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Increased Quinolone-Resistant Mutations of *gyrA* and *parC* Genes after Pouchitis Treatment with Ciprofloxacin

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Keywords
Pouchitis · Ulcerative colitis · Ciprofloxacin · *gyrA* · *parC*

Abstract
Background: Oral antibiotics, such as ciprofloxacin (CFX), are widely used for the treatment of acute and chronic pouchitis. Most bacterial mutations that confer quinolone resistance are at Ser-83 and Asp-87 in the *gyrA* gene and Ser-80 and Glu-84 in the *parC* gene. ***Methods:*** We obtained 51 stool samples from 43 patients who were diagnosed with ulcerative colitis and underwent ileal pouch-anal anastomosis. Patients were divided into 2 groups: 13 patients with CFX treatment of pouchitis and 30 patients without pouchitis. After extraction of fecal DNA, the amount of *Escherichia coli 16S rRNA*, *gyrA*, and *parC* gene DNA were measured using real-time polymerase chain reaction (PCR). Possible mutations at *gyrA* 83 and 87 and at *parC* 80 and 84 were investigated by PCR cloning and sequencing, and mutation rates were quantified by rapid PCR-restriction fragment length polymorphism. ***Results:*** Samples from both CFX-treated and -untreated patients had comparable levels of *gyrA* and *parC* gene DNA. Nucleic acid and amino acid mutations were identified at *gyrA* 83 and 87, and at *parC* 80 and 84. We successfully quantified mutation rates at *gyrA* 83 and 87, and at *parC* 84, all of which were significantly higher in samples from CFX-treated patients (70, 84, and 38%) than from CFX-untreated patients (13, 11, and 5%). ***Conclusion:*** *E. coli* in patient pouches may have mutations in their *gyrA* and *parC* genes that produce CFX resistance. Mutation rates of these genes were significantly higher in samples from CFX-treated patients. This study contributes to understanding the decrease and loss of CFX effectiveness against pouchitis.

Introduction

Pouchitis is an idiopathic mucosal inflammation that develops in a surgically constructed ileal pouch after total proctocolectomy for ulcerative colitis (UC) and, in rare cases, in patients with familial adenomatous polyposis (FAP). The incidence of pouchitis varies depending on how and when it is diagnosed, but it is about 20% in patients at 1 year and up to 40% in patients at 5 years after a patient's pouch starts to function [1].

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Metronidazole and ciprofloxacin (CFX) are the first choice of drugs for treatment of pouchitis and are usually effective for acute pouchitis. Treatment of chronic refractory pouchitis remains difficult and largely empirical. Meta-analysis has shown that antibiotics, in particular CFX, were able to induce remission in patients with chronic pouchitis [2].

CFX is a fluoroquinolone, which is a class of broad-spectrum antibiotics [3]. An oral dose of 500 mg CFX/day produces a CFX concentration high enough to kill bacteria, at least in the colon [4]. Quinolones inhibit the action of type II topoisomerases, DNA gyrase, and topoisomerase IV and impair bacterial DNA replication, transcription, recombination, and repair [5–7].

Four mechanisms of quinolone resistance are known: gyrase and topoisomerase gene mutations, alteration of quinolone efflux, cell membrane alterations, and plasmid-mediated resistance [8]. Of these, the major mechanism of quinolone resistance is mutations in DNA gyrase (i.e., the product of *gyrA* and *gyrB* genes) and in topoisomerase IV (i.e., the product of *parC* and *parE* genes) [7, 9]. Mutations that confer fluoroquinolone resistance are predominantly found in the quinolone resistance-determining region (QRDR) of *gyrA* and *parC*. Furthermore, most mutations in the QRDR are at *gyrA* Ser-83 and Asp-87 and at *parC* Ser-80 and Glu-84 [10, 11].

Antibiotic resistance has recently reached dangerously high levels in all parts of the world. In general, oral antibiotics affect the composition of gut microbiota and reduce its diversity in human adults [12, 13]. We believe quinolone resistance is a major public health concern and may be an unreported serious issue, specifically for patients with pouchitis treated with CFX. In the first step, we should investigate whether quinolone resistance and/or the aforementioned gene mutations are now spreading or not in fecal bacteria of patients with pouchitis. However, no information is available on *gyrA* and *parC* mutations in fecal bacteria of patients with and without CFX treatment for pouchitis.

In the present study, we investigated the presence and absence of chromosomal mutations in the QRDR of *Escherichia coli gyrA* and *parC* genes. Of the fecal bacteria, we selected *E. coli* for this study because the direct association of quinolone resistance and mutations in *E. coli* has been intensively studied. We show here that mutation rates in *gyrA* and *parC* QRDRs were significantly higher in samples from patients with a history of CFX treatment for pouchitis than in samples from patients without that history.

Table 1. Parameters of patients in this study

	CFX-untreated	CFX-treated
Patients, *n*	30	13
Male:female	18:12	7:6
Samples, *n*	30	21 (4)[a]
Active inflammation[b]		8/13[b]
Mean age, years	40.5	35.5
Range	19–66	18–61
Time after IC, years	3	4.5
Range	1–12.5	1.2–15
Duration of CFX treatment, months		13.9
Range		0.25–36
Probiotics	8/30[c]	12/21[c]

CFX, ciprofloxacin; IC, ileostomy closure. [a] Number of samples from patients who previously received CFX and were CFX-free for more than 3 months at the time of sampling. [b] Thirteen samples were obtained on the day of pouch endoscopic examination. We confirmed the presence of active inflammation in 8 samples. [c] Number of samples from patients taking probiotics/total number of samples.

Materials and Methods

Samples

We obtained 51 stool samples from 43 patients with diagnosed UC and who underwent total proctocolectomy and ileal pouch-anal (canal) anastomosis (IPAA) at Tohoku University Hospital, Japan, where a two- or three-step surgery was routine (Table 1). We collected samples from patients who had an ileostomy closure more than 1 year before sampling because pouch fecal flora change during the adaptation process and become stable after 1 year [14, 15]. As shown in Table 1, we obtained stool samples from 2 groups of patients based on whether or not a patient's history indicated CFX treatment of pouchitis. We routinely used daily doses of 400 mg CFX and 500 mg metronidazole for 2 to 4 weeks for patients with acute pouchitis because this combined therapy appeared to be most effective. A regular (400 mg/day) or low (200 mg/day or 200 mg every other day) dose of CFX was used for symptomatic patients with chronic pouchitis. Pouch endoscopy was carried out at least once for symptomatic patients, and diagnosis of pouchitis was based on the diagnostic criteria developed by the research committee on inflammatory bowel disease of the Ministry of Health, Welfare and Labor of Japan [16], which is compatible with a modified pouchitis disease activity index [17].

