qPCR-BASED ANALYSES OF INTESTINAL MICROBIOTA AFTER ORAL ANTIBIOTIC TREATMENT OF MICE

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KEYWORDS:
Intestinal microbiota, antibiotics, 16S rRNA, faecal bacteria, qPCR, faecal sample collection

SUMMARY:
Here we provide detailed protocols for the oral administration of antibiotics to mice, collection of faecal samples, DNA extraction and quantification of faecal bacteria by qPCR.

ABSTRACT:
The gut microbiota has a central influence on human health. Microbial dysbiosis is associated with many common immunopathologies such as inflammatory bowel disease, asthma or arthritis. Thus, understanding the mechanisms underlying microbiota-immune system crosstalk is of crucial importance. Antibiotic administration, while aiding pathogen clearance, also induces drastic changes in the size and composition of intestinal bacterial communities which can have an impact on human health. Antibiotic treatment in mice recapitulates the impact and long-term changes in human microbiota from antibiotic treated patients, and enables investigation of the mechanistic links between changes in microbial communities and immune cell function. While several methods for antibiotic treatment of mice have been described, some of them induce severe dehydration and weight-loss complicating the interpretation of the data. Here, we provide two protocols for oral antibiotic administration which can be used for long-term treatment of mice without inducing major weight-loss. These protocols make use of a combination of antibiotics that target both Gram-positive and Gram-negative bacteria and can be provided either \textit{ad libitum} in the drinking water or by oral gavage. Moreover, we describe a method for the quantification of microbial density in faecal samples by qPCR which can be used to validate the efficacy of antibiotic treatment. The combination of these
approaches provides a reliable methodology for the manipulation of the intestinal microbiota and the study of the effects of antibiotic treatment in mice.

INTRODUCTION:
The mammalian gastrointestinal mucosa is a unique environment colonized by a highly complex mixture of microorganisms that establish a mutualistic relationship with the host. The defense system of the intestinal mucosa comprises an epithelial layer and a plethora of immune cells that restrict commensals within the intestine while preserving their number and diversity. Conversely, commensal organisms are required for the development of a fully functional immune system. While interactions between host and commensal bacteria are normally beneficial, it is becoming increasingly clear that dysregulated immune system-microbiota crosstalk can favour the development of chronic inflammatory diseases, such as inflammatory bowel disease, arthritis or asthma. The gut microbiota can be altered by various factors, but perhaps the most drastic changes are induced by antibiotic treatment that severely alters both the size and composition of bacterial communities. While the benefits of antibiotics to treat infections are unquestionable, the microbiota changes induced by antibiotic exposure in humans can also modify immune defences which can lead to detrimental effects on health. For instance, antibiotic treatment in humans has been linked with an increased risk of Clostridium difficile-induced diarrhoea, asthma and certain types of cancer. Antibiotic treatment in mice recapitulates the impact and long-term alterations found in gut communities of antibiotic-treated patients, and has enabled investigation of the mechanistic links between changes in microbial communities and immune cell function. However, several reports have shown that administration of antibiotics on the drinking water ad libitum results in very noticeable weight loss as mice refrain from drinking water, presumably due to its foul taste. Thus, in these models the severe dehydration concomitant to oral antibiotic administration may complicate the interpretation of experiments aiming to identify the effect of antibiotic treatment in immune cell function.

Several approaches can be used to explore the size and composition of microbial communities in the intestinal compartment. Next generation sequencing technologies have provided invaluable data on this matter, however these methods are relatively expensive and require expert bioinformatic analyses for interpretation of the data. On the other hand, traditional microbiological culture methods allow detection of bacterial species, but they have low sensitivity and a big fraction of commensal bacteria (particularly anaerobes) are very difficult or impossible to cultivate with currently available methods. qPCR techniques are increasingly being used for quantification and identification of faecal bacterial species, as they provide a fast and reliable culture-independent measure of total microbial load. Accordingly, qPCR methods have proved useful to study changes in the microbiota associated with age or with progression of several diseases including inflammatory bowel disease. In line with this, qPCR methods provide a fast and cost-effective approach to validate the effect of various treatments (including antibiotics) in faecal bacterial loads and microbiota composition.
Here, we present a detailed step-by-step account of two distinct protocols for oral antibiotic administration to mice, faecal sample collection, DNA extraction, preparation of standards and quantification of bacteria in faecal samples by qPCR. These protocols provide a reliable method to manipulate the intestinal microbiota in mice and to study the effects of antibiotic treatment in intestinal homeostasis and disease.

