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High prevalence of pharyngeal bacterial pathogens among healthy adolescents and young adults

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The pharyngeal mucosa can be colonized with bacteria that have potential to cause pharyngotonsillitis. By the use of culturing techniques and PCR, we aimed to assess the prevalence of bacterial pharyngeal pathogens among healthy adolescents and young adults. We performed a cross-sectional study in a community-based cohort of 217 healthy individuals between 16 and 25 years of age. Samples were analyzed for Group A streptococci (GAS), Group C/G streptococci (SDSE), *Fusobacterium necrophorum*, and *Arcanobacterium haemolyticum*. Compared to culturing, the PCR method resulted in more frequent detection, albeit in most cases with low levels of DNA, of GAS (20/217 vs. 5/217; $p < 0.01$) and *F. necrophorum* (20/217 vs. 8/217; $p < 0.01$). Culturing and PCR yielded similar rates of SDSE detection (14/217 vs. 12/217; $p = 0.73$). *Arcanobacterium haemolyticum* was rarely detected (3/217), and only by PCR. Overall, in 25.3% (55/217) of these healthy adolescents and young adults at least one of these pathogens was detected, a rate that is higher than previously described. Further studies are needed before clinical adoption of PCR-based detection methods for pharyngeal bacterial pathogens, as our findings suggest a high incidence of asymptomatic carriage among adolescents and young adults without throat infections.

Key words: Adolescence; carriage; *Fusobacterium necrophorum*; pharyngeal bacteria; *Streptococcus dysgalactiae* subspecies equisimilis; *Streptococcus pyogenes*; young adulthood.

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INTRODUCTION

Streptococcus pyogenes (Group A streptococci; GAS) and *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE; also referred to as group C and G streptococci) have been regarded as the primary pharyngeal pathogens [1], with *Arcanobacterium haemolyticum* sometimes also regarded as a pharyngeal pathogen [2]. Recently, *Fusobacterium necrophorum* has been established as a pathogen present in pharyngotonsillitis [3–7]. In a systematic review of *F. necrophorum*-positive acute tonsillitis, patients showed higher rates of detection than asymptomatic controls, and detection of *F. necrophorum* peaked in the age group 15–25 years [8]. In this review use of PCR methods versus culture methods showed no difference in detection of *F. necrophorum* either among pharyngotonsillitis patients or asymptomatic controls. As none of the reviewed studies included usage of several detection methods for *Fusobacterium necrophorum,* a more direct comparison of methodology was not possible.

In the present study, we collected pharyngeal swab samples from a large community-based population of healthy adolescents and young adults. This age group is of particular interest because it is in these ages that recurrent pharyngotonsillitis is the most common, and it is also the typical age for invasive throat infections such as peritonsillar abscess and Lemierre's syndrome [9]. We used both

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PCR-based and culturing methods to detect presence of *F. necrophorum,* as well as of GAS, SDSE, and *A. haemolyticum*.

MATERIALS AND METHODS

Sample collection and transportation

We performed a cross-sectional community-based study in the city of Umeå in Västerbotten County, Sweden. Individuals 16–25 years of age were recruited by visiting secondary school classes, university classes, and a dental practice during routine oral health screenings. Potential participants were excluded if they had ongoing throat pain, antibiotic treatment within the last two weeks, or had undergone tonsillar surgery. Data were collected on 12 occasions between January 2014 and October 2016 (Table 1). All sampling was conducted by the same researcher. With a headlight used for vision, the participant's tongue was held down with an autoclaved metal depressor. Two swabs were held together, rubbed against both tonsils and then placed in its corresponding vial with liquid transport medium (eSwab collection kit art. no. 480CE, Copan Italia, Brescia, Italy). Samples were transported at room temperature to the research laboratory within three hours. One sample was then frozen at −20°C for subsequent DNA extraction and PCR analysis, and the other sample was processed for culturing.

