MOLECULAR IDENTIFICATION AND TYPING

Source of variation detected in ribotyping patterns of *Haemophilus influenzae*: comparison of traditional ribotyping, PCR-ribotyping and rDNA restriction analysis

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The pattern of *EcoRI* restriction fragments of chromosomal DNA that hybridise with a probe for genes encoding 16S and 23S rRNA is highly discriminatory for non-capsulate *Haemophilus influenzae* (NCHI). The source of variation detected by these probe-based ribotyping patterns was investigated by restriction analysis of rRNA operon (*rrn*) amplification products from nine representative strains. Digestion of *rrn* amplification products with *EcoRI* indicated one conserved *EcoRI* site within 16S rDNA and no *EcoRI* sites within the 16S–23S intergenic spacer region of the nine strains, and an *EcoRI* site at the 5' end of 23S rDNA from seven of the nine strains. Comparison of the *EcoRI* ribotyping patterns obtained with separate probes for 16S and 23S rDNA showed more variation with the 23S probe indicating variation in *EcoRI* sites downstream from the operon. Restriction analyses of 16S and 23S rDNA amplification products with *AluI*, *HhaI*, *HaeIII* and *TaqI* divided the nine 'traditional' ribotypes into a maximum of three and eight groups, respectively. Similar analyses of the 16S–23S intergenic regions (PCR-ribotyping) failed to distinguish any of the nine representative strains. Therefore, there is probably insufficient variation within the operon for it to form a good target for PCR-based typing methods. In contrast, 'traditional' ribotyping with cDNA from 16S plus 23S rRNA detects restriction site differences in the sequences flanking the operon, which show considerably more variation between strains. 'Traditional' ribotyping should therefore remain the standard for characterising NCHI in epidemiological investigations.

Introduction

The pattern of restriction fragments of chromosomal DNA that hybridise with probes for the operon encoding rRNA (*rrn*) has been applied successfully to both the inter- and intra-species characterisation of a wide range of bacteria [1]. With the appropriate choice of restriction endonuclease and probe for the species under investigation, this method (ribotyping) has been shown to detect sufficient heterogeneity within many species to provide useful epidemiological information [2]. The source of the heterogeneity in ribotyping

patterns is unknown [1], but may be caused by variations within *rrn*, i.e., in the genes encoding 16S rRNA, 23S rRNA, 5S rRNA or the two intergenic spacer regions (IGS), or in the regions flanking the multiple *rrn* copies that are present within most strains.

Ribotyping patterns obtained from *EcoRI*-digested chromosomal DNA hybridised with a cDNA probe for 16S and 23S rRNA-encoding genes (rDNA) have been shown to be a highly discriminatory method for characterising strains of non-capsulate *Haemophilus influenzae* (NCHI) from nosocomial outbreaks of respiratory infection [3]. However, ribotyping involves lengthy procedures for DNA extraction, digestion, blotting, hybridisation and detection, and often takes up to 1 week to provide a result; more rapid methods are desirable for hospital infection control purposes. PCR-based methods of characterisation based on randomly amplified polymorphic DNA (RAPD) have been shown to correlate well with ribotyping for NCHI [3], but inter-laboratory reproducibility of

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RAPD is poor [4]. PCR-based methods of strain characterisation that specifically target *rrn* sequences have been applied to the inter- and intra-species characterisation of bacteria. Amplified 16S rDNA restriction analysis (16S ARDRA) has been shown to differentiate species of mycobacteria [5], some *Acinetobacter* spp. [6], streptococci [7] and *Prevotella* spp. [8], and has been shown to discriminate between and within some fluorescent *Pseudomonas* spp. [9]. Amplification of the 16S–23S IGS, which may vary in length and sequence between the multiple copies of *rrn* that are usually present within a genome, has been shown to differentiate between genera and species [10], and also between unrelated strains within *Burkholderia* (*Pseudomonas*) *cepacia* [11, 12], *Clostridium difficile* [13] and *Neisseria meningitidis* [14]. This method has been referred to as PCR-ribotyping [15]. Restriction analysis of amplification products containing IGS plus a portion of 23S rDNA has been shown to differentiate strains of *Rochalimaea henselae* [16]. PCR-ribotyping and 16S ARDRA have been applied predominantly to inter-species characterisation, i.e., for identification rather than typing or strain characterisation purposes. The ability of these methods to detect heterogeneity within species, as required for epidemiological studies, has yet to be determined for many important pathogens, including *H. influenzae*. The relationship between the results of 'traditional' ribotyping with different probes, ARDRA and PCR-ribotyping has not been studied.

