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ORIGINAL ARTICLE

Differences in mortality in *Fusobacterium necrophorum* and *Fusobacterium nucleatum* infections detected by culture and 16S rRNA gene sequencing

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Abstract

Fusobacterium species are components of the normal microbiota of the oral cavity, gastrointestinal tract, and female genital tract. They are increasingly recognized as causative agents of oral, laryngeal, and tonsillar infections. Several fusobacterial species are involved in infections, with *F. necrophorum* and *F. nucleatum* being the most commonly cultured subtypes. In this study, we aimed to investigate clinical and prognostic differences in terms of mortality and association with malignancy between *F. necrophorum* and *F. nucleatum* detected by culture and 16S rRNA gene sequencing. This is a systematic, comparative, retrospective, non-interventional study. Data were extracted from the Department of Clinical Microbiology, Region Zealand, Denmark: all patients with *F. necrophorum* or *F. nucleatum* detected by culture or 16S rRNA gene sequencing from 1st of January 2010 to 30th of June 2015 were included. In total, *F. necrophorum* was detected in samples from 75 patients, and *F. nucleatum* in samples from 68 patients (total: $n = 143$). Thirteen patients had a current cancer diagnosis at the time of fusobacterial sampling. Multivariate analyses revealed a significant association of "current cancer" with 30-day mortality. Fusobacterial subtype was not associated with mortality neither in overall nor in subgroups with or without current cancer. Despite differences in clinical disease pattern between *F. necrophorum* and *F. nucleatum*, mortality was unaffected by fusobacterial subtype. Mortality was significantly related to comorbidity, especially a current diagnosis of cancer. Our data highlights the current debate whether fusobacterial involvement in cancer may have disease-altering properties, rather than being opportunistic pathogens secondary to cancer disease.

Keywords Fusobacteria · *F. necrophorum* · *F. nucleatum* · Infections · Mortality

Introduction

Fusobacterium species, obligate anaerobic Gram-negative pleomorphic rods, are components of the normal microbiota of the human oral cavity, gastrointestinal tract, and female genital tract. They are increasingly recognized as causative agents of oral, laryngeal, and tonsillar infections [1–4]. *Fusobacterium* spp. can escape detection, as they are difficult to culture and may require longer incubation times than other bacteria [1, 2, 5]. Culture is also hampered, if antibiotics are initiated before specimens are taken for culture.

Several Fusobacteria species are involved in human infections with *F. necrophorum* and *F. nucleatum* being the most commonly found subtypes. They have been linked to distinct anatomical sites and diseases.

Fusobacterium necrophorum is rarely found in children and middle-aged persons but increases with age > 60 years, in whom it is associated with severe infections and comorbidities such as previous or current cancer [5]. It is a common cause of tonsillitis (acute and recurrent), peritonsillar abscess [2, 3, 6], and the rare syndrome of Lemierre's disease (recent

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pharyngeal disease complicated by septic emboli and thrombosis of the internal jugular vein) [7].

Fusobacterium nucleatum is commonly found in the oral cavity, and is associated with periodontal and endodontal illnesses [1]. Furthermore, the *F. nucleatum* is associated with adverse pregnancy outcome (e.g., preeclampsia, preterm birth, miscarriage, and stillbirth) due to hematogenous transmission [1]. Additionally, *F. nucleatum* might have a causal role in the development of colorectal cancer [8].

The 16S rRNA gene sequencing and sequencing for detection of microbial agents in invasive infections allows detection of bacterial DNA, and may be applied as a compliment to culture for clinical samples where culture is hampered by prior use of antibiotics. The sensitivity is less affected by current antibiotic treatment [9].

In the current study, we aimed to investigate clinical and prognostic differences in terms of mortality and association with malignancy between *F. necrophorum* and *F. nucleatum* detected by culture and 16S rRNA gene sequencing.

Materials and methods

Design, data extraction, and ethics

This is a systematic, comparative, retrospective, non-interventional study. Data were extracted from the laboratory information system (LIMS) database at the Department of Clinical Microbiology, Region Zealand, Denmark. All patients with *F. necrophorum* or *F. nucleatum* detected by culture or 16S rRNA gene sequencing from 1st of January 2010 to 30th of June 2015 were included in the study. The following data were collected from the electronic medical files: gender, age, and co-morbidity for assessment of Charlson's index of co-morbidity (previous/current cancer, chronic heart failure, diabetes, chronic renal failure, chronic hepatic failure), date of discharge, intensive care admission, surgery performed, surgical complications present, and antibiotics provided during admission. All data were copied to predefined data record sheets and proofread by two investigators.