Media and growth conditions

Tonsillar samples were inoculated onto four different agar plates and incubated at different conditions. Double blood Columbia agar plates (BD Difco, Sparks, MD, USA), with the top layer containing 5% defibrinated horse blood (Håtunalab AB, Bro, Sweden) were incubated at 36°C in an anaerobic jar with a Gas Pak CO_2 generator (BD BBL, Sparks, MD, USA). GC agar plates (BD Difco) supplemented with 1% hemoglobin (Oxoid, Basingstoke, UK) and 1% isovitalex (BD BBL) were incubated at 36°C with 5% CO_2. For *Fusobacterium*-selective culture we used Fastidious Anaerobe Agar (LabM, Bury, UK) supplemented with 5% defibrinated horse blood, 50 mg/L nalidixic acid (Fluka, Buchs, Switzerland) and 40 mg/L vancomycin (Duchefa biochemie, Haarlem, The Netherlands). As a general anaerobic medium, we used Fastidious Anaerobe Agar supplemented with 5% lysed defibrinated horse blood (Håtunalab AB, Bro, Sweden) and two 30-µg gentamicin disks (Oxoid) placed on top of the primary inoculum. *Fusobacterium*-selective and general anaerobic agar plates were incubated in an anaerobic jar with Gas Pak.

Columbia blood agar and GC agar plates were evaluated after 16–20 h and 30–44 h. The Fastidious Anaerobe Agar plates were evaluated after 40–44 h and 64–68 h. Bacterial species identification was performed using a MALDI-TOF Microflex mass spectrometer and Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany). Lancefield typing of streptococci was performed using the Streptex rapid latex agglutination test (Remel, Thermo Fisher Scientific, Lenexa, KS, USA).

Real-time PCR

The frozen swabs were thawed, and the liquid transport medium was used for DNA extraction. The bacterial DNA was extracted from the liquid transport medium with the Viral NA Extraction Kit (DiaSorin, Saluggia VC, Italy) in an Arrow instrument (NorDiag ASA, Oslo, Norway). Real-time PCR was performed using hydrolysis probes (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) to detect tonsillar pathogens (Table 2). The GAS PCR detection method was previously described [10]. The SDSE and *A. haemolyticum* PCR assays were developed for this study and were evaluated together with the *F. necrophorum* subsp. *funduliforme* assay [7] (supplementary material). PCR reactions contained 900 nM of each primer, 250 nM probe, 9.6 µl DNA template, and TaqMan 1X Universal PCR Master Mix (Applied Biosystems) in a final volume of 25 µl. Using a QuantStudio 7 Flex Real-Time instrument (Applied Biosystems), the amplification cycles were as follows: 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. A fixed threshold was used to define Cq values and amplification curves were checked manually. As positive controls, we used DNA from the strains SDSE CCUG 7975, *A. haemolyticum* CCUG 33552, *F. necrophorum* CCUG 42162, and *S. pyogenes* CCUG 25571. Analytical sensitivity was tested using plasmids containing cloned PCR product, and analytical specificity was tested using DNA extracted from bacterial isolates, as detailed in the supplementary material.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, NY, USA). Descriptive statistics are presented as median

Table 1. Study population according to sampling site, gender, and age

Sampling site	Number of participants			Median age (25th–75th percentile)			Sampling calendar month (*n*)
	Female	Male	All	Female	Male	All	
Secondary school	60	40	100	17.6 (16.9–18.3)	17.5 (16.9–18.5)	17.6 (16.9–18.4)	Jan (24), March (56), June (20)
University campus	55	46	101	21.4 (20.0–23.4)	20.6 (19.6–22.8)	21.1 (19.7–23.0)	Jan (25), Sept (12), Oct (46), Nov (18)
Dental clinic	9	7	16	19.2 (19.1–19.7)	19.4 (19.4–19.5)	19.4 (19.1–19.6)	Sept (16)
Total	124	93	217	19.1 (17.6–21.1)	19.4 (17.7–20.6)	19.2 (17.7–20.8)	Jan (49), March (56), June (20), Sept (28), Oct (46), Nov (18)

