BACTERIAL PATHOGENICITY

Haemolysin-deficient variants of *Streptococcus pyogenes* and *S. dysgalactiae* subsp. *equisimilis* may be overlooked as aetiological agents of pharyngitis

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Variants of large colony β -haemolytic Lancefield group A, C and G streptococci that are non-haemolytic or α -haemolytic on sheep blood agar have been detected in clinical specimens due to their enhanced haemolytic activity when grown on a new selective and differential blood agar medium containing colistin, nalidixic acid and pH 7.5-adjusted PIPES buffer (CNA-P). The large colony Lancefield group C and G isolates were identified as *Streptococcus dysgalactiae* subsp. *equisimilis* by API 20 Strep classification and 16S rDNA profiling. The haemolytic activity of these variants on various blood agar media, including CNA-P, was closely similar to that of known streptolysin S-defective mutants of *S. pyogenes* and was blocked by addition of cholesterol, a specific inhibitor of the streptolysin O family of haemolysins. As haemolysin variants could be detected in large numbers in cultures from patients with clinical symptoms of pharyngitis it is suggested that they may function as primary pathogens in such infections. The high frequency with which haemolysin variants were isolated from clinical specimens during a 3-month trial (3%, 13% and 10%, respectively, of group A, C and G streptococcal isolates) indicated that a substantial proportion of streptococcal infections may go undetected if only conventional sheep blood agar media are used in clinical laboratories for the detection of β -haemolytic streptococci. As haemolysin variants have been implicated in the development of serious streptococcal sequelae, further investigation of the full extent of their contribution to streptococcal disease is indicated.

Introduction

β -Haemolytic streptococci of Lancefield groups A, C and G are commonly present in the human pharynx where they can adopt either a commensal or a pathogenic role. When grown on blood agar media, some of these streptococci typically form small colonies and others form relatively large colonies. The small colony forms belong to the *Streptococcus anginosus* group, whereas the larger colonies are generally *S. pyogenes* (group A), *S. equi* subsp. *zooepidemicus* (group C) or *S. dysgalactiae* subsp. *equisimilis* (group C or group G). The latter have been associated with a wide variety of diseases including septicaemia, endocarditis, pneumonia and meningitis

[1–4]. There is also the ever-present risk of development of glomerulonephritis [5] or of reactive arthritis [6, 7] subsequent to group C or G streptococcal infection. Although there have been numerous reports of epidemic group C and G streptococcal pharyngitis in adults [8–13], their significance as a cause of pharyngitis in children is less firmly established. Chowdhury *et al.* [14] considered them not to be significant, whereas Cimolai *et al.* [15] found large colony isolates to be significant when isolated in moderate to large numbers from a throat culture, and Martin *et al.* [12] demonstrated that group G streptococci of one DNA fingerprint pattern were isolated from 73% of cases during a paediatric outbreak of streptococcal pharyngitis.

Considerable research has been undertaken into the relatedness of large colony forms of α - and β -haemolytic strains of groups C, G and L streptococci of both animal and human origin [16–19]. Recent taxonomic studies have led to these organisms being

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grouped into the subspecies *S. dysgalactiae* subsp. *dysgalactiae*, comprising α -haemolytic and non-haemolytic strains, and *S. dysgalactiae* subsp. *equisimilis* which are usually β -haemolytic, react with Lancefield group A, C, G or L antisera and are most frequently isolated from human infections [18–21].

Although *S. pyogenes* is generally considered to be β -haemolytic, non-haemolytic variants have been isolated from disease outbreaks [22]. In one report, a non-haemolytic variant was associated with an epidemic of pharyngitis which resulted in six cases of rheumatic fever [23]. Non-haemolytic variants of *S. dysgalactiae* subsp. *equisimilis* have not been reported previously, possibly because such variants may have been presumed to represent isolates of the normally non- β -haemolytic *S. dysgalactiae* subsp. *dysgalactiae*.

