BACTERIAL CHARACTERISATION AND PATHOGENICITY

Haemolytic activity of the '*Streptococcus milleri* group' and relationship between haemolysis restricted to human red blood cells and pathogenicity in *S. intermedius*

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A collection of 297 clinically documented '*Streptococcus milleri*' strains, identified to the genotype level by 16S rRNA gene hybridisation, was screened for haemolysis of human and animal red blood cells. Forty-nine strains (65%) of the *S. intermedius* genotype displayed haemolysis restricted to human blood; they were named 'exclusive human haemolytic' (EHH) *S. intermedius* strains. The 26 remaining *S. intermedius* strains were named *S. intermedius* non-EHH strains. Quantitative studies on the haemolysis indicated that intermedilysin was the factor involved. The *S. intermedius* EHH strains represented the *S. intermedius* phenotype, whereas the *S. intermedius* non-EHH strains were phenotypically characteristic of *S. constellatus*. The complete 16S rRNA sequences of the *S. intermedius* EHH strains exhibited identity with *S. intermedius* strains ATCC 27335 (= NCDO 2227, NCTC 11324); the 16S rRNA sequences of the *S. intermedius* non-EHH strains were identical to *S. constellatus* strain ATCC 27823 (= NCDO 2226, NCTC 11325) except for positions 228 and 229 that carried an *S. intermedius* sequence signature. The 16S sequence similarities between the non-EHH strains and the *S. constellatus* and the *S. intermedius* type strains were 99.5% and 98.6%, respectively. Hybridisations of the complete 16S rRNA genes with oligonucleotide probes indicated a 16S rRNA homogeneity within the *S. intermedius* EHH and the non-EHH strains respectively. The *S. intermedius* EHH strains were isolated most frequently from infection- and abscess-related specimens. The present data emphasise the genetic variability within the *S. constellatus* species and redefine the *S. intermedius* species as a homogeneous group at the 16S rRNA level.

Introduction

Streptococcus intermedius, *S. anginosus* and *S. constellatus* belong to the '*Streptococcus milleri*' group (SMG) that forms a separate phylogenetic group recently designated as the 'anginosus group' [1, 2]. SMG strains are notorious because of their ability to cause purulent infections and abscesses and are known for their phenotypic heterogeneity, which makes them difficult to identify [3, 4]. A previous study described a collection of SMG strains that were identified to the 16S rRNA genotype level by hybridisation of the PCR-amplified partial 16S rRNA gene with species-specific

oligonucleotide probes. These probes were homologous to the 213–231-bp regions of the 16S rRNA sequences of the type strains *S. anginosus* ATCC 33397^T, *S. constellatus* ATCC 27823^T and *S. intermedius* ATCC 27335^T. That study revealed a distinct 16S rRNA ribosomal population that reacted with both the *S. intermedius*- and *S. constellatus*-specific probes: these strains, referred to as 'CI strains', were most closely related to *S. constellatus* strain ATCC 27823^T [5].

Although SMG strains are part of the 'viridans streptococci', they may exhibit all three types of haemolysis (α -, β - or γ -haemolysis). Several studies reported β -haemolytic '*S. milleri*' strains to be associated with purulent disease more frequently than non-haemolytic strains [6, 7], but this association was not found by others [8]. A study of 499 SMG clinical

Received 16 Feb. 1999; accepted 7 June 1999.

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strains consecutively isolated irrespective of their haemolytic behaviour found that non-haemolytic strains were isolated more frequently from abscess-related specimens than β -haemolytic strains [9]. Furthermore, it found that the *S. intermedius* phenotype was proportionally more associated with infection and abscesses than the *S. anginosus* and *S. constellatus* phenotypes. The data also confirmed the findings of Whiley and co-workers [10] who reported that the majority of *S. intermedius* strains are non-haemolytic on sheep blood agar. These findings apparently dispute the assumed association between β -haemolysis and pathogenicity. Recently, Nagamune and co-workers identified a cytotoxin in an *S. intermedius* strain, which they called intermedilysin [11]. They demonstrated that intermedilysin was strongly haemolytic against human red blood cells (RBC) but did not lyse RBC from non-primates. The authors raised the question as to whether intermedilysin analogues were expressed by the other SMG species, but no information on this subject has been reported to date. Moreover, it is not yet known if all *S. intermedius* strains produce this enzyme.