Table 2. Primer and probe sequences for the real-time PCR assays

Target organism and gene	Forward 5′-> 3′	Reverse 5′-> 3′	Probe 5′-> 3′	Amplicon size (bp)
Streptococcus pyogenes malF [10]	CTCGACAA GTCCTCAAT CAAACC	ATGAGTTGCGGAA ATTTGAGGTA	FAM- CATAGAGAATTTA TAACCGCACTC-MGBEQ	100
Streptococcus dysgalactiae subspecies *equisimilis lacI* (this study)	CGCGAATC ATTGCTCTG TGG	AACCGATGGTGTG GAAAGCA	TET-ACCGCAACCATT TCTCGATTTGCT-BHQ1	143
Fusobacterium necrophorum gyrB [7]	AGGATTGC ATGGAGT AGGAA	CCTATTTCATTTCG ACAATCCA	JOE-TCCGCTTTAGAG GCTGGAGAAACGAC-BHQ1	306
Arcanobacterium haemolyticum (this study)	CGCGAACG CTCTGGA AATT	GCCGTATCGCCAG CACTAGT	FAM-ATTTTACTGCG TGGCGCC-MGBEQ	90

values and percentiles. We compared the detection rates with culture and PCR methods using McNemar's test. A *p*-value of less than 0.05 was used to define statistical significance.

RESULTS

Our analysis consisted of a total of 217 asymptomatic individuals of 16–25 years of age, who were included and sampled at 12 separate occasions between January 2014 and October 2016. Table 1 presents the participants' demographic characteristics together with sampling sites and month. All samples were subjected to microbiological procedures as planned, with no missing data.

Performance of real-time PCR assays

Real-time PCR assays for SDSE, *A. haemolyticum*, and *F. necrophorum* were reactive for all tested target species, and non-reactive for all other tested bacterial strains (Tables S2–S5). *In silico* specificity screening revealed that the SDSE assay would likely detect the animal pathogen *S. dysgalactiae* subspecies *dysgalactiae* (SDSD), as there were only minor mismatches of the amplifying primers and a perfect match with the probe. However, we did not test an SDSD isolate as it is not a colonizer of the human pharyngeal mucosa. All assays were linear. The detection limits (LOD) per PCR reaction were 2.5×10^2 gene copies for GAS, 6×10^2 gene copies for SDSE, 2.5×10^2 gene copies for *A. haemolyticum*, and 1×10^4 gene copies for *F. necrophorum* (Table S1). Genomic DNA from bacterial strains was reproducibly amplified from 10-fold serial dilutions at $5 * 10^2$ genomes for GAS, $5 * 10^2$ genomes for SDSE, $5 * 10^2$ genomes for *A. haemolyticum* and $1 * 10^4$ genomes for *F. necrophorum*.

Detection of bacterial pharyngeal pathogens

Table 3 summarizes the detection rates of the different bacteria by PCR, cultivation, or the combination of both methods. Among all tested individuals, 25.3% (55/217) were found to carry at least one of GAS, SDSE, *F. necrophorum*, or *A. haemolyticum*. This carriage rate was 23.4% (29/124) among female participants, and 28.0% (26/93) among male participants. The detection rates significantly differed between the two methods ($p < 0.01$), with a carriage rate of 24.4% (53/217) as determined by PCR, compared to 11.5% (25/217) based on culture methods.

