Short Communication

Fusobacterium necrophorum Subsp. *funduliforme* in Tonsils from Various Patient Populations in Japan

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SUMMARY: *Fusobacterium necrophorum* has recently been suggested to be associated with tonsillopharyngitis, peritonsillar abscess, and recurrent tonsillitis. Between the 2 subspecies of *F. necrophorum*, subsp. *funduliforme* is known to be a major human pathogen. To better understand the epidemiology of *F. necrophorum* subsp. *funduliforme* (FNSF), we studied the prevalence of FNSF in the tonsils of patients undergoing elective tonsillectomy (TE) for different indications. Adult patients who underwent elective TE from October 2014 to November 2015 were included. The tonsils were sent for aerobic and anaerobic tissue culture within 30 min of excision; the presence of FNSF was detected using PCR with *gyrB* primers and 16S rRNA. A total of 32 patients were enrolled. The prevalence of FNSF identified by either culture or *gyrB* PCR did not significantly differ between infectious and noninfectious TE indications. The constant presence of FNSF might not be associated with recurrent pharyngotonsillitis.

Fusobacterium necrophorum is suggested to be associated with various clinical presentations (1), and *F. necrophorum* subsp. *funduliforme* (FNSF) is mainly associated with human infections (2). Previous studies suggested an association between FNSF and recurrent pharyngotonsillitis (RPT); however, data on FNSF isolated from tonsils are limited, and most of the information has been reported from European countries (3–6).

This study was conducted at the National Center for Global Health and Medicine (NCGM, a tertiary referral hospital in Tokyo, Japan) from October 2014 to September 2015. Patients aged ≥ 16 yr who underwent elective tonsillectomy (TE) were prospectively included. Our primary objective was to compare FNSF prevalence in tonsils from patients with different indications for TE. RPT included the following categories: i) a history of ≥ 2 episodes of acute pharyngotonsillitis per year, ii) persistent episodes for > 1 month, and iii) recurrent peritonsillar abscess. Information on the indications for tonsillectomy, ongoing antimicrobial treatment at the time of tonsillectomy, and recent antimicrobial exposure within 1 week was collected by reviewing the medical charts. This study was approved by the Human Research Ethics Committee of NCGM (NCGM-G-001662-00), and written consent was obtained from each subject.

The tonsils were sent for microbiological analyses within 30 min after excision. They were sectioned into pieces and rolled over 5% sheep blood agar, Brucella HK agar (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan), and PV Brucella HK agar (Kyokuto Pharmaceu-

tical) (7). Tonsillar tissue was stored at -80°C until molecular investigations were performed. Agar plates were incubated in canisters containing Anaero pack (Mitsubishi Gas Chemical Co., Tokyo, Japan). Phenotypic identification was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) criteria (M100-S22). *F. necrophorum* was identified using Rapid ID 32A (bioMérieux SA, Marcy l'Etoile, France) and confirmed using sequencing analysis of the 16S rRNA gene. The DNA solution from the colony was further sequenced in both directions using *gyrB* assay primers to identify the subspecies.

Susceptibility was evaluated using broth microdilution in accordance with CLSI criteria (M11-A8). β -Lactamase production was screened using the nitrocefin method.

PCR assays based on *gyrB* and *rpoB* to detect *F. necrophorum* DNA directly from the excised tonsil specimen were performed as previously reported (2,8).

Bivariate analyses were performed using Fisher's exact test or Mann-Whitney U tests. Two-sided *p*-values < 0.05 were considered statistically significant. These analyses were performed using SPSS version 20.

Thirty-two patients (14 men, 43.8%) participated (median age, 38 yr; interquartile range [IQR], 26–44 yr). The indications for TE were RPT in 26 patients and noninfectious diseases (NID) in 6 patients (Table 1). *F. necrophorum* were identified in the cultures of 6 patients (18.8%); all were FNSF based on sequence analyses of *gyrB*. PCR results of excised tonsils were positive for *gyrB* in 7 patients (21.9%) and *rpoB* in 5 patients (15.6%).