This study investigated the expression of the haemolytic activities of the different SMG species against RBC of human and animal origin and correlated the expression of haemolytic activity against human RBC with the clinical significance of the strains. In addition, it investigated the genotypic heterogeneity amongst strains that exhibited different haemolytic patterns against human and animal RBC.

Materials and methods

Bacterial strains, phenotypic and genotypic identification and clinical significance

The streptococci studied were isolated from clinical specimens submitted to the microbiology laboratory of the University Hospital of Maastricht. Their phenotypic and genotypic identification, site of origin and clinical significance have been described previously [5]. Briefly, streptococci were included in the study if they were identified as '*S. milleri*' by the API 20 Strep system supplied with the database 4.0 (bioMérieux, Marcy-l'Etoile, France). Phenotypic species identification was performed according to the scheme described by Whiley and co-workers [12]: strains were tested for production of glycosidase enzyme activities with 4-methylumbelliferyl-linked fluorogenic substrates (Sigma) and for production of hyaluronidase by a plate assay [13]. Genotypic identification was performed in a line blot assay: PCR-amplified 16S ribosomal RNA gene was hybridised with species-specific 5'-biotinylated oligonucleotide probes homologous to the 213–231-base region of the 16S rRNA gene sequences of the type strains *S. anginosus* ATCC 33397^T (= NCTC 10713), *S. constellatus* ATCC 27823^T (= NCDO 2226, NCTC 11325) and *S. intermedius* ATCC 27335^T

(= NCDO 2227, NCTC 11324). Strains were assigned to broad clinical categories (abscess-related, infection-related and questionable clinical significance) based upon the opinion of the clinician treating the patient [14].

The isolates were stored at -70°C on porous beads in a cryopreservative (Microbank, Pro-Lab Diagnostics, Richmond Hill, Ont, Canada). Before inclusion in the study, they were retrieved, checked for purity and subcultured. Two strains were not recoverable. All 75 strains genotypically identified as *S. intermedius* were included in the study, and 101 *S. anginosus* strains were randomly selected from the collection.

In the previous study, the PCR-amplified 16S rRNA genes of 41 SMG strains hybridised with the probes directed to the 213–231 base region of the 16S rRNA genes of both the *S. constellatus* ATCC 27823^T and the *S. intermedius* ATCC 27335^T type strains [5]. These 'CI strains' were presently grouped together with the 80 genotypic *S. constellatus* strains, to which they are most closely related. Reference strains included in the collection were the type strains *S. anginosus* ATCC 33397^T, *S. constellatus* ATCC 27823^T and *S. intermedius* ATCC 27335^T.

Detection and characterisation of haemolytic activity

For the qualitative detection of haemolytic activity, fresh RBC obtained from sheep, cow, horse, pig and rabbit were kindly provided by Dr van den Boogaard (Central Experimental Animal Facilities, Maastricht University, The Netherlands). Human RBC of group O Rh⁺ were obtained from one of the authors (J.A.J.). RBC were washed three times with phosphate-buffered saline (PBS) pH 7.4, and added (2.5% v/v) to Blood Agar Base No. 2 (Oxoid); 20 ml were dispensed into polystyrene Petri dishes. The plates were left at room temperature overnight to dry and then inoculated with 10^4 cfu/spot with a multipoint inoculator (Denley, Sussex) and incubated in air with CO₂ 7% at 35°C. After incubation for 18 h, the colonies were stabbed with a sterile toothpick and incubated in CO₂ 7% at 35°C for another 24 h. Haemolysis was read by observation of the subsurface growth, both α - and γ -haemolysis were recorded as 'non-haemolytic'. The effect of hot and cold incubation on the haemolysis of human RBC was studied on 12 strains by incubating inoculated blood plates at 35°C for 24 h, and subsequently transferring them to 4°C overnight. Phospholipase enzyme activities were assayed with Egg Yolk Emulsion (Oxoid 5% v/v) added to Blood Agar Base No. 2.

