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Journal of Drug Design and Research

Short Communication

Metronidazole Treatment Increased Intestinal Bifidobacterial Populations: A Pilot Study in Infants with Mild Gastrointestinal Disorders

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Submitted: 08 July 2010

Accepted: 22 September 2020

Published: 25 September 2020

ISSN: 2379-089X

Keywords
- Metronidazole
- Mild gastrointestinal disorders
- Bifidobacteria
- Mucin-degraders
- Infant intestinal microbial communities

Abstract

Background: Metronidazole (MTZ), a nitroimidazole antibiotic effective against anaerobes, is commonly used with success to reduce symptoms of gastrointestinal disorders in infants, but this treatment still remains empirical. A previous MTZ administration in rats resulted in a large increase in colonic mucus layer thickness and in caecal bifidobacterial populations.

Objective: In this pilot study, the colonic microbiota of four infants suffering from mild gastrointestinal disorders was monitored before and after 10 days of exposure to MTZ.

Methods: Quantitative PCR of several bacterial groups and high-throughput sequencing of bacterial 16S rRNA genes were used to investigate the intestinal microbiota.

Results: Bifidobacterial populations significantly increased at the end of treatment. Moreover, the counts of mucin-degraders decreased as well as gas-producing species belonging to *Clostridium* genus.

Conclusion: These alterations could have health benefits that would explain the well-being of infants after MTZ treatment.

