Original Research

Rapid Molecular Diagnosis of *Lactobacillus* Bacteremia by Terminal Restriction Fragment Length Polymorphism Analysis of the 16S rRNA Gene

*Jeffrey E. Christensen, PhD, *Clinical Research Center, Marshfield Clinic Research Foundation, Marshfield, Wisconsin*

Cory E. Reynolds, MS, *Clinical Research Center, Marshfield Clinic Research Foundation, Marshfield, Wisconsin*

Sanjay K. Shukla, PhD, *Clinical Research Center, Marshfield Clinic Research Foundation, Marshfield, Wisconsin*

Kurt D. Reed, MD, *Clinical Research Center, Marshfield Clinic Research Foundation, Marshfield, Wisconsin*

*Current affiliation: *Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado*

ABSTRACT

OBJECTIVE

Bacteremia due to lactobacilli is uncommon, yet it is increasing in frequency, especially among immunosuppressed patients. In the clinical laboratory, lactobacilli must be subcultured from positive blood cultures before identification by traditional biochemical methods. Delays in diagnosis are significant because the organisms are inherently resistant to vancomycin, a drug frequently prescribed for empiric therapy for gram-positive bacteremia. Recently, we developed a rapid terminal-restriction fragment length polymorphism (T-RFLP) diagnostic assay based on species-specific variations in the bacterial 16S rRNA gene. We sought to apply this technique to the identification of *Lactobacillus* spp. from three cases of bacteremia.

DESIGN

The results of the T-RFLP analysis are compared with two standard biochemical identification methods.

METHODS

Lactobacillus strains were isolated from positive clinical blood cultures. Initial suspect cultures were subcultured and characterized using an automated substrate hydrolysis system and *Lactobacillus* carbohydrate fermentation profiles. Further biochemical and molecular analyses were performed from isolates propagated in *Lactobacillus* MRS broth. DNA was extracted and the 16S rRNA gene sequenced. Two sets of fluorescent labeled primers targeting the 16S rRNA gene were used for polymerase chain reaction (PCR) with chromosomal preparations from reference strains and blood isolates. The PCR products were digested with restriction enzymes and terminal-restriction fragment profile analysis performed.

RESULTS

T-RFLP analysis correctly identified the *Lactobacillus* species in each case. T-RFLP analysis could be completed within 8 hours of obtaining a positive blood culture as compared to more than the 24 to 48 hours required for traditional culturing and biochemical characterizations.

CONCLUSION

T-RFLP analysis allows for rapid identification of *Lactobacillus* directly from positive blood cultures and circumvents the requirement for subculture. Reduced diagnostic time has implications for duration of infection, the cost of patient care, length of hospitalization, development of broad-spectrum antibiotic resistance, and mortality due to bacteremia. T-RFLP profiling represents a highly reproducible and predictive source for identification of many organisms associated with bacteremia.

RECEIVED: **NOVEMBER 20, 2003**

ACCEPTED: **DECEMBER 1, 2003**

REPRINT REQUESTS:

Kurt D. Reed, MD
Clinical Research Center
Marshfield Clinic Research Foundation
1000 North Oak Avenue
Marshfield, WI 54449
Telephone: 715-389-5478
Fax: 715-389-3319
Email: reed.kurt@mcrf.mfldclin.edu

KEYWORDS:

Lactobacillus; Bacteremia; PCR; 16S rRNA; Rapid diagnosis

GRANT SUPPORT:

Marshfield Clinic Research Foundation

INTRODUCTION

The detection and identification of bacterial infections of the bloodstream is one of the most important functions of the clinical microbiology laboratory. Bacteremia results in significant morbidity and mortality, especially among patient populations that are immunocompromised.¹⁻³ One of the current limitations of processing blood cultures in the laboratory is the requirement to subculture isolates in order to perform biochemical or other tests needed for bacterial identification. This often results in one or more days of delay during which it is often necessary to administer broad-spectrum antimicrobial therapy. This practice contributes to the continued emergence of drug-resistant strains of pathogenic bacteria. In addition, some species of bacteria associated with bacteremia are inherently resistant to commonly prescribed antimicrobial agents and may not be effectively treated with empiric therapy.^{4,5}

