Ribotyping of coagulase-negative staphylococci

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Summary. The discriminative capacity of ribotyping was initially assessed without knowledge of results obtained for the same isolates by use of more established typing methods. Forty-eight isolates of coagulase-negative staphylococci (CNS) from peritoneal fluids were studied. They were collected prospectively during 31 consecutive episodes of infection associated with peritoneal dialysis in 17 patients. DNA was digested by the restriction endonucleases *EcoRI* or *HindIII* and ribotyped by means of a biotinylated cDNA probe to 16S + 23S staphylococcal ribosomal RNA gene sequences. These methods in combination produced a total of 27 types which compared well with numbers of groups distinguished by other typing methods: limited biotype-antibiotic resistogram (ARB; 28), antibiotic resistogram alone (25), API-Staph (12), phage typing (9) and plasmid analysis (22). Ribotyping was highly reproducible and typed all isolates, including those that were not phage-typable (35) or did not contain plasmids (4). When used in a hierarchical manner with ARB, ribotyping results produced 13 additional types in comparison with the other three methods. When used hierarchically with all other typing systems, a further five types were found among isolates from two patients. However, some of the differences observed as a result of ribotyping could have been due to subtle changes produced by mutation, lysogenisation or gene transposition. Since the method requires additional time, expense and technical expertise, it is likely to be useful only when answers to specific epidemiological problems are required or as an initial screen before using other methods of genetic analysis.

Introduction

In recent years there has been increasing recognition of the ability of coagulase-negative staphylococci (CNS) to produce clinically significant infections. A commensurate rise in interest in the study of the epidemiology of CNS-related sepsis has occurred and resulted in the application of various typing systems to investigations of CNS reservoirs and modes of transmission between patients and staff.^{1,2} Bacteria have been typed by comparison of fragments obtained from restriction endonuclease digests of total or chromosomal DNA (restriction fragment length polymorphisms: RFLP) but this has often produced complex patterns that have been difficult to interpret.³ Ribosomal RNA genes (rRNA) are highly conserved in bacteria and analysis of restriction fragments which include ribosomal sequences—ribotyping³—has shown potential for the identification and typing of CNS isolates.^{4–6} However, results of previous studies with radiolabelled probes were not compared with simpler, more conventional typing methods, and techniques were not evaluated for comprehensive collections of epidemiologically related strains. We

have evaluated the use of a biotin-labelled probe to ribotype a large collection of strains which had already been characterised by other typing systems.¹

Materials and methods

Strains examined

A total of 48 isolates of CNS was studied. They were obtained during 31 consecutive episodes of peritonitis in 17 patients undergoing continuous ambulatory peritoneal dialysis (CAPD) as described previously.¹ Multiple isolates were obtained from 17 episodes of infection which occurred in eight patients. The isolates were typed by antibiotic resistance pattern, limited biotype and API-Staph profiles (API Products Ltd, Basingstoke, Hants), plasmid analysis and phage typing.¹ Eight isolates which were distinguished by these methods were selected as “reference isolates” for use in early studies that attempted to establish optimal methods (see below). Following analysis of all results, resolution of speciation of some isolates was attempted by use of the ATB32 Staph system (API Products Ltd).

DNA preparation, digestion and separation

Bacteria were lysed in 100 μ l of 0.1 M phosphate buffer, pH 7.0, that contained 60 μ g of lysostaphin (Sigma). After centrifugation for 10 min in a Microcentaur centrifuge (MSE) the resuspended pellet was incubated at 37°C for up to 1 h until viscous. The DNA was then prepared by the method of Pitcher *et al.*⁷ and the amount of DNA present was measured and assessed for purity by determination of absorbance ratios at 260 nm.⁷ DNA (8- μ g quantities) was digested with *Eco*RI and *Hind*III restriction endonucleases (Boehringer Mannheim, Germany), since these enzymes had produced satisfactory banding patterns in studies with the reference isolates; 10- μ l volumes of each digest were loaded on 0.8% w/v agarose gels and electrophoresed at 30 V for 16 h in pH 8 buffer. Biotinylated λ phage *Hind*III fragments (Gibco-BRL, Paisley) were used as molecular size standards. To assess the reproducibility of the method, the same preparation derived from each reference isolate was run at the periphery and the centre on two different gels, and single preparations of all isolates were run on separate gels. Two isolates with very different blot patterns (later revealed to be *Staphylococcus epidermidis*) were run on every gel. In addition, DNA from the eight reference isolates was extracted on two occasions and gel banding patterns were compared to determine inter-extract variability.

