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Identification of *Corynebacterium bovis* by Endonuclease Restriction Analysis of the 16S rRNA Gene Sequence

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ABSTRACT

Despite its high prevalence within the bovine mammary gland, *Corynebacterium bovis* is considered a minor pathogen and of limited clinical significance. It has been suggested that intramammary infection with *C. bovis* may protect quarters against subsequent infection with other pathogens. The literature has produced much conflicting data on the subject. A possible explanation for some of the divergence of opinion on the subject is incorrect identification of isolates in previous studies.

This paper describes a novel method for differentiating *C. bovis* from other lipophilic *Corynebacterium* species based on endonuclease restriction analysis. The 16S rRNA gene sequences for all known lipophilic *Corynebacterium* species were obtained from published data and analyzed. It was predicted that endonuclease restriction with *Hin*dIII and *Sma*I could be used to differentiate *C. bovis* from all other known lipophilic *Corynebacterium* species.

The method was successfully employed to identify 741 of 762 (97.2%) lipophilic *Corynebacterium* species as *C. bovis*. Twenty one (2.8%) were identified as species other than *C. bovis*. Using this technique, it was demonstrated that it is not safe to assume that all lipophilic coryneform organisms isolated from bovine milk samples are *C. bovis*.

This method is an alternative to more traditional methods of identification in large scale studies until methods such as 16S rRNA gene sequencing become more widely available.
(**Key words:** *Corynebacterium bovis*, identification, endonuclease restriction analysis, 16S rRNA)

INTRODUCTION

Corynebacterium bovis is one of the most frequently isolated organisms from the bovine mammary gland (Brooks et al., 1983). Despite its high prevalence, *C. bovis* is considered a pathogen of limited clinical significance, primarily associated with subclinical disease (Black et al., 1972; Bramley et al., 1976; Honkanen-Buzalski et al., 1984). Some authors have even suggested that *C. bovis* should be more properly termed a commensal organism (Brooks and Barnum, 1984a).

C. bovis is occasionally associated with disease in other species. It has recently been identified as the aetiological agent of hyperkeratotic dermatitis of athymic nude mice (Duga et al., 1998) and has sporadically been implicated as a cause of opportunistic disease in humans (Bolton et al., 1975; Vale and Scott, 1977; Gupta et al., 1986; Bernard et al., 2002).

Corynebacterium bovis is of interest to mastitis research workers because of the observation that quarters infected with *C. bovis* are significantly less likely to become infected with other more pathogenic organisms (Brooks and Barnum, 1984b; Lam et al., 1997; Schukken et al., 1999). Conversely other authors have demonstrated the opposite affect, i.e., infection with *C. bovis* predisposes the gland to infection with other organisms (Brooks et al., 1983; Hogan et al., 1988).

Corynebacterium species can be divided into lipophilic and nonlipophilic subgroups; the growth of members of the lipophilic subgroup being enhanced by the addition of free fatty acid (e.g., 1% Tween 80) to the growth medium (Funke et al., 1997). *Corynebacterium bovis* is a member of the much smaller lipophilic group (Funke et al., 1997) and is currently the only lipophilic *Corynebacterium* species that has been isolated from milk of bovine origin; although a new lipophilic species (*Corynebacterium mastitidis*) has been isolated from sheep with subclinical mastitis (Fernandez-Garayzabal et al., 1997).

Many laboratories presumptively diagnose *C. bovis* based on the presence of tiny off-white nonhemolytic colonies after 48 to 72 h growth on blood agar cultured at 37°C (Watts et al., 2000). Some strengthen their diagnosis based on Gram stain, morphology, catalase test, and demonstration of enhanced growth with the addition of Tween 80 to the culture media. None of these tests will, however, definitively identify *C. bovis*. For routine mastitis bacteriological examination, the

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misclassification of some isolates as *C. bovis* is of little consequence because of its limited clinical significance. However, for studies and trials that ascribe either pathogenicity or biological significance e.g., a "protective" effect, to the presence of *C. bovis* it is necessary to confirm the identity with more definitive tests.

Identification to a species level can be performed by comparing the biochemical reactions and fermentation profiles of test isolates to those of type strains (Funke et al., 1997). Two commercial identification kits based on these reactions are available (API Coryne and the Biolog system); however, they correctly identified only 88.0 and 54.0% of *C. bovis* isolates in a recent study (Watts et al., 2000), probably because biochemical reactions and fermentation profiles can vary even between members of the same species (Funke et al., 1997).