For species with insufficient IGS length heterogeneity, it may be possible to increase the discriminatory ability of PCR-ribotyping by restriction analysis of the products. In addition, ARDRA based on 23S rDNA—which is 60% larger than 16S rDNA and is associated with a faster rate of sequence change than 16S rDNA [17]—may be more discriminatory than 16S rDNA-based ARDRA. The present study investigated the source of variation detected in traditional ribotyping patterns to determine the suitability of *rrn* as targets for PCR-based methods of strain characterisation. This was achieved by restriction analysis of PCR-amplified 16S rDNA, 23S rDNA and 16S–23S IGS, and by comparative hybridi-

sation studies with three different probes: cDNA from 16S plus 23S rRNA, and separate PCR-generated probes for 16S and 23S rDNA. The methods were also compared for their intra-species characterisation of NCHI in epidemiological investigations.

Materials and methods

Isolates

As part of a survey of antimicrobial resistance in *H. influenzae* by Bath Public Health Laboratory, 20 β -lactamase-negative strains with reduced susceptibility to β -lactam agents were isolated and 20 β -lactam-susceptible strains were selected from age-matched patients as controls [18]. All isolates were received in Oxford on heated blood (chocolate) agar slopes and subcultured on to chocolate agar with overnight incubation at 37°C in a humidified atmosphere containing CO₂ 5%. The growth was harvested and stored at –80°C in Protect vials (Technical Services Consultants, Heywood, Lancs.). As part of the Bath survey, the 40 isolates had been characterised by *Eco*RI ribotyping with a digoxigenin-labelled cDNA probe made from purified 16S and 23S rRNA from *Escherichia coli* as described previously [3, 18]. These 40 isolates were further characterised by traditional ribotyping with a probe for 16S rDNA, as described below, and nine of these isolates with different ribotyping patterns were selected for further analyses. Total cellular DNA was extracted as described previously [19].

PCR-generated 16S rDNA probe

For the digoxigenin-labelled 16S rDNA probe, c. 1.5 kb of 16S rRNA-encoding DNA (corresponding to nucleotides 8–1540 in *E. coli*) was amplified from *H. influenzae* type b (HIB) strain Eagan [20] with primers 5'-AGAGTTTGATCATGGCTCAG-3' [21] (Fig. 1; F2) and 5'-AGGAGGTGATCCAACCGCA-3' [22] (Fig. 1; R2). Amplification was in a total volume of 25 μ l and contained 0.3 μ M of each primer, 250 μ M of each dNTP and 0.1 U of *Taq* polymerase (Advanced Biotechnologies Ltd, Leatherhead, Surrey) in the

Fig. 1. Schematic representation of a *rrn* operon showing the relative position and orientation of primers used in the present study (not to scale). E, stable *Eco*RI site; (E), unstable *Eco*RI site.

presence of the buffer supplied with the polymerase (buffer IV, containing 1.5 mM MgCl₂). This reaction mixture was overlaid with mineral oil, and *c.* 1 µg of target DNA was added. A control tube containing all reagents except target DNA was included in all amplification experiments. Amplification conditions comprised 25 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, followed by 10 min at 72°C. Amplification products (10 µl) were separated by electrophoresis in agarose 0.9% w/v gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na₂ EDTA), stained with ethidium bromide (1 µg/ml) and photographed under ultraviolet transillumination. A portion (1 µl) of the initial amplification products was used as target in a second round of amplification with the same reaction conditions except that dig-11-dUTP (Boehringer Mannheim) replaced 35% of the dTTP. The mobility of the resulting product in agarose gel electrophoresis was compared with the unlabelled product and shown to be retarded, indicating successful incorporation of digoxigenin.

