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ARTICLE

Molecular markers for discriminating *Streptococcus pyogenes* and *S. dysgalactiae* subspecies *equisimilis*

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Abstract Given the increasing aetiological importance of *Streptococcus dysgalactiae* subspecies *equisimilis* in diseases which are primarily attributed to *S. pyogenes*, molecular markers are essential to distinguish these species and delineate their epidemiology more precisely. Many clinical microbiology laboratories rely on agglutination reactivity and biochemical tests to distinguish them. These methods have limitations which are particularly exacerbated when isolates with mixed properties are encountered. In order to provide additional distinguishing parameters that could be used to unequivocally discriminate these two common pathogens, we assess here three molecular targets: the *speB* gene, intergenic region upstream of the *scpG* gene (IRSG) and virPCR. Of these, the former two respectively gave positive and negative results for *S. pyogenes*, and negative and positive results for *S. dysgalactiae* subsp. *equisimilis*. Thus, a concerted use of these nucleic acid-based methods is particularly helpful in epidemiological surveillance to accurately assess the relative contribution of these species to streptococcal infections and diseases.

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Introduction

Streptococcus pyogenes and *S. dysgalactiae* subspecies *equisimilis* are closely related Gram-positive bacteria that colonise the skin and respiratory tract of humans. *S. pyogenes* infection is associated with a wide range of diseases, which include relatively benign and common diseases (such as bacterial pharyngitis, impetigo and scarlet fever), potentially fatal invasive diseases (such as necrotising fasciitis and toxic shock syndrome) and rheumatic heart disease [1]. Together, these diseases are estimated to kill half a million people each year [2]. *S. dysgalactiae* subsp. *equisimilis*, which occupies the same tissue sites as *S. pyogenes*, was previously considered to be a less pathogenic organism and causes infections opportunistically [3]. *S. dysgalactiae* subsp. *equisimilis* expresses many of the same virulence factors (including the M-protein, C5a peptidase, streptokinase, fibronectin binding proteins and plasminogen receptors) as those expressed by *S. pyogenes* [4–9] and can cause a similar spectrum of diseases in humans as *S. pyogenes* [3, 10, 11].

In many populations, the *S. dysgalactiae* subsp. *equisimilis* colonisation rate in the throat and the rate of its recovery from pharyngitis exceed that of *S. pyogenes* [12]. The relative contribution by these species to the disease burden could vary considerably between communities and populations

[13, 14]. There is a paucity of information on streptococcal disease burden in many resource-poor nations [15]. The lack of definitive and rapid tests to differentiate these species and the perception among clinicians that *S. dysgalactiae* subsp. *equisimilis* is generally non-pathogenic may also have contributed to this paucity. Recent studies on invasive disease due to beta-haemolytic streptococci of groups other than A and B concluded distinct clinical manifestations in some populations [16–18]. These studies recommended increased awareness of *S. dysgalactiae* subsp. *equisimilis* as a human pathogen and emphasise the importance of the identification of beta-haemolytic streptococci at the species level.

It is generally difficult to distinguish *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis* on a morphological basis, as both species are usually large colony forming with similar haemolysis patterns. These and other beta-haemolytic streptococci are traditionally differentiated on the basis of differences in carbohydrate on the bacterial surface and sensitivity to bacitracin [19]. Additional diagnostics such as the PYR reaction (for L-pyrrolidonyl-β-naphthylamide aminopeptidase) may be used as confirmatory tests, but are not routine procedures in many clinical microbiology laboratories. Whereas the group A carbohydrate is found in *S. pyogenes*, it has also been found in *S. dysgalactiae* subsp. *equisimilis* in rare cases [5, 20]. Positive reactivity to group C or G antigen is given by *S. dysgalactiae* subsp. *equisimilis*, *S. dysgalactiae* subsp. *dysgalactiae*, *S. equi*, *S. canis* and *S. anginosus* [5, 20, 21]. The bacitracin sensitivity test can sometimes give false-positive results [19]. While the PYR test has excellent specificity and sensitivity in discriminating *S. pyogenes* from *S. dysgalactiae* subsp. *equisimilis*, other bacteria commonly found in the same habitat may also give a positive reaction [22], and the test performed less well in routine diagnostic laboratory settings, sometimes giving weaker reactions [23]. In order to provide additional distinguishing parameters that could be used to discriminate these species, we assess here three molecular targets.