Sequencing and phylogenetic analysis of the 16S rRNA gene is now recognized as one of the reference techniques for differentiating closely related species. It is particularly useful for *Corynebacterium* species because 16S rRNA gene sequence divergence rates are relatively high (Pascual et al., 1995; Ruimy et al., 1995). However, gene sequencing is still relatively expensive and not within the capabilities of many laboratories, which makes it an unsuitable technique in large-scale studies.

Gene sequencing is currently used extensively as a tool for differentiating closely related species and identifying novel species. Consequently the 16S rRNA gene sequences for the type strains of all currently recognised *Corynebacterium* species are published and available for analysis. This paper describes a technique for differentiating *C. bovis* from other lipophilic *Corynebacterium* species based on endonuclease restriction analysis of the 16S rRNA gene sequence.

MATERIALS AND METHODS

Bacterial Isolates

Aseptic quarter milk samples were collected for microbiological analysis from 505 cows at three sampling time points (end of lactation, at calving, and 7 to 14 d after calving). Samples were frozen immediately after collection and submitted to an accredited laboratory for culture and identification. Samples were cultured using standard techniques (Quinn et al., 1994). *Corynebacterium* species were presumptively identified based on Gram stain, morphology, haemolisin reaction, and catalase test.

Characterization of Coryneform Isolates as Lipophilic or Nonlipophilic

Coryneform isolates were subcultured onto brain heart agar (Merck, Darmstadt, Germany) with and without the addition of 1% Tween 80, vol/vol, to differentiate lipophilic and nonlipophilic isolates (Riegel et al., 1995). Plates were compared after aerobic incubation at 37°C for 48 and 72 h.

Lipophilic species were considered those that demonstrated no or minimal growth on agar without Tween 80 and increased or luxuriant growth on agar containing Tween 80.

Compilation of a 16S rRNA Gene Sequence Database

A 16S rRNA gene sequence database was created from published data for all lipophilic *Corynebacterium* species. Sequence data was imported from GenBank, held by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), into sequence manipulation software (Omiga 2.0, Genetics Computer Group, Inc., Madison, WI). At least one 16S rRNA gene sequence was available for the 11 currently validated lipophilic *Corynebacterium* species [(*C. accolens* (Funke et al., 1997), *C. afermentans* subsp. *lipophilum* (Riegel et al., 1993), *C. apendicis* (Yassin et al., 2002), *C. bovis* (Funke et al., 1997), group F-1 (Funke et al., 1997), group G (Funke et al., 1997), *C. jeikeium* (Funke et al., 1997), *C. lipophiloflavum* (Funke et al., 1997), *C. macginleyi* (Riegel et al., 1995), *C. mastitidis* (Fernandez-Garayzabal et al., 1997), and *C. urealyticum* (Riegel et al., 1992)].

Universal Primer Design

Sequences downloaded into the database were aligned using the sequence manipulation software. Forward and reverse primers were designed to be universal for all available 16S rRNA gene sequences from lipophilic *Corynebacterium* species, located outside the hypervariable regions and positioned close to either end of the 16S rRNA gene. They had a G + C content of 50 to 55%, similar melting temperatures (approximately 60°C) and at least one G or C at the 3′ end. Homopolymeric regions, sequence repeats, and self-complimentary areas were avoided. Forward primer: 5′ – GCG AAC GGG TGA GTA ACA CG – 3′; Reverse primer: 5′ – TCT GCG ATT ACT AGC GAC TCC G – 3′. All 16S rRNA gene sequences held within the database were shortened at the 5′ and 3′ ends, so that sequences began with the forward primer and ended with the reverse primer.

Prediction of Restriction Endonuclease Digest Patterns

Using the 16S rRNA sequence database, restriction endonuclease digest patterns (number and size of resul-

Table 1. Predicted number of restriction sites and size of fragments produced, in base pairs, after endonuclease restriction of lipophillic *Corynebacterium* species 16S rRNA gene sequences with *Hin*dIII and *Sma*I.

