Acute appendicitis is characterized by local invasion with Fusobacterium nucleatum/necrophorum

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Title

Acute appendicitis is characterized by local invasion with *Fusobacterium nucleatum/necrophorum*

Short title

Fusobacteria in acute appendicitis

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Abbreviations:

FISH - fluorescence in situ hybridizationAlexa488, Cy3, Cy5, DAPI – different fluorescent dyes corresponding to green, orange, dark red, and blue colours

FISH probes abbreviations used in text:

Ato291 - *Atopobium*,

Bac303 - *Bacteroides*,

Bif164 - *Bifidobacteriaceae,*

Chis150 - *Clostridium histolyticum,*

Ebac1790 - *Enterobacteriaceae,*

Ecyl1387 - *Eubacterium cylindroides,*

Erec482 - *Clostridium group* XIVa,

Eub338 - *Eubacteria* (virtually all bacteria),

Fprau - *Faecalibacterium prausnitzii*,

Fnec - *Fusobacterium necrophorum*,

Fnuc - *Fusobacterium nucleatum*,

Fuso - Fusobacteria,

Lab158 - *Lactobacillus,*

Muc1437 - *Akkermansia muciniphila*

Pasco - *Phascolarctobacterium faecium,*

Pnig657 - *Prevotella nigrescens,*

Ser1410 - *Serpulina,*

Veil223 - *Veillonella*

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ABSTRACT

Background: Acute appendicitis is a local intestinal inflammation with unclear origin. The aim was to test whether bacteria in appendicitis differ in composition to bacteria found in cecal biopsies from healthy and disease controls.

Methods and patients: We investigated sections of 70 appendices using rRNA-based fluorescence *in situ* hybridization. Four hundred cecal biopsies and 400 faecal samples from patients with inflammatory bowel disease and other conditions were used as controls. A set of 73 group-specific bacterial probes was applied for the study.

Results: The mucosal surface in catarrhal appendicitis showed characteristic lesions of single epithelial cells filled with a mixed bacterial population ("pinned cells") without ulceration of the surroundings. Bacteria deeply infiltrated the tissue in suppurative appendicitis. Fusobacteria (mainly *Fusobacterium nucleatum* and *necrophorum*) were a specific component of these epithelial and submucosal infiltrates in 62% of patients with proven appendicitis. The presence of Fusobacteria in mucosal lesions correlated positively with the severity of the appendicitis and was completely absent in cecal biopsies from healthy and disease controls. Main faecal microbiota represented by *Bacteroides, Eubacterium rectale* (*Clostridium* group XIVa), *Faecalibacterium prausnitzii* groups and *Akkermansia muciniphila* were inversely related to the severity of the disease. The occurrence of other bacterial groups within mucosal lesions of acute appendicitis was not related to the severity of the appendicitis. No Fusobacteria were found in rectal swabs of patients with acute appendicitis.

Conclusions: Local infection with *Fusobacterium nucleatum/necrophorum* is responsible for the majority of cases of acute appendicitis.

INTRODUCTION

Acute appendicitis is the most common surgical emergency. After more than 250 years of intensive research, the disease is clinically well characterized and appears uniform and simple from the clinical point of view. There is, however, astonishingly little known about causal factors for this disease. The most often mentioned cause listed in older textbooks is obstruction of the appendix by a foreign body. In the age of molecular genetics and modern imaging, however, obstruction as a cause can be ruled out.[1] Upon histopathological evaluation, evidence of obstruction of the appendix is demonstrable in only a minority of resected appendices and seems to be the result rather than the cause of appendiceal inflammation.[1] Bacteria have been repeatedly considered as protagonists of inflammation. However, while microbial cultures are efficient in isolating single bacterial species, they are unable to characterize polymicrobial processes. Previous studies confirmed the polymicrobial involvement in acute appendicitis, but were unable to designate a leading microorganism.[2,3] This is not surprising, because the diversity of the colonic microbiota is extremely high, with up to 5000 different species.[4] Cultures are not able to quantitatively or qualitatively cover this diversity. We studied the in situ composition of mucosal and invading bacteria in acute appendicitis using rRNA-based fluorescence in situ hybridization (FISH).

Our aim was to test whether bacteria in appendicitis differ in composition to bacteria found in cecal biopsies and faecal samples from healthy and disease controls, and whether the changes in composition of microbiota are local or can be found also in rectal swabs of individuals with acute appendicitis prior to surgery.

PATIENTS AND METHODS

Appendices

Materials from 70 appendices removed during laparoscopic emergent appendectomy were investigated. None of the patients had received pre-operative antibiotic treatment. All patients

were operated on within 24 hours after onset of symptoms for suspected acute appendicitis. They had no history of previous episodes, ruling out chronic appendicitis.

