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Novel Real-Time PCR Assays Using TaqMan Minor Groove Binder Probes for Identification of Fecal Carriage of *Streptococcus bovis*/*Streptococcus equinus* Complex from Rectal Swab Specimens

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Real-time PCR based on the *recN* and *gyrB* genes was developed to detect four *Streptococcus bovis*/*Streptococcus equinus* complex (SBEC) subspecies from rectal swab specimens. The overall prevalence was 35.2%: *Streptococcus gallolyticus* subsp. *gallolyticus* (11.1%), *S. gallolyticus* subsp. *pasteurianus* (13%), *Streptococcus infantarius* subsp. *coli* (20.4%), and *S. infantarius* subsp. *infantarius* (11.1%). To conclude, these real-time PCR assays provide a reliable molecular method to detect SBEC pathogenic subspecies from rectal swab specimens.

In an attempt to define the taxonomy of the *Streptococcus bovis*/*Streptococcus equinus* complex (SBEC), researchers have proposed renaming *S. bovis* biotypes as *Streptococcus gallolyticus* subsp. *gallolyticus* (*S. bovis* biotype I), *Streptococcus infantarius* subsp. *infantarius* (S. bovis biotype II/1), *Streptococcus infantarius* subsp. *coli* (*S. bovis* biotype II/1), and *Streptococcus gallolyticus* subsp. *pasteurianus* (*S. bovis* biotype II/2) (1, 2).

S. bovis bacteremia has been found to be related to colorectal cancer (CRC) for more than 30 years (3). Results have suggested that *S. gallolyticus* sp. is more frequently related to both endocarditis and colonic lesions than *S. infantarius* sp. (4, 5). Nevertheless, *S. infantarius* sp. is associated with noncolonic cancer, such as cancer of the pancreas and biliary tract, generally appearing as cholangitis (6).

Authors have claimed that different *S. bovis* subspecies should be named according to the classification proposed by Schlegel et al. (2) both in clinical practice and in scientific publications, which implies the use of molecular techniques for the subclassification of SBEC (7). Recently, the use of a partial *recN* gene sequence was explored in order to investigate taxonomy and phylogeny in the genus *Streptococcus*, and other genes (16S rRNA, *groEL*, *gyrB*, *rpoB*, and *sodA*) were compared to *recN*: interspecies and intrasubspecies similarities of *recN* and *gyrB* were lower (8).

Therefore, our aim was to use the *recN* and *gyrB* genes as targets to develop useful real-time PCR assays to detect *S. gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *pasteurianus*, *S. infantarius* subsp. *coli*, and S. *infantarius* subsp. *infantarius* from rectal swab specimens in individuals that underwent colonoscopy. The first assay is based on *recN*, which detects *S. gallolyticus* subsp. *gallolyticus* and *S. gallolyticus* subsp. *pasteurianus*. The second assay specifically detects *S. infantarius* subsp. *coli* and *S. infantarius* subsp. *infantarius* based on *gyrB*.

A total of 19 bacterial strains were evaluated in this study and are listed in Table 1. All five clinical isolates were identified using the technique of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) by using the Vitek MS system (bioMérieux, Marcy l'Etoile, France) (Table 1). Growth culture strains were resuspended in 500 μl of ultrapurified water and lysed by boiling for 15 min. Cells were pelleted, and the supernatant was separated and stored at −20°C.

Rectal swab specimens were collected in accordance with ethical guidelines established by the Ethical Principles for Medical Research Involving Human Subjects (Declaration of Helsinki, 1964) and stored at −80°C. All subjects gave written informed consent, and the study protocol was approved by our local Institutional Ethical Committee.

In a period ranging from June to September 2011, 54 rectal swab specimens were obtained from subjects who underwent colonoscopy at Santa Clara Hospital endoscopy service. Santa Clara Hospital is a general hospital in Porto Alegre, Brazil. Each swab (ESwab; Copan, Brescia, Italy) was suspended in 1 ml of modified liquid Amies medium and stored at −80°C. DNA isolation was performed using the BioPur Mini Spin extraction kit (Biometrix, Curitiba, Brazil).

Partial *recN* and *gyrB* nucleotide sequences were analyzed using the BLASTN algorithm to compare the sequences to a database. Afterwards, primers and fluorescent dye-labeled TaqMan MGB probes were designed using the Primer Express v3.0 software program (Applied Biosystems, Foster City, CA). The sequences of primers and probes and product sizes are listed in Table 2. A TaqMan 6-carboxytetramethylrhodamine (TAMRA) real-time PCR assay based on the β-globin gene (Applied Biosystems, Foster City, CA) was used for an internal control.

