Restriction endonuclease digest patterns of chromosomal DNA from group B β -haemolytic streptococci

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Summary. Scanning densitometry and computer-assisted numerical analysis were used to examine restriction endonuclease digest patterns (RDPs) of chromosomal DNA from 26 infecting strains and 44 vaginal isolates of group B β -haemolytic streptococci (GBS). At the 95% similarity level, *Hind*III RDPs of serotype Ia and III strains clustered into four and three RDP types, respectively. Nine of 10 strains from neonates with early-onset septicaemia belonged to two particular RDP types (Ia-3 and III-3). In contrast, serotype III GBS strains from meningitis cases were not characterised by particular RDP types. Associations between RDPs and certain phenotypic characteristics were also found.

Introduction

Group B β -haemolytic streptococci (GBS) are associated with various infections of man, particularly in the neonatal period. Type-specific capsular polysaccharide antigens allow GBS to be classified into serotypes Ia, Ib, II, III, IV and V, and a candidate serotype NT6.^{1–3} These serotypes may be further classified according to the presence of protein antigens c, R or X.^{4,5} Epidemiological studies of the serotype distribution of infecting GBS isolates show that more than half the strains from meningitis belong to serotype III,^{6,7} whereas the majority of cases of septicaemia with lower respiratory tract infection are caused by strains of serotype Ia.^{8–10} However, it is still unclear whether GBS strains of serotypes III and Ia isolated from infected infants belong to a specific subgroup within these serotypes.

Restriction endonuclease digest pattern (RDP) analysis of chromosomal DNA has been used successfully to characterise strains of various bacteria,¹¹ particularly for epidemiological typing, when other conventional methods cannot be used.

To improve the technique and increase objectivity, electrophoresis gels may be scanned by a densitometer¹² and the patterns analysed by computer-assisted numerical analysis.¹³ In the present study this approach was employed to compare GBS strains from infants with group B streptococcal infections with vaginal isolates from healthy women. The aim was to clarify whether infecting GBS isolates of serotypes III and Ia were characterised by a particular RDP.

Materials and methods

Bacterial strains

GBS strains, comprising 26 isolates from infected infants and 44 vaginal isolates from unrelated healthy women, are listed in table I. Organisms were stored at -80°C until use, as described previously.¹⁴

Serotyping

All strains were grouped serologically by the Phadirect streptococcus method (Pharmacia Diagnostics, Uppsala, Sweden). Serotyping was performed by the capillary precipitin method with antisera (Denka Seiken, Tokyo, Japan) to the polysaccharide antigens of types Ia, Ib, II, III, IV, V and NT6, and to the protein antigens c, R and X, according to the manufacturer's instructions.

Preparation of DNA

Bacteria grown in 20 ml of Todd-Hewitt Broth (BBL, Microbiology Systems Cockeysville, MD, USA) to late-exponential growth phase (OD_{600} , 0.6) were pelleted and washed with 4 ml Tris-EDTA (40 mM Tris-50 mM disodium edetate, pH 6.8). Cells were resuspended in 2 ml of mutanolysin (Sigma) solution (100 U/ml of Tris-EDTA) and incubated at 37°C until the suspension became translucent (1.5–2 h). Following the addition of 2 ml of lysing solution (sodium dodecyl sulphate 2% in Tris-EDTA), the mixture was allowed to stand at room temperature for 5 min. Salt precipitation was induced by adding 2 ml chilled (4°C) 3 M sodium acetate-acetic acid (pH 5.5) to the lysate

