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FINE FOCUS

AN INTERNATIONAL MICROBIOLOGY JOURNAL FOR UNDERGRADUATE RESEARCH

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Cover Art

Front Cover: Original watercolor piece painted by Sharon Par Mawi Cung depicting an endospore stain of *Bacillus* depiction of insect antimicrobial peptides, as rendered by artist Karen Hale.

Back Cover: American Goldfinch exploring the Lake of the Woods in Geneva, Indiana USA, near the Limberlost wetland area and the home of author and naturalist Gene Stratton-Porter. Photo credit: Bill Hubbard.

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Objective Lens:
Insect antimicrobial peptides as a promising source for antibiotic "substitutes"

John L. McKillip, *Fine Focus* Managing Editor

Currently, at least 2.8 million infections and over 700,000 deaths are reported from AMR bacterial infections globally (1,2). If no new antibiotics are isolated and made available by 2050, the CDC estimates that 10 million annual deaths will occur globally as a result of this inaction (5,8). Yet, surprisingly few have even heard of antibiotic-resistant bacteria, or understand the implications for global health. In fact, no new classes of antibiotics have been developed to treat microbial infections in well over 30 years, as pharmaceutical companies have instead pursued research and development of more lucrative drugs for non-infectious diseases. Since this trend is likely to continue into the foreseeable future, this crisis must be addressed using alternative creative approaches. Unfortunately, this problem is exacerbated poor antibiotic stewardship practices by healthcare providers and consumers for decades. The World Health Organization (WHO) has declared multidrug-resistant (MDR) bacterial and fungal pathogens to be one of the principal threats to global public health (5,7-9).

Alexander Fleming's discovery of penicillin in 1928 was met with his own prediction that bacterial resistance to this "miracle drug" would soon be documented (3,4). In the 70+ years since penicillin was introduced, overuse and misuse of antibiotics have contributed to the problem of MDR bacterial infections, as has the widespread use of antibiotics in agriculture for prophylaxis and growth promotion (8). In fact, the CDC has reported that over 70% of antibiotics used in the U.S. are in production animal environments (8-10). Clinically, antibiotic stewardship and surveillance programs have shown limited success in addressing the MDR crisis (9). In recent years, both the CDC and the White House have outlined clear goals and objectives for directly addressing antibiotic resistance in order to slow the spread of MDR bacteria, while offering a timeline on collaborative international efforts required to make this happen by 2020 (5,6). Unfortunately, this Executive Order signed by President Barack Obama (#13676) has not been addressed, during which time MDR bacterial infections have worsened and become more frequently diagnosed (6).

The global crisis of antimicrobial resistance (AMR) is fueled by antibiotic overuse in agriculture and human clinical applications. ESKAPE pathogens, identified by the World Health Organization as a priority for new antimicrobial drugs (11), are central to this pending catastrophe. However, it may be prudent to open a new line

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of investigation in ernest evaluating insects as a largely unexplored source for novel antimicrobial peptides (AMPs), which show tremendous promise as an alternative for the use of antibiotics.

With over one million described species, insects are the largest class of organisms on the planet, and thus represent tremendous untapped potential for describing novel antimicrobial compounds (12). Insects adapt in remarkable ways to constant changes in their environments, including to a wide range of pathogens. This adaptability is due in large part to their immune systems, which respond quickly to invading microorganisms that gain entry into the hemocoel. A variety of AMPs comprise the key elements of an insect's humoral immune response; some of these molecules are involved in binding to bacterial cells, and facilitating their inactivation or elimination in unique ways. For example, many insect AMPs are positively charged and are thus attracted to the negative charge of bacterial surfaces, where they may serve as membrane antagonists (disrupting bacterial cell structure), or are involved in a hemolymph coagulation response that prevents the bacterial pathogen from disseminating or growing within the insect host.

Insect AMPs are not only antibacterial, but have also been shown to have potential against fungal pathogens, some viruses, and cancer cell lines as well (12), although few if any of these molecules have been investigated more deeply with regard to their biotherapeutic practicality. Because these AMPs are unique in their mechanisms of action against target pathogens, little if any resistance issues have been observed, in stark contrast to traditional antibiotics (13), and this trait is arguably one of the most compelling reasons to investigate AMPs further as a promising means of treating the growing roster of drug-resistant bacterial pathogens. **This issue's cover features an artist's conceptualization of various insect AMPs.**

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oJN.1: A New Threat to Global Health

Neelabh Datta

Asutosh College

Affiliated with the University of Calcutta, West Bengal, India

Abstract

A new coronavirus variation known as JN.1 first appeared in Luxembourg in August 2023 and has since spread to a number of nations. It is distinguished by a high transmissibility, and it may outpace earlier Omicron waves in terms of infections, hospitalizations, ICU admissions, and fatalities. More research is required to establish the projected continued efficacy of current vaccinations, treatments, and testing. An outline of JN.1's epidemiological, clinical, and virological characteristics as well as its effects on world health are given in this brief communication.