In the course of evaluating a new selective medium (CNA-P) for isolation of β -haemolytic streptococci in a clinical laboratory setting [24], group A, C and G haemolytic variants that failed to produce β -haemolysis on traditional sheep blood agar media, but which were β -haemolytic on CNA-P were identified. They were examined for production of streptolysins O and S, and their API-20 Strep and 16S rDNA profiles were determined.

Materials and methods

Strains, media, and growth conditions

Laboratory strains were subcultured on Columbia agar base (Gibco, Madison, WI, USA) supplemented with human blood 5% v/v and CaCO₃ 0.1% (BA-Ca). BA-Ca is used routinely in this laboratory to minimise the effect of acid production on culture viability during strain storage. Strains used in this study included fresh clinical isolates of haemolytic variant *S. dysgalactiae* subsp. *equisimilis* of Lancefield group C (strains 0726, 1312, 1446 and 3610) and Lancefield group G (strain 0662). Previously described [25] haemolytic variant *S. pyogenes* strains 82-541 (streptolysin S-deficient) and Blackmore (streptolysin O-deficient) were used for comparative purposes. Other controls included the β -haemolytic *S. pyogenes* M-type 1 strain SF370 (from J. Ferretti, University of Oklahoma, OK, USA), *S. dysgalactiae* subsp. *dysgalactiae* strain 67 (J. Tagg culture collection) and *S. equi* subsp. *zooepidemicus* strain 4881 (J. Tagg culture collection). Clinical specimens were plated on to sheep blood agar (SBA), CNA and CNA-P. SBA consisted of Columbia agar base supplemented with defibrinated sheep blood (Life Technologies) 5% v/v. CNA-P consisted of Difco Columbia CNA agar (which contains colistin sulphate 10 mg/L and nalidixic acid 15 mg/L, supplemented with 100 mM PIPES (piperazine-N-N'-bis-[2-ethanesulphonic acid]; Sigma), adjusted to pH 7.5 before autoclaving and then supplemented with sheep blood 5% v/v [24]. CNA lacked PIPES buffer and the pH

was not adjusted before autoclaving. Cholesterol agar was prepared by adding cholesterol 1 g/L to CNA-P agar after autoclaving. Agar media were poured to a depth of 4 mm and stored at 4°C in plastic bags until required. Enhanced haemolysis (EH) medium (Difco Columbia blood agar base EH medium; product code 0790) was prepared by Fort Richards Laboratories, New Zealand. The component Bacto Bitone H Plus is responsible for the enhanced β -haemolytic reactions on this medium.

Clinical specimens

Throat swabs obtained from patients with clinical symptoms of pharyngitis and referred to Southern Community Laboratories by physicians in the Dunedin area were plated on SBA, CNA and CNA-P and examined for β -haemolytic streptococci. A total of 361 pharyngeal swabs received during March–May 1999 was tested. Each swab was plated on to SBA, CNA and CNA-P, with the order of plating rotated weekly. The inoculum was streaked into four quadrants for single colony isolation by a standardised procedure [26]. Plates were incubated at 37°C in an atmosphere of CO₂ 5% in air and examined after 24 and 48 h. β -Haemolytic colonies were semi-quantified and the degree of positivity recorded as 1+ (10 or fewer colonies), 2+ (11–100 colonies), 3+ (>100 colonies, with some present in the third quadrant of the plate) and 4+ (β -haemolytic colonies extending into the fourth quadrant).

Identification of isolates

Initial detection of β -haemolytic streptococci was based upon their typical haemolysis and colony morphology [26]. Representative β -haemolytic colonies were subcultured to SBA and their Lancefield group identity was established serologically by latex agglutination (Streptococcal grouping kit, Oxoid) with group A, B, C, D, F and G antisera. Small colony β -haemolytic streptococci belonging to the *S. anginosus* group were excluded from this study. The commercial API-20 Strep system (bioMérieux) was used for biochemical classification of isolates. Representative colonies that were β -haemolytic on CNA-P, but not on SBA and CNA, were subcultured on CNA-P and then re-cultured on to SBA and CNA to confirm the status of their haemolytic activity. Additionally, isolates were serially cultured at least four times on CNA-P to establish whether or not their haemolytic activity was a stable strain characteristic.