**PROTOCOL:**
Experiments described here were performed using 6-8 weeks old wild-type (C57BL6/J) mice maintained in a specific pathogen free (SPF) facility. All animal experiments were approved by the King’s College London and the Francis Crick Institute Animal Welfare and Ethical Review Body and the United Kingdom Home Office. Prior to beginning any animal procedure, ensure that the appropriate permission is obtained through the local institution/organization.

1. **Administration of antibiotics**
Two alternative methods for antibiotic treatment are provided: oral gavage (1.1) and administration of antibiotics in the drinking water (1.2).

1.1. **Oral gavage**
1. Prepare stocks of individual antibiotics by dissolving them in autoclaved water at the following concentrations: Ampicillin (100 mg/ml), Gentamicin (100 mg/ml), Neomycin (100 mg/ml), Metronidazole (10 mg/ml) and Vancomycin (100 mg/ml). Filter sterilize using a 0.45 µm filter. Aliquot and store at -20 °C.

2. Prepare cocktail of antibiotics by mixing the stocks prepared above. For a volume of 1 ml mix: 50 µl of Ampicillin (5 mg/ml final), 50 µl Gentamicin (5 mg/ml final), 50 µl Neomycin (5 mg/ml final), 500 µl Metronidazole (5 mg/ml final), 25 µl Vancomycin (2.5 mg/ml final) and 325 µl of water. Prepare cocktail fresh before use.

3. Fix an appropriate gauge gavage needle (20 G for 15-20 g mice) onto a 1 ml sterile syringe and load the antibiotic mix, eliminating any bubbles.

4. Gavage mice with 200 µl of antibiotic mix. Grab the skin over the mouse shoulder firmly, stretch the head and neck to make the oesophagus straight. Direct the ball-tip of the feeding needle along the roof of the mouth and toward the back of the pharynx, then gently pass down into the oesophagus and inject the 200 µl solution.

5. Administer the antibiotic cocktail once daily for the duration of the experiment

1.2 **Antibiotics in the drinking water**

CAUTION: Carefully monitor mouse weight daily for the first two weeks of antibiotic administration in the drinking water.
1. Prepare cocktail of antibiotics by dissolving the following in 1 L of autoclaved water: 1 g of Ampicillin (1 g/L final), 1 g of Neomycin (1 g/L final), 1 g of Metronidazole (1 g/L final), 0.5 g of Vancomycin (0.5 g/L final) and 8 sachets (0.75 g each) of the artificial sweetener (60 g/L final). Shake until dissolved. Store at 4°C.

NOTE: Sweetener is added to hide the flavour of antibiotics and prevent mice dehydration. While several sweetener brands may work, the final concentration required may be different for each specific brand.

2. Place antibiotic cocktail in water bottle (~100 ml/bottle) and place on mouse cage.

NOTE: Use brown bottles or cover bottles with foil to protect antibiotics from light.

3. Replace antibiotic cocktail with fresh stock twice a week for the duration of the experiment.

2. Collection of faecal samples from stool, ileum content and ileum wall.

1. Weigh and label 2 ml autoclaved tubes for sample collection.

2. For collection of fresh stool samples, place mice in a restrainer and collect faecal pellets directly from the anus in a collection tube. Samples can also be obtained by placing mice in a clean autoclaved cage, and collecting stool samples with clean sterilised forceps.

3. Euthanize mice with CO₂ asphyxiation followed by cervical dislocation.

4. Lay the mouse with the abdomen fully exposed and spray abdominal area with 70 % Ethanol.

5. Using sterilised forceps and scissors make a transverse incision in the abdomen to expose the peritoneum without damaging any internal tissues. Lift the peritoneum and make an incision to expose the intestines.

6. Remove the intestines (from colon to stomach) with the forceps and scissors and place in a sterile petri dish.

7. Carefully use forceps to tease the small intestine (SI) away from the mesenteric arteries and fat. Extend intestine and place on a clean tissue.

