Title: A Targeted Metabolomics Analysis Identifies Intestinal Microbiota-Derived Urinary Biomarkers of Colonization Resistance in Antibiotic-Treated Mice

Authors: Mark E. Obrenovich\textsuperscript{1,2,3}, MaryAnn Tima\textsuperscript{1}, Alex Polinkovsky\textsuperscript{1}, Renliang Zhang\textsuperscript{4}, Steven N. Emancipator\textsuperscript{2,5} and Curtis J. Donskey\textsuperscript{1,6}

Affiliations:
\textsuperscript{1}Research Service, Cleveland Veterans Affairs Medical Center, Cleveland, Ohio, United States of America
\textsuperscript{2}Pathology and Laboratory Medicine Service (PALMS), Cleveland Veterans Affairs Medical Center, Cleveland, Ohio, United States of America
\textsuperscript{3}Cleveland State University, Department of Chemistry, Cleveland, Ohio, United States of America
\textsuperscript{4}Mass Spectrometry II Core Lab, Cleveland Clinic Cleveland Clinic Foundation, Cleveland, Ohio, United States of America
\textsuperscript{5}Case Western Reserve University, Department of Pathology, Cleveland, Ohio, United States of America
\textsuperscript{6}Geriatric Research Education and Clinical Center, Cleveland Veterans Affairs Medical Center, Cleveland, Ohio, United States of America

*Corresponding author: Curtis J Donskey, Cleveland Veterans Affairs Medical Center, 10701 East Boulevard, Cleveland, Ohio 44106; Telephone: (216)-791-3800 ext 4788; Fax: (216)-707-5973; email: curtisd123@yahoo.com
Abstract

Antibiotics excreted into the intestinal tract may disrupt the microbiota that provide colonization resistance against enteric pathogens and alter normal metabolic functions of the microbiota. Many of the bacterial metabolites produced in the intestinal tract are absorbed systemically and excreted in urine. Here, we used a mouse model to test the hypothesis that alterations in levels of targeted bacterial metabolites in urine specimens could provide useful biomarkers indicating disrupted or intact colonization resistance. To assess in vivo colonization resistance, mice were challenged with oral *Clostridium difficile* spores at 3, 6, and 11 days after completion of 2 days of treatment with piperacillin/tazobactam, aztreonam, or saline. For concurrent groups of antibiotic-treated mice, urine samples were analyzed using liquid chromatography mass spectrometry (LC-MS/MS) to quantify 11 compounds targeted as potential biomarkers of colonization resistance. Aztreonam did not affect colonization resistance, whereas piperacillin/tazobactam disrupted colonization resistance 3 days after piperacillin/tazobactam treatment with complete recovery by 11 days after treatment. Three of the 11 compounds exhibited a statistically significant and greater than 10-fold increase (tryptophan metabolite N-acetyltryptophan) or decrease (plant polyphenyl derivatives cinnamoylglycine and enterodiol) in urinary concentration 3 days after piperacillin/tazobactam treatment followed by recovery to baseline that coincided with restoration of in vivo colonization resistance. These urinary metabolites could provide useful and easily accessible biomarkers indicating intact or disrupted colonization resistance during and after antibiotic treatment.
The gastrointestinal tract of adult mammals is inhabited by a complex microbial community that includes hundreds of bacterial species (1). These organisms complement host physiology by providing an array of metabolic functions that benefit the host (e.g., digestion of complex polysaccharides and proteins) (1-4). The indigenous microbiota of the colon also provide an important host defense, termed colonization resistance, by inhibiting growth of potentially pathogenic microorganisms such as Clostridium difficile (5-6). Antibiotics that are excreted into the intestinal tract may suppress the microbiota and disrupt bacterial metabolic functions and colonization resistance (5-9). In mice, we demonstrated that clindamycin or piperacillin/tazobactam treatment resulted in disruption of colonization resistance and marked changes in bacterial metabolites present in fecal specimens based on non-targeted metabolic profiling (9). Of 484 compounds analyzed, 146 (30%) exhibited a significant increase or decrease in concentration during antibiotic treatment followed by recovery to baseline that coincided with restoration of in vivo colonization resistance. Identified as potential biomarkers of colonization resistance, these compounds included intermediates in carbohydrate or protein metabolism that increased (pentitols, gamma-glutamyl amino acids and inositol metabolites) or decreased (pentoses, dipeptides) with antibiotic treatment.