Cecal biopsies and samples of faecal cylinders

Paraffin embedded cecal biopsies and faecal cylinders (400 each) that were previously investigated in our clinic for the study of mucosal[5] and faecal[6] microbiota, were used as controls. While selecting controls, we chose at random 100 patients with ulcerative colitis, 100 patients with Crohn's disease, 50 patients with self-limiting colitis, 50 patients with diverticulosis, 50 patients with irritable bowel syndrome and 50 healthy controls.

Rectal swabs

Rectal swabs were taken from 30 patients immediately before appendectomy.

Intraoperative isolation of bacteria

Intraoperative isolation of bacteria was performed in 5 patients with acute appendicitis. Pieces of appendices were washed with physiologic saline, changing the solution 4 times, and then hypotonically lysed. The lysates of appendiceal tissues were plated on Columbia and Schaedler blood agar in series of dilutions and incubated anaerobically for 48 hours. Then a smear from the plate without dilution was taken and hybridized. If Fusobacteria were found within this smear, single colonies (20 to 200) were tested until bacteria that positively hybridized with the *Fusobacterium* (Fuso,[7] Fnec, Fnuc[8]) probe were confirmed and isolated.

FISH

All materials (appendices, biopsies, heads of the rectal swabs) were fixed in Carnoy solution[5,6] and then embedded into paraffin using standard techniques. Sections of 4 µm thickness were placed on SuperFrost slides (R. Langenbrinck, Emmendingen, Germany**)**.

Multicolour FISH was performed according to previously described protocols for evaluation of tissue specimens and identification of bacteria[9] with a Nikon e600 fluorescence microscope (Nikon, Tokyo, Japan) and Nikon DXM1200 camera. Altogether 73 FISH probes (see supplementary material) were applied.[10,11]

The Cy3 labelled probe that characterized a bacterial group of interest (orange fluorescence) was simultaneously hybridized with the universal for virtually all bacteria *Eubacteria* specific Eub388[12] Alexa488 probe (green fluorescence) and a mix of Cy5 labelled probes (red fluorescence) that included the main faecal bacterial groups such as *Faecalibacterium prausnitzii* (Fprau[10]), *Bacteroides* (Bac303[13]) and *Clostridium* group XIVa (Erec482[14]). The fluorescence signals of specific probes, universal probe and probes for Fprau+Bac303+Erec482 groups were overlaid within the same microscopic field. The difference in colour allowed to calculate the proportion of the selected bacterial group (orange fluorescence) within all bacteria (Eub338, green signals) and to the main faecal bacterial groups (Fprau+Bac303+Erec482, red signals) and to allocate single bacteria groups spatially in relation to each other and histological structures.

The counter hybridizations with Alexa488 and Cy5 stained probes allowed further the exclusion of false positive signals. In case of reduced intensity of the specific hybridization signals and high background fluorescence it is often difficult to distinguish between true signals and unspecific binding of the probe to bacteria or complex eukaryotic cell structures. To avoid these biases, signals that were apparent both with the probe of interest and with the Fprau/Erec482/Bac303 probes were regarded as unspecific and not further evaluated. Only signals that had no counterpart with hybridization signals representing the main faecal bacterial groups were regarded as genuine and used for evaluation and referenced to the Eub338 signals.[9]

RESULTS

The clinical diagnosis of acute appendicitis was confirmed intra-operatively and after histopathological evaluation in 52 patients; 25 patients had catarrhal appendicitis and 27 had suppurative appendicitis. In 18 patients the appendix showed no histological signs of acute appendicitis.

Composition of the microbiota in the appendix

Dense bacterial masses typical for faeces were found in the lumen of 6 of the 18 patients without appendicitis, 2 of the 25 patients with catarrhal appendicitis and in none of the patients with suppurative appendicitis. In all other samples, the lumen was filled either with leukocytes or mucus. Despite lack of gut content, intestinal bacteria could be nevertheless abundantly seen either between luminal leukocytes (Figure 1), adherent to the mucosal surface and invading single epithelial cells (Figure 2) or spreading into the subepithelium (Figure 3).

The proportions of bacterial groups composing more than 1% of the total number of bacteria are presented in Table 1 for each of the investigated appendices. The Fusobacteria were mainly represented by *Fusobacterium nucleatum* (79%), *Fusobacterium necrophorum* (12%) and Fusobacteria (9%) that could not be determined at species level as they were positive with Fuso but negative with the Fnuc and Fnec FISH probes.

