Acute appendicitis in children is associated with an abundance of bacteria from the phylum Fusobacteria

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ARTICLE INFO

Article history:
Received 3 June 2013
Received in revised form 28 June 2013
Accepted 30 June 2013

Key words:
Appendicitis
Microbiome
Gut bacteria
Fusobacteria

ABSTRACT

Background: Although luminal obstruction has traditionally been viewed as the underlying cause of appendicitis, recent evidence has suggested that the disease may result directly from invasion by specific pathogens, e.g. Fusobacterium nucleatum. The purpose of this study was to survey microbial communities within pediatric appendectomy specimens using a culture-independent approach.

Methods: We performed 16S ribosomal gene sequence analysis to profile the microbiota present within luminal fluid obtained from 22 pediatric appendectomy specimens. These included 10 simple appendicitis cases, 5 perforated appendicitis cases, 2 interval appendectomies, and 5 incidental appendectomies.

Results: Samples could be divided into 2 distinct clusters based upon the composition of the appendecal bacterial communities. Appendicitis samples contained an increased abundance of Fusobacterium spp. and a reduced abundance of Bacteroides spp. relative to non-appendicitis cases. Appendicitis samples also contained variable amounts of other oral taxa such as Porphyromonas, Parvimonas, and Gemella, whereas these taxa were generally absent from non-appendicitis samples.

Conclusions: Acute appendicitis is associated with an abundance of Fusobacterium spp. and other pathogens commonly found in the oral cavity. Further research is needed to determine whether these organisms directly cause appendicitis or rather proliferate in the appendix as a secondary consequence of inflammation.

The role of microbes in the pathogenesis of appendicitis is uncertain. Although it has traditionally been stated that bacterial overgrowth in the appendix is a secondary consequence of appendiceal obstruction or inflammation [1,2], support for this concept is surprisingly limited. In fact, recent evidence has accumulated indicating that primary bacterial infection may be an initiating event in the pathogenesis of disease. The notion that appendicitis is primarily microbe-mediated is consistent with the reproducible observations that appendicitis occurs in clusters [3], varies seasonally [4], and rarely occurs in rural areas of the undeveloped world [5].

Over the past decade, it has been clearly demonstrated that molecular surveys of microbes allow for more accurate and higher throughput analysis of clinical specimens than traditional culture-based methods [6]. Most commonly, culture-independent surveys involve the analysis of bacterial 16S ribosomal RNA gene sequences [7]. However, until recently, the bacteriology of appendicitis had been investigated only with culture-based laboratory techniques. Organisms commonly isolated from both inflamed and normal appendiceal tissue have included Bacteroides fragilis, Escherichia coli, Pseudomonas aeruginosa, and Peptostreptococcus species [8–10]. Taken together, culture-based studies performed over several decades have failed to demonstrate a reproducible association between appendicitis and the presence of specific organisms.

To date, three studies have utilized culture-independent molecular techniques to comprehensively profile mucosa-associated microbes from the human appendix in the setting of appendicitis. In 2011, Swidsinski et al. reported the results of fluorescence in situ hybridization (FISH) analysis of 70 appendectomy specimens collected in Germany [11]. In this study, invasive species from the genus Fusobacterium (anaerobes from the phylum Fusobacteria, commonly found in the oral cavity) were demonstrated histologically to penetrate the epithelium and submucosal layers of appendicitis samples. They reported that Fusobacteria were present in most appendicitis samples but were missing completely from control samples. Furthermore, the proportion of Fusobacterium increased with the severity of appendiceal inflammation. In 2012, the same group evaluated a series of appendectomy samples from Russia and China with the same FISH technique. Remarkably, they again found a high incidence of invasive Fusobacterium in appendicitis samples and again demonstrated that these organisms were completely absent in controls [12]. In 2013, a group from Ireland published the results of the first study of deep sequencing of bacterial DNA obtained from human appendices [13]. This was a relatively small study, in which only 7 samples were evaluated. Both inflamed and uninflamed samples were found to
contain *Fusobacterium*, but *Fusobacterium* abundance was clearly highest in inflamed samples. Interestingly, other microbes commonly found in the oral cavity (e.g. *Gemella* and *Parvimonas*) were also found to be abundant in the appendicitis samples.

