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Generation of Antibiotic Resistant Mutants in the Minimal Pathogen Mycoplasma Pneumoniae

Bryant De Jesus

GENERATION OF ANTIBIOTIC RESISTANT MUTANTS IN THE MINIMAL PATHOGEN *MYCOPLASMA PNEUMONIAE* M129-B7

A Thesis

by

BRYANT JONATHAN DE JESUS

Submitted to Texas A&M International University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

August 2019

Major Subject: Biology

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Approved as to style and content by:

Co-Chairs of Committee,	Ruby A. Ynalvez Keith D. Combrink
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ABSTRACT

Generation of Antibiotic Resistant Mutants
in the Minimal Pathogen *Mycoplasma pneumoniae* M129-B7 (August 2019)

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Co-Chairs of Committee: Ruby A. Ynalvez and Keith D. Combrink

Mycoplasma pneumoniae (MPN) causes community acquired pneumonia (CAP). The inability to identify *M. pneumoniae* infections through common clinical screenings has added to the global antibiotic resistance problem. MPN characteristically lacks a cell wall, which prevents proper diagnosis in an infected patient. This has led to the inappropriate prescriptions of antibiotics at insufficient dosages, allowing these microorganisms to thrive at sub-lethal concentrations. The small genome size of MPN, makes it a viable candidate for studying and developing tools to understand the genetic mechanisms behind the acquisition of antibiotic resistance by microbes. This study aimed to generate MPN mutants through prolonged exposure to 19 commercially available antibiotics. The *objectives* of this study were (1) to determine the minimum inhibitory concentration (MIC) of different antibiotic groups including macrolides, aminocyclitols, aminoglycosides, amphenicols, ansamycins, cephalosporins, fluoroquinolones, glycopeptides, ketolides, nitrofurans, and tetracyclines against *M. pneumoniae* (MPN) wild type strain M129-B7 and (2) to generate antibiotic resistant mutants through exposure of *M. pneumoniae* M129-B7 to constant sub-inhibitory (SIC) antibiotic concentrations across subsequent generations.

MICs were determined qualitatively through color changes in the medium used for MIC assays. The averages of the MIC values indicate that the antibiotics: SPT, PUR, LVX, MXF, AZM, CLR, ERY, RXM, DOX, and TET are under the suggested breakpoint of ≤ 1 µg/mL, which supports the efficacy of these drugs against unaltered versions of MPN M129-B7. Alternatively, GEN, KAN, CHL, RFB, CFZ, CRO, CIP, CLI,

and NIT had MIC averages higher than 1 µg/mL. These MIC values were used to generate four separate sets of passages under decreasing sub-inhibitory concentrations (SIC). The antibiotics: SPT, GEN, KAN, PUR, CHL, CIP, LVX, MXF, CLI, DOX, and TET were tested on MPN M129-B7 and successfully generated five passages under antibiotic pressure for each. This study shows that antibiotic pressure from SPT, GEN, KAN, CIP, and MXF antibiotics can generate mutations in MPN M129-B7 within five passages. This indicates that we were able to further develop techniques to use on *M. pneumoniae* as a model system to lead to the exploration of the genetic mechanisms involved in antibiotic resistance development in mycoplasmas and possibly other microbes.

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INTRODUCTION

Background. Antibiotics have increased overall life expectancies by decreasing the morbidity and mortality burden in both first- and third-world countries (1). The main problem lies in the loss of efficacy of these drugs through the developed antibiotic resistance of bacterial pathogens. Massive misuse due to the accessibility to antibiotics led to a surge of super bug strains. This has increasingly alarmed the world and has resulted in an antibiotic resistance crisis. As of 2013, the Center for Disease Control and Prevention (CDC) estimated annually that about 2 million people develop infections with antibiotic-resistant pathogens with an estimated 23,000 deaths a year (2). According to the CDC, the four core actions required to reduce antibiotic-resistant infections are: 1- preventing infections and cross-transmission, 2- tracking the resistant bacteria of hospitalized patients, 3- improving the use of existing antibiotics through proper education of prescription and use, and 4- promoting the development of new antibiotics and diagnostic tests (2). Health-care providers are expected to be at the forefront of the implementation of these actions.

Bacterial infections from gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp., the multidrug resistant Gram-negative *Acinetobacter baumanii*, *Mycobacterium tuberculosis*, and *Neisseria gonorrhoeae* are in need of alternative treatments (3). The rate of antibiotic resistance cases in the world is increasing exponentially compared to the rate of new drugs being commercially developed for use. In this regard, there is a need to understand the genetic mechanisms in the development of antibiotic resistance on a specific organism; as this can provide more promising results in overcoming antibiotic resistance (4).

Mycoplasma pneumoniae is responsible for many cases of atypical community acquired pneumoniae (CAP) due to its improper diagnosis and spread. CAP is a general term for pneumoniae that elicits the general symptoms of cough, dyspnea, pleuritic pain, malaise, and fever or chills (5). *M. pneumoniae* is responsible for 4 to 8% of endemic community-acquired bacterial pneumonias and as high as 20 to 40% for general populations during an epidemic (6, 7, 8).

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This thesis follows the model of *American Society for Microbiology: Infection and Immunity*.

Symptoms can lead to hospitalization, but usually the infection goes undetected due to the mild nature of the infection.

Additionally, symptoms are similar to other causes of pneumonia, such as fever, shortness of breath, body aches, cough, and sore throat, and there is a lack of reliable diagnostic tests to confirm *M. pneumoniae* as the cause of illness (9). Limited surveillance studies and clinical trials provide some insights into the scale at which *M. pneumoniae* affects populations. However, current designed tests for assessing the etiologies of pneumonia are restricted to focus only on bacteria and the use of serology instead of more accurate techniques such as molecular based methods (10). European northern regions such as England, Germany, Finland, France, and Sweden have had higher instances of *M. pneumoniae* infections since 2010 (7, 11, 12,13) and similar epidemics have occurred in Chile, Brazil, South Korea, Israel, Japan, and China (13, 14, 15, 16, 17, 18). There is limited organized surveillance programs for *M. pneumoniae* in the U.S. which makes it difficult to get a better picture of *M. pneumoniae*'s resistance patterns and its regions of frequency in comparison to other countries. On the other hand, the CDC has managed to compile information on *M. pneumoniae* infections through serology, molecular-based detection, and strain typing techniques from studies done on outbreaks throughout the U.S. (19, 20, 21, 22).

Macrolides stand as some of the most effective drugs to date against *M. pneumoniae*. They are primarily bacteriostatic in their activity as they bind to the specific nucleotides in domains II and/or V of the 23S rRNA in the 50S bacterial ribosomal subunit. This binding causes the blocking of protein synthesis through the premature dissociation of peptidyl-tRNA from the ribosome (23). *M. pneumoniae* develops a natural resistance to macrolides through mutations in various positions of the 23S rRNA (24). Based on the *Escherichia coli* numbering system, these positions include C2611G, A2058G/C/T, and A2059G/C corresponding to positions 2617, 2063, and 2064 in *M. pneumoniae*. The transition mutation A2063G is the most common one reported. It must be noted that since the 23S rRNA gene is available in the *M. pneumoniae* genome as one copy then a single mutational event can change the macrolide susceptibility phenotype from antibiotic-sensitive to an antibiotic-resistant strain (24). Tests have shown that the position of the mutation has an effect on the resulting macrolide

resistance since a mutation at position 2617 produces a lower resistance than those at positions 2063 and 2064 (25). The high mutation rates exhibited by *M. pneumoniae* contributes to the increasing rates of resistant strains (23, 26, 27).

Improper diagnosis of *M. pneumoniae* infections coupled with antibiotic misuse and overuse are responsible for the worldwide emergence of macrolide-resistant *M. pneumoniae*. The studies on *M. pneumoniae* present a vehicle for better understanding of antibiotic resistance development due to access of the fully sequenced genome of *M. pneumoniae* M129 (ATCC 29342) (28, 29) and the development of macrolide-resistant *M. pneumoniae* rapid testing methods (i.e. real-time polymerase chain reaction high resolution melting [PCR-HRM], polymerase chain reaction restriction fragment length polymorphism [PCR-RFLP], pyrosequencing assays, and other direct molecular methods) for any 23S rRNA mutations (30, 31, 32, 33, 34, 35, 36, 37, 38, 39). Research on *M. pneumoniae* will further expand on the current knowledge of antibiotic resistance in bacterial pathogens.

This research is driven by the current knowledge on antibiotic (macrolides, lincosamides, streptogramin combinations and ketolides) resistance development in *Mycoplasma pneumoniae* (32, 40, 41); and is also driven by an effort to explore the effects on *M. pneumoniae* of other accessible antibiotics in the market. The current literature suggests that *M. pneumoniae* is associated with antibiotic resistance through antimicrobial target modifications, so our project is shaped by the fundamental understanding of this organism (40). The *objectives* of this study are: (1) to determine the minimum inhibitory concentration (MIC) of different antibiotic groups including macrolides, aminocyclitols, aminoglycosides, amphenicols, ansamycins, cephalosporins, fluoroquinolones, glycopeptides, ketolides, nitrofurans, and tetracyclines against *M. pneumoniae* (MPN) wild type strain M129-B7 and (2) to generate antibiotic resistant mutants through exposure of *M. pneumoniae* M129-B7 to constant sub-inhibitory (SIC) antibiotic concentrations across subsequent generations. We hypothesized the following: (1) exposure of MPN to constant sub-inhibitory concentrations of antibiotics will result in MIC shift as a result of mutations in specific candidate genes responsible for resistance development; (2) that antibiotic resistance genes in *Mycoplasma pneumoniae* M129-B7 will be expressed upon the 5th generation

of cells due to the small genome size of the strain. The *results of this research study aims* (1) to provide a better understanding of the resistance process in MPN, and (2) to provide a basis to find improved treatment practices for MPN infections and other pathogenic bacteria.

Antibiotic resistance crisis. Sir Alexander Fleming in 1928 ushered in the modern era of antibiotics with his discovery of penicillin from the fungus *Penicillin notatum* (42). Difficulties with making enough of the penicillin drug slowed the use of this drug until its better production during World War II. This had allowed for an effective treatment method for injured soldiers experiencing critical infections in the 1940s (43). Implementation of antibacterial therapy changed the medical landscape by allowing otherwise lethal cases of infection to be systematically treated or eradicated. By the 1950s, resistant cases such as those of methicillin-resistant *Staphylococcus aureus* (MRSA) had begun to arise prompting the search for better drugs (44). The pharmaceutical companies of that time were able to promptly deliver new medications by 1962. This made researchers of infectious diseases hopeful that bacterial diseases would be eradicated from humanity's list of problems (4).

In 1965, there were discussions on the reemergence of resistant bacteria. The pharmaceutical industry was able to alleviate the growing problem by expanding on the antibiotics available from the late 1960s through the early 1980s. However, bacteria subjected to antimicrobial therapy increased their resistance to the available drugs, while the development of antimicrobial countermeasures had decreased (4). In 2012, and in the United States alone, bacterial strains isolated from intensive care units were resistant to carbapenems (45). These included 10% of *Klebsiella* spp., 50% of *Acinetobacter baumannii*, and 20% of *Pseudomonas aeruginosa* (45). These types of trends foreshadow the dwindling effects antibiotics have on counteracting diseases caused by bacteria. This prompted the development of new approaches to tackle the resilient nature of these microbes.

The main cause of antibiotic resistance comes from the overuse and misuse of antibiotics. Biologically, an organism is genetically optimized for survival in a particular environment through selective pressures that result in adaptations. For bacteria, this means that there is a direct relationship between antibiotic use and the emergence and

dissemination of resistant bacterial strains (46, 47). Bacteria are capable of absorbing the genetic elements for resistance through horizontal gene transfer (HGT) from relatives or nonrelatives by absorbing plasmids (48). It is also possible for resistance to develop based on a random mutations. Antibiotics kill drug-sensitive bacteria but, upon misuse, leave behind resistant cells that can later propagate and thrive in the natural selection process (48).

Antibiotic consumption in the U.S. in 2012 was estimated at 22.0 standard units (a unit being one dose of antibiotic regardless of form) per person with the states running from the Great Lakes to the Gulf Coast having the most written prescriptions and the West Coast with the lowest (49, 50). An estimated 30% to 60% of antibiotics prescribed in intensive care units (ICUs) are inappropriate and unnecessary, thus further contributing to an increase in antibiotic resistant pathogens (51). Other countries experience mass antibiotic misuse through unregulated availability (52). Patients using sub-inhibitory, sub-therapeutic, or ineffective prescription medications promote the development of resistance by supporting the genetic alterations due to antimicrobial target modifications by acquired mutations and HGT. Detrimental effects of sub-inhibitory concentrations can be seen in the diversification and induced broad proteomic alternations as seen in *Pseudomonas aeruginosa* and *Bacteroides fragilis* (53).

In 2013, there was an estimation that 80% of antibiotics sold in the U.S. were used in livestock to promote the growth of and prevention of infection in the animals. This led to livestock's larger yields and higher-quality products (52, 54). Antibiotic use is high in the developed and the developing world. Studies have observed the antibiotic resistance development in the intestinal flora of farm animals and farmers alike (54). This likely comes from the use of antibiotics on healthy livestock. The antibiotics used on farm animals destroy the susceptible bacteria, but allow antibiotic-resistant bacteria to thrive. The resistant bacteria can be transmitted to humans through the consumption of that food supply with unforeseen side effects (55). It is also important to consider the adverse effects that antibiotics could have on the environmental microbiome; since up to 90% of the antibiotics taken by livestock are released into the environment. The antibiotics are released through urine and feces while being dispersed through fertilizer, ground water, and surface runoff (54). Some antibiotics, such as tetracycline and

streptomycin, are additionally used as pesticides for fruit trees in the western and southern U.S. further expanding the affected areas (54).

Epidemiology of *Mycoplasma pneumoniae*. The rate of *M. pneumoniae* infections is increasing worldwide. A study of 758 presumably healthy individuals showed that 13.5% of them had a dormant strain of *M. pneumoniae* with the percentage dropping to 4.6% in 499 volunteers after 11 months (56). This statistic is a good indicator of the fluctuating occurrence of this pathogen over time. A critical problem to tackle for *M. pneumoniae* infections is that strains can live in the respiratory tract after an infection has been clinically resolved with antibiotics (57). In the case of asymptomatic carriers, they provide a vehicle for the spread of the organism from one person to another without the knowledge of the individual (56, 58). Current data of the occurrence of *M. pneumoniae* infections is restricted to the United States, Europe, Japan, and China (59, 60, 61, 62, 37, 27). This bacterium has been shown to be responsible for an estimated 15-20% of all cases of community acquired pneumoniae, which created an endemic disease transmission linked to a cyclic epidemic every 3-5 years, over the course of 50 years from 1946 to 1995, in the United States and Europe (57, 63, 64, 65, 66).

It is projected that the incidence of cases will increase over time based on the emergence of drug resistant *M. pneumoniae* in Japan from 2003 to 2011 with an increase from 5.0% to 89.5% in resistant strains (67); in China in 2005 from 84.4% to 100% (32); in France from 1994 to 2007 from 2 of 155 strains to 10% (27, 37); in the US from 2007 to 2010 rising to 8.2% (38); and a few minimal but notable instances in Italy in 2010 (68) and Germany from 1991 to 2009 (61). This highlights the importance of understanding *M. pneumoniae* as a vehicle to reduce the problems created by improper antibiotic use and potentially contribute to a long term solution for these types of pathogens.

***Mycoplasma pneumoniae* (MPN M129-B7) and antibiotic resistance.** The pathogenic bacterium *Mycoplasma pneumoniae* (MPN) is an important model organism for studying the genetic development of antibiotic resistance. This is due to (1) its small genome size, (2) its growing clinical relevance as the cause of community-acquired pneumonia infections in children and immunocompromised adults, and (3) *M. pneumoniae's* increasing resistance to commonly prescribed antibiotics such as

macrolides and tetracyclines. *M. pneumoniae* (MPN) is a common respiratory pathogen that causes diseases of varying severity ranging from mild upper respiratory tract infections to severe atypical pneumonia (69). *M. pneumoniae* is responsible for most of the community acquired pneumoniae present in populations. *M. pneumoniae* lack a cell wall, which means that they cannot be targeted by more accessible β-lactam antibiotics. Generally, macrolides are the first-choice antibiotics to be prescribed for treating *M. pneumoniae* infections in both children and adults, but the rising concern is the fluctuation of infections in the population over time that could be indicative of a growing antibiotic resistance (68).

Research studies indicate that the most effective drugs of the macrolide class for this microbe are slowly losing their potency against the microbe, thus leading to the observed resistances in clinical strains (32, 43, 70, 71). Around the world, macrolide resistance cases affecting immunocompromised adults, the elderly, and children have been reported (59, 72, 73). Resistance development in MPN highlights the importance of a national action plan to combat the growing antibiotic resistance crisis (4, 46, 47). The growing relevance of *M. pneumoniae* and its genetic nature as a respiratory pathogen make *M. pneumoniae* a good candidate for understanding the development of antibiotic resistance. Clinical trials worldwide have shown a rise in *M. pneumoniae* drug resistance due to improper antibiotic use by patients.

Taxonomy and classification within Mycoplasma. *Mycoplasma pneumoniae* falls within the class *Mollicutes* which is comprised of about 200 known species that have been detected in vertebrate animals, humans, arthropods, and plants (43). Each of the currently categorized members of the *Mollicutes* class have distinctly small genomes with a single circular chromosome consisting of 0.58-2.22 Mbp with a 23-40 mol% G+C content, and lack of a cell wall (74). The genome size is attributed to *Mollicutes* undergoing a degenerative evolution process diverging from gram-positive, low G+C content *Streptococcus* branch of bacteria around 605 million years ago according to extensive 16S rRNA analyses (75). While the specifics of the selective pressure leading to the evolution of *Mollicutes* is not known, Mycoplasmas stand as the smallest self-replicating organisms in terms of cellular dimensions, genome size, and capability for a cell-free existence.

Genetic background, cell biology, and cytoadherence mechanisms of *M. pneumoniae.* Cells of *M. pneumoniae* are spindle-shaped, 1-2 µm long and 0.1-0.2 µm wide, which allows them to pass through up to 0.45 µm pore size filters. They do not produce any visible turbidity in liquid growth media and cannot be detected by light microscopy (43). Its minute size is accompanied by a genome of 0.816394 Mbp with 687 genes. *M. pneumoniae* uses the universal stop codon UGA as a codon for tryptophan as seen commonly in other *Mycoplasma* species (76). In comparison with *Escherichia coli* genome containing 0.460000 Mbp and an estimated 4,300 genes, MPN's genome makes MPN a better candidate as a genetic model for respiratory pathogen studies (29). The encoded genes present limited biosynthetic capabilities, such as their inability to synthesize peptidoglycan cell walls thus creating the sterol-based pleomorphism that makes them only biologically sustainable through the infection of a eukaryotic host.

The osmotically fragile, triple-layered Mycoplasma cell membrane makes them unable to exist as free living organisms in nature since they are overtly predisposed to desiccation (43). They use their parasitic relationship to supplement their metabolism and for the synthesis of essential components such as proteins, carbohydrates, and lipids. They are unable to synthesize purines or pyrimidines de novo and instead use a process that has optimized the fermentation of glucose to lactic acid by substrate phosphorylation through the use of phosphoglyceric acid kinase and pyruvate kinase to generate ATP (77). Further metabolic studies are required to fully understand the annotated enzyme systems present in *M. pneumoniae.* Mollicutes perform glycolysis but not the tricarboxylic acid cycle nor oxidative phosphorylation (43, 78). Additionally, *M. pneumoniae* uniquely reduces tetrazolium either aerobically or anaerobically based on old identification techniques prior to the use of PCR assays (77); it uses the enzyme arginine deiminase as an important factor in its ammonia production (79), which is linked to the detrimental effects on lung epithelial cells by making them nonviable (80). It is suggested to use its external capsular material as a subsistence strategy directly tied to its adherence to eukaryotic epithelium (81). Observed limitations within *M. pneumoniae* metabolic processes are related to its small number of encoded genes.

The small genome of *M. pneumoniae* has allowed for it to develop a specialized reproductive cycle. It was observed through electron microscopy that *M. pneumoniae* contains a protuberance on its plasma membrane. This protuberance is a specialized attachment organelle that has clusters of 170-Kda transmembrane protein, P1 adhesin that allows MPN to attach to host cells and glass surfaces (82, 83, 84, 85). The P1 adhesin protein is additionally found all over the remainder of the plasma membrane, but in lower concentrations and it is the keystone protein for creating the fundamental parasitic relationship with a host (86, 87, 88, 89). Despite the lack of findings on the mechanisms involved in the attachment organelle that allow for the motility of *M. pneumoniae*, it is active during the binary fission mode of replication of these microbes as it gets duplicated before it migrates to the opposite pole of an individual cell to prepare for the separation of the nuclei (83).

Studies that have tested the effect of inactivating the P1 activity by targeting it with monoclonal antibodies (90), trypsin treatment, or by inducing mutations (91) show that it leads to avirulence through reduced adherence of the Mycoplasmas to its eukaryotic targets. Research studies on the adherence properties of *M. pneumoniae* suggest that expression of the P1 protein alone is not enough for adhering to particular host cells and instead requires an amalgamation of proteins for the infection to occur (92, 93). The mediation of cytoadherence of *M. pneumoniae* comes in part from P30 as any damage or detrimental effect on its expression leads to the lack of hemadsorption (94). Other proteins involved include HMW1, HMW2, HMW3, HMW4, HMW5, P90, and P65. Although these proteins have been more directly linked to the formation of the polar structure that contribute to the independently assembled complex of proteins B, C, and P1, that leads to the creation of the attachment organelle (83, 95). The method of intracellular invasion and replication in vivo is not clear, but there are some suggestions as to how it occurs.

