Denaturing Gradient Gel Electrophoresis of PCR-Amplified *gki* Genes: a New Technique for Tracking Streptococci[†]

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Viridans group streptococci (VGS) are a well-known cause of infections in immunocompromised patients, accounting for severe morbidity and mortality. *Streptococcus mitis* group species (*Streptococcus mitis*, *Streptococcus pneumoniae*, *Streptococcus oralis*) are among the VGS most often encountered in clinical practice. Identifying the portal of entry for *S. mitis* group strains is crucial for interventions preventing bacterial translocation. Unfortunately, tracking the source of *S. mitis* group strains is dependent on a combination of extremely laborious and time-consuming cultivation and molecular techniques (enterobacterial repetitive intergenic consensus-PCR [ERIC-PCR]). To simplify this procedure, a PCR analysis with newly designed primers targeting the household gene glucose kinase (*gki*) was used in combination with denaturing gradient gel electrophoresis (DGGE). This *gki*-PCR-DGGE technique proved to be specific for *S. mitis* group strains. Moreover, these strains could be detected in samples comprised of highly diverse microbiota, without prior cultivation. To study the feasibility of this new approach, a pilot study was performed. This confirmed that the source of *S. mitis* group bacteremia in pediatric patients with acute myeloid leukemia could be tracked back to the throat in five out of six episodes of bacteremia, despite the fact that throat samples are polymicrobial samples containing multiple *S. mitis* group strains. In contrast, using the classical combination of cultivation techniques and ERIC-PCR, we could detect these strains in only two out of six cases, showing the superiority of the newly developed technique. The new *gki*-PCR-DGGE technique can track the source of *S. mitis* group strains in polymicrobial samples without prior cultivation. Therefore, it is a valuable tool in future epidemiological studies.

Viridans group streptococci (VGS) are a notorious cause of bacteremia in immunocompromised cancer patients (1, 3, 5). In pediatric patients with acute myeloid leukemia (AML), up to 62.5% of all infections are caused by VGS, accounting for serious morbidity and mortality (8, 18).

The group of VGS is divided into 24 different species, of which *Streptococcus mitis* is one of the most encountered in clinical practice. Compared to the other 23 VGS species, *S. mitis* is associated with higher levels of bacteremia, a higher risk of infectious complications, and more resistance to antibiotics in cancer patients (5, 9).

Despite the clinical relevance of *S. mitis* infections, the portal of entry for these bacteria remains questionable. However, to study the effect of interventions to prevent *S. mitis* translocation, it is essential to know the source of these disease-causing streptococci. Comparison of streptococcal strains from different sampling sites of the human body is crucial to detect the portal of entry for these VGS. This comparison is hindered by the fact that the throat, and to a lesser extent the large intestine, is inhabited by many different VGS strains, between which it is hard to discriminate. Moreover, strain detection is

dependent on a combination of laborious culturing techniques and molecular typing. Furthermore, due to variable and inconclusive biochemical reactions, it is often difficult to name VGS to the species level, let alone to the strain level. Especially the differentiation between *S. mitis*, *Streptococcus oralis*, and *Streptococcus pneumoniae*, further referred to as the *S. mitis* group, has been proven to be very hard and has led to the introduction of DNA-based approaches such as multilocus sequence typing (7, 14). However, to investigate the source of *S. mitis* group strains causing infections, a method of discriminating *S. mitis* group members at the strain level is needed. Using conventional culturing techniques combined with molecular typing, it is nearly impossible to compare *S. mitis* group strains isolated from surveillance samples of various locations, as it is very laborious. Until now, no culture-independent molecular detection technique exists that directly discriminates between *S. mitis* group strains.