8. With a ruler measure and cut 4 cm of the distal ileum of the SI (the closest part to the cecum). Cut and discard the 1 cm of intestine proximal to the cecum. There will be a 3 cm portion of ileum left which will be used to collect intestinal bacteria

9. Hold the ileum portion (3 cm) over a 2 ml sterile tube. Collect the intestinal content by directly extruding the intestine and collecting the sample in the tube. This sample will have bacteria from the ileum content.
10. Prepare a 20 ml syringe with cold Phosphate buffered saline (PBS) and flush the ileum portion (discard the flow-through).

11. Place the ileum portion on a clean tissue and open longitudinally with scissors.

12. Scrape the inside of the ileum wall with a scalpel.

13. Collect any bacteria on the scalpel by washing the scalpel with 1 ml of PBS over a clean tube. Spin at 8000 x g for 5 min to pellet the bacteria and discard the supernatant. This sample will contain bacteria from the ileum wall.

NOTE: Faecal samples from stool, ileum content and wall can be frozen and stored at -80 °C until use.

14. Weight the tubes containing the samples from stool and ileum content, and subtract the weight from the empty tubes (from step 2.1) to obtain the grams of faeces in each sample.

15. Extract bacterial DNA from stool, ileum content and ileum wall samples using commercially available kits. Store DNA samples at -20 °C until use.

3. Quantification of intestinal microbiota by qPCR
This procedure includes the generation of a standard (3.1) and the method for qPCR set-up for standard and faecal samples (3.2)

3.1 Generation of standard for qPCR

1. Amplify by PCR the 16S rRNA gene from genomic DNA extracted from a bacterial culture using the reagents and PCR conditions detailed in Table 1.

2. Run PCR product on agarose gel (1.5 %) and purify the DNA band using a commercially available kit.

3. Ligate the purified DNA fragment in an appropriate plasmid (containing antibiotic resistance and β-galactosidase (LacZ)-based blue/white colony selection) and transform DH10 competent E. coli following the manufacturer’s instructions. Plate transformation onto Ampicillin (100 µg/ml), X-gal (20 µg/ml) Luria Bertani (LB) agar plates (Tryptone 10 g/L, Yeast Extract 5 g/L, NaCl 10 g/L, Agar 15 g/L). Incubate O/N at 37 °C.

4. Select a single positive (white) colony from the plate. Inoculate it in 5 ml LB broth containing 100 µg/ml Ampicillin and incubate O/N at 37 °C, shaking at 250 rpm.
5. From the O/N culture, isolate and purify the plasmid using a commercial kit according to manufacturer's instructions.

NOTE: It is important to sequence the plasmid insert at this stage, to ensure that the plasmid contains only one copy of the 16S rRNA gene and to determine its length in base-pairs (bp).

6. Linearize the plasmid with a restriction enzyme which cuts the plasmid only once.

7. Purify linearized plasmid using commercially available kits.

8. Determine the concentration of plasmid by measuring absorbance at 260 nm using a spectrophotometer.

9. Calculate the number of plasmid copies/µl of sample using the following formula:

\[
\text{number of copies} = \frac{\text{amount} \times 6.022 \times 10^{23}}{\text{length} \times 1 \times 10^9 \times 650}
\]

where \text{amount} is the DNA concentration obtained in step 8 (in ng/µl); and \text{length} is the total length of the plasmid (with the insert) in bp. The \text{number of copies} will be obtained as copies/µl

NOTE: There are online tools based on the above formula that enable easy calculation of the number of plasmid copies

10. The standard can be aliquoted and kept at -20 °C until use.

3.2 qPCR set-up for standard and faecal samples

1. Thaw qPCR standard (from step 3.1.10), faecal samples DNA (from step 2.15) and qPCR reagents (from a commercially available kit) on ice. qPCR reagents used in this example include the SYBR Green qPCR reaction mix and forward and reverse primers (Table 2).