Fecal samples from hospital outpatients were collected at clinical visits by excretion into toilets designed for sample collection. Fecal samples were obtained after informed consent, and this study was approved by the Ethics Committee of Tohoku University, Graduate School of Medicine. The samples were frozen immediately and stored at −80°C until DNA extraction.

DNA Extraction from Fecal Samples

Stool DNA was extracted using a QIAamp DNA Stool Mini Kit (QIAGEN Co., Tokyo, Japan) according to the manufacturer's

Table 2. Primer pairs used for PCR of *Eubacteria* and *E. coli* DNA [18, 28, 29]

Target	Primer	Sequence	Reference
Eubacteria 16S rRNA	331F 797R	5′-TCCTACGGGAGGCAGCAGT-3′ 5′-GGACTACCAGGGTATCTATCCTGTT-3′	Nadkarni et al. [28]
E. coli	Eco1	5′-GACCTCGGTTTAGTTCACAGA-3′	Wang et al. [29]
16S rRNA	Eco2	5′-CACACGCTGACGCTGACCA-3′	
E. coli gyrA	*gyrA* UP *gyrA* DN	5′-GTACACCGTCGCGTACTTTA-3′ 5′-TGCGCCATGCGGACGACCGT-3′	Nakano et al. [18]
E. coli parC	*parC* UP *parC* DN	5′-TTTAAAAAATCCGCCCGTACCGT-3′ 5′-GCTGCGCCATCAGGAGAATCGC-3′	Nakano et al. [18]

protocol. The DNA concentration of each sample was estimated from its spectrophotometric absorbance at 260 nm.

Polymerase Chain Reaction Amplification, Cloning, and Sequencing of E. coli gyrA and parC DNA Fragments

DNA fragments of the *gyrA* and *parC* genes were obtained by polymerase chain reaction (PCR) using samples from CFX-treated and -untreated patients and specific primer pairs (Table 2). Each reaction mixture (12.5 μL) included 4 ng DNA, 1X buffer supplied by the manufacturer, 0.2 mm dNTP, 0.5 μm up- and downstream primers, and 0.25 unit Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., Tokyo, Japan). "Touchdown PCR" was performed using the following conditions: initial denaturation at 98°C for 30 s, 7 cycles of denaturation at 98°C for 5 s, annealing at 67–61°C for *gyrA* or 70–64°C for *parC* for 30 s, and extension at 72°C for 30 s, followed by 35 cycles of denaturation at 98°C for 5 s, annealing at 60°C for *gyrA* or 63°C for *parC* for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. A 10-μL sample of each PCR product was analyzed by agarose gel electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, visualized with a UV transilluminator, and photographed. Target DNA of expected sizes were cloned into pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen Co., Tokyo, Japan). Plasmids from bacterial clones containing the target DNA were purified using a Miniprep DNA Purification Kit (Takara Bio Inc., Kusatsu, Japan) and sequenced using a BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturers' protocol and analyzed using an ABI 3100 sequencer (Applied Biosystems Japan).

Quantification of the Eubacteria *16S rRNA Gene and* E. coli *16S rRNA,* gyrA*, and* parC *Genes*

The quantification method for the *Eubacteria* 16S rRNA gene was as previously described [15]. For the *E. coli* 16S rRNA, *gyrA*, and *parC* genes, duplicate samples of 10 ng stool DNA were used for gene quantification with the SYBR Green real-time PCR master mix (Toyobo Co., Ltd., Osaka, Japan) and the ABI 7500 real-time PCR system (Applied Biosystems Japan) according to the manufacturers' protocol. The amplification program for the *E. coli* 16S rRNA gene was one cycle at 95°C for 1 min, 40 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s. The amplification program for the *E. coli gyrA* gene was one cycle at 95°C for 1 min, 45 cycles of 95°C for 15 s, 67°C for 30 s, and 72°C for 1 min. The amplification program for the *E. coli parC* gene was one cycle at 95°C for 1 min, 45 cycles of 95°C for 15 s, and 70°C for 1 min. To establish standard curves for quantification, plasmids with the cloned genes were used with 5-fold serial dilutions. The amount of *E. coli* 16S rRNA, *gyrA*, and *parC* gene DNA was expressed relative to the respective plasmid DNA controls per microgram stool DNA. The amount of *Eubacteria* 16S rDNA was also expressed relative to the plasmid DNA control per microgram stool DNA. When *Eubacteria* 16S rRNA gene DNA was measured, the amount of *E. coli* 16S rRNA, *gyrA*, and *parC* gene DNA was expressed relative to the amount of *Eubacteria* 16S rRNA gene DNA.

Quantitative Analysis of Genetic Mutations in E. coli gyrA *and* parC *Genes*

We established a semi-quantitative method to evaluate *gyrA* and *parC* mutations based on the rapid PCR-restriction fragment length polymorphism (RFLP) method developed by Nakano, but with minor modifications [18]. In brief, 2 primer sets were designed for PCR-RFLP to detect mutations in *gyrA* residues 83 and 87 and in *parC* residues 80 and 84 by introducing an artificial restriction cleavage site into their PCR products (Table 2; Fig. 1a, b). The QRDR is known to be well-conserved among bacteria, so we designed the primers to selectively bind to the *E. coli gyrA* and *parC* genes. The *gyrA* reverse primer was designed with two mismatched nucleotides to create a *Tth*111I site (GACNNNGTC) in the *gyrA* sequence containing the codon for Asp-87 (GAC). The *parC* reverse primer was designed with two mismatched nucleotides to create an *Xmn*I site (GAANNNNTTC) in the *parC* sequence containing the codon for Glu-84 (GAA). Therefore, if the mutations in these genes are absent, restriction endonuclease digestion of the PCR products produces shorter DNA fragments in this system (Fig. 1c, d).