The study did not need approval from the Ethics Committee system due to the retrospective design. The study and data collection was approved by the Danish Data Protection Agency (reg-28-2016).

Culture

For blood culture, three BacT/ALERT† bottles (BioMerieux, Marcy l'Etoile, France) including one anaerobic bottle were used, according to the manufacturer, with 5.6 days of incubation and detection. *Fusobacterium* spp. were routinely identified by finding growth of Gram-negative rods from the anaerobic sub-culture plates. If *Fusobacterium* spp. were suspected, cultures were examined for kanamycin susceptibility, green fluorescence in UV-light and smell of butyric acid. If these traits were present, further phenotypic methods including MALDI-TOF were used for species identification according to a Danish guideline (17). However, only *F. necrophorum* or *F. nucleatum* were reported to the species level.

16S rRNA gene sequencing

16S rRNA gene sequencing was performed using Molzym *UMD-SelectNA* kit (Molzym GmbH, Germany). All enzymes, reagents and the PCR-water are supplied from Molzym GmbH and are free from DNA contaminants. DNA extraction consists of a manual pre-treatment of the swaps, liquid samples, or tissue samples and automated pathogen DNA isolation. The pre-treatment ends up with a mix of human, bacterial, and/or fungal cells. The suspension of cells is loaded to the SelectNA. The DNA eluates were mixed with the supplied matermixes for broad-range 16S and 18S rDNA analysis. Real time PCR was then performed on AriaMx (Agilent Technology, USA). Melting curves analysis was performed on the AriaMx to detect the pathogen DNA. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany). The purified samples were then sent to GATC Biotech (Germany) for sequencing. Three sequencing primers—one for Gram-positive, one for Gram-negative, and one for fungi—were applied. The primer sequences were provided by Molzym. The achieved DNA sequences were compared to sequences deposited in the NCBI database by using the BLAST search engine to identify potential pathogens.

Statistics

Descriptive and analytical statistics were performed using SPSS (version 21.0, IBM, Chicago, United States). Continuous data were reported as median with 10- and 90%-tiles and categorical data as absolute values. Differences between patients infected with *F. necrophorum* vs. *F. nucleatum* were analyzed using Mann-Whitney's U test *resp*. chi-squared test. To assess predictors of 30-day mortality and 12-month mortality, multivariate regression analyses (backward conditional elimination) were performed including variables that were significantly associated with mortality in univariate analyses (chi-squared test or ANOVA were appropriate). Significance was reached if $p < 0.05$. Since date of death was not available, it was not possible to ascertain differences in time-to-death in survival curves.

Results

In total, *F. necrophorum* was detected in samples from 75 patients, and *F. nucleatum* in samples from 68 patients (total:

$n = 143$). Table 1 shows baseline demographic and clinical data, including differences in comorbidity and clinical symptoms between the two bacterial subgroups. Seven samples were detected by 16S rRNA gene sequencing (three *F. Necrophorum* and four *F. Nucleatum*), the rest ($n = 136$) were detected by culture.

In total, 13 patients had a current diagnosis of malignancy at time of fusobacterial sampling: metastatic lung cancer ($n = 3$), metastatic colorectal cancer ($n = 2$), metastatic esophagus cancer, metastatic pancreas cancer, thyroid cancer, metastatic breast cancer, vulva cancer, prostate cancer, acute myeloid leukemia with relapse, and myelomatosis ($n = 1$ each).

Sites of infection and treatment

Table 2 shows that the Fusobacterial subtypes were associated with significant different disease patterns with significantly different anatomical preference sites. Despite this, we found no differences concerning surgical or invasive procedures between the two subspecies. The majority of patients received antibiotic treatment but significantly more patients with *F. necrophorum* infections received dual therapy including clindamycin (Table 2). The number of treated patients admitted to hospital did not differ whereas length-of-stay was significantly higher in patients infected with *F. nucleatum*.

Mortality

When comparing the 30-day-mortality group with the non-mortality group, we found no significance difference between patients infected with *F. necrophorum* (2.7%) vs. *F. nucleatum* (8.8%; $p = 0.11$), whereas the difference reached significance concerning 12-month-mortality (*F. necrophorum* 6.7% vs. *F. nucleatum* 17.6%; $p = 0.043$).