Restriction endonuclease	Lipophilic *Corynebacterium* species																				
	A[2]	B[2]	C[2]	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
*Hin*dIII	1[3]	1	1	2	0	0	0	0	2	2	1	0	0	0	0	0	2	2	2	0	0
	346[4]	346	345	48					48	48	347						48	48	48		
	888[4]	886	889	299					45	58	888						58	299	58		
				888					1132	1132							1131	888	1131		
*Sma*I	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0
	488	488	487									494	493	485	472	483					
	746	746	747									747	747	737	732	740					

[1]A = *C. bovis* (AJ222965), B = *C. bovis* (X84444), C = *C. bovis* (AF311389), D = *C. accolens* (X80500), E = *C. afermentans* (X81874), F = *C. afermentans* (X82054), G = *C. afermentans* (X82055), H = *C. apendicis* (AJ314919), I = C. F-1 (X81904), J = C. F-1 (X81905), K = C. G-2 (X80498), L = *C. jeikeium* (X84250), M = *C. jeikeium* (X82062), N = *C. jeikeium* (U87815), O = *C. jeikeium* (U87816), P = *C. jeikeium* (U87823), Q = *C. lipophiloflavum* (Y09045), R = *C. macginleyi* (X80499), S = *C. mastitidis* (Y09806), T = *C. urealyticum* (X81913), and U = *C. urealyticum* (X844439).

[2]The sequence data from 49 *C. bovis* isolates was available for analysis, 3 representative isolates are presented here, data for the other 46 isolates is not shown.

[3]Predicted number of restriction sites with each enzyme.

[4]Predicted size of fragments (in base pairs) after endonuclease restriction.

tant fragments) were predicted for all lipophilic *Corynebacterium* species using the following common enzymes: *Alu*I, *Bam*HI, *Bg*II, *Bg*III, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hinf*I, *Kpn*I, *Mlu*I, *Msp*I, *Nco*I, *Not*I, *Nru*I, *Pst*I, *Pvu*II, *Rsa*I, *Sal*I, *Sfi*I, *Sma*I, *Sph*I, *Taq*I, *Xba*I, and *Xho*I.

Based on the number and size of fragments, it was predicted that endonuclease restriction with *Sma*I and *Hin*dIII could be used to differentiate *C. bovis* from all other lipophilic species (Table 1).

Extraction of Genomic DNA

DNA was extracted from all lipophilic isolates using a method similar to that described by Vaneechoutte et al. (1995). Briefly cellular mass was emulsified in double distilled water and DNA was released from cells by placing the solution in a boiling water bath for 10 min. The resultant solution was allowed to cool and centrifuged briefly to pellet cellular debris.

Polymerase Chain Reaction

Two and a half microliters of boiled product to provide DNA template was amplified by PCR using *Taq* DNA polymerase in a PCR premix solution (*Taq* PCR Mastermix, Qiagen, Crawley, West Sussex, UK). The premix solution provided a final concentration of 1.5 m*M* magnesium chloride and 200 μM of each dNTP. Amplification of the 16S rRNA gene sequence was performed using 50 ng of each of the primer pair described previously, using a Techne Genius FGEN02TD thermocycler (Cambridge, Cambridgeshire, UK) with a heated lid using the following temperature regime: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2.5 min, and concluding with 72°C for 10 min.

PCR Product Purification

The resulting PCR products were purified using a commercially available filter kit (Microcon PCR Centrifugal Filter Devices, Millipore Corporation, Bedford, MA), according to the manufacture's instructions. PCR product was recovered in 20 μL of ultrapure water and frozen at –20°C until required.

Gel Electrophoresis

Purified PCR products were visualized by gel electrophoresis in 1% (wt/vol) agarose gels (Agarose, Transgenomic, Crewe, Cheshire, UK). Electrophoresis was carried out in Tris-acetate buffer at between 4 and 6 V/cm. Gels were stained with 5 μg of ethidium bromide (Sigma, Poole, Dorset, UK), visualized by ultraviolet transillumination and photographed using a gel documentation system (UVP, GDS 7500, Cambridge, Cambridgeshire, UK). Gel images were captured and manipulated using GRAB IT 2.59 (Synoptics Ltd., Cambridge, Cambridgeshire, UK).

