Assessing Lyme Disease Relevant Antibiotics through Gut Bacteroides Panels

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Abstract: Lyme borreliosis is the most prevalent vector-borne disease in the United States caused by the transmission of bacteria *Borrelia burgdorferi* harbored by the *Ixodus scapularis* ticks (Sharma, Brown, Matluck, Hu, & Lewis, 2015). Antibiotics currently used to treat Lyme disease include oral doxycycline, amoxicillin, and ceftriaxone. Although the current treatment is effective in most cases, there is need for the development of new antibiotics against Lyme disease, as the treatment does not work in 10-20% of the population for unknown reasons (X. Wu et al., 2018). Use of antibiotics in the treatment of various diseases such as Lyme disease is essential; however, the downside is the development of resistance and possibly deleterious effects on the human gut microbiota composition. Like other organs in the body, gut microbiota play an essential role in the health and disease state of the body (Ianiro, Tilg, & Gasbarrini, 2016). Of importance in the microbiome is the genus *Bacteroides*, which accounts for roughly one-third of gut microbiome composition (H. M. Wexler, 2007). The purpose of this study is to investigate how antibiotics currently used for the treatment of Lyme disease influences the *Bacteroides* cultures in vitro and compare it with a new antibiotic (antibiotic X) identified in the laboratory to be effective against *B. burgdorferi*. Using microdilution broth assay, minimum inhibitory concentration (MIC) was tested against nine different strains of *Bacteroides*. Results showed that antibiotic X has a higher MIC against *Bacteroides* when compared to amoxicillin, ceftriaxone, and doxycycline, making it a promising new drug for further investigation and *in vivo* studies.

I. Introduction

Lyme borreliosis is the most prevalent vector-borne disease in the United States, where it is transmitted by *Ixodes scapularis* ticks carrying the bacteria *Borrelia burgdorferi*, the causative agent of Lyme disease (Sharma, Brown, Matluck, Hu, & Lewis, 2015). *B. burgdorferi* belongs to the phylum Spirochaetes, which is characterized by highly motile, spiral-shaped bacteria. Since a standardized case definition of Lyme disease was issued in 1991, the geographic range of ticks carrying *B. burgdorferi* has expanded past the mid-Atlantic and New England regions to encompass the entire East coast, majority of the West coast, and much of the North-central portions of the United States (X. Wu et al., 2018). Similar spirochetes, such as *Borrelia duttonii* and *Borrelia garinii*, cause Lyme disease in Europe and Asia (X. Wu et al., 2018). While approximately 30,000 cases of Lyme disease are reported every year in the U.S., the true incidence might range from 300,000 to 1,000,000 cases per year (Stricker & Johnson, 2014).

The most common sign of Lyme disease is erythema migrans, which is an expanding rash occurring in roughly 80% of patients (Cairns, 2020; Shapiro, 2014). When detected at an early, localized stage of Lyme disease, the typical antibiotic treatment of oral doxycycline (100 mg twice daily for 21 days) cures the disease in most patients (Cairns, 2020). Other common antibiotics used include amoxicillin and ceftriaxone (Shapiro, 2014). However, when the pathogen delocalizes and enters the bloodstream, patients can experience Bell's Palsy (facial paralysis), arthritis, nerve pain, vision loss and severe ear pain (Cairns, 2020). While most patients are cured and no longer experience symptoms 2 to 4 weeks following antibiotic treatment, some patients still experience symptoms, particularly arthritis, in which a second course of antibiotics is recommended (Weitzner et al., 2015). Roughly 10 to 20% of Lyme disease patients continue to experience varying levels of muscle and joint pain after treatments, a condition referred to as post-treatment Lyme disease syndrome (PTLDS) (Weitzner et al., 2015).

Very little is currently known about PTLDS, and efforts to isolate *Borrelia* from patients with PTLDS in the past have had no success (Klempner et al., 2001). It is not known what factors may predispose

one to PTLDS and why only some patients develop it (X. Wu et al., 2018). Some animal models of Lyme disease do suggest that residual bacteria or bacterial products remain after antibiotic treatment, but whether this has any effect on PTLDS is currently unknown (Jutras et al., 2019; X. Wu et al., 2018). Despite the lack of knowledge concerning PTLDS, more effective management of *B. burgdorferi* would decrease the ability of bacterial recalcitrance and inflammation-inducing bacterial products. The pressing need for a more efficacious antibiotic regimens to kill growing and non-growing forms of *B. burgdorferi* is clear.