For quantitative assessment of the haemolytic activities of the culture supernatant fluids, streptococci were grown in 10 ml of Todd-Hewitt Broth (THB; Oxoid) in screw-capped culture tubes incubated for 24 h at 35°C

and 1 ml of culture from each tube was transferred to another THB and incubated for 24 h. Cultures were centrifuged in an 'EBA 12' bench microcentrifuge (Hettich, Tuttlingen, Germany) at 22 000 g for 5 min. Culture supernates were collected aseptically, filtered through a 0.2- μ m pore size filter (Acrodisc 32, Gelman Sciences, Ann Arbor, MI, USA) and then stored at -70°C until analysed. Citrated human blood was washed three times with PBS and used within 1 week of collection. Spectrophotometric analysis was performed with 150 μ l of an erythrocyte suspension (5% v/v in PBS) and an equal volume of culture supernate in sealed Eppendorf tubes. The reaction mixtures were incubated in a water bath at 37°C for 1 h with frequent inversion of the tubes. The reaction was stopped by centrifugation at 22 000 g for 5 min, and 200 μ l of the reaction mixture were transferred to a flat-bottomed microtitration plate (Hospidex, Nieuwkoop, The Netherlands). The released haemoglobin was measured at 540 nm with a Novopath microplate reader (BioRad, North Yorkshire). All assays were performed in duplicate and corrected for turbidity of the culture supernate and non-specific haemolysis due to the THB.

For further characterisation of haemolytic activity against human RBC, spectrophotometric assays were performed on supernates from four strains which were diluted in PBS until they displayed an optical density at 540 nm (OD_{540}) between 0.600 and 0.800. The supernates were incubated with cysteine-HCl (Merck, Darmstadt, Germany; final concentration 15 mM), DTT (dithiothreitol, Merck; concentrations 2, 10 and 50 mM), DNTB (5.5'-dithio-bis-(2-nitrobenzoic acid), Sigma; 0.2, 1 and 5 mM), H_2O_2 (Merck; 0.09, 0.018 and 0.36% v/v) and proteinase K (Boehringer Mannheim, Mannheim, Germany; 10 and 100 $\mu\text{g}/\text{ml}$). Results were corrected for solvent effects. Additionally, the culture supernates were heated at 100°C for 15 min. Finally, the RBC were pre-incubated with trypsin (ICN Biomedicals, Costa Mesa, CA, USA; concentrations 0.1, 1.0 and 10 mg/ml), washed twice with PBS and then used in the haemolytic assay.

Total 16S rRNA sequencing and reverse line blot hybridisation

For further characterisation to 16S rRNA level, isolates were retrieved onto blood agar base (Oxoid) with sheep blood 5% v/v, checked for purity and streaked on secondary plates. The cells were transferred to a microcentrifuge tube and spun twice, first in a 100- μ l volume and then in a 900- μ l volume of 10 mM Tris, 150 mM NaCl buffer, pH 8.0. The supernate was discarded and the cells were resuspended in 100 μ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and heated at 99°C for 10 min.

Primers corresponding to residues 8–27 (primer 16S8FE) and 1523–1542 (biotin-labelled primer B-16S1523 RB) of the *Escherichia coli* 16S rRNA gene

sequence were used to amplify the entire 16S rRNA gene sequence. Amplification was performed in 25- μ l reaction volumes, in an OmniGene Thermal Cycler (Hybaid Omnigene, Biozym, Landgraaf, The Netherlands). Each sample contained 10 pmol of both primers, 0.25 units of super Taq DNA polymerase (HT Biotechnology, Cambridge) and standard amounts of amplification reagents (200 μM of each deoxynucleoside triphosphate, 50 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl_2 , gelatin 0.01%, Triton X-100 0.1%). A 25- μ l overlay of sterile mineral oil was added to the tubes. The PCR programme used was 3 min at 95°C , followed by 25 cycles of amplification that consisted of 20 s at 95°C , 30 s at 55°C and 30 s at 72°C .