Table I. Sources and characters of GBS strains

Strain no.	Source	Serotype	RDP type	SA content	Tc susceptibility	Growth kinetics*
1	V	Ia/c	Ia-1	L	S	—
2	V	Ia	Ia-1	L	S	—
3	V	Ia/c	Ia-1	L	S	—
4	V	Ia	Ia-1	L	S	—
5	B	Ia/c	Ia-2	H	R	—
6	CSF	Ia/c	Ia-2	H	R	—
7	V	Ia/c	Ia-2	M	R	—
8	V	Ia/c	Ia-2	H	R	—
9	V	Ia/c	Ia-2	L	R	—
10	V	Ia/c	Ia-2	M	R	—
11	B	Ia	Ia-3	H	S	—
12	B	Ia/c	Ia-3	L	S	—
13	B	Ia/c	Ia-3	H	R	—
14	B	Ia/c	Ia-3	H	R	—
15	B	Ia/c	Ia-3	H	R	—
16	V	Ia/c	Ia-3	H	S	—
17	V	Ia/c	Ia-3	L	S	—
18	V	Ia	Ia-3	M	S	—
19	CSF	Ia	Ia-4	L	R	—
20	CSF	Ia	Ia-4	L	R	—
21	V	Ia	Ia-4	M	R	—
22	CSF	III	III-1	L	S	—
23	V	III	III-1	L	S	—
24	V	III	III-1	L	S	—
25	V	III	III-1	L	S	—
26	V	III	III-1	L	S	—
27	V	III	III-1	L	S	—
28	CSF	III/R	III-2	L	R	—
29	B	III/R	III-2	L	R	—
30	CSF	III/R	III-2	L	R	—
31	CSF	III/R	III-2	L	R	—
32	B	III/R	III-2	L	R	—
33	B	III/R	III-2	L	R	—
34	B	III/R	III-2	L	R	—
35	CSF	III/R	III-2	L	R	—
36	CSF	III/R	III-2	L	R	—
37	V	III	III-2	H	R	—
38	V	III/R	III-2	L	R	—
39	V	III/R	III-2	L	R	—
40	V	III/R	III-2	L	R	—
41	B	III/R	III-3	M	R	+
42	B	III/R	III-3	M	R	+
43	CSF	III/R	III-3	L	S	+
44	CSF	III/R	III-3	M	R	+
45	CSF	III/R	III-3	M	R	+
46	B	III/R	III-3	M	R	+
47	B	III/R	III-3	L	R	+
48	V	III/R	III-3	L	R	+
49	V	III/R	III-3	M	R	+
50	V	III/R	III-3	L	R	+
51	V	Ib/c	Ib-1	M	R	—
52	V	Ib/c	Ib-1	L	S	—
53	V	Ib/c	Ib-1	L	S	—
54	V	Ib/c	Ib-1	L	S	—
55	V	Ib/c	Ib-1	L	S	—
56	V	II/R	II-1	L	S	—
57	V	II/R	II-1	L	R	—
58	V	II/R	II-1	L	R	—
59	V	II/R	II-1	L	R	—
60	V	II/R	II-2	L	R	—
61	V	V/R	V-1	L	R	—
62	V	V/R	V-1	L	R	—
63	V	V	V-2	L	R	—
64	V	V	V-2	L	R	—
65	V	V	V-2	L	R	—
66	V	NT6/c	NT6-1	L	S	—
67	V	NT6	NT6-1	L	S	—
68	V	NT6/c	NT6-1	L	S	—
69	V	NT6	NT6-1	L	S	—
70	V	NT6	NT6-1	L	S	—

V, vagina; B, blood; CSF, cerebrospinal fluid; SA, sialic acid; L, low; M, moderate; H, high; Tc, tetracycline; S, susceptible; R, resistant.

* Growth kinetics in FMC-200P medium; +, lag time growth; —, normal growth.

and the material was centrifuged at 2100 *g* at 4°C for 1 h. The supernate was treated with an equal volume of phenol-chloroform (v:v, 1:1) and two volumes of chilled (–20°C) ethanol 95% was added to the mixture. After standing at –20°C for 1 h, extracted DNA was pelleted by centrifugation at 2100 *g* at 4°C for 10 min, and resuspended in 200 µl Tris-acetic acid-EDTA (10 mM Tris acetic acid-2 mM disodium edetate, pH 8.0). RNAase A (2 µl, 1 mg/ml) was added to the DNA sample and the reaction mixture was held at 0°C for 30 min. After purification by treatment twice with phenol and chloroform,¹² the DNA sample was mixed with 50 µl 1 M sodium acetate and DNA was precipitated with ethanol 95% and centrifuged, as described above. The DNA pellet was washed with chilled ethanol 70%, dried under nitrogen and then dissolved in 20 µl of Tris-acetic acid-EDTA and stored at 4°C until use.