Antibiotics, however, are no longer considered solely beneficial to humans, as repeated use of these drugs are linked to numerous disorders associated with microbiota imbalance (Ianiro, Tilg, & Gasbarrini, 2016). Most antibiotics on the market have a wide array of action, impacting not only harmful bacteria, but also healthy bacteria. Furthermore, repeated abuse of antibiotics has been shown to have a direct link to the development of antibacterial resistance (Jernberg, Lofmark, Edlund, & Jansson, 2010). The primary negative effect of current antibiotics is decreased diversity of gut microbiota, which are of clinical importance due to the wide range of disorders associated with their imbalance (Lange, Buerger, Stallmach, & Bruns, 2016). Microbial life prospers in the anaerobic environment of the human gastrointestinal tract, representing "one of the densest microbial communities known to nature" (A. G. Wexler & Goodman, 2017). Bacteria in the gut play a major role in critical bodily functions, from immunity to digestion to protection against disease (Jernberg et al., 2010; Lange et al., 2016; G. D. Wu et al., 2011). Modulation of gut microbiome has been linked to obesity, inflammatory bowel disease (Crohn's disease and ulcerative colitis), type 2 diabetes, and numerous other metabolic disorders (Hills et al., 2019; Lange et al., 2016; Rinninella et al., 2019). Recent studies have shown that early life appears to be a critical period for maturation of metabolic function (Ianiro et al., 2016). Therefore, use of antibiotics that may cause unnatural fluctuations of the gut microbiota in children may be particularly worrisome. The goal of effective antibiotics against Lyme disease in any population should be to kill the pathogenic *B. burgdorferi* while simultaneously leaving minimal impact on healthy gut microbes.

Although the human gut microbiome consists of thousands of bacteria, the most predominant genus found in lower intestinal tract isolates from the United States and Canada—accounting for roughly one-third of gut microbiome composition—is *Bacteroides* (King et al., 2019; Nishijima et al., 2016; Sears, 2005). The genus *Bacteroides* is the principal component of the phylum Bacteroidetes, which constitutes roughly half of the relative abundance of bacteria in the gut (Garcia-Bayona & Comstock, 2019). Laitinen and Mokkala (2019) found that the median relative abundance of *Bacteroides* in the gut is 31.16%, with some individuals having a microbiome composition of up to 40.83% *Bacteroides*. To put into perspective, as many as 1010–1011 cells of *Bacteroides* are found in one gram of human feces (Hong, Wu, & Liu, 2008). Numerous species of *Bacteroides* are present in the intestines, with the most common being *B. fragilis* (A. G. Wexler & Goodman, 2017). As mutualists and commensals, *Bacteroides* generally have a beneficial and complex relationship with its host. *Bacteroides* can break down food to produce valuable energy, digest complex sugars, and help in other metabolic activities of the colon (Karlsson, Ussery, Nielsen, & Nookaew, 2011; H. M. Wexler, 2007). Studies have shown that people with healthier diets and less-obese individuals have a higher composition of *Bacteroides* in their microbiome (Gorvitovskaia, Holmes, & Huse, 2016).

Despite their many benefits, imbalances in *Bacteroides* levels are responsible for infections such as intraabdominal sepsis, appendicitis, and gynecological infections that cause significant morbidity and mortality (Johnson, Heaver, Walters, & Ley, 2017). In fact, most anaerobic human infections have an abnormal prevalence of *B. fragilis. B. fragilis* related disorders have an associated mortality rate of 19%, increasing to 60% when left untreated (H. M. Wexler, 2007).

Given the importance of microbiome balance to the health of individuals and the prevalence of *Bacteroides* in the gut microbiome, antibiotic dosages that strongly impact *Bacteroides* should be closely monitored. Studies have attempted to discern the range of *Bacteroides* species present in the gut but have yielded varied results due to individual

differences in geography, diet, lifestyle, and other factors that contribute to the composition of the gut microbiome (Aldridge & Johnson, 1997; Snydman et al., 2011; Yim et al., 2015).

Nevertheless, Table 1 attempts to summarize known minimum-inhibitory concentration (MIC) values of ten antimicrobial agents against *B. fragilis* (Fernandez-Canigia et al., 2012). Clinical isolates in this study were recovered from several body sites, with 58% recovered from the human gut. MIC values refer to the minimum concentration of drug (in μg/mL) needed to kill a certain percentage of bacteria. Limited information is known about *Bacteroides* MIC values of specific species (i.e. *B. vulgatus, B. nordii*, etc.) with respect to clinically relevant antibiotics for Lyme disease.