Comparison of haemolytic activity with use of various media and incubation conditions

Pure cultures of haemolytic variants were compared with reference strains of haemolytic group A and C streptococci and with known streptolysin S or streptolysin O mutants for haemolytic activity on various

media. Subcultures of each strain to be tested were freshly grown on BA-Ca at 35°C in an atmosphere of CO₂ 5% in air for 18 h. Samples of these cultures were swabbed on each test medium, streaked for isolated colonies, and a stab was made into the confluent zone for examination of subsurface haemolysis. The media tested were CNA-P, CNA, BA-Ca, SBA and EH agar. Cultures were incubated aerobically, anaerobically and in CO₂ 5% in air. Colonies were examined for surface and subsurface haemolysis after 18 h and 48 h. The pH of the cultures after growth was measured with a surface pH electrode (Radiometer Copenhagen). Cholesterol-containing medium was used to specifically block streptolysin O activity.

PCR of 16S rDNA and *emm* genes

Nucleic acids were extracted by a boiling lysis method [27] and 1 µl of supernate was used in subsequent 50-µl PCR reactions. Universal eubacterial primers P0mod and P3mod were used to amplify *c.* 800 bp at the 5' terminus of the 16S rDNA as described by Wilson *et al.* [28]. Amplicons were purified with a Qiaquick PCR purification kit (Qiagen) and subjected to automated sequencing with an ABI 377 sequencer (Centre for Gene Research, Otago University). DNA-STAR software was used for alignment with 16S streptococcal sequences [16].

Emm-typing was accomplished with the group A streptococcus 'all-M' PCR primers as described previously [27, 29]. PCR products were digested with *Hae*III and *Hinc*II restriction endonucleases. Fragment sizes were compared and a representative isolate of each unique restriction profile was subjected to automated sequencing. Homology searches were performed on the GenBank database (BLAST) and the *emm* database (Centers for Disease Control and Prevention, Atlanta, GA, USA; <http://www.cdc.gov/ncidod/biotech/strep/strepindex.html>).

Results

Isolation of haemolytic variants from clinical specimens

Of 361 throat cultures examined, 104 (29%) were positive for large colony β -haemolytic streptococci and these comprised 64 (61%) Lancefield group A, 30 (29%) group C and 10 (10%) group G isolates (Table 1).

Seven of these isolates were β -haemolytic on CNA-P, but not on either CNA or SBA and in each case, the haemolytic variants represented a major portion of the bacterial growth on the plate (3+ or 4+ positivity). Two isolates were group A, four group C and one group G. Non- β -haemolytic colonies from the companion CNA and SBA plates were detected that exhibited β -haemolysis when re-streaked to CNA-P. Conversely, pure isolates of β -haemolytic colonies from CNA-P were found to be non- β -haemolytic when re-streaked to CNA and SBA. The group C and G isolates were identified as *S. dysgalactiae* subsp. *equisimilis* by the API 20 Strep system. The two group A variants were isolated early in the study and were inadvertently discarded. A group A haemolytic variant isolated in this laboratory in an earlier study was identified as an M-type 4 *S. pyogenes* (data not shown).