After PCR amplification of the *gyrA* and *parC* genes as described earlier, 2.5 μL of the *gyrA* amplicons was digested with restriction endonuclease *Hinf*I (Takara Bio Co.) or *Tth*111I (Takara Bio Co.), and 2.5 μL of the *parC* amplicons was digested with restriction endonuclease *Xmn*I (New England Biolab Japan Inc., Tokyo, Japan). In addition to the restriction endonuclease treatment, 2.5 μL of the amplicons with and without nucleotide mutations was kept on ice. To ensure the efficacy of the restriction enzyme digestion, amplicons of plasmids containing *gyrA* and *parC* gene

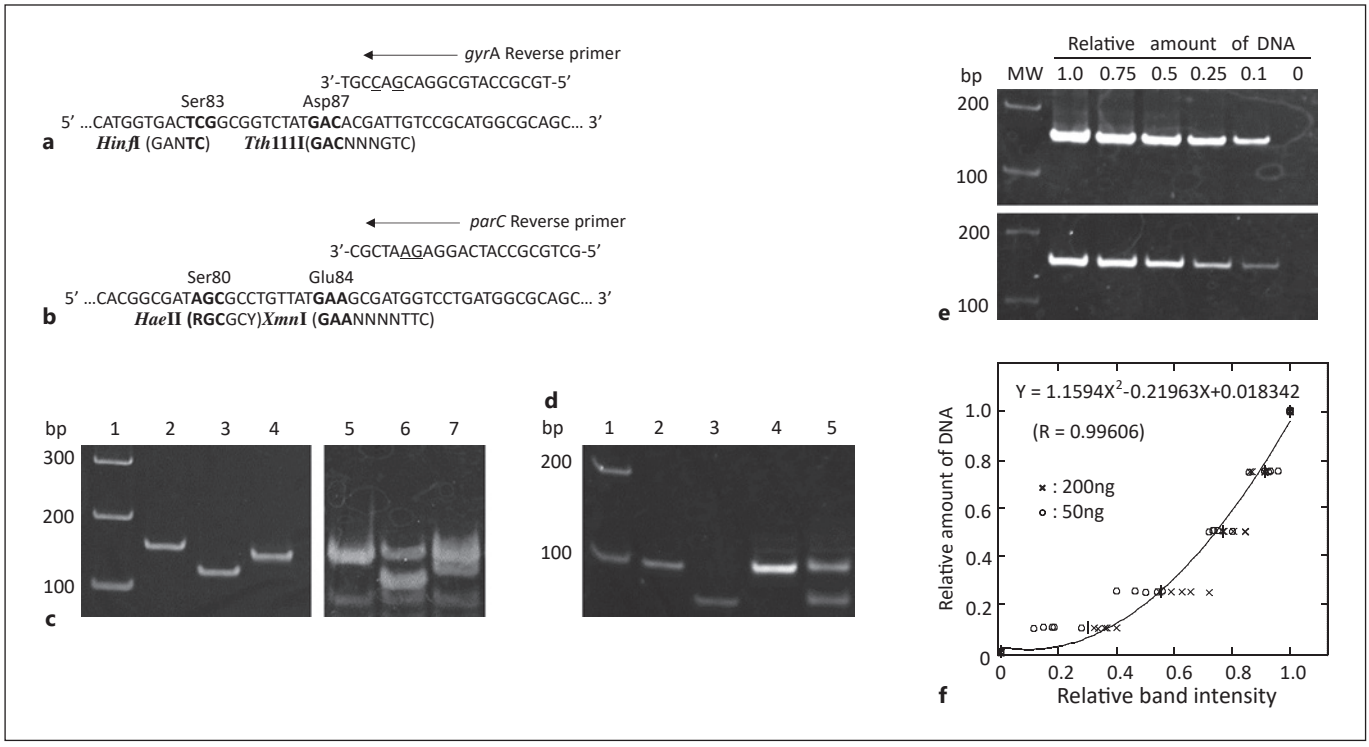

Fig. 1. Analysis of *gyrA* and *parC* amplicons. Sequences of *gyrA* and *parC* genes and reverse primers for *gyrA* (**a**) and *parC* gene amplification and their annealing sites (**b**). The codons for *gyrA* residues Ser-83 and Asp-87 and *parC* residues Ser-80 and Glu-84 are in bold. Sequences of the restriction endonuclease sites of the enzymes in this study are shown below the *gyrA* or *parC* sequences. The codon at *parC* 80 Ser was AGT in this study and not AGC, which was the sequence reported by Nakano et al. [18]. **c** PCR-RFLP analysis of the *gyrA* gene. Lane 1: Molecular weight marker. Lane 2: Amplicons of a control plasmid containing a DNA fragment of the wild-type *gyrA* gene. Lane 3: Amplicons of a control plasmid containing a DNA fragment of the wild-type *gyrA* gene and digested with *Hinf*I. Lane 4: Amplicons of a control plasmid containing a DNA fragment of the wild-type *gyrA* gene and digested with *Tth*111I. Lane 5: Sample containing amplicons of both wild-type and mutant *gyrA* genes. Lane 6: Sample containing amplicons of both wild-type and mutant *gyrA* genes and digested with *Hinf*I. Lane 7: Sample containing amplicons of both wild-type and mutant *gyrA* genes and digested with *Tth*111I. **d** PCR-RFLP analysis of the *parC* gene. Lane 1: Molecular weight marker. Lane 2: Amplicons of a control plasmid containing a DNA fragment of the wild-type *parC* gene. Lane 3: Amplicons of a control plasmid containing a DNA fragment of the wild-type *parC* gene and digested with *Xmn*I. Lane 4: Sample containing amplicons of both wild-type and mutant *parC* genes. Lane 5: Sample containing amplicons of both wild-type and mutant *parC* genes and digested with *Xmn*I. **e** Serial dilutions of 200 (upper panel) and 50 ng (lower panel) of *gyrA* PCR amplicons were analyzed by gel electrophoresis and stained with SYBR Green I, and band intensity was measured. MW, molecular weight markers. **f** Determination of the relationship between DNA band intensity and DNA amount in amplicons of *gyrA* gene. The regression curve was constructed with the parameters shown. Serial dilutions of *parC* PCR amplicons were similarly analyzed and the regression curve was constructed (data not shown).

fragments without mutations were used as reaction controls. Nakano et al. also established a protocol to detect mutations at *parC* 80 using restriction endonuclease *Hae*II, which cuts at the A(G) GCGCT(C) sequence. However, we did not carry out *Hae*II digestion in this study because the nucleotide sequence at *parC* 80 was AGT (Ser), not AGC (Ser), in the sequencing experiment, indicating that *Hae*II would not cut at this site.