PCR-generated probe for 23S rDNA

A segment (*c.* 2.6 kb) of 23S rDNA (corresponding to nucleotides 188–2744 in *E. coli*) was amplified from HIB strain Eagan [20] with primers previously designated 11a (5'-GGAAGTGAACATCTAAGTA-3') and 97ar (5'-CCCGCTTAGATGCTTTCAGC-3') [23] (Fig. 1; F3 and R3, respectively). Amplification reactions (25 µl) contained 0.5 µM of each primer; other reagents were as above for 16S rDNA. Amplification conditions comprised 25 cycles of 1 min at 94°C, 1 min at 55°C and 2.5 min at 72°C. The product (1 µl) was used as the target in the PCR labelling reaction, as described above for the 16S rDNA probe.

Ribotyping with PCR-generated probes for 16S and 23S rDNA

Southern blots of total cellular DNA digested with *EcoRI* were hybridised with the PCR-generated probe for 16S rDNA with the same highly stringent conditions as traditional ribotyping [3]. Nine strains showing different ribotyping patterns (when hybridised with the cDNA probe), including two pairs indistinguishable when hybridised with the 16S rDNA probe, were investigated further by ribotyping with the PCR-generated probe for 23S rDNA and by the techniques below.

Restriction analysis of 16S rDNA and 23S rDNA

A segment (*c.* 1.5 kb) of 16S rDNA was amplified from each strain with the same primers and conditions used to make the 16S rDNA probe, except that reagents were scaled up to give final reaction volumes of 100 µl. After amplification, a 5-µl portion was electrophoresed on an agarose 2% w/v gel in TBE containing ethidium bromide 1 µg/ml to check the amount and purity of product. For restriction analysis, 8 µl of the product

were digested with one of four restriction endonucleases with four-base recognition sequences (*AluI*, *HaeIII*, *HhaI* and *TaqI*) and *EcoRI* (the enzyme used for ribotyping) and the conditions recommended by the manufacturer (Life Technologies, Paisley). Digestion products were electrophoresed initially on agarose 2% w/v gels, followed by polyacrylamide 10% w/v if products of *<c.* 200 bp were detected. Amplification and digestion products were occasionally stored at –20°C before electrophoresis. Amplification of 23S rDNA was as described above for 23S probe preparation, except that the reaction volume was increased to 100 µl. Digestion products were separated on Metaphor™ Agarose (FMC Products, Flowgen Instruments, Sittingbourne, Kent) 2% w/v gels in TBE to increase the resolution of fragments of 100–1000 bp. All other conditions were as described for 16S rDNA restriction analysis. All strains were analysed on at least three separate occasions.

PCR ribotyping

The 16S–23S rDNA IGS was amplified with primers 5'-GAAGTCGTAACAAGG-3' and 5'-CAAGGCATC-CACCGT-3' [10] (Fig. 1; F1 and R1, respectively). Amplifications were performed in total reaction volumes of 100 µl as described previously [10]. A 5-µl sample of the products was electrophoresed on agarose 1.6% w/v gels in TBE containing ethidium bromide (1 µg/ml). An 8-µl portion of these products was digested with one of five restriction endonucleases, as above.

Other amplifications

The 23S rDNA product amplified above lacked 187 bp at the 5'-end and, although *c.* 20 bp were included in the IGS amplification product [10], *c.* 170 bp were not examined for *EcoRI* restriction sites. To detect *EcoRI* sites within this region, primers F1 and F2 were each used with R3. For the larger product (F2–R3; *c.* 5 kb), the proof-reading enzyme complex BIO-X-ACT (kindly provided by Bioline UK Ltd, London) was used. Amplification conditions comprised an initial 5 min at 94°C, followed by 20 cycles of 1 min at 94°C, 1 min at 55°C and either 3 min (for the F1–R3 product) or 5 min (for the F2–R3 product) at 72°C, and a final extension period of 10 min at 72°C. Products were digested with *EcoRI* as described above. The F2–R3 product was also digested with *HaeIII* and the products were separated on Metaphor™ agarose 2% w/v gels in TBE.