Evaluation of haemolytic activity

The absence of β -haemolytic activity was examined further by plating pure cultures of the variant isolates and of several previously characterised haemolytic variants on five media containing blood and incubating under different atmospheric conditions (Table 2). Four of the media contained sheep blood and the fifth (BA-Ca) incorporated human blood. All four group C isolates were β -haemolytic on CNA-P medium and non- β -haemolytic on CNA, SBA and EH. On BA-Ca there was some slight variation in the haemolytic activity and terminal pH of these isolates. As both BA-Ca and CNA-P contained additional buffering agents, it was possible that differences in culture pH were responsible for variations in haemolytic activity, as it is known that the pH should be maintained between 6.8 and 7.4 to avoid acidic pH-mediated streptolysin O inactivation or proteolytic degradation by proteases that are most active below pH 6.8 [30]. When the terminal pH of the group C streptococcal culture on BA-Ca was <6.9 no haemolysis was evident, whereas at pH values $\geq$ 6.9 some β -haemolysis was detected. This observation was consistent with the anticipated haemolytic activity of isolates that produced streptolysin O but not streptolysin S.

The haemolytic activity of the group G clinical isolate differed from that of the group C isolates in that β -haemolysis was observed on all five media when incubated anaerobically. However, as observed with the group C isolates, the group G strain was β -haemolytic

Table 1. Detection of β -haemolytic Lancefield group A, C and G streptococci in pharyngeal cultures when grown on CNA-P and other blood agar media

Medium	Number (%) of 361 pharyngeal cultures positive for β -haemolytic streptococci of Lancefield group			
	A	C	G	Total
All blood agar tested	64 (18)	30 (8)	10 (3)	104 (29)
CNA-P only	2 (0.5)	4 (1)	1 (0.2)	7 (2)

Table 2. Effect of blood agar medium composition and incubation atmosphere on haemolytic activity of streptococcal cultures

Culture medium	Incubation atmosphere	Presence of β -haemolysis and terminal pH of cultures*									
		Group C strains†		Group G strain 0662		<i>S. pyogenes</i> strain 82-541 (SLS-negative)		<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i> strain 67		<i>S. equi</i> subsp. <i>zooepidemicus</i> strain 4881	
		β	pH	β	pH	β	pH	β	pH	β	pH
CNA-P	Anaerobic	+	7.3	+	7.4	+	7.2	—	7.2	+	7.2
	CO ₂ 5%	+	7.3	+	7.3	+	7.3	—	7.2	+	7.4
	Aerobic	+	7.4	+	7.3	+ weak	7.3	—	7.3	+	7.4
CNA	Anaerobic	—	6.1	+	6.7	—	5.9	—	6.0	+	6.6
	CO ₂ 5%	—	6.4	—	6.8	—	6.5	—	6.6	+	6.6
	Aerobic	—	6.6	—	6.8	—	6.5	—	6.5	+	6.8
BA-Ca	Anaerobic	+ / —‡	6.7–6.9	+	7.0	—	6.7	—	6.6	+	6.8
	CO ₂ 5%	+ weak	7.0	+	7.2	—	6.9	—	7.0	+	6.9
	Aerobic	+ weak	7.1	+	7.2	—	7.0	—	7.0	+	6.9
SBA	Anaerobic	—	6.5	+	6.4	—	6.4	—	6.7	+	6.4
	CO ₂ 5%	—	6.8	—	6.5	—	6.8	—	6.9	+	6.7
	Aerobic	—	6.9	—	6.6	—	6.8	—	6.9	+	6.9
EH	Anaerobic	—	6.0	+	6.2	—	5.9	—	5.7	+	5.9
	CO ₂ 5%	—	6.2	—	6.5	—	6.3	—	6.1	+	6.1
	Aerobic	—	6.2	—	6.4	—	6.2	—	6.1	+	6.3

*Terminal pH in the confluent zone and presence or absence of β -haemolysis on each medium was noted.

†Group C strains 0726, 1446, 3610 and 1312 gave identical results except as noted below and for convenience are recorded as a composite.

‡At pH values of <6.9, β -haemolysis was not observed.

in all atmospheres when grown on CNA-P or on BA-Ca (terminal pH range 7.0–7.2) and it was non-haemolytic on CNA, SBA and EH media when cultured in an aerobic or CO₂-enriched atmosphere.

