The effect of sub inhibitory concentrations of antibiotics on bacterial evolution

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Key words: antibiotic resistance, sub-inhibitory, antibiotic pollution, SOS response, evolution

Chapter 2 follows the guidelines set out for publication in Emerging Infectious Diseases. Some sections have been expanded for ease of comprehension

Submitted 10th October 2014 as part of the requirements for completion of the Masters of Research degree

Abstract

Antibiotics and the genetic elements that confer resistance to them are both disseminated into aquatic environments via human waste streams and agricultural run-off. Antibiotics are not readily broken down or degraded and therefore persist in the environment at low concentrations. Such pollution with antibiotics establishes a selection gradient, but may also raise the frequency of events that can generate resistance: point mutations; recombination; and lateral gene transfer. The effect of clinical levels of antibiotics on the evolution of antibiotic resistance has been extensively studied. This study examined the response of bacteria to sub inhibitory levels of antibiotics, such as those found in aquatic environments subject to human pollution. Two species, *Pseudomonas aeruginosa* and *Pseudomonas protogens* were exposed to subclinical levels of the antibiotics kanamycin, tetracycline and ciprofloxacin. The minimum inhibitory concentration (MIC) of the bacteria to these antibiotics was determined, and then bacteria were subjected to 1/10 the MIC in a serial streaking experiment over 40 generations. Repetitive Element polymerase chain reactions were carried out to monitor changes in genomic DNA every five generations. Significant changes in the banding patterns of both species were discovered, even at five generations, suggesting that 1/10 the MIC induces mutation and/or recombination events. Lines exposed to sub-clinical levels of antibiotics also exhibited phenotypic effects. There were significant changes to colony morphology in exposed lines. The final MICs at generation 40 were significantly higher in some lineages of *Ps. protogens,* showing that even subclinical levels of antibiotics can select for resistance. In conclusion, these experiments demonstrate that exposure to sub-clinical levels of antibiotics, such as those found in waste streams, can induce significant genotypic and phenotypic changes.

Chapter 1

Introduction

In early 2014 the World Health Organisation identified Antibiotic Resistance as one of the greatest threats to human Health for the 21st century [1]. There are fears that if current trends in antibiotic resistance continues, we may face a post-antibiotic era where infections are harder, and in some cases, impossible to control. Standard treatments for infections will no longer be sufficient, there will be a high risk of spread of infection, illnesses will take longer to treat, and the risk of death will be greatly increased. The practice of modern medicine is itself threatened, since invasive surgeries may become too risky to perform, and common infections and minor injuries could kill [2].

The risks of widespread antibiotic resistance were known before antibiotics were widely used. The first multi-drug resistant strain appeared as early as 1955 [3]. Overuse and misuse of antibiotics in the medical and agricultural sectors have contributed to the problem, and it is now estimated that 70% of pathogens exhibit resistance to at least one antibiotic [4]. Development of new, stronger antibiotics will help in the short term, but will only continue to feed the growing problem. However, few new antibiotics have been produced over the last 30 years, as there is little incentive for pharmaceutical companies to develop antibiotics that will likely become ineffective, or be reserved as a last line of defence [1, 5].

Antibiotic resistance is a global threat and needs to be treated as such. In April 2014, the World Health Organisation released a Global Report on Surveillance which compiled data from 114 countries, examining seven common, yet serious pathogenic bacterial species. Resistance to 3rd generation cephalosporins, fluoroquinolones, carbapanems, β-lactams and penicillin antibiotics was reported. In most cases, the risk of death doubled if the individual was infected with a resistant strain of bacteria. In the United States in 2013, there were 23,000 confirmed deaths due to antibiotic resistance (US CDC) and Europe reported 25,000 death per year (2007, ECDC).

The primary use of antibiotics is medical, where they are used to treat a range of bacterial infections. However, misuse of antibiotics is contributing to the development of resistance. Often antibiotics are prescribed for viruses, such as the common cold, which cannot be treated by antibiotics [6]. They are also used in higher than necessary concentrations when a lower dosage

or alternative treatments would be equally as effective [7, 8]. Antibiotics are often misused; examples include unfinished courses of antibiotics and daily antibiotic supplements, both of which contribute to the development of resistance [6, 9].

Antibiotics are extensively used in agriculture and aquaculture to prevent disease, infections, and as a growth promoter [10, 11]. It has been estimated that 50-70% of antibiotics produced in the United States of America are used in agriculture [12, 13]. Antibiotics use in agriculture not only contribute to the level of antibiotics in the environment but also pose a direct threat to human health through contamination [13]. This was seen in 2013, in California, when a multidrug resistant strain of *Salmonella* saw 300 people fall ill, with 40% hospitalised following consumption of chicken from a farm using antibiotic supplemented feed (CDC 2013). This was the largest outbreak of a multidrug resistant bacterial strain in a western country and sparked attempts to control antibiotic-supplemented feed [10].

The faecal waste from animal agriculture is often reused as manure for crops. However if the animals have been maintained on antibiotics, there will be antibiotics and antibiotic resistance genes present in the manure which could then threaten human health. Following one application of manure, antibiotics and antibiotic resistance genes can persist in the soil for approximately six months, depending on environmental conditions, during which time it could be dangerous to consume products that have had direct contact with the soil. [14]

Origins of antibiotics

Just as bacteria are naturally occurring, so are many of the compounds we use as antibiotics. The majority of antibiotics are produced by fungi, actinomycetes and other bacteria [4]. By purifying and concentrating these molecules, humans have transformed them into the powerful agents we use today.

There are two main hypotheses about the role that antibiotics may play in natural environments: as killing molecules or as signalling molecules. The theory that antibiotics may have been killing molecules comes from the idea that antibiotics are produced in stressful conditions in order to protect their producer against organisms such as fungi and other bacteria. This theory was supported in that the environments that bacteria inhabit, such as biofilms, are often very limited

in resources such as space and nutrients, therefore it would be advantageous to have a mechanism to remove competitors from the surrounding areas. Antibiotics may have played a role in this [15]. The second theory and the most likely theory, is that antibiotics are signalling molecules. As antibiotics occur in such low concentrations, it is unlikely that they would ever have reached concentrations high enough to have any significant killing effects. Low concentrations of antibiotics have been found to trigger transcriptional changes and regulate interactions within microbial communities [16].

Mechanisms of Antibiotic resistance

Antibiotics can be grouped into two categories: bactericidal antibiotics that kill bacteria or bacteriostatic antibiotics that affect the growth of bacteria [17, 18]. They can be further classified by their mechanism of action, such as inhibition of cell wall synthesis, preventing synthesis of protein, RNA or DNA, or attacking the cell membrane [19].

Organisms can be intrinsically resistant to antibiotics [20] or they can acquire resistance through two main mechanisms; mutation of existing genes or lateral gene transfer (LGT) of genetic material between individuals and species. Intrinsic resistance is a phenotypic feature common to all individuals of a particular species. A bacterial species may exhibit intrinsic antibiotic resistance via outer membrane permeability or efflux mechanisms. Outer membrane permeability is mediated by two cell wall structures: the first being the positively charged nature of lipopolysaccharides, which prevent the entry of negatively charged antibiotic molecules; and the second being highly selective outer membrane proteins called porins which control the influx of molecules and can prevent the entry of antibiotics. Bacterial cells also utilise efflux pumps to actively move antibiotics out of the cell [20]. Efflux pumps can be specific to one drug, or more general and capable of transporting several drugs out of the cell.

Bacteria can generate resistance through mutations to existing genetic material, which can lead to resistance via modification of target sites which then prevents the antibiotic from binding in the cell. Like intrinsic resistance, mutations can alter efflux pumps that actively remove antibiotics from the bacterial cell [21]. Bacteria can also modify enzymes so that they can directly inactivate the antibiotic. This is most common for β-lactam and aminoglycoside antibiotics [22].

Bacteria can acquire and disseminate genes conferring antibiotic resistance via lateral gene transfer (also known as horizontal gene transfer) via conjugation, transduction, or transformation [15]. Genetic material such as plasmids can be transferred between bacteria by conjugation. DNA molecules are transferred via a structure called the pilus which forms between two bacterial cells [23]. Transformation is the uptake of DNA fragments and genetic elements directly from the environment. This can occur when a bacterium dies, it breaks apart and the fragments can be absorbed by nearby bacteria which incorporate the DNA into their own genetic material. Genetic material may be transferred via a vector, commonly a virus, which infects the bacteria and in doing so introduces the resistance gene along with its own DNA. [6, 24].

Lateral gene transfer allows the genetic information within a microbial environment to be shared between individuals. It is in this way that resistance can be disseminated on a global scale. This form of gene exchange means that bacteria is not reliant on random beneficial mutations as are the vast majority of eukaryotes [25].

Antibiotic pollution

A relatively small amount of the antibiotics consumed by humans and animals are actually absorbed into the body, 30-90% of antibiotics pass through unchanged and are excreted into waste treatment facilities [26]. Antibiotics, along with heavy metals, disinfectants and genes conferring resistance are disseminated into the environment via human waste streams, agricultural run-off [27] and effluent from antibiotic production factories [28, 29]. Although these are usually filtered through waste treatment facilities, the current mechanisms used to treat waste is often unable to remove these substances from water and the water is either reused as reclaimed water [30] or released into the environment via rivers [31, 32], estuaries and the ocean [30, 33]. . Manuring of land for agriculture can also result in antibiotics entering the soil or washing into waterways [34-36]. The release of these substances into the environment should be thought of as a significant component of water pollution.

Waste water treatment facilities and aquatic environments can become hotspots for the generation and acquisition of resistance. The presence of selective agents such as antibiotics, heavy metals and disinfectants, combined with genes conferring resistance, mobile elements such

as integrons, and diverse microorganisms creates an optimal environment to generate resistance through mutation or lateral gene transfer.

The time that an antibiotic can persist in the environment differs depending on the class of antibiotic and the environmental conditions [57]. Closed bottle tests provide a simple way to measure the biodegradability of antibiotics and indicate whether or not the antibiotic will readily degrade in aquatic environments. Classes of antibiotics such as the β-lactams, tetracyclines, macrolides, lincosamides, aminoglycosides, carbapenems, nitroimidazoles, quinolones, sulphonamides and glycopeptides have been found to persist over a 28 day testing period. [37, 38] High temperatures and exposure to UV light can cause degradation of some antibiotics. Fluoroquinolone antibiotics can degrade in sunlight, however they are readily absorbed onto sediments, where they have been documented persisting up to 80 days with less than 1% of degradation [39]. It would be optimal if resistant organisms destroyed or inactivated the antibiotics, however the mechanisms that usually allow for resistance involve mutation of binding sites and efflux pumps meaning that the antibiotics may persist in the environment [40]. It is clear that antibiotics persist in the environment for longer than previously thought and that this could have serious implications for the development of antibiotic resistance in natural environments.

Sub inhibitory concentrations of antibiotics

As mentioned above, 30-90% of antibiotics pass through humans and animals and are introduced to the environment via a number of pathways. Many studies have investigated the effect of clinical levels, or inhibitory levels, of antibiotics on the generation of antibiotic resistance. However, there is increasing speculation that sub inhibitory levels of antibiotics may be having significant effects on bacterial populations. A gradient of antibiotic concentration forms within all human-dominated environments. Within the human microbiome there may be a gradient along the digestive tract. Dissemination of antibiotics via waste water will cause a gradient of antibiotic concentration spreading outwards from human population centres.

SOS Response

The SOS response is a broad response to DNA damage that has been documented in many bacterial species. It may play a significant role in the generation of antibiotic resistance, as it can increase the rates of mutation and lateral gene transfer [41]. It is ultimately triggered by an increased occurrence of single stranded DNA occurring due to DNA damage or inhibition of the processes involved in DNA replication. The SOS response is mediated by the LexA repressor. Under normal conditions, LexA prevents SOS genes from being expressed. Under stressful conditions, the protein, RecA is recruited onto single stranded DNA where it stimulates cleavage of the LexA repressor, inactivating it and therefore allowing the expression of approximately 40 SOS genes. SOS genes are mainly involved in DNA repair [41, 42].

It is well documented that lethal concentrations of antibiotics can induce the SOS response in bacteria [43, 44]. It has been suggested that sub-inhibitory levels of antibiotics, as those discussed above, may be more relevant to the problem of antibiotic resistance than lethal concentrations of antibiotics [5, 8, 42]. Lethal concentrations exert a strong selective pressure on bacteria whereby they either die or they acquire mutations allowing them to survive. When exposed to sub-inhibitory levels of antibiotics, bacteria survive with little effect on growth, and the SOS response is initiated. This, in turn, increase rates of mutation and lateral gene transfer. The diverse mutations or lateral transfers generated via this mechanism can become fixed within populations of bacteria. [41]

How sub-inhibitory levels of antibiotics may increase resistance mutations

While clinical concentrations of antibiotics select for resistant strains of bacteria, sub-clinical or sub-inhibitory levels of antibiotics may do so indirectly, encouraging the emergence of novel mutations that provide resistance. Sub-inhibitory antibiotic concentrations induce the SOS response, causing expression of error prone DNA polymerases, which consequently increases the likelihood of mutations occurring, including those that might confer antibiotic resistance [5, 45, 46].

How sub-inhibitory levels of antibiotics may increase lateral gene transfer

Mobile elements containing genes conferring resistance, such as plasmids, integrons, transposons

and insertion sequences are transferred between organisms via transformation, conjugation or transduction. The propensity for these mechanisms to occur is greatly increased by sub-inhibitory concentrations of antibiotics, since they initiate the SOS response which, in turn, promotes the expression of genes involved in lateral gene transfer [42].

Antibiotics and molecular evolution – are we selecting for lineages of bacteria with increasing ability to evolve?

Traditionally, it has been thought that the rate at which populations generate genetic variation is constant and largely dictated by the generation time of a species, where species with short generations are able to evolve more rapidly than species with long generations. However there is an emerging hypothesis that the rate at which evolution occurs may be more flexible than once thought and could be dictated by both the generation time of a species and the intrinsic rate at which genetic variation can be generated within a population. Central to this idea is that just as traits such as body size and color are under selection pressure, so also is the rate at which the raw material for evolution (variation) can be generated [47]. The ability of a population to evolve and the speed at which this can take place, also needs to be thought of as a trait that is under selective pressure and may be altered or changed in any given environment, this trait being termed 'evolvability' [48]. The rate at which genetic variation is generated is under balancing selection, whereby in fluctuating or changing environments, the problems associated with the loss of genetic integrity that is produced by mutation, recombination and gene transfer, are outweighed by the demand for novelty. Novel traits may be advantageous for individuals in rapidly changing environments.

Humans provide a huge selective pressure on bacteria through the excessive use of antibiotics in both the medical and agricultural fields, resulting in strong selection for bacteria that acquire traits to avoid or withstand the deleterious effects of antibiotics. It is thought that humans may be inadvertently selecting for lineages of bacteria with a greater ability to evolve through increased rates of mutation and lateral gene transfer [49].

It may be that the sub-inhibitory concentrations of antibiotics that pollute areas surrounding human activity are affecting: (i) the rates at which bacteria can generate variation; and (ii) the

rates at which advantageous mutations fix in natural environments. However, there has been little or no empirical evidence to address these hypotheses.

In this thesis, I have passaged two species of *Pseudomonas* as single colony transfers, on media containing 1/10 the minimum inhibitory concentration of three different classes of antibiotics. In comparison to control lines, experimental lines showed changes to their genome, and phenotypic changes to colony morphology and antibiotic resistance traits. These results suggest that environmental pollution with antibiotics may be having significant effects on the genotypes and phenotypes of environmental bacteria.

Chapter 2

Exposure to sub-clinical concentrations of antibiotics induces genomic and phenotypic changes and promotes antibiotic resistance

This chapter is written as a manuscript for submission to Emerging Infectious Diseases and is formatted accordingly.

In these experiments, I show that passage of bacterial colonies on media containing sub-inhibitory concentrations of antibiotics induces genotypic and phenotypic change: including increased resistance. The implications of these results are clear: exposure of the environmental microbiome to antibiotic pollution will induce similar changes, including generating newly resistance species that may be of significant concern for human health.

Manuscript number: xxxx

Article Summary Line: Low concentrations of antibiotics, such as those polluting natural environments, can help generate resistance and threaten human health.