PCR amplicons of the *gyrA* and *parC* genes with and without restriction endonuclease digestion were analyzed by electrophoresis on 6% polyacrylamide gels. The gels were then stained with SYBR Green I (Takara Bio Co.), visualized with UV light, and photographed. Intensities of the 150-bp *gyrA* band and the 100-bp *parC* band were measured using Image J (version 1.46). To establish the relationship between band intensity and the amount of DNA, serial dilutions of *gyrA* or *parC* amplicons were analyzed by gel electrophoresis, the band intensities were measured, and a regression curve was constructed (Fig. 1e, f). Mutation rates of the *gyrA* and *parC* genes were defined as the mutation rates of the respective amplicons because the PCR amplification efficiency of the *gyrA* and *parC* genes was essentially the same for their wild-type and mutant genes. Therefore, the mutation rates of the amplicon DNA were calculated from the ratio of the band intensity between

Table 3. Nucleotide changes in *E. coli gyr*A and *par*C genes

*gyr*A residue			*parC* residue		
83 (Ser)	87 (Asp)	clones, *n*	80 (Ser)	84 (Glu)	clones, *n*
TCG (Ser)	GAC (Asp)	13	AGT (Ser)	GAA (Glu)	16
TTG (Leu)	GAC (Asp)	18	ATC (Ile)	GAA (Glu)	7
TTG (Leu)	AAC (Asn)	2	ATT (Ile)	GTA (Val)	6
Total = 33 clones			Total = 29 clones		

undigested *gyrA* and *parC* amplicons relative to the band intensity of the *gyrA* and *parC* amplicons after *Hinf*I, *Tth*111I, or *Xmn*I digestion, as appropriate.

Statistical Analysis

Data were expressed as median and percentile values within each group. A Wilcoxon rank sum test was used to compare two groups, with significance at $p < 0.05$.

Results

We investigated the presence or absence of mutations in the QRDRs of *E. coli* isolated from IPAA patients by cloning and sequencing DNA fragments from *E. coli gyrA* and *parC* genes. All clones had 98–100% homology to *E. coli gyrA* or *parC* genes in a BLAST database search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (data not shown). Thirteen of 33 clones (39.4%) of *gyrA* PCR amplicons had the wild-type DNA sequence (i.e., *gyrA* 83 TCG [Ser] and *gyrA* 87 GAC [Asp]), which are found in *E. coli* sensitive to quinolones (Table 3). Eighteen clones had TTG (Leu) at *gyrA* 83. Two clones had mutations at both *gyrA* 83 (i.e., TTG, Leu) and 87 (i.e., AAC, Asn), which increase quinolone resistance [19]. A silent nucleic acid mutation was found at *parC* 80 (i.e., AGT [Ser]), which did not change the amino acid, in 16 of 29 clones (55.2%). The wild-type nucleic acid sequence at *parC* 80 (i.e., AGC [Ser]), reported by Nakano et al., was not found in any of the clones examined (Table 3). Seven clones had a nucleotide mutation at *parC* 80, with an amino acid change to ATC (Ile). Six clones had nucleic acid mutations, with amino acid changes at both *parC* 80 and 84 to ATT (Ile) and GTA (Val), respectively.

The amount of *E. coli* 16S rRNA, *gyrA*, and *parC* gene DNA was measured and expressed per microgram stool DNA or relative to the amount of *Eubacteria* 16S rDNA (Fig. 2). Samples from both CFX-treated and CFX-untreated patients had comparable levels of *gyrA* and *parC* gene DNA. We then compared the prevalence of mutations at *gyrA* 83 and 87 and at *parC* 84. When the 150-bp amplicons of plasmids containing wild-type *gyrA* gene fragments were digested with a restriction endonuclease, we found shorter DNA fragments: these shorter fragments were 110 bp after *Hinf*I digestion and 130 bp after *Tth*111I digestion (Fig. 1c, d). Analysis of the restriction endonuclease digestion of *gyrA* genes from patient samples often found 150 bp amplicons along with shorter fragment amplicons, suggesting that *E. coli* in patients contain both wild-type and mutant *gyrA* genes. When the 100-bp amplicons of plasmids containing wild-type *parC* gene fragments were digested with restriction endonuclease *Xmn*I, a shorter 80-bp DNA fragment was found. A similar analysis of *parC* genes from patient samples showed that the 100-bp amplicons of the *parC* genes were often found along with shorter fragment amplicons after restriction endonuclease digestion, suggesting that *E. coli* in patients also contain wild-type and mutant *parC* genes.

To determine the mutation rate of the total amplicons (i.e., of the wild-type genes plus the mutant genes), a regression curve was constructed for the relationship between DNA band intensity and the relative amount of both *gyrA* and *parC* amplicons (Fig. 1e, f). The mutation rates of the *gyrA* 83 and 87 residues and the *parC* 84 residue in samples from CFX-treated patients were significantly higher than that in samples from CFX-untreated patients (Fig. 3a–c). Since the mean value of the mutation rates in samples from CFX-untreated patients was about 20% at the 3 residues, we counted the number of residues with mutation rates over 20% at 1, 2, or 3 positions in the residues (Fig. 3d). Two-thirds of samples from CFX-treated patients showed a mutation rate of over 20% at all 3 positions in *gyrA* 83 and 87 and in *parC* 84. In addition, the mutation rate at all 3 positions was over 50% in 7 samples from 4 CFX-treated patients: none of these samples was from a CFX-untreated patient. We did not find any correlation between the amount of *E. coli* 16S rRNA gene DNA and the mutation rate of the *gyrA* or *parC* gene (data not shown).