Keywords: JN.1, coronavirus, variant, outbreak, global health

Corresponding author email: neelabhdatta@gmail.com

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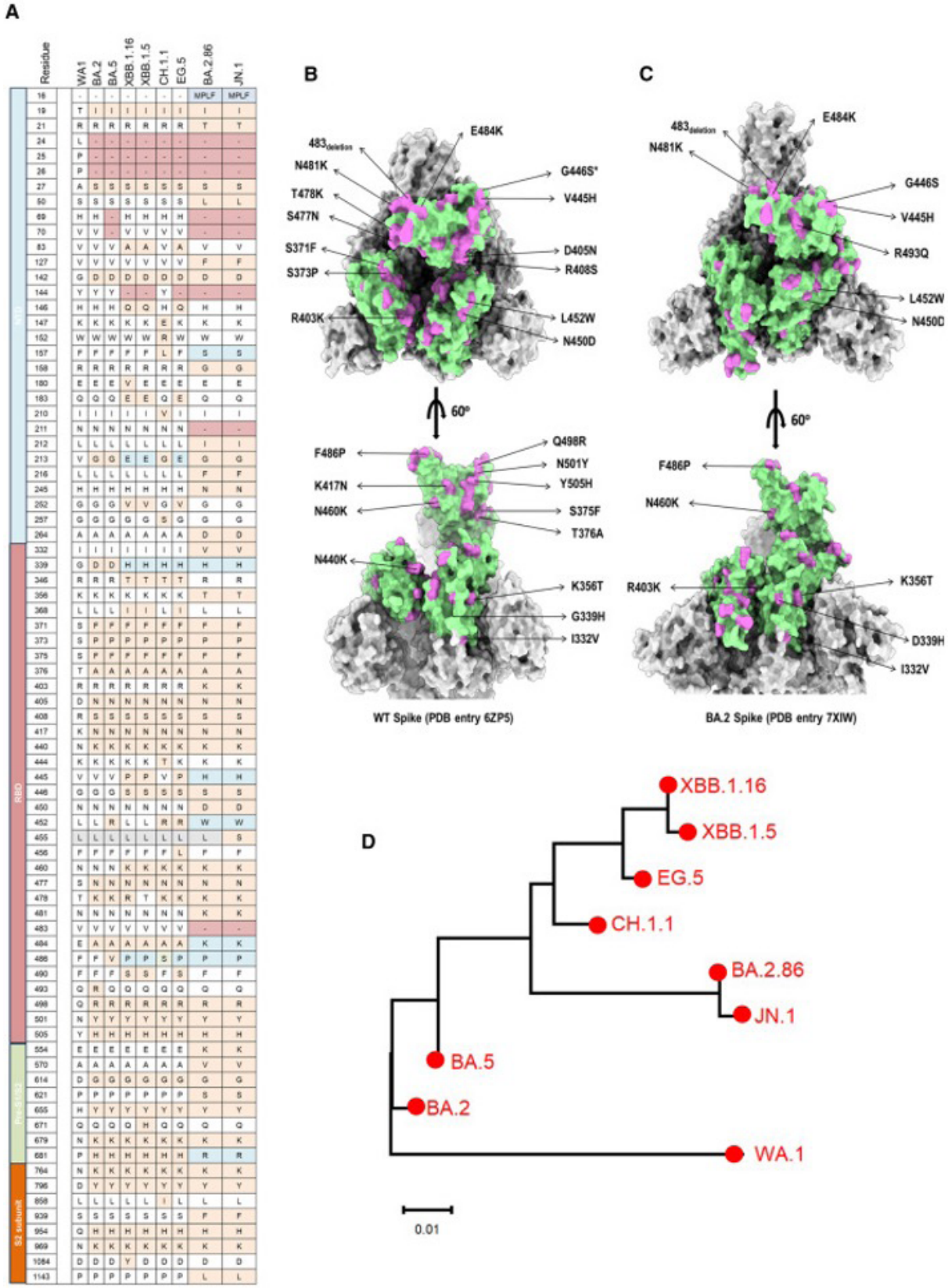
The JN.1 variant of COVID-19 is a new sub-variant that has emerged in late 2023 and has become the dominant and most transmissible strain of the virus in the world. It has currently affected more than 41 countries, and is expected to cause a bigger wave of infections and deaths than the previous ones, according to global experts. The World Health Organisation (WHO) has classified JN.1 as a variant of interest (VOI) because of its rapid spread and its ability to evade the immune system, which is causing a global surge of infections, hospitalisations, and ICU admissions (1). JN.1 was first detected in Luxembourg in August 2023 and is now present in many countries. It is derived from another VOI, BA.2.86, which was first identified in the same month. BA.2.86 is a lineage of SARS-CoV-2 that is phylogenetically distinct from the Omicron XBB variant lineages that were circulating before, such as EG.5.1 and HK.3. The genomic sequence of SARS-CoV-2 is known to code for a total of 29 distinct proteins. This set of proteins is categorized into three groups: 16 non-structural proteins that are crucial for the viral replication cycle, 4 structural proteins that form the virus particle, and 9 accessory proteins that modulate the host's cellular environment. As part of the global scientific community's response to the pandemic, researchers have determined the three-dimensional structures of these viral proteins (2). So far, over 1,228 SARS-CoV-2 experimental structures have been added to the Protein Data Bank (PDB). These entries include two different functional domain structures of the nucleocapsid (N) protein and the structures for all 16 of the non-structural proteins. Additionally, numerous structures of various protein complexes have been deposited. When these structures are compared to those of the closely related SARS-CoV, which caused the SARS outbreak in 2003, a high degree of structural similarity is observed. (2)