In univariate analyses, 30-day mortality was significantly associated with increasing age, number of comorbidities,

Table 1 Baseline and clinical differences between patients with positive 6S for *Fusobacterium necrophorum* vs. *Fusobacterium nucleatum*

	Fusobacterium necrophorum	*Fusobacterium nucleatum*	*p* value
Female gender, *n* (%)	26 (35)	31 (46)	*ns*
Age in years, median (10%–90%-tile)	22 (15–74)	53 (19–77)	< 0.0001
Comorbidity			
Heart failure, *n* (%)	9 (13)	14 (22)	*ns*
COPD, *n* (%)	3 (5)	2 (3)	*ns*
Stroke/TCI, *n* (%)	6 (8)	2 (3)	*ns*
Renal failure, *n* (%)	1 (1)	6 (9)	0.06
Hepatic failure, *n* (%)	0	4 (6)	< 0.05
Diabetes, *n* (%)	7 (9)	12 (19)	*ns*
Former cancer, *n* (%)	4 (6)	7 (11)	*ns*
CCI* 0/1/≥2, *n* (%)	60/5/10	40/15/13	< 0.05
Fever, *n* (%)	41 (56)	21 (32)	< 0.01
Respiratory symptoms, *n* (%)	7 (10)	15 (22)	< 0.05
Empyema/lung abscess, *n* (%)			
Sore throat, *n* (%)	55 (76)	6 (9)	< 0.0001
Current cancer diagnosis, *n* (%)	6 (8)	7 (11)	*ns*
CRP, median (10%–90%-tile)	149 (12–309)	98 (7–300)	*ns*
Leukocytes × 10^9, median (10%–90%-tile)	16.0 (1.6–68.8)	26.0 (11.0–34.0)	*ns*
Specimen			0.07#
Blood, *n* (%)	11 (15)	12 (18)	
Pus, *n* (%)	61 (81)	46 (68)	
Pleural fluid, *n* (%)	1 (1)	9 (13)	
Surgical specimen, *n* (%)	1 (1)	1 (2)	
Bronchial wash, *n* (%)	1 (1)	0	

ns $p > 0.1$, **CCI* Charlson's index of comorbidity, # *p* for trend, *COPD* chronic obstructive pulmonary disease, *TCI transitory cerebral ischemia*, *CRP* C-reactive protein

Table 2 Baseline and clinical differences between patients with positive 6S for *Fusobacterium necrophorum* vs. *Fusobacterium nucleatum*

	Fusobacterium necrophorum	*Fusobacterium nucleatum*	*p* value
Anatomical location of infection			< 0.0001#
Head-neck, *n* (%)	53 (71)	18 (27)	
Lung-pleura, *n* (%)	7 (9)	14 (21)	
Abdomen, *n* (%)	8 (11)	22 (32)	
Urology, *n* (%)	1 (1)	1 (2)	
Gynecological tract, *n* (%)	3 (4)	5 (7)	
Extremity, *n* (%)	2 (3)	6 (9)	
Septicemia from unknown focus, *n* (%)	1 (1)	2 (3)	
Diagnosis			< 0.0001#
Peritonsillary abscess, *n* (%)	42 (56)	2 (3)	
Other infection in head/neck, *n* (%)	11 (14)	16 (24)	
Infection in lung/pleura	7 (8)	14 (21)	
Infection in abdomen/pelvic region, *n* (%)	12 (16)	28 (42)	
Infection in extremity joint/skin, *n* (%)	2 (3)	6 (9)	
Septicemia from unknown focus, *n* (%)	1 (1)	2 (3)	
Antibiotics prescribed, *n* (%)	70 (93)	60 (88)	*ns*
≥ 2 drugs, *n* (%)	54 (72)	28 (41)	< 0.0001
Surgical treatment, *n* (%)			*ns*#
Abscess drainage, *n* (%)	42 (56)	33 (49)	
Fistula surgery, *n* (%)	3 (4)	4 (6)	
Pleural drainage, *n* (%)	3 (4)	11 (16)	
Tonsillectomy, *n* (%)	11 (15)	6 (9)	
Amputation, *n* (%)	4 (5)	0	
In-patients, *n* (%)	69 (92)	48 (87)	*ns*
Days in hospital, median (10%–90%-tile)	3 (1–14)	10 (2–25)	< 0.0001
Mortality			
30 days, *n* (%)	2 (3)	6 (9)	0.11
12 months, *n* (%)	5 (7)	12 (18)	< 0.05

ns: $p > 0.1$, **CCI* Charlson's index of comorbidity, # *p* for trend

former cancer diagnosis, current cancer, heart, or renal failure, site of the infection other than head and neck, need for surgery, presence of surgical complications, pulmonary symptoms, and inversely related to symptoms from the throat. Additional to these variables, 12-month mortality was associated with presence of diabetes, liver failure, and fever.

In multivariate analyses, 30-day mortality was only significantly associated with a diagnosis of current cancer), whereas 12-month mortality was significantly associated with both current cancer and hepatic failure and age > median (= 39 years. In patients without current cancer, 12-month mortality that was additionally associated with an increased Charlson's index of comorbidities (CCI 0: 2.2%, CCI 1: 6.7%, CCI ≥ 2: 35.3%). Fusobacterial subtype was not associated with mortality neither in overall nor in subgroups with or without current cancer.