Running title: Effects of sub-inhibitory antibiotic concentrations

Keywords: antibiotic resistance, sub-inhibitory, antibiotic pollution, SOS response, evolution

Exposure to sub-clinical concentrations of antibiotics induces genomic and phenotypic changes and promotes antibiotic resistance

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Abstract

Antibiotics and the genetic elements that confer resistance to them are disseminated into environments via human waste streams and agricultural run-off. Where they can persist at low, but biologically relevant, concentrations. Antibiotic pollution establishes a selection gradient for resistance and may also be raising the frequency of events that generate resistance: point mutations; recombination; and lateral gene transfer. This study examined the response of bacteria to sub-inhibitory levels of antibiotics. *Pseudomonas aeruginosa* and *Pseudomonas protogens* were exposed to subclinical levels of the antibiotics kanamycin, tetracycline and ciprofloxacin. Bacteria were subjected to 1/10 the minimal inhibitory concentration (MIC) in a serial streaking experiment over 40 generations. REP-PCR monitored changes in DNA every five generations. Significant changes in the DNA finger prints of both species were noted. Experimental lines also displayed variant colony morphologies. The final MICs were significantly higher in some experimental lineages of *Ps. protogens* suggesting that 1/10 the MIC induces mutation and/or recombination events that can generate resistance.

Introduction

Antibiotic resistance has been identified as one of the greatest threats to human health for the 21st century by the World Health Organisation [1]. Overuse and misuse of antibiotics in the medical and agricultural sectors have contributed to the problem, and it is estimated that 70% of pathogens now exhibit resistance to at least one, if not more, antibiotics [2]. In most cases the risk of death is doubled if the individual is infected with a resistant strain of bacteria. In the United States in 2013, there were 23,000 confirmed deaths due to Antibiotic resistance (US CDC) and Europe reports 25,000 deaths per year (2007, ECDC).

The primary use of antibiotics is medicinal, where they are used to treat a range of bacterial infections. However, misuse and overuse of antibiotics are contributing to the development of antibacterial resistance. Incorrect prescription of antibiotics, unnecessarily high dosages and over-use are all promote resistance [3-6]. Antibiotics are also extensively used in agriculture and aquaculture to prevent disease and infection, and as a growth promoter [7, 8]. It has been estimated that 50-70% of antibiotics produced in the United States of America are used in agriculture [9, 10].

A relatively small amount of the antibiotics consumed by humans and animals are actually absorbed into the body, 30-90% of antibiotics pass through unchanged and are excreted into waste treatment facilities or into the environment[11]. Antibiotics, along with heavy metals, disinfectants and genes conferring resistance are disseminated into the environment via human waste streams, agricultural run-off [12] and effluent from antibiotic production factories [13, 14]. Although these are usually filtered through waste treatment facilities, the current mechanisms used to treat waste are often unable to remove these substances from water and the water is either reused as reclaimed water [15] or released into the environment via rivers [16, 17], estuaries and

the ocean [15, 18]. The release of these substances into the environment should be thought of as a significant component of soil and water pollution.

Many studies have investigated the effect of clinical levels, or inhibitory levels, of antibiotics on the generation of antibiotic resistance. However, there is increasing speculation that sub inhibitory levels of antibiotics may be having significant effects on bacterial populations. A gradient of antibiotic concentration forms around human activities. Within the human microbiome there may be a gradient along the digestive tract, while dissemination of antibiotics via waste water will cause a gradient of antibiotic concentration spreading outwards from human population centres.

Sub inhibitory levels of antibiotics are known to trigger the SOS response, a broad response to DNA damage that has been documented in many bacterial species. It may play a significant role in the generation of antibiotic resistance, as it can increase the rates of mutation and lateral gene transfer [19]. It is ultimately triggered by an increased occurrence of single stranded DNA resulting from DNA damage or inhibition of the processes involved in DNA replication. The SOS response is mediated by the LexA repressor. Under normal conditions, LexA prevents SOS genes from being expressed. Under stressful conditions, the protein RecA is recruited onto single stranded DNA where it stimulates cleavage of the LexA repressor, inactivating it and therefore allowing the expression of approximately 40 SOS genes. SOS genes are mainly involved in DNA repair. [19, 20].

It is well documented that lethal concentrations of antibiotics can induce the SOS response in bacteria [21, 22]. It has also been suggested that sub-inhibitory levels of antibiotics, as those discussed above, may be more relevant to the problem of antibiotic resistance than lethal concentrations of antibiotics [4, 20, 23]. Lethal concentrations exert a strong selective pressure

on bacteria whereby they either die or they acquire mutations allowing them to survive. When exposed to sub-inhibitory levels of antibiotics, most bacteria survive with little effect on growth, and the SOS response is initiated. This, in turn, increases general rates of mutation and lateral gene transfer amongst all bacteria in a population. It is also thought that humans may be inadvertently selecting for lineages of bacteria with a greater ability to evolve through increased rates of mutation and lateral gene transfer [24].

It may be that the sub-inhibitory concentrations of antibiotics polluting areas surrounding human activity are affecting: (i) the rates at which bacteria can generate variation; and (ii) the rates at which advantageous mutations fix in natural environments. However, there has been little or no empirical evidence to test these ideas.

In this study, two species of *Pseudomonas* were passaged as single colony transfers on media containing 1/10 the minimum inhibitory concentration of three different classes of antibiotics. This experiment was designed to test the genotypic and phenotypic effect of realistic levels of antibiotic pollution.

Materials and Methods

Bacterial isolates

Isolates of two species were selected for this study: *Pseudomonas aeruginosa* strain PA14; and *Pseudomonas protegens* strain PF-5. *Pseudomonas* is an ideal study genus as it encompasses human and environmental bacteria and has been extensively studied. Both strains have been genome sequenced (Genbank: AY273869.1 GenBank: CP000076.1. [25, 26]). *Ps. aeruginosa* PA14 is an opportunistic bacterium that causes infections in hospitals and cystic fibrosis patients. *Pseudomonas protegens* PF-5 (formally *Pseudomonas fluorescens* PF-5) is a common soil bacterium studied for its potential biocontrol properties [27].

Pseudomonas aeruginosa PA14 was obtained from Professor Joyce Loper, Oregon State University and *Pseudomonas protegens* PF-5 was obtained from Professor Ian Paulsen, Macquarie University. Bacteria were maintained on LB Agar plates (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride, 0.015% Agar) at 25°C. A second isolate of *Pseudomonas protegens* PF-5 was obtained that had been routinely maintained of 100µg/ml ampicillin, which is a common laboratory practice. This isolate was studied in order to determine whether maintenance on ampicillin affects the resistance of *Pseudomonas protegens* PF-5 and will be referred to as *Pseudomonas protogens* PF-5A. Single colonies were re-suspended in equal parts 30% glycerol and M9 salts and held at -80°C for long term storage.

Antibiotic treatments

Three antibiotics were selected for this study, each with different modes of action: kanamycin; tetracycline; and ciprofloxacin. Kanamycin is an aminoglycoside antibiotic which binds to the

30S ribosomal subunit and inhibits prevents protein synthesis [28]. Tetracycline is a polyketide antibiotic that is similar to kanamycin in that it binds to the 30S ribosomal subunit, however it prevents aminoacyl-tRNAs attaching to the ribosome, which in turn prevents addition of amino acids to growing polypeptide chains [29]. Ciprofloxacin is a second generation fluoroquinolone used to treat a broad spectrum of infections. It inhibits DNA gyrase, which in turn prevents DNA replication [30].

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined for each isolate against the three antibiotics following the methodology outlined in Weigand et.al. (2008) [31]. MICs were determined in microtitre trays containing a serial dilution of the relevant antibiotic in Luria-Bertani medium (0.01% tryptone, 0.005% yeast extract, 0.005% sodium chloride). Wells were inoculated with bacteria that was prepared from an overnight culture and diluted to an optical density of 0.01. The concentration of antibiotic in test wells ranged from 32mg/L to 0.0156mg/L for ciprofloxacin and 512mg/L to 0.0156mg/L for tetracycline and kanamycin. A growth control containing only the suspension of bacteria and a sterility control containing only medium were included on each plate. Plates were incubated at 25°C for 24hr and then the optical density was read on a Pherastar FS spectrometer at 540 nm. Relative optical density was plotted against antibiotic concentration to determine the MICs.

To determine statistical significance of differences in MIC, a one way analysis of variance (ANOVA) was performed. Growth data were expressed as the ratio of growth in the presence of antibiotics against growth in the control. This standardised the data prior to the ANOVA

153 *DNA extraction*

154 DNA was extracted from bacterial cultures using a bead-beating method [32, 33]. Briefly, a
155 single, well isolated colony from an overnight culture was resuspended in a lysing matrix tube
156 with sodium phosphate buffer and MT buffer (MP Biomedicals) or with CLS-TC buffer (MP
157 Biomedicals). Preliminary testing indicated no significant difference between sodium phosphate
158 buffer and MT buffer and CLS-TC buffer, therefore CLS-TC buffer was used for the remainder
159 of the study as it was the more economical of the two (Appendix 1). Cells were physically lysed
160 by treatment in a FastPrep FP120 (BIO 101 Savant) machine for 30s at 5.5m/s before being
161 centrifuged in an Eppendorf 5417C, for 5 minutes at 14,000x g. Protein precipitation, binding
162 and washing of DNA, and subsequent elution in TE buffer were as previously described [32, 33].
163 Purified DNA was stored at -20°C.

164

165 *Repetitive Element PCR*

166 DNA fingerprints were generated using ERIC-PCR, REP-PCR or BOX-PCR [34, 35] with the
167 modifications outlined in Gillings and Holley (1997a, b [36]). One µL of DNA was mixed with
168 9µL of Genereleaser ™ (Bioventures Inc.) in a 0.5mL PCR strip tube, and heated on high for 7
169 min in a 650 W microwave oven with a microwave sink. Tubes were then held at 80°C for 5 min
170 in an Eppendorf Master Cycle Epigradient S PCR machine, before 40µL of PCR master mix was
171 mixed into each tube. The PCR master mix per reaction was as follows: 11 µL PCR water, 25 µL
172 GoTaq® white (Promega), 2.5 µL 25 mM $MgCl_2$, 0.5 µL 1 mg/ml RNAse, 1µL 50µM BOXA1R
173 primer. Negative controls containing Genereleaser™ only and water only were included in each

PCR. The appropriate PCR cycle was then performed (Table 1). BOX, ERIC and REP primers were synthesised by Sigma-Aldrich Inc.

Table 1: Thermal cycling programs and primers used to generate DNA fingerprints using Rep-PCR

Rep PCR	Primers	Thermal Cycle
BOX	5'CTACGGCAAGGCGACGCTGACG	94°C 3 mins 94°C 30s 52°C 30s 68°C 8 mins x 35 68°C 15mins 4°C hold
ERIC	ERIC1R: 5'ATGTAAGCTCCTGGGGATTCAC ERIC 2: 5'AAGTAAGTGACTGGGGTGAGCG	94°C 3 mins 94°C 30s 52°C 30s 68°C 8 mins x 35 68°C 15mins 4°C hold
REP	REPR: 5'TTCGCYGGCAAGCCRGCTCC REP F: 5'GGCTTGCCRGCGAARRGGCC	94°C 3 mins 94°C 30s 65°C 30s 72°C 8 mins x 35 72°C 15mins 4°C hold

ERIC sequences Hulton et al (1991) [58], BOX sequences Martin et al (1992) [59]

182 *Agarose Electrophoresis*

183 PCR products were separated on 2% agarose gels poured in Tris-Borate-EDTA (TBE) buffer
184 [37]. DNA samples were loaded with one quarter volume of bromophenol blue loading dye
185 (0.45M Tris-borate, 0.01 EDTA, 40% sucrose, 0.25% bromophenol blue). A 100 base pair ladder
186 (Crown Scientific) was included on each gel. Gels were run in Tris-Borate-EDTA (TBE) at 110
187 volts for 50 to 80 min. Gels were stained with GelRed ™ (Biotium) and DNA visualized under
188 UV light. Gel images were captured using a Gel logic 2200 PRO camera and Carestream MI
189 computer software.

190

191 *Serial Plating Experiments*

192 A single colony of each species was used to inoculate a series of triplicate plates: control LB agar
193 plates; LB plates containing 1/10 the MIC for kanamycin; LB plates containing 1/10 the MIC for
194 tetracycline; and LB plates containing 1/10 the MIC for ciprofloxacin. Plates were incubated at
195 25°C for 48 hr, referred to here, for convenience, as one generation.

196 After incubation for 48hr, a single well-separated colony from each plate was used to continue
197 the serial plating. After five generations, three single colonies were randomly selected from each
198 plate for DNA extraction and PCR analysis, the first of these three would also be used to
199 continue the serial plating. Repetitive Element PCRs were carried out to monitor changes in
200 DNA patterns and to monitor for possible contamination of the cultures.

201

Table 2: Concentrations of antibiotics used in serial plating experiments

Bacterial species / Antibiotic	PA14	PF5	PF5A
Kanamycin	25.6 mg/L	0.8 mg/L	0.8 mg/L
Tetracycline	25.6 mg/L	25.6 mg/L	25.6 mg/L
Ciprofloxacin	0.0125 mg/L	0.025 mg/L	0.025 mg/L

DNA banding analysis

Images captured of the gels were analysed to identify changes in the banding patterns, indicative of changes in the genome of the sample. Changes were scored against a control profile to calculate the similarity coefficient (F) using the formula devised by Nei and Lei (1979) [38]:

$$F = 2N_{xy} / (N_x + N_y)$$

Where Nx and Ny are the number of bands in lane x and lane y respectively and Nxy is the number of bands that lane x and lane y share. Samples with an F value of 1 are identical while a value of 0 indicates no similarity. Scoring of the bands was carried out blind by an individual not involved in the Rep-PCR process to remove the possibility of bias. The F-values for antibiotic treatments were plotted as a scatter graph to illustrate the spectrum of variation.

Changes in Colony Morphology

To examine colony morphology at the end of the experiment, colonies of all lines from generation 40 were streaked onto LB agar plates and incubated for 48 hr at 25°C. Images of single colonies were captured using a Motic BA300 compound microscope with a 4x lens,

mounted with a Moticam 2 2.0MP camera and were analysed using DigiLabII-C and Motic Images Plus 2.0 computer programs.

Results

Colony morphology changes

Images captured of colonies at generation 40 show significant morphological changes between treatment groups. The three control lines of *Ps. aeruginosa* PA14 displayed no significant changes, kanamycin line 2, tetracycline lines 2 and 3, and ciprofloxacin line 3 exhibited significant changes to their colony morphology (Fig. 1a). The three control lines of *Ps. protegens* PF-5 displayed little change, kanamycin line 3 and tetracycline line 3 exhibited significant morphological differences. The three ciprofloxacin lines were relatively unchanged (Fig. 1b). The three control lines and three tetracycline lines of *Ps. protegens* PF-5A had similar colonies. All three kanamycin lines had significantly changed colonies, as had lines 2 and 3 of the ciprofloxacin treatment (Fig. 1c).

Detectable genome changes

BOX, ERIC AND REP-PCRS were carried out to detect genome changes. The basis of these PCRs is explained in Gillings & Holley (1997) [36], but, in brief, relies on amplification of regions between two random, but reproducible priming sites. Consequently, amplicons are sensitive to mutations in the priming sites and indels across the amplified regions. After testing both species with ERIC, REP and BOX primers, BOX-PCR was determined as the best method to examine changes. BOX-PCRs every five generations showed changes in the banding patterns

242 in the samples exposed to 1/10 MIC antibiotics while the control lines remained the same
243 indicating that the changes were due to antibiotic exposure (Fig. 2). A scatter plot of the f-
244 statistic highlighted the uniformity of control lines and the variation seen among antibiotic
245 treatment groups, both species that were exposed to 1/10 MIC of tetracycline and ciprofloxacin
246 exhibited several variants while only *Ps. protegens* PF-5 that had been treated with kanamycin
247 showed variation (Fig. 3).