In this paper, we describe a new *gki*-PCR-denaturing gradient gel electrophoresis (DGGE) technique consisting of specific PCR primers, targeting the household gene *glucose kinase* (*gki*), used in combination with DGGE to discriminate between different *S. mitis* group strains. To test the feasibility of this new approach, we compared *S. mitis* group strains isolated from blood cultures with strains in throat swabs, gastric fluids, and fecal samples in immunocompromised AML patients, independent of culturing techniques and biochemical analyses. We demonstrate that this new *gki*-PCR-DGGE technique can be used to detect *S. mitis* group strains in surveillance samples,

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which may contain a high level of bacterial diversity. Moreover, this new technique can be used to track the source of invasive *S. mitis* group bacteria.

MATERIALS AND METHODS

PCR. Recently, Ip et al. performed a phylogenetic analysis based on both *gdh* and *gki* loci to separate *S. pneumoniae*, *S. oralis*, and *S. mitis* at the strain level (7). Especially the *gki* household gene showed considerable heterogeneity between members of these three species. To make a gene eligible for DGGE, the combination of considerable heterogenic regions and stable regions for primer alignment is obligatory. Regions on the *gki* gene fulfilled both criteria, and thus we chose to develop a *gki*-PCR-DGGE technique. The nucleotide sequences of the *gki* primers were based on the alignments of the previously published *S. mitis* group sequences (7).

To make the *gki*-PCR product eligible for DGGE, a previously described GC clamp was attached to the reverse primer (11).

The PCR mixture contained 5 μ l of reaction buffer (100 mM Tris-HCl [pH 8.8], 500 mM KCl, 15 mM MgCl₂), 400 nM of both forward primer *gki*-F (TCTCCGCAGCTGACAC) and reverse primer *gki*-R (GCATCRCTTCRT ATTC A), 200 μ M of deoxynucleoside triphosphates, 2.5 U of *Taq* polymerase (Takara Bio Inc., Otsu, Japan), 1.0 μ l of template DNA, and H₂O to a total volume of 50 μ l. The temperature profile for the *gki*-PCR included a first denaturing step of 1 min at 94°C, followed by 35 cycles of a denaturing step at 94°C for 45 s, a primer annealing step at 55°C for 60 s, and an extension step at 72°C for 90 s. The final step consisted of an extension step at 72°C for 5 min.

Clinical isolates and patient samples. To test the specificity of the newly developed *gki* primers for *S. mitis* group strains, PCR analysis was performed using DNA of the following 25 previously isolated bacterial species: *Clostridium histolyticum*, *Clostridium innocuum*, *Clostridium perfringens*, *Clostridium difficile*, *Clostridium sporosphaeroides*, *Clostridium cadaveris*, *Clostridium bifermentans*, *Carnobacterium divergens*, *Bacteroides vulgatus*, *Bacteroides uniformis*, *Bacteroides fragilis*, *Eubacterium tenue*, *Eubacterium plautii*, *Eubacterium ventriosum*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Lactococcus lactis*, *Bifidobacterium adolescentis*, *Ruminococcus hansenii*, *Ruminococcus productus*, *Streptococcus sanguinis*, *Streptococcus mutans*, *Streptococcus anginosus*, and *Streptococcus salivarius*. Determination of the streptococci mentioned above was confirmed at the species level by 16S rRNA sequencing. As a quality control, the DNA of the same strains was amplified using universal bacterial primers as described previously (17).

To test the feasibility of the new *gki*-PCR-DGGE technique, all blood cultures positive for VGS in nine pediatric patients with AML were included in this study. The study was approved by the local ethical committee and carried out in accordance with the Declaration of Helsinki, and informed consent was given accordingly. All included patients were treated according to the AML protocol of the Dutch Childhood Oncology Group/MRC 12 protocol (high-dose cytarabine, daunorubicin, etoposide, amsacrine, and mitoxantrone) (4). All patients received selective gut decontamination (oral colistin, neomycin, and amphotericin B or ciprofloxacin and itraconazole), VGS prophylaxis (oral phenethicillin), and *Pneumocystis jirovecii* prophylaxis (oral cotrimoxazole). Throat swabs and fecal samples were collected from all patients. Gastric fluids were collected only in patients receiving nasal gastric tube feeding, as it was considered not to be ethical to put in a nasal tube for research purposes only. The throat swabs and gastric fluids were frozen for long-term storage at -20°C. Fecal samples were frozen for long-term storage at -80°C.

