JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1985, p. 562–564
0095-1137/85/040562-03$02.00/0

Vol. 21, No. 4

DNA Restriction Endonuclease Analysis of *Mycobacterium bovis* and Other Members of the Tuberculosis Complex

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Received 3 October 1984/Accepted 31 December 1984

DNA preparations from 24 New Zealand isolates, two reference strains of *Mycobacterium bovis*, and one reference strain each of *Mycobacterium microti*, *Mycobacterium africanum*, and *Mycobacterium tuberculosis* were characterized by restriction endonuclease analysis. Twenty-five restriction enzymes were investigated. The clearest differences in *M. bovis* patterns were obtained with the enzymes *Bst*EII and *Bcl*I. These produced four and five different patterns, respectively, for the 24 local isolates. When the results from both enzymes were considered, seven different combinations were obtained. The patterns produced for the two reference strains of *M. bovis* could be distinguished from each other and also from the patterns produced for the local isolates. All patterns were reproducible and are now being used for typing *M. bovis* isolates. With either enzyme, the patterns produced for the *M. tuberculosis*, *M. bovis*, and *M. africanum* strains had many features in common, but all the *M. bovis* patterns were clearly more similar to each other than to the *M. tuberculosis* patterns. The patterns produced for the *M. microti* strain were markedly different from those produced for the other species. Restriction endonuclease analysis is clearly a useful method for inter- and intraspecific classifications of the tuberculosis complex.

The tuberculosis complex (17, 19) consists of four closely related species: *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium microti*, and *Mycobacterium africanum*. Although this classification has historical significance and some epidemiological uses, all members of the complex have so many features in common that their status as separate species is widely disputed (15, 16, 18).

Although *M. bovis* was legitimately accorded species status in 1970 (8), it has been recognized as a separate entity for many years. It is the cause of endemic tuberculosis in cattle on a global scale (10) and is pathogenic for many other animals, including humans. Concerted efforts to control bovine tuberculosis have been made in many countries (9), but complete eradication has proved difficult to achieve. In New Zealand, this lack of success has been attributed to reservoirs of *M. bovis* in feral animals, principally brush-tailed possums (*Trichosurus vulpecula*) (3). The absence of firm evidence is due to the difficulty in observing transmission under natural conditions and to the lack of a sensitive and reliable technique for intraspecific typing of *M. bovis* (3).

Phage susceptibility has provided a typing system for *M. tuberculosis*, but efforts to extend this technique to *M. bovis* have been unproductive (14). Attempts were made to type *M. bovis* strains on the basis of their ability to remove amino acids from a solution, but the results were only partially reproducible (1). Recently, we reported the use of restriction endonuclease analysis to identify different types of *M. tuberculosis* (4). In this technique, high-molecular-weight DNA is digested with a restriction enzyme, and the fragments produced are separated by gel electrophoresis. Strains are characterized on the basis of their fragment patterns. The technique was highly reproducible. This report describes the application of the technique to cultures of *M. bovis* isolated from 24 feral and pastoral animals and to reference strains of all four species of the tuberculosis complex.

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MATERIALS AND METHODS

Bacteria. The 24 local isolates of *M. bovis* were cultured from animal tissues submitted for routine isolation and identification of mycobacteria. The samples were chosen to include the animals most commonly infected in New Zealand (cattle, deer, pigs, and brush-tailed possums) and to cover as wide a geographical area as possible. *M. bovis* isolates were identified by their acid-fast staining, slow growth, colony characteristics, growth on pyruvate-supplemented medium but not on glycerol-supplemented medium, and sensitivity to isoniazid (10 μg/ml) and 2-thiophenecarboxylic acid hydrazide (10 μg/ml). *M. tuberculosis* TMC 102 (ATCC 27294, type strain, H37Rv), *M. microti* TMC 1601 (ATCC 11152), and *M. bovis* TMC 410 (ATCC 19210, type strain) were obtained from the National Institutes of Health, Bethesda, Md.; *M. africanum* ATCC 25420 (type strain) was obtained from the American Type Culture Collection, Rockville, Md.; and *M. bovis* BCG was a commercial vaccine preparation produced by Glaxo Laboratories Ltd., London, England.

