i>B. suis</i> biovar 1	1330 (human)	NADC
<i>B. suis</i> biovar 2	Danish sow 160	NADC
<i>B. suis</i> biovar 3	NADC 710	NADC
<i>B. suis</i> biovar 4	IAB 2579	NADC
<i>B. suis</i> biovar 5	513	WHO ^b

^aNADC, National Animal Disease Center.
^bWHO, World Health Organization.

Figure 2. Differentiation of *Brucella* strains based on point mutations found within IS711 DNA sequences. RNA hybrid cleavage products from 18 *Brucella* strains were resolved by agarose gel electrophoresis as described in the text. Lane 1 = 100-μb ladder as size marker.

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Table 2. PCR Primers

Primer	Sequence (5'→3')
First-stage PCR primers	
IS711A-L	GCT-GCG-ATT-GGT-TAA-AGC-GCG-A
IS711A-R	ATA-AAA-AAC-AAT-TCG-TAA-TGA-TG-GG
Second-stage (nested) PCR primers	
<div><div><div>T7 promoter</div><div>Inverted Repeat</div><div>IS711</div></div></div>	
T7-IR1	GAT-AAT-ACG-ACT-CAC-TAT-AGG-GCG-TGT-CTG-CAT-TCA-ACG-TAA-CCA
<div><div><div>SP6 promoter</div><div>Inverted Repeat</div><div>IS711</div></div></div>	
SP6-IR2	TCA-TTT-AGG-TGA-CAC-TAT-AGG-AGG-GCT-TGT-CTG-CAT-TCA-AGG-ATT-CCC

samples, transcription was done at 37°C for 2 h. Each 10-μL sample and the reference antisense RNA (20× = 210 μL) were diluted 1:1 (vol:vol) with hybridization buffer. An equal volume

of the reference RNA (20 μL) was added to each sample, heated to 95°C for 3 min, then cooled to room temperature for approximately 20 min before storage at -20°C overnight.

Four microliters of each thawed hybridized sample were added to 16 μL of RNase digestion solution, which had been prepared as described (2). Samples were digested for 40 min at 37°C

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and then mixed with 4 μ L stop/dye solution. The entire 24 μ L of each sample was loaded into one well of a 2.5% (wt/vol) MetaPhor[®] Agarose Gel (FMC BioProducts) prepared in 0.5 $\times$ TBE (50 mM Tris, 90 mM boric acid, 1 mM EDTA, pH 8.4) and pre-soaked in ethidium bromide (0.5 mg/L). Samples were electrophoresed for 4 h at 45 V (16 mA). DNA was visualized and photographed under UV illumination.

RESULTS

Transcripts of IS711A from 18 *Brucella* biovars were hybridized to the antisense transcript of the original IS711 element described from *B. ovis*. Figure 2 shows the RNase cleavage products derived from the heteroduplexes. Based on the cleavage patterns, all six species can be differentiated. Many biovars within a single species also can be subtyped on the basis of cleavage products. The seven biovars of *B. abortus* can be subtyped into three groups: biovars 1, 2 and 4; biovars 3, 5 and 9; and biovar 6.

Most of these fingerprints are easily distinguished; a few however are subtle. As an example, the five biovars of *B. suis* can be easily differentiated from one another; although, the fingerprints for *B. suis* biovar 3 and *Brucella neotomae* are very similar. The major differences are that (i) *B. neotomae* has two minor cleavage fragments migrating at approximately 200 bp, which are not present in *B. suis* biovar 3, and (ii) that *B. suis* biovar 3 has two cleavage products of about 400 bp, while *B. neotomae* has only one. In another case, the fingerprint for *Brucella melitensis* biovar 3 is very similar to the fingerprints of *B. abortus* biovars 3, 5 and 9. In this case, the *B. melitensis* biovar 3 fingerprint differs by having a single cleavage fragment of about 100 bp, while the *B. abortus* biovars have two products this size.

DISCUSSION

Previously, we were able to differentiate some subtypes (biovars) by Southern blot hybridization probed with IS711 DNA (2). However, differentiation based on RNA cleavage at point

mutations is more sensitive than any previously published molecular-based assays. For example, this is the first assay, to our knowledge, which can differentiate among *B. canis*, *B. suis* biovar 4 and *B. abortus* biovar 3. The assay is sensitive enough to differentiate between DNA sequences from two copies of IS711 at different loci within the same genome (the *B. ovis* IS711A fingerprint has a minor cleavage product about 200 bp in size, which is absent from the control). Furthermore, the RNA mismatch cleavage procedure is faster and easier than Southern blot analysis because purified genomic DNA is not necessary for PCR.

Mismatch cleavage fingerprints were reproducible with duplicate assays. Occasionally, fingerprint patterns were somewhat obscured by a high background smear, possibly due to hybridization of truncated transcripts. The background was only problematic if comparing nearly identical fingerprints. For this reason, it could be helpful to analyze two distinct targets for each strain. We intend to investigate other loci in *Brucella* to further increase our differentiation of *B. abortus* strains.

In conclusion, point-mutation detection could be a valuable epidemiological tool. Many samples can be analyzed concurrently. We believe that our experience is applicable to other bacteria where strain differentiation has been difficult due to low DNA sequence divergence levels.

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Betsy J. Bricker
National Animal Disease Center
USDA
Agricultural Research Service
Ames, IA, USA