Results

Traditional probe-based ribotyping

The cDNA probe for 16S and 23S rDNA had previously identified 24 patterns among the 39 typable isolates: 16 patterns for 19 susceptible isolates and

eight different patterns for 20 resistant isolates [18]. The 16S PCR-generated probe used in the present study identified 22 patterns among the 39 isolates: 15 patterns for the 19 susceptible isolates and eight patterns for the resistant isolates. There was excellent correlation between the results of the two techniques but, in contrast to the cDNA probe, the 16S probe did not distinguish between two ampicillin-susceptible strains or between one ampicillin-susceptible strain and an ampicillin-resistant strain. Nine representative strains with different ribotyping patterns (obtained following hybridisation with the cDNA probe for 16S and 23S rDNA), including the four isolates that gave indistinguishable patterns when hybridised with the probe for 16S rDNA alone, were characterised by hybridisation with a probe for 23S rDNA. These nine strains were all distinguishable by ribotyping with the 23S rDNA probe. The ribotyping patterns of these nine strains obtained following hybridisation with 16S and 23S cDNA probe, the 16S rDNA probe and the 23S rDNA probe are shown in Figs. 2a and b. The nine strains were further characterised by restriction analysis of 16S rDNA and 23S rDNA, and amplification and restriction analysis of 16S–23S IGS.

Restriction analysis of 16S rDNA

A 1.5-kb 16S rDNA product was amplified from the nine study strains and digested with five different restriction endonucleases. *EcoRI* digestion resulted in two bands of *c.* 700 bp and *c.* 800 bp; there was no

variation in the sizes of these two fragments between the different strains (data not shown). The enzymes with four-base recognition sequences each gave one to three different patterns (each consisting of three to seven bands in the *c.* 150–500 bp size range) for the nine strains (Fig. 3; Table 1).

Fig. 3. Restriction analysis of 16S rDNA amplification products. Digestion with *AluI* gave two restriction patterns (A1 and A2; lanes 1 and 2, respectively). Digestion with *HaeIII* gave three restriction patterns (E1, E2 and E3; lanes 3, 4 and 5, respectively). Digestion with *HhaI* gave a single restriction pattern (H1; lane 6). Digestion with *TaqI* gave two restriction patterns (T1 and T2; lanes 7 and 8, respectively). M, molecular size marker (Marker VIII, Boehringer).

Fig. 2. **a**, Traditional ribotyping patterns of NCHI investigated in the present study showing Southern blots of *EcoRI*-digested DNA hybridised with a cDNA probe from 16S and 23S rRNA. Lanes 1–9 correspond to strains 1–9 (see Table 1). M, molecular size markers (digoxigenin-labelled *HindIII* fragments of bacteriophage λ). **b**, Ribotyping patterns of NCHI probed with separate PCR-generated probes for entire 16S rDNA (left) and 23S rDNA (right). Lanes as for **a**.

Table 1. Differentiation of NCHI strains by probe-based ribotyping, PCR-ribotyping and restriction analysis of *rrn* amplification products (column entries indicate the assigned pattern number)

Strain	Original number*	Ribotype†			16S rDNA restriction‡				23S rDNA restriction§				F2-R3 Product <i>Hae</i> III
		cDNA	16S	23S	<i>Alu</i> I	<i>Taq</i> I	<i>Hha</i> I	<i>Hae</i> III	<i>Alu</i> I	<i>Taq</i> I	<i>Hha</i> I	<i>Hae</i> III	
1	219	1	I	i	A2	T2	H1	E2	a1	t1	h1	e1	L1
2	40	2	I	ii	A2	T2	H1	E2	a1	t2	h2	e1	L1
3	230	3	II	iii	A2	T2	H1	E2	a2	t3	h3	e2	L2
4	280	4	II	iv	A2	T2	H1	E2	a3	t4	h4	e3	L3
5	307	5	III	v	A2	T2	H1	E2	a1	t5	h5	e4	L4
6	354	6	IV	vi	A2	T2	H1	E2	a4	t6	h6	e5	L5
7	51	7	V	vii	A2	T1	H1	E3	a5	t7	h7	e6	L6
8	139	8	VI	viii	A1	T1	H1	E1	a6	t7	h8	e7	L7
9	178	9	VII	ix	A2	T2	H1	E3	a6	t7	h8	e8	L8
Total number of patterns		9	7	9	2	2	1	3	6	7	8	8	8