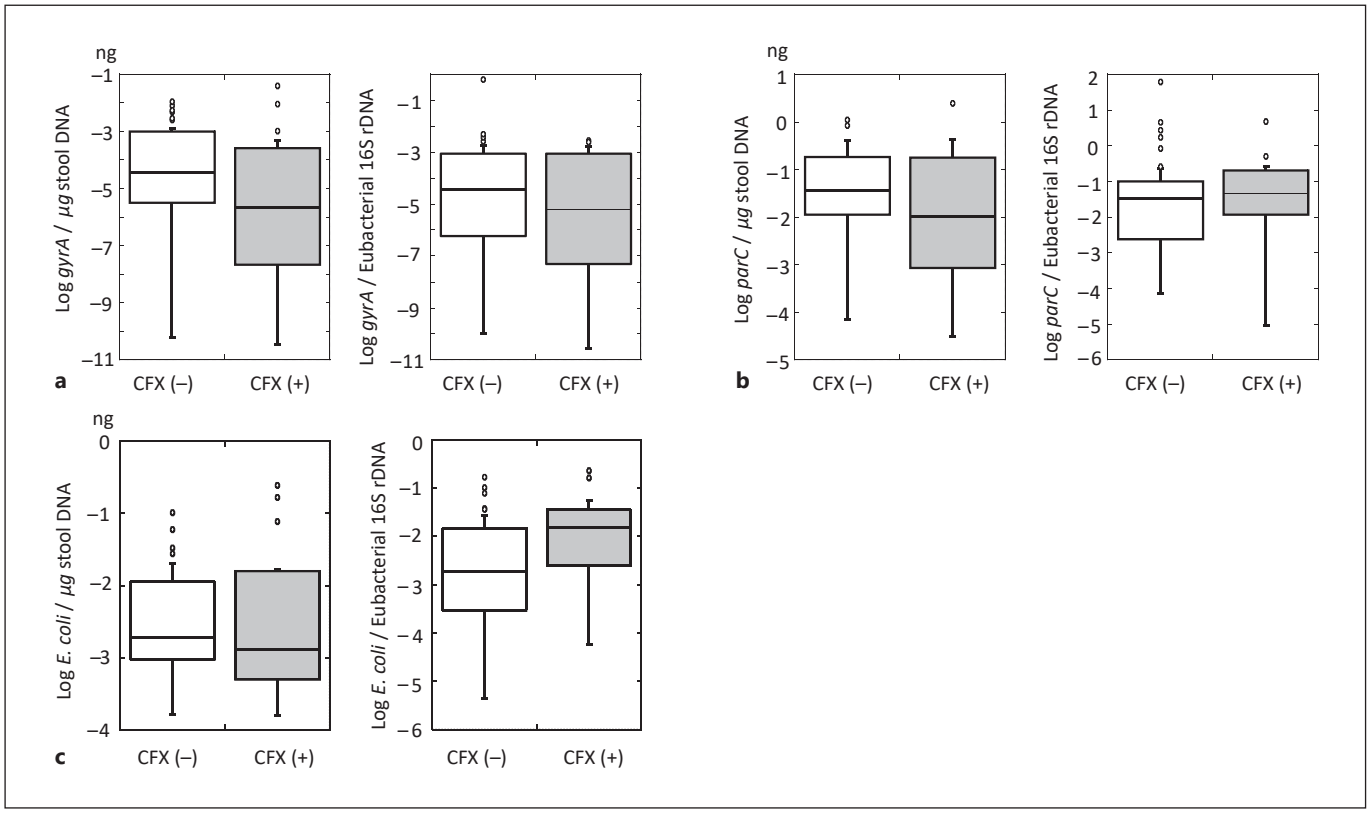

Fig. 2. Measurement of the amount of *E. coli* 16S *rRNA*, *gyrA*, and *parC* genes using samples from pouch stools of ciprofloxacin-untreated and -treated patients. Data were expressed by a box plot. **a** Amount of *E. coli gyrA* gene DNA per microgram stool DNA (*left figure*) and relative to the amount of *Eubacteria* 16S rDNA (*right figure*). **b** Amount of *E. coli parC* gene DNA per microgram stool DNA (*left figure*) and relative to the amount of *Eubacteria* 16S rDNA (*right figure*). **c** Amount of *E. coli* 16S rRNA gene DNA per microgram stool DNA (*left figure*) and relative to the amount of *Eubacteria* 16S rDNA (*right figure*). CFX (–), ciprofloxacin-untreated patients; CFX (+), ciprofloxacin-treated patients.

We also analyzed 3 paired samples from patients before and just after the start of CFX treatment (Fig. 4a–c). These data showed a decrease in the amount of *gyrA* and *parC* gene DNA, together with an increase in mutation rates at *gyrA* 83 and 87 and at *parC* 84. We quantified the mutation rate in samples from 2 patients with chronic pouchitis who continuously received a low dose of CFX (200 mg/2–4 days) for 12 or 36 months. As expected, all 3 mutation rates remained high (Fig. 4d).

Discussion

Continual use of CFX should increase the pressure for selection of quinolone-resistant bacteria, although this risk has not been well-studied. The loss of antibiotic efficacy after repeated administration of CFX is not rare, but the mechanism for this loss needs further study.

In this study, we investigated possible mutations in the QRDRs of *E. coli gyrA* and *parC* genes. The relationship between *gyrA* and *parC* mutations and quinolone resistance has been well-characterized in *E coli*, although *E. coli* may not be directly linked to the pathology of pouchitis [10, 11, 19, 20]. The acquisition of plasmid genes encoding quinolone resistance proteins (i.e., QnrA1, QnrB1, QnrS1, QnrC, and QnrD) is also known to be a factor for enhancing quinolone resistance [21, 22]. However, the plasmid *qnrA*, *qnrB*, *qnrC*, and *qnrS* genes were not detected by PCR in the fecal DNA samples examined in this study (data not shown).

Our initial experiments showed that *E. coli gyrA* and *parC* point mutations that produced amino acid changes in their gene products were found in fecal samples from IPAA patients. For *E. coli* with wild-type codons at *gyrA* Ser-83 (TCG) and Asp-87 (GAC) and at *parC* Ser-80 (AGC) and Glu-84 (GAA), the minimum inhibitory con-