FNSF prevalence based on culture was similar among groups with different indications for TE (comparison of each indication, $p = 0.241$; RPT vs. NID, $p = 0.99$) (Table 1). The median age was similar between RPT (35 yr [IQR: 25–44]) and NID (42 yr [IQR: 28–51]) patients ($p = 0.371$) or between patients with and without FNSF (32 yr [IQR: 26–37] vs. 26 yr [IQR: 22–34], $p = 0.219$). The prevalence in men was also similar between the RPT and NID groups (11 [42%] vs. 3 [50%],

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Table 1. Comparison between the prevalence of *Fusobacterium necrophorum* isolated from excised tonsils among various indications for tonsillectomy

		Indications for tonsillectomy						
		Recurrent pharyngo tonsillitis ¹⁾ (<i>n</i> = 26)			IgA nephropathy (<i>n</i> = 3)	PFAPA syndrome (<i>n</i> = 1)	Sleeping apnea syndrome (<i>n</i> = 1)	Chronic urticaria (<i>n</i> = 1)
	Total (<i>n</i> = 32)	≥ 2 episodes of pharyngo- tonsillitis per year (<i>n</i> = 22)	Persistent episodes for > 1 month (<i>n</i> = 1)	Recurrent peritonsillar abscess (<i>n</i> = 3)				
Culture and colony identification								
<i>F. necrophorum</i> ²⁾	6 (18.8%)	5 (19.2%)			0	1 (100%)	0	0
		4 (18.2%)	1 (100%)	0				
<i>F. necrophorum</i> subsp. <i>funduliforme</i> ³⁾	6 (18.8%)	5 (19.2%)			0	1 (100%)	0	0
		4 (18.2%)	1 (100%)	0				
Group A <i>Streptococcus</i>	1 (3.1%)	1 (3.8%)			0	0	0	0
		1 (4.5%)	0	0				
Group B <i>Streptococcus</i>	1 (3.1%)	1 ⁴⁾ (3.8%)			0	0	0	0
		1 (4.5%)	0	0				
Group C/G <i>Streptococcus</i>	1 (3.1%)	1 (3.8%)			0	0	0	0
		1 (4.5%)	0	0				
PCR from excised tonsil								
<i>F. necrophorum</i> (<i>gyrB</i>)	7 (21.9%)	5 (19.2%)			1 ⁵⁾ (33.3%)	1 (100%)	0	0
		4 (18.2%)	1 (100%)	0				
<i>F. necrophorum</i> (<i>rpoB</i>)	5 (15.6%)	4 (15.4%)			0	1 (100%)	0	0
		3 (13.6%)	1 (100%)	0				

Abbreviation: PFAPA, periodic fever with aphthous pharyngitis and adenitis.

¹⁾: Including the following 3 categories: i) a history of ≥ 2 episodes of acute pharyngotonsillitis per year, ii) persistent episodes for > 1 month (after infectious mononucleosis due to cytomegalovirus), iii) recurrent peritonsillar abscess.

²⁾: Identified using Rapid ID 32A (bioMérieux SA) with confirmation by sequencing analysis of the 16S rRNA gene.

³⁾: Identified by *gyrB* sequence analysis.

⁴⁾: Also positive for FNSF.

⁵⁾: The excised tonsil was positive for *gyrB*, but negative for *rpoB*. *F. necrophorum* PCR of the culture colony was positive for both *gyrB* and *rpoB*. Sequencing analysis of the 16S rRNA gene of the cultured colony revealed the isolate was *F. gonidiaformans*.

$p = 0.99$). Three RPT patients had positive results for β -hemolytic streptococci: 1 each in Groups A, B, and C/G. Results of FNSF culture and *F. necrophorum* PCR of the excised tonsil were both positive in a patient with RPT after infectious mononucleosis due to cytomegalovirus infection. No patient had recurrent episodes of pharyngotonsillitis for at least 1 year after surgery.

None of the isolates produced β -lactamase. Except for fluoroquinolone, all isolates had low MICs to β -lactam antibiotics, clindamycin, chloramphenicol, and metronidazole. Three isolates had high MICs to levofloxacin and moxifloxacin (> 8 mg/ml).

Three aspects of this study differ from previous studies: i) inclusion of patients who underwent TE for various indications, ii) identification of subspecies using PCR, and iii) inclusion of patients in Japan. Previous studies revealed a high prevalence of *Fusobacterium* species in the tonsils of patients with recurrent/chronic pharyngotonsillitis (4,5) and a higher *F. necrophorum* prevalence in patients with RPT than in those with acute pharyngitis or asymptomatic controls (9,10). However, we found a similar prevalence of FNSF between the RPT group and NID group. Klug et al. also reported similar recovery rates of *F. necrophorum* from patients with (22%) and without (30%) RPT (6). Bjork et al. reported

the prevalence of tonsillar colonization of *F. necrophorum* in patients who underwent a scheduled TE due to chronic/RPT or recurrent peritonsillar abscess (3). *F. necrophorum* was prevalent before (28%) and after (16%) surgery based on throat swab cultures, indicating that *F. necrophorum* is not alone causative of the symptoms (3). Unlike our study, these previous studies used conventional culture methods to identify the *F. necrophorum* rather than *gyrB* PCR (4–6,9–11) (one study also used MALDI-TOF, (3)), which might have affected the results of the prevalence of *F. necrophorum* in each study.