Macrolevel effects of *Mycoplasma pneumoniae* infections. *M. pneumoniae* infections can proliferate in either the upper or lower respiratory tract, or both. PCR and culture techniques have allowed for the observation of *M. pneumoniae* in extra pulmonary sites such as synovial fluid and cerebrospinal fluid, pericardial fluid, skin lesions, and blood (96, 97, 98). Symptoms of *M. pneumoniae* infections develop over

time for several days and can persist for weeks to months. Some of the more common symptoms include: hoarseness, fever, sore throat, headaches, chills, myalgias, ear ache, coryza, a cough that eventually yields small to moderate levels of non-bloody sputum, and general malaise (99, 100, 101, 102). Extent of symptoms vary depending on the age of the affected patient. Children younger than 5 years of age experience coryza and wheezing with the development of pneumoniae being uncommon while those from 5-15 years old develop bronchopneumonia, in one or more lobes, that could require hospitalization (99, 102). Adults experience more commonly mild infection and asymptomatic conditions with bronchopneumonia affecting one or more lobes developing in 3-10% of cases (103). On the other hand, the elderly exhibit similar symptoms with the developed pneumoniae that may require hospitalization (104, 105). It is also possible for infection to lead to extra pulmonary complications regardless of whether or not there is a respiratory illness at any point after inoculation.

Long term damage to the central nervous system (CNS) is the most common extra pulmonary effect (96, 97, 98). Patients serologically tested to have contracted pneumonia due to *M. pneumoniae* have experience the following complications: cerebral syndrome and polyradiculitis, cranial nerve palsies, encephalitis, acute disseminated encephalomyelitis, coma, optic neuritis, diplopia, mental confusion, aseptic meningitis or meningoencephalitis, mental confusion, and acute psychosis secondary to encephalitis (106, 107, 108, 109, 110). Some of the motor deficiencies include cranial nerve palsy, choreoathetosis, Gullain-Barré Syndrome, and brachial plexus neuropathy (111, 112, 113, 114). Typically, patients manifest neurological complications from 1 to 2 weeks after the respiratory signs, or even if there are none, with the effects on children likely being higher (115, 116, 117).

Less severe extra pulmonary effects of *M. pneumoniae* include dermatological disorders such as vesicular and erythematous maculopapular rashes. However, it is possible for severe conditions of conjunctivitis, ulcerative stomatitis, bullous exanthems and Stevens Johnson syndrome to develop by inoculation via cutaneous lesions (118, 119, 120). Bones and joints can also be affected by causing osteomyelitis (121). In some instances cardiac complications have been tied to *M. pneumoniae* by finding the organism in the pericardial fluid of affected patients (122, 123, 124). A mixture of

nonspecific ear symptoms, renal failure, IgA nephropathy, and acute glomerulonephritis to name a few elucidate the need to further explore the extent to which a mycoplasma infection can harm its human target (102, 125, 126). These direct extrapulmonary invasions need to be studied further as most aspects of the disease are rarely evaluated for clinical trials and updated information is sparse.

Pathology of the parasitic relationship with mammals. Mycoplasma species are established mucosal pathogens capable of living extracellularly on epithelial surfaces. There is data that suggests that there are Mycoplasma species that are able to fuse with and enter the host cells that are not phagocytic by exploiting the fluidity of their cell wall-free plasma membranes (95). This is based on observations of *Mycoplasma penetrans* being isolated from the urine of patients with human immunodeficiency virus. *Mycoplasma fermentans* are able to embed themselves into host cells. *M. pneumoniae* have the ability to survive, thrive, and replicate in cell culture systems over a period of 6 months (127). These characteristics likely give *M. pneumoniae* the potential to establish a latent state, prevent biodegradation via mycoplasmicidal immune mechanisms, easily diffuse through mucosal barriers to further internalize itself into deeper tissues, and overall decrease the effectiveness of drug treatment therapies (128, 129, 130). The introduction of this pathogen with the host could induce the release of hydrolytic enzymes together with its mycoplasmal membrane components. This could alter receptor recognition sites to hamper the induction and expression of cytokines, but further research is required to elucidate the extent of this activity (95). Once embedded into the respiratory epithelium, *M. pneumoniae* begins to degrade host's cells through its biochemical and immunological properties, which ongoing experiments are trying to explore.

For the infection to take place, *M. pneumoniae* requires a close proximity to the target epithelial cells for it to attach with P1 and the rest of the adhesin proteins in order to disrupt the tissue and induce its cytotoxicity. The *M. pneumoniae* exotoxins are not expressed, but instead produce toxicity through superoxide radicals and hydrogen peroxide. The cytotoxins are created as products of the flavin-terminated electron transport chain as opposed to the virulence enzymes like catalase and dismutase present in other bacteria (130). These components combine with the endogenous toxic

oxygen molecules generated by the host cells create the oxidative stress in the respiratory epithelium leading to its damage. As observed in erythrocytes, peroxide causes ultrastructural effects on the host cells such as a loss of reduced glutathione, peroxidation of erythrocyte lipids, denaturation of hemoglobin, and the eventual lysis of the cell (131, 132). The superoxide anions produced by *M. pneumoniae* could be the reason behind the inhibition of catalase in host cells. This leads to the reduction of enzymatic breakdown of peroxides generated endogenously as the parasite causes the host cell to become prone to more oxidative damage (43, 130).

Another potential source for the damage to host cells could be the forced acquisition of lactoferrin. This generates highly reactive hydroxy radicals by placing iron complexes in a locally acidic microenvironment. This locally acidic environment also includes hydrogen peroxide and superoxide anion, which upon buildup could potentially damage the host cell membrane and thus will allow for entry of *M. pneumoniae* (133). The infection of *M. pneumoniae* in mammalian cells creates deterioration of the cilia in the respiratory epithelium at the structural and functional level possibly leading to the chronic effects experienced by carriers. Parasitized cells can lose cilia entirely and can become vacuolated. They exhibit reduced oxygen consumption, uptake of amino acids, glucose usage, and macromolecular synthesis; all of which result in the slow loss of some or all of the parts of the infected cells (101, 134). As the infection progresses into the lower respiratory tract, the elicited immune response can be better observed.

The immunomodulatory effects of *M. pneumoniae*. Based on in vitro studies and the use of animal models, the amplification and prevalence in alveolar fluid and serum of the immune response is associated with the production of immunoglobulins, lymphocyte proliferation, release of tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-y), and several interleukins such as interleukin 1β [IL – 1β], 1L-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-18 (135, 136, 137, 138, 139, 140, 141, 142, 143). This infection process is also associated with the activation of macrophages that enter the site of infection and begin phagocytosis due to the high number of neutrophils and lymphocytes ($CD4^+$ T lymphocytes and B lymphocytes) present in alveolar fluid as they infiltrate the lungs (144, 145). In human and animal studies, it is unclear whether the spike in cytokines and activation of lymphocytes is due to the infection or if it a response to aid the host in

defending itself through its biochemical mechanisms (142). It is possible this change in cytokine levels could worsen the effects of the disease through immunological lesion development (142, 146, 147). This is based on how the clinical illness and pulmonary injury gets more severe and more vigorous as excited lymphocytes levels increase (146, 147). The immune system responds by quickly producing antibodies that peak after 3 to 6 weeks, which gradually decrease over time (138, 148).

Protein and glycolipid antigens on the surface of *M. pneumoniae* allow the body to respond at the onset of infection. While the P1 protein is the main target of the antibodies produced as a response by the host, the inherent mechanisms for Mycoplasma to integrate itself into the epithelium prevents it from being completely eradicated by the immune system. Those at the beginning stages have the body elevate its *M. pneumoniae*-specific immunoglobulin M (IgM) levels within 1 week of interaction with the pathogen during an acute infection (149). Around 2 weeks post infection, the body begins to generate the immunoglobulin G (IgG) antibody (150). Clinical studies on children and adults focus on the variation in IgM and IgG levels throughout the course of the infection (151, 152). Immunoglobulin A (IgA) may be a better candidate for analysis to determine initial infection as their peak levels and degeneration occurs within a much narrower time frame than IgM or IgG (153, 154). Additionally, there could be better assessment of infection by exploring the development of cross-reactive antibodies during a *M. pneumoniae* infection (155).

Genome sequence of *M. pneumoniae* revealed the extensive sequence homology (20.7%) for a microbe present in the segments responsible for the adhesin proteins and glycolipids of MPN's cell membrane with those of mammalian tissues (43). This is a good example of molecular mimicry behind autoimmune disorders involving multiple organ systems by having antibodies that attack substances like myosin, keratin, kidney, smooth muscle, and lung tissues (156). Further comparison to human sequences shows that the mycoplasmal adhesins have homologies in their amino acid sequence with that of the CD4 and class II major histocompatibility complex lymphocyte proteins responsible for producing autoreactive antibodies to induce cell destruction and immunosuppression (157).

Mycoplasmas, as a species, can affect the immune responsiveness of the host through perpetual mitogenic stimulation of B and T lymphocytes to cause autoimmune disease through activation of polyclonal B lymphocytes or anti-self T cells (128). All of these properties contribute to the induced production of cytokines during the beginning of the acute inflammatory response. Likewise, these properties add to the difficulty in fully removing the pathogen from its host. Such an evolved and efficient set of mechanisms have allowed for *M. pneumoniae* to successfully thrive as a parasite by optimizing its intracellular localization and immunomodulatory activities.

MATERIALS AND METHODS

***Mycoplasma pneumoniae* (MPN M129-B7) passage generation.** *Mycoplasma pneumoniae* MPN M129-B7 (product name, *Mycoplasma pneumoniae* Somerson *et al.;* ATCC® 29342™*)* was obtained from American Type Culture Collection (ATCC). Cell passages were propagated by pipetting 400 µL of MPN M129-B7 in a 750 mL culture flask with 100 mL of modified Hayflick (MHF) (Table A1). This medium was incubated at 32℃ with the flat portion of the flask facing downwards over a period of 4 days to create a passage of the strain. After the incubation period the used medium was discarded, the cells were washed twice with 10 mL of 1X phosphate-buffered saline (PBS) Ph 7.4 (Table A2), and submerged in 12 mL of freshly made MHF. The cells were collected from the flask's flat surface with a cell scraper. The mixture of the new MHF with the collected MPN were pipetted into a 15 mL tube for storage at -80℃. Subsequent passages used 400 µL of the previous 12 mL of MPN in MHF to start the 100 mL culture in preparation for the creation of the new passage. Procedures as detailed below were carried out on the 5th passage of MPN, as was randomly chosen to prevent restocking problems with the strain.

Spectrophotometry as a metric for growth for tube cultures. Bacterial concentration of cultured strains of *M. pneumoniae* were determined through spectrophotometry. Readings at the 550 nm wavelength have shown to be capable of identifying the growth of MPN (158). Cell growth measurements were obtained from the 12 mL frozen stocks by allowing them to thaw on ice, then gently inverting to fully dissolve clumps of cells, taking 200 µL from the liquid MPN mixed in MHF and placing it into a sterile 1.5 mL microcentrifuge tube. The tube was placed in a centrifuge for 5 minutes at 10,000 x g and 4℃ to allow for the removal of the supernatant, so that the cells could be washed and then resuspended in 200 µL of PBS. Once resuspended, the cells were transferred to a cuvette containing 600 µL of 1x PBS Ph 7.4 and mixed lightly by pipetting the solution up and down. The blank was a cuvette with 800 µL of 1x PBS Ph 7.4. Measurements were taken in absorbance, ABS, with a target optical density, OD, value of 0.005 at OD_{550}. Optical density of a sample was determined based on the following equation:

Final volume of cell suspension (350 µL) X OD_{550} (0.005) = Volume of cell culture (X µL) X OD_{550}(measured X 4)] X 16

Actual inoculation volume was 16X higher to compensate for the dilution factor in 96-well plate for the minimum inhibitory concentration (MIC) assays. Total volume did not exceed 350 µL. A specific cell culture volumes (from cryogenic tube) was used based on OD_{550} (0.005) to inoculate 350 µL modified Hayflick (HF) medium (containing 20% horse serum, 1% glucose, and 5,000 U penicillin) using the formula above.

Preparation of antibiotic stocks as a selective pressure for *Mycoplasma pneumoniae*. Antibiotic stocks were prepared in 1.5 mL microcentrifuge tubes by dissolving the solid substance into Millipore water or DMSO based on the solubility of each antibiotic at 5, 10, and 15 mg/mL concentrations. The following antibiotics, with their abbreviations, were tested on MPN M129-B7: spectinomycin (SPT), gentamicin (GEN), kanamycin (KAN), puromycin (PUR), chloramphenicol (CHL), rifabutin (RFB), cefrazolin (CFZ), centriaxone (CRO), ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), clindamycin (CLI), azithromycin (AZM), clarithromycin (CLR), erythromycin (ERY), roxithromycin (RXM), nitrofurantoin (NIT), doxycycline (DOX), and tetracycline (TET). Each antibiotic was stored in a -20℃ freezer for long term storage. Stocks were used after they had been fully thawed and stored on ice during short term usage.

Minimum inhibitory concentration (MIC) assays in 96-well plates for *Mycoplasma pneumoniae*. Passage 5 (P5) MPN M129-B7 unaltered (UA) cells were thawed on ice before being quantified through spectrophotometry for the minimum inhibitory concentration assay (MIC). Procedures were the same as described above with the use of the equation to find the required volume to pipette from the passage to create a 0.005 at OD_{550} concentration of cells in the 96-well plate. When MPN was not in use, it was stored on ice to slow down its growth. MIC assay procedures were replicated 12 times total for MPN M129-B7 UA, and 3 times for each passage generated at sub-inhibitory concentrations (SIC) described later in this procedure.

Each 96-well plate was designated two rows (labeled A-H) per antibiotic with the ability to test up to 4 antibiotics simultaneously (Table A3). The numbered wells (1-12) served as an indicator of the dilution quantities taking place for the MICs. Each antibiotic

being tested had its highest concentration in the first well. Every well after that had a two-fold dilution up to the 10th well and then continued on the second row to its 10th well. Well 11 served as the positive control with media and MPN cells, and well 12 served as the negative control (with media only). Wells 1-11 were filled with 160 µL of MHF media and the 12th wells had the total volume desired of 200 µL. The rest of the volumes were increased to the desired total volume of 200 µL by adding MHF and MPN M129-B7 cells at the target 0.005 at OD_{550}.

Antibiotics tested were diluted in a 1.5 mL microcentrifuge tube containing MHF. The dilution was based on the antibiotic concentration and total volume of MHF used. The total volume was 180 µL to work with the required small concentrations. The highest concentration being tested was adjusted to 2x to allow for the concentration to halve in accordance with this two-fold dilution setup (e.g. if the highest concentration being tested is 256 µg/mL, dilute to 512 µg/mL). The following equation was used to calculate the required amount of antibiotic for the MIC assay:

[Final volume of antibiotic highest concentration solution

(180 µL) X Antibiotic highest concentration $\left(\frac{\mu g}{mL}\right)$ = Volume of antibiotic stock solution

(X µL) X Antibiotic stock concentration $\left(\frac{\mu g}{mL}\right)$ X 1.25

Actual antibiotic highest concentration value were slightly higher (1.25X) to compensate for the dilution factor in 96-well plate after adding cells. Total volume did not exceed 180 µL.

The prepared diluted antibiotic was pipetted into the first well, mixed 5-8 times at the first stop to ensure the liquid was not fully dispensed, then diluted 5-8 times on the next well and so on until the 10th well of that row. Once the 10th well was reached, the dilution series was continued on the following row up to the 10th well on it. The tip was then discarded along with any remaining antibiotic dilution. Any remaining antibiotics pending to test on that plate were done in the manner just described.

MPN M129-B7 was diluted based on the previous calculations obtained for the OD_{550} in a 1.5 mL microcentrifuge tube. Once obtained, 12.5 µL of the diluted MPN were dispensed in wells 1-11 starting from well 11 and working backwards to well 1 to prevent increasing the dilution series antibiotic concentrations. Then 27.5 µL of MHF

was added from 1-11 starting from the last well, to bring up the total volumes in all of the wells to 200 µL. Plates were covered, labeled, and stored in an incubator at 37℃ for 7 days. After the incubation period, the results were read qualitatively by observing the 96-well plates, visually determining the first well to lack turbidity in the 200 µL of MHF or the lowest concentration of antibiotic without a color change in the medium, and recording the value assigned to that particular well from the two-fold dilution setup as the MIC value for the tested drug (Fig. 1). This change in color is due to the Ph change caused by the released acids of the developed bacteria (159).

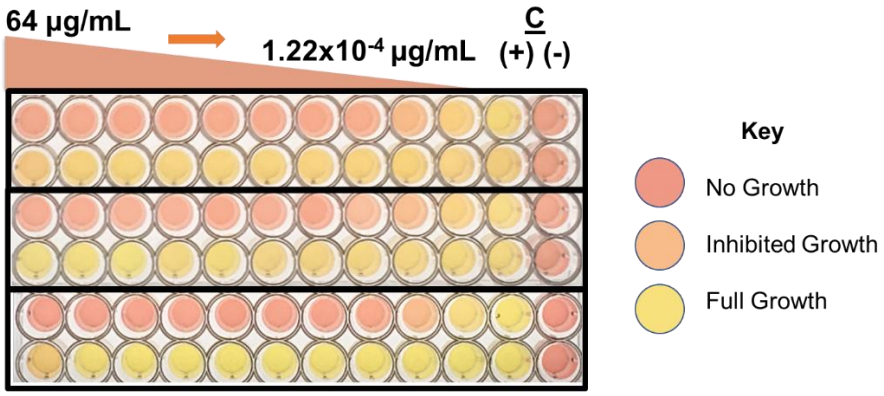

FIG 1 Example of qualitative visual assessment criteria for determining MIC through phenol red coloration changes in MHF in 96-well plate MIC assay series dilution. Each two-fold dilution within a plate was designated two rows per antibiotic. Here we illustrate a plate containing a highest concentration of 64 µg/mL and lowest concentration of $1.22x10^{-4}$ µg/mL for three separate tests. After 7 days of incubation at 32℃, we compared our red colored negative control (indicating no growth) and yellow colored positive control (indicating full growth) against the wells with MPN cells and antibiotic. The first well to show orange coloration (indicating inhibited growth) was considered as a sub-inhibitory concentration (SIC), so the MIC was determined as the well containing one assigned antibiotic concentration higher. The presented MIC setup shows inhibited growth in well 9 (0.250 µg/mL of antibiotic concentration), so we determined the MIC value to be 0.500 µg/mL, from well 8 in all three tests.

MPN M129-B7 passage creation with antibiotic pressure in culture flask protocol. MIC values were used as a reference to create MPN M129-B7 passages under sub-inhibitory antibiotic stress. Each of these passages was abbreviated as (PA[number of

passage]) to categorize them as the passages with antibiotic pressure. Once the MIC value was determined it was used as a starting point to grow MPN under the pressure of a single antibiotic at a time at four sub-inhibitory concentrations (SIC1, SIC2, SIC3, SIC4) from the MIC based on the values from the two-fold setup. Four sub-inhibitory concentrations were picked due to the nature of the visual assessment of growth through cell attachment in the culture flasks. Doing this allowed us to harvest at least one set of passages under sub-inhibitory antibiotic stress. There were four sets of data gathered from these passages as each set per antibiotic had constant SIC MIC values being used throughout the duration of the passage creation process. The passages were made in 250 mL flasks with 50 mL of MHF. The preparation recipe from Chanock *et al.*, 1992 (160) (Table A1) prepares 51.5 mL of modified media, thus 1.5 mL was discarded, or set aside for potential use for any required dilutions, as the calculations were made with 50 mL total volume.

The MPN M129-B7 MHF solution was used to prepare 0.005 concentration of cells. This concentration was determined spectrophometrically at OD_{550}. With the calculated volume required, that amount was transferred into the prepared tube with MHF alongside the antibiotic to bring up the volume to 50 mL. The labeled culture flask(s) were placed in a 37℃ incubator with the bottom surface(s) downwards, and observed over the course of 7 days initially. Range of observation of growth was extended up to 10 days with variations among antibiotics to avoid having low cell yields to work from. Any remaining passage cells from the MHF stock were stored in the -80℃.

Harvesting MPN M129-B7 passage(s) with antibiotic pressure from culture flask for long term storage. Once the 7-10 day incubation period for the created passage(s) ended, and close monitoring showed visible MPN cell attachment to the bottom of the 250 mL flask(s), the culture flask(s) were removed from the incubator. The used media was discarded, cells were washed with 5 mL of PBS twice before adding 12 mL of freshly made MHF media with the same concentration of antibiotic initially used to create the passage. Cells were scraped off the bottom surface of the culture flask and pipetted into a 15 mL tube to prepare for storage in the -80℃ freezer. The OD_{550} reading were obtained immediately before storage or during the next experiment. Every set of passages, SIC 1-4, per antibiotic was tested on a new MIC assay on a 96-well plate at

the same highest concentration used to determine the initial MIC value. MPN M129-B7 UA strains were retested for each drug alongside the SIC 1-4 tests to direct qualitative comparisons.

Quantitative analysis of MIC value shifts to consider antibiotic resistance mutation within created passages under constant antibiotic stress. Studies working with *Mycoplasma pneumoniae* use the previously described visual technique of determining the MIC value to a particular antibiotic by identifying the lowest concentration of antimicrobial agent that prevents a change in coloration of the Hayflick's medium (161, 162). We decided to use a single factor analysis of variance (ANOVA) on the qualitatively determined MIC values in order to determine significant differences among the antibiotic treatments on MPN M129-B7 UA and SIC1-4 passages based on disk diffusion assays of antimicrobial susceptibility tests on *E. coli* (163). Our null hypothesis was that there would be no change in MIC value in the antibiotic stressed passages with that of the unaltered (UA) MPN M129-B7, therefore signifying no antibiotic resistance development. Our alternative hypothesis was that there would be a change in MIC value in at least one antibiotic stressed passage with that of the unaltered (UA) MPN M129-B7, therefore signifying antibiotic resistance development. To narrow down which passage(s) had significant differences from the MIC of MPN M129-B7 UA, we performed a two-tailed *t* test of two samples assuming unequal variance. The reason we chose to do a two-tailed *t* test instead of a one-tailed was due to the possibility of misreading the MIC values qualitatively. This could then consider significant changes in MIC means from the UA strain for each passage as possible alterations to the phenotype, and as a results a genotypic variability caused by the drug pressure. All statistical tests for ANOVA and *t* tests used an alpha value of 0.05 ($\alpha = 0.05$) to avoid Type I, or rejecting a true null hypothesis, and Type II, or rejecting a false null hypothesis, errors. All statistical analyses were executed on Microsoft ® Excel.

Effects of induced stress on the MPN M129-B7 genome and sequencing goals. By creating controlled resistance in the MPN M129-B7 strain, we were able to generate a library of antibiotic resistant strains that could be sent out for sequencing. Additionally, we also optimized stressed MPN cell yields by expanding this protocol to enable future experiments to replicate our results. MPN SIC1-4 strains that had MIC value shifts in

comparison to those of their MPN M129-B7 UA were considered as expressing phenotypes of antibiotic resistance development. This is accordance with similar studies (40), and generated strains that can be sent out for genome sequencing.

