A rapid method for identification of *Mycobacterium* species by polyacrylamide gel electrophoresis of soluble cell proteins

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Summary. The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of an easily and rapidly prepared soluble protein fraction were used in conjunction with conventional techniques to identify different strains of *Mycobacterium tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. avium*, *M. kansasii*, *M. marinum*, *M. gastri*, *M. simiae* and *M. malmoense*. Complete concordance of results from both methods was obtained with all species except those of the *M. tuberculosis* complex. With the SDS-PAGE technique, all strains of the *M. tuberculosis* complex were recognised as belonging to one species. By visual analysis of the SDS-PAGE polypeptide profiles, only minor differences between strains of the same species were seen and each species showed a characteristic polypeptide profile. Quantitation of the data by calculation of the Dice coefficient of similarity of the band positions obtained by densitometry indicated that the similarity between different strains of one species was 90–100% and the similarity between the species was in the range 30–45%. The results indicate that SDS-PAGE is a simple and rapid method for identifying mycobacterial strains.

Introduction

In a routine laboratory, the identification of slowly growing mycobacterial strains on the basis of various biochemical reactions is very time consuming.^{1,2} Only strains belonging to the *Mycobacterium tuberculosis* complex are fairly easily recognised biochemically, or by hybridisation with a DNA-probe³ or by use of monoclonal antibodies.⁴ However, the number of strains isolated from clinical material and not belonging to the *M. tuberculosis* complex is increasing.⁵ In our laboratory up to 40% of mycobacterial isolates do not belong to the *M. tuberculosis* complex; all these isolates must be identified, because not all species that do not come within the *M. tuberculosis* complex are potentially pathogenic in man. Moreover, in the clinical setting this needs to be done easily and rapidly. Furthermore, the study of infectious diseases depends on accurate identification of isolates.

For all these reasons, we sought a suitable method for identifying all mycobacterial species. Several investigators have used sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of soluble bacterial polypeptides successfully as a taxonomic tool,^{6,7} and others have shown that densitometric analysis of stained gels provides data suitable for numerical taxonomy.^{8,9} The aim of this study was to evaluate the usefulness of SDS-PAGE as a tool for identifying mycobacterial isolates. This paper de-

scribes our results with SDS-PAGE of soluble polypeptides of strains of the *M. tuberculosis* complex and of seven other mycobacterial species.

Material and methods

Bacterial isolates and culture conditions

A total of 191 clinical isolates was studied, comprising strains of *M. tuberculosis* (50 isolates), *M. bovis* (16), *M. africanum* (12), *M. bovis* BCG (13), *M. avium* (50), *M. kansasii* (9), *M. marinum* (11), *M. gastri* (10), *M. simiae* (3), *M. xenopi* (7), *M. malmoense* (10), together with the following reference strains—*M. tuberculosis* ATCC 35836, *M. gastri* ATCC 15754, *M. marinum* ATCC 927, *M. simiae* ATCC 25275 and *M. xenopi* ATCC 19250. The reference strains were obtained from the American Type Culture Collection, Rockville, MD. All strains were identified at our laboratory as described elsewhere.^{10,11}

Mycobacterial strains were grown routinely on Coletsos slants (Diagnostics Pasteur, Marnes-la-Coquette, France)¹² or in Modified Dubos Broth (MDB: Middlebrook 7H9 Broth, Difco, 4.7 g; Bactocasitone, Difco, 0.5 g; L-asparagine 4.0 g; L-glutamic acid 1.0 g; sodium pyruvate 1.6 g; yeast extract 1.0 g; biotin 5.0 mg; glycerol 50 ml; distilled water 950 ml; pH 6.6 at 25°C; after sterilisation at 121°C for 15 min, 50 ml inactivated new born calf serum, Gibco, was added). All chemicals were from Sigma, St Louis, MO, USA

unless otherwise stated. Acrylamide was from Serva Feinbiochemica, Heidelberg, Germany.

Preparation of samples for SDS-PAGE

Bacterial suspensions were prepared either by scraping growth from Coletsos slants or by growing the bacteria in 25-ml volumes of MDB at 35°C for 1–6 weeks and harvesting by centrifugation at 5000 *g* for 15 min at 4°C. In both cases, the cell mass was washed twice in 25 ml of 10 mM Tris-HCl buffer, pH 8.2 at 4°C. The cell suspension was transferred to a 1.5-ml Eppendorf vessel and the wet weight of the cell mass was determined after centrifugation and removal of the supernate. Then, for each 1 mg of cell mass, 2 µl of buffer (5 mM Tris-HCl, pH 7.3) was added and the bacterial cells were resuspended by sonication for 30 s at 60% pulse and 20% maximal power in an Ultrasonics type W225 sonifier (Heat Systems-Ultrasonics Inc., Farmingdale, NY, USA) equipped with a microtip. This procedure yielded a homogeneous suspension.