Although measurement of bacterial metabolites in stool could provide useful biomarkers of colonization resistance, collection and processing of stool specimens can be challenging. In healthcare settings, urine or blood specimens are much more frequently used for diagnostic testing due to ease of collection and the ability to collect specimens in a timely manner. Because many metabolites produced by intestinal bacteria are absorbed with subsequent excretion in urine (10-14), we hypothesized that urine...
metabolites might provide useful indicators of intact or disrupted colonization resistance.

To test this hypothesis, we used a mouse model to determine if the timing of recovery of
in vivo colonization resistance to C. difficile after antibiotic treatment correlated with
changes in concentrations of selected metabolites of intestinal bacteria in urine.

RESULTS

Restoration of in vivo colonization resistance after antibiotic treatment. Figure 1
shows the results of the assessment of in vivo colonization resistance to C. difficile.
Aztreonam-treated mice maintained intact colonization resistance with no increase in the
density of C. difficile colonization in comparison to saline controls (P>0.92).
Piperacillin/tazobactam-treated mice had altered colonization resistance when challenged
with C. difficile 3 days after the final antibiotic dose with high-density colonization in
comparison to the controls and aztreonam-treated mice (P<0.001); at 6 days after the
final antibiotic dose, there was a non-significant trend toward increased density of
colonization in the piperacillin/tazobactam versus control and aztreonam-treated mice
(P=0.07). At day 11 after the final dose, piperacillin/tazobactam-treated mice
demonstrated intact colonization resistance.

Identification of urinary biomarkers of colonization resistance. Figure 2
shows the effect of antibiotic treatment on the concentrations of the 11 urinary
compounds studied. Five of the 11 (45%) compounds differed significantly between the
piperacillin/tazobactam-treated and control or aztreonam-treated mice, including N-acetyl
tryptophan, indole-3 propionic acid, cinnamoylglycine, enterodiol, and 3,4-
dihydroxyphenylacetic acid. Three compounds, including N-acetyl tryptophan,
cinnamoylglycine, and enterodiol were identified as potential biomarkers of colonization resistance because they exhibited a 10-fold difference between piperacillin/tazobactam and saline- or aztreonam-treated mice on day 3 after treatment with normalization by day 11 after treatment in conjunction with recovery of colonization resistance. The mean concentration of N-acetyltryptophan was greater than 10-fold higher in urine of piperacillin/tazobactam-treated mice than in saline controls or aztreonam-treated mice on day 3 after antibiotic treatment; 7 of 8 piperacillin/tazobactam-treated mice had increased levels of N-acetyltryptophan in comparison to the pre-treatment levels. The concentrations of cinnamoylglycine and enterodiol were greater than 100-fold lower in urine of all of the piperacillin/tazobactam-treated mice in comparison to the saline controls or aztreonam-treated mice on day 3 after antibiotic treatment.

**DISCUSSION**

Our findings are consistent with previous studies in demonstrating that piperacillin/tazobactam treatment causes disruption of colonization resistance, whereas aztreonam, an agent lacking activity against anaerobes, does not.\(^7\)\(^-\)\(^9\) The rapid restoration of colonization resistance against *C. difficile* by 6 to 11 days after treatment highlights the resilience of the microbiota. By correlating the timing of functional recovery of colonization resistance with changes in concentrations of selected urine metabolites, we identified 3 potential urinary biomarkers of colonization resistance (N-acetyl-tryptophan, cinnamoylglycine, and enterodiol).