ABBREVIATIONS

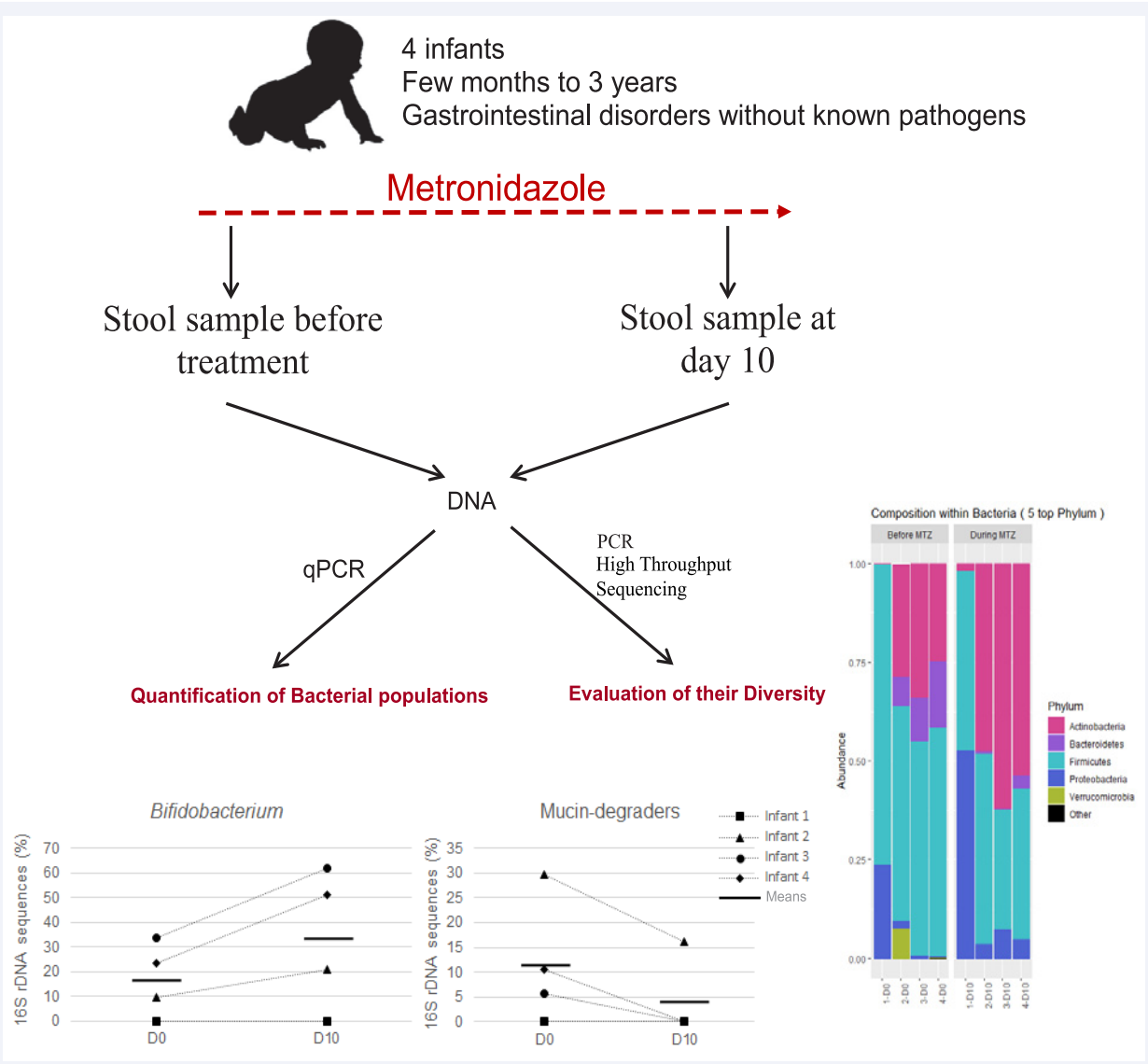
MTZ: Metronidazole

INTRODUCTION

Digestive disorders such as flatulence, abdominal distension and loose stools, are very common in infants. These are mild digestive problems that have no impact on the infant growth. The main consequence is the child pain leading to crying and therefore to the anxiety of the parents. In absence of established etiologic factors and in the hypothesis of a microbiota dysbiosis, pediatricians treated these episodes by oral courses of metronidazole (MTZ), a nitroimidazole antibiotic having specific activity against anaerobic bacteria and protozoa. However, this treatment still remains mainly empirical [1].

We previously observed that a 7-day oral administration of MTZ in rats resulted in a two-fold increase in colonic mucus layer thickness correlated with a dramatic increase in *Bifidobacterium* colonic populations [2]. More recently, we showed, using the same model, that a similar increase in mucus thickness was obtained following the oral administration of viable *Bifidobacterium* cells *(B. pseudolongum* strain Patronus). This change was associated with both a significant increase in Bifidobacterium populations and a decrease in mucus-degrading species *Akkermansia muciniphila* in the rat colonic microbiota [3].

If similar changes occur in the colonic microbiota of MTZ treated infants leading to increased protection by thickening colonic mucus layer, this could in part explain the observed improvement of the symptoms. To our knowledge, no scientific



Graphical Abstract

publication detailed, at molecular level, human colonic microbiota during MTZ treatment. The objective of this pilot study was to characterize changes in colonic microbiota of infants suffering from mild gastrointestinal disorders and treated with MTZ.

MATERIALS AND METHODS

Subjects

Four infants were included (3 boys and 1 girl), all born at term and in good health. Three of them were under 1-year-old and 1 was 2.5-year-old. They were suffering from mild gastrointestinal disorders, i.e. with abdominal pain (4/4), mild abdominal distension (4/4), gas (3/4) and diarrhea (1/4). They were treated with 20-25 mg/kg/day dose of metronidazole (MTZ) for 10 days. Fresh faecal samples were collected before (D0) and the last day of the treatment (D10) in sterile containers during routine clinical care and stored at 4°C before a rapid transfer to the laboratory where they were stored at - 20°C for less than 2 months. All the parents gave informed consent to the protocol which was approved by the ethics committee of Necker-Enfants Malades (CENEM 2019-FC-6).

Characterization of colonic microbiota

Total DNA was extracted and purified from 125 mg aliquot of faecal samples and used to quantify twelve bacterial groups using qPCR (Total bacteria, , *Bacteroides-Prevotella*, *Clostridium* cluster I (*Clostridiaceae* 1, i.e. with *Clostridium perfringens*, *C. butyricum*, ...), *enterobacteria*, *Clostridium coccoides* group (*Lachnospiraceae*), *Clostridium leptum* group (*Ruminococcaceae*)), four genera (*Bifidobacterium*, *Lactobacillus, Staphylococcus* and *Enterococcus)* and two species of interest (*Clostridium difficile* and *Akkermansia muciniphila*)) and to perform high-throughput sequencing of bacterial 16S rRNA genes as previously described [3].