Lactobacilli belong to a diverse group of bacteria that are gram-positive or gram-variable, facultatively anaerobic, non-spore forming, and produce lactic acid as a major product of carbohydrate fermentation. These bacteria are ubiquitous in the environment and propagate in ecological niches, such as plant surfaces and decaying plant material. In humans they colonize the oral cavity, gastrointestinal tract, and vagina.⁶ *Lactobacillus casei* appears to be the most frequently isolated species within the genus,⁷ although *L. paracasei* and *L. rhamnosus* are also encountered in clinical situations. However, isolates are often only identified by genus and most automated identification systems are not capable of accurate differentiation of *Lactobacillus* species. In general, lactobacilli are avirulent or of low virulence, but are recognized as opportunistic pathogens involved in bacteremia,⁸⁻¹¹ as well as other significant infections such as amnionitis,¹² abscesses,¹³ and endocarditis.^{7,9,14} Lactobacilli are also considered emerging pathogens in patients with chemotherapy induced neutropenia.^{15,16}

One important clinical feature of lactobacilli is their inherent resistance to vancomycin, a drug frequently given as empiric therapy for bacteremia due to gram-positive bacteria.¹⁷ Therefore, rapid identification of lactobacilli from blood cultures would allow a more timely prescription of appropriate antimicrobial therapy. We recently described a rapid identification procedure for positive blood cultures that is based on terminal-restriction fragment length polymorphism (T-RFLP) profile analysis of the 16S rRNA gene.¹⁸ This molecular diagnostic procedure is applied directly to positive blood culture bottles and circumvents the need to subculture the isolate prior to identification. The assay is robust and is applicable to a broad range of bacterial pathogens. In this report we demonstrate the utility of T-RFLP in three cases of *Lactobacillus* bacteremia and compare the results with two conventional methods of bacterial identification.

MATERIALS AND METHODS

Bacterial strains and isolation

The *Lactobacillus* strains characterized in this study were isolated from positive blood cultures at Marshfield Laboratories as part of routine diagnostic testing. Marshfield Laboratories uses the BacT/Alert blood culturing system (Organon Teknika Corp., Durham, NC) and performs Gram staining for all positive blood cultures. Positive blood bottles were cultured on trypticase soy–5% sheep blood or chocolate agar at 37°C under the appropriate atmospheric conditions. Blood bottle isolates were initially characterized using the automated VITEK system (bioMérieux Vitek Inc., Hazelwood, MO) and standard biochemical methods. Additional biochemical and molecular analyses described below were performed from isolates propagated in *Lactobacillus* MRS broth (Difco Laboratories, Detroit, MI).

Anaerobe identification card

The VITEK anaerobe identification card was used in conjunction with the VITEK system for the automated identification of each strain by characterizing the ability to hydrolyze 28 substrates. Following initial isolation and identification of each strain, cultures were propagated through MRS broth and plated on blood agar at 37°C under anaerobic conditions. Identification of isolates was repeated with the anaerobe identification cards as per the manufacturer's protocol.

Lactobacillus carbohydrate fermentation profile

The API 50 CH test kit and the API CHL medium (bioMérieux Vitek Inc.) were used to determine the species of each *Lactobacillus* isolate by characterizing the ability to ferment 49 carbohydrates. Strains were grown for 18 hours to stationary phase in MRS broth and 1 mL of culture was harvested by centrifugation at 20,000 × g for 1 minute. The cell pellets were washed with 1 mL sterile saline, suspended in 0.5 mL API CHL medium, and then further diluted 100-fold in API CHL medium. The culture dilutions were then loaded to the API 50 CH test strips as per the manufacturer's protocol, the capules were covered with mineral oil, and the test strips were incubated at 37°C for up to 48 hours. Test strips information was read and recorded at 16 and 40 hours. Carbohydrate fermentation profiling was performed in duplicate for each strain. Species identification was made by comparison of the results from API 50 CH test strips with known carbohydrate fermentation profiles of lactobacilli as indicated in Bergey's Manual of Systematic Bacteriology.¹⁹

DNA extraction

DNA was extracted from cultured colonies using a modification of the tissue protocol for the QIAmp DNA Mini Kit (Qiagen, Inc., Valencia, CA). Briefly, 200 µL of a cell suspension in H₂O (containing ~5 to 10 mg cells) was mixed with 200 µL of QIAmp Buffer AL and 10 µL of proteinase K (20 mg/mL). Samples were incubated for 15 minutes at 56°C and then for 15 minutes at 95°C. Two hundred microliters of ethanol was added and mixed by

vortexing. The samples were loaded, washed, and eluted in 200 µL from QIAmp spin columns, as indicated in the remainder of the manufacturer's protocol.