*Designation in James *et al.* [18].†*Eco*RI restriction patterns when hybridised with each probe indicated. These patterns are shown in Fig. 2a and b.‡Pattern numbers indicate the lane position in Fig. 3 (i.e., A2 = 2nd lane of *Alu*I digests).§*Hha*I and *Hae*III patterns of 23S rDNA are shown in Figs. 4 and 5, respectively.

Restriction analysis of 23S rDNA

A c. 2.5-kb 23S rDNA product was amplified from all the study strains. *Eco*RI did not digest the product. *Hha*I produced a major fragment (an intensely staining

Fig. 4. *Hha*I restriction analysis of 23S rDNA amplification products. Lane numbers correspond to strain numbers.

band) of 1.0 kb, and one to four minor fragments (less intensely staining bands) of 0.5–1 kb, resulting in eight different patterns for the nine strains (Fig. 4). These patterns were not easy to read or compare as only two different bands with similar mobilities were detected with some strains. *Hae*III produced six to nine bands of 70–700 bp, giving eight patterns for the nine strains (Fig. 5). Digestion with *Taq*I resulted in one major fragment of 1.0 kb in all strains, plus seven to nine minor products, of 100–500 bp, giving seven patterns. *Alu*I digestion yielded a 600-bp product and seven to nine products of 100–400 bp from all strains, giving six patterns. Patterns were compared on the basis of the presence or absence of specific bands, regardless of intensity (i.e., reproducible faint bands were included). The distribution of patterns obtained with the different restriction endonucleases is shown in Table 1.

Fig. 5. *Hae*III restriction analysis of F2-R3 (left) and 23S rDNA (right) amplification products. Lane numbers correspond to strain numbers. M, molecular size markers (marker VIII, Boehringer). U, undigested 23S rDNA and F2-R3 products.

PCR-ribotyping and restriction analysis of the 16S–23S IGS

PCR-ribotyping resulted in two major bands of *c.* 570 bp and *c.* 820 bp, and a variable faint band in all nine strains. The faint, variable band was always at the same position in all patterns on any given gel, usually between the two major bands, but occasionally above the larger product. When amplification products from experiments in which the fainter band was in different positions were re-run on the same gel, the fainter bands were all in the same position. This phenomenon has been documented previously [10] and suggests that these bands were not fragments of double-stranded DNA. The nine strains were indistinguishable by PCR-ribotyping. The major products were not digested by *EcoRI*, but an apparent increase in size of the faint band was observed. Digestion of the products with four restriction endonucleases with four-base recognition sequences resulted in indistinguishable patterns of two to four bands in all isolates. Fig. 6 shows the amplification products and the patterns obtained after digestion.

Restriction of 5'-region of 23S rDNA

As expected, amplification with primers F1 and R3 gave products of *c.* 3.3 kb and *c.* 3.5 kb for each strain. *EcoRI* digestion yielded products of *c.* 2.7 kb, *c.* 0.9 kb and *c.* 0.7 kb for seven strains, but did not appear to cut the amplification products from the remaining two strains. The other primer pair (F2, R3) gave two products of *c.* 5 kb for each strain, as expected. For seven strains, digestion with *EcoRI* resulted in products of *c.* 2.7 kb, *c.* 1.8 kb, 1.5 kb and 700 bp, while two strains gave fragments of 700 bp and two fragments of *c.* 4 kb (Fig. 7). As *HaeIII* produced the most patterns from 16S rDNA and 23S rDNA products, the F1–R3 product was also digested with this enzyme. Eight patterns, comprising 11–14 bands of 0.1–*c.*1.7 kb, were detected (Fig. 5).