Detection of plasmid DNA

Chromosomal DNA was denatured by adding 50 µl alkaline solution (equal volumes of 100 mM Tris-2 mM disodium edetate and 0.2 N NaOH) to 2 µl of DNA extract. After neutralisation with 25 µl 3 M sodium acetate-acetic acid (pH 5.5), ethanol precipitation and drying under nitrogen (as described above) the material was dissolved in 10 µl of sterile water. Plasmid DNA was detected by electrophoresis as previously described.¹²

Restriction endonuclease digestion

The extracted DNA was treated with each of five endonucleases, *Bam*HI, *Bgl*II, *Hind*III, *Kpn*I, and *Sal*I (Takara Shuzo Co., Kyoto, Japan), under the conditions specified by the manufacturer. The reaction mixture (total volume 100 µl), containing 10 µl of DNA solution and 100 U of endonuclease, was allowed to react for 4 h. After the addition of 25 µl of 1 M sodium acetate, digested DNA was precipitated in ethanol as described above and dissolved in 20 µl of sterile water. Phage λ DNA (4 µg/ml) was digested with *Hind*III, and used as the migration distance standard.

Agarose gel electrophoresis

The electrophoretic technique for densitometric scanning¹² was used for the separation of digested DNA samples. Electrophoresis was performed in agarose gel 1.0% at 9.2 V/cm (120 V/gel) for 6 h. Gels were stained with ethidium bromide and photographed on a shortwave transilluminator (type TS36; Ultra-Violet Products Inc., San Gabriel, USA).

Densitometric scanning and numerical analysis

Densitometric scanning was performed as previously described,¹² and numerical analysis was done

by the method of Kersters and De Ley,¹³ except that the OD value of the area outside the sample lanes on the film was removed as background. Electrophoretograms on negative film were scanned with a scanning densitometer (Cliniscan, Helena Laboratories, Beaumont, USA) at 610 nm with a slit size of 2.5 × 0.1 mm. OD values at regular intervals along the direction of migration, (72 measurement points/10 mm of gel length) were recorded on a magnetic disk. Phage λ DNA-*Hind*III digests—mol. wts (10⁶) 15.27, 6.21, 4.33 and 2.88—run on adjacent lanes were used as markers to correct for distortion of migration distance on the scanning pattern. One of the densitometric patterns of the phage λ DNA-*Hind*III digests was designated as the reference pattern. Correction of the migration distance of the pattern (expansion or compression) to that of the reference pattern was done for each strain within each segment defined by the markers of the next lane. Similarity was calculated over a mol. wt range of (15.27–2.88) × 10⁶ comprising 218 measurement points; this excluded the low mol. wt regions containing low-intensity bands. The Pearson product moment correlation coefficients between all possible pairs of scanning corrected patterns were calculated on the basis of the OD value of the overlapping portion between a pair of the patterns, and similarity was expressed as a percentage value (multiplied by 100) for convenience. Patterns were clustered by the method of unweighted pair group average linkage (UPGMA).¹⁵ Data handling and analysis were performed with the aid of a locally written software package.

Quantitation of sialic acid

Cell-wall sialic acid (SA) was extracted from freeze-dried bacterial cells by acid-heat treatment as described previously.¹⁴ SA was determined by the thiobarbituric acid method¹⁶ with N-acetylneuraminic acid as the standard. According to previously described criteria¹⁴ SA levels were classified as low (<9 µg/mg bacterial cell dry weight), moderate (9–12 µg/mg), and high (>12 µg/mg).

Determination of MIC of tetracycline

The MIC of tetracycline (Tc) against GBS isolates was determined by the microdilution broth method¹⁷ with an inoculum of 10⁶ cfu/ml in Mueller-Hinton Broth (BBL). *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. GBS strains with MICs <1 µg/ml were classified as susceptible and strains with MICs >8 µg/ml as resistant.¹⁸

Growth kinetics in phosphate-containing media

The method of Maurer and Mattingly,¹⁹ with a chemically defined medium²⁰ containing 200 mM

phosphate (FMC 200P medium), was followed. Growth kinetics were measured by recording absorbance with a spectrophotometer (Ultraspec Plus 4054, Pharmacia LKB Biochrom, Cambridge) at a 675 nm. According to previously described criteria, isolates were classified as strains exhibiting lag time or normal growth kinetics.¹⁴

Results

Plasmid DNA

Plasmid DNA was not detected in alkali-denatured extracts of any of the 70 GBS strains tested.