Discussion

The main findings of this study, when comparing the two subgroups of bacteria, included no significant differences with respect to localization as well as treatment, with *F. necrophorum* being primarily localized to the head and neck, and causing deep infections, whereas *F. nucleatum* did not show any prevalence as to site and depth of infection. Interestingly, these differences did not influence the mortality of the patients in the two bacterial subgroups. The only factor significantly associated with mortality was a current diagnosis of malignancy at the time of the bacterial sampling, and not the Fusobacterial subtype.

Data from a total of 143 patients' samples were evaluated. We collected data from all patients with *F. necrophorum* or *F. nucleatum* detected by culture and by 16S rRNA gene

sequencing from 1st of January 2010 to 30th of June 2015 in region Zealand, Denmark, which makes the study comprehensive, as it includes all samples taken over a 5-year period in a specific region of Denmark.

Seven samples from six patients were detected by 16S rRNA gene sequencing after culture did not show any growth, and provides additional evidence that this method is a good supplement to standard culture in case of recent antecedent and current antimicrobial treatment [10].

Several studies have evaluated the disease/mortality risk in both Fusobacteria as a whole, and more specifically in the *F. nucleatum* subtype. Mitsuhashi et al. found that Fusobacteria found within a pancreas tumor was independently associated with a worse prognosis of the pancreatic cancer, and thus speculated that Fusobacteria could be a prognostic biomarker in cancer [11]. Other studies have also more frequently found Fusobacteria as a group, and more specifically *F. nucleatum* in colorectal cancer tissue, as compared to normal colorectal tissue [12–14]. Another study found *F. nucleatum* to be a risk factor for disease progression in colorectal cancer, possibly affecting the survival of the patient, and again suggested the Fusobacteria as a diagnostic and prognostic biomarker of colorectal cancer [15]. A few studies have investigated the underlying pathomechanisms for the apparent prevalence of Fusobacteria in cancers. The spread of oral Fusobacteria to the colorectal cancer is thought to be enabled by a transient bacteremia, such as those seen in *Fusobacterium* infections [16]. The *Fusobacterium* recognizes the cancer by binding to the tumor-displayed D-galactose-b(1-3)-*N*-acetyl-*D*-galactosamine (Gal-GalNAc) [16]. Kostic et al. found that Fusobacteria were able to generate a proinflammatory microenvironment that would promote the progression of the colorectal cancers [17]. The bacteria did this through recruitment of tumor-infiltrating immune cells [17]. Similarly, Nosho et al. found that in colorectal cancer, *F. nucleatum* expands the myeloid-derived immune cells, which inhibits T cell proliferation and induce apoptosis in the colorectal cancer [18]. This indicates that *F. nucleatum* inhibits human T cell responses, by immunosuppressive qualities of the bacteria [18]. Ahn et al. found that the human gut microbiome in colorectal cancer had a decreased overall diversity of bacteria, with a higher abundance of Fusobacteria and *Porphyromona*s, and a lower finding of Clostridia [19].

All the above studies, including our study, suggest that Fusobacteria play an important part in the human microbiome of the gut, and the pathomechanisms regarding colorectal cancer evolution and growth. However, very few studies have been conducted regarding lung cancer and the lung microbiome. We found three patients with lung cancer and a *Fusobacterium* infection. All three patients died during the study period. These data suggest a link, also in lung cancer, between the bacterial constitution and the cancer. To support this, a recent study compiled data that suggest that, through the Gal-GalNAc, Fusobacteria may be linked to many different cancers including breast, lung, and esophagus cancers [16]. Further studies in lung cancer are required to confirm this hypothesis.

In conclusion, despite differences in clinical disease pattern between *F. necrophorum* and *F. nucleatum*, mortality was unaffected by Fusobacterial subtype. Mortality was significantly related to comorbidity, especially a current diagnosis of cancer. This supports recent evidence that Fusobacterial involvement in cancer may have disease-altering properties, rather than merely an opportunistic pathogen secondary to cancer disease. Future studies should aim at the clinical impact of targeting Fusobacteria in cancer disease.

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Contributions KJ analyzed data and drafted the manuscript; SK provided data, did lab work, and revised the manuscript; NG compiled all data and revised the manuscript; XN did lab work and wrote part of the methods section as well as revised the manuscript; TB conceptualized the manuscript and revised it and UB conceptualized the manuscript, analyzed data, and revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest to report.

Access to data Access to data will be granted by request to those eligible for this.

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