Ten of the 72 investigated group and species specific FISH probes hybridized with ≥10% of the total bacteria in at least one appendix: *Bacteroides* (Bac303), *Clostridium* group XIVa (Erec482), *Faecalibacterium prausnitzii* (Fprau), Fusobacteria (Fuso, Fnec, Fnuc), *Enterobacteriaceae* (Ebac1790),[15] *Bifidobacteriaceae* (Bif164),[16] *Akkermansia muciniphila* (Muc1437)[17] and *Serpulina* (Ser1410).[18] All other investigated bacterial groups were found in less than 5% of the samples and in concentrations usually around 1% or less: *Clostridium histolyticum* (Chis150),[14] *Veillonella* (Veil223),[19] *Atopobium* (Ato291),[20] *Lactobacillus*

(Lab158),[21] *Prevotella nigrescens* (Pnig657),[22] *Eubacterium cylindroides* (Ecyl1387)[19] *Phascolarctobacterium faecium* (Phasco).[19] The occurrence (Table 2) and mean proportion of Fusobacteria (Table 3) increased significantly with increasing severity of the inflammation. The mean proportions of main faecal microbiota represented by a pool of *Bacteroides, Eubacterium rectale* and *Faecalibacterium prausnitzii* groups (Bac303+Fprau+Erec482) to the total bacteria (Eub338) were inversely related to the severity of the appendicitis (Table 3). With increasing degree of inflammation the cumulative proportion of these three main faecal groups fell from 87% in patients with no appendicitis to 51% in patients with suppurative appendicitis ($P<0.001$). When investigated using singular FISH probes, the reduction of *Faecalibacterium prausnizii* (Fprau) was more profound than that of the *Clostridium* group XIVa (Erec482). *Bacteroides* was less affected. In some cases particular habitual bacterial groups were completely eradicated (Table 1). *Bacteroides* was absent only in two patients with catarrhal and one patient with suppurative appendicitis. *Faecalibacterium prausnizii* was completely depleted in 11% of the patients with no appendicitis, in 28% of patients with catarrhal and 54% of patients with suppurative appendicitis. *Clostridium* group XIVa (Erec482) was completely depleted in 4% of patients with catarrhal and 33% of patients with suppurative appendicitis.

The mean proportions of *Akkermansia muciniphila*, similar to the habitual faecal bacterial groups, were inversely related to the severity of the appendicitis (Table 3).

The occurrence and mean proportions of all other groups did not correlate with the severity of disease (Table 1).

Occurrence of single bacterial groups in lumen and mucosal lesions of patients with acute appendicitis and controls

Bacterial groups that composed more than 10% of the bacterial population in the lumen were usually found in mucosa adjacent regions and within mucosal lesions of the same sample. The

occurrence of single bacterial groups in lumen and mucosal lesions of patients with acute appendicitis and controls differed markedly, despite the polymicrobial nature of the bacterial infiltrates and the high diversity of bacteria in each patient. Invasive Fusobacteria (Fnec, Fnuc, Fuso) were the only bacteria, which were present in the mucosal lesions of patients with acute appendicitis but not in controls (Table 2). The occurrence of Fusobacteria that infiltrated the submucosa increased with the progression of the disease from catarrhal to suppurative appendicitis (Table 2). Fusobacteria were found in the mucus of only 2 of the 400 cecal biopsies and in none of the stool samples investigated from disease controls and healthy controls, and they were not invasive. One of the two patients was diagnosed with irritable bowel syndrome and the other with Crohn's disease.

Histomorphologic appearance of the bacterial infiltrates

The most frequent lesion in acute appendicitis was a needle like infiltration of single epithelial cells by a mixed bacterial population within an intact appearing epithelial layer. We called them "pinned cells" because of the marked appearance of these infiltrates (Figure 2). No such lesions were observed in repeated hybridizations of cecal biopsies in controls. The "pinned cells" contained a bacterial mix, with a significant part being Fusobacteria (Fnec, Fnuc or Fuso positive bacteria). The long filamentous form of the Fusobacteria gave the impression of bacteria needling the epithelial cell layer. The "pinned cells" were most common in acute catarrhal appendicitis. The superficial defects of single epithelial cells were less often seen in suppurative appendicitis, due to the progressive destruction of the epithelial surface, but the bacterial infiltration of the submucosa was increased (Figure 3, Table 2).

One of the appendices removed for suspected acute appendicitis and without obvious histo-pathologic signs of appendicitis had pinned cells with invasion of Fusobacteria, indicating that the preoperative clinical diagnosis was probably correct: the operation was probably performed at a time when the inflammation had not reached its climax (Table 1).