Materials and methods

S. pyogenes and *S. dysgalactiae* subsp. *equisimilis* isolates were collected from two different geographical regions, India and Australia. The three isolates with unusual characteristics were from Europe. The bacteria were grown on Todd–Hewitt agar supplemented with 2% horse blood. Group carbohydrates were determined using the Prolex Streptococcal Grouping Latex Kit (Pro-Lab Diagnostics). The PYR test was performed according to the manufacturer's instructions (Becton Dickinson). DNA was extracted using purification kits (QIAGEN Inc.). The polymerase chain reaction (PCR) conditions were as follows. For *speB*: denaturation 94°C/30 s, annealing 55°C/30 s, extension 72°C/90 s; forward and reverse primers were GGTTCTGCAGG TAGCTCTCG and TGCCTACAACAGCACTTTGG. For the intergenic region upstream of the *scpG* gene (IRSG): the conditions were the same as above and the primers were CAACACATAACCACCTTCTGGA and TTGCAAGTGCG TCACAAGAT. The primers and conditions for virPCR were as described previously [24, 25]. Sequence typing (*emm* typing) was performed as described by Beall et al. [26] and sequencing the gene for rRNA was carried out after amplifying with universal primers [27].

Results and discussions

The rationales for the selection of the targets are as follows. Comparative microarray analysis showed that the gene encoding streptococcal cysteine protease, *speB*, found ubiquitously in *S. pyogenes* isolates [28, 29], is absent in *S. dysgalactiae* subsp. *equisimilis* [30]. Furthermore, a *speB* orthologue has not been reported in other bacteria. Hence, the PCR primers targeting this gene are expected to be specific for *S. pyogenes*. Indeed, a PCR for *speB* has been suggested as a diagnostic test for the *S. pyogenes* aetiology of necrotising fasciitis [31]. The remaining two targets are based on the sequence differences in *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis* upstream of the gene for C5a peptidase (*scpA* and *scpG*, respectively). The physical locations of *scpA*, *emm* (gene for M protein) and the regulator *mga* in the *S. pyogenes* genome are close to each other and are amplifiable by PCR (virPCR; [24, 25]). Depending on the architecture of this chromosomal region, the amplicon arising from virPCR is about 4–7 kb. All *S. dysgalactiae* subsp. *equisimilis* isolates possess *emm* and *scpG* genes [10, 32]. However, previous studies [7, 33] revealed that the *emm* and *scpG* genes in the *S. dysgalactiae* subsp. *equisimilis* genome are not physically linked and the

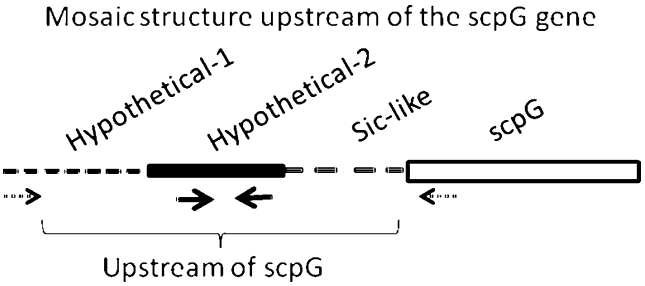

Fig. 1 Organisation of the intergenic region upstream of *scpG* (IRSG) of *Streptococcus dysgalactiae* subsp. *equisimilis*. The virPCR primers (*dashed arrows*) and the IRSG primers (*solid arrows*) are shown. The length virPCR amplifiable region is somewhat variable, but in more than 98% of *S. dysgalactiae* subsp. *equisimilis* isolates, it is about 2 kb. Within this region, the segment defined by IRSG PCR primers is highly conserved and targets a hypothetical protein

Table 1 Molecular and biochemical tests carried out on 167 *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis* isolates from Australia and India

Group carbohydrate	Number of isolates	Number of *emm* types or subtypes	PYR test	IRSG PCR	*speB* PCR	virPCR size (kb)			
						~2	3	4	>4–7
A	58	41	+	−	+	0	0	1	57
G	98	31	−	+	−	98	0	0	0
C	11	5	−	+	−	9	1	1	0

sequences immediately upstream of *scpG* exhibit mosaic structure [34]. However, the virPCR designed for *S. pyogenes* also gave a product with *S. dysgalactiae* subsp. *equisimilis*, but the amplicon was only about 2 kb [33–35], suggesting that the primer binding sites may be conserved. We sequenced the virPCR products from diverse *S. dysgalactiae* subsp. *equisimilis* strains and found that an open reading frame for a hypothetical protein within the IRSG is highly conserved in all of the strains (Fig. 1) and is absent in *S. pyogenes*. This allowed us to design *S. dysgalactiae* subsp. *equisimilis*-specific IRSG PCR primers.