Fluorescence-labelled dideoxynucleotide technology was used for DNA sequencing reactions (Perkin-Elmer, Applied Biosystems Division, Gouda, The Netherlands). PCR products were purified with Qiaquick PCR purification kits (Qiagen, Hilden, Germany). Sequence reactions were analysed on an ABI 377 automated DNA sequencer (Perkin-Elmer). Sequencing was performed with various 16S rRNA specific primers. The collected sequences were assembled, edited and analysed with the DNASTar package (DNASTar, Madison, WI, USA).

The PCR-amplified 16S ribosomal RNA genes were hybridised with 5'-amino-linked oligonucleotide probes (Isogen Bioscience, Maarssen, The Netherlands) directed to the species-specific positions of *S. intermedius* ATCC 27335^T and *S. constellatus* ATCC 28223^T. The sequences of these probes are listed in Table 1. Hybridisation reactions were performed by a reverse line blot (RLB) assay as described by Kaufhold and co-workers [15]. Oligonucleotide probes were covalently linked to an activated Biodyne C membrane (Pall Filtron, Breda, The Netherlands) by the 5'-amino-group link. For this purpose, the membrane was activated by incubation for 15 min in 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide (Sigma) 16%, rinsed with water and placed in a miniblott system (Immunetics, Cambridge, MA, USA). Slots were filled with 150 μ l of an oligonucleotide suspension, which consisted of (depending on the probe) 50–1600 pmol of oligonucleotide probe in 500 mM NaHCO_3 , pH 8.4. After incubation for 1 min, excess solution was aspirated, and the blot was removed and inactivated by incubation with 100 mM NaOH for 10 min in a rolling bottle. The blot was then washed with 150 ml of $2 \times \text{SSPE}$ (360 mM NaCl, 20 mM NaH_2PO_4 , $^*\text{H}_2\text{O}$ 2 mM EDTA, pH 7.2) sodium dodecyl sulphate (SDS) 0.1% w/v, for 5 min at 60°C . For hybridisation, the filter was again mounted into the miniblott in a 90° rotated position, resulting in a perpendicular position of the slots on the lines which contained the oligonucleotide probes. The slots were filled with 150 μ l of heat-denatured biotinylated PCR products (10 μ l of the amplification product in 140 μ l of $2 \times \text{SSPE}$ -SDS 0.1% w/v heated

Table 1. Oligonucleotide sequences of primers and probes used in PCR and reverse line blot assay

Designation	Target specificity	Nucleotide sequence	Position in 16S rRNA gene
Primers for PCR			
16S8FE	Eubacterial 16S rRNA gene	GGA ATT CAG AGT TTG ATC MTG GYT CAG	8–27
B-16S1523 RB	Eubacterial 16S rRNA gene	5'-Biotin-CGG GAT CCA AGG AGG TGA TCC ADC CVC A	1523–1542
Amino-linked oligonucleotide probes for reverse line blotting			
Con 210	<i>S. constellatus</i> ATCC 27823 ^T	5'-amino-link GTG CAA GAG CAT CAC TAC	229–246
Con 280	<i>S. constellatus</i> ATCC 27823 ^T	5'-amino-link GCT CAC CAA GGC AAC G	288–303
Con 1030	<i>S. constellatus</i> ATCC 27823 ^T	5'-amino-link TCG GGG CAG AGG TGA	1052–1066
Int 210	<i>S. intermedius</i> ATCC 27335 ^T	5'-amino-link GTG CAA ATG CAT CAC TAC	229–246
Int 280	<i>S. intermedius</i> ATCC 27335 ^T	5'-amino-link GCT CAC CTA GGC GAC	288–303
Int 1030	<i>S. intermedius</i> ATCC 27335 ^T	5'-amino-link TCG GTA CAT CGG TGA CA	1053–1069

at 99°C for 10 min and chilled on ice) and incubated at 42°C for 60 min. Afterwards, the slots were aspirated and the blot was removed from the miniblitter and washed twice at 52°C for 10 min with 2 × SSPE-SDS 0.5% w/v. The membranes were incubated with streptavidin-peroxidase conjugate (Boehringer Mannheim, Mannheim, Germany; diluted 1 in 4000) at 42°C for 45 min in 10 ml of 2 × SSPE-SDS 0.5% w/v and then washed twice (10 min each) with 150 ml of 2 × SSPE-SDS 0.5% w/v at 42°C. After a final 5-min wash step with 2 × SSPE, the membranes were incubated with ECL detection reagents (Amersham International, 's Hertogenbosch, The Netherlands) for 1 min and used to expose an ECL hyperfilm (Amersham) for 10 min to visualise hybridisation. After each hybridisation and detection step, the PCR products were stripped from the membrane by washing it twice in SDS 1% w/v at 80°C for 30 min and thereafter the blot was incubated in 20 mM EDTA, pH 8.0, sealed moist in plastic and stored at 4°C until re-used.