BA.2.86 has more than 30 mutations in the spike (S) protein, which is the part of the virus that attaches to human cells and is targeted by vaccines and antibodies. These mutations indicate that BA.2.86 has a high potential to escape the immune response and infect new hosts (3). JN.1 is similar to BA.2.86, but has one additional mutation in the spike protein, L455S, and three other mutations in non-S proteins. The Leu455Ser (L455S) mutation is a hallmark of JN.1 and is also found in HK.3 and other variants that are called "FLip" variants (3). These variants have the Leu455Phe (L455F) mutation, which is similar to L455S, but with a different amino acid change. An infection assay was conducted by Tamura er al. (4) using HIV-1-based pseudoviruses to evaluate the infectivity of different spike (S) protein mutations. The findings indicated that pseudoviruses carrying the S proteins from the B.1.1 or EG.5.1 variants demonstrated a marked increase in infectivity compared to those with the BA.2 S protein (Figure 1). However, the infectivity of pseudoviruses with the BA.2.86 S protein was found to be on par with those bearing the BA.2 S protein (4).

In order to analyse the effects of individual mutations on infectivity in more detail, Tamura er al. (4) created 33 different BA.2 derivatives, each of which included a different set of mutations identified in the BA.2.86 variant. The infectivity of the BA.2 S pseudoviruses was not significantly affected by the bulk of these alterations. However, it was shown that two mutations in the receptor-binding domain (RBD), N460K and F486P, increased infectivity. Notably, infectivity was significantly boosted by three unique mutations—F157S, N211del, and A264D—that were found in the N-terminal region of the BA.2.86 S protein. Six mutations specific to BA.2.86 increased the infectivity of BA.2, whereas sixteen mutations had the reverse impact, according to the mutagenesis assays, which revealed a complex interaction of mutations influencing infectivity. In spite of this, BA.2.86's overall infectivity was comparable to that of BA.2. This points to a possible evolutionary path for BA.2.86, in which the S gene may have mutated to avoid the humoral immunological response of the host, then acquired enhancing mutations to compensate for any infectivity lost as a result of immune evasion.