Given the apparent impact of environmental variables on the incidence of appendicitis, it is significant that these three prior studies reported similar findings despite collecting samples across multiple countries. To our knowledge, a culture-independent analysis of bacterial DNA obtained from appendectomy samples has not yet been performed in the United States. Here, we report analysis of 16S ribosomal RNA gene sequences present in appendectomy specimens taken from children with and without appendicitis in Pittsburgh, Pennsylvania. Similar to the abovementioned studies, we found that both simple and complicated appendicitis samples harbored an abundance of microbes typically found in the mouth (*Fusobacterium, Gemella*, and *Parvimonas*) and a corresponding depletion of *Bacteroides* species.

### 1. Methods

#### 1.1. Subject selection

During a 1-month period in June and July 2012, we sampled fresh luminal fluid from appendectomy specimens obtained from children less than 18 years of age at the Children’s Hospital of Pittsburgh of University of Pittsburgh Medical Center. This pilot study was performed with institutional approval according to the following protocol. When patients were taken to the operating room for appendectomies, the operating surgeons notified the study investigators. No review of the patient chart or electronic records was performed. In the operating room, the attending surgeon was asked to characterize the appendix specimen. For children with appendicitis, the indication for surgery was classified as simple appendicitis (SA), perforated appendicitis (PA), or interval appendectomy (IntA). Interval appendectomies were performed electively on children that had been hospitalized during the prior 3 months for non-operative management of complicated perforated appendicitis. Samples obtained from children without gross evidence of appendicitis were referred to as incidental appendectomies (IA), and the operating surgeon was asked to comment on the indication for surgery (e.g. chronic abdominal pain). These cases were used as a reference for comparison.

#### 1.2. Sample processing

Immediately following removal of the appendix, a sterile cotton swab was inserted into the appendix to sample luminal fluid. Samples were cryopreserved until later analysis. Subsequently, microbial DNA was extracted from each swab using the MO BIO PowerSoil DNA Isolation kit. Samples were added directly into bead tubes containing 60 μL of Solution C1 and then incubated at 65 °C for 10 min. Tubes were then shaken horizontally on a lab mixer for 10 min at maximum speed using a MO BIO vortex adapter. All remaining steps followed the manufacturer’s protocol.

PCR amplification of the V2–V4 region of the small subunit ribosomal RNA gene (16S rRNA) was performed in triplicate 25 μL reactions. Amplicons were produced utilizing fusion primers adapted for the GS FLX Titanium pyrosequencing platform (Roche). Primers incorporated either the A linker, key and a 10 nucleotide barcode (forward primer) or the B linker and key (reverse primer), followed by a sequence targeting a conserved region of the bacterial 16S rRNA gene. Individual PCR amplicons were gel purified, quantified, pooled in equimolar ratios, and then a gel-purified pool of libraries was submitted for pyrosequencing on the Roche/454 GS FLX + System.

After low quality bases were removed, the raw pyrosequencing reads were processed using QIIME ([http://qiime.sourceforge.net/](http://qiime.sourceforge.net/)) followed by removal of chimeric sequences identified by UChime [14]. QIIME, utilizing the Uclust software, was used to cluster unique reads at a 0.97 operational taxonomic unit (OTU) threshold, and cluster taxonomic identification was assigned via the RDP classifier [15]. QIIME was used to generate taxa summaries and perform rarefactions, calculate alpha entropy (diversity) indices within samples, beta entropy (diversity) indices between samples, and to generate UPGMA trees of the jackknifed beta diversity.

Comparison of sample composition and identification of statistically significant differences was performed with 2 sided t-tests assuming unequal variance in Microsoft Excel.