Restriction Endonuclease Cleavage

Purified PCR products were cleaved using *Hin*dIII and *Sma*I restriction endonuclease enzymes. Digests were performed in the buffer supplied by the manufacturer [*Hin*dIII (Buffer SB) and *Sma* I (Buffer SA),

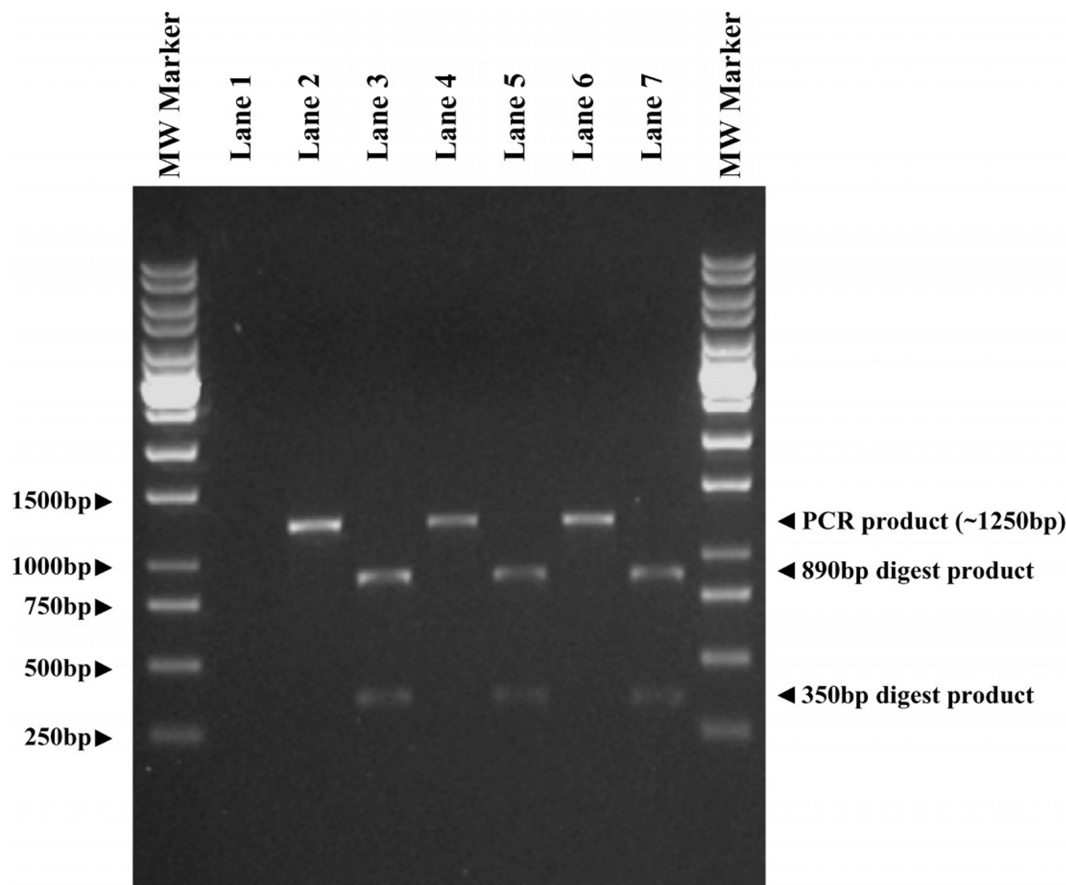

Figure 1. Agarose gel electrophoresis image of the 16S rRNA PCR product of *Corynebacterium bovis* after endonuclease restriction with *Hin*dIII. *Hin*dIII digestion of the 16S rRNA PCR product of *C. bovis* produced 2 digest products (MW ~ 350 and 890 bp), visualized on a 1% agarose gel. Lane 1: Negative control (*Hin*dIII digest reaction with no DNA), Lane 2: Isolate 47 (-ve control, no *Hin*dIII), Lane 3: Isolate 47 after *Hin*dIII digestion, Lane 4: Isolate 462 (-ve control, no *Hin*dIII), Lane 5: Isolate 462 after *Hin*dIII digestion, Lane 6: Isolate 3933 (-ve control, no *Hin*dIII), and Lane 7: Isolate 3933 after *Hin*dIII digestion.

Sigma, Poole, Dorset, UK], using 2.5 units of enzyme (*Hin*dIII and *Sma*I restriction endonucleases, Sigma). The concentration of DNA in the PCR product was estimated by comparison to a known standard marker in the DNA molecular weight ladder (GeneRuler 1-kb DNA Ladder, MBI Fermentas, Vilnius, Lithuania); approximately 500 ng of DNA was used in each restriction reaction. Reactions were incubated for at least 6 h at 37°C (*Hin*dIII) and 25°C (*Sma*I). Cleavage products were separated and visualized in 1% agarose gels as previously described. The size of cleavage products was estimated by comparison to a DNA molecular weight ladder (GeneRuler 1-kb DNA Ladder, MBI Fermentas).