DNA extraction. DNA from both throat swabs and gastric fluid samples was extracted using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany), according to the protocol provided by the manufacturer. DNA from fecal samples was extracted using the QIAamp DNA stool mini kit (Qiagen GmbH, Hilden, Germany), according to the protocol provided by the manufacturer, with two minor modifications. First, the lysis temperature was increased from 70°C to 95°C to improve the lysis of gram-positive bacteria. Second, the amount of elution buffer was decreased to 30 μ l instead of 200 μ l to increase the final DNA concentration.

DGGE. DGGE of the PCR products was performed as described by Muyzer et al., by means of a PhorU system (Ingeny, Goes, The Netherlands) (11). A total of 5.0 μ l of PCR product was loaded on an 8% (wt/vol) polyacrylamide gel in 0.5 $\times$ TAE (20 mM Tris base, 10 mM acetic acid, and 0.5 mM EDTA, pH 7.5) with a 20% to 50% denaturant gradient (100% denaturant equals 7 M urea and 40% formamide), with 10 ml of stacking gel without denaturant added on top. Electrophoresis was performed at 100 V and at 60°C for 16 h. Hereafter, the gel was removed from the PhorU system and was stained with silver nitrate as described previously (12).

***gki* gene sequencing.** The sequences of the *gki*-PCR products were determined using the *gki*-forward primer by an external company (BaseClear, Leiden, The Netherlands). Sequences were aligned using a biomedical software program (BioEdit; Ibis Biosciences, Carlsbad, CA).

Culturing techniques. Throat swabs from all patients, as well as bacteria isolated from blood cultures, were plated on blood agar (Mediaproducts, Groningen, The Netherlands) and incubated aerobically at 37°C in 5% CO₂. The microorganisms were identified as streptococci based on gram-positive reaction, morphology, negative catalase test, and alpha-hemolysis. Strain identification by the API 20 Strep tool kit (bioMérieux, Buxel, The Netherlands) was performed according to the manufacturer's protocol.

Penicillin susceptibility testing. MICs were determined using the Etest methodology.

Pneumolysin PCR. For further identification of isolated strains, a PCR specific for pneumolysin was performed as described previously (15) with the following primers: 5'-CAGTCGCCTCTATCCTGGAG-3' and 5'-AGCCAACAATCG TTTACCG-3'.

ERIC-PCR. Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) analysis was used to compare *S. mitis* group strains isolated from blood cultures and throat cultures in all patients. ERIC-PCR consists of the amplification of DNA enclosed between conserved repetitive regions scattered over the bacterial genome. The number and location of ERIC sequences vary between strains of the same species (6). Therefore, electrophoresis of amplified fragments provides band patterns specific for different bacterial strains. ERIC-PCR was performed according to Moissenet et al. (10) with the following primers described previously (16): ERIC1R (5'-ATGTAAGATCCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'). The samples were amplified using the following temperature profile: 95°C for 5 min; 5 cycles at 25°C for 1 min and 72°C for 1 min; and 40 cycles of 94°C for 30 s, 40°C for 50 s, 72°C for 1 min, and finally 72°C for 10 min. Amplified products were analyzed by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and detected by UV transillumination.