Sequencing of the 16S rRNA gene

The template DNA for sequencing of the 16S rRNA gene was amplified using primers FD1 (5'-AGA GTT TGA TCC TGG CTC AG) and RD1 (5'-AAG GAG GTG ATC CAG CC), resulting in PCR products of ~1500 bp. The reaction mixtures contained 1 x polymerase chain reaction (PCR) buffer, 200 µM each deoxynucleoside triphosphate, 3.0 mM MgCl₂, 0.5 µM each primer species, 1.0 U *Taq* DNA polymerase, and 1.0 µL chromosomal preparation per 15 µL reaction. The PCR products were used for cycle sequencing reactions without further purification.

Cycle sequence reactions were prepared with BigDye Terminator mix (Applied Biosystems, Foster City, CA) as per supplier's protocol using primer FD1 (see above), RD1 (see above), 515F (5'-TGC CAG CAG CCG CGG TAA), 806R (5'-GGA CTA CCA GGG TAT CTA AT), 91E (5'-TCA AAK GAA TTG ACG GGG GC), or 13B (5'-AGG CCC GGG AAC GTA TTC AC). Sequence determination was done on an Applied Biosystems model 377XL automated DNA sequencing instrument. DNA sequences were aligned using Lasergene99 software (DNASTAR Inc., Madison, WI). The nucleotide sequences, determined from a minimum of 4-fold coverage, were deposited in GenBank under the accession numbers AY299486, AY299487, and AY299488. Sequence analysis was performed using the BLAST program for database searches at the National Center for Biotechnology Information Web site²⁰ and the sequence match program at the Ribosomal Database Project (RDP-II) Web site.²¹ Multiple sequence alignments were performed using the ClustalW program.²²

PCR primers for determining T-RFLP

Independent reactions with two sets of fluorescent labeled primers targeting the 16S rRNA gene were used for PCR with chromosomal preparations from reference strains and blood isolates. The primers were chosen as described in Christensen et al.¹⁸ based on evaluations of domain specific primers and maximization of matches received from queries using the TAP T-RFLP on-line analysis of RDP-II. As indicated, primers were labeled at the 5' end with the dyes 6-carboxyfluorescein (6-FAM), 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein (HEX), or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA).

The 5' region (encompassing hypervariable regions V1-V3) of the 16S rRNA gene was amplified with S-D-Bact-0045-b-S-20 (5'-6-FAM-GCY TAA CAC ATG CAA GTY GA) and S-D-Bact-0785-a-A-19 (5'-HEX-CTA CCA GGG TAT CTA ATC C). The 3' region of the 16S rRNA gene was amplified with S-D-Bact-0785-a-S-19 (5'-6-FAM-GGA TTA GAT ACC CTG GTA G) and S-D-Bact-1371-a-A-20 (5'-HEX-AGG CCC GGG AAC GTA TTC AC). PCR

products from both sets of primers were predicted to be 600-750 bp, with some variability dependent on the organism from which the template DNA is extracted.

PCR conditions for T-RFLP

Fluorescent dye labeled PCR products for T-RFLP were prepared as described previously.¹⁸ The reaction mixtures for PCR contained 1 x PCR buffer, 200 µM each deoxynucleoside triphosphate, 3.0 mM MgCl₂, 0.5 µM each primer species, 1.0 U *Taq* DNA polymerase, and 1.0 µL chromosomal preparation per 15 µL reaction. Due to approximately 4-fold higher detection sensitivity of the fluorescence emission from the 6-FAM dye relative to HEX dye, a ratio of 1 part 6-FAM labeled primer (0.125 µM) to 3 parts unlabeled primer (0.375 µM) was used for primers S-D-Bact-0045-b-S-20 and S-D-Bact-0785-a-S-19. DNA was amplified using a model 9600 thermal cycler (Perkin-Elmer, Norwalk, CT) with the following program: 15 minutes at 94°C for denaturation and *Taq* activation, 35 cycles consisting of denaturation (45 seconds at 94°C), annealing (30 seconds at 52°C), extension (60 seconds at 72°C), and a final extension for 5 minutes at 72°C. All PCR reactions were analyzed by gel electrophoresis and stained with ethidium bromide to assure product formation prior to restriction digest.

Restriction digests of PCR products

PCR products were digested with restriction enzymes (Invitrogen, Carlsbad, CA and New England BioLabs, Beverly, MA) without further purification. Each 10 µL restriction digest contained 1.0 µL of PCR product, 0.5 µL of restriction enzyme, and 8.5 µL of prepared stock containing 2.5 ng/µL each appropriate size/digest control DNA in 1 x buffer. The PCR product of 6-FAM labeled S-D-Bact-0045-b-S-20 and HEX labeled S-D-Bact-0785-a-A-19 (amplifying the 5' region of the 16S rRNA gene) was digested in separate reactions with *AluI*, *HhaI*, *MspI*, and *RsaI*. The PCR product of 6-FAM labeled S-D-Bact-0785-a-S-19 and HEX labeled S-D-Bact-1371-a-A-20 (amplifying the 3' region of the 16S rRNA gene) was only digested with *HhaI*. Restriction digests were performed in a model 9600 Perkin-Elmer thermal cycler programmed for a 1 hour incubation at 37°C followed by 20 minutes at 65°C for enzyme inactivation. The restriction digest products were purified with DyeEx 96-well spin columns (Qiagen) as per manufacturer's protocol.