Restriction endonuclease analysis. DNA was prepared as described previously (4, 12). Briefly, cultures were grown in 200 ml of liquid medium for 6 to 10 weeks and then heat killed. The cells (0.2 to 1.0 g [wet weight]) were washed, treated with lysozyme, and then lysed with sodium dodecyl sulfate in the presence of nonspecific proteases. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and purified by dialysis. Its concentration in the final preparation was measured by fluorimetry (11). DNA samples (4 μg) were digested with 20 to 40 U of each of the following restriction enzymes at the temperature and with the buffer specified by the supplier: *Apa*I, *Bam*HI, *Bcl*I, *Bgl*I, *Bst*EII, *Bst*XI, *Cla*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Kpn*I, *Mlu*I, *Nae*I, *Not*I, *Pst*I, *Sac*I, *Sal*I, *Sca*I, *Sfi*I, *Sma*I, *Tth*111I, *Xba*I, *Xho*I, and *Xmn*I. Restriction enzymes were obtained from New England Biolabs, Beverly, Mass., with the exception of *Hae*III, *Sal*I, and *Sma*I, which were obtained from Sigma Chemical Co., St. Louis, Mo. Digests were analyzed by gel electrophoresis on 330-mm-long gels of 1% agarose (ultrapure DNA grade; Bio-Rad Laboratories, Richmond,

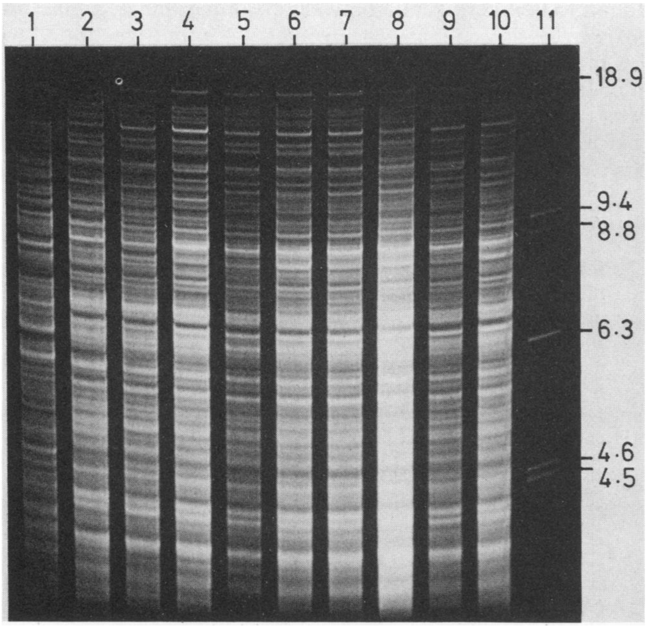

FIG. 1. Fragment patterns after *BclI* digestion of DNA from the following: lane 1, *M. microti* TMC 1601; lane 2, *M. tuberculosis* H37Rv; lane 3, *M. africanum* ATCC 25420; lane 4, *M. bovis* TMC 410; lane 5, *M. bovis* BCG; lanes 6 through 10, *M. bovis* local isolates; and lane 11, λ phage. The sizes of the λ phage fragments are given in kilobase pairs.