RESULTS

***gki*-PCR-DGGE technique.** To simplify the extremely time-consuming procedure of conventional culturing and molecular typing, a specific PCR targeting the *gki* gene was developed, which was used in combination with DGGE (*gki*-PCR-DGGE). DGGE analysis of DNA amplified using these *gki* primers showed separate product bands at different heights for three different *S. mitis* group reference strains as well as separate bands for 14 of the 15 different *S. mitis* group strains isolated from blood cultures and cultured throat swabs of included patients (Fig. 1). Sequence analysis showed that product bands detected at the same height on the DGGE gel indeed had identical *gki* sequences (see Fig. S1 and S2 and file S3 in the supplemental material). Although all clinical isolates belonged to the same species as one of the three reference strains, they did have different *gki* gene sequences, resulting in bands at different heights on the DGGE gel. This confirms the previous results of Ip et al. (7) and indicates that the *gki* primers in combination with DGGE can be used as a molecular technique to discriminate between *S. mitis* group strains. To test the specificity of the newly developed *gki*-PCR-DGGE technique, the *gki* primers were tested using DNA extracted from 25 bacterial species. PCR analyses using the *gki* primers were performed on DNA from both aerobic and anaerobic bacteria, including *S. sanguinis*, *S. mutans*, *S. anginosus*, and *S. salivarius*, and did not show any amplification of bacterial DNA. Amplification with universal bacterial primers, however, did yield PCR products, indicating that the *gki* primers are specific for strains belonging to the *S. mitis* group (*S. mitis*, *S. oralis*, and *S. pneumoniae*) (data not shown).

Tracking the source of *S. mitis* group bacteremia using *gki* primers. To test the feasibility of the new *gki*-PCR-DGGE

FIG. 1. *gki*-PCR-DGGE analysis of 14 *S. mitis* group strains cultured from clinical samples. Different strains show *gki*-PCR bands at different heights, although all strains belong to the *S. mitis* group, indicating that *gki*-PCR-DGGE can be used to discriminate between *S. mitis* group strains. Numbers represent patients; a and b are different episodes of bacteremia. T, throat; B, blood; M, marker consisting of *gki* genes amplified from the three reference strains (clinical *S. mitis* isolate MMB1, clinical *S. oralis* isolate MMB2, and *S. pneumoniae* ATCC 6303).

technique, a pilot study to track the source of *S. mitis* group bacteremia was performed. During the pilot study, in four out of nine patients a total of six blood cultures were positive for *S. mitis* group strains.

gki-PCR-DGGE was performed on bacterial DNA isolated from all six blood cultures as well as on DNA isolated from throat swabs, gastric fluids (if applicable), and feces of the same patients, taken during the same time period and without prior cultivation (Fig. 2). DGGE bands reflecting *S. mitis* group strains isolated from blood cultures were compared to *S. mitis* group strains detected in throat swabs, gastric fluids, and feces. One to four different members of the *S. mitis* group could be identified in DNA isolates from throat swabs (for

example, two *S. mitis* group members in patient 1), whereas no *S. mitis* group strains could be detected in the gastric fluids of patients receiving nasal tube feeding ($n = 3$), nor in the collected fecal samples. In five out of six episodes of *S. mitis* group bacteremia, *gki*-PCR-DGGE bands were detected at the same height in throat and blood samples. In other words, the same *S. mitis* group strain was present in the throat swab and blood culture during the development of bacteremia in five out of six episodes. Most interestingly, in two patients the *S. mitis* strains isolated from two different blood cultures, drawn during two episodes of febrile neutropenia 4 to 6 weeks apart, were classified as identical based on *gki*-PCR-DGGE analysis (Fig. 2).

FIG. 2. DGGE analysis showing that *S. mitis* group strains causing bacteremia can be detected prior to infection in the throat, but not in gastric fluids or feces, in five out of six episodes of bacteremia. T, throat; G, gastric fluids; F, feces; B, blood.

TABLE 1. Characteristics of streptococci isolated from blood and throat samples covering six episodes of bacteremia by using conventional culturing and determination techniques and genotyping^a