The majority of patients were asymptomatic at the time of elective surgery. Patients with recurrent peritonsillar abscess had active episodes at least a few weeks to 2 months before surgery. A previous study also reported that tonsil cultures obtained during the asymptomatic period from patients with RPT did not show a higher *F. necrophorum* prevalence than patients without RPT (6). Patients with RPT would likely have a low *F. necrophorum* prevalence during the asymptomatic phase.

In our study, no patient was undergoing antibiotic treatment at the time of tonsillectomy, nor did they receive antibiotic treatment within 1 week. An antibiotic (flomoxef) was preoperatively infused as surgical site

prophylaxis, and the time between antibiotic administration and TE was short. We performed *F. necrophorum* PCR to minimize the effects of administered antibiotics, which revealed similar prevalence in the culture methods.

The only patient with positive FNSF in the NID group had periodic fever adenitis pharyngitis aphthous ulcer (PFAPA) syndrome. Previously, differences in tonsillar microbiota between patients with PFAPA and controls have been suggested, although differences in *F. necrophorum* prevalence have not been reported (12). Further study is necessary to identify the association of PFAPA syndrome with *F. necrophorum*.

In our study, all cultured colonies were identified as FNSF, and no patient had *F. necrophorum* subsp. *necrophorum*. Culture and *F. necrophorum* PCR results of excised tonsils were mostly concordant, except in 2 patients. In 1 patient with RPT, the cultured colony was positive for FNSF, and the excised tonsil was positive for *gyrB* by PCR, but negative for *rpoB*. This could be due to the false-negative result of *rpoB* PCR. In another patient, *F. necrophorum* PCR results of excised tonsil were positive for *gyrB*, but negative for *rpoB*; however, *F. necrophorum* PCR results of cultured colony were positive for both *gyrB* and *rpoB*. Based on sequencing analysis of the 16S rRNA gene of the cultured colony, the isolate was identified as *F. gonidiaformans*. A previous report tested the *rpoB* primer for *F. gonidiaformans* ATCC 25563^T isolate and suggested a negative result (8). The discordant results might be because we used a clinical *F. gonidiaformans* isolate. In our study, the culture was as sensitive as *gyrB* PCR detection and more sensitive than *rpoB* PCR. In a previous study, culture methods identified much lower *F. necrophorum* than *gyrB* PCR; however, the authors concluded that the anaerobic agar medium used was not very effective for detecting *F. necrophorum* (2). In a more recent study, 1 *F. necrophorum* isolate was detected only by *rpoB* PCR, whereas 9 other *F. necrophorum* isolates were identified by both *rpoB* PCR and culture methods (13), suggesting similar findings as our study. The culture methods could be as sensitive as PCR for detecting *F. necrophorum*, if appropriately conducted.

A major limitation of this study is the small number of patients resulting in underpowered statistical analyses, and thus, statistical comparison between RPT and NID should be interpreted accordingly.

Although there have been conflicting reports (9,14), the majority of studies suggested that *F. necrophorum* is most prevalent among cases of pharyngotonsillitis at the age of 15 to 25 yr (14,15). In this study, the cohort with median age of 38 yr (IQR 26–44 yr) might not have reflected the patient population with the highest *F. necrophorum* prevalence.

We did not quantitatively analyze FNSF using real-time PCR. For MIC determination, the broth, not agar, microdilution method was used. Information on recent antimicrobial exposure was collected retrospectively by reviewing the medical charts. Undocumented antimicro-

bial exposure might have been overlooked, resulting in the underestimation of *F. necrophorum*.

In conclusion, our findings suggest that during the asymptomatic phase, the prevalence of FNSF and β -hemolytic streptococci seems to be similar between the RPT and NID groups. FNSF was present regardless of the indications for TE among the patients who underwent elective TE. The constant presence of *F. necrophorum* might not be necessary for RPT.

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