GAS and *F. necrophorum* were equally common, each detected (by any method) in 22 individuals (10.1%). Both organisms were detected significantly more often by PCR than culture: GAS in 20/217 versus 5/217 ($p < 0.01$), and *F. necrophorum* in 20/217 vs. 8/217 ($p < 0.01$). The amounts of the target GAS and *F. necrophorum* DNA were likely low, as indicated by the high Cq values (Table S6), which were often higher than the average Cq values at the LOD (Table S1). SDSE was detected in 17 individuals (7.8%), and its detection rate did not significantly differ between PCR and culture (14/217 vs. 12/217, respectively; $p = 0.73$). Five SDSE isolates were Lancefield Group C, and seven were Group G streptococci. *Arcanobacterium haemolyticum* was not detected in any participants using our non-selective culturing method, and was a rare finding in PCR analyses, only detected in three individuals (1.4%). In seven individuals, the PCR results were negative, while target bacteria grew from the swab used for culture. Repeated PCR analysis using DNA extracts that were diluted 10-fold and 100-fold revealed GAS in one of these individuals, indicating that this sample was affected by PCR inhibition, while the remaining six samples remained PCR negative. In nine

Table 3. Number and percentages of individuals with pharyngeal pathogens detected by culture and PCR out of a total number of 217 tested individuals

Species	PCR and/or culture		PCR		Culture	
	n	(%)	*n*	(%)	*n*	(%)
GAS	22	(10.1)	20	(9.2)	5	(2.3)
GAS and SDSE	1	(0.5)				
GAS and *F. necrophorum*	2	(0.9)				
SDSE	17	(7.8)	14	(6.5)	12	(5.5)
SDSE and GAS	1	(0.5)				
SDSE and *F. necrophorum*	5	(2.3)				
F. necrophorum	22	(10.1)	20	(9.2)	8	(3.7)
F. necrophorum and GAS	2	(0.9)				
F. necrophorum and SDSE	5	(2.3)				
F. necrophorum and A. haemolyticum	1	(0.5)				
A. haemolyticum	3	(1.4)	3	(1.4)	0	(0)
A. haemolyticum and *F. necrophorum*	1	(0.5)				
At least one pathogen above	55	(25.3)	53	(24.4)	25	(11.5)

GAS, Group A streptococci; *Streptococcus pyogenes*; SDSE, *Streptococcus dysgalactiae* subsp. *equisimilis*; Group C/G streptococci.

Table 4. Recent studies reporting asymptomatic carriage of GAS, SDSE, and *F. necrophorum*

	Centor et al. 2015 [3]	Hedin et al. 2015 [6]	Nygren et al. 2021 [11]	Agerhäll et al. (present)	
Number of asymptomatic individuals	180	128	100[a]	217	
Age range in years	15–30 (Mean, 24)	16–46 (Median, 31)	15–25 (Median, 22)	16–25 (Median, 19)	
Included population	University students, USA	Primary healthca re patients, Sweden	Health education students, acute orthopedic patients, Sweden	Secondary school and university students, routine oral health screening, Sweden	
Exclusion	Ongoing sore throat, ongoing antibiotic treatment	Visiting primary health care for infection	Sign of throat infection, antibiotic treatment last 4 weeks, previous tonsillectomy	Ongoing sore throat, antibiotic treatment last 2 weeks, previous tonsillar surgery	
Diagnostic method	PCR	Culture	PCR	Culture and PCR	PCR
GAS	1.1% (2/180)	2.3% (3/128)	N/A	10.1% (22/217)	9.2% (20/217)
SDSE	3.9% (7/180)	7.8% (10/128)	N/A	7.8% (17/217)	6.5% (14/217)
FN	9.4% (17/180)	3.1% (4/128)	21.0% (21/100)	10.1% (22/217)	9.2% (20/217)
Any above	14.4% (25/180)	13.3%[a] (17/128)	N/A	24.4% (53/217)	23.5% (51/217)

FN, Fusobacterium necrophorum; GAS, Group A streptococci; Streptococcus pyogenes; SDSE, Streptococcus dysgalactiae subsp. equisimilis; Group C/G streptococci.

[a]The study of Nygren et al. 2021 [11] has two arms with participants in Sweden and Zambia respectively, with the sampling performed in different periods in the two countries. We have only included the part performed in Sweden.

individuals, we detected a combination of two tonsillar pathogens, eight of whom were carrying *F. necrophorum* (Table 3).