Patient no.	Episode	Sample site or strain	API 20 Strep identification (%)	API 20 Strep ^b							Pneumolysin PCR result ^c	MIC of penicillin	ERIC-PCR results	gki sequence similarities
				βGAL	PAL	AMD	RAF	ESC	RIB	TRE	INU			
1		Blood	<i>S. mitis</i> (82)	+	+							–	Throat isolates are identical; no resemblance between throat and blood isolates	T1 has 96% similarity with the blood isolate; no sequence was determined for T2
		Throat1	<i>S. mitis</i> (82)	+	+							–		
		Throat2	<i>S. mitis/S. oralis</i>	+	+	+						–		
2	a	Blood	<i>S. mitis</i> (82)	+	+							–	Throat isolates are identical; high level of resemblance between blood isolates; high level of resemblance between throat isolates and both blood isolates	All four strains had identical sequences
	a	Throat	<i>S. mitis</i> (82)	+	+							–		
	b	Blood	<i>S. mitis</i> (82)	+	+							–		
	b	Throat	<i>S. mitis</i> (82)	+	+							–		
3		Blood	<i>S. mitis</i> (98)	+	+	+			+		+	+	Throat isolates are identical; no resemblance between throat and blood isolates	T1 and T2 are identical and have 96% similarity with the blood isolate
		Throat1	<i>S. mitis</i> (82)	+	+	+						–		
		Throat2	<i>S. mitis/S. oralis</i>		+	+	+					–		
4	a	Blood	<i>S. mitis</i> (82)	+	+							–	Throat isolates T and T1 are identical; blood isolates are identical; no resemblance between throat and blood isolates	T and T1 are identical and have 92% similarity with blood isolate a; T2 has 93% similarity with other T strains and 94% with blood isolate a; no sequence was determined for blood isolate b
	a	Throat	<i>S. mitis/S. salivarius</i>				+	+		+		–		
	b	Blood	<i>S. mitis</i> (82)	+	+							–		
	b	Throat1	<i>S. mitis/S. salivarius</i>				+	+		+		–		
	b	Throat2	<i>S. oralis</i>	+	+	+	+					+		

^a “API 20 Strep” indicates the biochemical tests used to identify the isolated streptococcal strains, and percentages depict the confidence of API testing. The MIC of penicillin was determined by Etest. ERIC-PCR results represent the interpretation of the data (see Fig. S4 in the supplemental material). *gki* sequence similarities are based on sequences from the sequence alignment (see Fig. S1 and S2 in the supplemental material). T, Throat strain; T1, Throat1 strain; T2, Throat2 strain.
^b All strains were positive for leucine aminopeptidase and lactose. All tested strains were negative for arginine hydrolysis, mannitol, sorbitol, and urea hydrolysis, and the Voges-Proskauer test result was negative. βGAL, β-galactopyranoside; PAL, 2-naphthyl phosphate; AMD, starch; RAF, raffinose; ESC, esculin; RIB, ribose; TRE, trehalose; INU, inulin. +, positive result.
^c An additional optochin resistance test was negative for all strains; results for arginine hydrolysis and the Voges-Proskauer test were confirmed to be negative by using standard tests. –, negative result.

Tracking the source of *S. mitis* group bacteremia using conventional techniques. All six strains isolated from blood were identified as *S. mitis* by conventional biochemical testing. However, a specific pneumolysin PCR was positive for the strain isolated in one patient, which was suggestive for *S. pneumoniae*, another member of the *S. mitis* group (Table 1). This illustrates one of the problems in the identification of strains belonging to the *S. mitis* group. Penicillin resistance was tested in all cultured strains. MIC values ranged from <0.016 to 1.0 mg/liter, indicating that no resistance against penicillin was found (MIC > 2 mg/ml).

Using conventional culturing techniques, streptococci were isolated from the throat swabs. Biochemical testing classified the isolated strains, which were all members of the *S. mitis* group according to the new *gki*-PCR-DGGE technique, as *S. mitis*, *S. mitis*/*S. oralis*, and *S. mitis*/*S. salivarius* strains (Table 1). To compare the strains isolated from blood cultures and throat swabs, ERIC-PCR was used (Table 1; see Fig. S4 in the supplemental material). ERIC-PCR showed different patterns for multiple bacterial colonies cultured from a single throat swab, making it obligatory to test every cultured bacterial colony when studying the source of *S. mitis* group strains causing invasive infections. ERIC-PCR analysis showed a high resemblance between streptococci isolated from the throat and *S. mitis* group strains isolated from positive blood cultures in only one patient with two episodes of *S. mitis* bacteremia, whereas *gki*-PCR-DGGE analysis was able to track the source of the *S. mitis* strain in five out of six episodes.