In addition to infectivity assays, the cleavage efficiency of the S protein was assessed through

Figure 1



Comparative analysis reveals mutations in the spike (S) proteins of Omicron subvariants BA.2, BA.5, XBB.1.5, XBB.1.16, CH.1.1, EG.5, BA.2.86, and JN.1, with reference to the ancestral SARS-CoV-2

Caption continues on page 8

Caption continued from page 7

strain WA1. The RBD of BA.2.86 exhibits specific amino acid changes when compared to both the original SARS-CoV-2 and BA.2. These mutations are highlighted in structural models with PDB IDs 7XIW (BA.2) and 6ZP5 (wild type). A phylogenetic tree of RBD sequences illustrates the evolutionary relationships and genetic divergence among SARS-CoV-2 WA1, its variants, and subvariants, including BA.2.86 and JN.1, with corresponding GeneBank accession numbers provided for their S protein sequences. Image credit to Liu et al. (5)

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western blot analysis of cells used for pseudovirus production (4). The results revealed that cells expressing the BA.2.86 S protein exhibited a higher band intensity for the S2 subunit compared to those expressing the BA.2 S protein. Analysis of point mutants based on the BA.2 S protein indicated that several mutations contributed to an increased cleavage efficiency of the S protein. Moreover, the level of the S2 subunit within virions pseudotyped with the BA.2.86 S protein was greater than that in virions with the BA.2 S protein. Among the BA.2-based point mutants, the three mutants—F157S, A264D, and N460K—showed not only increased cleavage efficiency in cells but also a higher incorporation of S2 protein into the viral particles released, suggesting a correlation between cellular cleavage efficiency and virion incorporation levels.

The presence of a four amino acids insertion (17MPLF) in the spike protein of JN.1 was reported by Chakraborty, (6). This insertion might enhance the transmissibility of JN.1 by compensating for the loss of eight amino acids (24LPP, 69HV, 145Y, 211N and 483V) in the spike. JN.1 was first detected in South India in December, 2023, and it was warned by scientists that it could become the dominant variant with 20–30% of all omicron infections worldwide in 2024. Lower affinity for the ACE-2 receptor but higher immune evasion than BA.2.86.1, was shown by JN.1 (6). A driving force for the higher transmission, compensating for the eight amino acids deletions, was JN1 17MPLF spike insertion. A more compact spike was predicted by SWISS-Model by Chakraborty, (6), although the surface amino acids for interaction with ACE-2 receptor were changed. The side chains of basic amino acid might interact with viral RNA for quick complete inclusion of virus into lung cells and thus might be involved in rapid spread among the people whose immunity to COVID-19 vaccines was lost considerably. More mutations and deletions and less protection to COVAXIN and COVISHILD vaccines were shown by spike protein of JN.1 along with more resistance to antibody of previously coronavirus-infected individual (6). Involvement in interaction with the ACE-2 receptor was indicated by the data for amino acids Glu484, Phe486, Gln474, Lys417, Tyr453 and Asn501 in the RBD of spike. But amino acids His442, Pro482, Lys480, Lys478 might be important in this aspect, as suggested by Chakraborty, (6) with JN.1 spike model. The first to interact were Arg346, Phe486, Lys444, Gly446, Val445 and Tyr449 (Figure 2).

A role for M-protein 3-D structure in the higher transmission of BA.2.86 and JN.1 subvariants was speculated by the finding of conserved M-protein mutations (6). A high degree of structural rigidity in a simple lipid bilayer was shown by molecular dynamics simulations and a role for M homodimers in scaffolding viral assembly was supported. An important electropositive cytosolic surface that might be important for interactions with N, S, and viral RNA was displayed by M. The charges in its surface should be changed by the D3H, A63T, A104V mutations in the M protein. NTD, TMI, TMII, TMIII and CTD were designated as the M protein domains AA 1–19, AAs 20–40, AAs 51–71 and AAs 80–100 and AAs 101–222 respectively. C-terminal Phe103, Arg107, Met109, Trp110, Arg131, and Glu135 were suggested as the common interacting residues of the M-protein with S and N proteins. However, an interacting role with Ser186 of N protein changing M-N interaction in JN.1 and BA.2.86 variants was found for Ala104 of M-protein (6).

JN.1 is believed to be associated with the second-biggest wave of infections in the US and unprecedented levels of wastewater viral load in several European countries (8). Global experts predict that JN.1 may result in a bigger wave than the previous ones, possibly exceeding the Omicron waves in 2022. Professor Christina Pagel of University College London (UCL) stated that the JN.1 wave has not yet reached its peak and may do so in mid-January, either next week or the week after. She expressed her certainty that this wave will rival or surpass the first two Omicron waves in 2022 (9). Professor Eric J. Topol of Scripps Research wrote in an opinion piece in the Los Angeles Times that JN.1 is now the main cause of infections in the US, based on the wastewater levels of the virus (8). He explained that the actual number of infections is difficult to track since most people either test at home or do not test at all, but he estimated that about 2 million Americans are getting infected each day. It was noted that several countries in Europe have reached unprecedented levels of wastewater viral load, exceeding Omicron. This allowed him to conclude that this virus variant, with its plethora of new mutations, has continued its evolution with mutations adapted for infecting or reinfecting us (10). Professor Peter Openshaw, a virus expert at Imperial College London, was quoted as saying to The Sun Health that the coming weeks could see a major surge in infections and that the wave could be bigger than anything we have seen before. A recent study by Qu et al. showed that BA.2.86 can infect cells in the lower lung and can enter cell membranes more efficiently than other versions of Omicron, raising the concern that JN.1 may have a similar or higher tendency to infect human lung epithelial cells, which could pose a serious threat to public health (11). The current vaccines, treatments and testing are expected to remain effective

