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Metagenomic analysis of the impact of nitrofurantoin treatment on the human faecal microbiota

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Objectives: The objective was to study changes in the faecal microbiota of patients with uncomplicated urinary tract infections (UTIs) treated with nitrofurantoin and of non-treated healthy controls using 16S rRNA analysis.

Methods: Serial stool samples were collected from patients receiving nitrofurantoin treatment at different time-points [before treatment (day 1; T1), within 48 h of end of treatment (days 5–15; T2) and 28 days after treatment (days 31–43; T3)], as well as from healthy controls. Direct DNA extraction (PowerSoil DNA Isolation Kit, MoBio Laboratories, Carlsbad, CA, USA) from stool samples was followed by pyrosequencing (454 GS FLX Titanium) of the V3–V5 region of the bacterial 16S rRNA gene.

Results: Among UTI patients, mean proportions of the Actinobacteria phylum increased by 19.6% in the first follow-up sample (T2) in comparison with the pretreatment baseline stool sample (T1) ($P=0.026$). However, proportions of Actinobacteria reversed to 'normal' pre-antibiotic levels, with a mean difference of 1.0% compared with baseline proportions, in the second follow-up sample (T3). The increase in Actinobacteria was specifically due to an increase in the Bifidobacteriaceae family (*Bifidobacterium* genus), which constituted 81.0% (95% CI ±7.4%) of this phylum.

Conclusions: No significant impact was observed other than a temporary increase in the beneficial *Bifidobacterium* genus following nitrofurantoin treatment, which supports its reintroduction into clinical use.

Keywords: 16S rDNA, compositional changes, antibiotic resistance, urinary tract infections, 16S rRNA, culture independent, stool, faecal flora, gastrointestinal flora

Introduction

Nitrofurantoin is a synthetic nitrofuran compound that has been used for decades for the effective treatment of lower uncomplicated urinary tract infections (UTIs).[1] Upon oral administration, most of the nitrofurantoin is rapidly absorbed in the small intestine and excreted by the kidneys into urine, where it reaches high therapeutic concentrations (200 μg/mL).[2] So far, clinically significant resistance in most uropathogens is uncommon.[3] However, as a small amount (6%–13%) of orally administered nitrofurantoin also reaches the colon,[1,4] it might impact the bacterial composition of the gastrointestinal tract. Nitrofurantoin-resistant strains have been detected previously in the faeces of both nitrofurantoin-treated patients and healthy volunteers, although with a very low prevalence (0.6%–2%).[5] As nitrofurantoin targets important bacterial nitroreductases,[3] this low recovery of nitrofurantoin-resistant strains might be ascribed to an associated fitness cost, especially in the presence of nitrofurantoin.[5] As part of a prospective cohort study in ambulatory care, we studied the impact of nitrofurantoin treatment on the gastrointestinal flora of patients with uncomplicated UTIs (ISRCTN 26797709). An initial screening of these samples on culture detected very low prevalences (1.6%–3.3%) of nitrofurantoin-resistant Enterobacteriaceae in the gastrointestinal flora of nitrofurantoin-treated patients both

pre- and post-treatment (A. Stewardson, N. Adriaenssens, S. Coenen, M. Godycki-Cwirko, H. Goossens, B. Huttner, A. Kowalczyk, C. Lammens, S. Malhotra-Kumar, J. Vervoort and S. Harbarth, unpublished data). Furthermore, differences in colony counts of Enterobacteriaceae (susceptible or resistant) either between UTI patients and controls or between the different timepoints of the patient and control groups were also unremarkable. The stability of the culturable gastrointestinal flora under nitrofurantoin treatment prompted us to investigate in the present study potential changes in the non-cultured fraction utilizing culture-independent screening methods.