Rectal swabs

Rectal swabs from 30 patients taken prior to appendectomy contained faecal flora but no Fusobacteria.

Bacterial culture from material of resected appendices

Fusobacterium nucleatum was cultured from 3 of the 5 washed acutely inflamed appendices. The bacteria had long filaments in the FISH hybridizations that were identical to *Fusobacterium nucleatum* observed in similar cases within appendices.

Controls

Although *Fusobacterium nucleatum* could be observed in 0.5% of the cecal biopsies and 2% of the faecal cylinders from controls, their proportion never consisted of more than 1% of the total population. Fusobacteria were never invasive in any of the control biopsies (Table 2,3). The difference to patients with acute appendicitis was highly significant $p<0.001$.

DISCUSSION

We found that the presence of Fusobacteria in mucosal lesions correlates positively with the severity of the acute appendicitis when comparing spatial distribution of bacteria within 70 appendices, 400 cecal biopsies, and 400 faecal samples from healthy and inflammatory controls with a large set of fluorescence in situ hybridization probes specific for different bacterial groups. Invasive Fusobacteria were completely absent in the controls. Despite clear traits, the interpretation of appendicitis being an infectious disease remains difficult.

The aetiology of infectious diseases is usually deduced from the so called Koch's postulates: 1. the pathogen should occur in diseased but not in healthy subjects. 2. the pathogen must be isolated and 3. the pathogen should lead to disease after transfection. Koch himself never

spoke about postulates but rather investigative criteria that he continuously adopted. We should do the same.

1. In case of indigenous microbiota, the isolation of bacteria from diseased persons is of no significance. To be of importance, indigenous bacteria should be found in locations were they are normally absent, reach concentrations they never normally achieve or be accompanied by unique histomorphologic changes. In other words, there must be a clear link between a pathogen and disease.

2. The molecular genetic research of the last 50 years demonstrated that the colonic flora of each person contains about 5000 different species.[4] Most of them can not be cultured or quantitatively characterized by culture methods. In modern terms, the second postulate would be therefore not the isolation, but the identification and characterization of the pathogenic microorganism. These could be achieved by culture and phenotypical characterization, but also by PCR, DNA sequencing, FISH or any other reliable method.

3. Ethical restrictions do not allow us to fulfil the third of Koch's postulates as it was originally formulated, as transfection is unethical in human beings. Animal experiments are not always transferable to humans and increasingly difficult to justify ethically as well. The essence of the third postulate is, however, not a transfection but positive evidence of the chain of infection. This proof may come from individual transfections or it can also be performed on an epidemiologic basis (for example in the case of *Helicobacter pylori* infection).

4. A feature which was not mentioned by Koch, but implemented throughout his work, is the impact of knowledge of a specific pathogen for development of new diagnostic methods and treatment.

So is appendicitis an infectious disease? We believe that our data strongly indicate that this is the case.

1. **A link between disease and pathogen:** The infiltration of the epithelial layer by Fusobacteria in acute appendicitis had a marked appearance we termed "pinned cells". This

appearance is unknown in any other mucosal pathology. Although Fusobacteria may be indigenous in the human oral cavity, in patients with acute appendicitis Fusobacteria were found in proportions that were much higher than those observed in healthy subjects and disease controls. The Fusobacteria were found in 52% of patients with catarrhal and 70% of patients with suppurative appendicitis. The mean ± SD proportion of Fusobacteria of the total intestinal microbiota in patients with suppurative appendicitis was 24±29%. The proportion of invasive Fusobacteria reached 70-90% in 6 patients. In contrast, in 400 cecal biopsies and 400 faecal cylinders from healthy and disease controls, Fusobacteria could be detected by FISH only in 2 cecal biopsies and 8 faecal cylinders. In all these cases, the proportion of Fusobacteria was ≤ 1% and Fusobacteria were located strictly within the colonic lumen. Besides Fusobacteria, seven other faecal bacterial groups were found within epithelial or subepithelial lesions, however Fusobacteria (*Fusobacterium nucleatum* and less often *Fusobacterium necrophorum* and other Fusobacteria) were the only bacterial groups that positively correlated to the severity of appendicitis and were absent in cecal biopsies from healthy and inflammatory controls. The proportions and occurrence of bacterial groups representing the main faecal microbiota within mucosal lesions correlated inversely to the severity of appendicitis, indicating the secondary nature of their involvement.