### 2. Results

#### 2.1. Study subjects

We analyzed 22 appendectomy specimens. As noted, all patients were less than 18 years of age. Ten samples were characterized by the attending surgeons as simple appendicitis (SA). Five samples were characterized as perforated appendicitis (PA). Two interval appendectomies (IntA) and five incidental appendectomies (IA) were included in the study. Three of the incidental appendectomies were performed during laparoscopy for abdominal pain of uncertain etiology. In 2 of these cases, hemorrhagic ovarian cysts were felt to be the cause of the pain but in the third case no etiology for the pain was identified. The remaining two IA’s were performed at the time of a Ladd’s procedure for malrotation and a resection of a Meckel’s diverticulum. Per hospital protocol, all patients undergoing appendectomy received at least one preoperative dose of a broad-spectrum antibiotic, most commonly cefoxitin.

#### 2.2. Overall composition of microbial communities

After processing, a total of 75,238 16S rRNA gene sequences were obtained from the 22 samples utilizing 454 pyrosequencing. We obtained an average of 3420 sequences per sample (range 1061–7513). The average length per sequence was 532 bp (range 160–688 bp).

Phylum and genus level taxonomic assignments for all sequences are shown in Fig. 1. Overall, 94.1% of all sequences were assigned to the three bacterial divisions *Fusobacterium* (40.5%), *Bacteroidetes* (33.8%), and *Firmicutes* (19.8%). The abundance of *Fusobacteria* sequences stands in contrast to most studies of human fecal samples [16,17], but is consistent with recent molecular studies of the appendiceal microbiome [11–13]. An additional 2.7% of sequences were assigned to the bacterial kingdom but could not be assigned to a phylum. Only 1.8% of sequences were assigned to the phylum *Proteobacteria*, which contains many commonly observed Gram-negative organisms such as *E. coli* and *P. aeruginosa*.

*Fusobacterium* and *Bacteroides* were clearly the most abundant genera. In most samples, organisms from these genera were present as a major community member (defined as abundance greater than or equal to 1.0%). Remarkably, 19 of the 22 total microbial communities were found to contain a dominant member, defined as a taxon representing at least 30% of all sequences [18]. In several cases the relative abundance of these dominating organisms exceeded 70%. The genera *Fusobacterium* (14 samples) and *Bacteroides* (4 samples) were commonly observed to be dominant. The only other genera found to be dominant were *Porphyromonas* (sample P1) and *Streptococcus* (sample S9). Other genera observed at lower frequency can be seen in Fig. 1B; only rarely were sequences assigned to the genera *Bifidobacterium* or *Lactobacillus*, which contains many species associated with probiotic qualities.
2.3. Comparison of appendicitis and non-appendicitis microbial communities

To assess the similarities and differences in community composition across samples, we generated a UPGMA tree of the jackknifed beta diversity using the weighted Unifrac distances among the samples [19] (Fig. 2), which enabled us to cluster the samples based on their pairwise similarities to each other. We identified 2 primary clusters of samples, which were clearly distinguished by variation in the relative abundances of the bacterial phyla Bacteroidetes and Fusobacteria (Fig. 3).

Cluster 1 was a homogenous cluster of 4 IA samples. Importantly, no appendicitis samples were in this cluster. These samples were highly enriched with taxa from the phylum Bacteroidetes (mean relative abundance 71.3% vs 25.4% in cluster 2, \(t\)-test \(p=0.0004\)), and relatively enriched with taxa from the phylum Firmicutes including several taxa from the class Clostridia. *Fusobacterium* was either absent (1 sample) or present at relatively low abundance (3 samples) in each of these samples. The high abundance of Bacteroidetes and Firmicutes within samples from the non-inflamed appendices was similar to commonly observed diversity patterns in normal human stool samples.