PCRs for the presence/absence of the *recN* and *gyrB* genes were carried out with 4 μl of extracted DNA and 10 μl of master mix, 800 nM (each) primer, and 200 nM probes. DNA was amplified using the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA). All reaction conditions included an initial denaturation of 95°C for 10 min, followed by amplifications using 40 cycles of 95°C for 15 s and 60°C for 1 min. The positive specimens were determined as follows: amplification yielded a positive

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TABLE 1 Reference strains and clinical isolates used as controls for development of real-time PCR assays targeting the *recN* and *gyrB* genes

Group	Species	Strain[a]	qPCR result
Streptococci	*Streptococcus gallolyticus*	ATCC 9809	*recN* SGG (+)
	Streptococcus gallolyticus subsp. *gallolyticus*	IMPG RS-51	*recN* SGG (+)
	Streptococcus infantarius subsp. *coli*	IMPG RS-52	*gyrB* SCI (+)
	Streptococcus infantarius subsp. *infantarius*	IMPG RS-54	*gyrB* SII (+)
	Streptococcus agalactiae	ATCC 13813	No amplification
	Streptococcus salivarius	ATCC 7073	No amplification
	Streptococcus mutans	ATCC 25175	No amplification
	Streptococcus oralis	ATCC 10557	No amplification
	Streptococcus parasanguinis	ATCC 903	No amplification
	Streptococcus pyogenes	ATCC 19615	No amplification
	Streptococcus pneumoniae	ATCC 33400	No amplification
Other bacteria	*Enterococcus faecalis*	ATCC 29212	No amplification
	Enterococcus faecium	ATCC 6569	No amplification
	Escherichia coli	ATCC 25922	No amplification
Clinical isolates	*Streptococcus gallolyticus* subsp. *gallolyticus*	HMV 317831	*recN* SGG (+)
	Streptococcus gallolyticus subsp. *pasteurianus*	HUSM 2011	*recN* SGP (+)
	Streptococcus gallolyticus subsp. *pasteurianus*	HMD 6075	*recN* SGP (+)
	Streptococcus gallolyticus subsp. *gallolyticus*	HMD 6925	*recN* SGG (+)
	Streptococcus gallolyticus subsp. *gallolyticus*	HMD 305	*recN* SGG (+)

[a] Sources of reference strains: INCQS, National Institute of Quality Health Control—Fiocruz; IMPG, Culture Collection of Paulo de Góes Microbiology Institute—UFRJ.

result, and the threshold cycle (C_T) deviation for the replicate was not higher than 0.5, with a C_T cutoff at 35.

Five clinical isolates and four reference strains (Table 1) were correctly identified using the primer and probe sets designed in these real-time PCR assays. No false-positive amplification reactions were observed when the real-time PCR assays were evaluated against various closely related *Streptococcus* species, *Enterococcus* species, and *Escherichia coli* (Table 2). We ensured adequate primer and probe specificity by alignment of partial sequences retrieved from the GenBank database.

Fifty-four subjects were enrolled (19 males [mean age, 64.6 years; range, 46 to 76] and 35 females [mean age, 57.9; range, 44 to 80]). The overall prevalence of any SBEC subspecies was 35.2% (for males, $n = 3$; for females, $n = 16$). For 10 subjects, a single SBEC subspecies was found (2 with *S. gallolyticus* subsp. *gallolyticus*, 2 with *S. gallolyticus* subsp. *pasteurianus*, and 6 with *S. infantarius* subsp. *coli*). Nine subjects harbored multiple species; 7 subjects had two subspecies each. Of the former, two had *S. gallolyticus* subsp. *gallolyticus* and *S. infantarius* subsp. *infantarius*, two had *S. gallolyticus* subsp. *pasteurianus* and *S. infantarius* subsp. *coli*, and one had *S. gallolyticus* subsp. *pasteurianus* and *S. infantarius* subsp. *infantarius*. The two remaining subjects had three subspecies: one with *S. gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *pasteurianus*, and *S. infantarius* subsp. *infantarius* and another with *S. gallolyticus* subsp. *gallolyticus*, *S. infantarius* subsp. *coli*, and *S. infantarius* subsp. *infantarius*.