The distribution and number of patterns obtained after restriction analysis of the various amplification products are summarised in Table 1. The position of the *EcoRI* sites in the operon and the relative positions of the primers used in the present study are indicated in Fig. 1.

Discussion

The present study investigated the source of variation detected in traditional probe-based ribotyping patterns of NCHI. Previous ribotyping studies of *H. influenzae* from the UK showed that, of 12 restriction endonucleases tested, *EcoRI* gave the most discriminatory patterns [19, 24]. The variation in these ribotyping patterns must, therefore, be a result of differences in *EcoRI* sites, either within the operons or flanking them.

Fig. 6. Amplification and restriction analysis of 16S–23S IGS. M, molecular size markers (1-kb ladder); U, undigested product; lanes 1–5 show products digested with *EcoRI*, *HhaI*, *HaeIII*, *TaqI* (partial digest showing undigested product) and *AluI*, respectively.

Fig. 7. Restriction analysis of F2–R3 product with *EcoRI*. Lane numbers 1–9 represent undigested product from strains 1–9; + indicates *EcoRI* fragments obtained from the product in the preceding track. M, molecular size markers (1-kb ladder).

Amplification and restriction analysis of *c.* 5 kb of the *rrn* operon, comprising almost the entire 16S rDNA, 23S rDNA and 16S–23S IGS, showed that there was one *EcoRI* site in 16S rDNA (conserved in all nine strains examined) and another *EcoRI* site at the 5'-end

of 23S rDNA; this was conserved in seven of nine strains (Fig. 1). No other *EcoRI* sites were detected. The variation in *EcoRI* ribotyping patterns must, therefore, result from variation in *EcoRI* sites outside the *c.* 5-kb region examined. The positions of these *EcoRI* sites were conserved in all *rrn* copies present within a genome, as indicated by the sizes of the *EcoRI* restriction fragments of the various amplification products (i.e., the sum of the fragment sizes was equal to the size of the product, and no product was left undigested).

Comparison of the ribotyping patterns obtained with separate probes for 16S rDNA and 23S rDNA, together with details of the positions of the *EcoRI* sites within the operon, confirmed that most variation lay outside *rrn*. Ribotyping patterns obtained with a 16S rDNA-specific probe showed two intense hybridisation bands at *c.* 1.5 kb and 1.8 kb to be common to all strains with an *EcoRI* site in the 23S gene (Fig. 2b, left); these represent all copies of the 3'-16S + IGS fragments (Fig. 1). Therefore, the other hybridisation bands in these strains correspond to fragments containing a common 3'-end (the conserved *EcoRI* site in 16S rDNA) and the 5'-end of each copy of 16S rDNA plus its associated flanking sequence. Variation in these bands must represent variation in DNA upstream from 16S rDNA. Two bands were shared by all nine strains studied, and two other bands were present in the majority of strains, indicating that there is relatively little variation in *EcoRI* sites upstream from the operons. This may be related to the presence of several tRNA genes upstream. Fleischmann *et al.* [25] demonstrated three tRNA genes upstream and two tRNA gene downstream from *rrn* in *H. influenzae* strain Rd. The two intense 16S hybridisation bands, representing 3'-16S + IGS, have been shown to be present in all HIB isolates examined by this laboratory to date [24] and in the vast majority of NCHI isolates [19; unpublished observations], suggesting that the two *EcoRI* sites are conserved in most *H. influenzae* strains. The difference in size of these hybridisation bands corresponds to the two different lengths of IGS characterised in the present study, which probably results from a difference in the number of tRNA genes present in the spacer [26]. Fleischmann *et al.* [25] demonstrated that the spacers are 478 bp or 723 bp long and contain one or two tRNA genes, respectively, in strain Rd. For patterns obtained with the 23S rDNA-specific probe, restriction fragments that hybridised must each have a common 5'-sequence—the *EcoRI* site at the 5'-end of 23S rDNA (or, if this is absent, the site in 16S rDNA)—and the 3'-end of each copy of 23S rDNA plus its associated flanking sequence. Variation in these bands must indicate variation in flanking DNA downstream from the operons. Fewer hybridisation bands were shared by these patterns than by 16S rDNA hybridisation patterns, indicating that the variation in *EcoRI* sites is 3' to the region. The size of 23S rDNA hybridisa-

tion fragments (all >3 kb) indicates that the variable sites are all outside the operons (Fig. 2b, right).