S. pyogenes (n=58) and *S. dysgalactiae* subsp. *equisimilis* (n=109) isolates in this study belong to 41 and 36 distinct *emm* (sub)types, respectively, suggesting that they are highly diverse (Table 1). The collection also represents multiple isolates of the same *emm* type. We have shown previously that horizontal gene transfers mediated by phages and conjugative transposons between these species are ongoing events and occur with greater frequencies among isolates from highly endemic regions [35]. Therefore, it is all the more necessary to show species-specific distribution of the target genes among the isolates from highly endemic regions. Clonal diversity and two distinct geographical sources of isolates, one of them (India) being highly endemic for streptococcal infection, fulfil the requirement in this regard.

All of the strains listed in Table 1 were beta-haemolytic on blood agar plates and possessed group A, C or G carbohydrate. The isolates expressing group A carbohydrate were PYR-positive, and the isolates expressing the group C or G antigen were PYR-negative. All of the isolates presumptively identified as *S. pyogenes* based on the above reactions were positive for the *speB* amplicon, whereas none of the *S. dysgalactiae* subsp. *equisimilis* isolates were positive for this reaction. The IRSG assay also proved highly discriminatory, as all *S. dysgalactiae* subsp. *equisimilis* isolates tested produced the expected product of about 500 bp, and none of the *S. pyogenes* isolates gave this amplicon. All PCRs were performed with appropriate positive controls and template-negative controls. Thus, *speB* and IRSG PCRs together unequivocally distinguish *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis*.

As reported earlier, the virPCR gave products with both the *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis* isolates in this study (Table 1). As expected, DNA from all of the *S. pyogenes* isolates gave approximately 4–7-kb product. By contrast, the reaction gave approximately 2-kb products from 107 of the 109 *S. dysgalactiae* subsp. *equisimilis* isolates. One *S. dysgalactiae* subsp. *equisimilis* isolate yielded a 3-kb product and another a 4-kb product. Both of these isolates expressed group C carbohydrate. Sequence analysis of these variant virPCR products (data not shown) revealed insertions within the mosaic regions upstream of the C5a peptidase gene. However, the region of the hypothetical open reading frame targeted by the IRSG primers is conserved (Fig. 1).

Ongoing cross-species horizontal gene transfers [33, 35, 36] could give rise to isolates with mixed characteristics. In this study, three isolates with mixed characteristics were tested. Two isolates (2005-0193 and 2006-0098) that were *S. dysgalactiae* subsp. *equisimilis* as judged by negative reaction to the PYR test but showed agglutination reaction with the group A reagent were also tested by PCRs (Table 2). Both were positive for IRSG PCR and negative for *speB* PCR. The size of the virPCR amplicons were 2 kb. We further confirmed the identity of these isolates as *S. dysgalactiae* subsp. *equisimilis* by 16S rRNA gene sequencing (data not shown). Another isolate (2007-0217) was *S. pyogenes* as shown by PYR positivity, group A carbohydrate reactivity and rRNA gene sequence. However, 2007-0217 belonged to *emm* stg1750, a type normally found in *S. dysgalactiae* subsp. *equisimilis*, suggesting horizontal transfer of the *emm* gene. This isolate was *speB*-positive and IRSG-negative (Table 2). Furthermore, the virPCR product is 7 kb long and revealed physical proximity of the *emm* locus to the *scpA* gene (data not shown).

Table 2 Molecular and biochemical tests carried out on three unusual *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis* isolates

Strain name	Group carbohydrate	*emm* type	PYR test	IRSG PCR	*speB* PCR	virPCR (kb)
2005-0193	A	stg6	−	+	−	~2
2006-0098	A	stg6	−	+	−	~2
2007-0217	A	stg1750	+	−	+	~7

Recently, a real-time PCR and a low-density microarray were developed to distinguish *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis*. These assays are based on a common target for both species and another one specific for *S. pyogenes* [37]. The authors recognised that their diagnostic system does not have a positive test for *S. dysgalactiae* subsp. *equisimilis*. By choosing specific targets for each of the species (*speB* and IRSG PCRs for *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis*, respectively), we have overcome this limitation. It may be noted that none of the molecular markers thus far described differentiate between group G and C carbohydrate expressing *S. dysgalactiae* subsp. *equisimilis*, suggesting that the acquisition of differences in group carbohydrate may have preceded that of molecular markers.

In summary, the three PCRs proposed here offer excellent discriminatory power in identifying common beta-haemolytic streptococcal human pathogens. The tests could be particularly useful in epidemiological surveillance to accurately assess the relative contribution of *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis* to streptococcal infections and diseases. Current clinical management of infections owing to these species are not different, as both have remained sensitive to penicillin. However, resistance to fluoroquinolone in *S. pyogenes* has been found to be horizontally acquired from *S. dysgalactiae* [38, 39]. Horizontal gene transfers have the ability to rapidly change in population structure, which, in turn, may require tailored management in the future. In the light of these observations, we believe that it would be prudent to identify the definitive aetiology of streptococcal infections and diseases.

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