Statistical analysis

For statistical evaluation, proportions were compared by the χ^2 test.

Nucleotide sequences and accession numbers

The sequences of the strains investigated in the study are available from Gen-Bank under the following accession numbers: *S. intermedius* ATCC 27335, AF104671; *S. intermedius* EHH strain no. 125,

AF104672; *S. intermedius* EHH strain no. 488, AF104673; *S. intermedius* non-EHH strain no. 535, AF104674; *S. intermedius* non-EHH strain no. 539, AF104675, *S. constellatus* ATCC 27823, AF104676; *S. constellatus* strain no. 206, AF104677; *S. anginosus* ATCC 33397, AF104678; *S. anginosus* strain no. 1012, AF1046679.

Results

Detection and characterisation of haemolytic activities, phenotypic and clinical characteristics of the strains

A total of 297 strains was included in the study. Table 2 presents the results of both the 16S rRNA genotype and the phenotype in relation to each other. Table 3 displays the haemolytic activities of the SMG strains (identified as 16S rRNA genotypes and phenotypes) against human and animal RBC, and the oligonucleotide probes to which the strains hybridised in the RLB assay.

When screened by the quantitative plate method, 124 strains produced β -haemolysis on sheep blood agar and 173 strains did not. With the exception of a few discrepancies, the results of the haemolysis on sheep blood agar were identical to those obtained on agar with RBC from cow, horse, pig and rabbit. The haemolytic pattern of the strains that lysed animal blood was similar for the RBC of all animal species tested: a zone of haemolysis that was best discernible on bright light transmission underneath a glass plate

Table 2. Comparison of phenotype and 16S rRNA genotype of SMG strains

Phenotype*	Number of strains identified to the genotype†			Total
	<i>S. anginosus</i>	<i>S. constellatus</i> ‡	<i>S. intermedius</i>	
<i>S. anginosus</i>	101	1	0	102
<i>S. constellatus</i>	0	113	22	135
<i>S. intermedius</i>	0	7	53	60
Total	101	121	75	297

*Identified by a panel of glycosidase enzymes [12].

†Identified by hybridisation of partial 16S rRNA gene with species-specific oligonucleotide probes [5].

‡The previously described CI strains (41) [5] are included within the *S. constellatus* 16S rRNA genotype.

Table 3. Haemolytic activities of strains with distinct 16S rRNA genotypes against RBC of animal and human origin

16S rRNA genotype and designation	Haemolysis on blood agar plate		Probes that hybridised in RLB	Phenotype
	Animal	Human		
<i>S. anginosus</i> (101)	Narrow zone (25) No haemolysis (76)	Narrow zone (25) No haemolysis (76)	ND	<i>S. anginosus</i> (101)
<i>S. constellatus</i> (121)	Narrow zone (74) No haemolysis (47)	Narrow zone (74) No haemolysis (47)	Con 210, Con 280, Con 1030 (121)	<i>S. anginosus</i> (1) <i>S. constellatus</i> (113) <i>S. intermedius</i> (7)
<i>S. intermedius</i> EHH strains (49)	Narrow zone (1) No haemolysis (48)	Large zone (1) Large zone (48)	Int 210, Int 280, Int 1030 (49)	<i>S. intermedius</i> (49)
<i>S. intermedius</i> non-EHH strains (26)	Narrow zone (24) No haemolysis (2)	Narrow zone (24) No haemolysis (2)	Int 210, Con 280, Con 1030 (26)	<i>S. constellatus</i> (22) <i>S. intermedius</i> (4)

ND, not done; (), number of strains.