DISCUSSION

Here, we performed a community-based survey among adolescents and young adults of asymptomatic carriage of bacteria that can cause pharyngotonsillitis and demonstrated a high rate, 24.4% of individuals detected with Group A streptococci, SDSE, or *F. necrophorum* (25.3% when including *A. haemolyticum)*. Several previous studies have described carriage rates in similar age groups; however, our present investigation comprised a unique combination of strengths—including a representative community-based population sampled over a long

time period and with consistent sampling technique and concomitant performance of cultivation and PCR. In Table 4, we present our present findings in relation to the findings of prior studies [3, 6, 11]. Our culture-based detection rate was similar to these studies. However, our inclusion of PCR-based detection yielded a more than two-fold higher rate due to the much higher detection of GAS and *F. necrophorum*. Notably, the PCR results were weakly positive for a majority of these samples, indicating low amounts of GAS and *F. necrophorum*. Interestingly, Nygren et al. noted an even higher rate of PCR- based detection of *F. necrophorum* than in our study [11].

We made efforts in sample collection, transportation, and culturing to optimize bacterial detection, particularly of *F. necrophorum*. A recent review showed that culture and PCR had equal rate of detection of *F. necrophorum* in pharyngotonsillitis patients as well as in asymptomatic controls [8]. In contrast, PCR had a significantly higher rate of detection of *F. necrophorum* in our cohort of asymptomatic adolescents and young adults. This was despite a low technical sensitivity of the *F. necrophorum* PCR assay in our hands, likely attributed to the 306 bp long amplicon and serving as a built-in cut-off level that prevented detection of very low levels of *F. necrophorum* DNA. Quantitative PCR may be used to determine a cut-off value that correlates with clinical infection. However, since tonsillar swab sampling is difficult to standardize in clinical practice, it is inherently difficult to reproduce and interpret the bacterial load in the sample. PCR and culture yielded similar SDSE detection rates, despite the use of non-selective culture media. It is unclear why PCR did not increase the detection rate of SDSE as with the other pathogens. All PCR assays used in this study were highly specific, as evaluated in our present study and in previous studies, with no amplification from related bacterial species. There was an *in silico* prediction of a lack of discrimination between the subspecies *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) and *Streptococcus dysgalactiae* subsp. *dysgalactiae* (SDSD), this is, however, clinically insignificant since the animal pathogen SDSD is not a human commensal and is rarely a cause of human infections [12]. *Arcanobacterium haemolyticum* was rarely found and was only detected by PCR. We did not use a selective culture method for *A. haemolyticum*, which likely reduced the chance of detection, and together with the low number of findings makes a comparison of detection techniques impossible for this species. Our participants largely comprised of clusters of secondary school and university classmates, and the relative closeness within these groups could have resulted in increased inter-individual transmission. While laboratory based culturing may underestimate the carriage rate of viable bacteria *in vivo*, PCR-based methods will also detect genetic material of non-viable bacteria from a recent transient colonization, perhaps overestimating the rate of individuals at risk of a pharyngeal infection.

Importantly, the presently reported high detection rate of GAS and *F. necrophorum* DNA among asymptomatic adolescents and young adults most likely corresponds to a high rate of asymptomatic carriage of these bacteria among sore throat patients of the same age group. Pharyngotonsillitis can be caused by bacteria or viruses, as well as non-infectious causes [6]. The high frequency of asymptomatic carriage of bacteria that can cause tonsillitis has to be considered to avoid overuse of antibiotics.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Technical performance of the PCR assays.
Table S2. Strains used to test the specificity of the SDSE, *A. haemolyticum* and *F. necrophorum* real-time PCR.
Table S3. *Streptococcus* strains used to test the SDSE real-time PCR.
Table S4. Strains used to test the *A. haemolyticum* real-time PCR.
Table S5. Strains used to test the *F. necrophorum* real-time PCR.
Table S6. Cq values and corresponding culture results for paired swab samples with bacterial finding.