The biomarkers identified are biologically plausible and our findings for these compounds are consistent with previous studies in germ-free mice and in antibiotic-
treated mice or humans.\textsuperscript{4,9,15-20} Cinnamoylglycine is a glycine conjugate of cinnamic acid that has previously been shown to be abundant in the serum of conventional mice, but present in minimal concentrations in the serum of germ-free mice.\textsuperscript{4} Cinnamic acid is present in a variety of foods or can be produced from phenylalanine metabolism, and cinnamoylglycine has been shown to be excreted in the urine of mice and humans.\textsuperscript{16-17}

Dietary tryptophan is metabolized to indole in the colon by tryptophanase produced by enteric bacteria, with subsequent conversion by other bacterial enzymes to indole-3-propionic acid.\textsuperscript{4} Our finding of increased N-acetyl-tryptophan and decreased indole-3-propionic acid in urine of piperacillin/tazobactam-treated mice is consistent with prior data from stool of antibiotic-treated mice and from serum of germ-free mice.\textsuperscript{4,9,15} Finally, dietary lignans are plant-derived polyphenols that are metabolized by anaerobic intestinal bacteria to produce the enterolignans enterodiol and enterolactone.\textsuperscript{18-19} Oral antibiotic therapy has been associated with reduced serum enterolactone concentrations in humans.\textsuperscript{20} It is not clear why enterodiol, but not enterolactone, was significantly suppressed by piperacillin/tazobactam treatment in our study.

Our findings have important clinical implications. There is a need for biomarkers that can be used to monitor whether colonization resistance is intact. The finding that urinary biomarkers may potentially be useful to monitor the functional status of the gut microbiota is significant because collection of urine samples is relatively easy compared to collection of stool specimens and samples may be collected in a timely manner. Although we only identified 3 candidate biomarkers of colonization resistance, we anticipate that it may be feasible to identify many additional urinary biomarkers if larger scale non-targeted and targeted studies are conducted. Previous studies have
demonstrated antibiotic-induced changes in several other urinary metabolites (e.g., urobilin, short-chain fatty acids, and trimethylamine-N-oxide). The availability of urine biomarkers of colonization resistance could be useful for a wide range of studies examining the impact of antibiotic treatment on the intestinal microbiota.

Our study has several limitations. First, we did not examine changes in the intestinal microbiota that correlated with recovery of colonization resistance. However, we previously demonstrated that recovery of colonization resistance after piperacillin/tazobactam treatment in mice correlates with recovery of bacteria from the families Lachnospiraceae and Ruminococcaceae (phylum Firmicutes, order Clostridiales) in stool specimens. Organisms from the phylum Firmicutes have been associated with colonization resistance against C. difficile. Second, additional studies are needed to determine which bacterial species are responsible for production of specific metabolites. Given the degree of functional redundancy of the intestinal microbiota, it is possible that multiple families of bacteria may be able to carry out the metabolic conversions required to produce the metabolites identified here. Third, findings in mice may differ from findings in humans given differences in diet and microbiota. Finally, we cannot exclude the possibility that metabolites that differed significantly between piperacillin/tazobactam- and saline-treated mice on day 3 after treatment (i.e., indole-3 propionic acid and 3,4-dihydroxyphenylacetic acid) without achieving a 10-fold difference could be useful as biomarkers. However, these compounds exhibited substantial day-to-day variation in concentrations in the saline control group which would make them potentially less valuable as biomarkers of colonization resistance.
In summary, using a targeted approach we identified N-acetyltryptophan, cinnamoylglycine, and enterodiol as potential urinary biomarkers of colonization resistance. Such compounds could provide useful and easily accessible biomarkers indicating intact or disrupted colonization resistance during and after antibiotic treatment. Studies are needed to determine if antibiotic treatment in humans results in alteration of these urinary metabolites. Ultimately, studies will be needed to identify or design compounds metabolized by the intestinal microbiota that provide colonization resistance that would provide a unique urine or serum indicator of colonization resistance.

MATERIALS AND METHODS

*C. difficile* strain. VA17 is an epidemic North American pulsed-field gel electrophoresis type 1 (NAP1) *C. difficile* strain. For VA17, the minimum inhibitory concentrations (MICs) of piperacillin/tazobactam and aztreonam are 2 µg/mL, and >256 µg/mL, respectively (8). *C. difficile* spores were prepared as previously described (23).