Ribotyping procedure

S. aureus NCTC 10442 was the source of 16S and 23S rRNA used to transcribe a biotinylated cDNA probe.⁸ A Southern blot of the agarose gels was performed with the VacuGene system (Pharmacia, Sweden).

Analysis of typing results

Results were analysed by two workers (PS and BC) without knowledge of results obtained with other typing methods. Migration distances of the DNA fragments containing copies of ribosomal RNA cistronic sequences were measured to the nearest 0.5 mm and entered into the DNASIZE program.⁹ The range of measurements of band migrations obtained in duplicated runs was used to establish the validity of single band loci. Dice coefficients¹⁰ were used to compare RNA gene profiles by scoring positive or negative matches in all loci, and clustering was based on the unweighted pair group arithmetic average algorithm.¹¹

Results

Analysis of ribotypes

While it was not possible to analyse RFLP patterns

Fig. 1. Computer generated representation of the 20 *Eco*RI pattern types (profiles) and API types of the CNS isolates.

Fig. 2. Computer generated representation of the 22 *Hind*III pattern types (profiles) and API types of the CNS isolates.

due to poor resolution of the fragments, the biotinylated probe produced distinct bands above a mol. wt of *c.* 1 kb. There were 9–19 *Hind*III and 7–17 *Eco*RI bands. Preparations derived from individual isolates produced the same number of bands in all intra-extract and inter-extract reproducibility studies. However, mol.-wt ranges for individual band loci were much smaller when samples were run on the same gel. Data from such runs produced a total of 47 *Eco*RI and 41 *Hind*III loci, compared with 38 and 35, respectively, when data from different gels were analysed. These latter loci were used to analyse the whole CNS collection. As the presence of all loci was reproducible, a one-locus difference rule, adopted in the previous study,⁴ was used to define the pattern type produced by each enzyme. This resulted in 20 *Eco*RI and 22 *Hind*III pattern types (figs. 1 and 2). When the two pattern types were combined there were 27 such types, called here “ribotypes”, amongst the 48 isolates.

The discrimination produced by each enzyme was assessed when the identities of the organisms were revealed to BC and PS. *Eco*RI subdivided two *Hind*III pattern types containing a total of 17 *S. epidermidis* isolates into two and three *Eco*RI pattern types, respectively and also produced subdivisions among five *S. simulans* isolates (figs. 1, 2 and 3). *Hind*III

subdivided three *Eco*RI pattern types among 21 *S. epidermidis* isolates, into two, two and four *Hind*III pattern types, respectively. The additional information provided by the *Hind*III preparation clearly distinguished one *S. haemolyticus* isolate from one *S. epidermidis* isolate which had been identified at only 24% certainty by API-Staph.

The separation of the isolates produced by Dice coefficient analysis of the ribotypes is shown in fig. 3. The analysis revealed good separation at the species level, apart from three *S. epidermidis* isolates for which both enzymes produced a large number of loci (figs. 1 and 2). These three isolates were related more distantly (mean dice coefficient 51.4%; range 33–77%) to other *S. epidermidis* isolates, although they were often biochemically indistinguishable (fig. 3). As shown in fig. 3, *S. epidermidis* isolates with identical biochemical properties, isolated from the same patient, did not always cluster together.

No species-specific bands were observed, but the distribution of the bands proved to be helpful. Five *S. simulans* isolates (T7/T8) were distinct from all other isolates at the 75% level and split into two groups (95% similarity) which were also distinct biochemically. The group of four *S. simulans* (T7) isolates were from the same patient over an 88-day period. One

Fig. 3. Dendrogram of Dice similarity coefficients of ribotypes (combined *EcoRI* and *HindIII* pattern types). API-Staph (ATB32 Staph) identifications and percentage probabilities: T1–6, *S. epidermidis* 86–99.7%; T7, *S. simulans* 97%; T8, *S. simulans* 56% (*S. simulans* 99.9%); T9, *S. hominis I* 93% (*S. warneri* 99.9%); T10, *S. epidermidis* 24% (*S. haemolyticus* 99.9%); T11, *S. haemolyticus* 43% (*S. haemolyticus* 94.9%); T12, *S. haemolyticus* “very doubtful profile” (*S. haemolyticus* 99.9%).

isolate of *S. haemolyticus* (T11) was related at the 75% level to an isolate which was identified by API-Staph as “*S. epidermidis* (T10) with a 24% certainty”, and at the 11% level to another (but biochemically distinct) *S. haemolyticus* isolate (T12). An isolate of *S. hominis I* (T9) was quite distinct from all other isolates (24% similarity).