RESULTS

Minimum inhibitory concentration assays provided a starting point for the generation of the antibiotic resistant strains. *Mycoplasma pneumoniae* could be useful in understanding the genetic mechanisms involved in antibiotic resistance development. By using *Mycoplasma pneumoniae* (ATCC® 29342™), with a median length of 0.81718 Mbp, we attempted to generate independently resistant strains to the following classes of antibiotics: aminocyclitol, aminoglycoside, amphenicol, ansamycin, cephalosporin, fluoroquinolone, lincosamide, macrolide, nitrofuran, and tetracycline. With MPN M129-B7 consisting of an underdeveloped genome in terms of genetic repair mechanisms, we predicted to observe qualitatively different MIC values using a colorimetric data gathering procedure, and to observe a quantitatively significant increases in MIC values within the first five generations under antibiotic stress. The strains that showed MIC value increases will be candidate strains for sequencing analysis. Here, we hypothesized that these strains will have mutations in any of the genes directly involved in the antibiotic resistance development process.

MPN M129-B7 unaltered (UA) strains were grown across five passages before starting the two-fold dilution MIC assays at 64 µg/mL for each antibiotic (Table A3a). This initial concentration allowed for a screening process to identify the MIC values against each drug. Spectinomycin (SPT), gentamycin (GEN), kanamycin (KAN), puromycin (PUR), chloramphenicol (CHL), ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), clindamycin (CLI), nitrofurantoin (NIT), doxycycline (DOX), and tetracycline (TET) MICs were identified on the 64 µg/mL highest concentration MIC setup. All MIC assays with no visible difference within their setup to the negative control, representing no growth, were repeated on assays at a concentration of 1 µg/mL (Table A3b). Azithromycin (AZM), clarithromycin (CLR), erythromycin (ERY), and roxithromycin (RXM) MICs were identified on the 1 µg/mL highest concentration MIC setup. Alternatively, the MIC value data with no discernable qualitative difference within an experiment in relation to the positive control, representing full growth, were retested at concentrations of 256 µg/mL, 512 µg/mL, and 1,024 µg/mL for reassessment. Cefazolin (CFZ) and ceftriaxone (CRO) MICs were identified on the 512 µg/mL highest concentration MIC setup. The rifabutin (RFB) MICs were identified on the MIC assay

with 1,024 µg/mL as the highest concentration. Succeeding experiments excluded drugs with MIC values higher than 64 µg/mL. Every MIC assay was performed six times before using results as a reference to begin the generation of the antibiotic resistant strains.

An additional six MIC assay experiments were carried out for MPN M129-B7 UA in tandem with the MIC assays for the generated passages under independent stress from each drug. The data presented here is collected from 12 MIC assays for each antibiotic (Table 1) on P5 of MPN M129-B7 UA. The numerical values for the MICs were taken directly from Table A3 setups by identifying the first well with qualitative color changes from the negative control. The first well to show slight discoloration from the negative control was used as the MIC value for that particular antibiotic. The 12 replicates for the MIC results were averaged to establish a quantifiable relationship between the obtained MIC values for every drug. Rifampicin (RIF), Imipenem (IPM), vancomycin (VAN) were also tested on MPN M129-B7, but MIC values could not be determined in the tests performed, so they were excluded from the antibiotic resistant passage generation experiments. By finding consistencies in the MIC values for the antibiotics tested we were able to find a starting point to begin growing MPN M129-B7 cells under antibiotic stress at sub-inhibitory concentrations. We did this by using the well values below the identified MIC from the setup carried out on Table A3 the sub-inhibitory concentrations for every antibiotic to expose MPN M129-B7 across five passages. The Chemotherapy Working Team of the International Research Program on Comparative Mycoplasmology (IRPCM) suggest to use the general guideline of considering MICs of ≤ 1 µg/mL for Molliculite species as viable indicators of the efficacy of that particular antibiotic for treatment (151). Based on this guideline, we determined that SPT (mean MIC = 0.396 µg/mL), PUR (mean MIC = 0.667 µg/mL), LVX (mean MIC = 0.750 µg/mL), MXF (mean MIC = 0.203 µg/mL), AZM (mean MIC = 0.001302083 µg/mL), CLR (mean MIC = 0.005289714 µg/mL), ERY (mean MIC = 0.040104167 µg/mL), RXM (mean MIC = 0.072916667 µg/mL), DOX (mean MIC = 0.156 µg/mL), and TET (mean MIC = 0.160 µg/mL) are effective in treating MPN M129-B7 UA.

TABLE 1 Compiled results of *Mycoplasma pneumoniae* M129-B7 (ATCC 29342) minimum inhibitory concentration assay using 19 antibiotics from 12 replicates

Antibiotic Class	Antibiotic	MIC (µg/mL) Mean	Standard Deviation
Aminocyclitol	Spectinomycin (SPT)	0.396	0.129
Aminoglycoside	Gentamicin (GEN)	7.000	3.464
	Kanamycin (KAN)	18.667	6.228
	Puromycin (PUR)	0.667	0.246
Amphenicol	Chloramphenicol (CHL)	5.333	1.969
Ansamycin	Rifabutin (RFB)	2.500	1.000
Cephalosporin	Cefrazolin (CFZ)	192.000	73.901
	Ceftriaxone (CRO)	448.000	128.000
Fluoroquinolone	Ciprofloxacin (CIP)	2.333	0.778
	Levofloxacin (LVX)	0.750	0.452
	Moxifloxacin (MXF)	0.203	0.149
Lincosamide	Clindamycin (CLI)	4.167	1.337
Macolide	Azithromycin (AZM)	0.001302083	0.000480826
	Clarithromycin (CLR)	0.005289714	0.003735117
	Erythromycin (ERY)	0.040104167	0.028882886
	Roxithromycin (RXM)	0.072916667	0.069665054
Nitrofuran	Nitrofurantoin (NIT)	5.000	2.000
Tetracycline	Doxycycline (DOX)	0.156	0.057
	Tetracycline (TET)	0.160	0.116

Exposure to decreasingly lower sub-inhibitory (SIC) in some antibiotics led to consistent mutations with higher cell yields. Growth conditions under antibiotic stress for MPN M129-B7 with each antibiotic were at ½ the determined MIC, as per our two-fold MIC assay setup (Table A3). For example, SPT had a well value MIC of 0.500 µg/mL, so the growth condition at SIC would be 0.250 µg/mL. Initial yields from the 6 day incubation period were too low to continue passage creation under the same antibiotic stress, so incubation time was increased to 7-10 days with daily visual assessment of MPN attachment to the 250 mL culture flask(s) before harvesting. Four sub-inhibitory concentrations (SIC1, SIC2, SIC3, and SIC4) below the obtained MIC values were used in an effort to consistently generate antibiotic resistant passages within five passages (PA1 to PA5) with enough cell growth to be able to continue

experiments without the need to frequently replenish antibiotic stressed MPN cell stocks (Table 2).

TABLE 2 Sub-inhibitory (SIC) concentrations used to generate MPN M129-B7 antibiotic resistant strains from MPN M129-B7 UA P5 MIC values

Drug	Drug Concentration (µg/mL)	Drug	Drug Concentration (µg/mL)	Drug	Drug Concentration (µg/mL)
SPT		CIP		CLR	
SIC1	0.25	SIC1	0.5	SIC1	39.06×10^{-4}
SIC2	0.125	SIC2	0.25	SIC2	19.53×10^{-4}
SIC3	6.25×10^{-2}	SIC3	0.125	SIC3	9.77×10^{-4}
SIC4	3.13×10^{-2}	SIC4	6.25×10^{-2}	SIC4	4.88×10^{-4}
GEN		LVX		ERY	
SIC1	2	SIC1	0.25	SIC1	31.25×10^{-3}
SIC2	1	SIC2	0.125	SIC2	15.63×10^{-3}
SIC3	0.5	SIC3	6.25×10^{-2}	SIC3	7.81×10^{-3}
SIC4	0.25	SIC4	3.13×10^{-2}	SIC4	3.91×10^{-3}
KAN		MXF		RXM	
SIC1	8	SIC1	6.250×10^{-2}	SIC1	62.50×10^{-3}
SIC2	4	SIC2	3.13×10^{-2}	SIC2	31.25×10^{-3}
SIC3	2	SIC3	1.56×10^{-2}	SIC3	15.63×10^{-3}
SIC4	1	SIC4	0.78×10^{-2}	SIC4	7.81×10^{-3}
PUR		CLI		DOX	
SIC1	0.25	SIC1	2	SIC1	62.50×10^{-3}
SIC2	0.125	SIC2	1	SIC2	31.25×10^{-3}
SIC3	0.063	SIC3	0.5	SIC3	15.63×10^{-3}
SIC4	3.13×10^{-2}	SIC4	0.25	SIC4	7.81×10^{-3}
CHL		AZM		TET	
SIC1	2	SIC1	9.77×10^{-4}	SIC1	62.50×10^{-3}
SIC2	1	SIC2	4.88×10^{-4}	SIC2	31.25×10^{-3}
SIC3	0.5	SIC3	2.44×10^{-4}	SIC3	15.63×10^{-3}
SIC4	0.25	SIC4	1.22×10^{-4}	SIC4	7.81×10^{-3}

Abbreviations for antibiotics: spectinomycin (SPT), gentamycin (GEN), kanamycin (KAN), puromycin (PUR), chloramphenicol (CHL), rifabutin (RFB), cefrazolin (CFZ), ceftriaxone (CRO), ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), clindamycin (CLI), azithromycin (AZM), clarithromycin (CLR), erythromycin (ERY), roxithromycin (RXM), nitrofurantoin (NIT), doxycycline (DOX), tetracycline (TET).

We were unable to generate passages beyond the first or second for macrolides AZM (SIC1 = 9.77×10^{-4} µg/mL, SIC2 =4.88×10^{-4} µg/mL,SIC3 = 2.44×10^{-4} µg/mL, SIC4 = 1.22×10^{-4} µg/mL), CLR (SIC1 = 39.06×10^{-4} µg/mL, SIC2 = 19.53×10^{-4} µg/mL,SIC3 =

$9.77x10^{-4}$ µg/mL, SIC4 = $4.88x10^{-4}$ µg/mL), ERY (SIC1 = $31.25x10^{-3}$ µg/mL, SIC2 = $15.63x10^{-3}$ µg/mL, SIC3 = $7.81x10^{-3}$ µg/mL, SIC4 = $3.91x10^{-3}$ µg/mL), and RXM (SIC1 = $62.5x10^{-3}$ µg/mL, SIC2 = $31.25x10^{-3}$ µg/mL,SIC3 = $15.63x10^{-3}$ µg/mL, SIC4 = $7.81x10^{-3}$ µg/mL) in any of the sub-inhibitory concentrations used, so we could not asses changes induced by antibiotic stress on MPN M129-B7. All other antibiotics were tested (see Table 2) and had a corresponding MPN stock under antibiotic stress at the four assigned sub-inhibitory concentrations. These SIC passages were retested with MIC assays under the same concentration corresponding to their identified MIC value to compare well value shifts. The new MIC assay tests including SIC passages were replicated three times for each drug. Well value shifts were considered as phenotypic changes representative of a development of antibiotic resistance to that particular antibiotic. Observations based on the values designated for each well were recorded and analyzed with analysis of variance (ANOVA) statistical tests with an alpha value of 5% ($\alpha = 0.05$). Significant differences among the means reinforced phenotypic well value shift considerations for the development of antibiotic resistance to the antibiotic being tested for that sample set.

Similar to the initial identification of the starting point MIC, from which we derived the SICs used, we observed qualitatively similar MIC trends for each passage for every drug. The data was compiled and analyzed through single factor ANOVA tests. ANOVA tests resulted in significant ($P < 0.05$) mean changes in MIC value for aminocyclitol spectinomycin, SPT, (Table 3) ($F = 44.122$, F crit= 1.761, $P < 0.0001$).

TABLE 3a: SPT MIC (µg/mL) comparison between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5) ANOVA output

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Mean (µg/mL)	Variance
MPN M129-B7 Unaltered	12	4.750	0.396	0.017
SIC1-PA1	4	160.000	40.000	256.000
SIC1-PA2	4	256.000	64.000	0.000
SIC1-PA3	4	256.000	64.000	0.000
SIC1-PA4	4	256.000	64.000	0.000
SIC1-PA5	4	256.000	64.000	0.000
SIC2-PA1	3	28.000	9.333	37.333
SIC2-PA2	3	80.000	26.667	85.333

TABLE 3a (Continued)

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Mean (µg/mL)	Variance
SIC2-PA3	3	160.000	53.333	341.333
SIC2-PA4	3	192.000	64.000	0.000
SIC2-PA5	3	192.000	64.000	0.000
SIC3-PA1	3	20.000	6.667	5.333
SIC3-PA2	3	24.000	8.000	0.000
SIC3-PA3	3	40.000	13.333	21.333
SIC3-PA4	3	64.000	21.333	85.333
SIC3-PA5	3	112.000	37.333	597.333
SIC4-PA1	3	7.000	2.333	2.333
SIC4-PA2	3	40.000	13.333	21.333
SIC4-PA3	3	80.000	26.667	85.333
SIC4-PA4	3	192.000	64.000	0.000
SIC4-PA5	3	192.000	64.000	0.000

Spectinomycin (SPT) sub-inhibitory concentrations (SICs) used were: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = 6.25×10^{-2} µg/mL; SIC4 = 3.13×10^{-2} µg/mL. MPN M129-B7 was determined to have significant ($P < 0.0001$) variation between UA strain and SIC1-4 PA1 to PA5 for SPT MIC (µg/mL) through Single Factor ANOVA.

TABLE 3b: SPT Single Factor ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	52518.706	20	2625.935	44.122	3.84×10^{-27}	1.761
Within Groups	3332.849	56	59.515			
Total	55851.555	76				

Spectinomycin (SPT) sub-inhibitory concentrations (SICs) used: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = 6.25×10^{-2} µg/mL; SIC4 = 3.13×10^{-2} µg/mL. MPN M129-B7 was determined to have significant ($P < 0.0001$) variation between UA strain and SIC1-4 PA1 to PA5 for SPT MIC (µg/mL) through Single Factor ANOVA.

The initial MIC value for MPN M129-B7 UA was 0.396 µg/mL with fluctuations from that mean MIC varying as high as 64 µg/mL in SIC1-PA2 to PA5. The lowest MIC concentration obtained was 2.333 µg/mL for SIC4-PA1, which is still higher than the unaltered MIC mean. The two-fold dilution performed is responsible for the high amount of variability observed, but the results were consistent across the three replicates performed for each passage. We can better observe the trends in MIC value changes from MPN M129-B7 UA MIC by plotting the SPT data (Fig. 2).

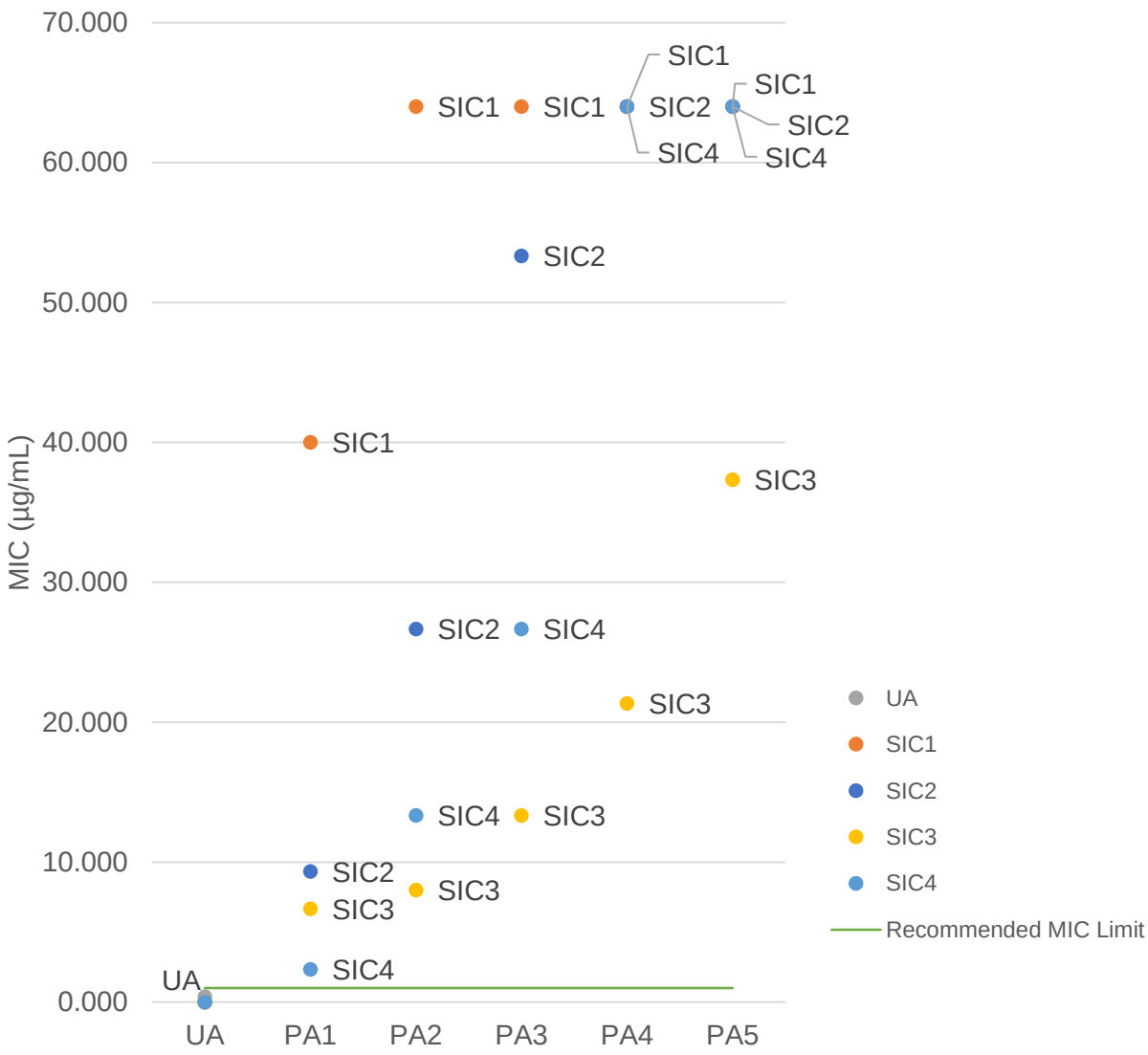

FIG 2 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under spectinomycin (SPT) stress. SPT sub-inhibitory concentrations (SICs) used: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = $6.25x10^{-2}$ µg/mL; SIC4 = $3.13x10^{-2}$ µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was determined to have significant ($P < 0.0001$) variation between UA strain and SIC1-4 PA1 to PA5 for SPT MIC (µg/mL) through Single Factor ANOVA.

We can see a positive increase in the MIC value of our SIC MPN M129-B7 strains when compared to the UA strain. Despite the variability among MIC shifts from varying concentrations, our data shows that higher SIC concentrations lead to higher and faster changes in MIC values when MPN M129-B7 is exposed to SPT.

The aminoglycoside gentamicin, GEN, caused significant ($P < 0.05$) MIC value changes (Table 4) ($F = 16.081$, $F\ crit = 1.780$, $P < 0.0001$)

TABLE 4a: GEN MIC (µg/mL) comparison between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5) ANOVA output

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Mean (µg/mL)	Variance
MPN M129-B7 Unaltered	12	84.000	7.000	12.000
SIC1-PA1	3	32.000	10.667	21.333
SIC1-PA2	3	80.000	26.667	85.333
SIC1-PA3	3	96.000	32.000	0.000
SIC1-PA4	3	128.000	42.667	341.333
SIC1-PA5	3	160.000	53.333	341.333
SIC2-PA1	3	16.000	5.333	5.333
SIC2-PA2	3	20.000	6.667	5.333
SIC2-PA3	3	32.000	10.667	21.333
SIC2-PA4	3	48.000	16.000	0.000
SIC2-PA5	3	80.000	26.667	85.333
SIC3-PA1	3	12.000	4.000	0.000
SIC3-PA2	3	12.000	4.000	0.000
SIC3-PA3	3	12.000	4.000	0.000
SIC3-PA4	3	12.000	4.000	0.000
SIC3-PA5	3	16.000	5.333	5.333
SIC4-PA1	3	16.000	5.333	5.333
SIC4-PA2	3	16.000	5.333	5.333
SIC4-PA3	3	12.000	4.000	0.000
SIC4-PA4	3	16.000	5.333	5.333
SIC4-PA5	3	16.000	5.333	5.333

Gentamycin (GEN) sub-inhibitory concentrations (SICs) used: SIC1 = 2 µg/mL; SIC2 = 1 µg/mL; SIC3 = 0.500 µg/mL; SIC4 = 0.250 µg/mL. MPN M129-B7 was determined to have significant ($P < 0.0001$) variation between UA strain and SIC1-4 PA1 to PA5 for GEN MIC (µg/mL) through Single Factor ANOVA.

Table 4b: GEN Single Factor ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	12603.778	20	630.189	16.081	1.62×10^{-15}	1.780
Within Groups	1998.667	51	39.190			
Total	14602.444	71				

Gentamycin (GEN) sub-inhibitory concentrations (SICs) used: SIC1 = 2 µg/mL; SIC2 = 1 µg/mL; SIC3 = 0.500 µg/mL; SIC4 = 0.250 µg/mL. MPN M129-B7 was determined to have significant ($P < 0.0001$) variation between UA strain and SIC1-4 PA1 to PA5 for GEN MIC (µg/mL) through Single Factor ANOVA.

GEN had its UA MIC value at 7 µg/mL. The highest MIC value observed was at 53.333 µg/mL for SIC1-PA5 while the lowest MIC value observed was 4 µg/mL for SIC3-PA1 to PA4, and SIC4-PA3. MIC values for GEN were plotted (Fig. 3).