Reference Strains

Six *C. bovis* reference strains were obtained from a culture collection held by the Veterinary Laboratories Agency, England, and processed in the manner outlined above.

RESULTS

Coryneform isolates (n = 939) were identified in the milk samples collected. Of these, 6 were not available for further analysis. Of the 933 isolates examined 762 (81.7%) were lipophilic, and 171 (18.3%) were nonlipophilic.

A PCR product of the predicted size (approximately 1250 bp) was generated by all 762 lipophilic isolates. From these, 741 (97.2%) restricted in the manner predicted for *C. bovis*, i.e., *Hin*dIII and *Sma*I each cut the PCR product once producing fragments of approximately 350 and 890 bp (representative examples are shown in Figure 1) and 490 and 750 bp (representative examples are shown in Figure 2), respectively. Twenty one (2.8%) lipophilic isolates did not restrict in the pattern predicted for *C. bovis*.

Atypical Lipophilic Isolates

Twenty one isolates did not restrict in the pattern predicted for *C. bovis*. Of these, 18 were not cut by

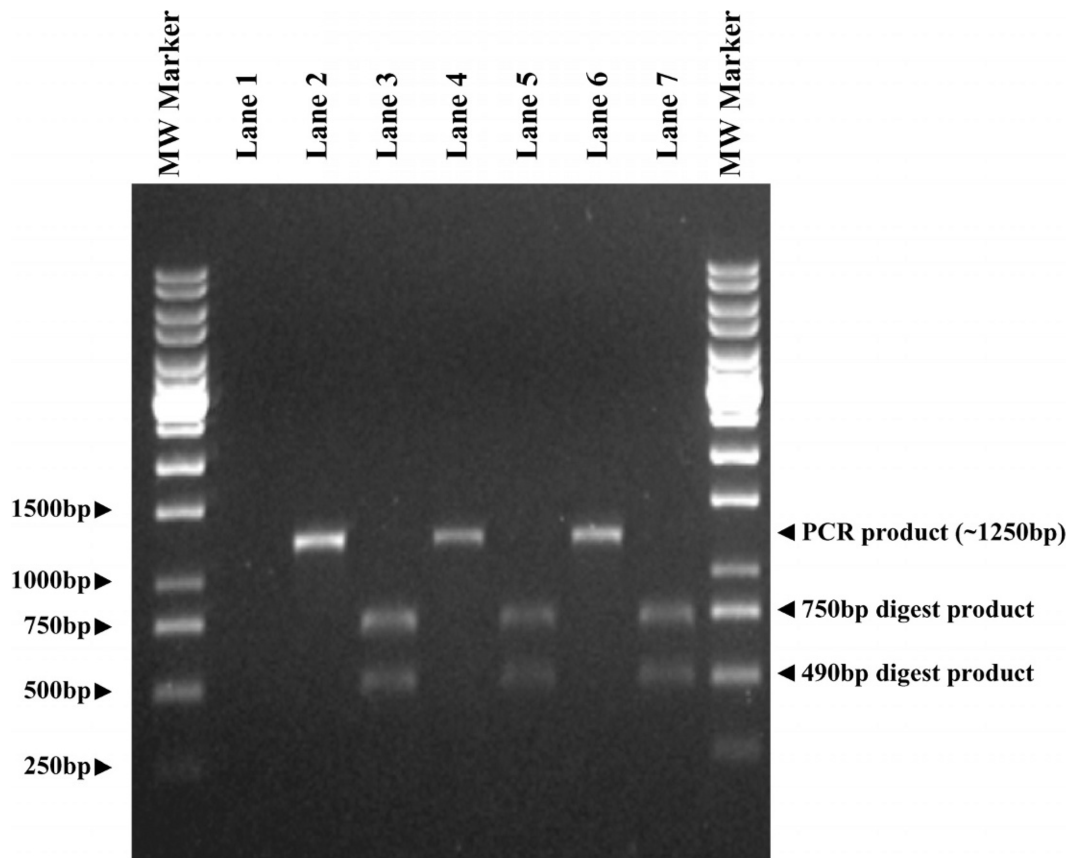

Figure 2. Agarose gel electrophoresis image of the 16S rRNA PCR product of *Corynebacterium bovis* after endonuclease restriction with *Sma*I. *Sma*I digestion of the 16S rRNA PCR product of *C. bovis* produced 2 digest products (MW ~ 490 and 750 bp), visualized on a 1% agarose gel. Lane 1: Negative control (*Sma*I digest reaction with no DNA), Lane 2: Isolate 47 (-ve control, no *Sma*I), Lane 3: Isolate 47 after *Sma*I digestion, Lane 4: Isolate 462 (-ve control, no *Sma* I), Lane 5: Isolate 462 after *Sma*I digestion, Lane 6: Isolate 3933 (-ve control, no *Sma*I), and Lane 7: Isolate 3933 after *Sma*I digestion.