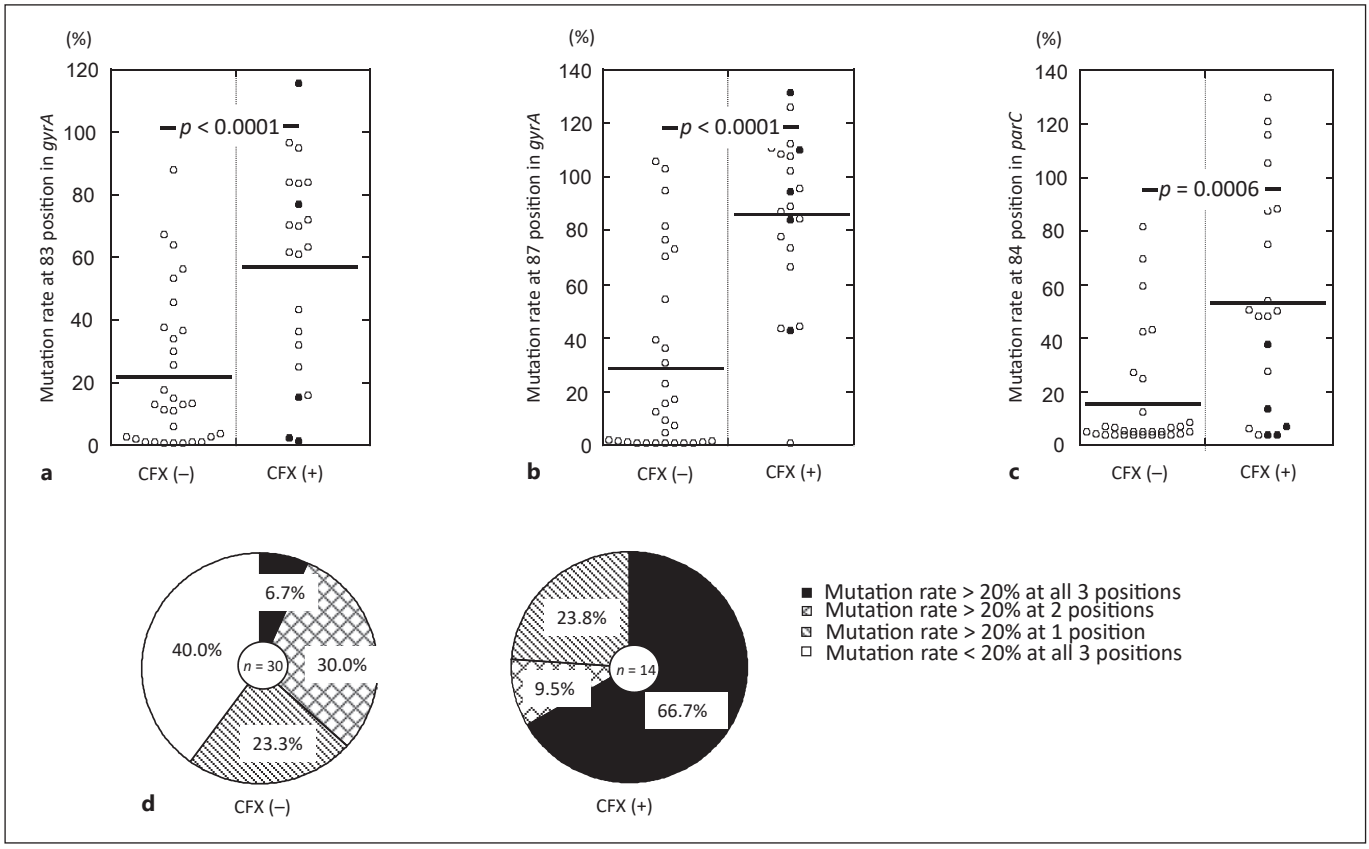

Fig. 3. Mutation rates at *gyrA* 83 (**a**), *gyrA* 87 (**b**), and *parC* 84 (**c**). Filled circles denote samples from patients who previously received ciprofloxacin and were ciprofloxacin free for more than 3 months at the time of sampling. CFX (–), ciprofloxacin-untreated patients; CFX (+), ciprofloxacin-treated patients. **d** Fraction of samples with mutation rates over 20%. Two-thirds of samples from ciprofloxacin-treated patients had mutations at all three positions with a rate over 20%. CFX (–), ciprofloxacin-untreated patients; CFX (+), ciprofloxacin-treated patients.

centration (MIC) of CFX is mostly below 0.008 μg/mL [19]. In this study, we found several nucleic acid changes leading to amino acid changes at those residues. In particular, the codons for *gyrA* 83 Leu (TTG) and 87 Asn (AAC) and for *parC* 80 Ile (ATC or ATT) and 84 Val (GTA) were also found. These four mutations dramatically increase CFX resistance to an MIC over 8 μg/mL [19]. All the mutations reported so far at *gyrA* 83 and 87 and at *parC* 84 lead to the failure of DNA cleavage by restriction endonucleases *Hinf*I, *Tth*111I, and *Xmn*I, respectively [18]. Therefore, we developed a semi-quantitative system based on the PCR-RFLP protocol of Nakano et al. [18] with minor modifications.

We measured the amount of *E. coli* 16S rRNA, *gyrA*, and *parC* gene DNA in pouch stools from CFX-treated and CFX-untreated patients. When the amount of these DNA was compared in 3 patients before and just after the start of CFX treatment, the amount of *gyrA* and *parC* gene DNA was found to be dramatically decreased by CFX treatment and the mutation rates of these genes increased. These observations strongly suggested that *E. coli* with such mutations in its *gyrA* and *parC* genes was more resistant to CFX and able to survive CFX treatment. But *E. coli* with wild-type *gyrA* and *parC* genes was sensitive to CFX, and the amount of *E. coli* 16S rRNA DNA decreased at an early stage of CFX treatment. In this study, CFX-treated patients were mainly chronic pouchitis patients who received long-term CFX treatment (13.9 months was the mean treatment time). No difference was observed in the amount of *E. coli* 16S rRNA, *gyrA*, and *parC* gene DNA from patients with and without CFX treatment. This observation suggested that during long-term CFX treatment, the *E. coli* titer may have increased or returned to levels comparable to that before CFX treatment. However, since the amounts of *E. coli* 16S rRNA, *gyrA*, and *parC* gene DNA were expressed per microgram stool