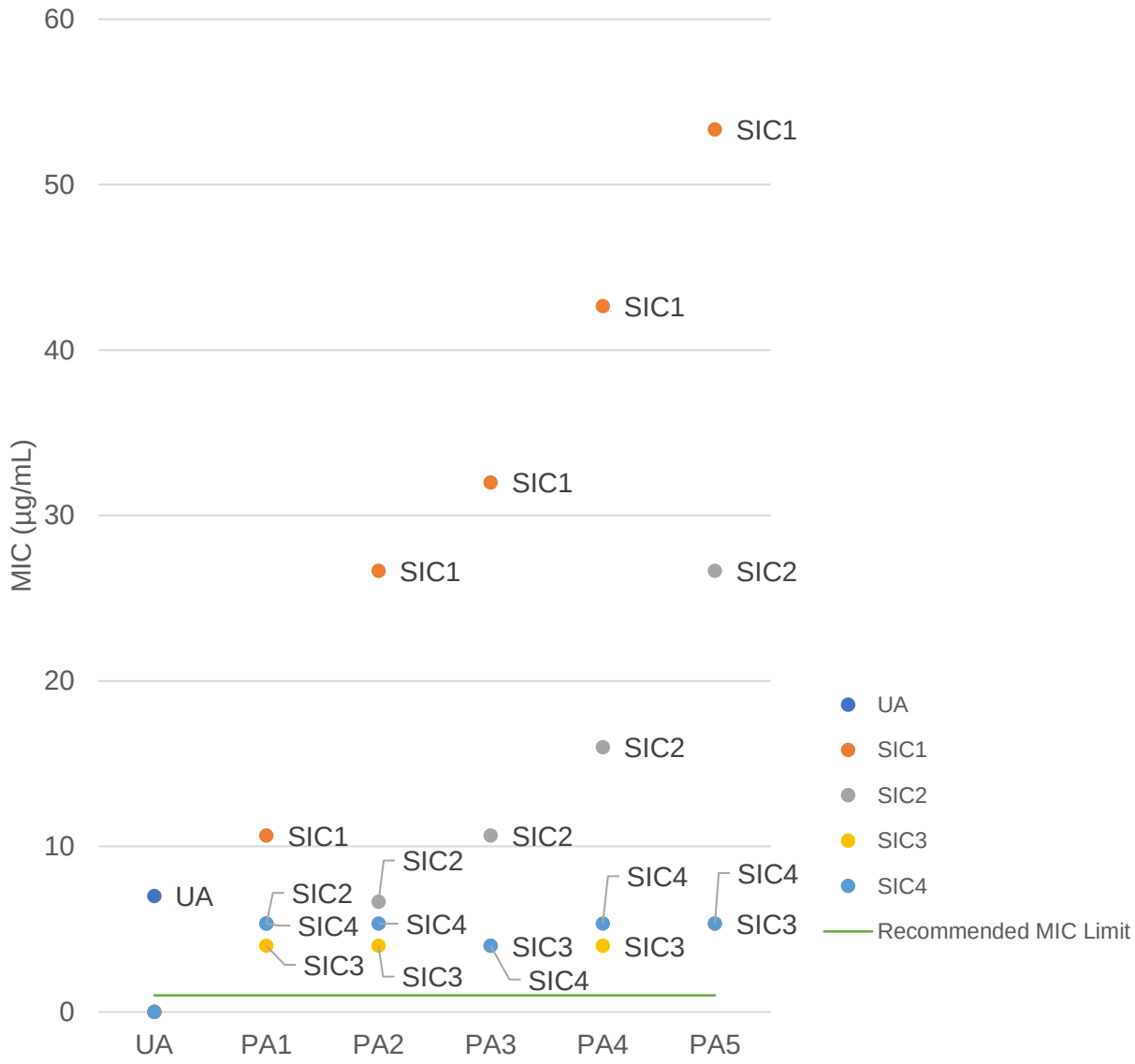

FIG 3 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under gentamycin (GEN) stress. GEN sub-inhibitory concentrations (SICs) used: SIC1 = 2 µg/mL; SIC2 = 1 µg/mL; SIC3 = 0.500 µg/mL; SIC4 = 0.250 µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was determined to have significant ($P < 0.0001$) variation between UA strain and SIC1-4 PA1 to PA5 for GEN MIC (µg/mL) through Single Factor ANOVA.

The aminoglycoside Kanamycin, KAN, caused significant ($P < 0.05$) MIC value changes (Table 5) ($F = 2.957$, $F\ crit = 1.780$, $P < 0.01$).

TABLE 5a: KAN MIC (µg/mL) data comparison with comparison between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5) ANOVA output

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Average (µg/mL)	Variance
MPN M129-B7 Unaltered	12	224.000	18.667	38.788
SIC1-PA1	3	48.000	16.000	0.000
SIC1-PA2	3	64.000	21.333	85.333
SIC1-PA3	3	80.000	26.667	85.333
SIC1-PA4	3	96.000	32.000	0.000
SIC1-PA5	3	128.000	42.667	341.333
SIC2-PA1	3	64.000	21.333	85.333
SIC2-PA2	3	48.000	16.000	0.000
SIC2-PA3	3	64.000	21.333	85.333
SIC2-PA4	3	80.000	26.667	85.333
SIC2-PA5	3	80.000	26.667	85.333
SIC3-PA1	3	48.000	16.000	0.000
SIC3-PA2	3	48.000	16.000	0.000
SIC3-PA3	3	48.000	16.000	0.000
SIC3-PA4	3	64.000	21.333	85.333
SIC3-PA5	3	48.000	16.000	0.000
SIC4-PA1	3	48.000	16.000	0.000
SIC4-PA2	3	48.000	16.000	0.000
SIC4-PA3	3	48.000	16.000	0.000
SIC4-PA4	3	64.000	21.333	85.333
SIC4-PA5	3	48.000	16.000	0.000

Kanamycin (KAN) sub-inhibitory concentrations (SICs) used: SIC1 = 8 µg/mL; SIC2 = 4 µg/mL; SIC3 = 2 µg/mL; SIC4 = 1 µg/mL. MPN M129-B7 was determined to have significant ($P < 0.01$) variation between UA strain and SIC1-4 PA1 to PA5 for KAN MIC (µg/mL) through Single Factor ANOVA.

TABLE 5b: KAN Single Factor ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2869.333	20	143.467	2.957	9.33×10^{-4}	1.780
Within Groups	2474.667	51	48.523			
Total	5344	71				

Kanamycin (KAN) sub-inhibitory concentrations (SICs) used: SIC1 = 8 µg/mL; SIC2 = 4 µg/mL; SIC3 = 2 µg/mL; SIC4 = 1 µg/mL. MPN M129-B7 was determined to have significant ($P < 0.01$) variation between UA strain and SIC1-4 PA1 to PA5 for KAN MIC (µg/mL) through Single Factor ANOVA.

Variation from the UA MIC mean for KAN (18.667 µg/mL), went as high as 42.667 µg/mL for SIC1-PA5, and as low as 16 µg/mL for SIC1-PA1, SIC2-PA2, SIC3-PA3-PA1 to PA3, and SIC4-PA1 to PA3 and PA5. The plotting of the data still shows that the highest of the subinhibitory concentrations used lead to higher MIC value changes in less passages (Fig. 4).

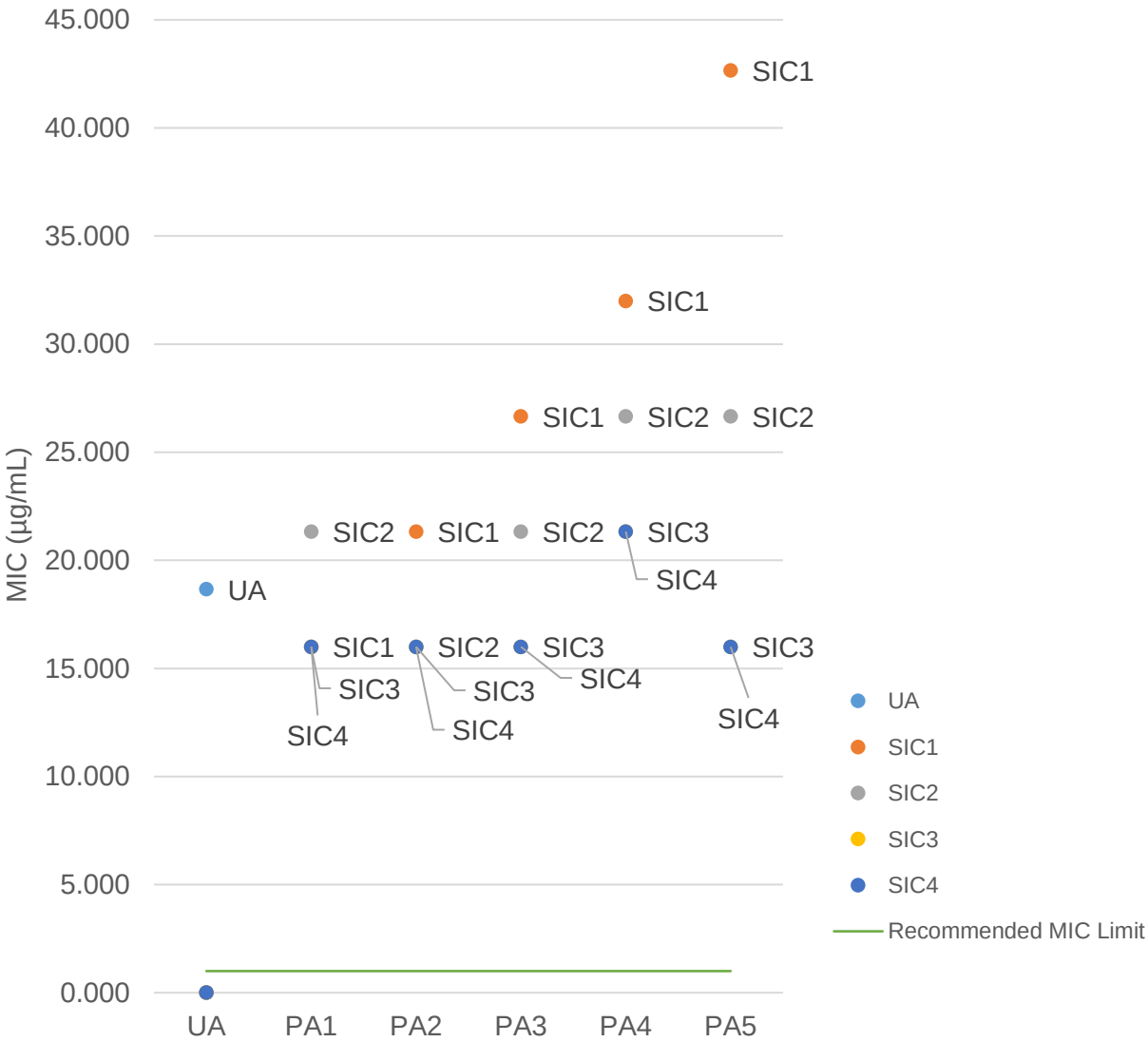

FIG 4 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under kanamycin (KAN) stress. KAN sub-inhibitory concentrations (SICs) used: SIC1 = 8 µg/mL; SIC2 = 4 µg/mL; SIC3 = 2 µg/mL; SIC4 = 1 µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was determined to have significant ($P < 0.01$) variation between UA strain and SIC1-4 PA1 to PA5 for KAN MIC (µg/mL) through Single Factor ANOVA.

The aminoglycosides puromycin, PUR, caused significant ($P < 0.05$) MIC value changes (Table 6) ($F = 2.255$, $F\ crit = 1.780$, $P < 0.05$).

TABLE 6a: PUR MIC (µg/mL) data comparison with comparison between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5) ANOVA output

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Average (µg/mL)	Variance
MPN M129-B7				
Unaltered	12	8.000	0.667	0.061
SIC1-PA1	3	2.000	0.667	0.083
SIC1-PA2	3	2.000	0.667	0.083
SIC1-PA3	3	2.500	0.833	0.083
SIC1-PA4	3	3.000	1.000	0.000
SIC1-PA5	3	4.000	1.333	0.333
SIC2-PA1	3	1.500	0.500	0.000
SIC2-PA2	3	2.000	0.667	0.083
SIC2-PA3	3	2.000	0.667	0.083
SIC2-PA4	3	2.000	0.667	0.083
SIC2-PA5	3	3.000	1.000	0.000
SIC3-PA1	3	1.500	0.500	0.000
SIC3-PA2	3	2.000	0.667	0.083
SIC3-PA3	3	2.000	0.667	0.083
SIC3-PA4	3	1.500	0.500	0.000
SIC3-PA5	3	1.500	0.500	0.000
SIC4-PA1	3	2.000	0.667	0.083
SIC4-PA2	3	1.500	0.500	0.000
SIC4-PA3	3	2.000	0.667	0.083
SIC4-PA4	3	1.500	0.500	0.000
SIC4-PA5	3	1.500	0.500	0.000

Puromycin (PUR) sub-inhibitory concentrations (SICs) used: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = 0.063 µg/mL; SIC4 = $3.13x10^{-2}$ µg/mL. MPN M129-B7 was determined to have significant ($P < 0.05$) variation between UA strain and SIC1-4 PA1 to PA5 for PUR MIC (µg/mL) through Single Factor ANOVA.

TABLE 6b: PUR Single Factor ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2.653	20	0.133	2.255	$1.01X10^{-2}$	1.780
Within Groups	3	51	0.059			
Total	5.653	71				

Puromycin (PUR) sub-inhibitory concentrations (SICs) used: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = 0.063 µg/mL; SIC4 = $3.13x10^{-2}$ µg/mL. MPN M129-B7 was determined to have significant ($P < 0.05$) variation between UA strain and SIC1-4 PA1 to PA5 for PUR MIC (µg/mL) through Single Factor ANOVA.

The PUR UA MIC mean was 0.667 µg/mL with the highest fluctuation from that mean being 1.333 µg/mL for SIC1-PA5, and the lowest being 0.500 µg/mL for SIC2-PA1, SIC3-PA1, PA4 to PA5, SIC4-PA2, PA4 to PA5 (Fig. 5). The SIC1 set of stressed passages still expressed a higher MIC value shift. The remainder were under or too close to the UA MIC mean to be considered significantly different.

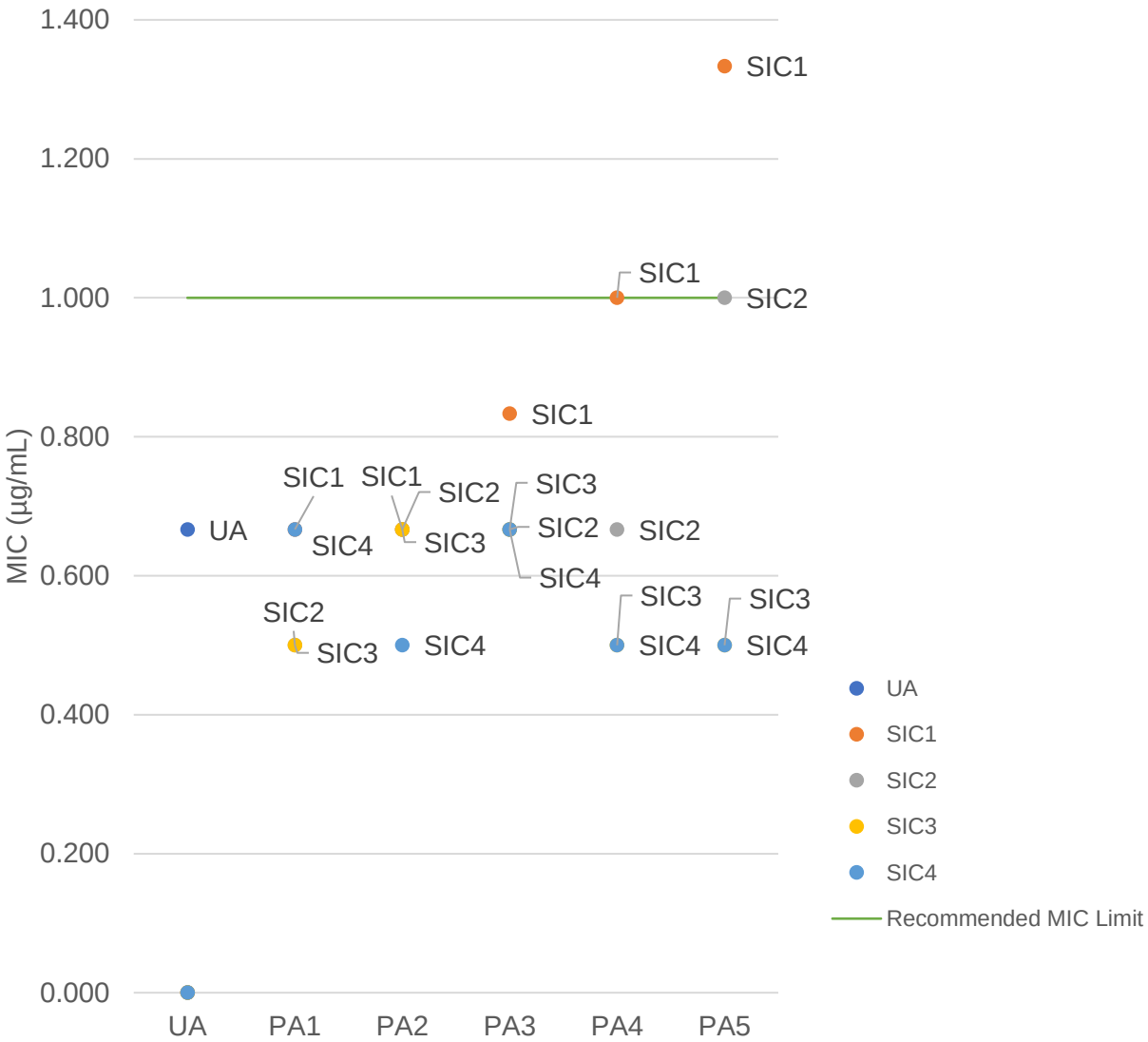

FIG 5 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under puromycin (PUR) stress. PUR sub-inhibitory concentrations (SICs) used: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = 0.063 µg/mL; SIC4 = $3.13x10^{-2}$ µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was determined to have significant ($P < 0.05$) variation between UA strain and SIC1-4 PA1 to PA5 for PUR MIC (µg/mL) through Single Factor ANOVA.

The amphenicol chloramphenicol, CHL, did not cause significant (P > 0.05) MIC value changes (Table 7) (F = 1.712, F crit = 1.780, P > 0.05).

TABLE 7a: CHL MIC (µg/mL) data comparison with comparison between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5) ANOVA output

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Average (µg/mL)	Variance
MPN M129-B7 Unaltered	12	56.000	4.667	2.424
SIC1-PA1	3	16.000	5.333	5.333
SIC1-PA2	3	12.000	4.000	0.000
SIC1-PA3	3	16.000	5.333	5.333
SIC1-PA4	3	16.000	5.333	5.333
SIC1-PA5	3	32.000	10.667	21.333
SIC2-PA1	3	16.000	5.333	5.333
SIC2-PA2	3	12.000	4.000	0.000
SIC2-PA3	3	16.000	5.333	5.333
SIC2-PA4	3	16.000	5.333	5.333
SIC2-PA5	3	16.000	5.333	5.333
SIC3-PA1	3	12.000	4.000	0.000
SIC3-PA2	3	16.000	5.333	5.333
SIC3-PA3	3	12.000	4.000	0.000
SIC3-PA4	3	16.000	5.333	5.333
SIC3-PA5	3	12.000	4.000	0.000
SIC4-PA1	3	12.000	4.000	0.000
SIC4-PA2	3	12.000	4.000	0.000
SIC4-PA3	3	16.000	5.333	5.333
SIC4-PA4	3	12.000	4.000	0.000
SIC4-PA5	3	16.000	5.333	5.333

Chloramphenicol (CHL) sub-inhibitory concentrations (SICs) used: SIC1 = 2 µg/mL; SIC2 = 1 µg/mL; SIC3 = 0.500 µg/mL; SIC4 = 0.250 µg/mL. MPN M129-B7 was not determined to have significant (P > 0.05) variation between UA strain and SIC1-4 PA1 to PA5 for CHL MIC (µg/mL) through Single Factor ANOVA.

TABLE 7b: CHL Single Factor ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	125.333	20	6.267	1.712	6.24×10^{-2}	1.780
Within Groups	186.667	51	3.660			
Total	312	71				

Chloramphenicol (CHL) sub-inhibitory concentrations (SICs) used: SIC1 = 2 µg/mL; SIC2 = 1 µg/mL; SIC3 = 0.500 µg/mL; SIC4 = 0.250 µg/mL. MPN M129-B7 was not determined to have significant (P > 0.05) variation between UA strain and SIC1-4 PA1 to PA5 for CHL MIC (µg/mL) through Single Factor ANOVA.

The MIC UA against CHL was 4.667 µg/mL. The biggest increase in MIC value was at an MIC value of 10.667 µg/mL. The lowest MIC value observed was at 4 µg/mL, which is seen frequently below the UA MIC mean. The plotted trends show us that what the ANOVA analysis suggest, that there is no significant variation in MIC value shifts for MPN M129-B7 stressed passages at the four tested SICs with CHL (Fig. 6).