Selection of restriction endonucleases

To select appropriate enzymes for RDP analysis, preliminary experiments were performed with five restriction endonucleases on DNA from four GBS strains (strains 5, 6, 44 and 45). RDPs produced by *Bam*HI, *Bgl*II, *Kpn*I and *Sal*I, were characterised by a broad band with segments of different sizes in the high mol. wt region. Such segments separate poorly with ordinary agarose gel electrophoretic techniques and these four enzymes were not used for further analysis. In contrast, RDPs produced by *Hind*III consisted of lower mol. wt segments ($<13 \times 10^6$) and *Hind*III was adopted for the main study.

Reproducibility of densitometric patterns

To check the reproducibility of RDPs, electrophoresis was performed on three different gels with DNA from GBS strains 5, 6, 44 and 45, and similarities were calculated for each strain. The RDPs were highly reproducible between gels; average similarities for GBS strains 5, 6, 44 and 45 were 98.8 SD 0.4, 99.1 SD 0.5, 98.7 SD 0.4 and 98.8 SD 0.5, respectively.

Numerical analysis

Agarose gel electrophoretograms of *Hind*III digests of DNA from representative GBS strains are shown in fig. 1. Numerical analysis results of *Hind*III RDPs, based on the determinations of similarity and UPGMA clustering, are summarised in fig. 2. Comparison at the 95% similarity level, where strains of different serotypes remained unclustered, revealed a total of 13 phenons, which were designated as RDP types. The average similarity of densitometric patterns within each of the RDP types Ia-1, Ib-1, II-1, V-1, V-2 and NT6-1 was $>98\%$. For RDP types Ia-2, Ia-3, Ia-4, III-1, III-2, and III-3, the average similarity level of the densitometric patterns within each of the RDP types was $>96\%$ but $<98\%$, and the patterns in the low-mol. wt region were visually identical within each of the RDP types.

Fig. 1. Agarose gel electrophoretogram of *Hind*III digests of representative GBS strains. Migration distances of mol. wt standards 15.27×10^6 (left hand triangle) and 2.88×10^6 (right hand triangle) were used to correct patterns before calculations of similarity.

RDP types and phenotypic characteristics

RDP types, SA cell wall content, susceptibility to tetracycline, and growth kinetics in FMC 200P medium of serotype Ia and III GBS strains are shown in table II. The correlation of RDP type with each of these phenotypic characteristics was poor except that RDP types Ia-1 and III-1 consisted exclusively of strains that had low cell-wall SA levels and were tetracycline-susceptible and all strains of RDP type

Fig. 2. Dendrogram of cluster analysis based on *Hind*III RDPs. Percentage similarities were determined by the correlation coefficient ($\times 100$) and UPGMA clustering. The densitometric RDP of a phage λ DNA-*Hind*III digest mol. wt (15.27×10^6 , 6.21×10^6 , 4.33×10^6 and 2.88×10^6 , left to right) is shown at the bottom of the figure. Only representative (similarity $> 98\%$) corrected densitometric RDPs are shown. Phenons (RDP types) were formed at the 95% similarity level.

III-3 were unique in exhibiting lag time growth kinetics in FMC 200P medium.

from meningitis did not belong to any particular RDP type.

RDP types and GBS disease patterns

For each of the 26 infecting isolates, RDP type and clinical disease classification were compared (table III). In cases of septicaemia occurring in the first 5 days of life, of 10 isolates, five were RDP type Ia-3, and four belonged to RDP type III-3. Isolates derived

Discussion

RDPs of chromosomal DNA between strains may vary in only a few fragments and the methods used in this study may not have detected such differences, particularly among the minor bands. Despite this,

Table II. Phenotypic characters and RDP types of serotype Ia and III GBS strains

RDP type	Number of strains							
	Total	Inf	col	SA content			Tc susceptible	Lag time* growth
				H	M	L		
Ia-1	4	0	4	0	0	4	4	0
Ia-2	6	2	4	3	2	1	0	0
Ia-3	8	5	3	5	1	2	5	0
Ia-4	3	2	1	0	1	2	0	0
III-1	6	1	5	0	0	6	6	0
III-2	13	9	4	1	0	12	0	0
III-3	10	7	3	0	6	4	1	10

Inf, infecting isolates; col, colonising isolates; SA, sialic acid; H, high; M, moderate; L, low; Tc, tetracycline.