The cell suspension in the Eppendorf vessel was now quickly frozen in cold (–70°C) ethanol and then thawed in water at 40°C. This freeze and thaw procedure was repeated three times to release membrane proteins. The suspension was then centrifuged at 10 000 *g* for 10 min at 4°C and the clear supernate, called the soluble protein fraction, was used for electrophoresis. The soluble protein fractions were stored at –70°C.

The soluble protein fraction was mixed in 3:1 proportions with 0.2 M Tris-HCl buffer, pH 6.8, containing SDS 8% w/v, sucrose 40% w/v, 2-mercaptoethanol 20% v/v and bromophenol blue 0.016% w/v. Before application to the gel, the samples were boiled for 10 min and centrifuged for 5 min at 10 000 *g* and the supernate was used for gel electrophoresis.

Gel electrophoresis

SDS-PAGE analysis of the soluble protein fraction sample was performed by a modified version of the technique of Laemmli.¹³ A slab gel (160 × 120 × 1.0 mm), built up as a vertical 10–20% gradient (acrylamide: bisacrylamide, ratio 28:1) in 0.375 M Tris-HCl, pH 8.8, SDS 0.1% w/v, was used. The 10-mm stacking gel was acrylamide: bis-acrylamide (ratio 29:1 3% in 0.125 M Tris-HCl, pH 6.8, SDS 0.2% w/v. A sample (10 µl, containing 60–80 µg of protein estimated by quantitative densitometry) of the soluble protein fraction was applied to each well. The gel was run for 16 h at a constant current of 10 mA without cooling or for 4 h at a constant current of 40 mA with cooling (10°C) in a Biorad Protean II gel apparatus. The gel was removed and stained in Coomassie Brilliant Blue R-250 0.25% w/v in methanol: acetic acid: water (25:10:65 v:v:v) and destained in the same solvent mixture. Low mol. wt protein markers (Pharmacia, Uppsala, Sweden) were run in the same gel to allow the estimation of mol. wts. Densitometric

analysis was performed with an LKB 2202 Ultrosan Laser Densitometer with Nelson software.

Comparison of polypeptide profiles

The average similarity between any two strains was assessed by use of the coefficient of Dice¹⁴ whereby: average percentage similarity (%S) = (number of matching bands × 2) / (total number of bands in both strains). The calculation of similarity was based on a visual comparison of the densitometric profiles and was done independently by two individuals. Extracts of strains were run several times to confirm the results.

Results

The complexities of the polypeptide profiles obtained by the method used were similar to those obtained after cell disruption by high energy sonication (5 min, 50% pulse, 80% of a maximum power of 45 W)(data not shown). Furthermore, the size of the sample of the soluble protein fraction did not affect greatly the complexity of the electrophoretogram. For instance, if the size of the sample of the soluble protein fraction of *M. tuberculosis* strains was increased from 30 µg to 250 µg of protein, the number of protein bands in the electrophoretogram decreased from 43 to

Fig. 1. Soluble protein fraction polypeptide profiles of *M. tuberculosis* tub 1159 cultured on five different media. Track 1: mol. wt markers—I. phosphorylase b (94 Kda); II. bovine albumin (67 Kda); III. ovalbumin (43 Kda); IV. carbonic anhydrase (30 Kda); V. trypsin inhibitor (20.1 Kda); VI. lactalbumin (14.4 Kda). 2–6: The profile obtained after culture (2) on 7H10 slants, (3) on Coletsos slants (bovine type), (4) on Coletsos slants (human type), (5) in MDB, (6) on MDB with agar 1% w/v slants.

Fig. 2. Soluble protein fraction polypeptide profiles of species of the *M. tuberculosis* complex. Track 1: mol. wt markers (see fig. 1); 2-4: *M. tuberculosis* strains tub 1129, tub 1159 and tub 1013 respectively; 5-7: *M. bovis* strains bov 1225, bov 1250 and bov 980; 8-10: *M. africanum* strains afr 3001, afr 3055 and Myc 4470; 11 and 12: *M. bovis*-BCG strains BCG 1193 and BCG 1228 (strain BCG 1228 is the Dutch vaccine strain).

Fig. 3. Soluble protein polypeptide profiles of eight mycobacterial species and *Streptococcus pyogenes*. Track 1: mol. wt markers (see fig. 1); 2: *M. tuberculosis* ATCC 35836; 3: *M. avium* AV 942; 4: *M. kansasii* KANS 991; 5: *M. marinum* ATCC 927; 6: *M. gastri* ATCC 15754; 7: *M. simiae* ATCC 25275; 8: *M. xenopi* ATCC 19250; 9: *M. malmoense* MYC 6138; 10: *S. pyogenes*.

39 and the Dice coefficient of similarity between the electrophoretograms was >90%. For that reason, 60–80 µg of protein was routinely loaded. Also, the duration of incubation (up to 6 weeks) or the culture medium used (see *Materials and methods*) did not affect the electrophoretogram significantly. In fig. 1, electrophoretograms are shown of an *M. tuberculosis* strain cultured on five different media. Apart from some differences in the amount of protein in some bands, only one extra protein band of 26 Kda was seen in the electrophoretogram obtained from the 7H10 slant. Similar results were obtained with other mycobacterial species.