Terminal restriction fragment (TRF) profile analysis and organism identification

Samples were prepared for electrophoresis by combining 1.0 µL of restriction digest product, 0.50 µL of X-rhodamine MapMarker 1000 XL (BioVenture, Inc., Muffreesboro, TN), and 14 µL of Hi-Di formamide (Applied Biosystems Instruments). The size standard contains single strands of DNA with a single ROX fluorophore at 50, 75, 100, 125, 150, 200, 250, 300, 350, 400, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, and 1000 bases. Samples were mixed by pipetting, denatured at 94°C, and immediately

cooled to 4°C. The lengths of TRFs derived from amplified DNA products were determined with an Applied Biosystems model 3100 capillary DNA sequencing instrument operating in GeneScan™ mode. The samples were loaded to a 50 cm capillary with an injection voltage of 1 kV for 22 seconds and run at 12.2 kV for 2.2 hours at 50°C. The sizes of control and sample TRFs were calculated by comparison with the internal standard using the local Southern method.²³ Fragment lengths ≥ 20 but < 50 bases were calculated by linear extrapolation from the migration times of the 50 and 75 base standards.

Identification of bacteria was accomplished through the use of a program for calculating terminal fragment sizes and a searchable terminal restriction fragment profile database (TRFPD).¹⁸ Briefly, the maximum area 6-FAM and HEX peak data for each sample are identified and organized according to the respective primer set and restriction digest combination. The program is then used to compare the sample TRF profile with 2435 TRF profiles representing 921 different species of potential relevance to bacteremia in the TRFPD. The results from a Gram stain can also be selected in the data input window to limit the search. The

Figure 1. Representative TRF profile of the PCR products from the 16S rRNA gene of *Lactobacillus rhamnosus* MCRF-412. Each panel shows a PCR product/restriction digest electropherogram and the applicable range of the standard curve (50-850 bases). The single major 6-FAM (blue peaks) and HEX (green peaks) fragment of each digest are identified by their respective nucleotide size. TRFs from PCR products obtained with (6-FAM)-S-D-Bact-0045-b-S-20 and (HEX)-S-D-Bact-0785-a-A-19 are shown in panels A (*AluI*), B (*HhaI*), C (*MspI*), and D (*RsaI*). TRFs from PCR products obtained with (6-FAM)-S-D-Bact-0785-a-S-19 and (HEX)-S-D-Bact-1371-a-A-20 are shown in panel E (*HhaI*). The presence of a single peak size > 700 bases for both the 6-FAM and HEX fragments in panel D indicates the PCR product was not digested with *RsaI*.

Table 1. VITEK anaerobe identification.

Substrate	Isolate, substrate hydrolysis, and identification ^a	
	MCRF-271 & MCRF-412	MCRF-284
p-nitrophenyl phosphate	-	-
p-nitrophenyl phosphate choline	-	-
p-nitrophenyl-β-D-galactopyranoside	Positive	Positive
p-nitrophenyl-α-D-galactopyranoside	-	-
p-nitrophenyl-β-D-glucopyranoside	Positive	Positive
p-nitrophenyl-α-D-glucopyranoside	Positive	Positive
p-nitrophenyl-β-D-glucuronide	-	-
p-nitrophenyl-β-D-lactoside	-	-
p-nitrophenyl-α-D-mannopyranoside	Weak	-
p-nitrophenyl-α-L-fucopyranoside	-	-
p-nitrophenyl-β-D-fucopyranoside	-	-
p-nitrophenyl-β-D-xylopyranoside	-	-
p-nitrophenyl-α-L-arabinofuranoside	-	-
p-nitrophenyl-N-acetylglucosaminide	Positive	Positive
N-benzoyl-DL-arginine p-nitroanilide	-	-
L-leucine p-nitroanilide	Positive	Positive
L-proline p-nitroanilide	-	-
L-alanine p-nitroanilide	Positive	Positive
L-lysine p-nitroanilide	Positive	Positive
γ-glutamyl p-nitroanilide	-	-
Triphenyl tetrazolium	-	-
Arginine	-	-
Urea	-	-
Glucose	Positive	Positive
Trehalose	Positive	Positive
Arabinose	-	-
Raffinose	-	-
Xylose	-	-
VITEK Identification	<i>L. casei</i>	<i>L. casei</i>