*In vivo mouse model of colonization resistance.* We used a mouse model described previously to evaluate recovery of colonization resistance after antibacterial treatment (9). Female CF-1 mice weighing 25 to 30 g (Harlan Sprague-Dawley, Indianapolis, IN) were housed in individual micro-isolator cages and fed sterilized Teklad Global 18% Protein Extruded Rodent Diet (Harlan Teklad, Madison, WI). Mice (32 per group) received daily subcutaneous injections (0.1-mL total volume) of saline, piperacillin/tazobactam (8 mg/day), or aztreonam (3 mg/day) for 2 days. The antibiotic
dose was equal to the usual human doses administered over a 24-hour period (milligrams of antibiotic per gram of body weight).

To assess in vivo colonization resistance, subgroups of the antibiotic-treated mice were challenged with $10^4$ colony-forming units (CFU) of C. difficile VA17 spores by orogastric gavage either before or 3, 6, or 11 days after completion of antibiotic treatment (8 mice from each treatment group were challenged at each time point). Fresh stool specimens were collected 2 days after gavage and the concentration of C. difficile was measured by plating serially diluted samples on selective agar as previously described (9). Colonization resistance was deemed intact if there was no significant increase in concentrations of C. difficile in the stool of antibiotic-treated mice in comparison to the control mice.

**Urine specimen collection and processing.** For the group of mice challenged with C. difficile on day 11 (8 mice), fresh urine specimens were collected in sterile eppendorf tubes before antibiotic treatment and on days 3, 6, and 11 after antibiotic treatment. For urine collection, individual mice were moved into clean cages with no bedding for 1 hour periods and observed continuously for production of urine or stool. Urine specimens were collected with a pipette tip immediately after they were produced. Urine was collected for analysis only if there was no contact with stool pellets. Mice were moved into new clean cages as needed to avoid stool contamination of urine. Urine specimens were centrifuged at 13,000 g for 15 min at 8°C. To reduce glucuronides and sulfates in urine that can interfere with analyses, urine supernatants were digested with beta-glucuronidase and aryl sulfatase for 24 hours according to manufacturer’s recommendation (Roche Diagnostics GmBH, Mahnheim, Germany). Digested urine
supernatants were frozen at -80ºC prior to analysis at the Cleveland Clinic Small Molecule Mass Spectrometry Core Facility.

**Liquid chromatography (LC)-mass spectrometry (MS)/MS (LC/MS/MS) analysis.** Targeted metabolic profiling by LC/MS/MS was used to quantify 11 urinary metabolites that were hypothesized to be associated with disrupted versus intact colonization resistance. The 11 metabolites included tryptophan metabolites (N-acetyl tryptophan, indole-3 propionic acid, and indoxyl sulfate), plant polyphenyl derivatives (cinnamoylglycine, hippuric acid, enterolactone, enterodiol, 3,4-dihydroxyphenylacetic acid, and phenylpropionylglycine), DL-tyrosine, and taurine. These metabolites were chosen because they were identified as potential biomarkers of colonization resistance in our recent non-targeted study of mouse fecal specimens or in previous studies by others (4,9-11,13,15).

Twenty μL aliquots of urine supernatant were injected onto a Waters 2690 high-performance liquid chromatography (HPLC) system (Quattro Ultima Micromass, Beverly, MA) and the metabolites were separated using a C18 column (Phenomenex, Rancho Palos Verdes, CA) under gradient conditions at a flow rate of 0.2 mL/min. A gradient was formed by mixing mobile phase A (water containing 5mM ammonium acetate) and B (acetonitrile containing 0.2% acetic acid and 5 ammonium acetate) as follows: isocratic elution with 100% A from 0 to 2 min, increasing to 100% B from 2 to 8, kept at 100% B for 10 min and then equilibrated for 10 min with 100% A.