*Hin*dIII but were cut in an identical fashion to *C. bovis* isolates with *Sma*I (Representative examples are given in Figures 3 and 4). Two were not cut by either enzyme and the remaining isolate was not cut by *Sma*I but was cut in an identical fashion to *C. bovis* isolates with *Hin*dIII.

Reference Strains

All 6 reference isolates restricted in the manner predicted for *C. bovis*.

DISCUSSION

The enzymes *Alu*I, *Cfo*I and *Rsa*I (Vaneechoutte et al, 1995), and *Hpa*I and *Pst*I (Wattiau et al., 2000) have been used to investigate the *Corynebacterium* genus by previous research workers. In both cases, the authors made no attempt to predict the outcome of restriction with the enzymes they used; they simply relied on different species restricting with different patterns. To the authors knowledge this study is the first in which members of the *Corynebacterium* genus have been differentiated from other species using enzymes for which the number and size of resultant DNA fragments was predicted prior to restriction. This has only become possible because of the increasingly widespread use of 16S rRNA gene sequencing as a method of identifying new species. Consequently, the sequences for all currently recognized lipophilic members of the *Corynebacterium* genus are freely available for analysis. Predicting the number and size of DNA fragments makes restriction analysis a much more powerful diagnostic tool because it enables enzymes to be selected for specific purposes, e.g., differentiating one species from all others within a group. It also becomes possible to create decision trees, which allow all members of a group or genus to be identified by the progressive application of different