Results were expressed as $\log_{10}$ 16S rRNA gene copies per gram wet weight, relative abundance (specific group/Bacteria 16S rRNA gene copy number x 100) or percentages of total

bacterial sequences (means ± standard error of the mean). The raw reads were deposited into the SRA database (https://www.ncbi.nlm.nih.gov/sra/PRJNA501836). Sequences were analyzed and normalized with the pipeline FROGS (Find Rapidly OTU with Galaxy Solution) [4].

RESULTS AND DISCUSSION

Infants

Clinical symptoms were improved for all infants at the end of the MTZ treatment.

Quantitative analysis of microbiota using qPCR (Table 1)

Total bacteria decreased after 10 days of MTZ treatment in faecal samples of 3 out of 4 infants (11 ± 0.1 $\log_{10}$ 16S copies g^{-1} before MTZ exposure and 10.6 ± 0.1 $\log_{10}$ after) and increased from 10.0 to 10.4 $\log_{10}$ gene copies g^{-1} for one infant.

In faecal samples from the youngest infant (infant 1, 1.5 month old), the *Bifidobacterium* genus was not detected, neither before, nor during the MTZ treatment.

For the three others, the proportion of bifidobacteria increased during MTZ treatment by a two-fold factor. In all infants, MTZ treatment induced significant increase in enterobacterial and enterococcal faecal populations. Conversely, proportion of four bacterial groups was decreased: *Bacteroides - Prevotella, Clostridium coccoides, Clostridium leptum* and *Clostridium* Cluster I. *Clostridium difficile* was detected in the microbiota of only one infant before treatment (0.1% of the total colonic microbiota). *A. muciniphila* was detected in the faecal microbiota of two infants and decreased during the treatment from 10% and 0.8% to 0.1% and 0% respectively.

Qualitative analysis of microbiota using high-throughput sequencing (Figure. 1)

Before treatment, all microbiota were dominated by five phyla, namely *Firmicutes* (60.5% ± 5.2%), *Actinobacteria* (21.8% ± 7.5%), *Bacteroidetes* (8.8% ± 3.5%), *Proteobacteria* (6.8% ± 5.7%), and *Verrucomicrobia* (2.0% ± 1.9%). Treatment clearly affected their ratios. After MTZ exposure, the microbiota of the infants was characterized by increased proportions of *Actinobacteria* (41.2% ± 13.5%) and *Proteobacteria* (17.3% ± 11.9%) associated with decreased proportions of of *Firmicutes* (40.6% ± 4.0%), *Bacteroidetes* (1.0% ± 0.8%) and *Verrucomicrobia* (0.0%).

At the genus level, the microbiota was notably enriched by bacteria belonging to *Bifidobacterium* (from 16.7% to 33.5%). This genus was mainly represented by three species i.e. *Bifidobacterium pseudocatenulatum*, *Bifidobacterium longum* and *Bifidobacterium breve*. *Escherichia* increased (from 6.4 to 15.0%, mostly *E. coli*) as well as *Enterococcus* (from 0.5% to 18.5%). On the contrary, there was a sharp decrease of *Akkermansia* (from 2.0% to 0%) and *Bacteroides* (from 7.6% to 0.02%, especially *Bacteroides vulgatus*, *Bacteroides thetaiotaomicron* and *Bacteroides fragilis*) and of the species *Ruminococcus torques* (from 5.8% to 4%). Metronidazole treatment led to a significant decrease in bacterial populations known as mucin-degraders populations (*A. muciniphila*, *B. vulgatus*, *B. fragilis*, *B. thetaiotaomicron* and *R. torques*) from 11.5% to 4.0%.