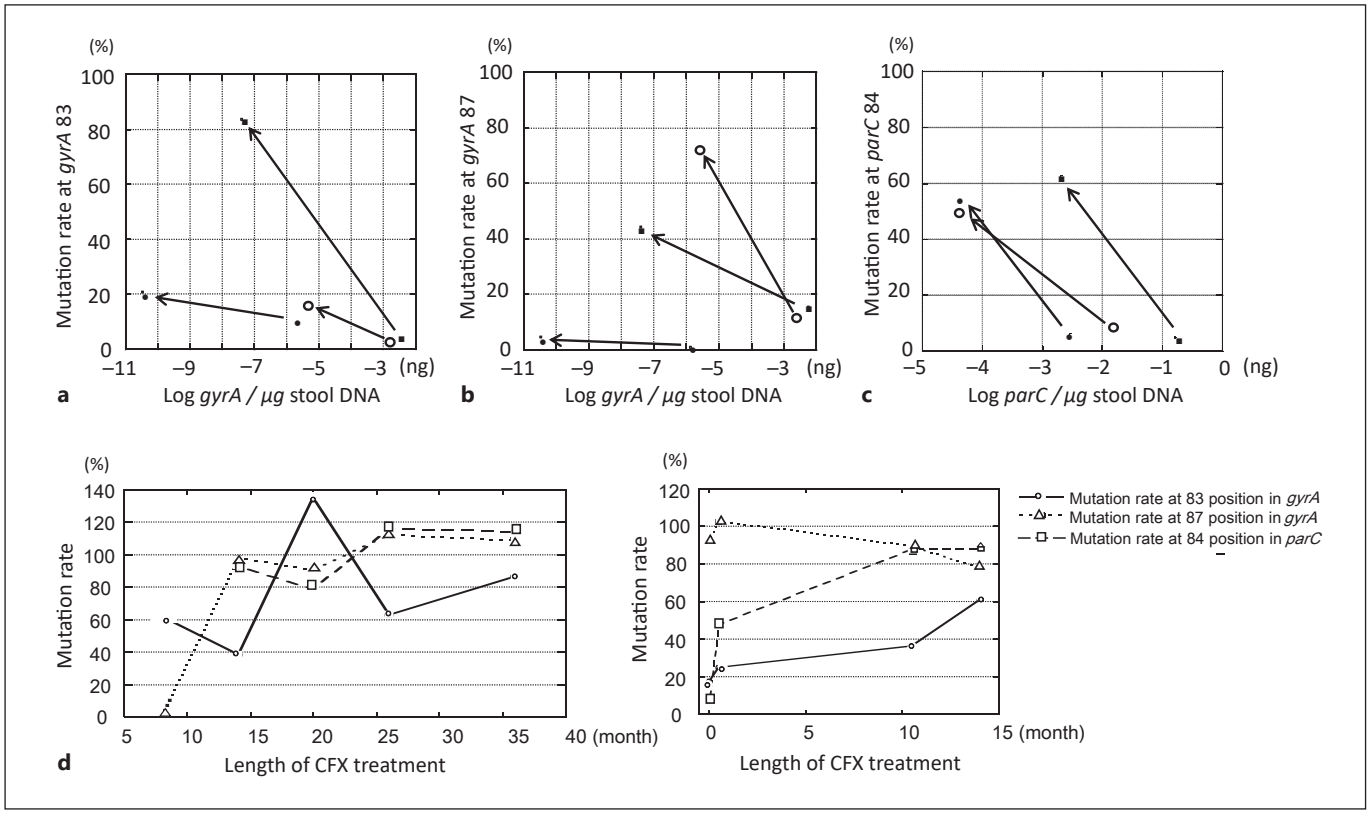

Fig. 4. Mutation rates at *gyrA* 83, *gyrA* 87, and *parC* 84 in individual cases. Mutation rates at *gyrA* 83 (**a**), *gyrA* 87 (**b**), and *parC* 84 as a function of the amount of *gyrA* and *parC* gene DNA (**c**). Each of the three different symbols (filled circles, unfilled circles, and filled squares) denotes samples from one of three different patients. For each patient, the arrows indicate the change from before to just after CFX treatment. **d** Mutation rates at *gyrA* 83, *gyrA* 87, and *parC* 84 during CFX treatment of 2 patients. Each of the three different symbols (circles, triangles, and squares) denotes mutation rates at one of the three residues.

DNA or relative to *Eubacteria* 16S rDNA, the *E. coli* titer expressed per gram stool may have decreased in the CFX-treated patients in this study.

The mechanism for the high mutation rate in samples from CFX-treated patients was not known. We speculated that each patient may have had several *E. coli* strains in their pouch. Major *E. coli* strains have wild-type *gyrA* and *parC* genes, which result in CFX sensitivity. Minor *E. coli* strains carry some *gyrA* and *parC* mutations, which can result in CFX resistance. Growth of *E. coli* strains carrying mutations in their *gyrA* and *parC* genes might gradually increase as growth of the strains with wild-type *gyrA* and *parC* genes decreases during CFX treatment. This hypothesis is consistent with the results in this study that there was a transient decrease in the amount of *gyrA* and *parC* gene DNA and an increase in their mutation rates after the start of CFX treatment. A few samples from CFX-treated patients had low mutation rates (below 5%) at *gyrA* 83, *gyrA* 87, and/or *parC* 84. In this case, *E. coli* with all three mutations may not have been in the patients' pouch. We also measured mutation rates of those residues in samples from 4 FAP patients who underwent IPAA and found similar mutation rates at *gyrA* 83 and *gyrA* 87 but not at *parC* 84 to those in samples from CFX-untreated patients (data not shown), suggesting that a further study is required.

The reason for CFX effectiveness for treating pouchitis is not well-understood. Although pouchitis presents as a heterogeneous medical problem, CFX is effective for treating patients with acute pouchitis and some patients with chronic pouchitis, in particular patients who are antibiotic dependent. It is generally believed that pouchitis results from alterations in the composition and quantity of enteric flora, which generate induction and exacerbation of the mucosal immune response in genetically susceptible hosts [23]. The mechanisms of CFX resistance due to *gyrA* and *parC* mutations in *E. coli* appear to be relevant to other species, for example *Haemophilus influ-*

enzae, *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Klebsiella pneumoniae* [11, 20]. If presently unknown bacteria specifically impair the mucosal barrier system and cause mucosal inflammation in the pouch, the pathogenesis of "chronic" or CFX-refractory pouch inflammation may be explained by the presence of CFX-resistant bacterial strains with *gyrA* and/or *parC* mutations. However, if other specific bacteria play an essential role in the maintenance of mucosal defense, the loss of CFX effectiveness may be explained by the absence of strains with mutant *gyrA* and *parC* genes.

There are some factors that were a concern in this study. The first factor is that we used samples from patients who were also treated with metronidazole together with CFX. Metronidazole is active against Gram-negative anaerobic bacteria, but its effects on *E. coli* in the human gut have not been intensively studied. Pendland et al. [24] reported that metronidazole was not active against *E. coli* in an anaerobic environment in single and polymicrobial cultures in vitro. In a study of nine healthy individuals, Krook [25] reported that 2 weeks of metronidazole treatment increased *E. coli* in fecal bacteria, but without pathological significance. We speculated that metronidazole did not have direct effects on the mutation rate of *E. coli gyrA* and *parC* genes, but may have had indirect and significant effects on the bacterial composition in a pouch. The second factor is that the history of antibiotic use in each patient in this study was not necessarily complete. Some samples from CFX-untreated patients had high mutation rates in their *E. coli gyrA* and *parC* genes. It is possible that these patients had received CFX treatment earlier in their lives. Alternatively, mutant *E. coli* strains may have been major residents in these patients' guts. Several investigators have reported that *E. coli gyrA* and *parC* mutations were detected in environmental strains (e.g. from chickens [26] and a pristine lake [27]), suggesting that there is always a risk of *E. coli* with *gyrA* and *parC* mutants being introduced into the gut. The third factor is that we did not investigate whether CFX treatment caused the loss of growth inhibition in *E. coli* with mutant *gyrA* and/or *parC* genes. As has been reported, the type and combination of mutations at *gyrA* 83 and 87 and at *parC* 80 and 84 are important for the degree of CFX resistance [10, 11, 18]. An additional study is required to investigate the relationship between the coexistence of each mutation and CFX resistance in *E. coli* strains. The forth factor is that previous or ongoing mucosal inflammation in a patient may by itself affect the bacterial composition in pouch stools, but probably not affect *E. coli gyrA* and *parC* mutations. Finally, an important question is whether or not mucosa-associated bacteria also had the *gyrA* and/or *parC* gene mutation. We quantified the mutation rates of the *gyrA* and *parC* genes using biopsies from 9 patients and compared the mutation rates between fecal and biopsy samples (see online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000504750). Positive correlations were found in all of the 3 mutation rates of *E. coli gyrA* and *parC* genes between them, indicating the possibility that mutations of quinolone-resistant genes are present similarly in both fecal and mucosa-associated bacteria.