A promising antibiotic—hereafter referred to as antibiotic X—has been identified by researchers at the Antimicrobial Discovery Center at Northeastern University. Antibiotic X has been found to be active against *B. burgdorferi* and shows signs of selectivity against spirochetes. Antibiotic X was kindly provided for this study by researchers led by Dr. Kim Lewis at the Antimicrobial Discovery Center. The purpose of this study is to investigate how antibiotic X influences *Bacteroides*, the predominant genus of bacteria in humans, as compared to other clinically relevant antibiotic treatments for Lyme disease.

B. fragilis **(n=198)**	**MIC_{50} (μg/mL)**	**MIC_{90} (μg/mL)**
Ampicillin-sulbactam	1	8
Piperacillin-tazobactam	0.25	2
Cefoxitin	16	32
Ertapenem	0.25	4
Imipenem	0.13	0.5
Doripenem	0.25	1
Clindamycin	1	>256
Metronidazole	0.5	1
Moxifloxacin	0.5	2
Tigecycline	0.13	1

Table 1: MIC values of ten antimicrobial agents against *B. fragilis*. MIC values represent the minimum concentration (in μg/mL) necessary to kill 50% (MIC_{50}) and 90% (MIC_{90}) of bacteria. 198 samples of *B. fragilis* were tested. Table adapted from Fernandez-Canigia et al. (2012).

II. Results

16S sequencing analysis showed that majority of the tested samples were not contaminated. Various *Bacteroides* isolates provided by the Antimicrobial Discovery Center were taken from lab freezer stock for use in the present study. However, freezer stocks are prone to contamination, so the identity of the isolates must first be genetically confirmed. Once bacterial colonies were cultured, the colony used in MIC analysis for each strain of *Bacteroides* was assessed to ensure that contaminated samples of bacteria were not used in this study. 16S rRNA gene sequencing was done to confirm the species of bacteria. 16S rRNA gene sequencing is valuable because the 16S rRNA gene region is highly conserved among bacteria, allowing for taxonomic resolution at the species and strain level. 27F and 1492R universal primers encompass nearly the entire 16S rRNA gene, which is about 1,500 base pairs long. These universal primers can amplify any gene in any organism. Gel electrophoresis results (Figure 1) show that PCR yielded the desired product length and was used for sequencing. All PCR product in lanes 2-6 are located around the 1,500 bp stain mark of the DNA ladder in lane 1, confirming gDNA isolation and PCR were successfully conducted. 16S sequencing analysis was conducted on four separate occasions. As shown in Table 2, of the 24 samples of *Bacteroides* tested, seven were shown to be contaminated. Multiple samples of *B. stercoris* and *B. dorei* were shown to

Figure 1: Gel electrophoresis results depicting the PCR products used for 16S rRNA gene sequencing Lane 1 represents the NEB 1kb ladder of known lengths, with desired region of DNA from different Bacteroides samples in lanes 2-6.

be of other species, suggesting that the freezer stock of these strains may be contaminated. Common contaminants were *Streptococcus parasanguinis* and *Aneurinibacillus aneurinilyticus*. Once correct strains of *Bacteroides* were confirmed via 16S rRNA gene sequencing, 96-well MIC assays could be conducted on the correctly identified strains to determine drug susceptibilities to the different antibiotics relevant to Lyme disease.

Expected *Bacteroides* Strains	No. of times used in study	No. of times 16S sequencing results were different	Contaminants (if different)
B. fragilis	2	0	N/A
B. vulgatus	3	0	N/A
B. xylanisolvens	3	0	N/A
B. thetaiotaomicron	2	2	*Lactobacillus paracasei, Aneurinibacillus aneurinilyticus*
B. ovatus	2	0	N/A
B. strecoris	2	2	*B. vulgatus*
B. cellulosilyticus	2	0	N/A
B. eggerthii	2	0	N/A
B. nordii	4	1	*Aneurinibacillus aneurinilyticus*
B. dorei	2	2	*Streptococcus parasanguinis*

Table 2: 16S rRNA gene sequence analysis was performed on different freezer stocks of *Bacteroides*. At 7 (of 24) occasions, the sequencing results were different than expected.