S. equi subsp. *zooepidemicus* 4881 (a β -haemolytic group C animal isolate) was strongly β -haemolytic on all five media tested under the three different atmospheric conditions. The largest haemolytic zones were produced on EH medium. The terminal pH ranged from a low of 5.9 on EH under anaerobic conditions to a high of pH 7.4 on CNA-P under CO₂ 5%. *S. dysgalactiae* subsp. *dysgalactiae* strain 67 (non-haemolytic group C animal isolate) was generally non-haemolytic, but did produce some α -haemolysis under a few test conditions within the confluent growth zones of these cultures.

Strains of *S. pyogenes* known to be deficient in production of either streptolysin O or streptolysin S were also tested for their haemolytic activity. *S. pyogenes* strain 82-541 has a *Tn916* insertion that abolishes streptolysin S activity and it only produces streptolysin O. The strain is β -haemolytic on CNA-P under anaerobic and CO₂ 5% incubation, but only has a small inner β - and wide outer α -haemolytic zone on CNA-P when incubated in air. No haemolysis was observed on any of the other media under the conditions tested. The Blackmore strain (SLO mutant) and wild-type M-type 1 strains were β -haemolytic under all test conditions, with activity and terminal pH values similar to those produced by *S. equi* subsp. *zooepidemicus* 4881 (data not shown). Strain Blackmore displayed enhanced zones of haemolysis on EH

medium comparable to zones observed with wild-type *S. pyogenes* and *S. equi* subsp. *zooepidemicus*.

These results suggested that the enhanced haemolysis observed on CNA-P was due to increased streptolysin O activity, whereas the increased haemolysis on EH agar was streptolysin S-dependent. To confirm that the CNA-P-specific haemolytic activity of the streptococci isolated in the present study was due to streptolysin O, the strains were plated on CNA-P containing cholesterol. Cholesterol and related sterols with a β -hydroxyl group on carbon 3 bind to SLO and prevent its absorption to susceptible cell membranes [30]. In the presence of cholesterol, β -haemolysis by the group C and G clinical isolates and by *S. pyogenes* 82-541 was abolished, whereas the haemolysis by *S. pyogenes* M-type 1 and *S. equi* subsp. *zooepidemicus* 4881 was unaffected.

Human but not animal isolates of group C and G streptococci have been shown to contain *emm*-like genes (*emmL*) that encode M proteins with biological and structural features similar to those observed for M proteins found in *S. pyogenes* [31, 32]. The group C and G isolates were tested for the presence of an *emmL* gene by PCR with *S. pyogenes*-specific primers. All five isolates yielded a product c. 1300–1350 bp, whereas *S. dysgalactiae* subsp. *dysgalactiae* and *S. equi* subsp. *zooepidemicus* were PCR negative. Three of the four group C streptococcal PCR amplicons had identical *Hae*III/*Hinc*II digest products of 1000 bp and 350 bp (designated 1446) and the fourth isolate (0726) had products of 1000 bp and 300 bp. The group G streptococcal PCR amplicon did not cut with *Hae*III

and *HincII* restriction endonucleases. The 5' ends of the three unique PCR products were sequenced and BLAST searches were conducted. The 1446 and 0726 sequences were identical to each other through the 450 bp sequenced, with the exception of an additional 42 bp located within the first 200 bp of the 1446 sequence. None of the three sequences showed significant homology to sequences in the *emm* database (Centers for Disease Control and Prevention, Atlanta). The 0726 and 1446 sequences did not match any GenBank sequences. Interestingly, the highest homology of the group G 0662 sequence was to a novel *emm*-type sequence from a putative *S. pyogenes* strain isolated recently in Thailand (GenBank AF104408). Species identification methods were not reported in the GenBank entry. In light of the recent report by Brandt *et al.* [20] of isolates of *S. dysgalactiae* subsp. *equisimilis* possessing the Lancefield group A antigen, classification by serogrouping should be viewed with caution if used as the sole method for identification. The three unique partial *emm* sequences have been entered in GenBank with the accession numbers AF226694 (strain 0726), AF226695 (strain 1446) and AF226696 (strain 0662).