Methods

Study design and sampling

This study was part of a prospective cohort study carried out in ambulatory patients with uncomplicated UTI visiting general practices in Antwerp (Belgium) and Lodz (Poland) between January 2010 and August 2013. Serial stool samples were collected from patients receiving nitrofurantoin treatment (100 mg three times daily for 3–15 days, $n=61$) before treatment (day 1; T1), within 48 h of end of treatment (days 5–15; T2) and 28 days after treatment (days 31–43; T3). In parallel, stool samples were also collected from non-antibiotic-treated control patients presenting either with minor trauma or for a gynaecological examination to the outpatient clinics. Of these, five nitrofurantoin-treated patients and four controls were included in the present study. Exclusion criteria for UTI patients were: treatment with systemic antibiotics within 2 months or hospitalization within 30 days; residence in a long-term care facility; current urinary catheter; and renal transplant or renal replacement therapy. The controls and household contacts received no antibiotics during the study period or 2 months before it. Approval was granted by the Medical Ethics Committee, University of Antwerp Hospital (B30020109056) and by the Bioethics Committee, Medical University of Lodz (RNN/127/10/KE). Subjects provided informed consent.

DNA extraction and 16S rDNA sequencing

We performed a 16S rDNA metagenomic analysis of 15 stool samples from five nitrofurantoin-treated patients and 12 stool samples from four controls. Total bacterial DNA was directly extracted with a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to an adapted protocol of the Human Microbiome Project.[6] From each stool sample, 2 g was homogenized with 5 mL of MoBio lysis buffer in a stomacher, followed by centrifugation for 5 min at 1500 **g**. Of the resulting supernatant, 1 mL was transferred to a MoBio Garnet Bead tube and processed according to the manufacturer's protocol. DNA concentration was measured with a NanoDrop instrument (Thermo Scientific, Waltham, MA, USA). If the DNA concentration was <10 ng/μL, an additional concentration step was included, consisting of precipitation with 0.1 volumes of 3 M sodium acetate and 2 volumes of 100% ethanol followed by a washing step with 70% ethanol. 16S rDNA V3–V5 regions were amplified by PCR using primers V345_341F (CCT ACG GGR SGC AGC AG) and V345_909R (TTT CAG YCT TGC GRC CGT AC). Purified and quantified PCR products were pooled in equimolar amounts and sequenced on a quarter plate/segment with a 454 GS FLX Titanium Sequencer (Roche, Basel, Switzerland) using Titanium FLX reagents, resulting in 3500 reads with a 400–600 bp read length on average per sample (Microsynth, Balgach, Switzerland).

Data analysis

Data were analysed using the online server MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST).[7] Raw reads were preprocessed with default parameters and searched against the rRNA database using BLAT for rRNA identification,[8] with a cluster cut-off value of 97% identity. The longest sequence of each cluster was used as a representative for a BLAT similarity search performed against the M5rna database, integrating RDP II,[9] Greengenes[10] and SILVA[11] (maximum e-value cut-off, 1×10^{5}; minimum identity cut-off, 97%; minimum alignment length cut-off, 15 bp). Mean proportions and 95% CIs were used to describe changes in proportions of the 16S rDNA reads assigned to the different phyla present in the stool samples of UTI patients and controls over the three timepoints. The raw sequence reads generated from this study were deposited in the European Nucleotide Archive with accession number PRJEB8375. The effect of nitrofurantoin use on mean proportions of the different phyla (based on 16S rDNA reads) between and within study groups for different timepoints was statistically analysed using a generalized linear mixed model in SAS (version 9.4).

Results

At baseline (T1), the three most highly represented phyla in the patient group were the Firmicutes, Verrucomicrobia and Proteobacteria, with mean proportions of 56.9% (95% CI ±21.6), 18.6% (95% CI ±31.8%) and 11.2% (95% CI ±12.1%), respectively. In the control group the three most highly represented phyla were the Firmicutes, Actinobacteria and Verrucomicrobia, with mean proportions of 46.0% (95% CI ±16.6%), 21.9% (95% CI ±15.1%) and 15.1% (95% CI ±20.8%), respectively. However, large variations in proportions of the different phyla existed between individuals, as is evident from the wide 95% CIs, regardless of study group or timepoint.