Figure 1a. *Pseudomonas aeruginosa* PA14 colony morphology at generation 40

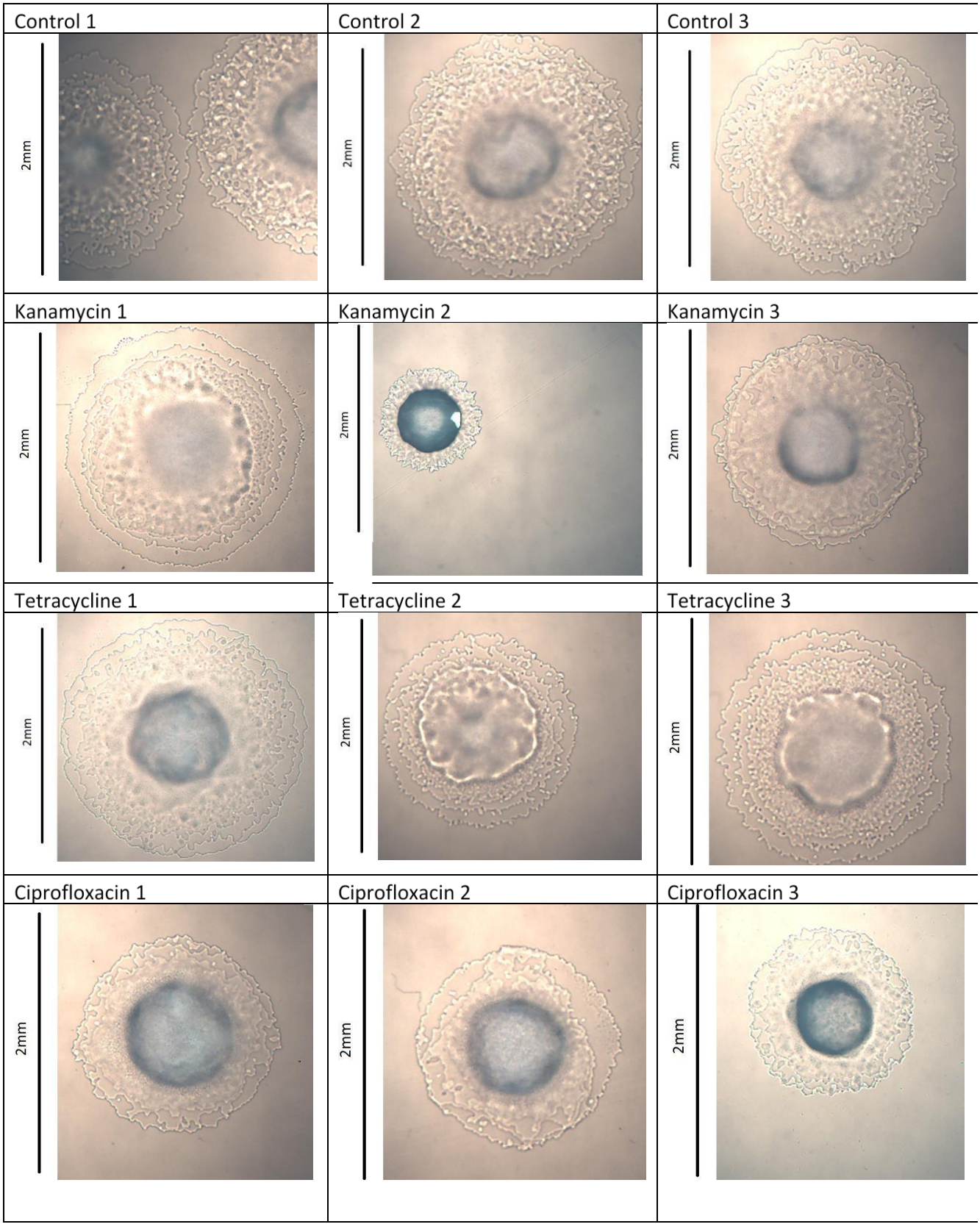


Figure 1b. *Pseudomonas protegens* PF-5 colony morphology at generation 40

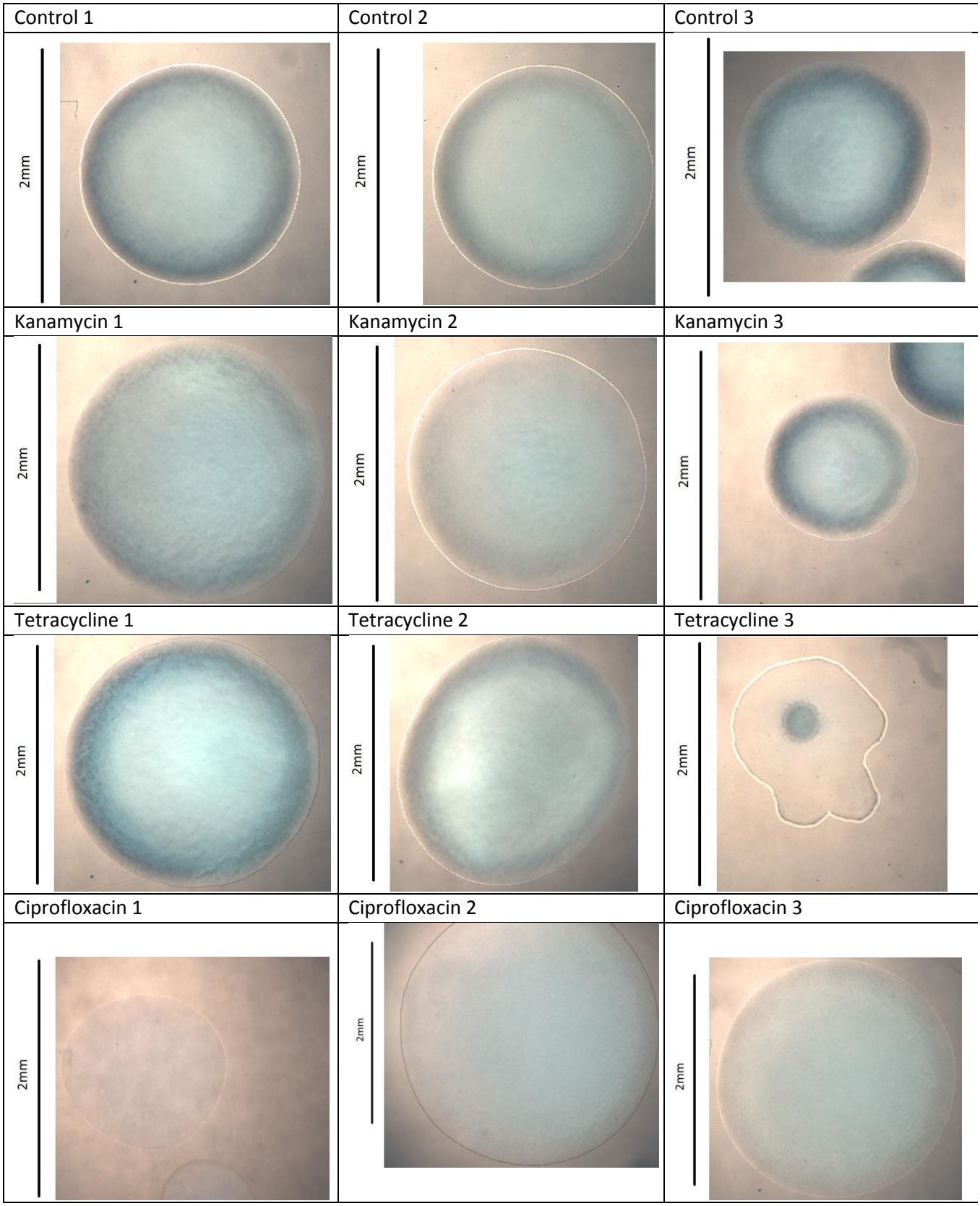


Figure 1c. *Pseudomonas protegens* PF-5A colony morphology at generation 40 245

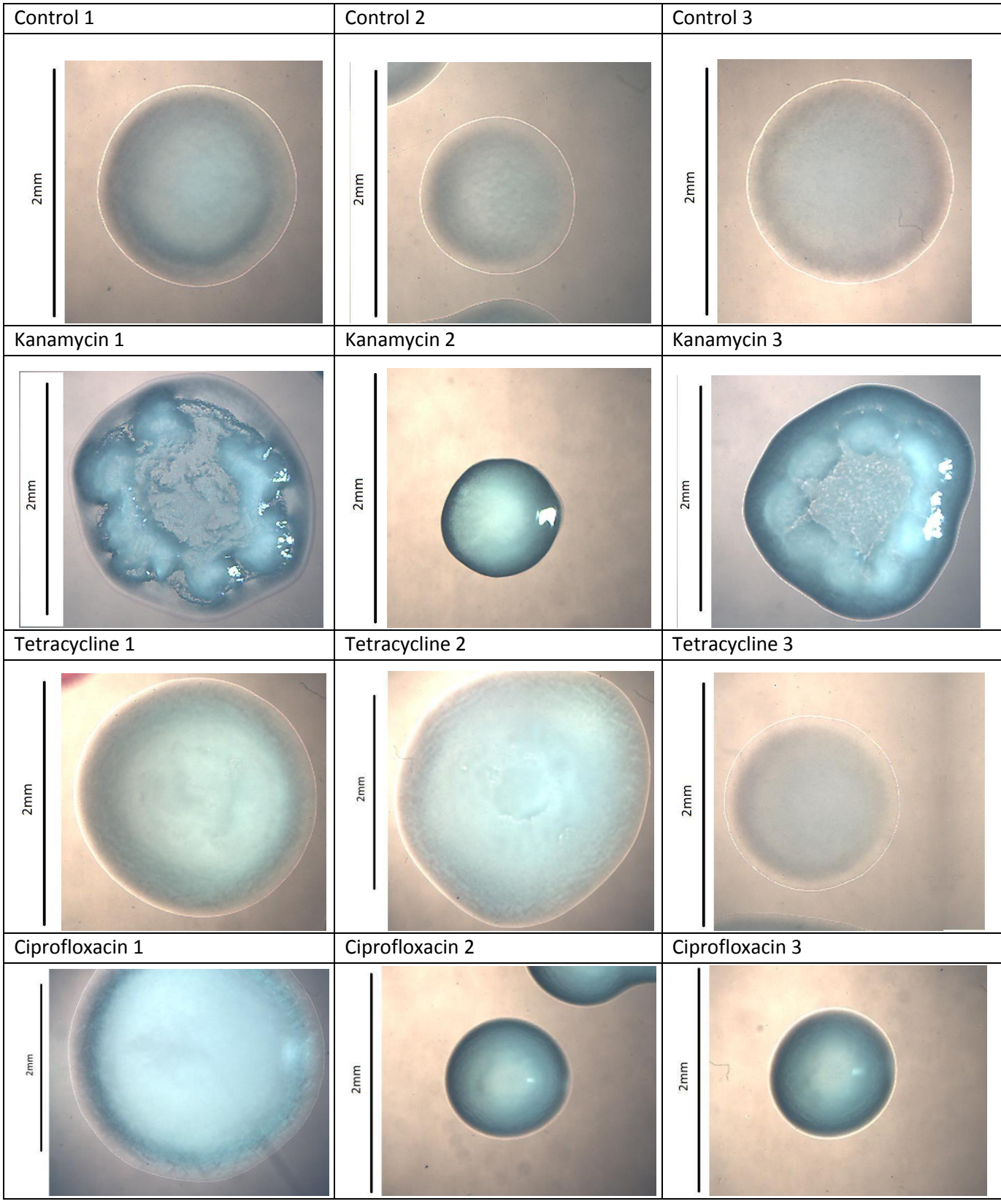
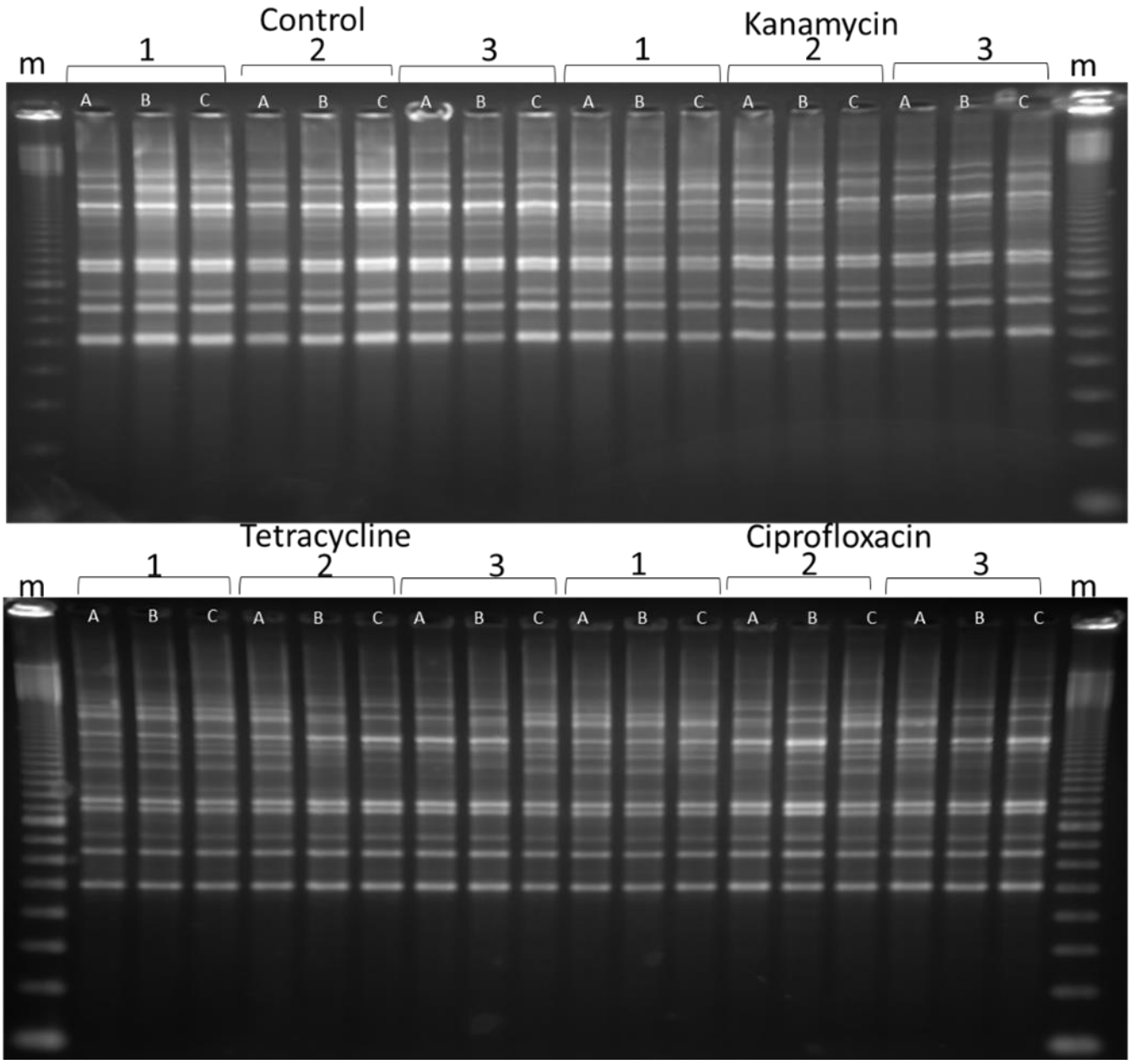


Figure 2. A representative sample of BOX-PCR products. BOX-PCR was performed on generation 40 Ps. Protegens PF-5. Lanes are labelled as follows: m=100bp ladder. Antibiotic treatments are noted as independent lines within each treatment (1, 2, or 3). Three colonies were tested from each line. For further examples see appendix 2.