In the present study, two TaqMan-MGB real-time PCR assays were designed to specifically detect subspecies of the SBEC based on sequence analysis of the *recN* and *gyrB* genes. To our knowledge, these are the first applicable real-time PCR assays to detect all clinically significant SBEC subspecies. Recently, a multiplex PCR/restriction fragment length polymorphism (RFLP) assay

TABLE 2 Primer and probe sets

Name	Target	Sequence (5′ → 3′)	Label	Amplicon size (bp)
F-recN SGG/P	*recN*	1055-GATTTTCAAGTCCAATTCACCAAAG-1080[a]	None	98
R-recN SGG/P	*recN*	1135-GGTTYGTTGAAATGTAAAATTCAACAG-1107	None	
Pf-recN/SGG	*recN*	1085-TTCAATCGTGATGGCAA-1102	FAM[d]	
Pv-recN/SGP	*recN*	1086-TCAACCGTGATGGAAA-1102	VIC	
F-gyrB SIC	*gyrB*	194-CGTATTCAGGAACTTGCTTTCTTG-217[b]	None	65
R-gyrB SIC	*gyrB*	258-CCTTCACGTTTGTCAGTGATTGA-236	None	
Pf-gyrB/SIC	*gyrB*	219-ACCGCGGTTTGCGTAT-234	FAM	
F-gyrB SII	*gyrB*	86-TTGAAAGTTATTGGTGATACAGATCGT-112[c]	None	69
R-gyrB SII	*gyrB*	154-AAAGATTTCACCGTCTGGAGTGA-132	None	
Pv-gyrB/SII	*gyrB*	115-CGGTACAACCGTTCAC-130	VIC	

[a] Sequence numbers based on NCBI accession no. EU917270.1 and EU917274.1 for *S. gallolyticus* subsp. *gallolyticus* and *S. gallolyticus* subsp. *pasteurianus recN* partial gene sequence.
[b] Sequence numbers based on NCBI accession no. EU003729.1 for *S. infantarius* subsp. *coli gyrB* partial gene sequence.
[c] Sequence numbers based on NCBI accession no. EU003767.1 for *S. infantarius* subsp. *infantarius gyrB* partial gene sequence.
[d] FAM, 6-carboxyfluorescein.

based on 16S rRNA proposed SBEC species identification of isolates from dairy microbial communities. However, this assay was not able to discriminate at the subspecies level (9). Previously, a group developed a PCR/RFLP assay based on *groESL* for the identification of SBEC subspecies evaluated in reference strains and clinical isolates (10). The combination of excellent sensitivity and specificity, low contamination risk, ease of performance, and speed has made real-time PCR an appealing alternative in the clinical microbiology laboratory (11). These real-time PCR assays could be useful for SBEC subspecies identification in different clinical samples, such as blood, cerebrospinal fluid, and colonic tissues.

We determined that 35.2% of the 54 subjects were colonized with SBEC subspecies, actually *S. bovis*. This fecal carriage rate is higher than those observed in prior studies (3, 12, 13). Notably, a recently published study (14) showed that the fecal carriage rate of SBEC subspecies was 4.6% among adult subjects with tumorous lesions, nontumorous lesions, and normal colonoscopy results. Those authors used a biomolecular technique and identified SBEC subspecies in the feces of 12 of 259 subjects, with the following distribution: *S. lutetiensis* (*S. infantarius* subsp. *coli*, $n = 9$; *S. gallolyticus* subsp. *pasteurianus*, $n = 2$; and *S. gallolyticus* subsp. *gallolyticus*, $n = 1$ (14). Our results were in agreement with theirs, with *S. infantarius* subsp. *coli* (formerly *S. bovis* biotype II) being the most commonly identified subspecies (20.4%), and the *S. gallolyticus* subsp. *gallolyticus* and *S. gallolyticus* subsp. *pasteurianus* fecal carriage rates were similar (11.1% and 13%, respectively). We identified almost half of SBEC-colonized subjects as having concomitant colonization with two or three subspecies. This finding has not been reported previously, possibly due to the fact that in most former studies, fecal culturing was performed for bacterial isolation. We analyzed clinical samples directly after collection of rectal swab specimens; therefore, underestimation of the actual fecal carriage rate was greatly minimized.

The use of molecular techniques for the subclassification of *S. bovis/S. equinus* complex bacteria is required in order to fully understand the clinical value of SBEC subspecies infections/colonization as early signaling of colonic malignancy or other gastrointestinal diseases (7, 15).

In summary, these real-time PCR assays provide a reliable molecular method to detect SBEC subspecies and quantify *S. gallolyticus* species from rectal swab specimens. These assays might be a useful tool for screening for and surveillance of colonization by this bacterial complex in individuals subjected to a routine colonoscopy.

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