We did not find Fusobacteria in 38% of patients with histologically proven acute appendicitis. However, since appendicitis is a clinical diagnosis, its uniformity can hardly be assumed. The absence of Fusobacteria in some cases does not contradict the causal role of Fusobacteria, but rather indicates that the symptoms of acute intestinal inflammation may be shared between different infections and diseases and that other pathogens may also be involved.

2. **Identification of pathogen:** The identification of Fusobacteria was performed with three Fusobacteria related FISH probes. Bacteria positive for *Fusobacterium necrophorum* (Fnec) and *Fusobacterium nucleatum* (Fnuc) were also positive with the group specific Fusobacteria (Fuso) probe and with the universal Eub338 probe, but were negative for the other 69 probes.

The results were reproducible. *Fusobacterium nucleatum* was the most often involved bacterium followed by *Fusobacterium necrophorum* and other Fusobacteria, which could not be identified at species level at present. We could also cultivate Fusobacteria from 3 of 5 randomly investigated acute inflamed appendices. The cultivation of Fusobacteria from inflamed appendices was also reported by other groups,[23, 24] in up to 44% in acute appendicitis. [24]

3. **Infectious properties**: The epidemiologic data indicate that acute appendicitis does occur in outbreaks and temporal clustering of geographically close cases.[25,26] We do not know how Fusobacteria spread and how it comes to the local infection, however, the infectious potential of *Fusobacterium necrophorum* in human and animal is well known.[27,28] Of the periodontal species that are statistically associated with periodontal disease, it is the most common in clinical infections of other body sites. It has been isolated from several parts of the body and from infections such as tropical skin ulcers, peritonsillar abscesses, pyomyositis and septic arthritis, bacteremia and liver abscesses, intrauterine infections, bacterial vaginosis, urinary tract infections, pericarditis and endocarditis and lung and pleuropulmonary infections.[29]

The pathogenic mechanisms of Fusobacteria are complex. Several toxins or secreted products, such as leukotoxin, endotoxin, hemolysin, hemagglutinin, proteases, porin, polyglutamate and adhesin, etc., have been implicated as virulence factors. The major virulence factor produced by *Fusobacterium necrophorum* appears to be leukotoxin, a secreted protein of high molecular weight, active specifically against leukocytes and induces signaling for apoptosis in neutrophils. *Fusobacterium nucleatum* does not express a true leukotoxin, but it can adhere to epithelial cells and invade them by exploiting the cell signalling and the cytoskeletal elements of the host cells. [29,30,31]

4. **Utility:** How can the knowledge of the infectious nature of appendicitis contribute to its treatment and diagnosis? Preliminary data demonstrate that the use of antibiotics is an

effective prophylaxis[32] in acute nonperforated appendicitis. However, the applicability of such studies to clinical practice was previously very low and the data scarce. If Fusobacteria are responsible for acute appendicitis, their detection in stool could be of diagnostic significance and would make the infectious nature of acute appendicitis even more likely. Unfortunately, our considerable efforts to develop a preoperative test for detection of Fusobacteria in stool of patients with suspected acute appendicitis failed. Because of intensive abdominal pain, patients with acute appendicitis repeatedly visit the toilet prior to hospital admission in search of relief. Because of their empty rectums, it proved impossible to obtain stool samples from patients with acute appendicitis pre-operatively for example by using digital investigation. We therefore investigated the mucus obtained by rectal swabs taken from patients preoperatively but were not able to find Fusobacteria in those samples. We do not think that it is likely due to the isolation technique, since using the same protocol, we were able to culture Fusobacteria from 3 of 5 appendices. It appears that the increase in the proportion of Fusobacteria in acute appendicitis is locally restricted and can not be seen in the rectum. We are not discouraged. Most of infectious diseases that were described at the time of Koch remained untreatable for 50 years. However, the awareness of their infectious nature led to the development of prophylactic measures and therapies, which in the end completely eradicated many of such diseases. Similarly to tracheotomy for croup due to diphtheria, which was once the only life saving measure, the resection of acute appendicitis presently has no real alternative. And like tracheotomy for the treatment of diphtheria or stomach surgery for the treatment of peptic ulcer in the past, today the removal of infectious foci of any kind is anachronistically and indicates a continuous imperfection of our present medical abilities.