The HPLC column effluent was introduced onto an Electrospray Ionization Triple Quadrupole Mass Spectrometer (Waters Corporation, Milford, MA) and analyzed using negative electrospray ionization in the multiple reaction monitoring mode. The multiple
reaction monitoring precursor ion and product ion transitions are shown in supplemental Table 1. Internal standard calibration curves were used to quantify the metabolites in urine. Urine metabolite concentrations were normalized to urine creatinine and expressed as nM/mM creatinine. Deuterated internal standards (100 ng each) detected in negative mode were used for hippuric acid-d5 (Toronto Research Chemicals, North York, Canada) and indoxyl sulfate-d4 (Sigma-Aldrich).

**Data analysis.** Analysis of variance (ANOVA) was used to compare concentrations of *C. difficile* recovered from stool for control versus antibiotic-treated mice. For the LC/MS/MS analyses, data processing was done using MassLynx software v4.1 from Waters. For the metabolite analysis, a two-way ANOVA was performed to assess differences in log-transformed concentrations across days and antibiotic treatments to identify compounds that differed significantly between control and antibiotic groups at specific points in time. Contrast tests performed to compare antibiotic groups on specific days. Significance was assessed at alpha = 0.05. As these steps were hypothesis generating, no adjustments were made for multiple comparisons. Compounds were assessed as possible biomarkers of colonization resistance if there was a significant difference in concentration between the piperacillin/tazobactam-treated and control mice on day 3 after piperacillin/tazobactam treatment but not at baseline or day 11; this choice of was based on the finding that colonization resistance was disrupted on day 3 after piperacillin/tazobactam treatment but not at baseline or on day 11 after treatment. For the compounds that differed significantly from saline controls, those that exhibited a 10-fold difference between the piperacillin/tazobactam-treated and control mice on day 3 but not at baseline or on day 11 were identified as potential biomarkers of colonization.
resistance; a 10-fold difference was chosen in order to identify potential biomarkers with marked differences that were robust to log transformation. Concentrations of the metabolites were analyzed for aztreonam-treated mice to determine if there were differences from saline-treated mice, but metabolites with differences among these groups were not considered potential biomarkers of colonization resistance because aztreonam did not disrupt colonization resistance.

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Competing Interests: The authors have declared that no competing interests exist.

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References


14 microbiota-derived biomarkers of colonization resistance in clindamycin-treated mice.


Figure legends

**FIG 1.** Effect of antibiotic treatment on *in vivo* colonization resistance to *Clostridium difficile*. Mice received subcutaneous antibiotic treatment for 2 days and subgroups (8 per group) were challenged by gastric gavage with 10,000 colony-forming units (CFU) of *C. difficile* spores on before treatment or on days 3, 6, and 11 after antibiotic treatment. SE, standard error. *, P<0.001.

**FIG. 2.** Effect of antibiotic treatment on the concentrations of the 11 urinary compounds. Compounds that differed significantly between the piperacillin/tazobactam-treated and control mice included N-acetyl tryptophan, indole-3 propionic acid, cinnamoylglycine, enterodiol, and 3,4-dihydroxyphenylacetic acid. N-acetyl tryptophan, cinnamoylglycine, and enterodiol were identified as potential biomarkers of colonization resistance because they exhibited a 10-fold difference between piperacillin/tazobactam and saline-treated mice on day 3 after treatment with normalization by day 11 in conjunction with recovery of colonization resistance. SE, standard error. *, P<0.05; **, P<0.001.
FIG. 1

![Graph showing the change in bacterial load over time after antibiotic treatment. The x-axis represents days after antibiotic treatment (Pre-treatment, 3, 6, 11 days), and the y-axis represents the log_{10} colony-forming units/gm stool. The graph compares Saline, Piperacillin/tazobactam, and Aztreonam treatments.]
FIG. 2

A. Cinnamoylglycine

Mean+/SE nM/mM creatinine

Pre-treatment 3 6 11
Days after antibiotic treatment

A.ztreonam  Piperacillin/Tazobactam  Saline
I. Indoxyl sulfate

Mean+/SE nM/mM creatinine

Days after antibiotic treatment

Pre-treatment 3 6 11

- Aztreonam
- Piperacillin/Tazobactam
- Saline