		Specific group/Total Bacteria 16S rRNA gene copy number x 100							
		**Infant 1		Infant 2		Infant 3		Infant 4	
Phylum	Specific group	D0*	D10*	D0	D10	D0	D10	D0	D10
	Other bacteria	79.4	16.5	32.9	51.7	21.1	24.4	38.2	48.3
Actinobacteria	*Bifidobacterium* genus	0	0	7.9	15.8	31.6	63.1	20	39.8
Proteobacteria	Enterobacteria	20	63.1	0.8	3.2	0.4	2.5	0	4
Bacteroidetes	*Bacteroides - Prevotella* genera	0	0	7.9	0.4	12.6	0	12.6	0
	Enterococcus genus	0	20	0.3	3.2	1.3	10	0	7.9
	Staphylococcus genus	0.2	0.4	0.1	0.4	0	0	0	0
	Lactobacillus genus	0	0	0	0	0	0	0	0
Firmicutes	*Clostridium coccoides* group	0	0	39.8	25.1	25.1	0	15.8	0
	Clostridium leptum group	0	0	0.2	0.1	7.9	0	12.6	0
	Clostridium Cluster I group	0.4	0	0	0	0	0	0	0
	Clostridum difficile species	0	0	0.1	0	0	0	0	0
Verrucomicrobia	*Akkermansia muciniphila* species	0	0	10	0.1	0	0	0.8	0

D0 = before MTZ*
D10 = after 10 days of MTZ treatment*

***Infant 1 = 1.5 months*
Infant 2 = 4.5 months
Infant 3 = 10 months
Infant 4 = 2,5 years

Table 1: Relative abundance of group-specific community measured by quantitative real-time PCR before and after 10d of metronidazole treatment.