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REFERENCES

1. Carr NJ. The pathology of acute appendicitis. *Ann Diag Pathol* 2000;4:46-58.
2. Roberts JP. Quantitative bacterial flora of acute appendicitis. *Arch Dis Child* 1988;63:536-40.
3. Moawad MR, Dasmohapatra S, Justin T, Keeling N. Value of intraoperative abdominal cavity culture in appendicectomy: a retrospective study. *Int J Clin Pract* 2006;60:1588-90.
4. Dethlefsen, L, Huse, S, Mitchell, L, et al. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol* 2008;November; 6(11): e280.
5. Swidsinski, A, Weber, J, Loening-Baucke, V, Hale LP, Lochs H. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol* 2005;43:3380-9.
6. Swidsinski, A, Loening-Baucke, V, Vaneechoutte, M, Doerffel Y. Active Crohn's disease and ulcerative colitis can be specifically diagnosed and monitored based on the biostructure of the fecal flora. *Inflamm Bowel Dis* 2008;14:147-61.
7. Sunde PT, Olsen I, Gobel UB, et al. Fluorescence in situ hybridization (FISH) for direct visualization of bacteria in periapical lesions of asymptomatic root-filled teeth. *Microbiology* 2003;149:1095-102.

8. Gmür R, Wyss C, Xue Y, Thurnheer T, Guggenheim B. Gingival crevice microbiota from Chinese patients with gingivitis or necrotizing ulcerative gingivitis. *Eur J Oral Sci* 2004;112, 33-41.

9. Swidsinski A. Standards for bacterial identification by fluorescence in situ hybridization within eukaryotic tissue using ribosoma rRNA-based probes. *Inflamm Bowel Dis* 2006;12:824-6.

10. Suau A, Rochet V, Sghir A, et al. Fusobacterium prausnitzii and related species represent a dominant group within the human fecal flora. *Syst Appl Microbiol* 2001;24:139-45.

11. Loy A, Maixner F, Wagner M, et al. probeBase - an online resource for rRNA-targeted oligonucleotide probes: new features 2007. *Nucleic Acids Res* 2007;35:800-4.

12. Amann R, Krumholz L, Stahl DA. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol* 1990;172:762-70.

13. Manz W, Amann R, Ludwig W, Vancanneyt M, Schleifer KH. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* 1996;142:1097-106.

14. Franks AH, Harmsen HJ, Raangs GC, Jansen GJ, Schut F, Welling GW. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* 1998;64:3336-45.

15. Bohnert J, Hübner B, Botzenhart K. Rapid identification of Enterobacteriaceae using a novel 23S rRNA-targeted oligonucleotide probe. *Int J Hyg Environ Health* 2002;203:77-82.

16. Takada T, Matsumoto K, Nomoto K. Development of multi-color FISH method for analysis of seven Bifidobacterium species in human feces. *J Microbiol Methods* 2004;58:413-21.

17. Derrien M, Collado MC, Ben-Amor K, Salminen S, de Vos WM. The mucin degrader *Akkermansia muciniphila* is an abundant resident of the human intestinal tract. *Appl Environ Microbio* 2008;74:1646-8.

18. Boye M, Jensen TK, Moller K, Leser TD, and Jorsal SE. Specific detection of the genus Serpulina, Serpulina hyodysenteriae and Serpulina pilosicoli in porcine intestines by fluorescent rRNA in situ hybridization. *Mol Cell Probes* 1998;12:323-30.

19. Harmsen HJ, Raangs GC, He T, Degener J E. and Welling GW. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. *Appl Environ Microbiol* 2002;68:2982-90.

20. Harmsen HJ, Wildeboer-Veloo AC, Grijpstra J, Knol J, Degener JE, Welling GW. Development of 16S rRNA-based probes for the Coriobacterium group and the Atopobiumcluster and their application for enumeration of Coriobacteriaceae in human feces from volunteers of different age groups. *Appl Environ Microbiol* 2000;66:4523-27.

21. Harmsen HJ, Elfferich P, Schut F, Welling GW. A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in fecal samples by fluorescent in situ hybridization. *Microbiol Ecol Health Dis* 1999;11:3-12.

22. Gmür R, Thurnheer T. Direct quantitative differentiation between Prevotella intermedia and Prevotella nigrescens in clinical samples. *Microbiology* 2002;148, 1379-87.

23. Hagelskjaer Kristensen L, Prag J. Localised Fusobacterium necrophorum infections: a prospective laboratory-based Danish study. *Eur J Microbiol Infect Dis* 2008 27:733-9.

24. Rautio M, Saxén H, Siitonen A, Nikku R, Jousimies-Somer H. Bacteriology of histopathologically defined appendicitis in children. *Infect Dis J* 2000;19:1078-83.

25. Andersson R, Hugander A, Thulin A, Nyström PO, Olaison GP. Clusters of acute appendicitis: further evidence for an infectious aetiology. *Int J Epidemiol* 1995;24:829-33.