Comparative analysis of partial 16S rRNA sequences of *Streptococcus* species by Bently *et al.* [16] revealed a region of sequence heterogeneity (*c.* bp 145–300 of the *Escherichia coli* numbering system) which allowed species discrimination and an assignment of intragenetic relationships. The 5' half of the 16S DNA was amplified and sequenced with conserved eubacterial 16S PCR primers. The sequence obtained from the group G isolate matched the 16S sequence of *S. dysgalactiae* subsp. *equisimilis* (GenBank AB008926) as did the sequences from the four group C isolates, except for a C/T transversion at bp 218 with respect to the GenBank sequence that was conserved in all four isolates.

Discussion

CNA-P was originally developed in this laboratory to minimise or abolish the bacteriocin activity of some strains of *S. salivarius* in order to enhance the detection of *S. pyogenes* when these are present in small numbers as in the carrier state [24]. On traditional isolation media, the growth of β -haemolytic streptococci is inhibited in the vicinity of bacteriocin-producing *S. salivarius* and bacteriocin-sensitive streptococci will only form characteristic colonies if they are outside the zone of influence of bacteriocin producers. In the course of the evaluation of CNA-P in a clinical laboratory setting, it was noted that in some specimens β -haemolytic colonies were isolated only on CNA-P irrespective of the concomitant presence of bacteriocin-producing *S. salivarius*. The previous observation that streptolysin S-deficient *S. pyogenes* produced β -haemolysis on CNA-P but not

on SBA [24] led to the suspicion that these isolates may also be SLS-deficient. During the short duration of this trial, seven group A, C or G streptococci were isolated that were not β -haemolytic on conventional blood agar media. The high frequency of pharyngitis-associated specimens that yielded these haemolysin variants and the high proportion of the streptococcal population that they represented in these specimens indicated that these bacteria could have considerable clinical significance.

The elimination of the β -haemolytic activity of the variant strains on CNA-P by addition of cholesterol to the medium suggested that streptolysin O was the only haemolysin produced by these strains. Variant strains were isolated in high numbers as the predominant organism in clinical samples from patients suffering symptoms of pharyngitis, which suggested a possible aetiological association with the disease. However, as it was not possible to access patient records or to obtain follow-up specimens during the course of the present study the possibility that these bacteria were sometimes carried in large numbers as commensals could not be excluded. Also, due to the short duration of the trial and the relatively small number of samples processed, it was not possible to predict whether variants would generally be present in such a high proportion of specimens or if these isolates (particularly those of group C) were representative of a localised outbreak.

A few reports in the very early literature on streptococci documented the occurrence of non-haemolytic variants of *S. pyogenes* [33–35]. Associations of non-haemolytic variants with disease outbreaks were reported by Coburn and Pauli [36] and Colebrook *et al.* [37]. Both episodes were caused by M-type 12 variants [22]. The strains isolated by Colebrook *et al.* produced only streptolysin O. More recently, James and McFarland (1971) reported an outbreak involving 118 air force recruits in Colorado, with six subsequent cases of rheumatic fever [23]. Interestingly, James and McFarland commented that although Colebrook *et al.* had speculated in 1942 that pathogenic streptococci were being missed in puerperal or surgical infections because of variations in haemolysin production, no further mention of non-haemolytic variants had appeared in the subsequent literature until their own report in 1971. Clearly, the question of the significance of these haemolysin variants as infectious disease agents has not been addressed adequately yet. With more widespread application of streptolysin O-enhancing media such as CNA-P the extent of their distribution in disease and carrier associations should be clarified.