Cluster 2 contained 18 samples total. All SA, PA, and IntA cases were present in this cluster in addition to one of the 5 IA cases. Three defining features (Fig. 3) characterized these samples at the genus level: a relative enrichment of *Fusobacterium* (mean relative abundance 48.6% vs 3.4% in cluster 1, \(t\)-test \(p=2.7E-6\)), a relative enrichment of *Prevotella* (5.1% vs 0.2%, \(t\)-test \(p=0.0068\)), and a corresponding depletion of *Bacteroides* (11.4% vs 52.9%, \(t\)-test \(p=0.017\)). Interestingly, many Cluster 2 samples also contained other pathogens that, like *Fusobacterium*, are commonly found in the oral cavity. For example, sample P1 contained an extremely high abundance of *Porphyromonas* (63%), a Gram-negative anaerobe frequently observed in the oral cavity; no other sample contained

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**Fig. 1.** Microbial communities observed within appendectomy specimens. Shown are relative abundances of bacterial taxa at the (A) phylum and (B) genus levels of phylogenetic classification.
this genus at an abundance greater than 1% and none of the non-appendicitis samples contained it at all. *Gemella*, a Gram-positive facultative anaerobe of the oral cavity and upper gastrointestinal tract, was observed at high abundance (27%) in sample S2 and lower abundance in several others within this cluster. Additionally, several samples within cluster 2 contained over 5% relative abundance of *Parvimonas*, a genus of obligate anaerobes commonly observed in children with severe periodontitis. Sample P5 notably contained a high abundance of *Jonquetella*, a Gram-negative strict anaerobe previously described in wound and abscess cultures [20]; no other samples contained this genus.

We found that alpha diversity, a measure of species richness and evenness within each sample, did not differ between clusters (t-test \( p = 0.11 \)).

### 2.4. Species level assignments

The QIME software package generally provides confident phylogenetic assignments of DNA sequences down to the taxonomic level of genus. To further define individual taxa of interest, we aimed to obtain species-level assignments by performing blastn searches of our observed OTUs against the NCBI GenBank database and against the curated Bacteria database of the RDP [15]. These searches revealed that all but one of the samples containing *Fusobacterium* contained the species *Fusobacterium nucleatum* (subsp. *nucleatum*). The remaining sample (Int2) contained the species *Fusobacterium necrophorum*. Additionally, we determined that the *Porphyromonas* sequences observed at high abundance in sample P3 specifically indicated the presence of *P. gingivalis*, which has commonly been identified as a causative pathogen in periodontitis. Finally, the *Streptococcus* sequences observed at high abundance in sample S9 and the *Gemella* sequences observed at high abundance in sample S2 could not be annotated at the species level. However, it is notable that members of both of these genera (*Streptococcus* and *Gemella*) are known oral pathogens that are occasionally identified in extra-oral infections.

### 3. Discussion

Since Wangensteen and others performed classic experiments exploring the relationship between appendiceal obstruction and inflammation [21], it has been generally stated that bacterial overgrowth in appendicitis is a secondary consequence of inflammation. Recently, however, this notion has been challenged [22–24]. Several investigators have reintroduced a longstanding hypothesis that appendicitis results from a fundamental disturbance in the appendiceal microbiota. This distinction is clinically relevant. Improved knowledge of the bacteriology of appendicitis could provide opportunities for earlier detection of disease and improved regimens for postoperative antibiotic therapy. Others have also argued that appendicitis, like diverticulitis, can be treated non-operatively with antibiotics alone [25].

The advent of high-throughput culture-independent experimental approaches has created unique opportunities to address the role of microbes in a wide range of clinical settings. As described above, three recent studies have identified a surprising abundance of *Fusobacterium* spp. in appendix specimens from children and adults with acute appendicitis [12,13,11]. Corresponding histologic studies of these appendix samples have suggested that Fusobacteria are invasive pathogens rather than innocent bystanders that simply proliferate after the onset of inflammation. None of the studies were performed in the United States, and the only study thus far to use deep DNA sequencing to construct a comprehensive profile of molecular diversity in the appendix was limited by a very small sample size. The deep sequencing in the latter study, however, did allow for detection of other oral pathogens accompanying Fusobacteria within appendicitis samples.