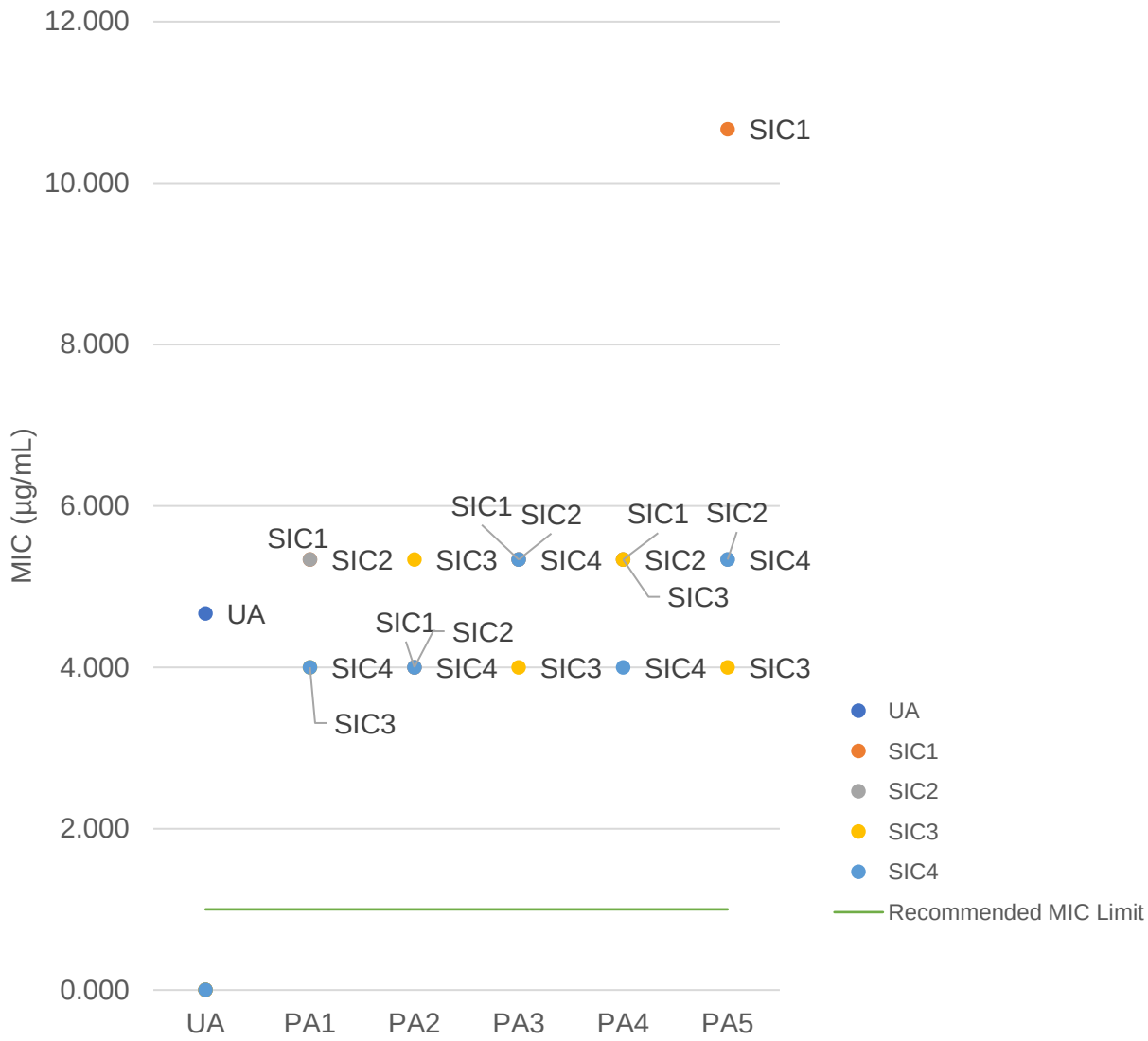

FIG 6 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under chloramphenicol (CHL) stress. CHL sub-inhibitory concentrations (SICs) used: SIC1 = 2 µg/mL; SIC2 = 1 µg/mL; SIC3 = 0.500 µg/mL; SIC4 = 0.250 µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was not determined to have significant ($P > 0.05$) variation between UA strain and SIC1-4 PA1 to PA5 for CHL MIC (µg/mL) through Single Factor ANOVA.

The fluoroquinolone ciprofloxacin, CIP, caused significant ($P < 0.05$) MIC value changes in MPN M129-B7 (Table 8) ($F = 3.113$, $F\ crit = 1.780$, $P < 0.01$)

TABLE 8a: CIP MIC (µg/mL) data comparison with comparison between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5) ANOVA output

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Average (µg/mL)	Variance
MPN M129-B7 Unaltered	12	28.000	2.333	0.606
SIC1-PA1	3	8.000	2.667	1.333
SIC1-PA2	3	10.000	3.333	1.333
SIC1-PA3	3	10.000	3.333	1.333
SIC1-PA4	3	12.000	4.000	0.000
SIC1-PA5	3	16.000	5.333	5.333
SIC2-PA1	3	6.000	2.000	0.000
SIC2-PA2	3	6.000	2.000	0.000
SIC2-PA3	3	6.000	2.000	0.000
SIC2-PA4	3	6.000	2.000	0.000
SIC2-PA5	3	8.000	2.667	1.333
SIC3-PA1	3	6.000	2.000	0.000
SIC3-PA2	3	6.000	2.000	0.000
SIC3-PA3	3	6.000	2.000	0.000
SIC3-PA4	3	8.000	2.667	1.333
SIC3-PA5	3	6.000	2.000	0.000
SIC4-PA1	3	6.000	2.000	0.000
SIC4-PA2	3	8.000	2.667	1.333
SIC4-PA3	3	6.000	2.000	0.000
SIC4-PA4	3	6.000	2.000	0.000
SIC4-PA5	3	8.000	2.667	1.333

Ciprofloxacin (CIP) sub-inhibitory concentrations (SICs) used: SIC1 = 0.500 µg/mL; SIC2 = 0.250 µg/mL; SIC3 = 0.125 µg/mL; SIC4 = 6.25×10^{-2} µg/mL. MPN M129-B7 was determined to have significant ($P < 0.01$) variation between UA strain and SIC1-4 PA1 to PA5 for CIP MIC (µg/mL) through Single Factor ANOVA.

TABLE 8b: CIP Single Factor ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	43.944	20	2.197	3.113	5.55×10^{-4}	1.780
Within Groups	36	51	0.706			
Total	79.944	71				

Ciprofloxacin (CIP) sub-inhibitory concentrations (SICs) used: SIC1 = 0.500 µg/mL; SIC2 = 0.250 µg/mL; SIC3 = 0.125 µg/mL; SIC4 = 6.25×10^{-2} µg/mL. MPN M129-B7 was determined to have significant ($P < 0.01$) variation between UA strain and SIC1-4 PA1 to PA5 for CIP MIC (µg/mL) through Single Factor ANOVA.

The MPN 129-B7 UA MIC value started at 2.333 µg/mL. From there it went as high as 5.333 µg/mL for SIC1-PA5, and as low as 2 µg/mL for SIC2-PA1 to PA4, SIC3-PA1 to PA3 and PA5, SIC4-PA1 and PA3 to PA4. Regardless of the fluctuations below the UA MIC mean, CIP shows rapid increase in MIC values at SIC1 across the five cell passages performed (Fig. 7).

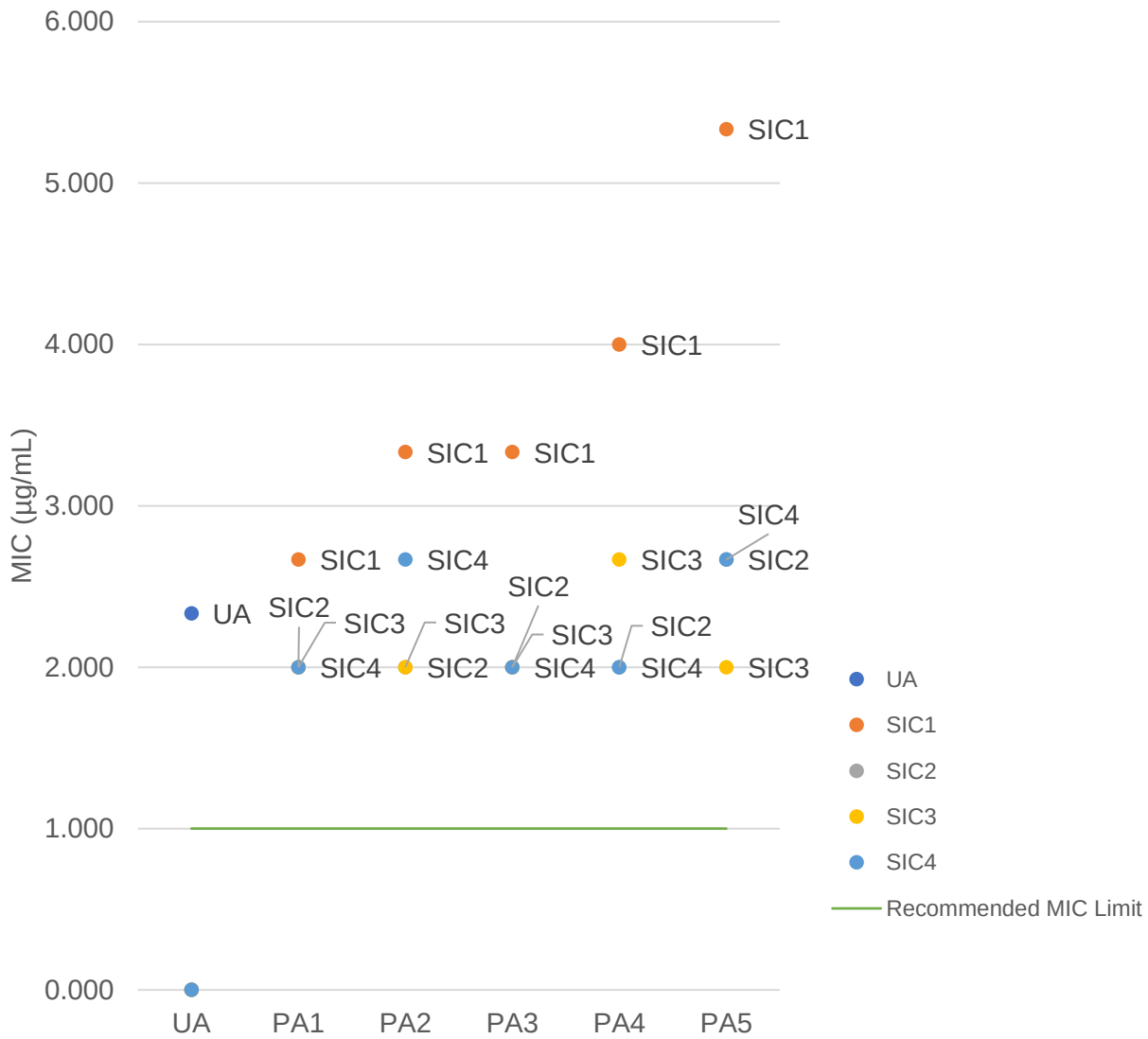

FIG 7 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under ciprofloxacin (CIP) stress. CIP sub-inhibitory concentrations (SICs) used: SIC1 = 0.500 µg/mL; SIC2 = 0.250 µg/mL; SIC3 = 0.125 µg/mL; SIC4 = 6.25×10^{-2} µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was determined to have significant ($P < 0.01$) variation between UA strain and SIC1-4 PA1 to PA5 for CIP MIC (µg/mL) through Single Factor ANOVA.

The fluoroquinolone levofloxacin, LVX, caused significant ($P < 0.05$) MIC value changes in MPN M129-B7 (Table 9) ($F = 5.106$, $F\ crit = 1.780$, $P < 0.0001$)

TABLE 9a: LVX MIC (µg/mL) data comparison with comparison between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5) ANOVA output

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Average (µg/mL)	Variance
MPN M129-B7 Unaltered	12	9.000	0.750	0.205
SIC1-PA1	3	2.000	0.667	0.083
SIC1-PA2	3	2.000	0.667	0.083
SIC1-PA3	3	4.000	1.333	0.333
SIC1-PA4	3	7.000	2.333	2.333
SIC1-PA5	3	8.000	2.667	1.333
SIC2-PA1	3	1.500	0.500	0.000
SIC2-PA2	3	1.500	0.500	0.000
SIC2-PA3	3	1.500	0.500	0.000
SIC2-PA4	3	2.500	0.833	0.083
SIC2-PA5	3	4.000	1.333	0.333
SIC3-PA1	3	1.500	0.500	0.000
SIC3-PA2	3	1.500	0.500	0.000
SIC3-PA3	3	1.500	0.500	0.000
SIC3-PA4	3	1.500	0.500	0.000
SIC3-PA5	3	1.500	0.500	0.000
SIC4-PA1	3	1.500	0.500	0.000
SIC4-PA2	3	1.500	0.500	0.000
SIC4-PA3	3	1.500	0.500	0.000
SIC4-PA4	3	1.500	0.500	0.000
SIC4-PA5	3	1.500	0.500	0.000

Levofloxacin (LVX) sub-inhibitory concentrations (SICs) used: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = 6.25×10^{-2} µg/mL; SIC4 = 3.13×10^{-2} µg/mL. MPN M129-B7 was determined to have significant ($P < 0.0001$) variation between UA strain and SIC1-4 PA1 to PA5 for LVX MIC (µg/mL) through Single Factor ANOVA.

TABLE 9b: LVX Single Factor ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	22.861	20	1.143	5.106	1.27×10^{-6}	1.780
Within Groups	11.417	51	0.224			
Total	34.278	71				

Levofloxacin (LVX) sub-inhibitory concentrations (SICs) used: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = 6.25×10^{-2} µg/mL; SIC4 = 3.13×10^{-2} µg/mL. MPN M129-B7 was determined to have significant ($P < 0.0001$) variation between UA strain and SIC1-4 PA1 to PA5 for LVX MIC (µg/mL) through Single Factor ANOVA.

Starting MIC value for UA was 0.750 µg/mL. The highest MIC was 2.667 µg/mL for SIC1-PA5. The lowest MIC was 0.500 µg/mL for SIC2-PA1 to PA3, SIC3-PA1 to PA5, and SIC4-PA1 to PA5. Once more the significant variations in MIC mean from the MIC UA mean occurred for SIC1 passages when placed under LVX antibiotic pressure (Fig. 8).

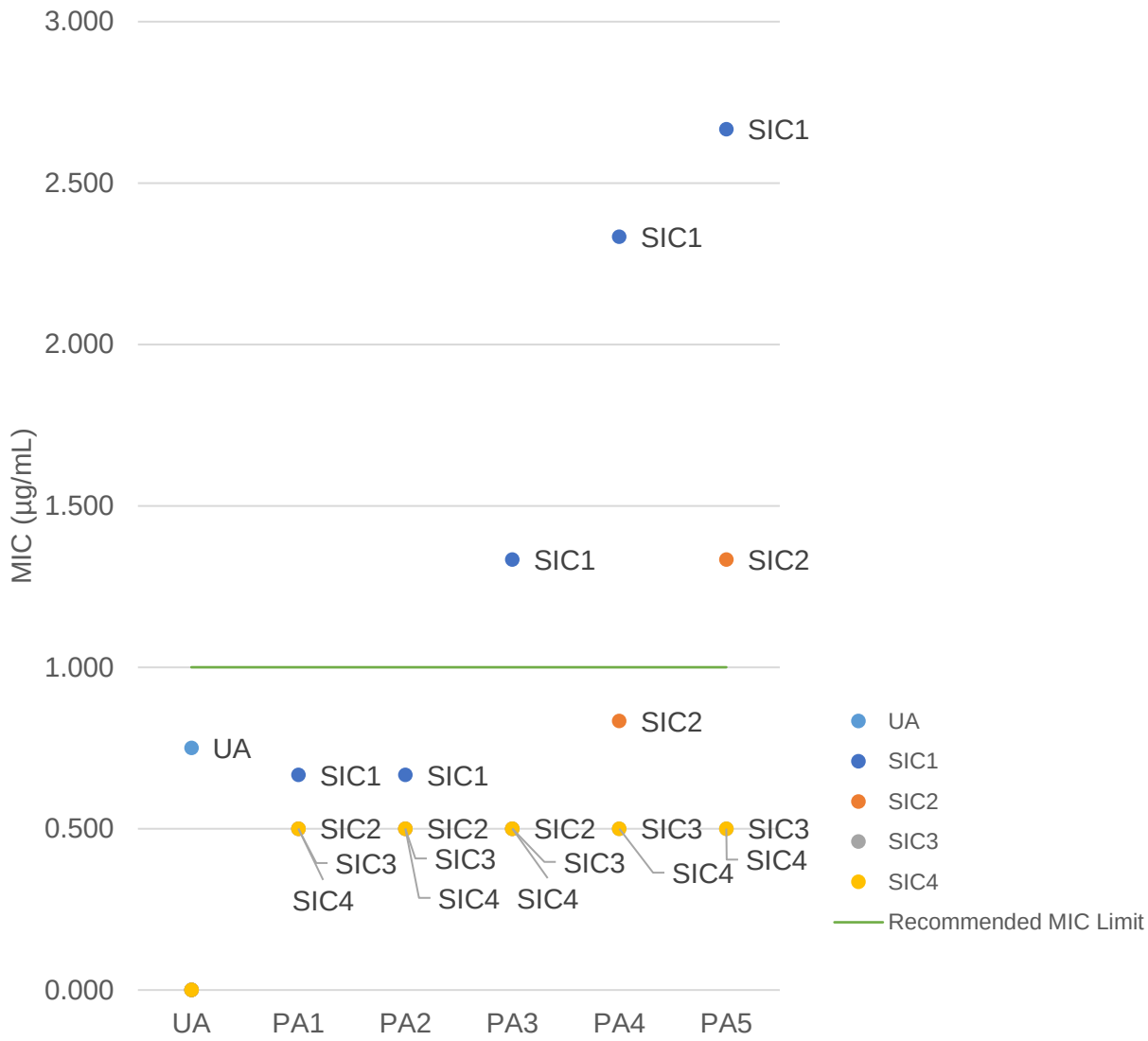

FIG 8 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under levofloxacin (LVX) stress. LVX sub-inhibitory concentrations (SICs) used: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = 6.25×10^{-2} µg/mL; SIC4 = 3.13×10^{-2} µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was determined to have significant ($P < 0.0001$) variation between UA strain and SIC1-4 PA1 to PA5 for LVX MIC (µg/mL) through Single Factor ANOVA.

The fluoroquinolone moxifloxacin, MXF, caused significant ($P < 0.05$) MIC value changes in MPN M129-B7 (Table 10) ($F = 13.459$ $F\ crit = 1.780$, $P < 0.01$).

TABLE 10a: MXF MIC (µg/mL) data comparison with comparison between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5) ANOVA output

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Average (µg/mL)	Variance
MPN M129-B7 Unaltered	12	2.438	0.203	0.022
SIC1-PA1	3	0.500	0.167	0.005
SIC1-PA2	3	1.000	0.333	0.021
SIC1-PA3	3	1.500	0.500	0.000
SIC1-PA4	3	2.000	0.667	0.083
SIC1-PA5	3	5.000	1.667	0.333
SIC2-PA1	3	0.375	0.125	0.000
SIC2-PA2	3	0.500	0.167	0.005
SIC2-PA3	3	0.500	0.167	0.005
SIC2-PA4	3	0.750	0.250	0.047
SIC2-PA5	3	2.000	0.667	0.083
SIC3-PA1	3	0.500	0.167	0.005
SIC3-PA2	3	0.375	0.125	0.000
SIC3-PA3	3	0.375	0.125	0.000
SIC3-PA4	3	0.500	0.167	0.005
SIC3-PA5	3	0.500	0.167	0.005
SIC4-PA1	3	0.375	0.125	0.000
SIC4-PA2	3	0.375	0.125	0.000
SIC4-PA3	3	0.375	0.125	0.000
SIC4-PA4	3	0.375	0.125	0.000
SIC4-PA5	3	0.500	0.167	0.005

Moxifloxacin (MXF) sub-inhibitory concentrations (SICs) used: SIC1 = 6.25×10^{-2} µg/mL; SIC2 = 3.13×10^{-2} µg/mL; SIC3 = 1.56×10^{-2} µg/mL; SIC4 = 0.78×10^{-2} µg/mL. MPN M129-B7 was determined to have significant ($P < 0.01$) variation between UA strain and SIC1-4 PA1 to PA5 for MXF MIC (µg/mL) through Single Factor ANOVA.

TABLE 10b: MXF Single Factor ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	7.661	20	0.383	13.459	6.17×10^{-14}	1.780
Within Groups	1.451	51	0.028			
Total	9.113	71				

Moxifloxacin (MXF) sub-inhibitory concentrations (SICs) used: SIC1 = 6.25×10^{-2} µg/mL; SIC2 = 3.13×10^{-2} µg/mL; SIC3 = 1.56×10^{-2} µg/mL; SIC4 = 0.78×10^{-2} µg/mL. MPN M129-B7 was determined to have significant ($P < 0.01$) variation between UA strain and SIC1-4 PA1 to PA5 for MXF MIC (µg/mL) through Single Factor ANOVA.

The initial MIC of MPN M129-B7 against MXF was 0.203 µg/mL. The highest MIC observed was 1.667 for SIC1-PA5. The lowest MIC was 0.125 µg/mL for SIC2-PA1, SIC3-PA2 to PA3, and SIC4-PA1 to PA4. The SIC1 caused a greater shift within the five passages. Most of the variability came from MIC values below the MIC UA (Fig. 9).

