Fusobacterium necrophorum subsp. funduliforme in tonsils from various patient populations in Japan

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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. Among 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 minutes 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 presence of FNSF might not be associated with recurrent pharyngotonsillitis.
Fusobacterium necrophorum (FN) is suggested to be associated with various clinical presentations (1), and FN 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 years 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; 1) a history of ≥2 episodes of acute pharyngotonsillitis per year, 2) persistent episodes for >1 month, 3) recurrent peritonsillar abscess. The information on the indications for tonsillectomy, ongoing antimicrobial treatment at the time of tonsillectomy, and recent antimicrobial exposure within 1 week was collected by the review of 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.

Tonsils were sent for microbiological analyses within 30 min after excision. Tonsils 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 Pharmaceutical) (7). Tonsillar tissue was stored at −80°C until molecular investigations were performed. Agar plates were incubated in canisters containing Anaeropac (Mitsubishi Gas Chemical Co., Tokyo, Japan). Phenotypic identification was
performed in accordance with the Clinical and Laboratory Standard Institutions (CLSI) criteria (M100-S22). FN was identified using Rapid ID 32A (bioMérieux SA) 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 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 FN 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 of <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 years; interquartile range [IQR], 26–44 years). The indications for TE were RPT in 26 patients and noninfectious diseases (NID) in 6 patients (Table 1). FN was identified in the cultures of 6 patients (18.8%); all were FNSF based on sequence analyses of gyrB. PCR of excised tonsils were positive for gyrB in 7 patients (21.9%) and for rpoB in 5 patients (15.6%).

FNSF prevalence based on culture was similar among groups of different indications for TE (comparison among each indication, p = 0.241; RPT vs. NID, p = 0.99) (Table 1). The median age were similar between RPT (35 years [IQR:25-44]) and noninfectious disease (42 years [IQR:28–51]) patients (p = 0.371) or between patients with and without FNSF (32 years [IQR:26–37] vs 26 years [IQR:22-34], p = 0.219). The
prevalence of men was also similar between RPT and noninfectious disease (11 [42%] vs. 3 [50%], p = 0.99). Three RPT patients had positive results for \( \beta \)-hemolytic streptococci: 1 each in Group A, B, and C/G. FNSF culture and FN 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 \( \beta \)-lactamase. Except for fluoroquinolone, all isolates had low MICs to the \( \beta \)-lactam antibiotics, clindamycin, chloramphenicol, and metronidazole. Three isolated had high MICs to levofloxacin and moxifloxacin (>8 mg/ml).

Three aspects of this study differ from previous studies: 1) inclusion of patients who underwent TE for various indications; 2) identification of subspecies using PCR; 3) inclusion of patients in Japan. Previous studies revealed a high prevalence of \textit{Fusobacterium} species in the tonsils of patients with recurrent/chronic pharyngotonsillitis (4, 5), and a higher FN prevalence in patients with RPT than in those with acute pharyngitis or asymptomatic controls (9, 10). However, we found the similar prevalence of FNSF prevalence between RPT group and NID group. Klug et al. also reported similar recovery rates of FN from patients with (22%) and without (30%) RPT (6). Bjork et al. reported the prevalence of tonsillar colonization of FN in patients who underwent a scheduled TE due to chronic/RPT or recurrent peritonsillar abscess (3). FN was prevalent before (28%) and after (16%) surgery based on throat swab cultures, indicating that FN is not alone causative of the symptoms (3). Unlike our study, these previous studies used conventional culture methods to identify the FN rather than gyrB.
PCR(4, 5, 9-11) (one study also used MALDI-TOF, (3)), which might have affected the results of the prevalence of FN 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 FN prevalence than patients without RPT (6). Patients with RPT would likely have a low FN prevalence during the asymptomatic phase.

In our study, no patient was undergoing antibiotic treatment at the time of tonsillectomy, neither did they receive the recent antibiotic treatment within 1 week. Antibiotic (flomoxef) was preoperatively infused as surgical site prophylaxis, the time between antibiotic administration and TE was short. We performed FN PCR to minimize the effects of administered antibiotics, which revealed the similar prevalence in the culture methods.

The only patient with FNSF positivity among the NID group had PFAPA syndrome. Previously, differences in tonsillar microbiota between patients with PFAPA and controls have been suggested, although differences in FN prevalence were not reported (12). Further study is necessary to identify the association of PFAPA syndrome with FN.