In the present study, we analyzed appendectomy specimens from 22 children. We elected to study fluid from the appendiceal lumen as a surrogate for appendiceal tissue. Similar to Guinane et al., we used deep pyrosequencing of 16S ribosomal RNA gene fragments to construct comprehensive profiles of microbial diversity that would not have been possible with a culture-based approach. Although the
sample size was small, our results correlate extremely well with the 3 recent studies of appendicitis. With one exception, each of the 17 appendicitis samples (10 SA, 5 PA, 2 IntA) in our study was characterized by a preponderance of *Fusobacterium* and a depletion of *Bacteroides*. Of the 5 non-appendicitis samples, only 1 contained *Fusobacterium* at a relative abundance greater than 10%. Additionally, we found that appendicitis samples contained variable amounts of oral pathogens previously implicated in extra-oral disease. We believe that our data, in conjunction with the reports by Swidsinski and Guinane, provide compelling evidence that appendicitis is associated with overgrowth of “oral” pathogens in the appendix and a corresponding loss of *Bacteroides* species. The abundance of Fusobacteria in the IntA samples suggests that the presence of Fusobacteria is not a consequence of active acute inflammation, since these samples were collected months after successful treatment of the acute inflammatory process.

An unresolved issue is whether the growth of these pathogens is a primary or secondary feature of appendicitis. Intriguing recent work has detailed how oral microbes can spread systemically and contribute to a surprisingly wide range of diseases including atherosclerotic disease, preterm labor and pregnancy loss, rheumatoid arthritis, and Crohn’s disease. Interestingly, *F. nucleatum* is the species most commonly implicated in extra-oral infections [26,27]. This Gram-negative anaerobe possesses a well-documented ability to adhere to and invade a variety of epithelial cell types [28]. Furthermore, Fusobacteria have been shown to promote mixed infections by facilitating tissue invasion by other coexisting bacterial species. Other well-studied oral pathogens implicated in extra-oral disease include *Porphyromonas spp.* and *Streptococcus spp.* [26], which were both observed in the appendicitis samples studied here. More work is needed to clarify the relationship between oral microbes and the pathogenesis of acute appendicitis.

This pilot study was intentionally performed with a limited scope of work, and it will be followed by a more detailed prospective study. The current study was limited by the fact that we did not have patient or family permission to review patient charts in conjunction with our sample processing and data analysis. Thus, we did not have access to surgical pathology results, and so we cannot exclude the possibility that surgeon assessment of appendiceal inflammation may have conflicted with the histologic review of the appendectomy specimens. Additionally, it is conceivable that the observed dysbiosis (i.e. alterations in microbial communities) in appendicitis results from administration of broad-spectrum antibiotics rather than the disease process itself. Importantly, however, in the Swidskinski 2010 study of 70 appendectomies, none of the patients received preoperative antibiotics and yet the authors still observed a very high abundance of invasive *Fusobacterium* species. Moreover, in our study and in the 2012 Guinane study [13], the IA patients also received preoperative antibiotics but generally did not possess an abundance of Fusobacteria or other oral microbes. Thus we do not believe that our data reflect exposure to antibiotics.

In conclusion, we have added to the growing body of literature demonstrating that acute appendicitis is associated with an altered appendiceal microbiome; specifically, appendicitis is characterized by

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**Fig. 3.** Mean relative abundances of bacterial (A) phyla and (B) genera in appendectomy clusters. The white boxes depict the 4 non-appendicitis samples from cluster 1, and the grey shaded boxes depict cluster 2 samples. Asterisks denote taxa that are significantly enriched or depleted within either cluster (p<0.05).
an abundance of *Fusobacterium* and other oral pathogens and a depletion of *Bacteroides* species. Larger prospective studies will be required to validate these associations, and must be accompanied by mechanistic research to clarify the relationship between oral microbes and appendicitis. An improved understanding of the contributions of microbes to the pathogenesis of appendicitis could yield improved methods of detecting the disease; for example, in a patient with a clinical examination that is equivocal for appendicitis, it might be possible to collect a rectal swab and test for the abundance of *Fusobacterium* species rather than utilizing computed tomography. Similarly, it might be possible to tailor postoperative antibiotic therapy to organisms known to be present during appendicitis in order to reduce the risk of postoperative complications, e.g. abscess formation. This line of inquiry demonstrates the power of applying molecular investigations of the microbiome to common medical and surgical problems.

References