251

MIC changes

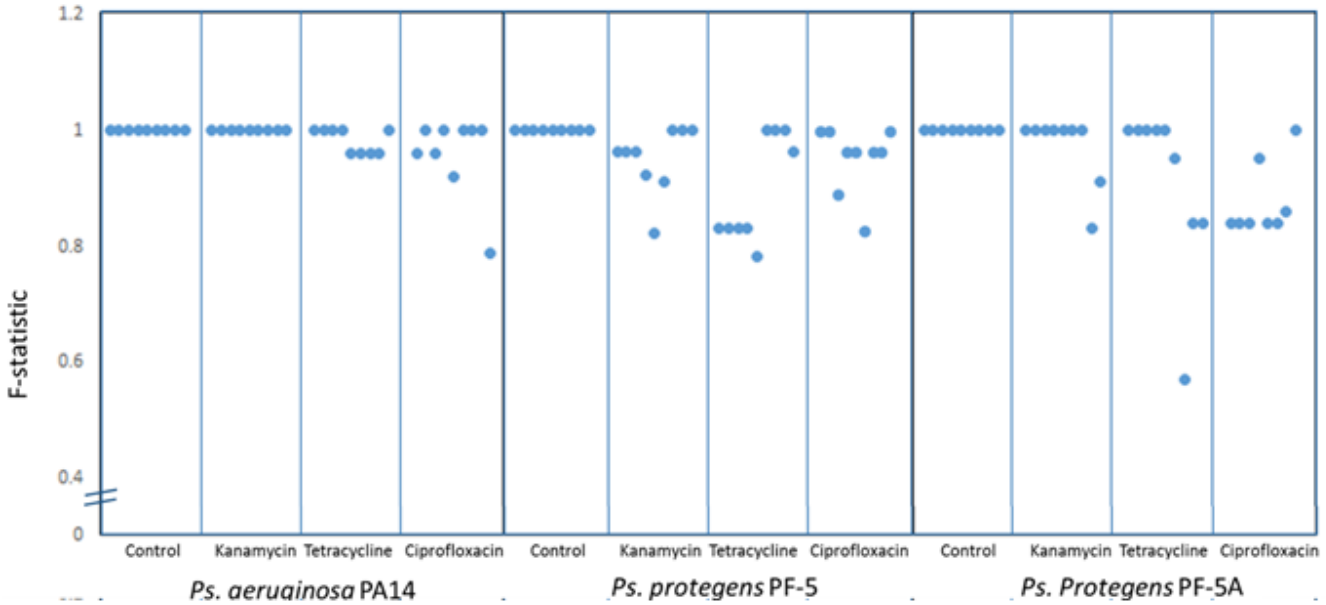
The MIC of each line was determined in order to detect significant differences in MICs from the control line. There were no significant differences in the MIC of *Ps. aeruginosa* PA14 for any of the treatment lines.

There were some significant differences in the MIC of *Ps. protegens* PF-5 and *Ps. protegens* PF-5A. A representative sample of MIC graphs are displayed in Figure 4. Figure 4a demonstrates the MIC of ciprofloxacin for *Ps. protegens* PF5, one line of *Ps. protegens* PF5 that had been exposed to 1/10 the MIC of ciprofloxacin over the serial plating experiment exhibited a 10-fold increase of MIC ($DF= 11$, $F\text{-value} = 11.94$, $P<0.0001$). A similar phenomenon was seen in *Ps. protegens* PF5 (Fig. 4b) and *Ps. protegens* PF5A (Fig. 4c) when tested on Kanamycin, where all 6 lines that had been treated with kanamycin over the serial plating experiment had a 4-8 fold increase in MIC ($DF= 11$, $F\text{-value} = 1.96$, $P >0.05$ and $DF=11$, $F\text{-value} = 46.04$, $P<0.0001$ respectively).

264 Figure 3. Similarity co-efficient (F) of BOX patterns from experimental lines at Gen 40

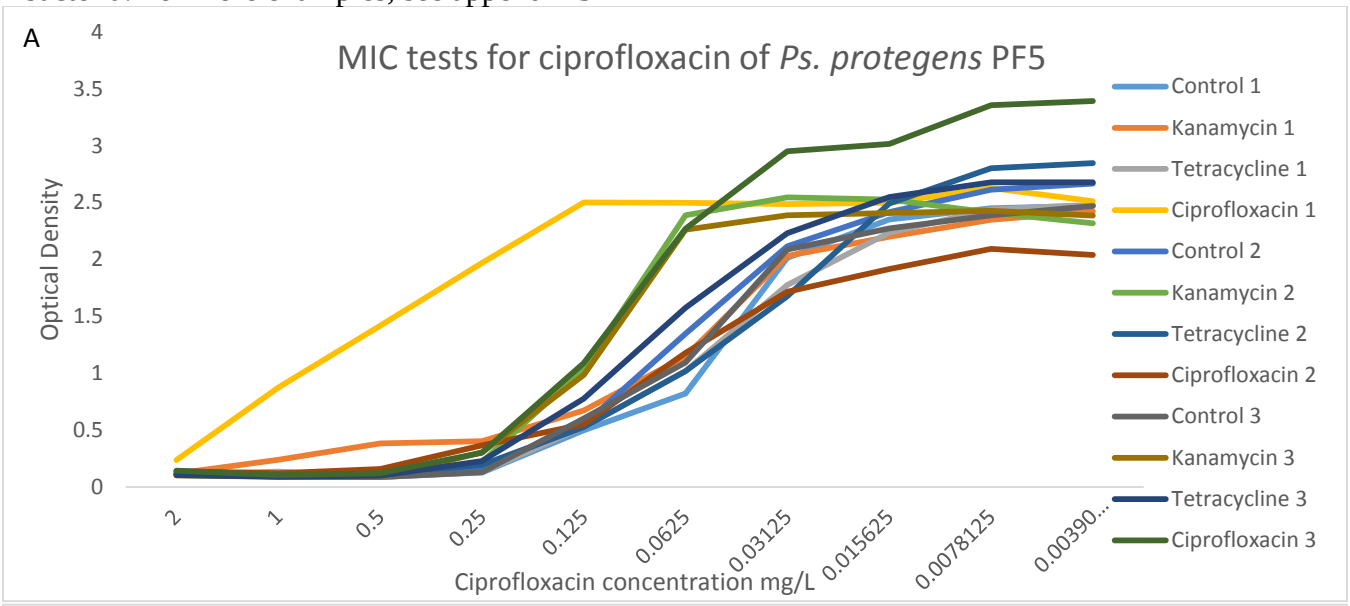
265 (F = 2Nxy / (Nx + Ny)) compared with control lines. Dots represent the average F-statistic of

266 three samples from each line and each box contains the 9 lines for each treatment.

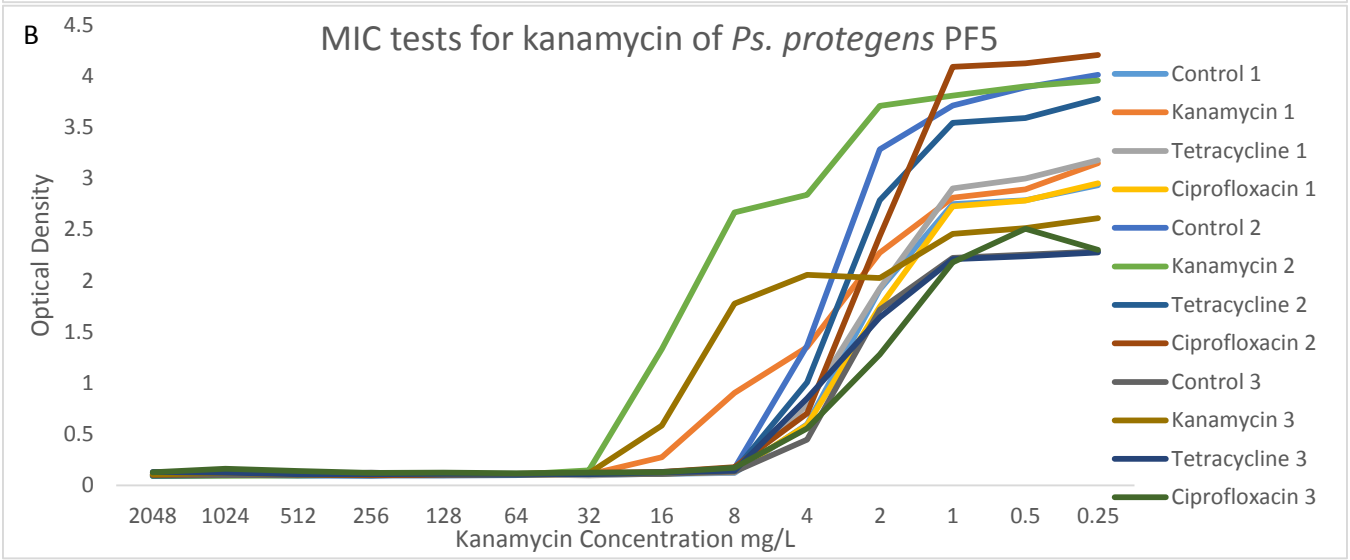


267

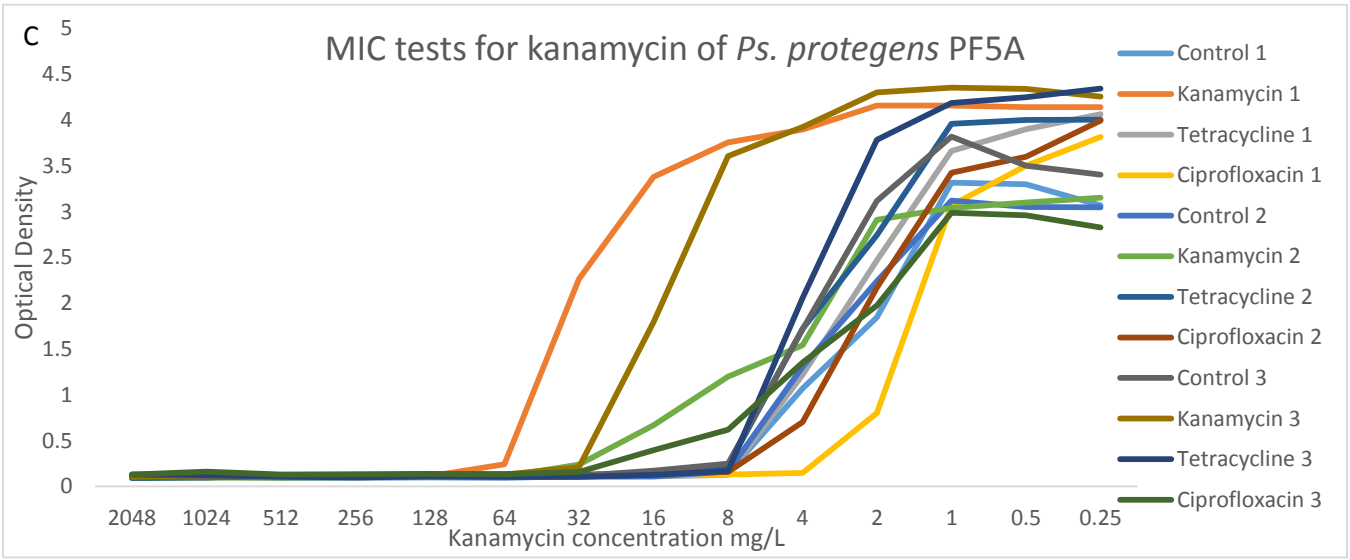
Figure 4. A representative sample of graphs displaying the MIC of selected antibiotics for the tested bacteria. For more examples, see appendix 3



268



269



270

Discussion

Exposure to 1/10 the MIC of the panel of antibiotics tested here had significant genotypic and phenotypic effects. Effects on the genomes were immediate and readily detectable. Even though BOX-PCR is a fairly insensitive measure of genetic variation, DNA fingerprint changes could be detected after only 5 serial transfers. By the end of the experiment, many of the lines exposed to 1/10 MIC exhibited significant changes in colony morphology. Perhaps of most significance, all six lines of Ps. protegens maintained on 1/10 MIC for kanamycin showed elevated kanamycin MICs by up to 8-fold. Similarly, one line held on 1/10 MIC for ciprofloxacin also showed an elevated ciprofloxacin MIC by 10-fold.

These findings suggest that similar phenotypic and genotypic changes will be occurring in all environments where antibiotics reach concentrations of 1/10 MIC, and that these effects will occur in all the environmental microbiomes. *Ps. protegens*, for instance, is a soil-dwelling environmental bacterium.

The concentrations of antibiotics used here may be typical of levels of antibiotic pollution. There is limited knowledge about the concentrations of antibiotics found in the environment however it is now known that antibiotics can persist in the environment longer than previously thought. The time that an antibiotic can persist in the environment differs depending on the class of antibiotic and the environmental conditions. Closed bottle tests provide a simple way to measure the biodegradability of antibiotics and indicate whether or not the antibiotic will readily degrade. Classes of antibiotics such as the β-lactams, tetracyclines, macrolides, lincosamides, penicillin, aminoglycosides, carbapenems, nitroimidazoles, polyene-antimycotics, quinolones, sulphonamides and glycopeptides have been found to persist over a 28 day testing period. [39, 40] High temperatures and exposure to UV light can cause degradation of some antibiotics.

Fluoroquinolone antibiotics can degrade in sunlight, however they are readily absorbed onto sediments, where they have been documented persisting up to 80 days with less than 1% of degradation [41]. It would be convenient if resistant organisms destroyed or inactivated antibiotics, however the mechanisms that usually allow resistance involve mutation of binding sites and efflux pumps meaning that the antibiotics are not physically altered and may persist in the environment [42]. Following one application of manure, antibiotics and antibiotic resistance genes can persist in the soil for approximately six months, depending on environmental conditions, during which time it could be dangerous to consume products that have had direct contact with the soil [43]. Given the significant time frame in which antibiotics can persist in the environment it is highly likely that they will exist at concentrations of, or close to, 1/10 the MIC.

The concentration at which antibiotics may occur in the environment is affected by several factors: substrate, proximity to source of antibiotics, environmental conditions and the antibiotics themselves. Testing of several rivers and some oceans have detected the presence of several antibiotic groups, most notably sulphonamides, quinolones which were found at high concentrations in a number of environments. Sulphonamides were detected in water (0.86 - 1563µg/L)[44-47] and quinolones were detected in sediments and plants (65.5-1166µg/kg and 8.37-6532 µg/kg respectively) [45]. The antibiotic concentration of 1/10 the MIC easily falls into the ranges of antibiotic pollution detected in several waterways which indicates that the results of this study are likely to be indicative of the rates of mutation and recombination that may be taking place in the environment.

Very small concentrations of common antibiotics can induce significant genotypic and phenotypic changes in bacterial species. Given the huge quantities of antibiotics that are entering the environment, it is likely that this antibiotic pollution is generating antibiotic resistant

organisms that may be a source of newly emerging opportunistic pathogens that will pose significant threat to human and animal life. Changes need to be made at every level of antibiotic use, from the individual, the medical practitioners, pharmaceutical companies and government monitoring and control, otherwise modern medicine is at a risk of facing a post antibiotic era where infections are harder and in some cases impossible to treat. Standard treatments for infections will no longer be sufficient, there will be a high risk of spread of infection and the risk death will be greatly increased.

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Chapter 3

Discussion and Major Conclusion

Exposure to 1/10 MIC of the antibiotics tested in this study had significant genotypic and phenotypic effects. It is likely that similar phenotypic and genotypic changes are occurring in all environments where antibiotic pollutants reach concentrations of 1/10 the MIC. Antibiotics are disseminated into the environment via a number of pathways and do not readily degrade allowing them to persist at varying concentrations. The concentration at which antibiotics may occur in the environment is affected by several factors: substrate, proximity to source of antibiotics, environmental conditions and the antibiotics themselves. Significant research has been done in China to monitor the levels of antibiotics in the environment as China is the world leader in antibiotic production and has been heavily impacted by the effects of multi drug resistant pathogens. In the Haihe River which runs through Beijing 10 out of 12 antibiotics tested for were detected, most notably sulphonamides which were present at high concentrations (24-385ng/L)[50]. Offshore waters of China in the Bohai and Yellow seas have 0.1-16.6ng/L of euythromycin, sulfamethoxazole and trimethoprim antibiotics [51] while sulphonamides were detected the freshwater Baiyangdian Lake (0.86-1563µg/L). Of further interest, Baiyangdian Lake sediment and plants appear to bioaccumulate quinolone antibiotics (65.5-1166µg/kg and 8.37-6532µg/kg respectively)[52]. High levels of quinolone antibiotics in sediment and plants is interesting as *Ps. protegens* PF-5 is a soil microorganism which is highly associated with numerous plant species. Quinolone antibiotics are a subset of fluroquinolone antibiotics of which ciprofloxacin belongs so it would be reasonable to suggest their response would be similar and as we have shown, ciprofloxacin at similar concentrations, can induce genomic and phenotypic changes to *Ps. protegens* PF-5. Antibiotics have also be detected in German rivers (0.2-2µg/L) [53] and Vietnamese rivers (7-360ng/L) [54] illustrating that antibiotic pollution is a global problem. The antibiotic concentration of 1/10 the MIC easily falls into the ranges of antibiotic pollution detected in several waterways which indicates that the results of this study are likely to be indicative of the rates of mutation and recombination that may be taking place in the environment.