^aSymbols for MCRF isolate results from the VITEK (VITEK (bioMérieux Vitek Inc., Hazelwood, MO) anaerobe identification: positive, positive for hydrolysis; weak, limited for hydrolysis; -, no detectable hydrolysis.

result of a search includes the number of matching fragment lengths (within chosen bp windows), ordering of closest matches, and links to best match TRF profiles. In addition, a sum of differences (SOD) score was determined for each TRF profile relative to the predicted fragments from the database. Specifically, the difference between the size of the experimentally determined TRF and the sequence predicted fragment size was calculated for each fragment/digest combination, and then the sum of these differences was calculated. A smaller SOD score is taken to represent a closer match to a given organism in the database.

RESULTS

Characterization of organisms from positive blood bottles

The three organisms described in this manuscript were isolated from positive anaerobic blood bottles in Marshfield Laboratories. Each culture was determined to contain either Gram positive or Gram variable rod-shaped cells. Isolates grew in 24 hours on trypticase soy–5% sheep blood or chocolate agar at 37°C under anaerobic conditions. Isolates were used for the identification of each organism using the following biochemical tests.

Table 2. Identification by carbohydrate fermentation profile.

Carbohydrate	<i>L. rhamnosus</i> ^a	MCRF-271 & MCRF-412 ^b	<i>L. casei</i> ^a	MCRF-284 ^b
Amygdaline	Positive	Positive	Positive	Positive
D-arabinose	C	Weak	-	-
Cellobiose	Positive	Positive	Positive	Positive
Esculine	Positive	-	Positive	-
D-fructose	Positive	Positive	Positive	Positive
Galactose	Positive	Positive	Positive	Positive
D-glucose	Positive	Positive	Positive	Positive
Gluconate	Positive	Weak	Positive	Weak
Lactose	Positive	Positive	C	Positive
Maltose	Positive	-	Positive	Positive
Mannitol	Positive	Positive	Positive	Positive
D-mannose	Positive	Positive	Positive	Positive
Melezitose	Positive	Positive	Positive	Positive
Melibiose	-	-	-	-
D-raffinose	-	-	-	-
Rhamnose	Positive	Positive	-	-
Ribose	Positive	Positive	Positive	Positive
Salicine	Positive	Positive	Positive	Positive
Sorbitol	Positive	Positive	Positive	Positive
Trehalose	Positive	Positive	Positive	Positive
D-xylose	-	-	-	-
L-xylose	-	-	-	-
Identification	<i>L. rhamnosus</i>		<i>L. casei</i>	

^a Symbols for indicated reference organism profile from Bergey's Manual¹⁹: positive, 90% or more strains positive for fermentation of the indicated carbohydrate; -, 90% or more strains negative; C, 11-89% strains positive.

^b Symbols for MCRF isolate results using the API 50 CH source strips: positive, positive for fermentation of indicated carbohydrate; weak, limited for fermentation; -, no detectable fermentation.

VITEK anaerobe identification

The substrate hydrolysis results from the anaerobe identification card are shown in table 1. The single difference found between the strains was the inability of MCRF-284 to use p-nitrophenyl- α -D-mannopyranoside, whereas strains MCRF-271 and MCRF-412 provided a weak reaction. All three strains were identified as *L. casei* on the basis of the results from Gram reaction, lack of indole production, and substrate hydrolysis on the anaerobic identification card.

Identification of Lactobacillus species by carbohydrate fermentation profile

Table 2 shows the API 50 CH results and the predicted carbohydrate fermentation profiles for *L. casei* and *L. rhamnosus* with 22 carbohydrates necessary for species differentiation and identification of most heterofermentative lactobacilli. Strains of *L. casei* and *L. rhamnosus* were differentiated by the ability of the latter isolates to ferment arabinose and rhamnose. In addition to the data shown in

table 2, all of the blood isolates were positive for fermentation of arbutine, β -gentiobiose, D-tagatose, inositol, N-acetyl-glucosamine, and saccharose. Also, strains MCRF-271 and MCRF-412 were positive for fermentation of L-fucose and L-sorbose, while no reaction was found with MCRF-284. Conversely, MCRF-284 was positive for fermentation of adonitol, D-turanose, and inuline, while no reaction was found with MCRF-271 and MCRF-412.