and did not extend >2 mm outside the colony edge. This pattern was shared by strains that belonged to all three 16S rRNA genotypes. For strains belonging to the *S. anginosus* and *S. constellatus* 16S rRNA genotypes, the results of the haemolysis on human blood agar plates were identical to those obtained on animal blood agar. In contrast, 48 strains belonging to the *S. intermedius* 16S rRNA genotype displayed no haemolysis on animal blood agar, but showed haemolysis on human blood agar. Their haemolytic pattern on human blood agar appeared as a clear, sharply delineated, distinctly large zone, that often reached up to 3 mm outside the edge of the colony. A single *S. intermedius* strain showed a narrow zone of haemolysis on animal blood agar, but displayed a large zone of haemolysis on human blood agar. This strain was grouped together with the 48 strains that did not show any detectable zone of haemolysis on animal blood agar. These 49 strains were designated 'S. intermedius exclusive human haemolytic' strains (*S. intermedius* EHH strains) and included the *S. intermedius* ATCC 27335 type strain. The remaining 26 *S. intermedius* strains were designated 'S. intermedius non-EHH' strains. Of these *S. intermedius* non-EHH strains, 24 displayed a narrow zone of haemolysis on the animal blood agar and two strains did not affect RBC of any species tested.

In the quantitative assay for haemolysis of human RBC, all *S. intermedius* EHH strains had OD₅₄₀ units

>0.550, whereas the *S. intermedius* non-EHH strains, as well as control strains belonging to *S. anginosus* (14) and *S. constellatus* (18), did not show any detectable haemolytic activity.

In the phenotypic identification method, all the *S. intermedius* EHH strains correlated with the *S. intermedius* phenotype. In contrast, the majority (22 of 26) of the *S. intermedius* non-EHH strains were characterised phenotypically as *S. constellatus* and thus accounted for the identification mismatch between the *S. intermedius* genotype and phenotype that is apparent from Table 2. In terms of individual phenotypic identification reactions, the *S. intermedius* EHH strains invariably displayed neuraminidase, β -D-N-acetylgalactosaminidase and β -D-N-acetylglucosaminidase enzyme activities, whereas the majority of the *S. intermedius* non-EHH strains (23, 23 and 25 strains, respectively) did not ($p < 0.001$).

Amongst the patients from whom the *S. intermedius* EHH strains were isolated, a male:female ratio of 5.6:1 was observed. The *S. intermedius* EHH strains were significantly more frequently associated with abscess- and infection-related samples than the *S. intermedius* non-EHH strains (Table 4). They were proportionally (but not significantly) recovered more frequently from the abdomen, the head and neck region, skin, soft tissues and the blood.

Table 4. Male:female ratio of the patients from whom the SMG strains were isolated and clinical significance of the samples from which the SMG strains were isolated*

16S rRNA genotype and designation	Male:female ratio	Number of strains that were	
		Abscess- or infection-related	Questionable clinical significance
<i>S. intermedius</i> EHH strains	5.6:1 ($p < 0.001$)	46	2 ($p < 0.001$)
<i>S. intermedius</i> non-EHH strains	1.2:1	8	18
<i>S. constellatus</i>	1.4:1	68	52
<i>S. anginosus</i>	0.7:1	49	51

*ATCC type strains were omitted from the comparison.

Anaerobic incubation did not enhance the haemolytic activity of the *S. intermedius* EHH strains against human RBC, and no increase in haemolysis against human RBC was found after hot rather than cold incubation. In the qualitative assay of haemolysis against human RBC, the haemolytic activities of the culture supernates of the *S. intermedius* EHH strains were not affected by DNTB and DTT, and not enhanced by incubation with cysteine-HCl. After heating at 100°C, the haemolytic activity of the culture supernates was completely abolished. Trypsinisation of the RBC resulted in a decrease of haemolytic activity, with total loss of haemolysis at the final concentration of 10 mg/ml. Phospholipase activity was not detected

in any of the 60 strains tested (20 strains of each SMG species were tested).

Determination of 16S rRNA sequence and reverse line blotting

The nearly complete 16S rRNA sequences of the three ATCC strains, two *S. intermedius* EHH strains, two *S. intermedius* non-EHH strains and a randomly chosen *S. anginosus* and *S. constellatus* strain were determined. Fig. 1 shows the genetic distances and a phylogenetic tree sequence similarity of these sequences. The *S. intermedius* EHH strains had $\geq 99.9\%$ sequence similarity with *S. intermedius* strain ATCC 27335^T.