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References

- Bradley SF, Gordon JJ, Baumgartner DD, Marasco WA, Kauffman CA. Group C streptococcal bacteremia: analysis of 88 cases. *Rev Infect Dis* 1991; **13**: 270–280.
- Carmeli Y, Ruoff KL. Report of cases of and taxonomic considerations for large-colony-forming Lancefield group C streptococcal bacteremia. *J Clin Microbiol* 1995; **33**: 2114–2117.
- Efstratiou A. Outbreaks of human infection caused by pyogenic streptococci of Lancefield groups C and G. *J Med Microbiol* 1989; **29**: 207–219.
- Salata RA, Lerner PI, Shlaes DM, Gopalakrishna KV, Wolinsky E. Infections due to Lancefield group C streptococci. *Medicine (Baltimore)* 1989; **68**: 225–239.
- Gnann JW, Gray BM, Griffin FM, Dismukes WE. Acute glomerulonephritis following group G streptococcal infection. *J Infect Dis* 1987; **156**: 411–412.
- Deighton C. Beta haemolytic streptococci and reactive arthritis in adults. *Ann Rheum Dis* 1993; **52**: 475–482.
- Jansen TLTA, Janssen M, de Jong AJL. Reactive arthritis associated with group C and group G beta-hemolytic streptococci. *J Rheumatol* 1998; **25**: 1126–1130.
- Baumgarten A, von Graevenitz A, Greco T. Serological evidence for a causative role of non-group A hemolytic streptococci in pharyngitis. *Zentralbl Bakteriol Mikrobiol Hyg [A]* 1981; **249**: 460–465.
- Cohen D, Ferne M, Rouach T, Bergner-Rabinowitz S. Food-borne outbreak of group G streptococcal sore throat in an Israeli military base. *Epidemiol Infect* 1987; **99**: 249–255.
- Corson AP, Garagusi VF, Chretien JH. Group C beta-hemolytic streptococci causing pharyngitis and scarlet fever. *South Med J* 1989; **82**: 1119–1121.
- Fox K, Turner J, Fox A. Role of beta-hemolytic group C streptococci in pharyngitis: incidence and biochemical characteristics of *Streptococcus equisimilis* and *Streptococcus anginosus* in patients and healthy controls. *J Clin Microbiol* 1993; **31**: 804–807.
- Martin NJ, Kaplan EL, Gerber MA *et al.* Comparison of epidemic and endemic group G streptococci by restriction enzyme analysis. *J Clin Microbiol* 1990; **28**: 1881–1886.
- Turner JC, Hayden FG, Lobo MC, Ramirez CE, Murren D. Epidemiologic evidence for Lancefield group C beta-hemolytic streptococci as a cause of exudative pharyngitis in college students. *J Clin Microbiol* 1998; **35**: 1–4.
- Chowdhury MNH, Kambal AM, Al-Eissa YA, Khaliq MRA, Al-Ayed IH, Al-Sanie AM. Non-group A streptococci: are they pathogens in the throat? *J R Soc Health* 1997; **117**: 160–163.
- Cimolai NB, Morrison BJ, MacCulloch L, Smith DF, Hlady J. Beta-haemolytic non-group A streptococci and pharyngitis: a case-control study. *Eur J Pediatr* 1991; **150**: 776–779.
- Bently RW, Leigh JA, Collins MD. Intrageneric structure of *Streptococcus* based on comparative analysis of small-subunit rRNA sequences. *Int J Syst Bacteriol* 1991; **41**: 487–494.
- Bert F, Branger C, Poutrel B, Lambert-Zechovsky N. Differentiation of human and animal strains of *Streptococcus dysgalactiae* by pulsed-field gel electrophoresis. *FEMS Microbiol Lett* 1997; **150**: 107–112.
- Vandamme P, Bot B, Falsen E, Kersters K, Devriese LA. Taxonomic study of Lancefield streptococcal groups C, G, and L (*Streptococcus dysgalactiae*) and proposal of *S. dysgalactiae* subsp. *equisimilis* subsp. nov. *Int J Syst Bacteriol* 1996; **46**: 774–781.