In our study, all cultured colonies were identified as FNSF, and no patient had FN subsp. necrophorum. Culture results and FN PCR of excised tonsils were mostly concordant, except in 2 patients. In 1 patient with RPT, cultured colony was positive for FNSF and 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 case, FN PCR of
excised tonsil was positive for \textit{gyrB}, but negative for \textit{rpoB}; however, FN PCR of cultured colony was positive for both \textit{gyrB} and \textit{rpoB}. Based on sequencing analysis of the 16S rRNA gene of the cultured colony, the isolate was identified as \textit{F. gonidiaformans}. A previous report tested the \textit{rpoB} primer for \textit{F. gonidiaformans} ATCC 25563\textsuperscript{T} isolate and suggested a negative result (8). The discordant results might be because we used a clinical \textit{F. gonidiaformans} isolate. In our study, culture was as sensitive as \textit{gyrB} PCR detection, and was more sensitive than \textit{rpoB} PCR. In previous study, culture methods identified much lower FN than \textit{gyrB} PCR, however, authors concluded that the anaerobic agar medium used was not very effective for detecting FN (2). In more recent study, one FN isolate was detected only by \textit{rpoB} PCR, whereas nine other FN isolates were identified by both \textit{rpoB} PCR and culture methods (13), suggesting the similar finding as our study; culture methods could be as sensitive as PCR for detecting FN 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 noninfectious disease should be interpreted accordingly.

Although there have been conflicting reports (9, 14), the majority of study suggested that FN are most prevalent among cases of pharyngotonsillitis at the age of 15 to 25 years (14, 15). The cohort of this study of median age as 38 years (IQR 26-44 years) might not have reflected the patients’ population with highest FN prevalence.

We did not quantitatively analyze FNSF using real-time PCR. For MIC determination, the broth, not agar, microdilution method was used. Information on the recent antimicrobial exposure was collected retrospectively by the medical chart review. Undocumented antimicrobial exposure might have been overlooked resulting in the
underestimation of FN.

In conclusion, our findings suggest that during the asymptomatic phase, the FNSF and β-hemolytic streptococci prevalence 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 FN might not be necessary for RPT.

Acknowledgement
The authors thank the clinical staff of Department of Otolaryngology for their help.

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Conflicts of interest
None declared.
References


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

<table>
<thead>
<tr>
<th>Indications for tonsillectomy</th>
<th>Total (n = 32)</th>
<th>Recurrent pharyngotonsillitis&lt;sup&gt;a&lt;/sup&gt; (n = 26)</th>
<th>IgA nephropathy (n = 3)</th>
<th>PFAPA syndrome (n = 1)</th>
<th>Sleeping apnea syndrome (n = 1)</th>
<th>Chronic urticaria (n = 1)</th>
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<tr>
<td>≥2 episodes of pharyngotonsillitis per year (n = 22)</td>
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<td>Persistent episodes for &gt;1 month (n = 1)</td>
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<tr>
<td>Recurrent peritonsillar abscess (n = 3)</td>
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</table>

**Culture and colony identification**

<table>
<thead>
<tr>
<th><em>F. necrophorum</em>&lt;sup&gt;b&lt;/sup&gt;</th>
<th>6 (18.8%)</th>
<th>5 (19.2%)</th>
<th>0</th>
<th>0</th>
<th>1 (100%)</th>
<th>0</th>
<th>0</th>
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<tbody>
<tr>
<td>(18.2%) (100%)</td>
<td>4</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
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</table>

<table>
<thead>
<tr>
<th><em>F. necrophorum subsp. funduliforme</em>&lt;sup&gt;c&lt;/sup&gt;</th>
<th>6 (18.8%)</th>
<th>5 (19.2%)</th>
<th>0</th>
<th>1</th>
<th>0</th>
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<th>0</th>
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<tbody>
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<td>(18.2%)</td>
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<td>(100%)</td>
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<td></td>
<td>(18.2%)</td>
<td>(100%)</td>
<td>(3.8%)</td>
<td>(19.2%)</td>
<td>(100%)</td>
<td>(33.3%)</td>
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<tr>
<td><strong>Group A Streptococcus</strong></td>
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<td>1 (3.1%)</td>
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<td>1 (4.5%)</td>
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<td>1</td>
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<td><strong>Group B Streptococcus</strong></td>
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<td>1 (3.1%)</td>
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<td>1 (4.5%)</td>
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<td>0</td>
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<tr>
<td><strong>Group C/G Streptococcus</strong></td>
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<td></td>
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<td>0</td>
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<td>1 (3.1%)</td>
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<td>1 (4.5%)</td>
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<tr>
<td><strong>PCR from excised tonsil</strong></td>
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<tr>
<td><strong>F. necrophorum (gyrB)</strong></td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
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<td></td>
<td>5 (19.2%)</td>
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<td>0</td>
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<td>4 (18.2%)</td>
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<td>(100%)</td>
<td>(100%)</td>
<td>(33.3%)</td>
<td>(100%)</td>
<td>(0%)</td>
</tr>
<tr>
<td>F. necrophorum (rpoB)</td>
<td>5 (15.6%)</td>
<td>4 (15.4%)</td>
<td>3 (13.6%)</td>
<td>1 (100%)</td>
<td>0</td>
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Abbreviation: PFAPA, periodic fever with aphthous pharyngitis and adenitis.

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

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

* Identified by gyrB sequence analysis.

* Also positive for FNSF.

* The excised tonsil was positive for gyrB, but negative for rpoB. FN 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 *Fusobacterium nucleatum*. 