Conventional culturing techniques combined with ERIC-PCR confirmed the close resemblance between *S. mitis* strains isolated from two blood cultures drawn 4 to 6 weeks apart, already detected by our new *gki*-PCR-DGGE technique, in both patients.

DISCUSSION

Until now, no culture-independent molecular detection technique existed to discriminate between different *S. mitis* group strains, making it very difficult to study and compare the variety of streptococcal strains from different parts of the human body. Using a DGGE-PCR with newly developed primers with their target on the *gki* gene (*gki*-PCR-DGGE), we were able to discriminate between phylogenetically closely related *S. mitis* group strains. The developed *gki* primers proved to be specific for the three different subtypes of the strongly related streptococci of the *S. mitis* group investigated (i.e., *S. mitis*, *S. pneumoniae*, and *S. oralis*).

To test the feasibility of the newly developed *gki*-PCR-DGGE technique, the *gki* primers were used to perform a pilot study looking for the source of *S. mitis* group bacteremia in pediatric AML patients. The *gki* primers could indeed be used to directly discriminate between *S. mitis* group strains in clinical samples comprising a highly diverse group of microorganisms, independent of culturing techniques. DNA of strains belonging to the *S. mitis* group was detected in the oropharynx prior to bacteremia in five out of six episodes of bacteremia. These data are consistent with the fact that the oropharynx is highly colonized with streptococci, making the throat a likely portal of entry for these bacteria in immunocompromised hosts (13). *S. mitis* group strains could not be detected in the

gastric fluids of patients receiving nasal tube feeding ($n = 3$), nor in the feces of these patients, emphasizing the importance of the oropharynx as a portal of entry for *S. mitis* group bacteremia. This finding contradicts literature suggesting that the lower gastrointestinal tract might be the portal of entry in patients receiving nasal tube feeding, due to a better survival of bacteria in the case of less gastric acidity (3, 13). Determination of the source of *S. mitis* group strains causing sepsis is essential for the regimen of prophylactically used antibiotics. A more topical use of prophylactic antibiotics to prevent *S. mitis* sepsis instead of the systemic treatment with pheneticillin used nowadays seems favorable if the oropharynx is the sole origin of *S. mitis* sepsis. The low incidence of VGS sepsis in a cohort of patients prophylactically treated with vancomycin mouth washes instead of oral pheneticillin also seems to be in favor of a more local form of prophylaxis (2). Topical use of prophylactic antibiotics can hopefully also put an end to the ongoing march of resistant intestinal bacteria frequently encountered in infectious complications.

Conventional culturing techniques combined with ERIC-PCR found a resemblance between the bacterial strain causing invasive infection and the strain isolated from the throat in only two episodes of bacteremia in one patient. This is probably due to the fact that we did not perform the laborious work of ERIC-PCR on all colonies of morphologically identical VGS isolated from throat swabs. Moreover, it is not certain that all VGS are detected using the current culturing techniques. Our results confirm that the newly developed *gki*-PCR-DGGE technique is superior to classical methods in tracking the source of *S. mitis* group bacteremia.

Recently, Ip et al. showed that multilocus sequencing can be used to differentiate between *S. mitis*, *S. pneumoniae*, and *S. oralis* (7). However, multilocus sequencing cannot be used to directly discriminate between strains belonging to these three species without culturing. The new *gki*-PCR-DGGE technique presented in this article can be used to discriminate between these streptococcal strains and therefore forms a promising technique to be used in epidemiological studies focusing on the source of *S. mitis* group bacteremia.

In conclusion, a newly developed PCR-DGGE of amplified *gki* genes can be used to detect *S. mitis* group strains in clinical samples containing a high level of bacterial diversity. The fact that this new *gki*-PCR-DGGE technique functions independently of culturing techniques is a great advantage over ERIC-PCR. Moreover, this technique can be used to compare strains of the *S. mitis* group from multiple sampling sites, making it a valuable tool for future research.

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