- Vieira VV, Teixeira LM, Zahner V *et al.* Genetic relationships among the different phenotypes of *Streptococcus dysgalactiae* strains. *Int J Syst Bacteriol* 1998; **48**: 1231–1243.
- Brandt CM, Haase G, Schnitzler N, Zbinden R, Lütticken R. Characterization of blood culture isolates of *Streptococcus dysgalactiae* subsp. *equisimilis* possessing Lancefield's Group A antigen. *J Clin Microbiol* 1999; **37**: 4194–4197.
- Bert F, Lambert-Zechovsky N. Analysis of a case of recurrent bacteraemia due to group A *Streptococcus equisimilis* by pulsed-field gel electrophoresis. *Infection* 1997; **25**: 250–251.
- Rammelkamp CH, Dingle JH. Pathogenic streptococci. *Annu Rev Microbiol* 1948; **2**: 279–304.
- James L, McFarland RB. An epidemic of pharyngitis due to nonhemolytic group A streptococcus at Lowry Air Force Base. *N Engl J Med* 1971; **284**: 750–752.
- Dierksen KP, Ragland NL, Tagg JR. A new alkaline pH-adjusted medium enhances detection of β -hemolytic streptococci by minimising bacterial interference due to *Streptococcus salivarius*. *J Clin Microbiol* 2000; **38**: 643–650.
- Tagg JR, Vugler LG. Enhancement of the hemolytic activity of group B streptococci and streptolysin S-deficient group A streptococci on blood agar medium containing staphylococcal beta-lysin. *J Microbiol Methods* 1986; **5**: 215–220.
- Johnson DR, Kaplan EL, Sramek J *et al.* Laboratory diagnosis of group A streptococcal infections. Geneva, World Health Organization. 1996.
- Beall B, Facklam R, Thompson T. Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci. *J Clin Microbiol* 1996; **34**: 953–958.
- Wilson KH, Blitchington RB, Greene RC. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *J Clin Microbiol* 1990; **28**: 1942–1946.
- Whatmore AM, Kapur V, Sullivan DJ, Musser JM, Kehoe MA. Non-congruent relationships between variation in *emm* gene sequences and the population genetic structure of group A streptococci. *Mol Microbiol* 1994; **14**: 619–631.
- Alouf JE. Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). *Pharmacol Ther* 1980; **11**: 661–717.
- Schnitzler N, Podbielski A, Baumgarten G, Mignon M, Kaufhold A. M or M-like protein gene polymorphisms in human group G streptococci. *J Clin Microbiol* 1995; **33**: 356–363.
- Bisno AL, Collins CM, Turner JC. M proteins of group C streptococci isolated from patients with acute pharyngitis. *J Clin Microbiol* 1996; **34**: 2511–2515.
- Cumming WM. On the pseudohaemolytic streptococci isolated from the sputum in pulmonary tuberculosis. *J Pathol Bacteriol* 1927; **30**: 279–300.
- Todd EW. The conversion of hemolytic streptococci to non-hemolytic forms. *J Exp Med* 1928; **48**: 493–511.
- Lancefield RC. Loss of the properties of hemolysin and pigment formation without change in immunological specificity in a strain of *Streptococcus haemolyticus*. *J Exp Med* 1934; **59**: 459–469.
- Coburn AF, Pauli RH. The interaction of host and bacterium in the development of communicability by *Streptococcus haemolyticus*. *J Exp Med* 1941; **73**: 551–570.
- Colebrook L, Elliot SD, Maxted WR, Morley CW, Mortell M. Infection by non-haemolytic group-A streptococci. *Lancet* 1942; **2**: 30–31.