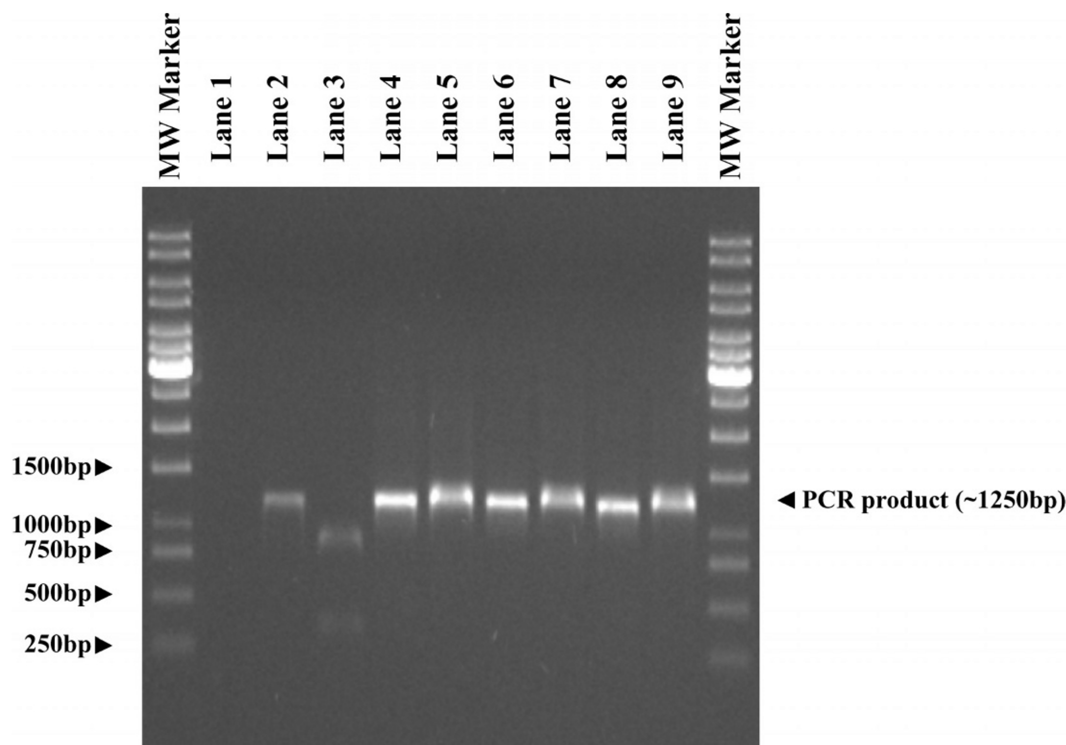

Figure 3. Agarose gel electrophoresis image of the 16S rRNA PCR product of isolates 4376, 4396, and 4465 after endonuclease restriction with *Hin*dIII. *Hin*dIII digestion of the 16S rRNA PCR product of isolates 4376, 4396, and 4465 produced no digest products, visualized on a 1% agarose gel. Lane 1: Negative control (*Hin*dIII digest reaction, no DNA), Lane 2: Positive control (*Corynebacterium bovis*, no *Hin*dIII), Lane 3: Positive control (*C. bovis* after *Hin*dIII digestion), Lane 4: Isolate 4376 (-ve control, no *Hin*dIII), Lane 5: Isolate 4376 after *Hin*dIII digestion, Lane 6: Isolate 4396 (-ve control, no *Hin*dIII), Lane 7: Isolate 4396 after *Hin*dIII digestion, Lane 8: Isolate 4465 (-ve control, no *Hin*dIII), and Lane 9: Isolate 4465 after *Hin*dIII digestion.

enzymes. This technique may be especially useful if species can be divided into small but distinct groups by the application of simple and reliable tests (e.g., demonstration of lipophilic nature in this study).

16S rRNA gene sequencing has recently been successfully employed to identify and differentiate *C. bovis* from other coryneforms present in milk (Watts et al., 2000) and is probably now the method of choice for definitively identifying *Corynebacterium* species. However, the technique is still relatively expensive and not within the capabilities of most diagnostic laboratories. Commercially available identifications kits are a cheaper alternative but correctly identified only 88.0 and 54.0% of *C. bovis* isolates in a recent study (Watts et al., 2000). Compared to sequencing, the endonuclease restriction analysis method outlined here is relatively cheap and easy to perform. Until 16S rRNA sequencing becomes more generally accessible, the method described here is a viable alternative to more traditional methods of identification (e.g., biochemical reactions and fermentation profiles) for the differentiation of *C. bovis* from other lipophilic *Corynebacterium* species. The method is particularly appropriate for large-scale field studies investigating the prevalence and significance of *C. bovis*. In these situations an accurate diagnosis is essential so that any protective or pathogenic affects demonstrated can be correctly accredited to *C. bovis*.

Endonuclease restriction analysis of the 16S rRNA gene sequence identified 97.2% of lipophilic coryneform isolates present in milk samples as *C. bovis*. The other 2.8% were identified as species other than *C. bovis*. To the authors' knowledge, this is the first time that lipophilic coryneform species other than *C. bovis* have been identified in bovine milk, although a novel lipophilic *Corynebacterium* species (*C. mastitidis*) has recently been identified in sheep with subclinical mastitis (Fernandez-Garayzabal et al., 1977). None of the isolates examined as part of the study described here restricted with the pattern predicted for *C. mastitidis*.

Ongoing work has demonstrated that 18 of the 21 isolates (not cut by *Hin*dIII but cut identically to *C. bovis* isolates with *Sma*I) that were identified as species other than *C. bovis* may well represent a novel lipophilic