When antibiotics at sub-inhibitory levels, such as 1/10 MIC are released into the microbiome they induce the SOS response of bacteria which in turn increases the rate of mutations that can

generate resistance and the propensity for lateral gene transfer. Mutations can transform environmental bacteria into pathogenic bacteria, likewise, through lateral gene transfer, previously harmless bacteria can acquire genetic material that generates pathogenicity which could pose threat to human health.

The use of antibiotics is continually placing selective pressure on bacteria and driving the generation and acquisition of genetic elements conferring resistance. Genetic diversity is generated by mutation or lateral gene transfer and the rates at which these mechanisms take place is under balancing selection, whereby in fluctuating or changing environments, the problems associated with the loss of genetic integrity that is produced by mutation and lateral gene transfer, are outweighed by the demand for novelty. Novel traits may be advantageous for individuals in rapidly changing environments. Under stable conditions, the generation of novel genetic elements is suppressed, however under stressful and unstable conditions, such as those created by antibiotics, it is likely that the basal rates of evolution will increase due to activation of the SOS response [55]. With continued exposure to antibiotics, it is likely that these increased basal rates will become fixed within populations and we will see lineages with higher basal rates of mutation and lateral gene transfer [49, 56]

Very small concentrations of common antibiotics can induce significant genotypic and phenotypic changes in bacterial species. Given the huge quantities of antibiotics that are entering the environment, it is likely that antibiotic pollution is generating antibiotic resistant organisms that may be a source of newly emerging opportunistic pathogens that will pose significant threat to human and animal life. Changes need to be made at every level of antibiotic use, from the individual, the medical practitioners, pharmaceutical companies and government monitoring and control, otherwise modern medicine is at a risk of facing a post antibiotic era. Further research in this study could extend to whole genome sequencing of the samples in order to score point mutations, transposition events, indels and/or recombination events. Following this, determination of the function of the gene mutations that occurred. It would be interesting to expand this study to encompass more species and different antibiotics.

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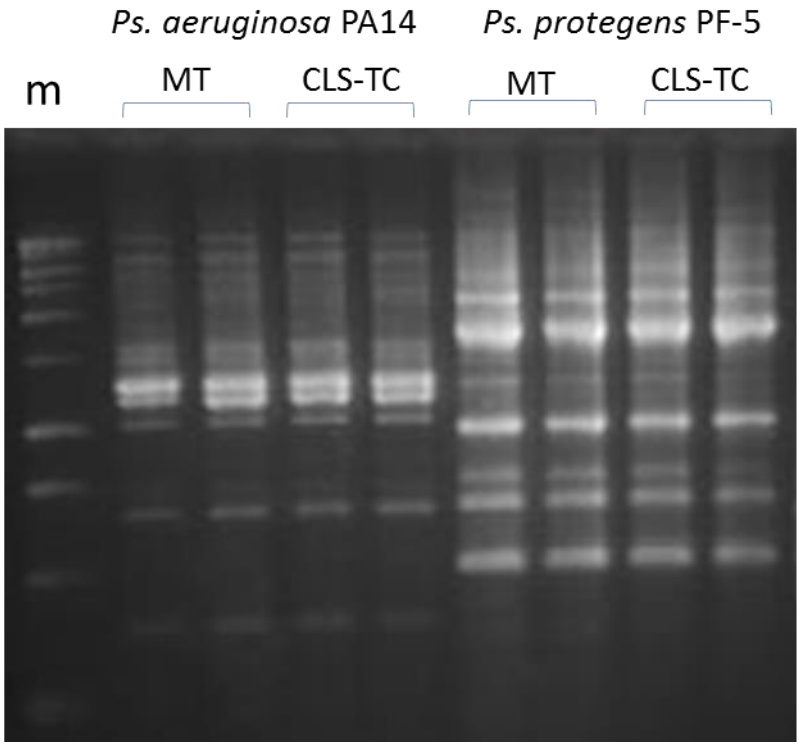
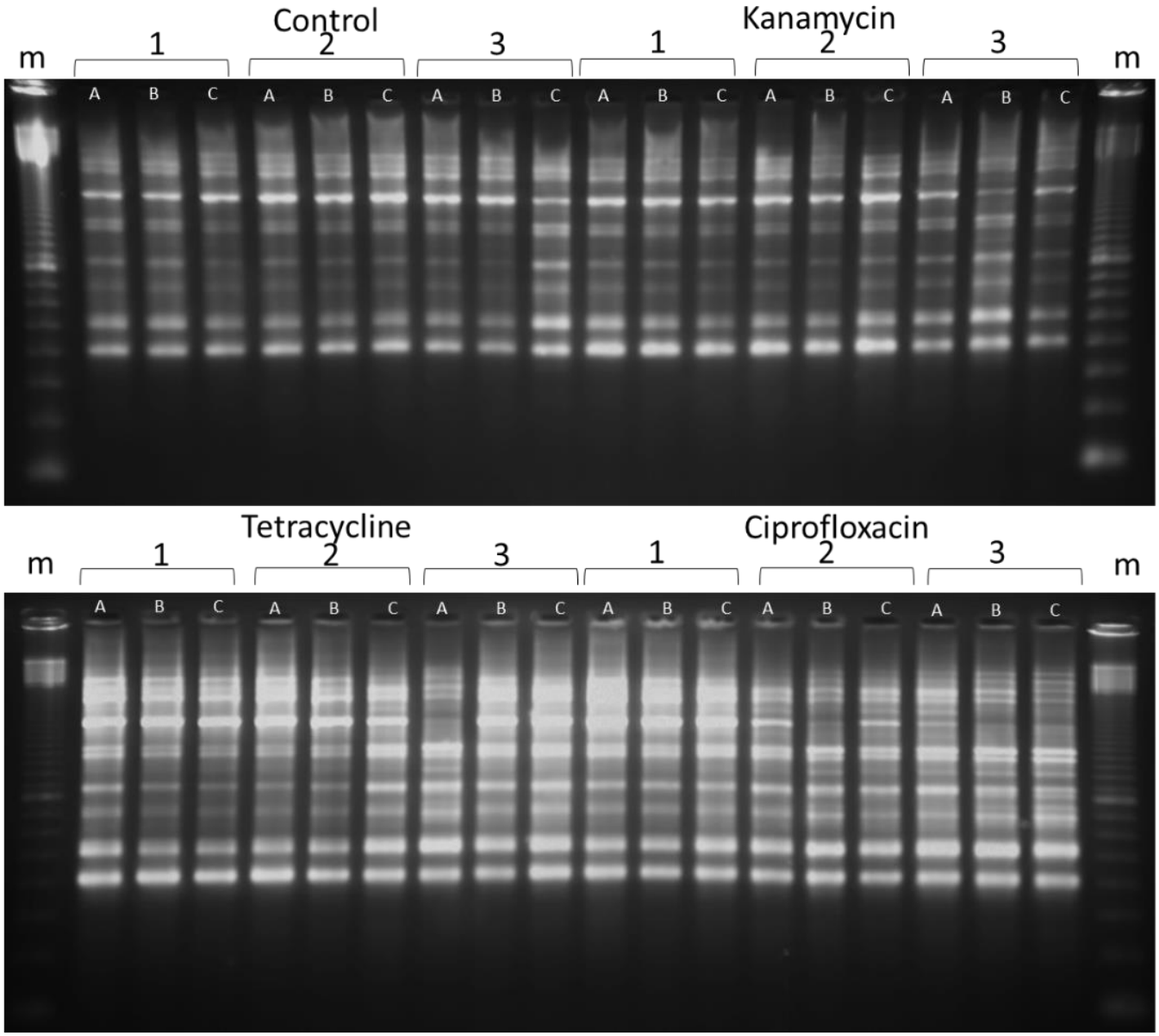
Appendices

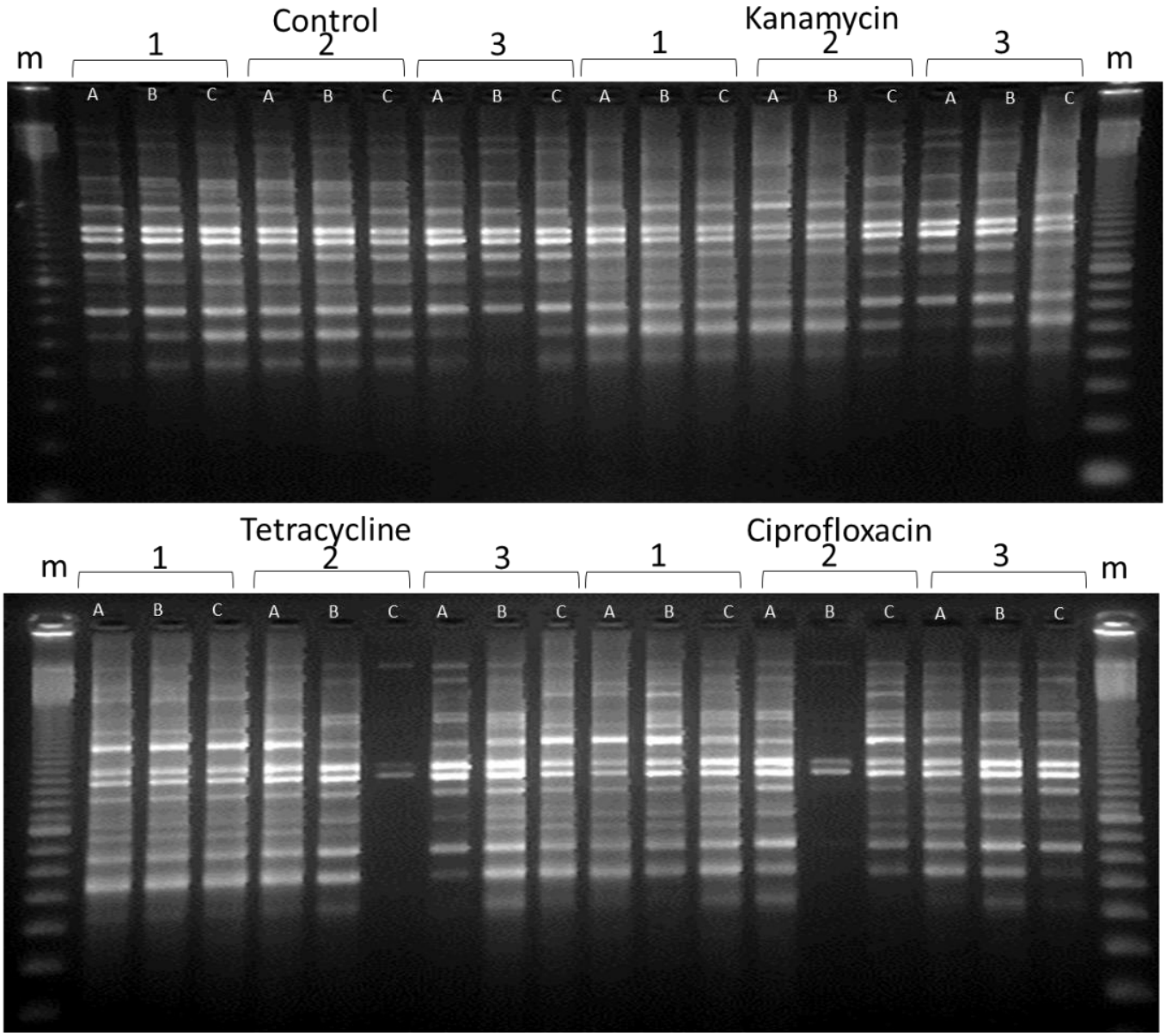
Appendix 1. BOX-PCR performed on Ps. Aeruginosa PA14 and Ps. Protegens PF-5 DNA extracted using two different methods: using MT buffer lanes 2, 3, 6 & 7 and using CLS-TC buffer lanes 4, 5, 8 & 9. Lane m is 100 base pair ladder. There is no significant difference between the two methods.



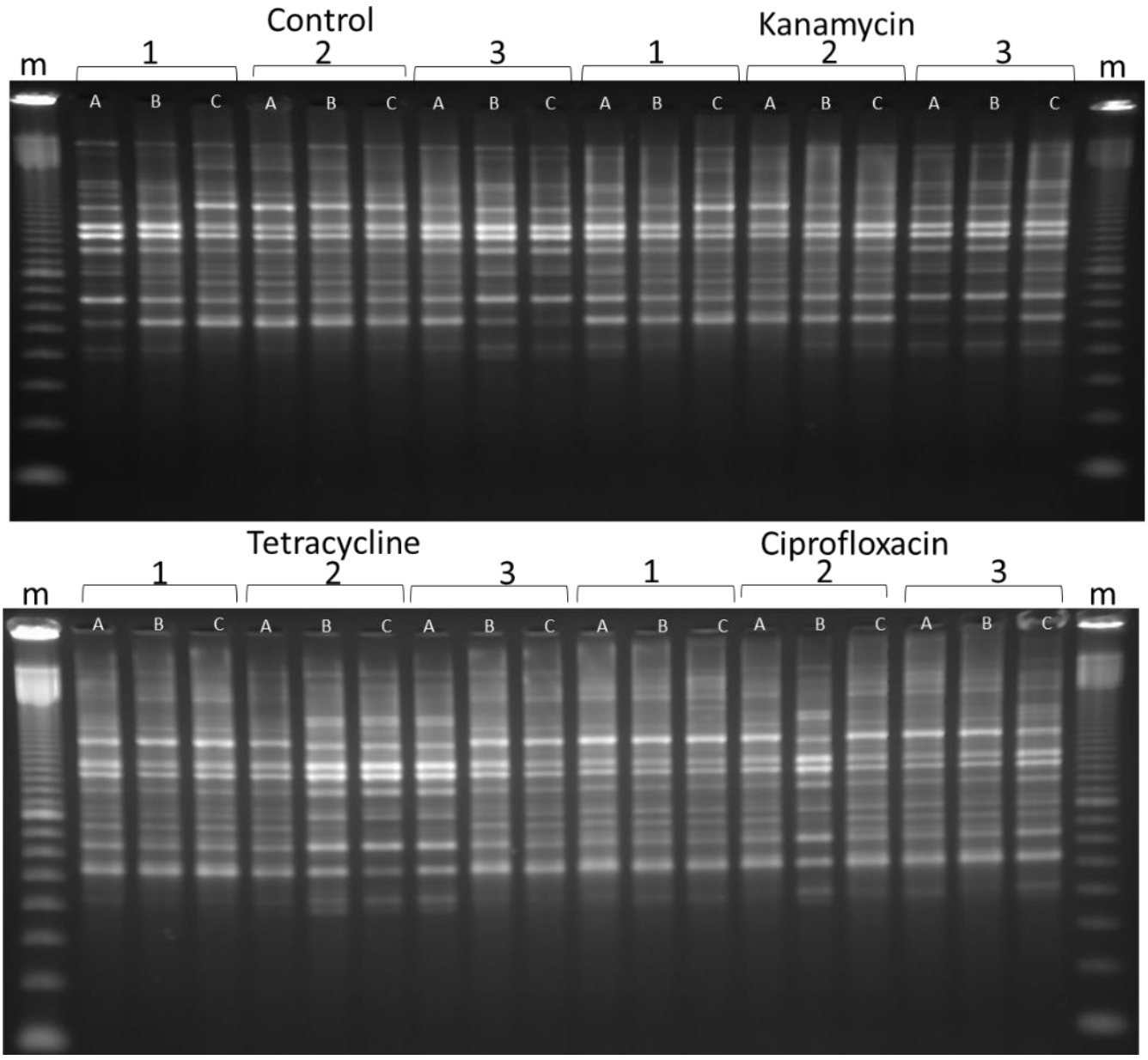
Appendix 2a. BOX-PCR of generation 5 *Ps. aeruginosa* PA14. Band m are 100bp ladder