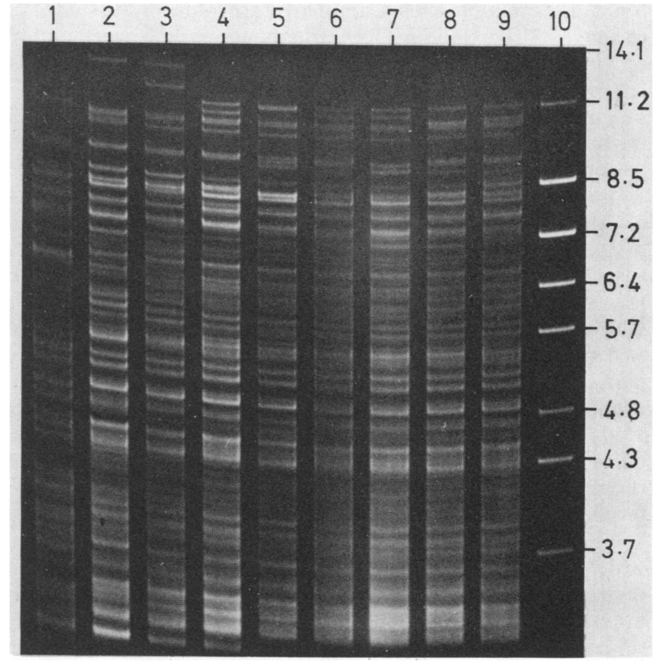

FIG. 2. Fragment patterns after *BstEII* digestion of DNA from the following: lane 1, *M. microti* TMC 1601; lane 2, *M. tuberculosis* H37Rv; lane 3, *M. africanum* ATCC 25420; lane 4, *M. bovis* TMC 410; lane 5, *M. bovis* BCG; lanes 6 through 9, *M. bovis* local isolates; and lane 10, λ phage. The sizes of the λ phage fragments are given in kilobase pairs.

Calif.) run for 24 h at 100 V in a horizontal electrophoresis tank (430 mm long and 200 mm wide). The buffer was recirculated from the anode compartment to the cathode compartment at 8 ml/min with a peristaltic pump. After electrophoresis, the gels were stained for 30 min in ethidium bromide (1 μg/ml), placed on a UV transilluminator, and photographed by means of a 120-format plate camera with a Wratten 23A gelatin filter.

RESULTS

The restriction enzymes which produced well-resolved patterns and which distinguished between the different species were *BclI*, *BglI*, *BstEII*, *ClaI*, *EcoRI*, *EcoRV*, *SmaI*, and *XhoI*. Of these, *BclI* and *BstEII* produced the best differentiation between *M. bovis* strains. The remaining 17 enzymes either did not digest the DNA completely or produced more fragments than could be resolved into a clear pattern with the electrophoresis system used. Figure 1 shows *BclI* digest patterns of DNA from the five reference strains together with the five different patterns that were found for the 24 local isolates of *M. bovis*. A *BclI* digest of λ phage DNA was run in parallel to indicate molecular sizes. The seven fragment lines of the largest molecular size appeared to be identical for *M. africanum* and all the *M. bovis* strains, but differences occurred below that region, particularly at fragment lines 8, 9, and 13 through 15 (numbering from the top of the gel). The *BstEII* digest patterns are shown in Fig. 2; a partial *BstEII* digest of λ phage DNA indicates molecular sizes. When this enzyme was used, four different patterns were found for the 24 local isolates of *M. bovis*. The patterns of the two reference strains of *M. bovis* were different from each other and from those of all the local isolates with either enzyme. When the results with both enzymes were considered together, the 24 local isolates were separated into seven different combinations (Table 1).

The 13 isolates which had the same combination of patterns were not restricted to any of the four animal species and were from all three areas of New Zealand that have a major problem with bovine tuberculosis. When DNA was prepared a second time from recultured strains representing each of these combinations and was then digested with the same enzymes, patterns identical to the original patterns were obtained.