2. Dilute standard in sterile DNA-free water in a range from 10^7 to 10^2 copies/µl (e.g. 1/5 serial dilutions from 10^7 to 6.4 x 10^2 copies/µl). Dilute faecal samples' DNA: 1/2, 1/5, 1/10

3. Make a master mix reaction for the total number of reactions plus 1 (Table 2)

4. Mix 30 µl of master mix and 5 µl of template (standard, sample or water for negative control)

5. Add 10 µl of this mix to each well in a 384-well optical qPCR plate. Perform each reaction in triplicate.

6. Seal the qPCR place, centrifuge briefly and load the plate on the qPCR machine programmed with the following cycling conditions: 95 °C for 20 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.
7. Obtain C_T values for standard and samples.

8. Generate a standard curve by plotting the C_T values for the standards vs the logarithm of the copy number of plasmid (as calculated in step 3.1.9). Calculate the lineal regression of the standard curve.

9. Calculate the 16S rDNA copy numbers for faecal samples by interpolating C_T values (obtained in step 3.2.2) in the standard curve. 16S rDNA copy numbers for each sample should be corrected considering the dilution factor of the sample (as prepared in step 3.2.2), the final volume nucleic acids were eluted (in step 2.15) and the quantity of faecal sample (calculated in step 2.14) to obtain gene copies per gram of faeces.

REPRESENTATIVE RESULTS:

Here we provide two alternative protocols for oral antibiotic treatment of mice. Figure 1 shows percentage of body-weight (related to original base line weight for each animal) in mice treated with antibiotics either by oral gavage (red) or in the drinking water (blue) for 10 consecutive days. No noticeable weight-loss is found in mice that receive antibiotics by oral gavage. However, when mice are treated with antibiotics ad libitum in the drinking water, they lose weight (~10 %) within the first few days of antibiotic administration, but recover normal weight gain thereafter (Figure 1). Nonetheless, around 5-10 % of mice receiving antibiotics in the drinking water can reach >20 % weight loss within the first week of treatment, in which case they are sacrificed.

The quantification of bacteria in faecal samples requires the use of an adequate standard curve which is obtained by plotting the log of copy number for the standard (as calculated in step 3.2.2) vs. the C_T values obtained in the qPCR (step 3.2.7). Figure 2A shows a representative example from a standard curve which meets the standard curve performance criteria with a R^2 value of 0.99827 a slope of -3.09 and a efficiency ((-1 + 10^(-1/slope)))*100) of 110 %. R^2 values of 0.99 and PCR efficiencies within the range of 90 to 110 % are preferred. Within the linear range, the regression analysis equation enables quantification of the 16S rDNA abundance within the faecal samples. Figure 2B shows number of 16S rDNA copies in faecal stool, SI content and SI wall. In Figure 2B data are shown as 16S rDNA copies/g of faecal sample for faecal stool and SI content. For SI wall, data are presented as total number of 16S rDNA copies obtained from bacteria recovered from the 3 cm of SI wall (as quantity of starting material is too small to obtain a precise weight).

To evaluate the effect of antibiotics on the density of bacteria in faecal samples, mice were treated with antibiotics by oral gavage daily for 10 days (days 1 to 10) and stool samples were collected before (day 0), at different time-points during antibiotic treatment (days 5 and 10) and 7 days after stopping antibiotic administration (day 17; Figure 3A). As shown in Figure 3B, antibiotic treatment induces a strong decrease in the number of 16S rDNA copies per gram of
faeces detected at days 5 and 10, while the density of bacteria in the faeces recovered normal
levels (comparable to pre-treatment) a week after antibiotic administration is stopped (day 17).

**DISCUSSION:**

Here we provide experimental protocols for oral administration of antibiotics to mice and
quantification of faecal bacteria by qPCR. The combination of antibiotics used in this protocol
(containing ampicillin, gentamicin, neomycin, metronidazole and vancomycin) targets both
Gram-positive and Gram-negative bacteria, offering a bactericidal activity against a full
spectrum of bacteria. Both oral gavage and administration of antibiotics in the drinking water
greatly decrease faecal bacterial load\(^5,6,12\). Moreover, both treatments have a profound effect
on the phenotype of the mice as they develop several characteristics typical of germ-free mice
including reduced spleen size and enlarged cecum. The selection of a particular method for
antibiotic administration may possibly depend on the length of the experiment as the oral
gavage method requires daily administration of antibiotics, being more labour-intensive and
possibly causing more discomfort to the animals on the long-run.