Fig. 1. Genetic relationships among the ATCC type strains *S. anginosus* 33397, *S. constellatus* 27823 and *S. intermedius* 27335, two *S. intermedius* EHH strains, two *S. intermedius* non-EHH strains and a randomly chosen strain each of *S. anginosus* and *S. constellatus*. Results are based on the comparison of the virtually complete 16S rRNA gene sequences (1523 bp). (a) Unrooted phylogenetic tree showing the inter-relationships between the different strains. (b) Levels of relatedness of the different strains.

The 16S rRNA gene sequences of the *S. intermedius* non-EHH strains shared the sequences of *S. constellatus* strain ATCC 27823^T except for positions 228 and 229 that carried an *S. intermedius* signature sequence. The *S. intermedius* non-EHH strains were most closely related to the *S. constellatus* strains, showing a 16S rRNA sequence similarity with the *S. constellatus* and *S. intermedius* type strains of 99.5% and 98.6%, respectively. RLB hybridisation with the oligonucleotide probes demonstrated genotypic homogeneity within the *S. intermedius* EHH strains and the *S. intermedius* non-EHH strains, respectively. The *S. intermedius* EHH strains reacted invariably with the three *S. intermedius* probes, whereas the *S. intermedius* non-EHH strains hybridised with the 229–246 *S. intermedius* probe and with the 288–303 and the 1052–1066 *S. constellatus* probes (Table 2).

Discussion

This study investigated the haemolytic activity of 297 SMG strains against RBC from man and various animal species. It demonstrated that some of the strains belonging to the *S. intermedius* 16S rRNA genotype (*S. intermedius* EHH strains) produced haemolytic activity restricted to human RBC, whereas others did not (*S. intermedius* non-EHH strains).

Although the study did not attempt to isolate the enzyme responsible for the haemolytic activity, the pattern of haemolysis of the *S. intermedius* EHH strains on human blood agar, together with the physiological properties of the haemolytic activities of the culture supernates, indicated that intermedilysin was the enzyme involved in haemolysis. According to Nagamune and co-workers, the most distinctive property of intermedilysin is its animal species specificity, as only human RBC were lysed by the enzyme [11]. In the present study, this particular characteristic was used to screen a collection of SMG strains. In agreement with the findings of Nagamune and co-workers, the haemolytic activity of the culture supernates in this study was heat-labile and was not influenced by either reducing or oxidising agents, precluding the possibility of a thiol-activated enzyme. Furthermore, none of the strains examined was active against lecithin, which suggested that the haemolysin did not fall into the phospholipase category of membrane-damaging haemolysins. Consequently, it may be concluded that intermedilysin activity is confined to the *S. intermedius* species as defined by both the complete 16S rRNA sequence and by the presence of neuraminidase, β -D-N-acetylgalactosaminidase and β -D-N-acetylglucosaminidase enzymes.

The relationship between abscess- and infection-related clinical samples on the one hand and the presence of *S. intermedius* EHH strains on the other suggested that the haemolysis of human RBC may be a factor in

pathogenicity. Further studies with defined mutants, deficient in haemolytic activities, are necessary to obtain direct evidence for the pathogenic nature of the haemolytic activity.

Most diagnostic laboratories use sheep or horse blood agar to screen for haemolysis and this may explain the paradoxical fact that non-haemolytic SMG strains have been found to be more associated with purulent infections than the β -haemolytic SMG strains [8, 9]. Indeed, from the present data, many of the non-(sheep) haemolytic SMG strains encountered in diagnostic practice represented *S. intermedius* strains that invariably expressed haemolysis against human RBC without affecting animal RBC. Furthermore, in diagnostic laboratories, β -haemolytic streptococci are investigated in detail more frequently than are non-haemolytic streptococci [4]. For this reason, the real incidence of *S. intermedius* in clinical samples might have been underestimated. According to the present findings, the lysis of human RBC could be explored as a possible taxonomic property and it may be used as a valuable diagnostic tool to facilitate recognition of *S. intermedius* strains among the accompanying flora in clinical samples.