The *M. tuberculosis* complex. A total of 91 strains belonging to different species of the *M. tuberculosis* complex was studied. All gave a similar band pattern by visual judgement (fig. 2); only minor differences in the relative amounts of protein in some bands were noted. Thus, irrespective of the biochemical classification, little variation of the band pattern was found between the strains. The number of bands varied from 41 to 43 and at least 90% of the bands were present in all strains investigated. The band position data obtained by densitometry were used to calculate the Dice coefficient of similarity and so the visual judgement in terms of co-migrating bands was given a quantitative assessment. The average Dice coefficient between strains was 96% (range 92–100%).

Other mycobacterial species. A total of seven other mycobacterial species was studied. Each species yielded a characteristic electrophoretogram; however, electrophoretograms of strains belonging to one species showed little variation by visual judgement. A more objective assessment was made by calculating the Dice coefficient of similarity from the densitometric profiles. The similarity between strains of one species was >90% (table).

Therefore, in this respect, the results were equivalent to those found with strains of the *M. tuberculosis* complex. The electrophoretograms showed 35–45 bands, depending on the species; each species gave a characteristic and distinct band profile (fig. 3). In fig.

Fig. 4. Representative examples of densitometric profiles of SDS-PAGE band profiles of (A) *M. tuberculosis* and (B) *M. simiae* as used to calculate the coefficient of Dice. Mol. wt markers: a, phosphorylase b (94 Kda); b, bovine albumin (67 Kda); c, ovalbumin (43 Kda); d, carbonic anhydrase (30 Kda); e, trypsin inhibitor (20.1 Kda); f, lactalbumin (14.4 Kda).

4, two representative examples of densitometric profiles are shown. Such densitometric profiles are more suitable for mutual comparison and, therefore, also more appropriate for identification than are electrophoretograms. The Dice coefficient of similarity between strains of different species varied from 30% to 45% (table).

Discussion

This study showed that all strains belonging to the *M. tuberculosis* complex had a similar SDS-PAGE band profile and, therefore, could not be differentiated from each other by SDS-PAGE band profiles. *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. bovis*-BCG strains were recognised as belonging to a single species with a high degree of homogeneity. This result is in agreement with the results of DNA homology¹⁵ and numerical classification studies.

Table. Percentage similarity (%S) of soluble protein profiles of mycobacterial species shown in fig. 3

	<i>M. tuberculosis</i>	<i>M. avium</i>	<i>M. kansasii</i>	<i>M. marinum</i>	<i>M. gastri</i>	<i>M. simiae</i>	<i>M. xenopi</i>	<i>M. malmoense</i>	<i>Streptococcus pyogenes</i>
<i>Streptococcus pyogenes</i> *	19	23	19	23	21	26	24	24	94†
<i>M. malmoense</i>	35	38	33	41	37	29	31	98†	
<i>M. xenopi</i>	34	33	35	39	28	40	91†		
<i>M. simiae</i>	30	41	45	36	33	97†			
<i>M. gastri</i>	31	37	34	31	95†				
<i>M. marinum</i>	35	38	41	94†					
<i>M. kansasii</i>	36	37	96†						
<i>M. avium</i>	34	92†							
<i>M. tuberculosis</i>	96†								

* *Streptococcus pyogenes* was used to determine the %S between an unrelated species and the mycobacterial species. The %S values between the mycobacterial species were not corrected for this "base line similarity".

† Average %S of the soluble protein profiles of a maximum of 10 strains of one species.

Our results with the other seven species show seven species-specific SDS-PAGE band profiles. These species-specific band profiles can easily be differentiated from each other and from that of the *M. tuberculosis* complex. The percentage similarity between these eight band profiles, as described in this paper, are significantly heterogeneous, ranging from 30% to 45%.

From these results we conclude that the *M. tuberculosis* complex, *M. avium*, *M. kansasii*, *M. marinum*, *M. gastri*, *M. simiae* and *M. malmoense* are eight distinct mycobacterial species. The low level of relatedness between *M. avium* and *M. marinum* (38%) or *M. simiae* (33%) that we found is not in agreement with the numerical results (91% relatedness) given by Tsukamura,¹ but is in agreement with results of DNA homology studies.^{15,16}

The development of a new method for determination

of mycobacterial species is desirable because a growing number of mycobacterial infections are caused by strains that do not belong to the *M. tuberculosis* complex. To come to a conclusion about the clinical significance of an isolate, it has first to be identified and in the clinical setting it is important to obtain an unambiguous result as soon as possible. The method of identification that we describe makes it possible to identify all mycobacterial isolates within 10 days. More work is necessary to evaluate the usefulness of the method for mycobacterial species other than those described in this paper. We have initiated studies to evaluate this approach with the *M. avium* complex and related species.

We thank Professor D. M. Maclaren of the Free University, Amsterdam for his support and Miss Karen Wichgers for typing the manuscript.

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