In view of the interesting relationships between certain isolates, they were also identified by a recently

introduced biochemical identification system (ATB32 Staph). This system (see key to fig. 3) confirmed that both *S. haemolyticus* isolates were correctly identified by API-Staph (although they still had distinct profiles) and that the dubious *S. epidermidis* (T10) isolate was also of this species. In addition, the distinct isolate thought to be *S. hominis I* was, in fact, more likely to be *S. warneri*.

Comparison with previous typing results

Ribotyping typed all isolates, unlike phage typing, which did not type 35 organisms and plasmid analysis which failed to reveal plasmids in four isolates examined. Ribotyping (27 types) produced almost as much discrimination as the antibiotic resistogram with limited biotype (ARB), which gave 28 types and was superior to the antibiotic resistogram alone (25), plasmid analysis (22), API-Staph (12) or phage typing (9). Ribotyping added more types (13) to those identified by ARB. Plasmid typing added seven, only one of which was not identified by ribotyping, and phage typing added three, only one of which was not identified by either ribotyping or plasmid typing. Results of application of API-Staph produced only five types in addition to those distinguished by ARB. The two isolates that were distinguished by plasmid analysis and not by ribotype were isolated from the same patient (38 days apart) and were also distinct by phage type. The nine isolates that were sub-typed by ribotyping alone were of eight ribotypes. These had been isolated from two patients on three occasions over 20-, 27- and 79-day periods, respectively. There were 2–10 band loci differences between isolates from the same patient. These eight ribotypes were of three types, as defined by a combination of all the other typing systems.

Discussion

The results of ribotyping reported here are difficult to compare with those published by other workers⁴⁻⁶ because there are methodological differences between studies. For example, it is unclear in previous studies whether capillary or vacuum blotting was performed; our initial work showed that the former method was less sensitive and produced more diffuse banding patterns. In addition, the use of a radioactive probe produces much sideways penetration, perhaps allowing double bands to be missed more easily. Bialkowska-Hobranska *et al.*⁶ used a different enzyme (*Cla*I). Moreover, a staphylococcal probe was used in the present study, whereas the other studies employed a 16S rDNA probe from *Bacillus subtilis*⁴ or a 16S + 23S rRNA *Escherichia coli* probe.^{5,6} All these factors might have contributed to differences observed in the banding patterns between the various studies. However, another important factor might be isolate selection. It is apparent that considerable variation within certain species can be obtained with this technique, but as this study and the three previous investigations each examined different isolates, it is impossible to comment objectively on this point.

Bialkowska-Hobranska *et al.*⁶ used a laser densitometer and computerised analysis of their blots but

did not state whether blots from gels run on different occasions were compared. We have shown that the number of loci was reduced when we compared results derived from single organisms obtained from different blots. Nevertheless, patterns observed were considered to be adequate for typing. The mol. wt of the higher restriction fragments that contained ribosomal sequences were measured only approximately because we were unable to fit the highest mol. wt λ band on the standard curve. However, this problem did not compromise discrimination because few very high-mol.-wt bands were obtained by our methods. Ribotyping appeared to be fairly good for species discrimination, agreeing well with the ATB32 Staph results used to resolve any problems due to the inability of API-Staph to identify certain species. Bialkowska-Hobranska *et al.*⁶ have found that digestion with other restriction endonucleases also provides discrimination between certain CNS species.

The ribotyping method used in this study was demanding of time, labour and materials and required more expertise than other typing methods as employed by Ludlam *et al.*¹ The cheap and rapid approach utilising antibiotic resistogram and limited biotype should answer many of the epidemiological questions which face clinical microbiologists. Few isolates will require to be typed by phage-susceptibility, plasmid analysis, ribotyping or RFLP analysis. When applied to this collection, ribotyping revealed no evidence of cross infection between patients. However, a collection of CNS from in-patients might not be typed satisfactorily by first-line typing systems and strain variability might result from acquisition or loss of transposons, phages or plasmids.

The discrimination produced by ribotyping, when both enzymes were used, was superior to that of any other single method. Nevertheless, changes to ribotypes might occur over a period of time or even in a single strain at different sites in the same patient, so that these factors require further assessment. For example, a point mutation inside a cistronic sequence could result in several band differences between isolates that appeared previously to be identical. Lyso-genisation with phages or insertion of certain transposons or plasmids into the chromosome does not seem to affect the ribotyping pattern of *S. aureus* isolates,¹² but this may not be true for CNS. Therefore, ribotyping may be of value as an additional tool to assist in the interpretation of differences seen with other simpler, cheaper and more rapid typing methods. It may also have a role to play as a screening method before progressing to more specialised investigations, e.g., to assist in the choice of isolates for gene banking, sequencing or other probing.

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