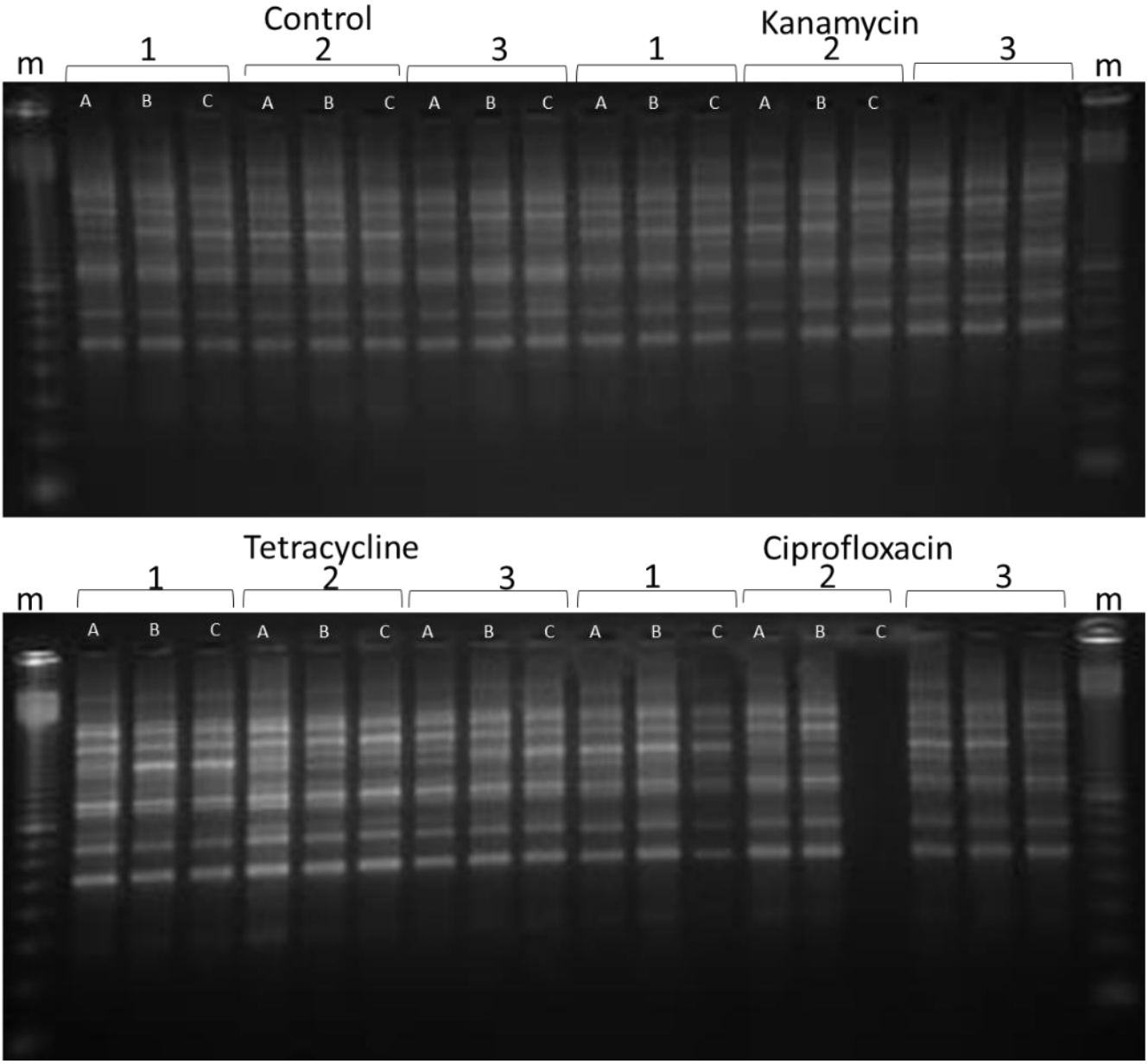
Appendix 2b. BOX-PCR of generation 20 *Ps. aeruginosa* PA14. Bands m are 100bp ladder



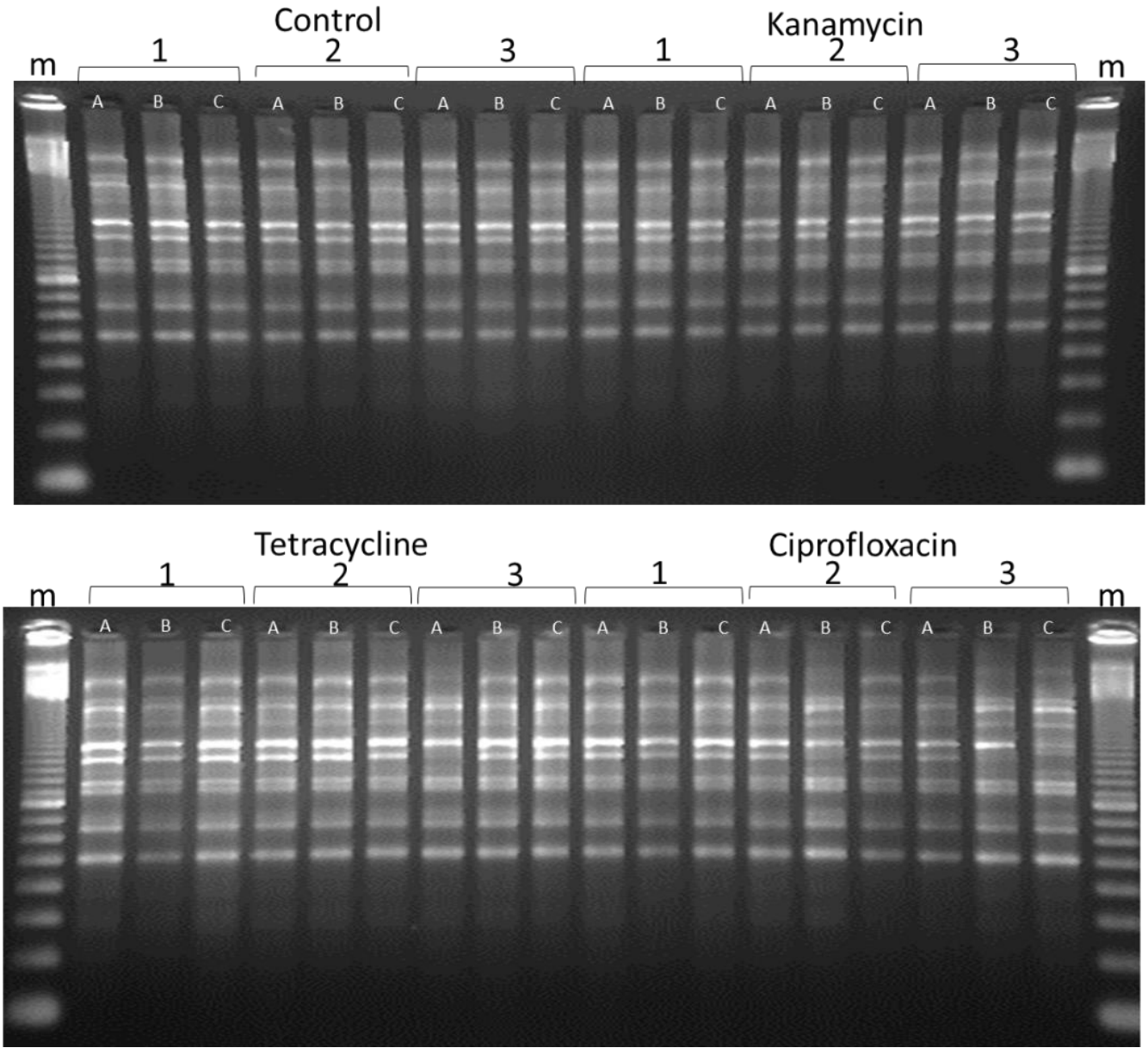
Appendix 2c. BOX-PCR of generation 40 *Ps. aeruginosa* PA14. Bands m are 100bp ladder



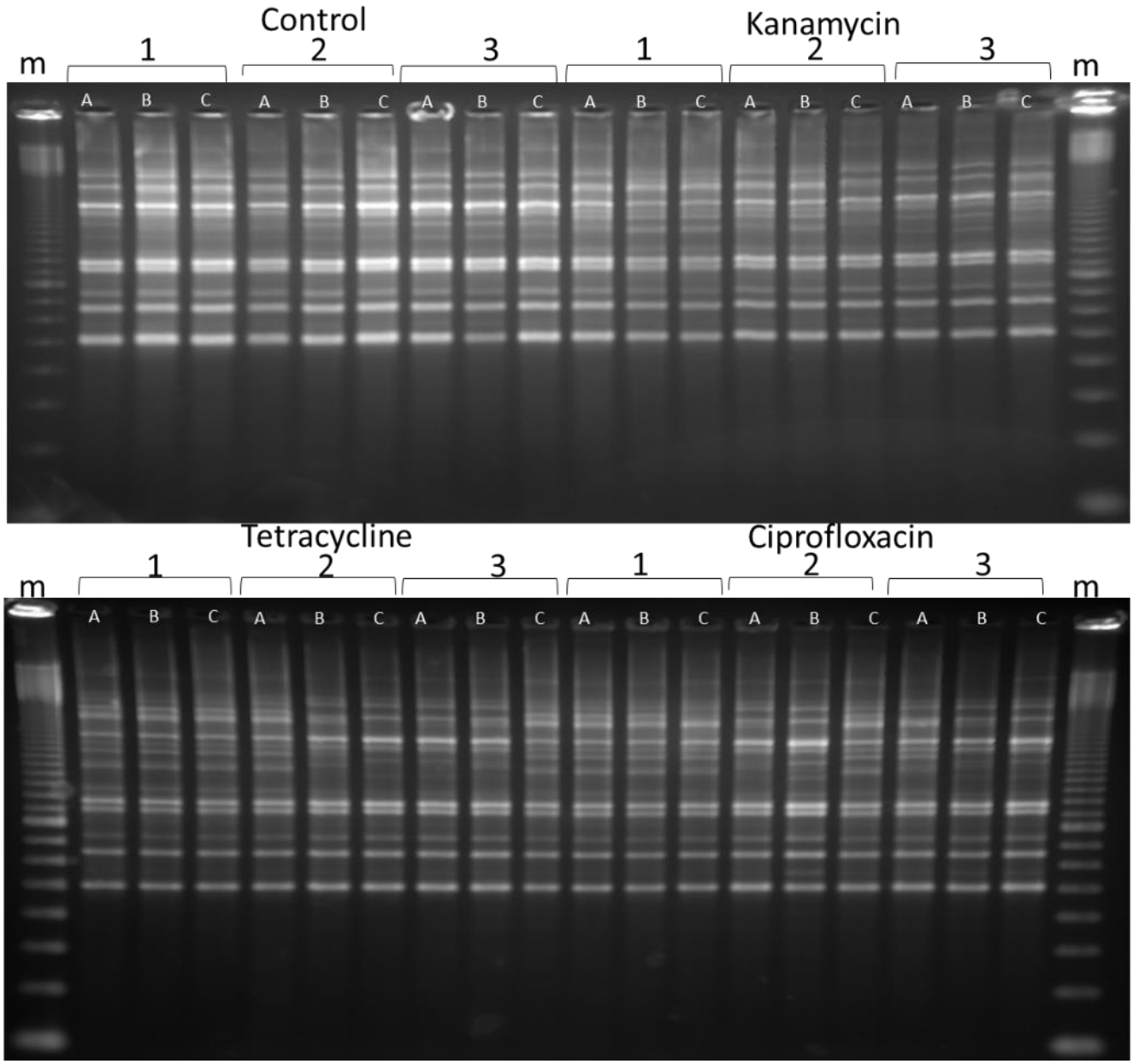
Appendix 2d. BOX-PCR of generation 5 *Ps. protegens* PF-5. Bands m are 100bp ladder



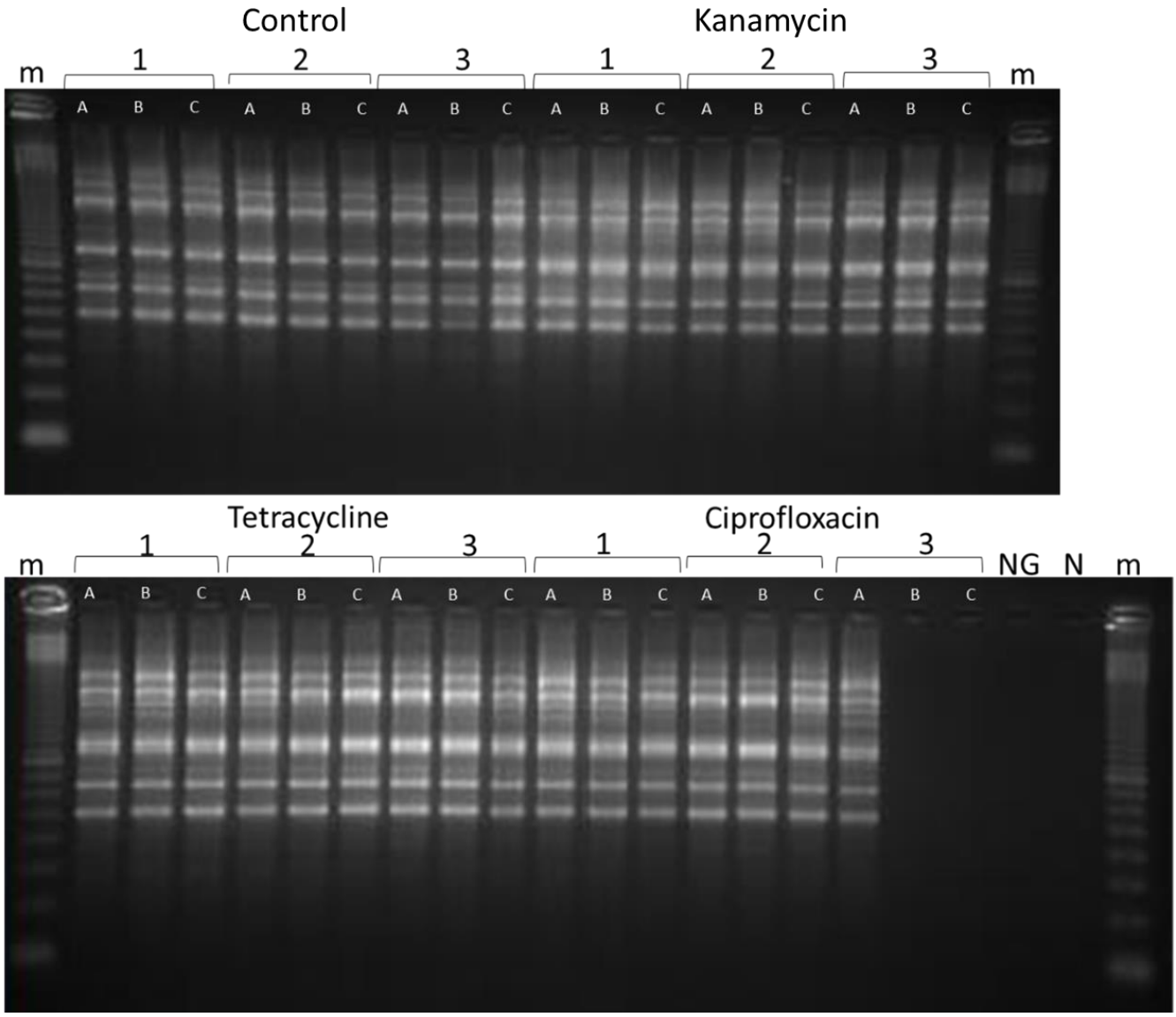
Appendix 2e. BOX-PCR of generation 20 *Ps. protegens* PF-5. Bands m are 100bp ladder