Among UTI patients, mean proportions of Actinobacteria increased by 19.6% in the first follow-up sample (T2) in comparison with the pretreatment baseline stool sample (T1) ($P=0.026$) (Table 1). However, proportions of Actinobacteria reverted to 'normal' pre-antibiotic levels, with a mean difference of 1.0% compared with baseline proportions, in the second follow-up sample (T3) (Table 1). Further analysis of the 16S rDNA reads revealed that for four of the five UTI patients the increase in the Actinobacteria phylum was specifically due to an increase in the Bifidobacteriaceae family, which constituted 81.0% (95% CI ±7.4%) of this phylum. Furthermore, within Bifidobacteriaceae, all 16S rDNA reads matched the genus *Bifidobacterium*, which are beneficial bacteria commonly used as probiotics in human medicine.[12] Besides Actinobacteria, no other remarkable changes in the faecal microbiota were noted either between or within the patient and control groups. A statistically significant change was observed in the phylum Bacteroidetes between the patient and control groups in the post-treatment T2 samples (Table 1); however, this change was also observed within the control group between the T1 and T2 samples, indicating that this might be ascribed to 'normal' variation. Furthermore, mean proportions of Bacteriodetes in the faecal microbiota at the three timepoints ranged from 1.6% to 4.6% and any variations therein were probably too minor to be of clinical relevance.

Of note, we did not utilize the 16S reads to calculate the absolute number of bacteria assigned to each phylum, but rather compared absolute numbers of 16S rDNA reads assigned to the different phyla to arrive at mean proportions of the phyla. This was because of (i) the lack of availability of sequencing data, and therefore of the 16S copy numbers, for several phyla or genera in the faecal microbiota, and (ii) the reported variations in 16S copy numbers at various taxonomic levels.[13] Furthermore, we

Table 1. Changes in mean proportions of 16S rDNA reads assigned to different phyla from baseline and between study groups

	UTI patients ($n=8$)		Controls ($n=5$)		UTI patients versus controls	
Phylum	difference in proportion (95% CI)	*P*	difference in proportion (95% CI)	*P*	difference in proportion (95% CI)	*P*
Actinobacteria						
T1	—	—	—	—	−16.5% (−35.6 to 2.5)	0.084
T2	19.6% (2.7 to 36.6)	**0.026**	−11.3% (−30.3 to 7.6)	0.221	14.4% (−4.7 to 33.4)	0.128
T3	1.0% (−15.9 to 18.0)	0.899	−14.7% (−33.6 to 4.3)	0.119	−0.8% (−19.9 to 18.2)	0.926
Bacteroidetes						
T1	—	—	—	—	0.1% (−2.2 to 2.3)	0.959
T2	0.5% (−1.3 to 2.2)	0.559	2.8% (0.9 to 4.8)	**0.008**	−2.3% (−4.5 to −0.1)	**0.044**
T3	−0.3% (−2.0 to 1.5)	0.740	1.0% (−0.9 to 3.0)	0.271	−1.3% (−3.5 to 1.0)	0.245
Firmicutes						
T1	—	—	—	—	10.9% (−19.8 to 41.6)	0.457
T2	−9.1% (−29.9 to 11.7)	0.365	2.0% (−21.3 to 25.3)	0.856	−0.2% (−30.9 to 30.5)	0.992
T3	−1.7% (−22.6 to 19.1)	0.862	8.5% (−14.8 to 31.8)	0.448	0.7% (−30.0 to 31.4)	0.960
Proteobacteria						
T1	—	—	—	—	8.2% (−1.0 to 17.3)	0.077
T2	−5.8% (−14.4 to 2.9)	0.175	−1.2% (−10.9 to 8.5)	0.795	3.6% (−5.6 to 12.8)	0.413
T3	−2.8% (−11.5 to 5.8)	0.496	0.2% (−9.5 to 9.9)	0.968	5.2% (−4.0 to 14.3)	0.247
Tenericutes						
T1	—	—	—	—	−2.1% (−5.2 to 1.0)	0.166
T2	0.2% (−2.3 to 2.7)	0.871	−1.5% (−4.1 to 1.1)	0.230	−0.4% (−3.6 to 2.8)	0.786
T3	2.4% (−0.4 to 5.1)	0.083	0.7% (−1.9 to 3.3)	0.553	−0.4% (−3.8 to 3.0)	0.801
Verrucomicrobia						
T1	—	—	—	—	0.9% (−34.2 to 36.1)	0.954
T2	−9.4% (−35.8 to 17.0)	0.447	8.8% (−13.2 to 30.8)	0.392	−17.3% (−54.2 to 19.7)	0.322
T3	4.8% (−18.9 to 28.4)	0.664	6.1% (−15.8 to 28.1)	0.548	−0.4% (−35.7 to 34.9)	0.979
Unclassified						
T1	—	—	—	—	−0.3% (−7.4 to 6.9)	0.936
T2	1.2% (−3.1 to 5.4)	0.557	0.3% (−4.5 to 5.1)	0.893	0.6% (−6.5 to 7.8)	0.856
T3	−1.2% (−5.5 to 3.1)	0.556	−1.9% (−6.6 to 2.9)	0.416	0.4% (−6.8 to 7.5)	0.909