Bacteroides Strain	Antibiotic X MIC (µg/mL)	Doxycycline MIC (µg/mL)	Amoxicillin MIC (µg/mL)	Ceftriaxone MIC (µg/mL)
B. fragilis	32	0.25	32	1
B. vulgatus	64	2	128	128
B. xylanisolvens	>512	0.5	32	64
B. nordii	8	0.25	32	1
B. ovatus	64	0.01	0.5	0.02
B. cellulosilyticus	8	2	128	128
B. eggerthii	64	0.01	0.5	ND

Table 3: MIC values of the different antibiotics against the lab isolates of *Bacteroides* strains cultivated under anaerobic conditions. ND = Not determined

Antibiotic X has comparatively higher MIC for Bacteroides. Compared to clinically relevant antibiotics for Lyme disease, higher concentrations of antibiotic X were required to kill the different *Bacteroides* species tested, suggesting that antibiotic X has minimal effect on the human microbiome. MIC was performed using microdilution broth assay on laboratory isolates of *Bacteroides* to determine the drug concentrations of antibiotic X, ceftriaxone, amoxicillin, and doxycycline. In a few instances, the MIC values of ceftriaxone and amoxicillin were high, suggesting that the strains used in this study may have developed resistance to them. Importantly, in four of the seven species tested, antibiotic X had a higher MIC value than all other antibiotics tested, and overall potency against all the strains were low. Of special significance is *B. fragilis*, whose imbalance is the cause of a variety of metabolic disorders. Antibiotic X and amoxicillin had the highest MIC values (32 µg/mL) of the four drugs against *B. fragilis*.

Mean MIC values against Bacteroides. Of the four drugs tested, doxycycline had the lowest overall MIC values, showing that even small concentrations of doxycycline have a large impact on *Bacteroides*. When the four drugs were ranked on their mean MIC values for *Bacteroides*, doxycycline had the smallest mean MIC (0.72 µg/mL), followed by amoxicillin (50.43 µg/mL), ceftriaxone (53.67 µg/mL), and then antibiotic X (107.43 µg/mL). Amoxicillin and ceftriaxone had comparable mean MIC values against *Bacteroides* in this study, both of which were still less than half the mean MIC for antibiotic X. The mean MIC for antibiotic X was more than 149 times higher than the mean MIC for doxycycline. Antibiotic X had a higher MIC than doxycycline in each of the species tested in this study. The low MIC values for doxycycline suggest that small dosages of doxycycline have the potential to dramatically shift the nature of the *Bacteroides* in the gut microbiome. Antibiotic X had a higher MIC value than the other clinically relevant treatments for Lyme disease. These results suggest that antibiotic X—when compared to doxycycline, amoxicillin, and ceftriaxone—has the least effect on *Bacteroides* in the human gut microbiome.

III. Discussion

The current study was aimed at identifying the effect of antibiotic X on *Bacteroides* species of the human gut microbiota as compared with clinically relevant antibiotics for Lyme disease. Results were obtained using laboratory isolates of a *Bacteroides* panel of seven distinct species of the genus. Importantly, the species most abundant in the human gut microbiome, *B. fragilis*, was present in the analysis.

MIC assays performed using microbroth dilution in a 96-well plate format showed that antibiotic X had minimal effect on the *Bacteroides* human microbiome when compared to doxycycline, amoxicillin, and ceftriaxone. MIC values for antibiotic X were significantly higher than the clinically relevant treatments for Lyme disease tested in this study, suggesting higher doses of antibiotic X

are needed to modulate the *Bacteroides* diversity in the microbiome. To the best of our knowledge, there are only a few recent studies assessing the MIC values of amoxicillin, ceftriaxone, and doxycycline against *Bacteroides*. However, one study from 1997 describes the MIC of ceftriaxone against unseparated isolates of 24 species of *Bacteroides*, which were predominantly *B. fragilis* (Aldridge & Johnson, 1997). Interestingly, the MIC values found in our study were different from the results from Aldridge and Johnsen (1997). Aldridge and Johnsen showed that ceftriaxone had an MIC of 128 μg/mL against the 24 species of *Bacteroides*. This study showed that ceftriaxone had an MIC of 1 μg/mL against *B. fragilis* and an average MIC of 53.67 μg/mL against all *Bacteroides*. This discrepancy may be explained by the fact that our study looked at *Bacteroides* on a species level rather than testing all *Bacteroides* together.