26. Guo Y, Xiao S-Y, Yan H, Sun N-D, Jiang MS, Liu Y. Cluster of acute hemorrhagic appendicitis among high school students in Wuhan, China. *Am J Surg* 2004;188:115-21.

27. Mira A, Pushker R, Legault BA, Moreira D, Rodriguez-Valera F. Evolutionary relationships of Fusobacterium nucleatum based on phylogenetic analysis and comparative genomics. *BMC Evol Biol* 2004;4:50.

28. Bolstad AI, Jensen HB, Bakken V. Taxonomy, biology, and periodontal aspects of Fusobacterium nucleatum. *Clin Microbiol Rev* 1996;9:55-71.

29. Huggan P, Murdoch D. Fusobacterial infections: Clinical spectrum and incidence of invasive disease. *J Infect* 2008;57:283-9.

30. Brazier JS Human infections with *Fusobacterium necrophorum*. *Anaerobe* 2006;12:65-72.

31 Roberts GL. Fusobacterial infections: an underestimated threat. *Br J Biomed Sci* 2000;57:156-62.

32. Busuttil RW, Davidson RK, Fine M, Tompkins RK. Effect of prophylactic antibiotics in acute nonperforated appendicitis: a prospective, randomised, double-blind clinical study. *Ann Surg* 1981;194:502-9.

Figure legends

Figure 1.

Section of appendix from a patient with catarrhal appendicitis. The lumen of the appendix is completely filled with leukocytes (Dapi stain, large blue nuclei, photograph above). The multicolor FISH of the same microscopic field (photograph below) demonstrates mixed microbiota located between leukocytes. Long bacterial rods of *Fusobacterium nucleatum* are orange, bacteria hybridizing with universal Eub338 probe are green. Other groups are not shown.

Figure 2.

Typical examples of "pinned" epithelial cells in a patient with catarrhal appendicitis. Single epithelial cells are filed with mixed bacteria within the otherwise intact appearing epithelial layer. Bacteria pierce the epithelial layer and start to spread subepithelially. *Fusobacterium necrophorum* (the panel above) and *Fusobacterium nucleatum* (the panel below) are orange, bacteria hybridizing with universal Eub338 probe are green. Other groups are not shown.

Figure 3.

Subepithelial infiltration by *Fusobacterium necrophorum* (photograph above) and *Fusobacterium nucleatum* (photograph below) in suppurative appendicitis. Bacteria of *Fusobacterium nucleatum* appear as long filaments, *Fusobacterium necrophorum* has the shape of shorter rods.

Table 1.

Percent of some bacterial groups to the total bacterial population in material resected for acute appendicitis

Pat. Nr.	Bac 303	EREC 482	Fprau	**Fnuc**	**Fnec**	Eubac 1790	Bif 164	Muc 1437	Others	Leuko. lumen	Infiltr. of epithel	Adherence	Infiltr. of crypt	Infiltr. of Sub-epith.
Catarrhal appendicitis														
1	10	5	2	0	0	10	10	0	30% Ser1410, Veil223					+
2	45	20	10	**15**	0	1	0	0	3% Ato291	+	+	+	+	+
3	50	10	30	0	0	1	0	2		+	+	+		
4	30	5	20	**40**	0	0	2	0	2% Chis150,Lab158, Ato291	++	++	+	+	++
5	40	10	0	**40**	0	1	0	0		+	+	+		++
6	40	25	0	**20**	0	1	0	0	3% Ato291, Pnig657	++	+	+		-
7	50	5	15	**10**	0	0	5	1		++	+	+		+
8	20	45	25	0	0	0	0	10		+	+	+		
9	30	45	20	0	0	0	0	5	Ecyl1387, Lab158	+		+		+
10	20	10	3	0	0	60	0	0		+		+		-
11	30	40	10	0	0	0	12	1		+	+	+	-	+
12	10	10	1	0	**70**	0	0	0		++	+	+	-	++
13	35	50	0	0	0	10	0	0		++	+			+
14	70	20	5	0	0	0	0	1		++		+		
15	40	40	15	0	0	5	0	1			+	+	++	+
16	30	12	0	**50**	0	2	0	0		++				+
17	0	15	1	0	0	60	0	3	Lab158	++		+		+
18	20	40	0	**1**	0	10	0	0		+	+			