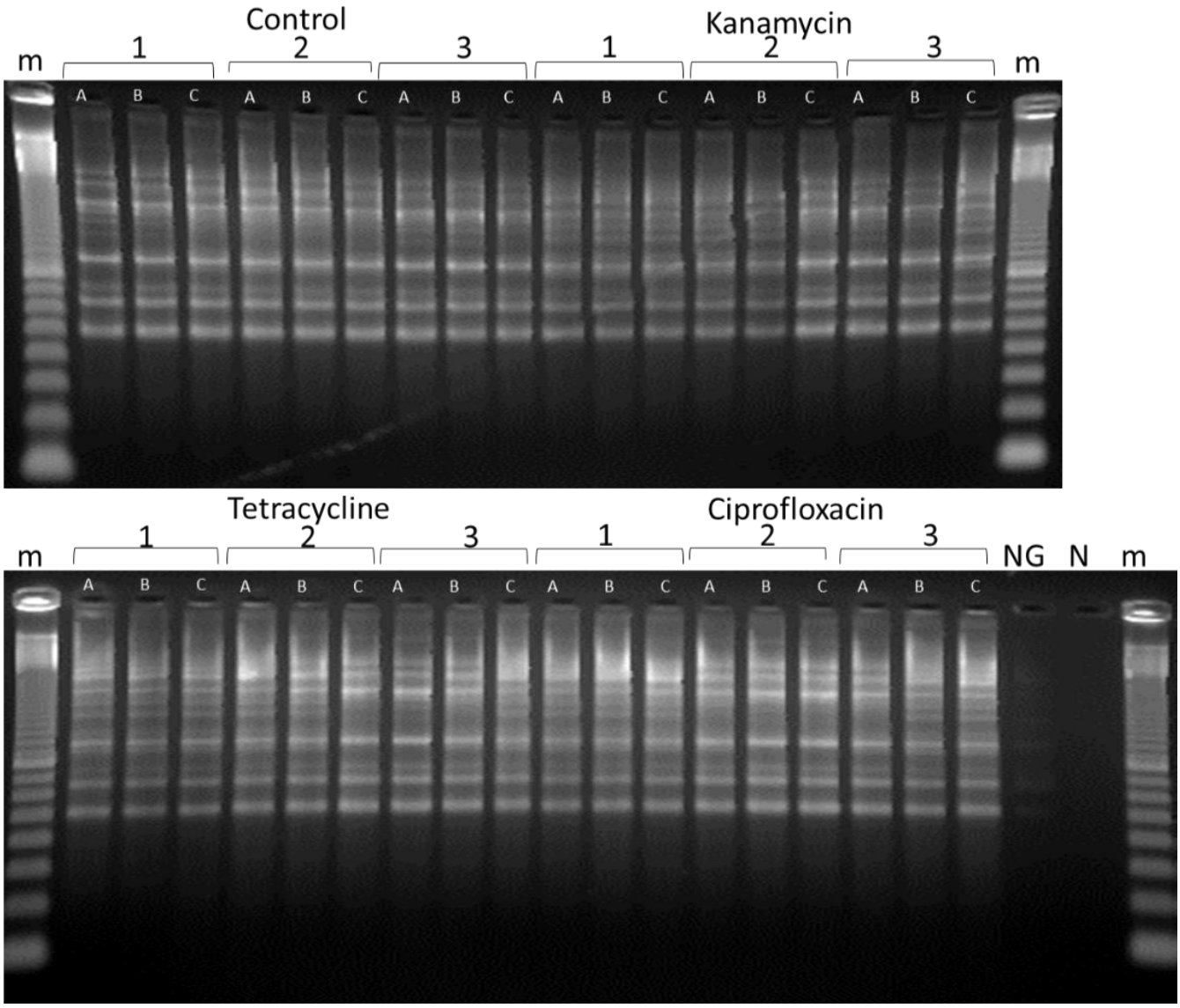
Appendix 2f. BOX-PCR of generation 40 *Ps. protegens* PF-5. Bands m are 100bp ladder



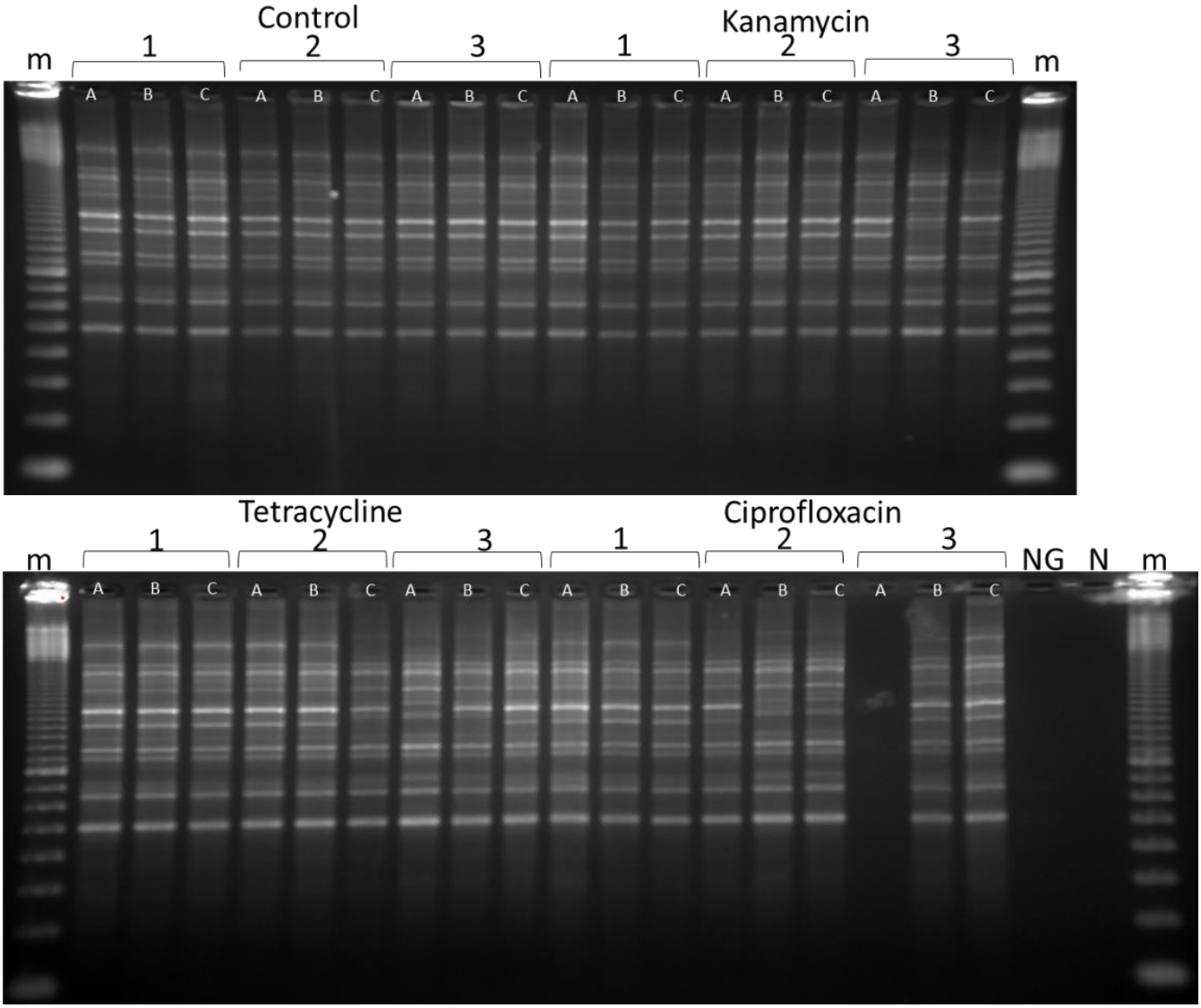
Appendix 2g. BOX-PCR of generation 5 *Ps. protegens* PF-5A. Bands m are 100bp ladder. The band marked NG is a negative control with genereleaser. The band marked N is a negative control without genereleaser



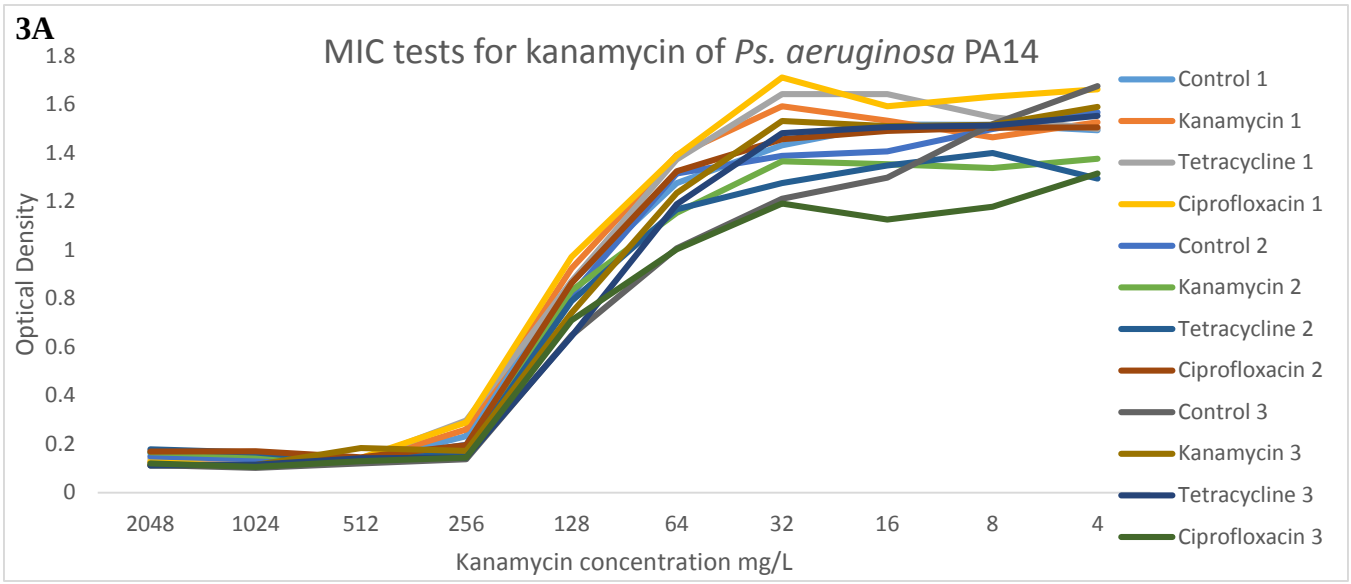
Appendix 2h. BOX-PCR of generation 20 *Ps. protegens* PF-5A. Bands m are 100bp ladder. The band marked NG is a negative control with genereleaser. The band marked N is a negative control without genereleaser

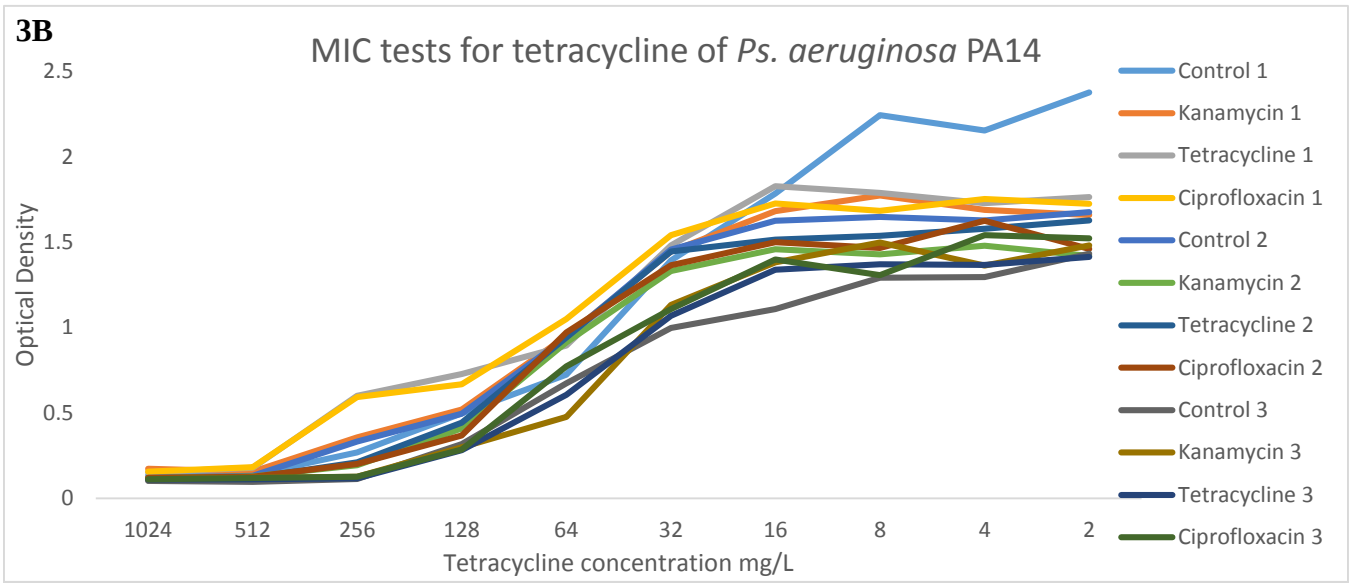


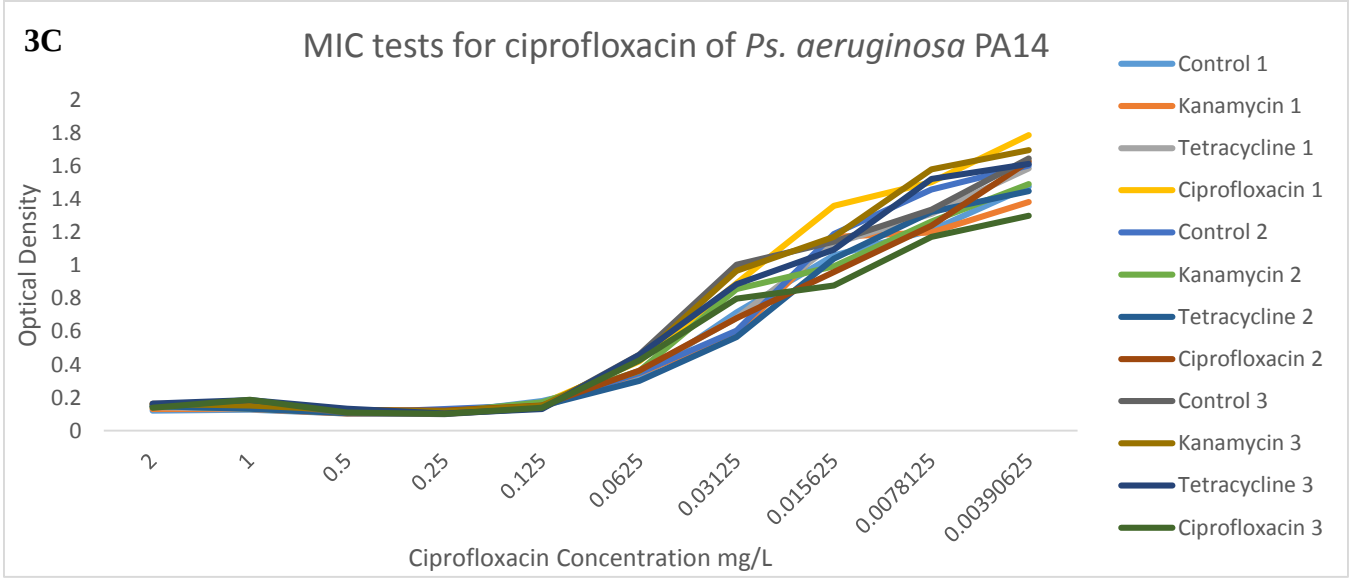
Appendix 2i. BOX-PCR of generation 40 *Ps. protegens* PF-5A. Bands m are 100bp ladder. The band marked NG is a negative control with genereleaser. The band marked N is a negative control without genereleaser



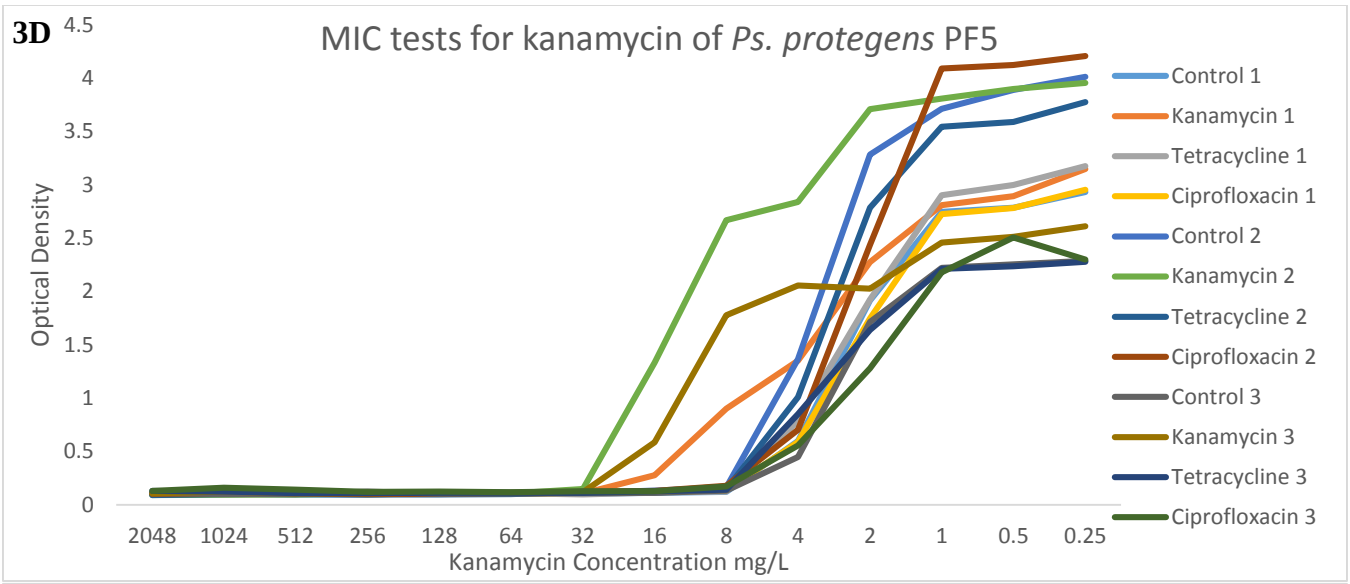
Appendix 3. MIC of antibiotics for the tested bacteria