In our study, 16S rRNA gene sequencing was performed with every experiment. 7 of the 24 sequencing results yielded unexpected results. The most common contaminants found after 16S sequencing were *Streptococcus parasanguinis* and *Aneurinibacillus aneurinilyticus*. These contaminations may arise during lab isolation of the strains from the source or due to non-sterile conditions that may have been present in the anaerobic chamber during the culturing of bacteria. MIC results for ceftriaxone against *B. eggerthii* were not determined due to a tailing-off effect in the MIC plates. The exact reason for this tailing off effect is unknown, but possibilities include incorrect pipetting of the drug into the 96-well plate or contamination of the 96-well plate. This tailing-off effect did not render a readable MIC value by visual search or plate reader.

Given the array of impacts antibiotics may have on the human gut microbiome, performing a high-throughput *Bacteroides* panel is crucial in the drug development pipeline, as the genus plays important roles in human health. The *in vitro* experiments performed in this study with individual assessment of different *Bacteroides* species allows for in-depth analysis of how drugs may impact the most prevalent *Bacteroides* found in the microbiota. However, this method does not consider the *in vivo* interaction of *Bacteroides*, both with the host and other microbes present in the body. Additionally, *in vitro* analysis of individual *Bacteroides* species does not mimic the true diversity and relative prevalence of *Bacteroides* in the proportions present in the human gut.

Considering that the typical dose of oral doxycycline prescribed to Lyme disease patients is 100 mg twice a day for ten to 21 days, the results found in this study suggest that doxycycline may have significant adverse effects on the *Bacteroides* population in the human gut microbiome. This artificial alteration of gut microbiota composition may have adverse health and lifestyle effects, in both the short and long term. *B. fragilis*, whose imbalance accounts for a majority of the adverse health impacts associated with gut microbiota, had an MIC of 0.25 μg/mL, compared to 32 μg/mL for antibiotic X. Of the four antibiotics tested, doxycycline had the greatest efficacy in killing the *Bacteroides* strains while antibiotic X had the least impact.

Although amoxicillin showed higher MIC values than doxycycline in this study, it suggests that many of the strains used in this study developed resistance to amoxicillin. Numerous studies have shown that it is not uncommon for *Bacteroides* to develop resistance towards amoxicillin. Nagy et al. (2011) showed that 10.4% of *Bacteroides* tested in his study developed resistance to amoxicillin. The typical dosage for amoxicillin in Lyme disease is 500 mg three times per day, compared to the 100 mg dosage of doxycycline twice a day (Wormser et al., 2000). Drug-induced rashes have also been common side effects of amoxicillin in the treatment of Lyme disease. As a result, treatment of Lyme disease with amoxicillin poses additional risk. In this study, we observe that antibiotic X had minimum impact on the *Bacteroides* strains tested when compared to clinically relevant antibiotics. Preliminary investigations have shown that antibiotic X is effective in selectively killing *B. burgdorferi,* the causative agent of Lyme disease. Taken together, these results suggest that antibiotic X may prove to be a more effective drug in the treatment of Lyme disease, as the antibiotic suppresses Lyme bacteria while preserving gut *Bacteroides*. Additional studies are required to investigate the exact *in vivo* effect of the different drugs on microbial diversity for a better understanding and approach towards the treatment of Lyme disease.

IV. Materials and Methods

Bacteria strains used in this study. Bacteroides strains listed in Table 4 are from lab isolates and were used in all the *in vitro* studies. Seven different species of *Bacteroides* were used for analysis and kept at -80°C.

***Bacteroides* Strains**
B. fragilis
B. vulgatus
B. xylanisolvens
B. ovatus
B. cellulosilyticus
B. eggerthii
B. nordii

Table 4: Strains of *Bacteroides* expected based on freezer stock.

Media preparation. To ensure optimal *Bacteroides* colony formation, careful attention was placed in preparing the nutrient-rich growth medium for strains to proliferate. Both solid BHIymch media and liquid BHIymch media were made. Directions for making each component of BHIymch media (both liquid broth and solid agar) are shown in Table 5.