Identification of species by sequence determination of 16S rRNA gene

The nucleotide sequence was determined for a 1480 base region of the 16S rRNA gene from each of the blood isolates (GenBank accession numbers AY299486, AY299487, and AY299488). The sequences determined for MCRF-271 and MCRF-412 were identical for all 1480 bases. The BLAST analysis of MCRF-271 and MCRF-412 nucleotide sequence indicated the highest identities with a *L. casei* (99.5%) and *L. rhamnosus* (99.2%) strain. The similarity scores from the

Table 3. TRF profiles for blood isolates and SOD scores for TRF database matches.

Isolate or organism ^a	Strain	Size (bases) of TRF obtained with:										Sum of differences (SOD) score ^d
		Alu I ^b		Hha I ^b		Msp I ^b		Rsa I ^b		Hha I ^c		
		6-FAM	HEX	6-FAM	HEX	6-FAM	HEX	6-FAM	HEX	6-FAM	HEX	
MCRF-271 / MCRF-412^e		193	560	561	223	537	247	UD	UD	321	284	NA
<i>L. rhamnosus</i>	DSM 20021	194	556	561	224	535	246	UD	UD	325	283	14
<i>L. rhamnosus</i>	JCM 1136	194	555	560	224	534	246	UD	UD	325	283	17
<i>L. plantarum</i>	NCDO 1752	175	558	562	224	536	246	UD	UD	320	283	26
<i>L. plantarum</i>	JCM 1149	175	556	560	224	534	246	UD	UD	325	283	33
<i>L. plantarum</i>	ATCC 8014	175	556	560	224	534	246	UD	UD	325	283	33
<i>L. manihotivorans</i>	LMG 18010	174	556	560	224	534	246	UD	UD	325	283	34
<i>L. pentosus</i>	JCM 1588	226	556	560	224	534	246	UD	UD	325	283	48
<i>L. casei</i>	ATCC 334	194	556	561	172	535	246	UD	UD	325	283	64
<i>L. casei</i>	ATCC 393	194	556	561	172	535	246	UD	UD	325	283	64
<i>L. zeae</i>	ATCC 15820	194	556	561	172	535	246	UD	UD	325	283	64
<i>L. casei</i>	JCM 1134	195	555	561	172	535	246	UD	UD	325	283	66
<i>L. paracasei</i>	JCM 1133	194	555	560	172	534	246	UD	UD	325	283	67
<i>L. paracasei</i>	JCM 8130	194	555	560	172	534	246	UD	UD	325	283	67
<i>L. paracasei</i>	JCM 1171	194	555	560	172	534	246	UD	UD	325	283	67
MCRF-284^e		193	560	561	172	537	247	UD	UD	321	284	NA
<i>L. casei</i>	ATCC 334	194	556	561	172	535	246	UD	UD	325	283	13
<i>L. casei</i>	ATCC 393	194	556	561	172	535	246	UD	UD	325	283	13
<i>L. zeae</i>	ATCC 15820	194	556	561	172	535	246	UD	UD	325	283	13
<i>L. casei</i>	JCM 1134	195	555	561	172	535	246	UD	UD	325	283	15
<i>L. paracasei</i>	JCM 1133	194	555	560	172	534	246	UD	UD	325	283	16
<i>L. paracasei</i>	JCM 8130	194	555	560	172	534	246	UD	UD	325	283	16
<i>L. paracasei</i>	JCM 1171	194	555	560	172	534	246	UD	UD	325	283	16
<i>L. rhamnosus</i>	DSM 20021	194	556	561	224	535	246	UD	UD	325	283	65
<i>L. paracasei</i>	JCM 1181	194	555	612	172	534	246	UD	UD	325	283	66
<i>L. rhamnosus</i>	JCM 1136	194	555	560	224	534	246	UD	UD	325	283	68

UD: Undigested PCR product (6-FAM and HEX fragment sizes were both >700 bases).

^aOrganisms are presented in ascending order of the SOD scores relative to the TRF results for each MCRF strain.

^bLabeled fragments derived from the PCR product of (6-FAM)-S-D-Bact-0045-b-S-20 and (HEX)-S-D-Bact-0785-a-A-19 or as predicted from sequence data.

^cLabeled fragments derived from the PCR product of (6-FAM)-S-D-Bact-0785-a-S-19 and (HEX)-S-D-Bact-1371-a-A-20 or as predicted from sequence data.

^dValue calculated from the differences between the size of the experimentally determined TRF and the sequence predicted fragment sizes for a restriction digest of organisms in the TRF database. The SOD for each organism was then calculated.