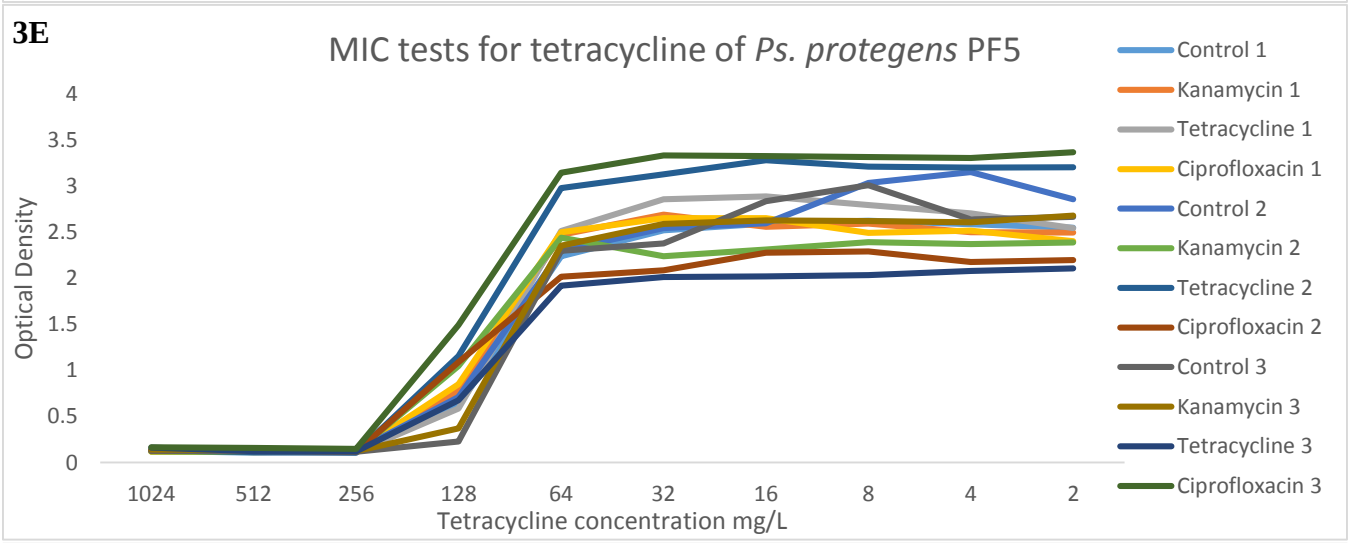




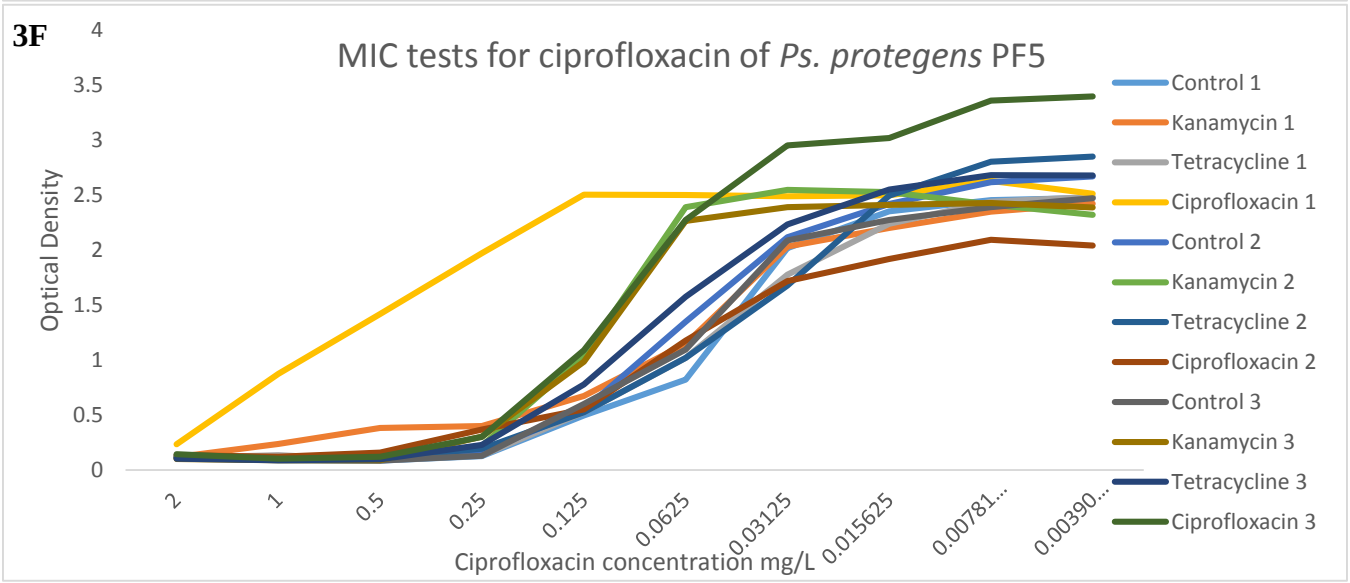
542


3D
MIC tests for kanamycin of *Ps. protegens* PF5
Optical Density
Kanamycin Concentration mg/L
2048 1024 512 256 128 64 32 16 8 4 2 1 0.5 0.25
Control 1
Kanamycin 1
Tetracycline 1
Ciprofloxacin 1
Control 2
Kanamycin 2
Tetracycline 2
Ciprofloxacin 2
Control 3
Kanamycin 3
Tetracycline 3
Ciprofloxacin 3

543

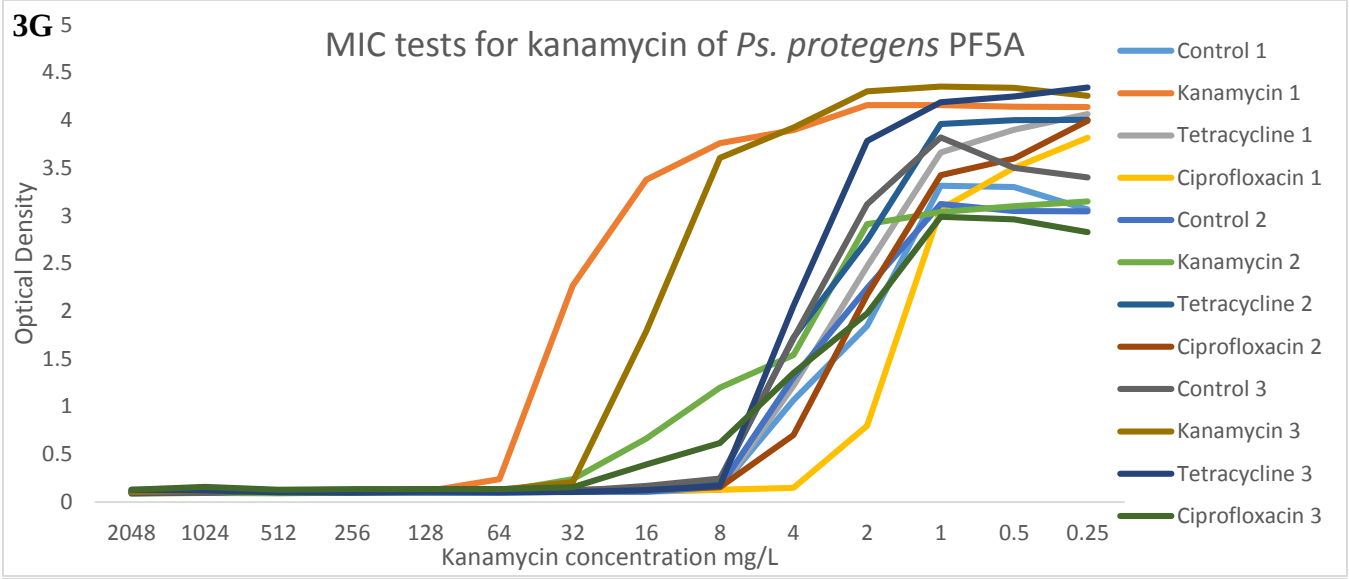

3E
MIC tests for tetracycline of *Ps. protegens* PF5
Optical Density
Tetracycline concentration mg/L
1024 512 256 128 64 32 16 8 4 2
Control 1
Kanamycin 1
Tetracycline 1
Ciprofloxacin 1
Control 2
Kanamycin 2
Tetracycline 2
Ciprofloxacin 2
Control 3
Kanamycin 3
Tetracycline 3
Ciprofloxacin 3

544

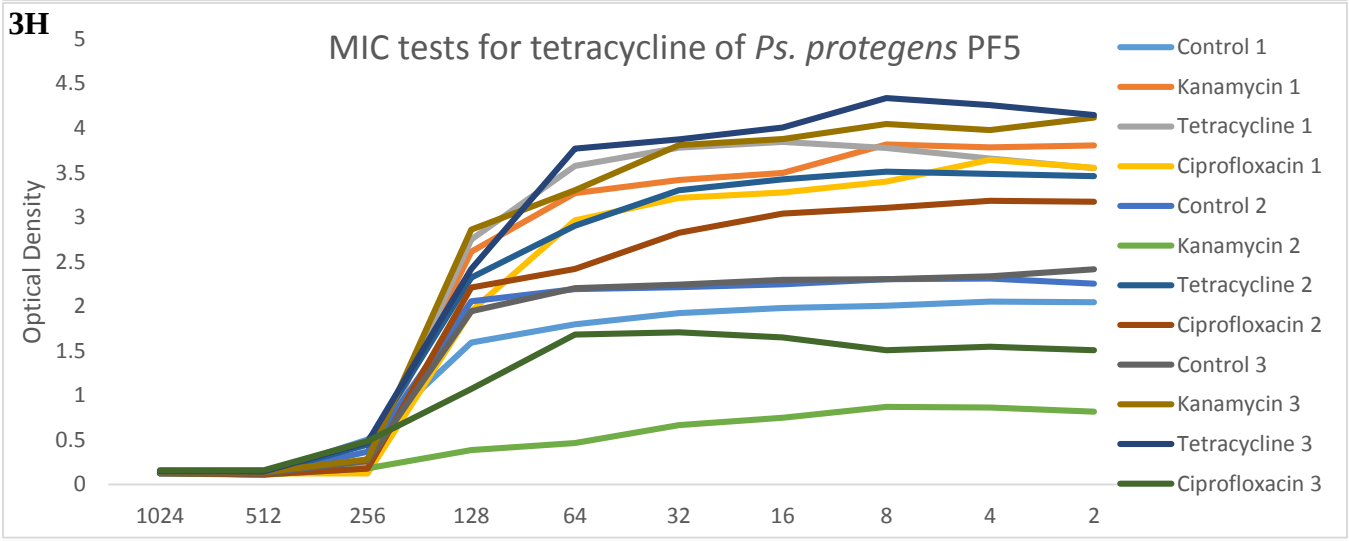

3F
MIC tests for ciprofloxacin of *Ps. protegens* PF5
Optical Density
Ciprofloxacin concentration mg/L
2 1 0.5 0.25 0.125 0.0625 0.03125 0.015625 0.00781... 0.00390...
Control 1
Kanamycin 1
Tetracycline 1
Ciprofloxacin 1
Control 2
Kanamycin 2
Tetracycline 2
Ciprofloxacin 2
Control 3
Kanamycin 3
Tetracycline 3
Ciprofloxacin 3

545

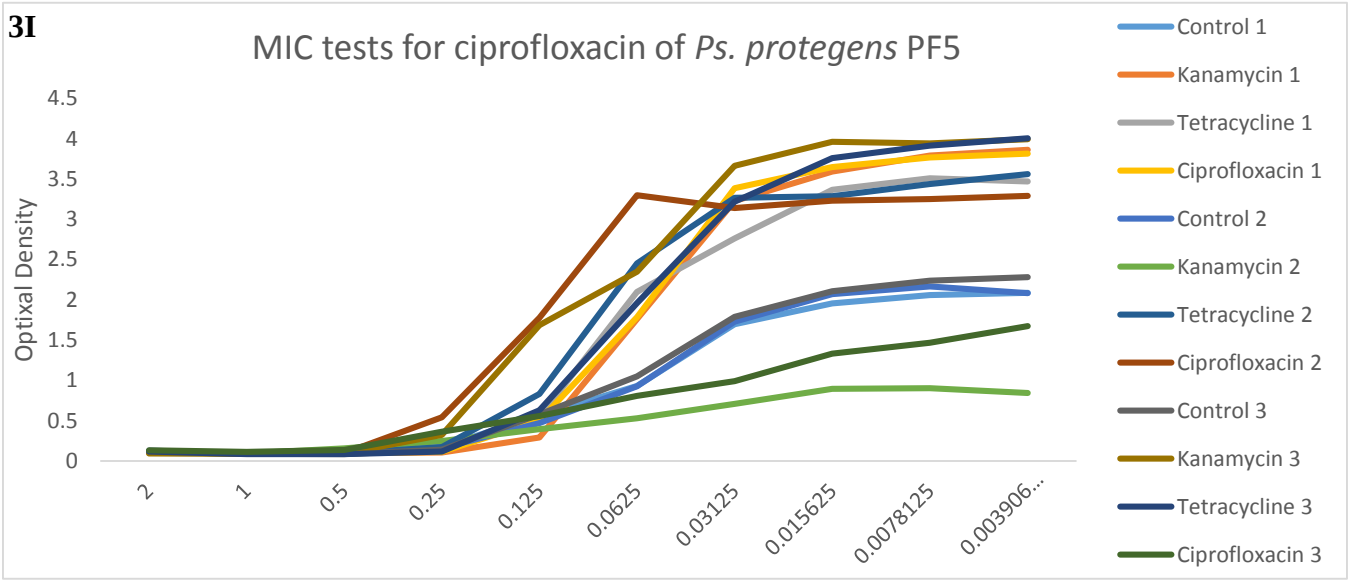
546


3G
MIC tests for kanamycin of *Ps. protegens* PF5A
Optical Density
Kanamycin concentration mg/L
2048 1024 512 256 128 64 32 16 8 4 2 1 0.5 0.25
Control 1
Kanamycin 1
Tetracycline 1
Ciprofloxacin 1
Control 2
Kanamycin 2
Tetracycline 2
Ciprofloxacin 2
Control 3
Kanamycin 3
Tetracycline 3
Ciprofloxacin 3

547


3H
MIC tests for tetracycline of *Ps. protegens* PF5
Optical Density
1024 512 256 128 64 32 16 8 4 2
Control 1
Kanamycin 1
Tetracycline 1
Ciprofloxacin 1
Control 2
Kanamycin 2
Tetracycline 2
Ciprofloxacin 2
Control 3
Kanamycin 3
Tetracycline 3
Ciprofloxacin 3

548


3I
MIC tests for ciprofloxacin of *Ps. protegens* PF5
Optixal Density
2 1 0.5 0.25 0.125 0.0625 0.03125 0.015625 0.0078125 0.003906...
Control 1
Kanamycin 1
Tetracycline 1
Ciprofloxacin 1
Control 2
Kanamycin 2
Tetracycline 2
Ciprofloxacin 2
Control 3
Kanamycin 3
Tetracycline 3
Ciprofloxacin 3

Acknowledgments

I would like to gratefully acknowledge the guidance and support I have received from my supervisor, Michael Gillings over the course of this study. Many thanks go to the EMMA lab, particularly Liette Vandine, for her support throughout the year and, Hilal Virinli and Marita Holley for all the help they have given me.

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