Restriction analysis of 16S rDNA, 23S rDNA and 16S–23S IGS amplification products with four other restriction endonucleases demonstrated no variation in the IGS and little variation in 16S rDNA. More variation was detected in 23S rDNA, although many fragments were common to several patterns, confirming the relative lack of variation within the operon. However, for strains 3 and 6, the sum of the *HaeIII* restriction fragments from 23S rDNA exceeded the size of the original product (Fig. 5) and, for some strains, some restriction fragments were present in much less quantity than others (Figs. 4 and 5), suggesting variation between *rrn* copies within a genome. Variation in the length of 23S rDNA and 16S rDNA between strains within species has been reported previously and has been shown to result from the presence of intervening sequences [27]. The results of the present study suggest the presence of variant *rrn* operons within the genomes of some *H. influenzae* strains.

The position of *EcoRI* sites within the operon, determined above, together with the results from the hybridisation studies with the separate probes, also enabled the number of *rrn* copies within each genome to be determined. The conserved *EcoRI* site in 16S rDNA must result in all 23S genes being on separate *EcoRI* fragments. Therefore, the number of hybridisation bands obtained with the probe for 23S rDNA represents the minimum number of copies of the operon in the genome, so that the present study indicates the presence of five or six copies of *rrn* in *H. influenzae*. The two strains of *H. influenzae* that have been mapped previously both possess six copies of *rrn* [25, 28, 29]. The five hybridisation bands detected with the 23S-specific probe for two strains in the present study suggest the presence of five operons. Alternatively, two separate fragments containing *rrn* operons may be of similar size and co-migrate under the conventional agarose gel electrophoresis conditions used in the present study. However, the presence of five *rrn* copies in strains 6 and 7, and six *rrn* copies in the remaining strains, was confirmed by the results with the 16S rDNA probe.

In addition to causing nosocomial outbreaks of respiratory infection [3], NCHI are recognised increasingly as an important cause of invasive disease [30] and are not expected to decline in response to the recent introduction of the vaccine against HIB [31]. Highly discriminatory methods of strain characterisation are therefore necessary for epidemiological investigations, and global studies require standardised, reproducible methods. The present study compared different *rrn*-based methods for their ability to discriminate NCHI strains. In traditional probe-based ribotyping of 40 isolates, the 16S probe was less

discriminatory than the classic combined probe for 16S and 23S rDNA. Nine strains with different traditional ribotyping patterns, including two pairs indistinguishable with the 16S rDNA probe, were investigated further. The 23S rDNA probe gave nine patterns, but fewer bands (which were less well distributed) than the combined probe, making pattern comparisons less sensitive but simpler. Restriction analysis of 16S rDNA and 23S rDNA amplification products classified the nine isolates into a maximum of three and eight patterns, respectively. Amplification of the 16S–23S IGS (PCR-ribotyping), with or without subsequent digestion, failed to distinguish any of the nine strains. The two pairs of strains indistinguishable by 16S rDNA probing were also indistinguishable by 16S rDNA restriction patterns. The 23S rDNA restriction fragment patterns distinguished these strains, although strains 1 and 2 were distinguishable only with *TaqI* and *HhaI*. This suggests that all 16S rDNA-based methods are poorly discriminatory for NCHI. The lack of perfect correlation between the results of the different methods is probably related to different selection pressures on sequences outside *rrn* and those within *rrn*. Although rapid methods are desirable for the investigation of outbreaks of infection in hospitals, none of the *rrn*-directed PCR-based methods investigated in the present study were as discriminatory for NCHI as traditional probe-based ribotyping. It was noticeable that the whole 16S rDNA or 23S rDNA PCR-generated probes produced bands of hybridisation with an even intensity, whilst the 16S + 23S cDNA probe produced bands of varying intensity. Differences in band intensity have been shown previously to be a stable and differential marker for the characterisation of HIB [24]. The PCR-generated probes consist of a whole piece of DNA (1.5 kb or 2.5 kb in length for 16S rDNA and 23S rDNA, respectively) containing digoxigenin-labelled dUTP. In contrast, the 16S + 23S cDNA probe is made by random priming and therefore consists of many smaller probes, also digoxigenin-labelled, but of perhaps only a few hundred bp. Following hybridisation at high stringency with the cDNA probe, the amount of rDNA in a specific chromosomal restriction fragment on the filter is directly proportional to the intensity of the hybridisation band. In contrast, the intensity of the hybridisation band with the PCR-generated probes is independent of the amount of rDNA in the fragment; when sufficient rDNA is present to form a stable hybrid, the resulting bands are of an even intensity as the probe is uniform. The cDNA probe for 16S rDNA and 23S rDNA detected the most heterogeneity and, therefore, should remain the standard for epidemiological investigations of NCHI.