^eEach TRF size reported was determined from the average of at least two digests.

RDP-II sequence match analysis of MCRF-271 and MCRF-412 were determined to be 0.991 for *L. casei* and 0.986 for *L. rhamnosus*.

The BLAST analysis of MCRF-284 nucleotide sequence indicated the highest identities with two *L. casei* and one *L. paracasei* strains (100% matches). The RDP-II similarity score obtained with the MCRF-284 sequence was determined to be 1.000 with two *L. casei* and one *L. paracasei* strains.

Identification of species by TRF profile

The TRF profiles obtained from restriction digest of the 16S rRNA gene PCR products for each of the blood isolates matched closely to those predicted from their respective nucleotide sequences (figure 1 and table 3). The TRF profiles for MCRF-271 and MCRF-412 were closest to those for both *L. rhamnosus* strains in the sequence predicted TRFPD, matching all 10 fragments within 5 bases and having a SOD score of 14 and 17 bases (table 3). The next closest matches were for three strains of *L. plantarum*, but the profiles matched only 9 of 10 fragments within 5 bases and had a higher SOD score (26-33 bases). The latter results were primarily due to the significant differences in the 6-FAM labeled *AluI* restriction digest product (18 base difference).

The TRF profile for MCRF-284 was closest to those for three *L. casei* strains and one *L. zeae* strain in the TRFPD, matching all 10 fragments within 5 bases for all four strains and having a SOD score of 13-15 bases. The next closest matches were for three strains of *L. paracasei*, again matching all 10 fragments within 5 bases, but with a slightly greater SOD score (16 base difference).

DISCUSSION

This study describes the use of TRF profiling for identification of *Lactobacillus* species from blood cultures and comparison of this technique with other methods of identification. In a previous study,¹⁸ we showed that TRF profiling was effective in differentiating many organisms responsible for bacteremia.

Biochemical characterization of the blood culture isolates using the VITEK anaerobe system resulted in identification of *L. casei* for all three strains, although the substrates available in the panel tests are not expected to differentiate all *Lactobacillus* species. To attempt more accurate identification of the blood culture isolates we tested their ability to ferment 49 carbohydrates in a commercially available kit for *Lactobacillus* speciation, which resulted in differentiation of the strains to *L. rhamnosus* and *L. casei* (table 2). This was primarily due to the ability of the *L. rhamnosus* strains

to ferment rhamnose and D-arabinose. Although the lack of differentiation of these strains using the VITEK anaerobe system is likely of limited clinical significance, the primary disadvantage of both biochemical identification systems is the requirement for isolated colonies to be grown out from the positive blood culture bottles before analysis.

The analysis of DNA sequence data from the 16S rRNA gene of the blood isolates clearly indicated all three strains were lactobacilli. However, differentiation of species was less obvious due to the high degree of BLAST identity scores and RDP-II similarity scores between the 16S rRNA sequences of some *L. casei* and *L. paracasei* strains, and also between some *L. rhamnosus* and *L. casei* strains. Since Genbank is a public database, there may be some ambiguity as a result of phenotypic misidentification of the *Lactobacillus* species for which 16S rRNA gene sequence have been submitted.

The blood culture isolates were clearly identified as lactobacilli using TRF profiling. The MCRF-271 and MCRF-412 TRF profile had a conclusive SOD score that identified them as *L. rhamnosus*. However, similar to identification by 16S rRNA gene sequencing, determination of species for MCRF-284 was not as obvious. The MCRF-284 TRF profile had similar SOD scores with *L. casei* and *L. zeae*, with a slightly greater difference from *L. paracasei*.

Commercially available biochemical identification systems often require isolation of a microorganism after growth in a blood culture bottle and generally require an additional 1 to 3 days before organism identification. Due to the fact that bacterial DNA can be obtained directly by extraction from positive blood culture bottles for the PCR step of TRF profiling, significant delays that result from the need for isolated colonies for biochemical identification methods are avoided. Also, TRF profiling for bacterial identification is more rapid than sequencing of the 16S rRNA gene, primarily because it does not require a second thermal cycler reaction to produce dye terminated products for sequencing. The analysis of 16S rRNA genes by TRF profiling allows the identification process to be completed within ~8 hours after obtaining a positive blood bottle sample. Reduced diagnostic time has implications for duration of infection, the cost of patient care, length of hospitalization, development of broad-spectrum antibiotic resistance, and mortality due to bacteremia. TRF profiling represents a highly reproducible and predictive source for identification of many organisms associated with bacteremia.