BHIymch (BHI broth or agar, supplemented with yeast extract, MOPS buffer, cysteine, and hemin)	
MOPS	• Prepare a 1.5M solution of MOPS free acid in dl water (30x stock solution) • Adjust pH to 7 • Filter sterilize with 0.22 um filters
Cysteine	• Prepare a 10% solution of cysteine-HCl in dl water (100x stock solution) • Filter sterilize with 0.22 um filters
Hemin	• Prepare 15 mg/mL hemin solution in DMSO (1000x stock solution)
Brain heart infusion with yeast	• Prepare BHI broth or agar as per instructions on bottle in dl water • Add yeast in this proportion: 2.5g for 500mL BHI • Sterilize by autoclavation and cool down in water bath

Table 5: Directions to prepare components for BHIymch media

Once autoclaved, the BHI-y media was cooled in a water bath. MOPS, cysteine, and hemin were added to their final concentration of 1X. To prepare the BHI-ymch plates, the media with agar was poured into petri dishes. All media and plates were stored in an anaerobic chamber.

Culturing conditions and microdilution broth assay for MIC determination. Bacteroides strains were removed from a -80°C freezer and taken to an anaerobic chamber. BHIymch agar plates were streaked with *Bacteroides* using a sterilized wire loop. Plates were incubated in an anaerobic chamber for 24-48 hours for colonies to appear. Single colonies were then used to transfer to the 3 mL BHI-ymch media and grown for 12-16 hours to allow cultures to proliferate in the liquid media.

Stock solutions of 50 times concentrated (i.e. 6.4 mg/ml) ceftriaxone, doxycycline and amoxicillin were prepared and serially diluted 2-fold. In a 96-well plate, 2 µL of the 50 times concentrated antibiotic solution was pipetted according to the scheme below (Table 6) in aerobic conditions. This gives a final concentration range of 128 µg/ml to 0.01 µg/ml to be tested.

µg/mL	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	128	64	32	16	8	4	2	1	0	0
C	0	0	0.5	0.25	0.13	0.06	0.03	0.02	0.01	0	0	0
D	0	0	128	64	32	16	8	4	2	1	0	0
E	0	0	0.5	0.25	0.13	0.06	0.03	0.02	0.01	0	0	0
F	0	0	128	64	32	16	8	4	2	1	0	0
G	0	0	0.5	0.25	0.13	0.06	0.03	0.02	0.01	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

Table 6: Schematic for final drug concentrations (in µg/mL) of amoxicillin, ceftriaxone, and doxycycline used for the MIC assay. Triplicates were used for each drug.

For MIC plates of antibiotic X, a similar procedure was performed with the final concentration range of 512 µg/mL to 0.002 µg/ml. A total of 4 MIC plates, each containing triplicates of the drug dilutions and drug controls per *Bacteroides* strain were prepared and placed in the anaerobic chamber. 100µL of the 1 in 100 dilution of the stationary phase cultures of the *Bacteroides* strains growing in the liquid BHI-YMCH media was then pipetted to each well using a multichannel pipette. The plates were incubated in the anaerobic chamber for 12-16 hours, and the MIC was determined as the lowest concentration of compound that inhibits growth of the bacteria as detected by the unaided eye.

Genomic DNA (gDNA) isolation. To verify species, a cell lysis-based method of DNA extraction consisting of a lysis solution of 500 mM EDTA, 1M Tris-HCl, and Triton X-100 was initially attempted. Polymerase chain reaction (PCR) was performed directly using the lysis solution. However, the PCR reaction did not yield any products. Therefore, gDNA

isolation was performed. gDNA was isolated from the *Bacteroides* strains used for MIC assay in order to perform 16S rRNA gene sequencing to confirm the species. Qiagen kits were used for gDNA isolation, and protocol from the Qiagen DNeasy Blood & Tissue handbook for gram-negative bacteria was followed.

1 µL of isolated gDNA was measured using a NanoDrop spectrophotometer to assess the concentration of the gDNA isolated in ng/µL. Using the DNA concentrations, 50 µL of 20 ng/µL gDNA stock solution was prepared in water. PCR using Q-load M0271S NEB was performed where each PCR tube with 25 µL reaction contained 0.2 µM of universal 27F forward primer (5' AGAGTTTGATCMTGGCTCAG 3'), 0.2 µM of universal 1492 reverse primer (5' TACGGYTACCTTGTTACGACTT 3'), 12.5 µL of 2X master mix, 6.5 µL of water, and 100 ng template gDNA. 35 cycles of PCR cycles were performed as follows: denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 70 seconds. Gel electrophoresis was conducted in 1% agarose gel to confirm PCR product was of desired length. Ethidium bromide was used to stain DNA in the gel and NEB 1kb ladder was used as marker. 20 µL PCR products were sent for sequencing (Macrogen), and the results were analyzed using NCBI nBLAST program.

V. References

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