BACTERIAL PATHOGENICITY

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

KAREN P. DIERKSEN and JOHN R. TAGG

Department of Microbiology, University of Otago, PO Box 56, Dunedin, New Zealand

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
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 pre-
sumed 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-decient) and
Blackmore (streptolysin O-decient) 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-ethanesul-
phonic 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 to 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 + (10–100 colonies), 2+ (>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 sub-
cultured to SBA and their Lancefield group identity
was established serologically by latex agglutination
(Streptococcal grouping kit, Oxioid) 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
to 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 strepto-
lysin 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 P1 mod and P3 mod 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 previously [27, 29]. PCR products were digested with HaeIII and HinfI 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 ≥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**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Number (%) of 361 pharyngeal cultures positive for β-haemolytic streptococci of Lancefield group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>All Blood agar tested</td>
<td>64 (18)</td>
</tr>
<tr>
<td>CNA-P only</td>
<td>2 (0.5)</td>
</tr>
</tbody>
</table>
Table 2. Effect of blood agar medium composition and incubation atmosphere on haemolytic activity of streptococcal cultures

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Incubation atmosphere</th>
<th>Group C strains</th>
<th>Group G strain 0662</th>
<th>S. pyogenes strain 82-541 (SLS-negative)</th>
<th>S. dysgalactiae subsp. dysgalactiae strain 67</th>
<th>S. equi subsp. zooepidemicus strain 4881</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>β</td>
<td>pH</td>
<td>β</td>
<td>pH</td>
<td>β</td>
</tr>
<tr>
<td>CNA-P</td>
<td>Anaerobic</td>
<td>+</td>
<td>7.3</td>
<td>+</td>
<td>7.4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CO₂ 5%</td>
<td>+</td>
<td>7.3</td>
<td>+</td>
<td>7.2</td>
<td>+</td>
</tr>
<tr>
<td>CNA</td>
<td>Anaerobic</td>
<td>–</td>
<td>6.1</td>
<td>+</td>
<td>6.7</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
<td>–</td>
<td>6.4</td>
<td>–</td>
<td>6.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>BA-Ca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Anaerobic</td>
<td>–</td>
<td>6.5</td>
<td>–</td>
<td>6.4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CO₂ 5%</td>
<td>+</td>
<td>6.8</td>
<td>–</td>
<td>6.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
<td>–</td>
<td>6.6</td>
<td>–</td>
<td>6.8</td>
<td>–</td>
</tr>
<tr>
<td>SBA</td>
<td>Anaerobic</td>
<td>–</td>
<td>6.0</td>
<td>–</td>
<td>6.2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CO₂ 5%</td>
<td>–</td>
<td>6.2</td>
<td>–</td>
<td>6.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
<td>–</td>
<td>6.2</td>
<td>–</td>
<td>6.4</td>
<td>–</td>
</tr>
</tbody>
</table>

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

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 strain 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.

In 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 *enm* 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 *enm*-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 *enm* 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 intragenic 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. pyogenes* 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. salivaruis* 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. salivaruis*. 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 Paul [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 purpural 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.

This research was supported by the Thrasher Foundation, the National Heart Foundation of New Zealand, the Community Trust of Otago and the Health Research Council of New Zealand. We thank Janet Wilson and Nadika Lyamarchey of Southern Community Laboratories for their assistance with this study and in particular to Megan Inglis and Nancy Rugland for technical assistance.