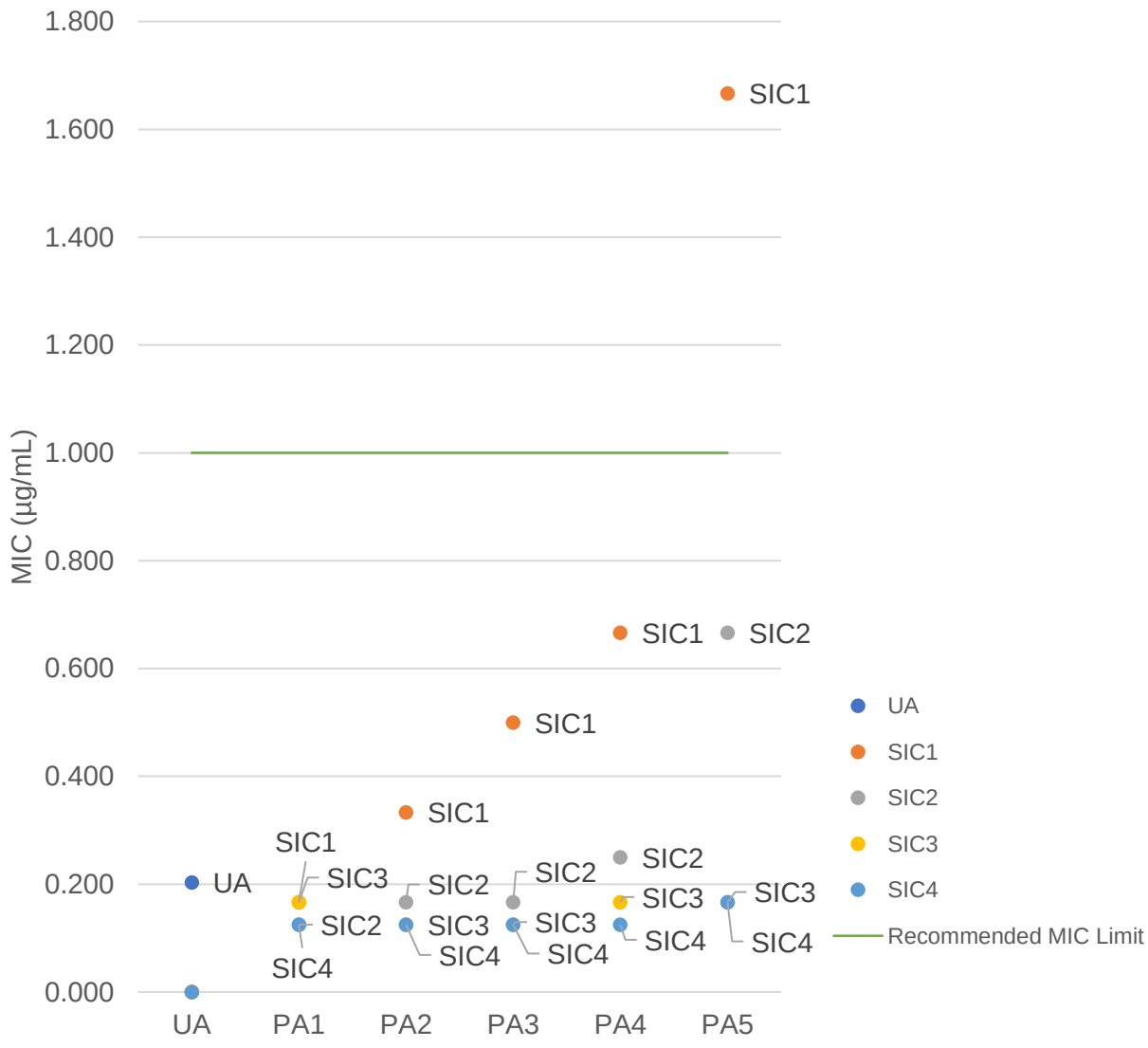

FIG 9 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under moxifloxacin (MXF) stress. MXF sub-inhibitory concentrations (SICs) used: SIC1 = 6.25 x10^{-2} µg/mL; SIC2 = 3.13 x10^{-2} µg/mL; SIC3 = 1.56x10^{-2} µg/mL; SIC4 = 0.78x10^{-2} µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was determined to have significant ($P < 0.01$) variation between UA strain and SIC1-4 PA1 to PA5 for MXF MIC (µg/mL) through Single Factor ANOVA.

The lincosamide clindamycin, CLI, did not cause significant (P > 0.05) MIC value changes (Table 11) (F = 0.434, F crit = 1.780, P > 0.05).

TABLE 11a: CLI MIC (µg/mL) data comparison with comparison between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5) ANOVA output

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Average (µg/mL)	Variance
MPN M129-B7 Unaltered	12	50.000	4.167	1.788
SIC1-PA1	3	12.000	4.000	0.000
SIC1-PA2	3	12.000	4.000	0.000
SIC1-PA3	3	12.000	4.000	0.000
SIC1-PA4	3	16.000	5.333	5.333
SIC1-PA5	3	12.000	4.000	0.000
SIC2-PA1	3	12.000	4.000	0.000
SIC2-PA2	3	12.000	4.000	0.000
SIC2-PA3	3	12.000	4.000	0.000
SIC2-PA4	3	12.000	4.000	0.000
SIC2-PA5	3	12.000	4.000	0.000
SIC3-PA1	3	12.000	4.000	0.000
SIC3-PA2	3	12.000	4.000	0.000
SIC3-PA3	3	12.000	4.000	0.000
SIC3-PA4	3	12.000	4.000	0.000
SIC3-PA5	3	12.000	4.000	0.000
SIC4-PA1	3	12.000	4.000	0.000
SIC4-PA2	3	12.000	4.000	0.000
SIC4-PA3	3	12.000	4.000	0.000
SIC4-PA4	3	12.000	4.000	0.000
SIC4-PA5	3	12.000	4.000	0.000

Clindamycin (CLI) sub-inhibitory concentrations (SICs) used: SIC1 = 2 µg/mL; SIC2 = 1 µg/mL; SIC3 = 0.500 µg/mL; SIC4 = 0.250 µg/mL. MPN M129-B7 was not determined to have significant (P > 0.05) variation between UA strain and SIC1-4 PA1 to PA5 for CLI MIC (µg/mL) through Single Factor ANOVA.

TABLE 11b: CLI Single Factor ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	5.167	20	0.258	0.434	0.978	1.780
Within Groups	30.333	51	0.595			
Total	35.500	71				

Clindamycin (CLI) sub-inhibitory concentrations (SICs) used: SIC1 = 2 µg/mL; SIC2 = 1 µg/mL; SIC3 = 0.500 µg/mL; SIC4 = 0.250 µg/mL. MPN M129-B7 was not determined to have significant (P > 0.05) variation between UA strain and SIC1-4 PA1 to PA5 for CLI MIC (µg/mL) through Single Factor ANOVA.

Fluctuations of the MIC for CLI on MPN M129-B7 from the UA MIC (4.167 µg/mL) were not sufficiently varied to determine a phenotypic change. The plotted data allows us to observe that SIC1-PA4 had the highest MIC at 5.333 µg/mL, and the lowest remaining at 4 µg/mLfor most of the passages (Fig. 10).

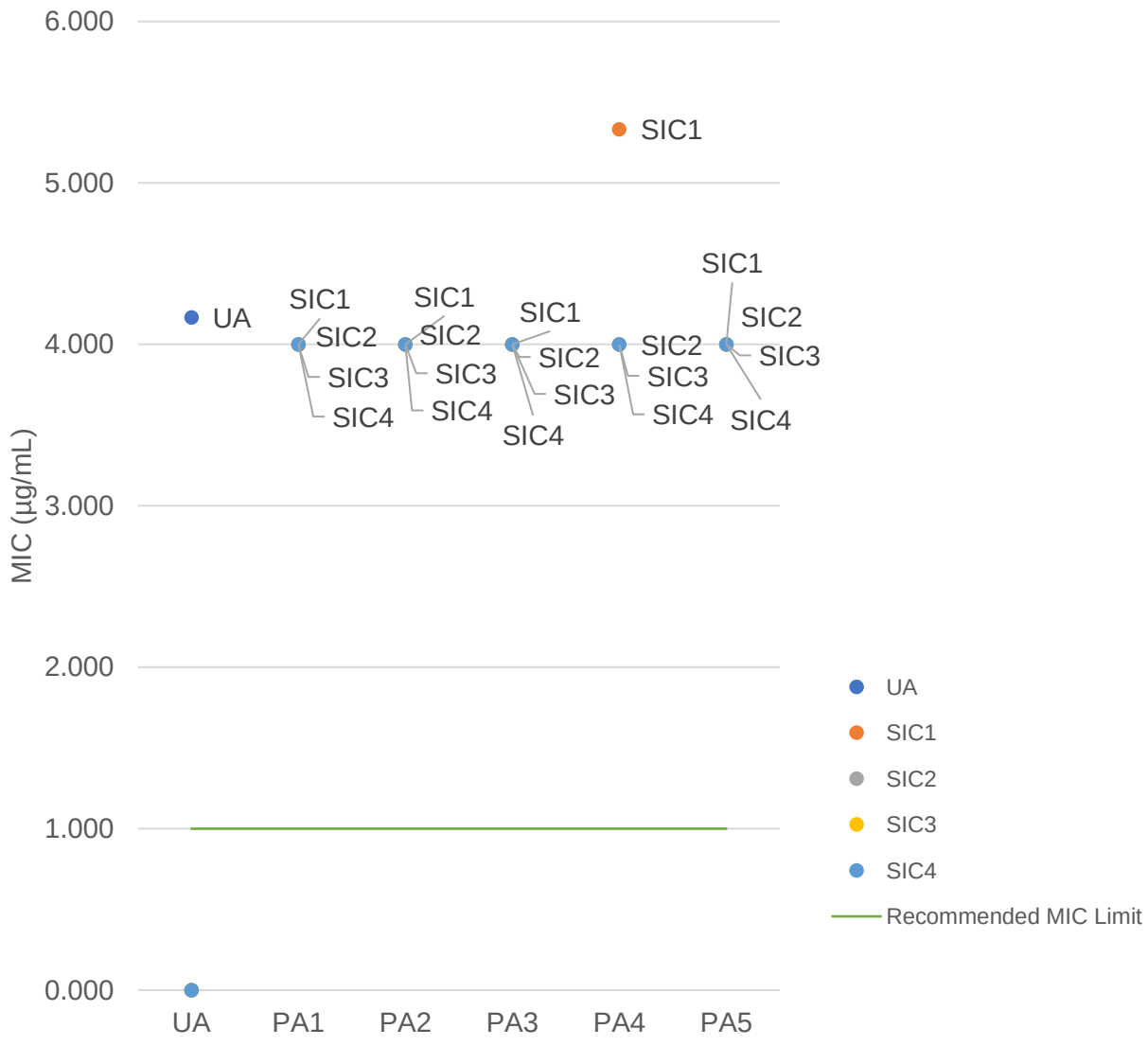

FIG 10 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under clindamycin (CLI) stress. CLI sub-inhibitory concentrations (SICs) used: SIC1 = 2 µg/mL; SIC2 = 1 µg/mL; SIC3 = 0.500 µg/mL; SIC4 = 0.250 µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was not determined to have significant ($P > 0.05$) variation between UA strain and SIC1-4 PA1 to PA5 for CLI MIC (µg/mL) through Single Factor ANOVA.

Lastly, the tetracyclines doxycycline, DOX, (Table 12), and tetracycline, TET, (Table 13) did not cause significant (P > 0.05) MIC value changes.

TABLE 12a: DOX MIC (µg/mL) data comparison with comparison between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5) ANOVA output

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Average (µg/mL)	Variance
MPN M129-B7 Unaltered	12	1.875	0.156	0.003
SIC1-PA1	3	0.375	0.125	0.000
SIC1-PA2	3	0.375	0.125	0.000
SIC1-PA3	3	0.500	0.167	0.005
SIC1-PA4	3	0.500	0.167	0.005
SIC1-PA5	3	0.500	0.167	0.005
SIC2-PA1	3	0.375	0.125	0.000
SIC2-PA2	3	0.500	0.167	0.005
SIC2-PA3	3	0.375	0.125	0.000
SIC2-PA4	3	0.500	0.167	0.005
SIC2-PA5	3	0.375	0.125	0.000
SIC3-PA1	3	0.375	0.125	0.000
SIC3-PA2	3	0.375	0.125	0.000
SIC3-PA3	3	0.500	0.167	0.005
SIC3-PA4	3	0.375	0.125	0.000
SIC3-PA5	3	0.500	0.167	0.005
SIC4-PA1	3	0.375	0.125	0.000
SIC4-PA2	3	0.375	0.125	0.000
SIC4-PA3	3	0.375	0.125	0.000
SIC4-PA4	3	0.375	0.125	0.000
SIC4-PA5	3	0.375	0.125	0.000

Doxycycline (DOX) sub-inhibitory concentrations (SICs) used: SIC1 = 62.50 $x10^{-3}$ µg/mL; SIC2 = 31.25 $x10^{-3}$ µg/mL; SIC3 = 15.63$x10^{-3}$ µg/mL; SIC4 = 7.81$x10^{-3}$ µg/mL. MPN M129-B7 was not determined to have significant (P > 0.05) variation between UA strain and SIC1-4 PA1 to PA5 for DOX MIC (µg/mL) through Single Factor ANOVA.

TABLE 12b: DOX Single Factor ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.026	20	1.3210^{-3}	0.625	0.875	1.780
Within Groups	0.108	51	$2.12X10^{-3}$			
Total	0.135	71				

Doxycycline (DOX) sub-inhibitory concentrations (SICs) used: SIC1 = 62.50 $x10^{-3}$ µg/mL; SIC2 = 31.25 $x10^{-3}$ µg/mL; SIC3 = 15.63$x10^{-3}$ µg/mL; SIC4 = 7.81$x10^{-3}$ µg/mL. MPN M129-B7 was not determined to have significant (P > 0.05) variation between UA strain and SIC1-4 PA1 to PA5 for DOX MIC (µg/mL) through Single Factor ANOVA.

TABLE 13a: ANOVA output of TET MIC (µg/mL) data comparison with MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Strain	Total Replicates	Sum of MICs (µg/mL)	MIC Average (µg/mL)	Variance
MPN M129-B7 Unaltered	12	1.925	0.160	13.35$x10^{-3}$
SIC1-PA1	3	0.500	0.167	5.21$x10^{-3}$
SIC1-PA2	3	0.500	0.167	5.21$x10^{-3}$
SIC1-PA3	3	0.625	0.208	5.21$x10^{-3}$
SIC1-PA4	3	0.625	0.208	5.21$x10^{-3}$
SIC1-PA5	4	1.875	0.469	0.15
SIC2-PA1	3	0.375	0.125	0.00
SIC2-PA2	3	0.375	0.125	0.00
SIC2-PA3	3	0.500	0.167	5.21$x10^{-3}$
SIC2-PA4	3	0.375	0.125	0.00
SIC2-PA5	3	0.500	0.167	5.21$x10^{-3}$
SIC3-PA1	3	0.375	0.125	0.00
SIC3-PA2	3	0.375	0.125	0.00
SIC3-PA3	3	0.375	0.125	0.00
SIC3-PA4	3	0.500	0.167	5.21$x10^{-3}$
SIC3-PA5	3	0.375	0.125	0.00
SIC4-PA1	3	0.375	0.125	0.00
SIC4-PA2	3	0.375	0.125	0.00
SIC4-PA3	3	0.375	0.125	0.00
SIC4-PA4	3	0.375	0.125	0.000
SIC4-PA5	3	0.500	0.167	5.21$x10^{-3}$

Tetracycline (TET) sub-inhibitory concentrations (SICs) used: SIC1 = 62.50 $x10^{-3}$ µg/mL; SIC2 = 31.25 $x10^{-3}$ µg/mL; SIC3 = 15.63$x10^{-3}$ µg/mL; SIC4 = 7.81$x10^{-3}$ µg/mL. MPN M129-B7 was not determined to have significant (P > 0.05) variation between UA strain and SIC1-4 PA1 to PA5 for TET MIC (µg/mL) through Single Factor ANOVA.

TABLE 13b: ANOVA output between MPN M129-B7 UA and respective stressed SIC strains (SIC1-4) across 5 passages (PA1-PA5)

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.433	20	0.022	1.658	0.074	1.776
Within Groups	0.679	52	0.013			
Total	1.113	72				

Tetracycline (TET) sub-inhibitory concentrations (SICs) used: SIC1 = 62.50 $x10^{-3}$ µg/mL; SIC2 = 31.25 $x10^{-3}$ µg/mL; SIC3 = 15.63$x10^{-3}$ µg/mL; SIC4 = 7.81$x10^{-3}$ µg/mL. MPN M129-B7 was not determined to have significant (P > 0.05) variation between UA strain and SIC1-4 PA1 to PA5 for TET MIC (µg/mL) through Single Factor ANOVA.

DOX had insufficient variability between strains to determine significant variation between their MIC values (F = 0.625, F crit = 1.780, P > 0.05). This is reflected in the plotted trends, which shows some variation in MICs above the UA 0.156 µg/mL (Fig. 11).

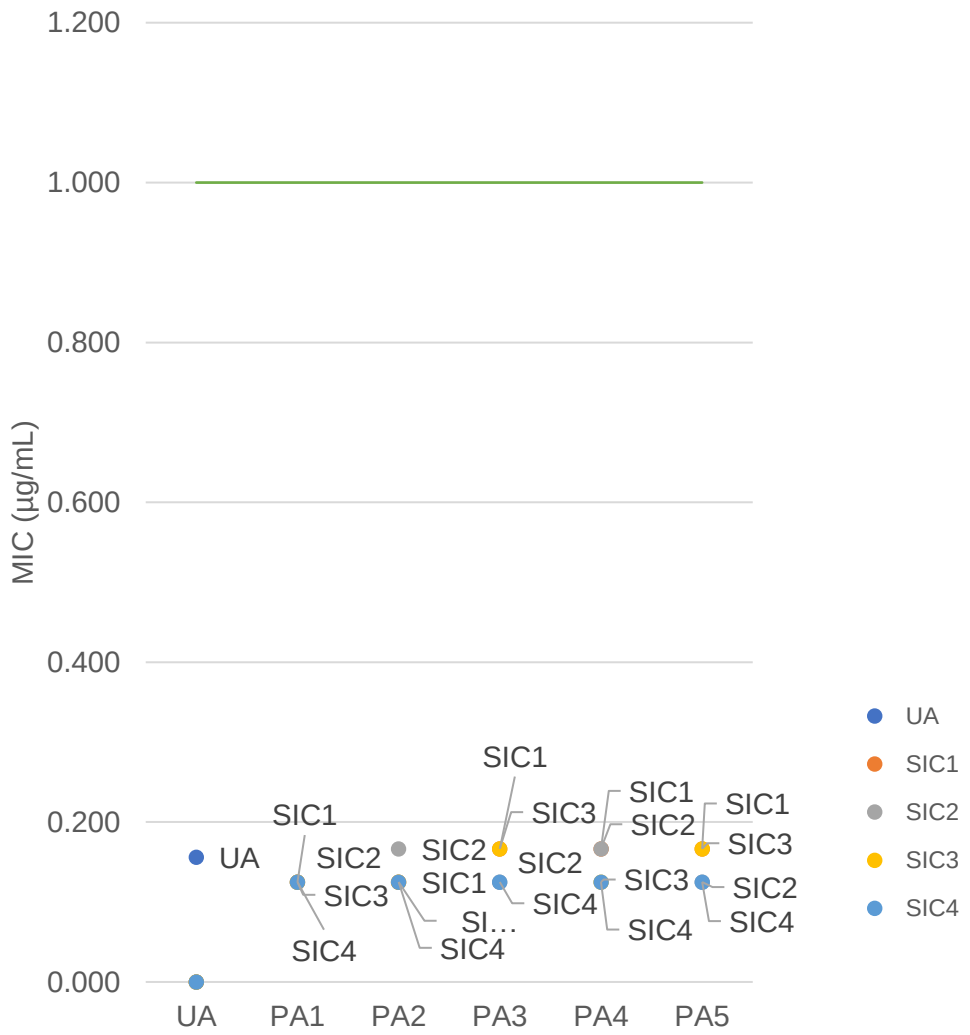

FIG 11 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under doxycycline (DOX) stress. DOX sub-inhibitory concentrations (SICs) used: SIC1 = 62.50 x10^{-3} µg/mL; SIC2 = 31.25 x10^{-3} µg/mL; SIC3 = 15.63x10^{-3} µg/mL; SIC4 = 7.81x10^{-3} µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was not determined to have significant (P > 0.05) variation between UA strain and SIC1-4 PA1 to PA5 for DOX MIC (µg/mL) through Single Factor ANOVA.

TET had slightly varied results to DOX as seen on (Fig. 12), but there was a much higher MIC value for SIC1-PA5 (0.469 µg/mL) than the UA MIC (0.160 µg/mL). There was also a more consistent build up of MICs throughout all SIC passages with increasing MIC values. SIC1 passages continued to result in higher MIC variation compared to SIC2-4.

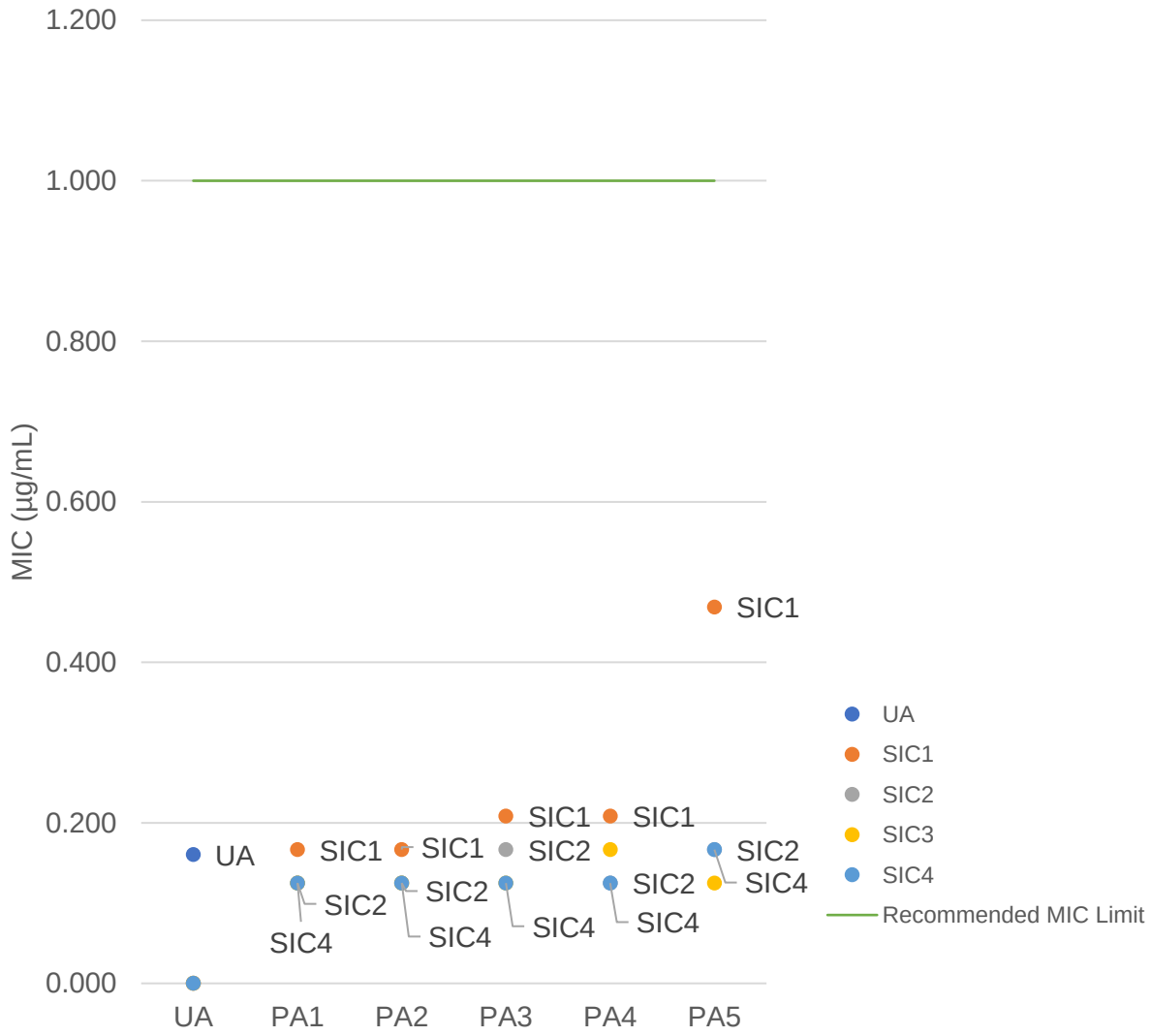

FIG 12 Plotted data comparison of MPN M129-B7 and SIC1-4 PA1 to PA5 MIC means under tetracycline (TET) stress. TET sub-inhibitory concentrations (SICs) used: SIC1 = 62.50 x10^{-3} µg/mL; SIC2 = 31.25 x10^{-3} µg/mL; SIC3 = 15.63x10^{-3} µg/mL; SIC4 = 7.81x10^{-3} µg/mL. The recommended MIC limit is 1 µg/mL as suggested by the IRPCM to determine if an antibiotic is viable in a clinical setting. MPN M129-B7 was not

determined to have significant (P > 0.05) variation between UA strain and SIC1-4 PA1 to PA5 for TET MIC (µg/mL) through Single Factor ANOVA.