				5						+				
19	40	20	20	**2**	0	0	0	1	5% Ato291		++			+
20	0	0	0	0	**90**	10	0	0		++	+	+		
21	20	30	40	0	0	0	2	1	4% Ato291			+		
22	40	20	35	**2**	0	0	0	0		+	+	+		
23	50	30	5	0	0	0	2	0	Ato291		+	+	+	++
24	30	10	0	**20**	0	20	0	0	Ato291	++				++
25	20	8	10	**50**	0	10	0	0		++	+	+	+	++
Suppurative appendicitis														
26	60	15	25	0	0	0	0	3		++		+		
27	95	0	0	**3**	0	0	1	0		++	+	+		
28	100	0	0	0	0	0	0	0		++				++
29	50	0	0	**30**	0	20	0	0		++	+			+
30	30	0	0	**50**	0	10	0	0	3% Ato291	+		+		+
31	70	5	5	**20**	0	0	0	0		++	+	+		+
32	5	20	0	**50**	0	0	0	0		+	+	+		++
33	50	20	0	0	0	0	0	0	10% Ecyl1387	+		+		+
34	20	60	0	0	0	20	0	0.1		+		+		++
35	35	15	30	0	0	10	0	0		no lumen				++
36	20	1	20	0	0	0	0	0	**40% Fuso**	no lumen		+		++
37	55	25	10	0	0	1	0	0.1	5% Veil223, Lab158, Ecyl	++		+	+	++
38	35	5	5	0	0	0	30	0		no lumen				++
39	70	20	0	0	**10**	0	0	0		no lumen				++
40	40	0	0	**35**	0	0	0	0		++	+			+
41	15	5	0	**70**	0	0	0	0	Veil, Phasco, Lab158	++				++
42	10	10	0	0	0	1	0	0	**70% Fuso**, Lab158	++				+
43	15	2	30	0	0	30	2	1	3% Ecyl1387, Lab158	++	+		+	+
44	10	1	15	**70**	0	1	0	0	Veil	++	++	+		++
45	30	0	0	**70**	0	0	0	0		++				++

46	0	0	10	**90**	0	0	0	0		+					++
47	20	10	10	**50**	0	1	0	0.1	Veil	++					++
48	10	0	0	0	0	1	0	0	**90% Fuso**	no lumen					++
49	12	0	20	**50**	0	8	0	1		++	++				++
50	30	15	0	**20**	0	20	0	0		++	++	+			++
51	10	30	1	0	**50**	0	0	0		+		+			++
52	20	20	0	**50**	0	1	0	0		++	+	+			++
No appendicitis															
53	40	40	0	0	0	20	0	0							
54	35	25	25	0	0	0	0	15		+					
55	60	25	10	0	0	0	0	3		+		+			
56	20	40	20	0	0	5	0	0	Veil			+	+		
57	40	10	25	**10**	0	10	0	1		+	+	+			++
58	40	30	25	0	0	0	0	5		+		+			
59	70	20	5	0	0	0	5	1		+		+			
60	50	„25	15	0	0	0	0	10		+					
61	5	50	10	0	0	0	0	0	Ato291, Veil						
62	40	10	40	0	0	5	0	3		+					+
63	50	10	0	0	0	30	0	0				+			+
64	30	30	30	0	0	0	0	10		+					
65	50	30	15	0	0	0	0	1		+					
66	40	30	25	0	0	0	0	5	Ato291						
67	40	40	10	0	0	1	0	8		++	+	+			+
68	30	40	20	0	0	0	0	0	Chis150						
69	30	40	15	0	0	0	0	10		++					
70	60	20	10	0	0	0	0	1				+	+		

Table 2.
Occurrence of Fusobacteria (Fnuc/Fnec/Fuso) at specific locations

	No appendicitis	Catarrhal appendicitis	Suppurative appendicitis	Cecal biopsy	Faecal cylinder
All locations % Number	6% (1/18)	52%* (13/25)	70%* (19/27)	0.5% (2/400)	2% (8/400)
Lumen	5%	44%*	52%*	0.5%	2%
Infiltration of epithelial cells	5%	40%*	29%*	0	

Submucosal infiltration	0	36%*	56%*	0	

*P< 0.01 from operated patients without appendicitis and from controls

Table 3.
Mean ±SD proportion of single bacterial groups on intestinal microbiota in appendicitis and controls

Bacterial group	No appendicitis N= 18	Catarrhal appendicitis N= 25	Suppurative appendicitis N=27	Cecal biopsy N=400	Faecal cylinder N=400
Fusobacteria	<1	11±17*	24±29*	<1	<1
Bac303+Fprau +Erec482	87±11	63±30**	51±29**	90±11	70±18
Muc1437	4.0 ±4.6	1.0 ±2.1**	0.2 ±0.6**	Not determined	Not determined
Ebac	3.9 ±8	8±16	5±8	Not determined	Not determined

*P< 0.01 from operated patients without appendicitis and from controls
**P<0.001 from operated patients without appendicitis