In conclusion, *E. coli* in the pouches of pouchitis patients may have nucleic acid and amino acid mutations in the QRDRs of their *gyrA* and/or *parC* genes, which confer CFX resistance on the bacteria. The mutation rates of these genes were significantly higher in samples from CFX-treated patients. The present study contributes to understanding the mechanism by which CFX is effective, but loses its efficacy against pouchitis.

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Statement of Ethics

This study was approved by the Ethics Committee of Tohoku University, Graduate School of Medicine.

Disclosure Statement

All of the authors do not have any conflict of interest.

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Author Contributions

K.F.: Study design and data analysis; T.S.: Data analysis; A.K. and K.W.: Sample collection.

References

1 Lohmuller JL, Pemberton JH, Dozois RR, Ilstrup D, van Heerden J. Pouchitis and extraintestinal manifestations of inflammatory bowel disease after ileal pouch-anal anastomosis. Ann Surg. 1990;211:622–7.
2 Segal JP, Ding NS, Worley G, McLaughlin S, Preston S, Faiz OD, et al. Systematic review with meta-analysis: the management of chronic refractory pouchitis with an evidence-based treatment algorithm. Aliment Pharmacol Ther. 2017;45:581–92.
3 Hooper DC. Clinical applications of quinolones. Biochim Biophys Acta Biomembr. 1998;1400:45–61.
4 Rashid M, Weintraub A, Nord CE. Comparative effects of the immediate and the extended release formulations of ciprofloxacin on normal human intestinal microflora. J Chemother. 2011;23:145–9.
5 Gellert M, Mizuuchi K, O'Dea MH, Itoh T, Tomizawa JI. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc Natl Acad Sci U S A. 1977;74:4772–6.
6 Sugino A, Peebles CL, Kreuzer KN, Cozzarelli NR. Mechanism of action of nalidixic acid: purification of Escherichia coli nalA gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc Natl Acad Sci U S A. 1977;74:4767–71.
7 Khodursky AB, Zechiedrich EL, Cozzarelli NR. Topoisomerase IV is a target of quinolones in Escherichia coli. Proc Natl Acad Sci U S A. 1995;92:11801–5.
8 Guan X, Xue X, Liu Y, Wang J, Wang Y, Wang J, et al. Plasmid-mediated quinolone resistance–current knowledge and future perspectives. J Int Med Res. 2013;41:20–30.
9 Smith JT. The mode of action of 4-quinolones and possible mechanisms of resistance. J Antimicrob Chemother. 1986;18 Suppl D:21–9.
10 Vila J, Ruiz J, Goni P, De Anta MT. Detection of mutations in parC in quinolone-resistant clinical isolates of Escherichia coli. Antimicrob Agents Chemother. 1996;40:491–3.
11 Weigel LM, Steward CD, Tenover FC. gyrA mutations associated with fluoroquinolone resistance in eight species of Enterobacteriaceae. Antimicrob Agents Chemother. 1998;42:2661–7.
12 Jernberg C, Lofmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J. 2007;1:56–66.
13 Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A. 2011;108 Suppl 1:4554–61.
14 Kohyama A, Ogawa H, Funayama Y, Takahashi K, Benno Y, Nagasawa K, et al. Bacterial population moves toward a colon-like community in the pouch after total proctocolectomy. Surgery. 2009;145:435–47.
15 Hinata M, Kohyama A, Ogawa H, Haneda S, Watanabe K, Suzuki H, et al. A shift from colon- to ileum-predominant bacteria in ileal-pouch feces following total proctocolectomy. Dig Dis Sci. 2012;57(11):2965–74.
16 Fukushima K, Fujii H, Yamamura T, Sugita A, Kameoka S, Nagawa H, et al. Pouchitis atlas for objective endoscopic diagnosis. J Gastroenterol. 2007;42:799–806.
17 Shen B, Achkar JP, Connor JT, Ormsby AH, Remzi FH, Bevins CL, et al. Modified pouchitis disease activity index: a simplified approach to the diagnosis of pouchitis. Dis Colon Rectum. 2003;46:748–53.
18 Nakano R, Okamoto R, Nakano A, Nagano N, Abe M, Tansho-Nagakawa S, et al. Rapid assay for detecting gyrA and parC mutations associated with fluoroquinolone resistance in Enterobacteriaceae. J Microbiol Methods. 2013;94:213–6.
19 Aoike N, Saga T, Sakata R, Yoshizumi A, Kimura S, Iwata M, et al. Molecular characterization of extraintestinal Escherichia coli isolates in Japan: relationship between sequence types and mutation patterns of quinolone resistance-determining regions analyzed by pyrosequencing. J Clin Microbiol. 2013;51:1692–8.
20 Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol Mol Biol Rev. 1997;61:377–92.
21 Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev. 2009;22:664–89.
22 Jacoby G, Cattoir V, Hooper D, Martinez-Martinez L, Nordmann P, Pascual A, et al. qnr gene nomenclature. Antimicrob Agents Chemother. 2008;52:2297–9.
23 Shen B. Acute and chronic pouchitis–pathogenesis, diagnosis and treatment. Nat Rev Gastroenterol Hepatol. 2012;9:323–33.
24 Pendland SL, Jung R, Messick CR, Schriever CA, Patka J. In vitro bactericidal activity of piperacillin, gentamicin, and metronidazole in a mixed model containing Escherichia coli, Enterococcus faecalis, and Bacteroides fragilis. Diagn Microbiol Infect Dis. 2002;43:149–56.
25 Krook A. Effect of metronidazole and sulfasalazine on the normal human faecal flora. Scand J Gastroenterol. 1981;16:587–92.
26 Lee YJ, Cho JK, Kim KS, Tak RB, Kim AR, Kim JW, et al. Fluoroquinolone resistance and gyrA and parC mutations of Escherichia coli isolated from chicken. J Microbiol. 2005;43:391–7.
27 Johnning A, Kristiansson E, Fick J, Weijdegard B, Larsson DG. Resistance mutations in gyrA and parC are common in Escherichia communities of both fluoroquinolone-polluted and uncontaminated aquatic environments. Front Microbiol. 2015;6:1355.
28 Nadkarni MA, Martin FE, Jacques NA, Weijdegard B, Larsson DG. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology. 2002;148(Pt 1):257–66.
29 Wang RF, Cao WW, Cerniglia CE. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl Environ Microbiol. 1996;62(4):1242–7.