All of the significant mean changes denoted by the ANOVA analysis were further analyzed through the use of the *t* test two-sample assuming unequal variances by comparing MIC value data for MPN M129-B7 UA with that of SIC1, SIC2, SIC3, and SIC4 to identify particular significant changes.

The SPT passages had significant (P > 0.05) variability between their MICs. Table 14 contains the comparison through *t*-test two-factor analysis. Most of the passages created had significant variation from the UA MIC mean when compared individually.

TABLE 14 Comparisons of SPT mean MIC variation between MPN M129-B7 UA and SPT SIC-PA strains with significant (P < 0.05) MIC mean shifts using *t* test two-sample assuming unequal variance

	SPT		
Strain	MIC Mean (µg/mL)	Standard Deviation	t calculated (vs. MPN M129-B7 UA)
MPN M129-B7 Unaltered	0.396	0.129	
SIC1-PA1	40.000	0.129	-4.950
SIC1-PA2	64.000	0.000	-1711.548
SIC1-PA3	IE	IE	IE
SIC1-PA4	IE	IE	IE
SIC1-PA5	IE	IE	IE
SIC2-PA2	26.667	9.238	-4.926
SIC2-PA3	53.333	18.475	-4.963
SIC2-PA4	IE	IE	IE
SIC2-PA5	IE	IE	IE
SIC3-PA1	6.667	2.309	-4.701
SIC3-PA2	8.000	0.000	-204.623
SIC3-PA3	13.333	4.619	-4.851
SIC4-PA2	13.333		-4.851
SIC4-PA3	26.667		-4.926
SIC4-PA4	64.000		-1711.548
SIC4-PA5	IE	IE	IE

Spectinomycin (SPT) sub-inhibitory concentrations (SICs) used: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = 6.25×10^{-2} µg/mL; SIC4 = 3.13×10^{-2} µg/mL. All presented data had individually significant (P <0.05) mean MIC variation between the mean MIC of the stressed PA strain against the mean MIC of MPN M129-B7 UA under SPT stress. There was insufficient evidence (IE) from our MIC assays for the reevaluation of SIC1-PA3 to P5, SIC2-PA4 to P5, and SIC4-PA5, but they were included in this table as the MIC value was observed higher than the 64 µg/mL highest concentration series dilution tests performed.

All of the tested aminoglycosides had significant (P > 0.05) variability between each other. The GEN SIC1-PA3 and PA5, SIC2-PA4, SIC3-PA1 to PA4, and SIC4-PA3 passages each had the significant variability form the MPN M129-B7 UA MIC to be considered phenotypically different (Table 15).

TABLE 15 Comparisons of GEN mean MIC variation between MPN M129-B7 UA and GEN SIC-PA strains with significant (P < 0.05) MIC mean shifts using *t* test two-sample assuming unequal variance

	GEN		
Strain	MIC Mean (µg/mL)	Standard Deviation	*t* calculated (vs. MPN M129-B7 UA)
MPN M129-B7 Unaltered	7.000	3.464	
SIC1-PA3	32.000	0.000	-25.000
SIC1-PA5	53.333	18.475	-4.325
SIC2-PA4	16.000	0.000	-9.000
SIC3-PA1	4.000	0.000	3.000
SIC3-PA2	4.000	0.000	3.000
SIC3-PA3	4.000	0.000	3.000
SIC3-PA4	4.000	0.000	3.000
SIC4-PA3	4.000	0.000	3.000

Gentamycin (GEN) sub-inhibitory concentrations (SICs) used: SIC1 = 2 µg/mL; SIC2 = 1 µg/mL; SIC3 = 0.500 µg/mL; SIC4 = 0.250 µg/mL. All presented data had individually significant (P <0.05) mean MIC variation between the mean MIC of the stressed PA strain against the mean MIC of MPN M129-B7 UA under GEN stress.

The SIC1-PA4 KAN passage was the only one that was varied enough from the UA MIC to be considered phenotypically different (Table 16).

TABLE 16 Comparisons of KAN mean MIC variation between MPN M129-B7 UA and KAN SIC-PA strains with significant (P < 0.05) MIC mean shifts using *t* test two-sample assuming unequal variance

	KAN		
Strain	MIC Mean (µg/mL)	Standard Deviation	*t* calculated (vs. MPN M129-B7 UA)
MPN M129-B7 Unaltered	18.67	6.23	
SIC1-PA4	32	0	-7.42

Kanamycin (KAN) sub-inhibitory concentrations (SICs) used: SIC1 = 8 µg/mL; SIC2 = 4 µg/mL; SIC3 = 2 µg/mL; SIC4 = 1 µg/mL. All presented data had individually significant (P <0.05) mean MIC variation between the mean MIC of the stressed PA strain against the mean MIC of MPN M129-B7 UA under KAN stress.

The last aminoglycoside we used, PUR, had more passages that were varied from the UA MIC, but many of them had established MICs below the 0.667 µg/mL (Table 17). The significantly ($P < 0.05$) lower MIC of 0.500 µg/mL suggests and improvement in drug function, but it is likely due to the qualitative nature of our procedure.

TABLE 17 Comparisons of PUR mean MIC variation between MPN M129-B7 UA and PUR SIC-PA strains with significant ($P < 0.05$) MIC mean shifts using *t* test two-sample assuming unequal variance

	PUR		
Strain	MIC Mean (µg/mL)	Standard Deviation	*t* calculated (vs. MPN M129-B7 UA)
MPN M129-B7 Unaltered	0.667	0.246	
SIC1-PA4	1.000	0.000	-4.690
SIC2-PA1	0.500	0.000	2.345
SIC2-PA5	1.000	0.000	-4.690
SIC3-PA1	0.500	0.000	2.345
SIC3-PA4	0.500	0.000	2.345
SIC3-PA5	0.500	0.000	2.345
SIC4-PA2	0.500	0.000	2.345
SIC4-PA4	0.500	0.000	2.345
SIC4-PA5	0.500	0.000	2.345

Puromycin (PUR) sub-inhibitory concentrations (SICs) used: SIC1 = 0.250 µg/mL; SIC2 = 0.125 µg/mL; SIC3 = 0.063 µg/mL; SIC4 = 3.13×10^{-2} µg/mL. All presented data had individually significant ($P < 0.05$) mean MIC variation between the mean MIC of the stressed PA strain against the mean MIC of MPN M129-B7 UA under PUR stress.

Fluoroquinolone CIP had only one passage, SIC1-PA4, with an MIC significantly different form the UA MIC (Table 18).

TABLE 18 Comparisons of CIP mean MIC variation between MPN M129-B7 UA and CIP SIC-PA strains with significant ($P < 0.05$) MIC mean shifts using *t* test two-sample assuming unequal variance

	CIP		
Strain	MIC Mean (µg/mL)	Standard Deviation	*t* calculated (vs. MPN M129-B7 UA)
MPN M129-B7 Unaltered	2.333	0.778	
SIC1-PA4	4.000	0.000	-7.416

Ciprofloxacin (CIP) sub-inhibitory concentrations (SICs) used: SIC1 = 0.500 µg/mL; SIC2 = 0.250 µg/mL; SIC3 = 0.125 µg/mL; SIC4 = 6.25×10^{-2} µg/mL. All presented data had individually significant ($P < 0.05$) mean MIC variation between the mean MIC of the stressed PA strain against the mean MIC of MPN M129-B7 UA under CIP stress.

Moxifloxacin, MXF, was the last antibiotic to yield us significant variability between the passages created under pressure from this antibiotic and the MPN M129-B7 UA strain. Two passages, SIC1-PA3 and SIC1-PA5, were sufficiently varied from the unaltered strain to consider them phenotypically different (Table 19).

TABLE 19 Comparisons of MXF mean MIC variation between MPN M129-B7 UA and MXF SIC-PA strains with significant (P < 0.05) MIC mean shifts using *t* test two-sample assuming unequal variance

	MXF		
Strain	MIC Mean (µg/mL)	Standard Deviation	*t* calculated (vs. MPN M129-B7 UA)
MPN M129-B7 Unaltered	0.203	0.149	
SIC1-PA3	0.500	0.000	-6.917
SIC1-PA5	1.667	0.577	-4.355

Moxifloxacin (MXF) sub-inhibitory concentrations (SICs) used: SIC1 = 6.25 $x10^{-2}$ µg/mL; SIC2 = 3.13 $x10^{-2}$ µg/mL; SIC3 = 1.56$x10^{-2}$ µg/mL; SIC4 = 0.78$x10^{-2}$ µg/mL. All presented data had individually significant (P <0.05) mean MIC variation between the mean MIC of the stressed PA strain against the mean MIC of MPN M129-B7 UA under MXF stress.

In addition, the incubation time for the antibiotics tested at lower concentrations was relatively shorter as ABS readings were higher, cell attachment to the 250 mL culture flask was more plentiful, and pellet formation after harvest was more visible.

MPN M129-B7 UA is capable of generating resistant mutant strains under the antibiotic pressure from the aminocyclitol, aminoglycoside, and fluoroquinolones tested within five passages under SIC antibiotic stress. The patterns in MIC value shifts under SPT antibiotic pressure indicate that SPT exposure towards MPN at SIC produced increases in MIC values from MPN M129-B7 UA in the PA strains (Fig. 2). Based on the results of our ANOVA analysis done on each passage created for each antibiotic, we proceeded to identify which passages were significantly different in mean MIC from their MPN M129-B7 UA counterpart.

Data gathered from the SPT trials showed that SIC1-PA3, SIC1-PA4, SIC-PA5, SIC2-PA4, SIC2-PA5, SIC4-PA5 had developed enough resistance to go beyond the tested SPT 64 µg/mL highest concentration. Further tests at a higher concentration would need to be carried out to establish a new MIC caused by these induced mutations. Fig. 2 shows a trend in SIC1 PA strains having higher mutation rates as

opposed to SIC2-4. Qualitatively, the distinction between MIC values is clear from the comparisons to the negative control representing full growth encompassing all of the rows dedicated to those tests. Through a two-tailed *t* test analysis we can infer that SIC1-PA1, SIC1-PA2, SIC2-PA2, SIC2-PA3, SIC3-PA1, SIC3-PA2, SIC3-PA3, SIC4-PA3, SIC4-PA4 had a significant ($P < 0.05$) MIC value shift to consider them as resistant when compared to those of MPN M129-B7 UA (Table 15). SPT ranged between 6.67-64.0 µg/mL in MIC value mean compared to the UA 0.396 µg/mL MIC mean. SPT SIC PA strains underwent a 16,061.62% increase from the MPN M129-B7 mean. All values from the excluded passages were not significantly different from the MPN M129-B7 UA MIC mean to be considered phenotypically nor perhaps genotypically altered by the drug exposure.

Trends in SIC1 for GEN, KAN, and PUR continued to show an exponential increase in MIC from MPN M129-B7 UA (Fig. 3-5). Single factor ANOVA data indicated significant ($P < 0.05$, $F > F\ crit$) MIC shifts for all of the aminoglycosides tested (Table 4-9). Our *t* test analysis suggests that we were able to generate significant difference from the mean of MPN M129-B7 UA to consider antibiotic resistance development in strains GEN (Table 14) SIC1-PA3, SIC1-PA5, SIC2-PA4, SIC3-PA1, SIC3-PA2, SIC3-PA3, SIC3-PA4, and SIC4-PA3; KAN (Table 15) SIC1-PA4; PUR (Table 16) SIC1-PA4, SIC2-PA1, SIC2-PA5, SIC3-PA1, SIC3-P4, SIC3-PA5, SIC4-PA2, SIC4-PA4, and SIC4-PA5. The means from the altered strains by GEN and PUR antibiotics vary within different ranges around the MIC mean for MPN M129-B7 UA. GEN MIC means (5.33-53.33 µg/mL) differ significantly from the UA (7 µg/mL mean) indicating a 661.86% increase, and PUR MIC means (0.500-1.00 µg/mL) differ significantly from the UA (0.67 µg/mL) with a 49.25% increase. Despite the qualitative nature of our main assessment of MIC values, we were able to account for any changes in phenotype/genotype by using a two-tailed *t* test with two-sample comparisons assuming unequal variances. This means that despite the fluctuations of these MIC shifts around the UA MIC, we can still consider those significant shifts as candidates for a genetic alteration that confers a shift caused by the induced stress of each antibiotic.

Lastly, SIC1 PA for LVX, CIP, and MXF had drastic changes from their respective MPN M129-B7 MICs (Fig. 7-9). Our ANOVA analysis indicated that there

was significant difference in the means of the MIC between LVX, CIP, and MXF. Further inspection of these results with our *t* test analysis indicated that LVX had no significant ($P > 0.05$) difference between means compared to MPN M129-B7 UA, but did have significant ($P < 0.05$) differences for a one-tailed test for all of the SIC3 and SIC4 passages. The analysis suggest that LVX could be more effective at lower inhibitory concentrations. CIP only had one significant ($P < 0.05$) difference from the MPN M129-B7 2.33 µg/mL MIC mean for SIC1-PA4 at a 4.00 µg/mL MIC mean indicating an increase of 71.67% (Table 18). In comparison, MXF had significant ($P < 0.05$) difference from the MXF 0.200 µg/mL MIC mean of MPN M129-B7 UA (Table 19). The significant differences from the UA MIC mean occurred in SIC1-PA3 at a mean of 0.500 µg/mL (a 150% increase), and SIC1-PA5 at 1.67 µg/mL (a 735% increase) for MXF, which indicate that they were phenotypically and likely genotypically altered by the drug stress.

This study successfully generated MPN M129-B7 antibiotic resistant strains for SPT, GEN, KAN, PUR, CIP, and MXF based on qualitatively gathered data from MIC assay MIC well value shifts, and quantitatively through single factor ANOVA and post hoc *t test* statistics. Significant difference from the UA mean for strain SPT (SIC1-PA1, SIC1-PA2, SIC1-PA3, SIC1-PA4, SIC1-PA5, SIC2-PA2, SIC2-PA3, SIC2-PA4, SIC2-PA5, SIC3-PA1, SIC3-PA2, SIC3-PA3, SIC4-PA2, SIC4-PA3, SIC4-PA4, SIC4-PA5), GEN (SIC1-PA3, SIC1-PA5, SIC2-PA4, SIC3-PA1, SIC3-PA2, SIC3-PA3, SIC3-PA4, SIC4-PA3), KAN (SIC1-PA4), PUR (SIC1-PA4, SIC2-PA1, SIC2-PA5, SIC3-PA1, SIC3-PA4, SIC3-PA5, SIC4-PA2, SIC4-PA4, SIC4-PA5), CIP (SIC1-PA4), and MXF (SIC1-PA3, SIC1-PA5) was determined through *t test* statistic with the guideline of t stat < -t critical two-tail.

DISCUSSION

Expanding the database of information on *Mycoplasma pneumoniae* for further research and practical application through MIC value assessment. There is a lack of antimicrobial susceptibility test (AST) interpretive criteria, otherwise known as breakpoints, for *M. pneumoniae*. AST is done in bacteria and fungi in order to predict the overall effects of antimicrobial therapy in individual patients. Additionally, it also performed to gather data on the susceptibility and resistance of local pathogens in order to create a foundation for empirical therapy procedures (164). With the AST information, researchers and clinicians can use it to detect infections in order to assign appropriate health measures. This will prevent further antibiotic resistance development within a microorganism in a population. Likewise, with the AST information, researchers and clinicians can observe the possible effects of issued countermeasures to slow, stop or reverse the measured dynamics of the development of resistance (164, 165, 166).

Clinical breakpoints are MIC values that allow for the prediction of the success or failure of an antibiotic therapy. In order to establish those clinical breakpoints one first requires data of an organism from clinical strain trials, resistance mechanisms, pharmacokinetics and pharmacodynamics dosage effects over time in target populations, and MIC distributions. With defined breakpoints it is possible to infer the effectiveness of the drug therapy by labeling it as S for 'Susceptible', R for 'Resistant', and I for 'Intermediate,' which is a strategy applied to clinical settings (164). Currently, the two major recommendations for breakpoints are taken from the European Committee on Antimicrobial Susceptibility Testing (EUCAST), which has more worldwide support with freely accessible guidelines, and the Clinical Laboratory Standards Institute (CLSI) (164, 167, 168, 169). Here we provide further data (Table 2-19) towards building a breakpoint criteria for *Mycoplasma pneumoniae* Somerson *et al.* (ATCC® 29342™) to be considered for addition into the general EUCAST or CLSI databases by providing MIC data against the tested aminocylitol, aminoglycosides, amphenicols, ansamycin, cephalosporins, fluoroquinolones, lincosamides, macrolides, nitrofuran, and tetracyclines.

On other hand, Mycoplasmal Chemotherapy Working Team of the International Research Program on Comparative Mycoplasmology (IRPCM) recommends to follow

the general guideline of considering MICs of ≤1 µg/mL for molliculite species to be good candidates for potentially effective treatment (151). Data obtained from our experiments on establishing MICs for MPN M129-B7 supports evidence of using aminocyclitol spectinomycin (SPT); aminoglycoside puromycin (PUR); fluoroquinolones levofloxacin (LVX) and moxifloxacin (MXF); macrolides azithromycin (AZM), clarithromycin (CLR), erythromycin (ERY), and roxithromycin (RXM); tetracyclines doxycycline (DOX), and tetracycline (TET) as feasible drugs of choice to fight MPN infections (Table 20). Reported minimum inhibitory concentration (MIC) for the closely related *Mycoplasma genitalium* and *Mycoplasma hominis* were included in Table 20 for comparison.

TABLE 20 Comparison between obtained MIC for unaltered *Mycoplasma pneumoniae* Somerson *et al.* (ATCC® 29342™) and literature reported means for *M. pneumoniae*, *M. genitalium*, and *M. hominis* against antibiotics tested

Antibiotic	MIC (µg/mL) Mean	Reported MIC (µg/mL) Means for:		
		M. pneumoniae	***M. genitalium***	***M. hominis***
SPT	0.396	-	<0.025	-
GEN	7.000	4.000	-	2-16
KAN	18.667	-	-	-
PUR	0.667	-	-	-
CHL	5.333	2.000	-	4-25
RFB	2.500	-	-	-
CFZ	192.000	-	-	-
CRO	448.000	-	-	-
CIP	2.333	1.000	1-2	0.1-4
LVX	0.750	0.120-1.000	0.5-1	0.1-2
MXF	0.203	$3.00x10^{-2}$-0.25	0.03-0.06	0.06-0.125
CLI	4.167	4.00	0.2-1	≤0.008-2
AZM	0.001302083	$2.00x10^{-3}$-$6.00x10^{-2}$	≤0.01	4-64
CLR	0.005289714	≤$4.00x10^{-3}$-0.125	≤0.01	16->256
ERY	0.040104167	$4.00x10^{-3}$-$3.00x10^{-2}$	≤0.01	32
RXM	0.072916667	≤$1.00x10^{-2}$	0.01	> 16
NIT	5.000	-	-	6-500
DOX	0.156	$2.00x10^{-2}$-0.500	≤0.01-0.3	0.2-2
TET	0.160	0.250-0.630	-	0.1-2

Literature MIC averages were compiled from several studies (40, 41, 151, 170, 171, 71, 172, 173, 174). Abbreviations for antibiotics: spectinomycin (SPT), gentamycin (GEN), kanamycin (KAN), puromycin (PUR), chloramphenicol (CHL), rifabutin (RFB), cefrazolin (CFZ), ceftriaxone (CRO), ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), clindamycin (CLI),

azithromycin (AZM), clarithromycin (CLR), erythromycin (ERY), roxithromycin (RXM), nitrofurantoin (NIT), doxycycline (DOX), tetracycline (TET).

Our experiments on *M. pneumoniae* MICs show that values obtained for AZM, CLR, ERY, LVX, MXF, DOX, TET were lower than reported averages (Table 20). Not all experiments in the literature were carried out with the same strain that we used, so it is expected to see some variability in MICs. It is also possible that pharmaceutical advancements in improving drug efficacy played a role in lowering these values (40). Obtained MICs for GEN, CHL, CIP, CLI, RXM were higher than the reported averages (Table 20). Consistent results against MPN M129-B7 UA to these drugs allows us to infer that there is a genetic mechanism within this particular strain that has decreased their effectiveness, but further genetic analysis would need to be carried out. GEN, CHL, CIP, and CLI have bacteriostatic properties, while RXM targets ribosomal proteins (40, 71). It is possible that some of these genetic responses for RXM could be involved with the predicted genes listed in Table 21, such as DUF 4011 domain-containing protein, ABC transporter ATP binding protein from the characteristic feature of the macrolide. As mentioned, *M. pneumoniae* has been observed to develop mutations associated with antibiotic resistance via mutations formed by antimicrobial target modifications, so there is a possibility that the bacteriostatic GEN, CHL, CIP, and CLI could be affecting the commonly altered A2058G, 16s RNA or 23S rRNA region observed in clinical strains (40). Results presented in our study adds to the unreported MICs of *Mycoplasma pneumoniae* Somerson *et al.* (ATCC® 29342™) for SPT, KAN, PUR, RFB, CFZ, CRO, and NIT.