ACKNOWLEDGMENTS

We thank the people at Marshfield Laboratories for provision of blood cultures and isolates for analysis. We also thank Marshfield Clinic Research Foundation for its support through the assistance of Alice Stargardt in the preparation of this manuscript.

REFERENCES

1. Elting LS, Rubenstein EB, Rolston KV, Bodey GP. Outcomes of bacteremia in patients with cancer and neutropenia: observations from two decades of epidemiological and clinical trials. *Clin Infect Dis* 1997;25:247-259.
2. Jarvis WR. Selected aspects of the socioeconomic impact of nosocomial infections: morbidity, mortality, cost, and prevention. *Infect Control Hosp Epidemiol* 1996;17:552-557.
3. Weinstein MP, Towns ML, Quartey SM, Mirrett S, Reimer LG, Parmigiani G, Reller LB. The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of the microbiology, epidemiology, and outcome of bacteremia and fungemia in adults. *Clin Infect Dis* 1997;24:584-602.
4. Rezende NA, Blumberg HM, Metzger BS, Larsen NM, Ray SM, McGowan JE Jr. Risk factors for methicillin-resistance among patients with *Staphylococcus aureus* bacteremia at the time of hospital admission. *Am J Med Sci* 2002;323:117-123.
5. Roghmann MC. Predicting methicillin resistance and the effect of inadequate empiric therapy on survival in patients with *Staphylococcus aureus* bacteremia. *Arch Intern Med* 2000;160:1001-1004.
6. Chassy BM, Murphy CM. *Lactococcus* and *Lactobacillus*. In: Sonenshein AL, Hoch JA, Losick R, eds. *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. Washington DC: American Society for Microbiology; 1993.
7. Husni RN, Gordon SM, Washington JA, Longworth DL. *Lactobacillus* bacteremia and endocarditis: review of 45 cases. *Clin Infect Dis* 1997;25:1048-1055.
8. Antony SJ, Stratton CW, Dummer JS. *Lactobacillus* bacteremia: description of the clinical course in adult patients without endocarditis. *Clin Infect Dis* 1996;23:773-778.
9. Bayer AS, Chow AW, Betts D, Guze LB. Lactobacillemia--report of nine cases. Important clinical and therapeutic considerations. *Am J Med* 1978;64:808-813.
10. Biocca E, Seppilli A. Human infections caused by lactobacilli. *J Infect Dis* 1942;84:112-115.
11. Schlegel L, Lemerle S, Geslin P. *Lactobacillus* species as opportunistic pathogens in immunocompromised patients. *Eur J Clin Microbiol Infect Dis* 1998;17:887-888.
12. Cox SM, Phillips LE, Mercer LJ, Stager CE, Waller S, Faro S. Lactobacillemia of amniotic fluid origin. *Obstet Gynecol* 1986;68:134-135.
13. Bantar CE, Relloso S, Castell FR, Smayevsky J, Bianchini HM. Abscess caused by vancomycin-resistant *Lactobacillus confusus*. *J Clin Microbiol* 1991;29:2063-2064.
14. Brouqui P, Raoult D. Endocarditis due to rare and fastidious bacteria. *Clin Microbiol Rev* 2001;14:177-207.
15. Cooper CD, Vincent A, Greene JN, Sandin RL, Cobian L. *Lactobacillus* bacteremia in febrile neutropenic patients in a cancer hospital. *Clin Infect Dis* 1998;26:1247-1248.
16. Fruchart C, Salah A, Gray C, Martin E, Stamatoullas A, Bonmarchand G, Lemeland JF, Tilly H. *Lactobacillus* species as emerging pathogens in neutropenic patients. *Eur J Clin Microbiol Infect Dis* 1997;16:681-684.
17. Felten A, Barreau C, Bizet C, Lagrange PH, Philippon A. *Lactobacillus* species identification, H₂O₂ production, and antibiotic resistance and correlation with human clinical status. *J Clin Microbiol* 1999;37:729-733.
18. Christensen JE, Stencil JA, Reed KD. Rapid identification of bacteria from positive blood cultures by terminal restriction fragment length polymorphism profile analysis of the 16S rRNA gene. *J Clin Microbiol* 2003;41:3790-3800.
19. Kandler O, Weiss N. Regular, nonsporing Gram-positive rods. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, eds. *Bergey's manual of systematic bacteriology*. Baltimore, MD: Williams & Wilkins; 1986. 1208-1234.

20. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403-410.
21. Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM, Tiedje JM; Ribosomal Database project. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* 2003;31:442-443.
22. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673-4680.
23. Southern EM. Measurement of DNA length by gel electrophoresis. *Anal Biochem* 1979;100:319-323.