This study used RLB to hybridise the SMG strains with oligonucleotide probes tailored to recognise sequences that were determined by sequencing the complete 16S rRNA genes of a panel of representative strains. The key feature of RLB in this setting was its ability to screen a large number of strains, omitting the need to sequence them. Furthermore, RLB offered additional advantages: only small amounts of PCR products were needed, the different probes could be hybridised concurrently and the blots with the covalently linked probes could be re-used, resulting in a 'labour-friendly' and standardised system. The genotypic identification results for the *S. intermedius* and *S. constellatus* strains in the present study differed in part from the results of the previous study (Table 2) [5]. However, in the previous study, sequence relatedness was calculated on only part of the 16S rRNA gene (i.e., position 0–487) and a diagnostic probe for identification was constructed complementary to part of this sequence (i.e., position 213–231). In contrast, in the present study the complete 16S rRNA gene was used for comparison. Genotypic identification based on the total 16S rRNA gene as presently performed resulted in a higher agreement with the phenotypic identification method, in particular because the majority (22 of 26) of the *S. intermedius* non-EHH strains (that phenotypically behave as *S. constellatus*) were identified as *S. constellatus* whereas in the previous study they were assigned to the *S. intermedius* 16S rRNA genotype.

In terms of 16S rRNA gene relatedness, phenotypic identification and haemolytic activity, the *S. intermedius* EHH strains were identical to *S. intermedius* strain ATCC 27335^T and, therefore, constitute a single

species. Additional features that distinguished the *S. intermedius* strains as a distinct species included their predilection for defined body sites such as the dental plaque as commensals, and liver and brain abscesses as pathogens [9, 10]. An intriguing but yet unexplained feature documented in this study was the high male:female ratio of the patients from whom the clinical samples containing the *S. intermedius* EHH strains were recovered. Most of these samples were associated with purulent infection. Reviewing the SMG strains implicated in purulent infections, Gossling also compiled a strong male bias, even if cases associated with trauma or alcoholism were subtracted [3]. Unlike *S. intermedius*, the *S. constellatus* species appeared to display genotypic variability, as suggested in a previous report [5]. A similar phenomenon of intra-species diversity has been observed for *S. anginosus* as currently defined, with the existence of distinct phenotypic groups and genomic subgroups at the 16S rRNA level [16, 17]. Based on the close 16S rRNA gene relatedness between the *S. intermedius* non-EHH strains and *S. constellatus* strain ATCC 27823^T as expressed in Fig. 1, we assume that the *S. intermedius* non-EHH strains should be allocated to the *S. constellatus* species. However, at such high levels of 16S rRNA gene sequence similarities, DNA-DNA re-association studies are needed to confirm this supposition [18]. Despite the 16S rRNA homogeneity within the *S. intermedius* non-EHH strains, the present study did not find either phenotypic or clinical consistency. Thus, the *S. intermedius* non-EHH strains resemble the previously described CI strains that were considered to be a distinct rRNA population for which phenotypic and clinical significance remained to be determined [5]. At present, further studies are being conducted to investigate the relationships between the *S. intermedius* non-EHH strains, the CI strains and the *S. constellatus* strains.

In conclusion, the present study demonstrated that, within the SMG, haemolysis of human RBC was confined to the *S. intermedius* strains. Circumstantial evidence indicated that intermedilysin was the putative factor for haemolysis and further emphasised the role of this enzyme in pathogenicity. Analysis of the total 16S rRNA gene and RLB with specific oligonucleotide probes resulted in a better delineation of the *S. intermedius* 16S rRNA genotype and in the identification of a distinct 16S rRNA population that encompassed strains most closely related to *S. constellatus* ATCC 27823^T. Further studies are needed to provide direct evidence that the haemolytic activity against human RBC is a virulence factor, and to elucidate the genotypic variability within the *S. constellatus* species.

We thank Dr R. A. Whiley, St Bartholomew's and the Royal London Hospital School of Medicine and Dentistry, London, and Dr J. D. A.

van Embden, National Institute of Public Health and the Environment for critical reading of the manuscript.

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