Figure 2

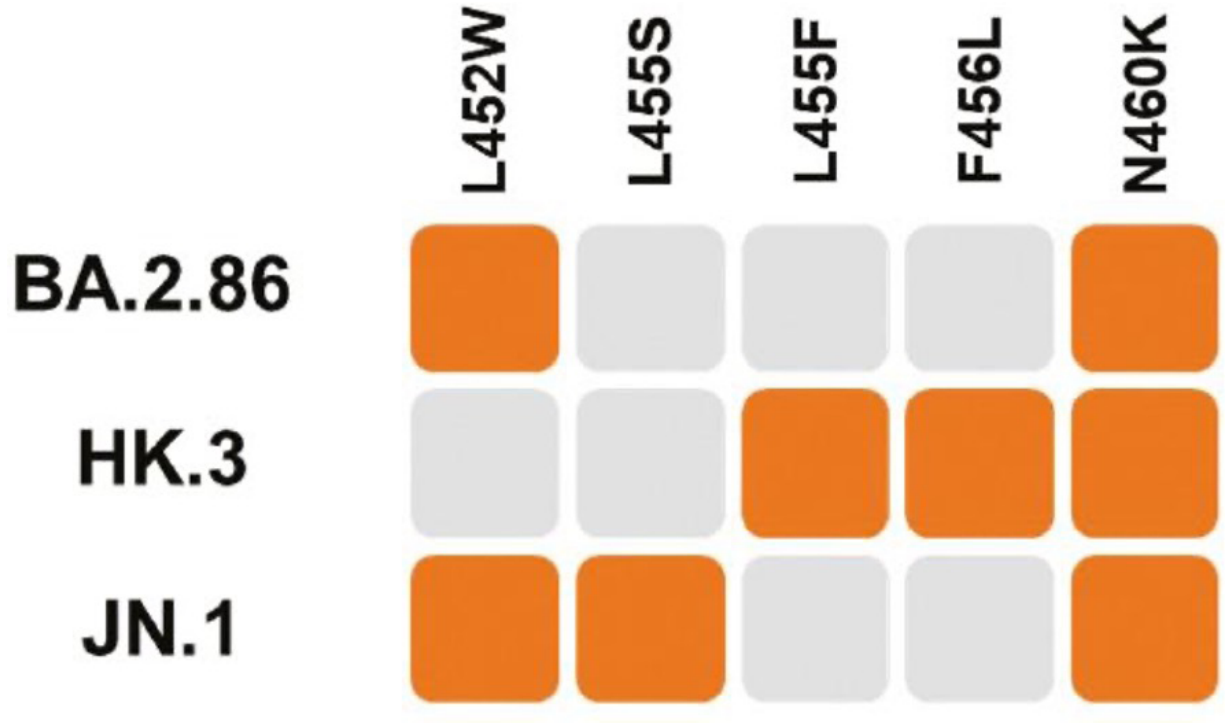

Figure 2 shows the various muations in variants of SARS-CoV-2. The orange cube shows the amino acid difference at the site between each variant and the reference strain Wuhan-Hu-1 (GenBank accession No. NC_045512.2) for the key mutations in the spike protein of BA.2.86, HK.3 and JN.1 variants, which are marked on the top. Image credit to Hang *et al.* (7)

against JN.1, but more rigorous and comprehensive studies are needed to confirm this (12). The experts advised people to take COVID-19 booster and wear masks in public again to help curb the spread of JN.1. The US Centre for Disease Control and Prevention (CDC) stated that there was only a single change between JN.1 and BA.2.86 in the spike protein, which is the part that existing vaccines target.