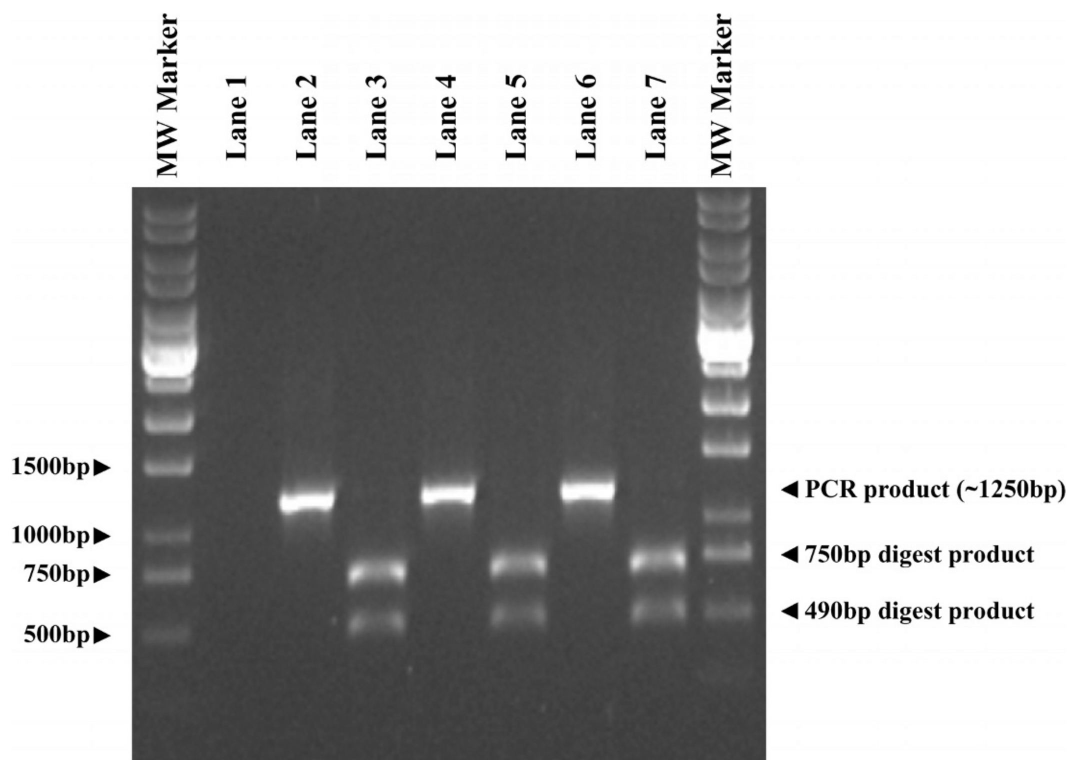

Figure 4. Agarose gel electrophoresis image of the 16S rRNA PCR product of isolates 4376, 4396, and 4465 after endonuclease restriction with *Sma*I. *Sma*I digestion of the 16S rRNA PCR product of isolates 4376, 4396, and 4465 produced two digest products (MW ~ 490 and 750 bp), visualized on a 1% agarose gel. Lane 1: negative control (*Sma*I digest reaction, no DNA), Lane 2: Isolate 4376 (-ve control, no *Sma*I), Lane 3: Isolate 4376 after *Sma*I digestion, Lane 4: Isolate 4396 (-ve control, no *Sma*I), Lane 5: Isolate 4396 after *Sma*I digestion, Lane 6: Isolate 4465 (-ve control, no *Sma*I), and Lane 7: Isolate 4465 after *Sma*I digestion.

Corynebacterium species. These isolates will be described in a future publication. Further restriction analysis of the remaining 3 isolates with *Pst*I, *Rsa*I, and *Pvu*II tentatively identified one as "*C. genitalium*", a *Corynebacterium* species not currently validated. The remaining 2 isolates did not restrict in a pattern predicted for any currently recognised lipophilic *Corynebacterium* species and remained untyped at the end of the study.

Corynebacterium bovis could be differentiated from all other currently recognized lipophilic species using the restriction endonucleases *Sma*I and *Hin*dIII. Of the 49 *C. bovis* 16S rRNA gene sequences suitable for analysis, 47 had identical predicted restriction patterns after digestion with these 2 enzymes, only 2 did not. One sequence (AF311397) contained an extra *Sma*I restriction site and another (AF311419) contained an extra *Hin*dIII restriction site. No lipophilic coryneform isolate analyzed during the course of this study restricted in the pattern predicted for either of these sequences. If the extra restriction sites identified in these 2 sequences are real, it would appear that the prevalence of strains carrying these sequence variations are low, or they may represent strains more prevalent in other geographical locations. In fact, it appears that most of the *C. bovis* 16S rRNA sequences currently published are from isolates originally identified in North America.

CONCLUSIONS

This paper describes a novel method for differentiating *C. bovis* from other lipophilic *Corynebacterium* species based on endonuclease restriction analysis of the 16S rRNA gene sequence with *Hin*dIII and *Sma*I. Results of the analysis suggest that it is not safe to assume that all lipophilic coryneforms isolated from milk are *C. bovis*. Three percent of isolates were identified as species other than *C. bovis* by this method. For routine analysis of bovine milk samples this error is of little consequence and therefore a presumptive diagnosis of *C. bovis* based on phenotypic and growth characteristics is adequate. However, for epidemiological studies, investigations of *C. bovis* clinical mastitis outbreaks and studies relating to the properties, characteristics, and

pathogenic interactions of *C. bovis*, further tests such as biochemical reactions, fermentation profiles, 16S rRNA gene sequencing or the endonuclease restriction technique described here are necessary to confirm species identity.

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