T1, day 1; T2, days 5–15; T3, days 31–43.
Bold type indicates a statistically significant difference ($P<0.05$); generalized linear mixed model.

utilized 454 sequencing (vis-à-vis Illumina) as it has an advantage in terms of producing longer read lengths covering multiple variable regions, which allows more reliable clustering for species determination.

Discussion

Utilizing a culture-independent approach, the present study did not show any significant impact of nitrofurantoin treatment on the faecal microbiota other than a temporary increase in the Actinobacteria phylum, more specifically in the beneficial *Bifidobacterium* genus. In addition to its low impact on the bacterial composition of the gastrointestinal flora, resistance selection by nitrofurantoin was also minimal, as observed by the low recovery of nitrofurantoin-resistant Enterobacteriaceae from urine and stools of treated patients.[5] This is most likely due to the fitness costs, observed as growth deficits, associated with acquisition of mutations in the target genes conferring nitrofurantoin resistance, as shown by us recently.[5] Furthermore, a recent epidemiological study on clinical nitrofurantoin-resistant *Escherichia coli* strains noted a lack of clonal spread of such isolates in the community, reiterating the high fitness costs associated with nitrofurantoin resistance in *E. coli*.[14]

However, despite its favourable profile, nitrofurantoin was earmarked as one of the older, potentially useful, but 'forgotten' antibiotics by the ESCMID Study Group for Antibiotic Policies (ESGAP) in 2006. ESGAP's review of literature regarding reasons for disappearance of such antibiotics from clinical use revealed a combination of market failures and failures in production and regulatory processes with non-availability of narrow-spectrum antibacterials, forcing clinicians to use broad-spectrum drugs and thus adversely influencing prudent antibiotic-use policies.[15–17] Reasons for shortages and market withdrawals of older antibiotics might in turn have been related to lack of profit for drugs in limited-market areas (small countries) and increasing regulatory requirements and

bureaucracy.[16,17] However, with the current escalation in antibiotic resistance rates and a lack of new antibiotics, nitrofurantoin's unique mechanism of action, site specificity, achievement of high urinary levels and low serum concentrations, and effectiveness against both Gram-negative and Gram-positive bacteria provide many advantages in UTI therapy that many of the newer agents do not.[18] Taken together, these data support the reintroduction of this antibiotic into clinical use and also prompt the development of nitrofurantoin derivatives or even of similarly highly 'targeted' antibiotics for other infection sites.

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Transparency declarations

None to declare.

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