* In FMC-200P medium.

Table III. Disease pattern and RDP type of 26 GBS infecting isolates

Strain no.	RDP type	Age of* patient (days)	Disease pattern	
			Septicaemia	meningitis
11	Ia-3	0	+	-
12	Ia-3	0	+	-
14	Ia-3	2	+	-
13	Ia-3	3	+	-
15	Ia-3	3	+	-
5	Ia-2	5	-	+
6	Ia-2	8	-	+
19	Ia-4	23	-	+
20	Ia-4	48	-	+
42	III-3	0	+	-
46	III-3	0	+	-
32	III-2	1	+	+
41	III-3	3	+	-
47	III-3	3	+	-
22	III-1	3	-	+
44	III-3	7	+	+
29	III-2	9	+	+
43	III-3	16	-	+
28	III-2	17	-	+
45	III-3	19	-	+
34	III-2	22	-	+
35	III-2	24	-	+
36	III-2	38	-	+
30	III-2	37	-	+
33	III-2	71	-	+
31	III-2	92	-	+

* Age at onset of infection.

numerical analysis results of *Hind*III RDPs of chromosomal DNA pointed to the effectiveness of this technique in separating GBS strains serotyped on the basis of capsular polysaccharide antigens. At similarity levels >95%, GBS strains with heterogeneous polysaccharide antigens were not linked; although strains of serotypes Ia, III, II and V could be subdivided into RDP types, numerical analysis data generally reflected serotyping. However, no correlation was demonstrated between protein antigens and RDP types, in some instances GBS strains sharing the

same polysaccharide antigen but with or without protein antigens showed similarities >95%.

The type-specific capsular polysaccharide of GBS is recognised as an anti-phagocytic factor.^{21,22} It has also been shown that amongst serotype Ia strains high levels of capsular SA correlates with resistance to tetracycline.¹⁸ In the present study, all GBS strains of RDP types Ia-1 and III-1 had low capsular SA levels and were susceptible to tetracycline; however, amongst other RDP types there was no correlation with SA levels or tetracycline susceptibility. GBS serotype III isolates from infected neonates or infants have been reported to exhibit lag time growth kinetics in FMC 200P medium.¹⁹ We found all GBS strains with this character were of RDP type III-3.

Systemic GBS infections can be broadly classified into two distinct disease patterns, septicaemia with respiratory tract infection and meningitis without respiratory symptoms.^{8,23} The former typically presents in the first 5 days of life and is normally the result of vertical transmission of the organism from the maternal genital tract.²⁴ Epidemiological studies have shown that more than half of strains from meningitis cases belong to serotype III.^{6,7} Such findings suggest that serotype III strains have a high tropism for the meninges or for the central nervous system. Most cases of early-onset septicaemia with respiratory distress are due to serotype Ia strains.⁸⁻¹⁰

In the present study, most GBS isolates from neonates with early-onset septicaemia were of RDP types Ia-3 and III-3. All the RDP type III-3 strains also exhibited lag time growth in FMC 200P medium. It has been suggested that this phenomenon is characteristic of strains with a high level of cell-wall lipoteichoic acid.¹⁹ Lipoteichoic acid has been reported to mediate specific adherence to human fetal cells.^{25,26} However, surface-associated proteins may also be important in mediating adherence.²⁷ We did not study directly the relationship between adherence of GBS and RDP type, but the strong association between strains belonging to RDP types Ia-3 or III-3

and early-onset disease is notable. As various RDP types were represented amongst GBS type III isolates from cases of meningitis, the tropism for the meninges suggested for serotype III strains does not appear to be linked to a particular RDP type but to be common to strains of this serotype.

In the study of GBS disease patterns and the characters of causative strains, our epidemiological analysis with *HindIII*-RDP typing provided some new information. However, to draw firmer conclusions, more GBS strains need to be studied. Southern blot hybridisation patterns of restriction digests

obtained with a 16S/23S ribosomal RNA gene probe have been used to identify strains of various bacterial species.¹¹ The main advantage of this technique is that it greatly reduces the number of bands in the RDP and so simplifies interpretation.¹¹ This technique may allow RDP differences between GBS isolates to be detected and analysed without the need for scanning by laser densitometry.

We thank Mr Y. Ida and Mr T. Maruko of Funabashi Medical Center for their co-operation.

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