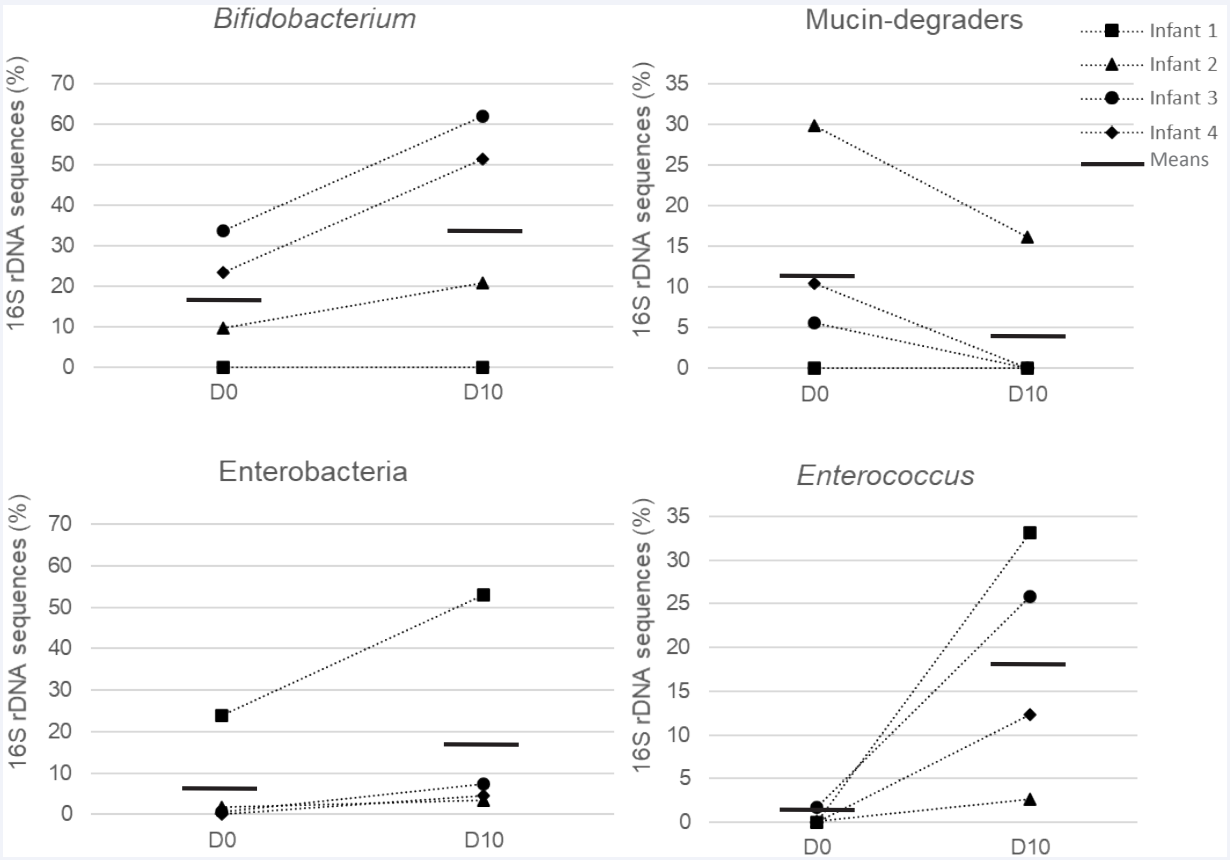


Figure 1: Impact of metronidazole treatment on several bacterial communities in faecal microbiota of infants with mild gastrointestinal disorders. The abundance is represented as percentages of sequences.

DISCUSSION

The main result obtained in this pilot study in infants with mild gastrointestinal disorders treated with MTZ was a significant increase in bifidobacterial populations associated with a decrease in mucin-degraders populations in the faecal microbiota. These results were obtained independently using two molecular techniques (qPCR and high throughput 16S rRNA gene sequencing).

These results were in agreement with experimental studies both *in vitro* [5] and *in vivo* animal models [2,3,6]. In a continuous fermentation model mimicking human colon, treatment with MTZ considerably affected the microbial composition, inducing a significant increase in bifidobacterial populations [5]. The administration of MTZ to five dogs during 14 days increased the proportions of *Bifidobacterium*, enterobacteria, enterococci and decreased the *Bacteroidetes* and Clostridiales in their faecal microbiota [6], as observed in our own study. In rats, the MTZ treatment led to an increase of bifidobacterial populations, an increase of enterococci and enterobacteria and a decrease of Clostridiales [3].

A decrease in the mucin-degrader *A. muciniphila* was observed in the present study after 10 days of MTZ treatment. Moreover, we observed in this study a decrease in *B. vulgatus*, *B. thetaiotaomicron*, *B. fragilis* and *R. torques*, four other species known to be mucin-degraders [7]. Mucin degradation could impair the protective mucin layer which lines the colon, increasing the vulnerability of the mucosa. Some studies suggested that the level of the mucin-degraders populations in microbiota could be inversely correlated with the inner mucus layer thickness [3,8], thus modulating intestinal sensitivity.

Another explanation could be that the mucus thickness is directly linked to the increased proportion of bifidobacteria *per se* rather than a decrease in mucin-degraders counts. Indeed, it has been shown in a recent study in mice that *Bifidobacterium longum* (strain NCC2705) restored colonic mucus growth in western diet fed mice [9].

Furthermore, the decrease of gas-producing bacteria such as *Clostridium* species due to MTZ treatment can also participate to the well-being of infants by decreasing flatulence.

CONCLUSION

In conclusion, MTZ likely participates in the equilibrium of the intestinal microbiota pattern by increasing bifidobacterial populations known to be beneficial for health. These results deserve further investigation to better characterize the pivotal link between the mucus layer, the colonic microbiota, particularly the mucus-degrading communities, and the mild gastrointestinal disorders in infants.

ACKNOWLEDGEMENTS

This study did not receive any specific funding.

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Cite this article

Mangin I, Pereira E, Suau A, Butel MJ, Pochart P, et al. (202)0 Metronidazole Treatment Increased Intestinal Bifidobacterial Populations: A Pilot Study in Infants with Mild Gastrointestinal Disorders. J Drug Des Res 7(1): 1075.