For administration of antibiotics in the drinking water caution must be taken with the addition
of the sweetener to the antibiotic mixture as this is a crucial factor to prevent mice
dehydration. Several groups have shown how administration of antibiotics in drinking water
(without addition of sweetener) leads to very severe and rapid weight-loss with all mice losing
more than 20% of initial body weight within the first few days of the experiment\(^5,6\). In our
protocol, the use of the saccharine-based Sweetn’ Low sweetener seems to be sufficient to
mask the antibiotic taste in the water as mice loose weight in the first few days after antibiotic
administration, but recover weight quickly after that (Figure 1). Nonetheless, in our

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Administration of antibiotics.** Mice received antibiotics either by oral gavage (red) or
in the drinking water (blue) for 10 consecutive days. Plot shows weights of mice throughout the
duration of the experiments related to original weight before antibiotic administration (day 0).
Data are shown as mean +/- SEM.

**Figure 2. 16S rRNA gene qPCR amplification of standards and faecal samples.** (A) Linear
regression of standard curve with standard curve descriptors; (B) Calculation of gene
abundances from faecal samples. Data are shown as mean +/- SEM.

**Figure 3. Faecal bacteria during antibiotic treatment.** (A) Schematic of the schedule for
antibiotic administration by oral gavage (Ab) and sample collection as depicted with *; (B) 16S
rDNA copies per gram of faeces in stool samples from mice collected at the indicated days. Data
are shown as mean +/- SEM.

**Table 1. PCR reagents and conditions.** This table depicts the reagents and PCR cycling
conditions to amplify 16S rRNA gene from bacterial culture for generation of a standard to use
in the qPCR assays. Primer sequences were originally published by Kruglov et al\(^13\).

**Table 2. qPCR master mix.** The volumes shown (final volume 35 µl) are for a single sample to be
run on triplicate (10 µl each) on a 384 well qPCR plate (accounting for 5 µl extra for pipetting
error). The amount may be scaled up according to the number of samples to be analysed.

**Error**
experiments still 5-10% of mice reach the human end-point of >20% loss of baseline body weight and need to be sacrificed. We have tested other sucralose-based sweeteners which completely failed to prevent mice dehydration (100% of mice lost >20% of weight) and other authors have published similar failures for aspartame-based sweeteners\textsuperscript{5,6}. Added to this, the age, genetic background and general health status of the mice used for the experiments should be considered, as they may influence weight-loss and animal well-being during antibiotic treatment. Thus, careful monitoring of mice weight and general health status should be performed daily during the first two weeks of oral antibiotic administration.

qPCR methods provide a fast and cost-effective approach for quantification of 16S rRNA in faecal samples. However, some limitations should be considered regarding this technique including: i) the requirement for a reliable high-quality standard; ii) the design and efficiency of the qPCR primers; iii) the fact that microorganisms may have different copy numbers of the 16S rRNA gene, thus gene copies may not directly equal cell counts\textsuperscript{15}. Nonetheless, qPCR is a robust and sensitive method which enables rapid analyses of faecal samples. This method can be particularly useful to quickly validate the effect of various treatments (including antibiotics) in faecal bacterial loads as detailed here. Moreover, although we provide a protocol for quantification of total 16S rRNA, this method can be easily adapted (by designing specific primers\textsuperscript{16}) to enable identification of individual bacterial taxa, thus providing both quantitative and qualitative information about microbiome size and composition.

In summary, we provide here two protocols for oral antibiotic treatment of mice and a qPCR-based method to quantify antibiotic-induced changes in faecal bacteria. Although these protocols can be further optimized and combined with other approaches according to individual experimental needs, they may serve as a quick, cost-effective and reliable tool to manipulate the murine intestinal microbiota and to study of the effects of antibiotic treatment in intestinal homeostasis and disease.

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DISCLOSURES:
The authors have nothing to disclose

REFERENCES:


Figure 1

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Figure 2

A

\[ y = -0.3086x + 9.2471 \]

\[ R^2 = 0.99827 \]

B

- 16S rDNA copies / g
- 16S rDNA copies

\[ 10^{11} \]

\[ 10^{10} \]

\[ 10^{9} \]

\[ 10^{8} \]

\[ 10^{7} \]

\[ 10^{6} \]

\[ 10^{5} \]

stool, SI

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Figure 3

A

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B

16S rDNA copies / g

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