We compared our obtained MIC values to those reported for *Mycoplasma genitalium* and *M. hominis* to observe differences in mycoplasma species (Table 20). Other data on *in vitro* effects of antibiotics suggest that there are similarities in susceptibilities between *M. pneumoniae* and *M. genitalium* (170, 175). *M. hominis* does not share as many similarities with *M. pneumoniae* in terms of drug treatment, but it has been included here to compare dissimilar mycoplasmas to the drugs we tested (151). All of these species belong to the Mollicute class, which means that any drug that targets cell walls will not work against these microbes (43). Therefore, macrolides, tetracyclines, streptogramins, lincosamides, aminoglycosides, fluoroquinolones, and

chloramphenicol should have at least some activity on these mycoplasmas. Our data suggests similar MICs for the *M. pneumoniae* M129-B7 strain with the reported MIC values for *M. genitalium*. It is possible that mutations in MPN M129-B7 caused by SIC antibiotic concentrations could affect similar genome regions in *M. genitalium* strains (Table 20). The MIC comparison to *M. hominis* supports the literature for the variability in the efficacy of the drugs used in our study against *M. pneumoniae*. It is also likely that target alterations in the *M. pneumoniae* genome could be associate with *M. hominis*, but genome sequencing of produced MPN M129-B7 DOX and TET antibiotic resistant mutants would need to be carried out. Clinical strains of *M. hominis* have been catalogued for tetracycline resistance through mediation by the *tet*M transposon (151), which could serve as a reference for tetracycline resistance development in *M. pneumoniae*.

Generation of *Mycoplasma pneumoniae* Somerson *et al.* (ATCC® 29342™) mutant strains under antibiotic stress. Addressing our second objective, generating mutations in our MPN M129-B7 unaltered strain initially posed a challenge for all drugs tested. By definition, the MIC is the least concentration of antibiotic required to kill bacteria. We incubated our MPN strains under antibiotic stress at sub-inhibitory concentrations at a halved value from the MIC based on our initial intended setup, however our cell yields were too low to continue propagating the cells under those conditions. Thus, we modified the protocol to include four sub-inhibitory concentrations (SIC1-4) to be able to generate enough MPN cells to work with. According to Pereyre *et al.*, 2016 (40) all strains of *Mycoplasma pneumoniae* acquire mutations associated with antibiotic resistance by antimicrobial target modifications, so we assumed that mutation rates would slow down as the sub-inhibitory concentrations would lower, but would be consistent while increasing MPN cell production. We observed higher cell yields after making these changes, but decided to add another modification. The 6 day-incubation was changed to 7-10 day-incubation with close monitoring in order to further increase cell yields. Therefore, the dose for each antibiotic was set empirically to give adequate bacterial growth for each passage with a given drug. We were unable to find a suitable concentration for the macrolides AZM, CLR, ERY, and RXM, which are the most effective drugs against *M. pneumoniae*.

All organisms belonging to the Mollicute class are inherently resistant to all β-lactams, trimethroprim, polymyxins, glycopeptides, rifampin, sulfonamides, and nalidixic acid due to the lack of a cell wall these antibiotics target (40, 151). *M. pneumoniae* is resistant to other antibiotics that include sulfonamides, trimethoprim, polymixins, rifampicin, and linezolids (41). *M. pneumoniae* is therefore susceptible to drugs like macrolides, lincosamides, streptogramin, ketolides (grouped together as MLSK), tetracyclines, and fluoroquinolones. These drugs are capable of reaching high intracellular concentration in cells to target intracellular Mycoplasmas (41). Out of all of them, macrolides are used as the primary method of treatment for respiratory tract infections caused by this pathogen. Macrolide usage is preferred for these microbes because of the low MIC against them. Our observations support the efficiency of AZM, CLR, ERY, and RXM against MPN M129-B7 UA. The practical problem is that clinical strains of *M. pneumoniae* have developed resistance to macrolides across 15 years worldwide (25, 40, 55). The latest reports state that within that time frame, there has been a population MPN macrolide resistance emergence between 0-15% in USA and Europe, about 30% in Israel, and 90-100% in Asia (40). As a result most of the research done on *M. pneumoniae* strains (unaltered or clinical isolates) are focused on their resistance development towards macrolides. This has allowed other researchers to identify the source of macrolide mutations coming from a transition in the peptidyl-transferase loop of domains II and V of the 23S rRNA, and the ribosomal proteins L4 and L22 (41, 176).

The candidate list of putative antibiotic stress resistant genes for *M. pneumonia* presented here could still prove useful in finding potential associations with other effective antibiotics when MPN is made resistant to them. For example, experiments exploring the genetic alterations caused by the lincosamide clindamycin (CLI) have also altered the ribosomal protein L4 in clinical strains (161, 177). From our results on CLI, we can say that for such a mutation in MPN M129-B7 UA we would need to take the passage pressure beyond five cell generations. The relatively higher MIC of CLI in comparison to any of the macrolides does not make it a viable solution in practical applications, so it makes sense that *in vitro* resistance studies are not as represented as those for macrolides. For now, high incidences of macrolide resistant *M. pneumoniae*

have placed infected patients under alternative antibiotic treatment with tetracyclines and fluoroquinolones.

Assessment of MIC value changes in *Mycoplasma pneumoniae* Somerson *et al.* (ATCC® 29342™) UA strains compared to MPN M129-B7 SIC1-4 in PA1-PA5.

Passages created under four sub-inhibitory concentrations for our tested tetracyclines and fluoroquinolones suggest that there is a significant difference in their MICs from their unaltered MPN M129-B7 counterparts. Our results suggest that fluoroquinolone LVX, and tetracyclines DOX and TET were slightly more effective *in vitro* across passages 1-5. Other experiments suggest that LVX could take up to a minimum of 7-15 passages before beginning to induce antibiotic resistance in MPN (174). Based on that, DOX and TET would also need to be used across more passages than we did before MIC changes for MPN M129-B7 can occur. This is supported by other *in vitro* studies working with tetracycline resistant strains in which doxycycline resistance mutations were induced at from 10-12 passages, yet MICs remained below ≤ 2 µg/mL across tetracycline, doxycycline and minocycline (173, 178). Passages under pressure from fluoroquinolones CIP and MXF had a significant MIC shifts from their unaltered versions. Those small shifts in MIC value from UA could possibly begin to cause more frequent mutations based on the patterns observed within this sample size (Table 2), and MPN being susceptible to antibiotic target modifications as described by the literature (40, 43). Fluoroquinolone mutations have been attributed to mutations in the conserved regions of the *gyrA*, *gyrB*, *parC*, and *Appe* genes otherwise known as the quinolone resistance-determining regions (174). This falls in line with our results, but the literature also suggest that mutation rates are still low for LVX and MXF with them ranging from $1{,}3x10^{-6}$ to $7x10^{-9}$ (174). Studies on CIP are not as explored due to their higher MICs in comparison to the other fluoroquinolones, so it is unclear if the mutation rates will be similar as for LVX and MXF despite them belonging to the same antibiotic class (40, 174). In terms of genetic alterations, our results could be further assessed through sequencing analysis. If we were to observe similarities within the mutations in the conserved regions of the *gyrA*, *gyrB*, *parC*, and *Appe* genes between CIP, LVX and MXF stressed passages, we could support the idea that a single drug pressure can

create a cascade effect of antibiotic resistance development towards chemically similar drugs in *M. pneumoniae*.

We observed significant MIC value changes in SPT, GEN, KAN, and PUR to consider MPN M129-B7 developing antibiotic resistance to these drugs. SPT and PUR begin at MICs ≤ 1 µg/mL in line with the recommendation of the Mycoplasmal Chemotherapy Working Team of IRPCM, which further warrants consideration of these antibiotics for use in patients. Exposure of MPN to SPT expresses rapid resistance development within the five passages despite SPT's low starting MIC. There are currently no studies on SPT and its effects on *M. pneumoniae*. Ideally, information for any organism should be derived from that particular specimen, but we can make educated assumptions from related species and information. The aminocyclitol spectinomycin is active against gram-negative bacteria, consists of bacteriostatic activity by binding to the bacterial 30S ribosomal subunit, stopping the initiation of protein synthesis and protein elongation, which causes the bacterial cell to die (179). A study exploring alternatives to treat a macrolide resistant strain of the sexually transmitted pathogen *Mycoplasma genitalium* was successful on a patient prescribed with spectinomycin (180). This could mean that there might be an associated antibiotic resistance gene between SPT and macrolides in the rest of the Molliculites. However, our *in vitro* experiments indicate that SPT would not be a reliable choice as an alternative since the resistance rate appears to be high within five cell generations.

Despite PUR having a low MIC similar to SPT, we observe a shift in the opposite direction that suggest an increased effectiveness in the antibiotic. Puromycin inhibits the translation process by disrupting the peptide transfer on ribosomes, which means that Mycoplasma species would not be able to grown when exposed to PUR even at low concentrations (181). From this we can conclude that PUR is more effective through the bacteriostatic effects of this antimicrobial agent by completely halting MPN growth in the cultures. This property of PUR, along with PUR's similarity to the 3'-terminal end of aminoacylated tRNAs, together with its attachment to carboxyl terminus of growing protein chains causes it to stop protein synthesis (181). This ability to avoid direct point mutation in Mycoplasmal species means that PUR has a potential to be used as a

genetic marker for future genetic studies involving our candidate antibiotic stress resistant genes.

The MICs obtained for GEN and KAN within MPN M129-B7 UA are greater than the recommendations that make a drug effective and preferable. The aminoglycoside gentamycin inhibits protein synthesis while damaging the cell membrane integrity by acting on the ribosomal 30S subunits of bacteria (32). Since part of its effect is on the cell wall of its targets, GEN has a limited effect on *M. pneumoniae*. Liu *et al.*, 2014 (32) reported that GEN has variably poor effects on different *M. pneumoniae* strains, so it is possible that the observed increases in MICs could be due to a promotion of growth through persistent exposure for this particular strain. More trials would need to be run to support this statement alongside sequencing the antibiotic stressed passages to determine any genetic changes. The aminoglycoside kanamycin is used against gram-positive and gram-negative bacteria, yet has some recorded effectiveness against Mycoplasmal species (182, 183). There is lack of recent studies involving KAN; this is likely due to the improvements in other drug therapy options.

Created passages at SIC concentrations for CHL, RFB, CFZ, CRO, and NIT showed no significant differences among them. CHL is considered a broad-spectrum drug capable of treating *M. pneumoniae* infections, but research on this antibiotic is limited because of its dangerous side effect of damaging bone marrow in patients (40, 41, 43). RFB is a spiro-piperidyl-rifamycin derived from rifamycin-S, and it is considered to have a broad-spectrum of antimicrobial activity as well (184, 185). Kunin (184) states that RFB is active against *Mycobacterium avium* complex (MAC), *Mycobacterium tuberculosis*, and *Mycobacterium leprae*. This explains the relatively low MIC and suggests that it is possible to produce resistance for analysis, but resistance development of MPN M129-B7 towards RFB requires more than five passages. Cephalosporin ceftriaxone and cefazolin are broad-spectrum antibiotics that bind to the bacterial penicillin-binding proteins in the same manner as β-lactams (186). Our MIC data is then supported by the high value for CFZ and CRO, thus indicating that there is no need to continue passage creation with them. Only a few studies have been done to include CFZ in combination with macrolides towards macrolide resistant *M. pneumoniae* cases, but none have worked (187, 188) Nitrofuran nitrofurantoin is an antibiotic often

prescribed to treat urinary tract infections with evidence of activity towards *Mycoplasma hominis* and *Mycoplasma fermentans* (189). There is a lack of recent studies in the pharmacodynamic properties of NIT, especially on the effects on *M. pneumoniae*, but this antibiotic is undergoing a resurgence in its use as an alternative solution to treat other resistant strains of bacteria (190, 191). Our MIC data can contribute towards that accumulation of knowledge on the efficacy of NIT against *M. pneumoniae*. From our results, more passages would have to be created to produce NIT induced mutations in our strain.

Conclusions and recommendations. This study successfully laid out an important foundation to produce reliable, consistent, long-term results for passages under perpetual antibiotic pressure at sub-inhibitory concentrations. The results from our applied statistical analysis strategies matched the results from other experiments on *M. pneumoniae* (Table 20), therefore supporting these reported studies. Thus, these analysis can be used to support the qualitative testing procedures presented here. It must be noted that the passages must be restocked after an estimated 3-4 replications, including spectrophotometric readings, on full 96-well plate MIC assays runs. This is considering the optimizations in the protocol towards increasing the 12 mL MPN cell culture yields within the 6-10 day time frame post inoculation. Repeats possible from obtained cell stocks for each passage vary depending on the antibiotic used, sub-inhibitory concentration used, and growth term post inoculation before harvesting. Generally, more time in incubation at 37℃ will allow for more cells to grow, which results in higher cell yields to work from. Lower sub-inhibitory concentrations will produce more cells for that passage in less incubation time compared passages at higher SICs (SIC1 < SIC2 < SIC3 < SIC4 in cell yields), but mutation rates are decreased. This means that one must passage them further to induce more antibiotic stress resistant mutations within the MPN cell culture.

To conclude, we were able to partially support our hypotheses. Our data partially supports that (1) exposure of MPN to constant sub-inhibitory concentrations of SPT, GEN, KAN, CIP, and MXF antibiotics, will result in a respective MIC shift as a possible result of mutations in specific candidate genes responsible for resistance development; and (2) that antibiotic resistance genes in *Mycoplasma pneumoniae* M129-B7 are

expressed within the 5th generation of cells exposed to SPT, GEN, KAN, CIP, and MXF. Future projects could use these observed MICs to elaborate on this dataset through more repetitions of the experiments. If more replicates are carried out for the stressed passage data collection, we could more confidently asses the significant MIC changes through prolonged sub-inhibitory antibiotic therapy. For CLI, AZM, CLR, ERY, RXM, DOX, and TET passages could be continued until resistance development occurs. We can then explore the genetic basis behind the antibiotic resistance mutations with reference to the putative antibiotic stress resistant genes. Table 21 presents the list of candidate genes that could be used as a basis for assessing the role of each gene in the development of antibiotic stress resistance.

TABLE 21 Candidate list of putative antibiotic stress resistance genes

Locus tag	Protein name
MPN018	ABC transporter ATP-binding protein
MPN019	ABC transporter ATP-binding protein
MPN035	Hypothetical protein; see; MPN036
MPN036	Hypothetical protein
MPN037	Hypothetical protein
MPN080	ABC transporter permease
MPN081	ABC transporter ATP-binding protein
MPN153	DUF4011 domain-containing protein
MPN159	Hlyc/CorC family tranporter
MPN234	Hypothetical protein
MPN333	ABC transporter permease
MPN334	ABC transporter ATP-binding protein
MPN335	ABC transporter permease
MPN683	ABC-type lipoprotein export system, ATPase component
MPN684	ABC-type antimicrobial peptide transport system, permease component.
MPN685	ABC-type lipoprotein export system, ATPase component

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APPENDICES

TABLE A1
Reagents' list to prepare modified Hayflick medium (Chanock *et al.*, 1962)

Reagent	**Volumes**
	400 mL
PPLO Broth	7.35 g
HEPES	11.92 g
Phenol Red (0.5 %)	2 mL
NaOH (2N)	14 mL
ddH_20	400 mL
pH 7.6-7.8	
Autoclave then, add:	
Horse Serum (Heat Inactivated)	100 mL
Penicillin (100,000 U/mL)	5 mL
Carbon Source (50%)	10 mL

Penicillin stock solution (100,000 U/mL). Dissolve 3.13 g of penicillin G sodium salt in 50 mL of ddH20 (using a 50-mL centrifuge tube). Sterilize the solution by filter sterilization. Store penicillin stock solution at 4℃.

TABLE A2
Reagents' list to prepare phosphate-buffered saline (PBS) solution

Reagent	**1 x Solution (1L)**	
	Amount to add	**Concentration**
NaCL	8.01 g	137 Mm
KCl	0.20 g	2.7 Mm
Na_2HPO_4 x 7 H_2O	2.68 g	10 Mm
KH_2PO_4	0.24 g	1,8 Mm

PBS can be made as a 1x solution or as a 10x stock solution. To prepare 1 L of either 1x or 10x PBS, dissolve the reagent listed above in 800 mL of ddH_20. Adjust the Ph to 7.4 (or 6.5, if required) with 1 N HCl, and then add ddH_20 to 1L. Dispense the solution into two 500 mL aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on cycle P13 (or by filter sterilization). Store 1x PBS at 4℃ and 10 x PBS at room temperature.

TABLE A3a

The 96-well plate mic two-fold dilution antibiotic concentration setup at 64 µg/mL highest concentration for SPT, GEN, KAN, PUR, CHL, CIP, LVX, MXF, CLI, NIT, DOX, and TET

	Well 1 (µg/mL)	Well 2 (µg/mL)	Well 3 (µg/mL)	Well 4 (µg/mL)	Well 5 (µg/mL)	Well 6 (µg/mL)	Well 7 (µg/mL)	Well 8 (µg/mL)	Well 9 (µg/mL)	Well 10 (µg/mL)	Well 11 (Control)	Well 12 (Control)
Test 1 A	64.000	32.000	16.000	8.000	4.000	2.000	1.000	0.500	0.250	0.125	C (+)	C(-)
Test 1 B	$6.25x10^{-2}$	$3.13x10^{-2}$	$1.56x10^{-2}$	$7.81x10^{-2}$	$3.91x10^{-3}$	$1.95x10^{-3}$	$9.77x10^{-4}$	$4.88x10^{-4}$	$2.44x10^{-4}$	$1.22x10^{-4}$	C (+)	C(-)
Test 2 C	64.000	32.000	16.000	8.000	4.000	2.000	1.000	0.500	0.250	0.125	C (+)	C(-)
Test 2 D	$6.25x10^{-2}$	$3.13x10^{-2}$	$1.56x10^{-2}$	$7.81x10^{-2}$	$3.91x10^{-3}$	$1.95x10^{-3}$	$9.77x10^{-4}$	$4.88x10^{-4}$	$2.44x10^{-4}$	$1.22x10^{-4}$	C (+)	C(-)
Test 3 E	64.000	32.000	16.000	8.000	4.000	2.000	1.000	0.500	0.250	0.125	C (+)	C(-)
Test 3 F	$6.25x10^{-2}$	$3.13x10^{-2}$	$1.56x10^{-2}$	$7.81x10^{-2}$	$3.91x10^{-3}$	$1.95x10^{-3}$	$9.77x10^{-4}$	$4.88x10^{-4}$	$2.44x10^{-4}$	$1.22x10^{-4}$	C (+)	C(-)
Test 4 G	64.000	32.000	16.000	8.000	4.000	2.000	1.000	0.500	0.250	0.125	C (+)	C(-)
Test 4 H	$6.25x10^{-2}$	$3.13x10^{-2}$	$1.56x10^{-2}$	$7.81x10^{-2}$	$3.91x10^{-3}$	$1.95x10^{-3}$	$9.77x10^{-4}$	$4.88x10^{-4}$	$2.44x10^{-4}$	$1.22x10^{-4}$	C (+)	C(-)

TABLE A3b

The 96-well plate mic two-fold dilution antibiotic concentration setup at 1 µg/mL highest concentration for AZM, CLR, ERY, and RXM

	Well 1 (µg/mL)	Well 2 (µg/mL)	Well 3 (µg/mL)	Well 4 (µg/mL)	Well 5 (µg/mL)	Well 6 (µg/mL)	Well 7 (µg/mL)	Well 8 (µg/mL)	Well 9 (µg/mL)	Well 10 (µg/mL)	Well 11 (Control)	Well 12 (Control)
Test 1 A	1.000	0.500	0.250	0.125	$6.25x10^{-2}$	$3.13x10^{-2}$	$1.56x10^{-2}$	$7.81x10^{-2}$	$3.91x10^{-3}$	$1.95x10^{-3}$	C (+)	C(-)
Test 1 B	$9.77x10^{-4}$	$4.88x10^{-4}$	$2.44x10^{-4}$	$1.22x10^{-4}$	$6.10x10^{-5}$	$3.05x10^{-5}$	$1.53x10^{-5}$	$7.63x10^{-6}$	$3.82x10^{-6}$	$1.91x10^{-3}$	C (+)	C(-)
Test 2 C	1.000	0.500	0.250	0.125	$6.25x10^{-2}$	$3.13x10^{-2}$	$1.56x10^{-2}$	$7.81x10^{-2}$	$3.91x10^{-3}$	$1.95x10^{-3}$	C (+)	C(-)
Test 2 D	$9.77x10^{-4}$	$4.88x10^{-4}$	$2.44x10^{-4}$	$1.22x10^{-4}$	$6.10x10^{-5}$	$3.05x10^{-5}$	$1.53x10^{-5}$	$7.63x10^{-6}$	$3.82x10^{-6}$	$1.91x10^{-3}$	C (+)	C(-)
Test 3 E	1.000	0.500	0.250	0.125	$6.25x10^{-2}$	$3.13x10^{-2}$	$1.56x10^{-2}$	$7.81x10^{-2}$	$3.91x10^{-3}$	$1.95x10^{-3}$	C (+)	C(-)
Test 3 F	$9.77x10^{-4}$	$4.88x10^{-4}$	$2.44x10^{-4}$	$1.22x10^{-4}$	$6.10x10^{-5}$	$3.05x10^{-5}$	$1.53x10^{-5}$	$7.63x10^{-6}$	$3.82x10^{-6}$	$1.91x10^{-3}$	C (+)	C(-)
Test 4 G	1.000	0.500	0.250	0.125	$6.25x10^{-2}$	$3.13x10^{-2}$	$1.56x10^{-2}$	$7.81x10^{-2}$	$3.91x10^{-3}$	$1.95x10^{-3}$	C (+)	C(-)
Test 4 H	$9.77x10^{-4}$	$4.88x10^{-4}$	$2.44x10^{-4}$	$1.22x10^{-4}$	$6.10x10^{-5}$	$3.05x10^{-5}$	$1.53x10^{-5}$	$7.63x10^{-6}$	$3.82x10^{-6}$	$1.91x10^{-3}$	C (+)	C(-)

Similar series dilution setups were done for cefazolin (CFZ) and ceftriaxone (CRO), but at 512 µg/mL highest concentration. The same was carried out for rifabutin (RFB) MIC assays, but at 1,024 µg/mL highest concentration. The table setups for CFZ, CRO, and RFB are not depicted due to their exclusion from further procedures. MIC assays performed with rifampicin (RIF), imipenem (IPM), and vancomycin (VAN) did not result in an MIC value within the range of antibiotic concentrations we tested.

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