Traditional ribotyping has been shown to be highly discriminatory for some species, but the use of different restriction endonucleases and probes for a single species makes inter-laboratory comparisons

difficult. The most appropriate restriction endonuclease (i.e., detecting the most variation) for a species may be difficult to standardise because bacterial populations may differ with geographical location; thus *EcoRI* ribotyping was shown to be highly discriminatory for enterococci from Oxford, but not for strains from London [32, 33]. Similarly, the distribution of subtypes within HIB has been shown to vary between host populations [34] and geographical regions [35]. Nevertheless, standardisation is vital for global comparisons of bacterial populations. The present study has shown that the major source of variation detected in ribotyping patterns is in the flanking DNA sequence. The use of probes containing a small part of the operon, e.g., an oligonucleotide probe for 16S rDNA [36], may be suitable if all the particular restriction sites and variations in these were outside *rrn*; however, the presence of conserved restriction sites within *rrn*, as shown in the present study, would decrease the discriminatory power of the method as all fragments would have a common end. Probes containing more of the operon inherently detect more variation, both within and outside the operon, and are therefore preferable. The use of the entire operon from one species as a universal, standard probe may be inappropriate as spacer regions vary within and between species and genera, as indicated by recent PCR-ribotyping studies [11, 13, 37]. The combined use of PCR-generated probes for 16S and 23S rDNA, as in the present study, gave essentially the same results as the cDNA probe, showing that either was satisfactory for ribotyping NCHI. The addition of 5S rDNA sequence to this probe may increase discrimination further for some species.

The multicopy, stable and essential nature of *rrn* makes it an ideal target for identification, but this study has shown that it is the dispersed location of *rrn* around the genome that makes ribotyping sufficiently discriminatory for epidemiological purposes; therefore, it may not be an ideal target for PCR-based strain characterisation as sequences within the operon are generally highly conserved. ARDRA and PCR-ribotyping are probably suited more to inter-species identification than intra-species characterisation for epidemiological studies. Analysis of 16S rRNA has been used extensively for phylogenetic studies [10, 38] and has shown that there is little sequence variation within species. Similarly, *rrn* spacer regions from *Staphylococcus aureus* have been shown recently to have a high degree of sequence conservation [26]. PCR-ribotyping, which has been shown to discriminate between strains within other species, failed to distinguish any of the strains in the present study. Restriction analysis of individual 16S, 23S and IGS products increased discrimination, but was still less discriminatory than cDNA probe-based ribotyping for NCHI. In contrast, restriction analysis of a single 5.5-kb amplification product from *rrn* ('long PCR ribotyping') has shown that internal *HaeIII* sites were

highly polymorphic within the operon [39]. These results may be a reflection of the strains investigated. For NCHI, the highly conserved nature of *rrn*, as demonstrated in the present study, shows that there is insufficient variation to make this a good target for PCR-based methods of strain characterisation. As *H. influenzae* has been shown to be one of the most heterogeneous bacterial species [40], these results suggest that this target is also unlikely to be suitable for characterising strains of other species. As the source of variation detected in traditional ribotyping is the DNA flanking the operons, it may be preferable for PCR-based methods of strain characterisation to target regions other than *rrn*.

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