DISCUSSION

The digestion of mycobacterial DNA with restriction endonucleases produced a large number of DNA fragments which were difficult to differentiate. The resolution of the fragments shown in Fig. 1 and 2 was an improvement over that obtained in our earlier study on *M. tuberculosis* and was achieved by carrying out electrophoresis on longer gels and

TABLE 1. Combinations of *BclI* and *BstEII* patterns for 24 New Zealand isolates of *M. bovis*

No. of isolates with same combination	Combinations of restriction enzyme patterns for *M. bovis* local isolates[a]	
	BclI pattern (Fig. 1)	*BstEII* pattern (Fig. 2)
13	6	6
3	9	8
3	9	9
2	6	9
1	7	7
1	8	8
1	10	8

[a] Numbers refer to lanes in the indicated figures.

for a longer time. This improved resolution was at the expense of the smaller DNA fragments, which were eluted off the end of the gel. However, when electrophoresis was carried out for a shorter time, no differences were observed in the smaller DNA fragments of the *M. bovis* strains. A third of the restriction enzymes used produced well-resolved patterns, but even the most discriminating enzymes, *Bcl*I and *Bst*EII, revealed relatively few differences in the DNAs of different *M. bovis* strains, including *M. bovis* BCG. The biological significance of these differences cannot be assessed from the present work. However, the finding of so few differences reflects the very close genetic similarity within this species. The differences in the *Bcl*I patterns of two of the *M. bovis* strains (Fig. 1, lanes 6 and 7) were particularly subtle and required careful comparison of the original gel photographs. As with the patterns of the other *M. bovis* strains, which could be distinguished more easily, these differences were entirely reproducible. The ability of restriction endonuclease analysis to detect intraspecific differences has also been noted for other bacterial genera (2, 5, 7, 12, 13), and it is probable that the technique will become widely used for epidemiological studies. Whether certain restriction enzymes will prove to be more suitable than others for studying a wide range of different bacterial species remains to be determined. However, it is interesting to note that *Bst*EII, which discriminated between *M. bovis* strains, was also used successfully in earlier work on *M. tuberculosis* (4) and *Campylobacter* species (5). In the present study, seven different pattern combinations were found for the 24 local isolates of *M. bovis*. These are being used as the basis for the first reliable typing system for this species.

The patterns of *M. bovis*, *M. africanum*, and *M. tuberculosis* shared many fragments of apparently identical size, particularly below 8 kilobase pairs. On careful inspection of the original photographs, some differences in these patterns could be seen down to fragments of 4 kilobase pairs, but the clearest differences were seen in the 15 fragments of the largest molecular size. *M. tuberculosis* H37Rv and two clinical isolates of *M. tuberculosis*, all of which were compared previously (4), had patterns more similar to each other than to the patterns of any of the *M. bovis* strains. These two limited studies based on genetic differences confirmed the conclusion of two large studies of phenotypic differences (6, 17) that *M. tuberculosis* and *M. bovis* strains are closely related but are distinct entities. Not enough experience has yet been gained with restriction endonuclease analysis to draw any firm conclusions about whether these entities should be accorded species status. The situation may become clearer when other mycobacterial species have been analyzed by the same technique. However, it is notable that there were fewer pattern differences between *M. bovis* and *M. tuberculosis* strains than there are within some species from other genera (2, 5, 12).

Only one strain each of *M. microti* and *M. africanum* was analyzed and, although these were reference strains, their restriction enzyme patterns may not be representative of their respective species. Nevertheless, it is interesting to note that although the *M. microti* patterns were very different from those of the other species, the *M. africanum* patterns had many fragment lines in common with both the *M. bovis* and the *M. tuberculosis* patterns. After *Bcl*I digestion, the *M. africanum* pattern was more similar to the patterns of the *M. bovis* strains, whereas after *Bst*EII digestion, it appeared more similar to the *M. tuberculosis* patterns. This particular strain of *M. africanum* was also found to be intermediate between *M. bovis* and *M. tuberculosis* by pyrolysis mass spectrometry (19) but was found to be more similar to *M. bovis* than to *M. tuberculosis* by numerical taxonomy (6). The present study indicates that, as well as its role in intraspecific typing, restriction endonuclease analysis has great potential for classifying closely related species.

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