Another recent study by Jeworowski et al. investigated the immune escape potential of the two SARS-CoV-2 variants, BA.2.86 and JN.1 that have emerged and become dominant in different regions of the world (13). The study revealed that both variants can evade the neutralizing antibodies generated by prior infection or vaccination with earlier variants of SARS-CoV-2, such as XBB.1.5 and EG.5.1. The immune escape ability of BA.2.86 and JN.1 is comparable and significantly higher than that of the previous variants which could account for the rapid spread and prevalence of these variants in the global population. However, the study also suggested that immune escape alone may not be sufficient to explain the recent surge in JN.1 cases, as this variant does not exhibit any enhanced immune escape relative to BA.2.86 (13). Therefore, other factors, such as viral fitness, host susceptibility, and environmental conditions, may also contribute to the increased transmissibility and infectivity of JN.1.The study also compares its results with two other studies that used a different cohort of participants, who had a higher exposure to XBB variants through infection or vaccination (13). It was found that the results were not consistent with those studies, as it observes a lower level of neutralizing activity against the newer variants, including XBB.1.5, EG.5.1, BA.2.86, and JN.1, in its cohort. This discrepancy may indicate a decline in vaccine- or infection-induced immunity over time, which could pose a risk of re-infection in the coming winter months in the northern hemisphere. The study's implications are relevant for the public health response to the COVID-19 pandemic, as the emergence and spread of new variants pose a challenge for the effectiveness of vaccines, treatments, and testing (13).

Because the JN.1 variation may avoid the immune system, non-immunologic preventive measures may be the best way to stop its spread. This implies that in addition to making sure that public spaces are properly ventilated and sanitized, individuals should also practice excellent personal hygiene, which includes often washing their hands, using masks, and maintaining a safe distance from others (7). To combat the spread of the JN.1 strain, viral testing and self-isolation protocols should be reinforced in order to promptly identify and contain affected persons. Developing vaccinations or booster tactics tailored to the JN.1 strain and its distinct features will strengthen immunity against this new danger. According to the CDC, the new COVID-19 vaccinations for 2023–2024 can help the immune system block BA.2.86 and that they expect JN.1 to have a similar response and that the current treatments and testing methods remain effective against these variants. This is based on the analysis conducted by the SARS-CoV-2 Interagency Group (14)

The World Health Organization (WHO) has emphasized that the current immunizations continue to protect against serious illness and death from JN.1 and other circulating variations. The WHO has also recommended individuals to maintain current vaccination records, particularly those who are at high risk of illness. In order to lessen the spread of the virus and its variations, the WHO has also advised individuals to keep up with preventative practices including mask use, social distance, and hand cleanliness. Preliminary tests on a revised monovalent vaccination targeting XBB.1.5, subvariant, provide cross-protection against JN.1 (12). Targeting the XBB.1.5 spike protein, a novel COVID-19 vaccine has been created (12). The vaccine's ability to protect people against other newly developing viruses is presently being tested and reported. Interestingly, the vaccine can also generate strong immune responses against JN.1. A preprint study by Wang et al. shows that the vaccine can produce high levels of antibodies that can neutralize JN.1, which may help prevent serious complications of COVID-19, such as hospitalizations and deaths (15).Since SARS-CoV-2 continues to mutate and transmit, it

is critical that worldwide communities collaborate in sharing data, exchanging experiences, and undertaking scientific study to acquire a more thorough knowledge of the emerging forms. This collaborative approach will allow for faster and better judgments in implementing effective public health initiatives.

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Analyzing selected FDA approved drugs for effects on template switch mutagenesis in *E. coli*

April Swain[1]

Emily Colburn[1] 0009-0001-6905-2381

Kamila De Andrade[2]

Pamela Rosales[2]

Sydney Addorisio[2] 0009-0001-3776-5870

Taylor Stuart[2]

Angelina Galvao[1]

Leigha Hubisz[2]

Margaret Ouellette[1]

Rana Hamssa[1]

Laura Laranjo[2] 0000-0002-1225-8821

Cara Pina[1] 0000-0002-8098-8148

[1]Framingham State University
Framingham, MA USA

[2] Salem State University
Salem, MA, USA

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Abstract

Quasipalindromes (QPs) are DNA sequences that are imperfectly mirrored, known to form secondary structures like hairpins and cruciforms. These sites have been linked with a specific type of mutation called template-switch mutation (TSM). Certain drugs like 5-Azacytidine, Azidothymidine (AZT), and Ciprofloxacin are known to induce TSM. This study aims to assess the impact of five FDA-approved drugs—three antitumor drugs (CPT-11, Doxorubicin hydrochloride, and Gemcitabine hydrochloride) and two anti-inflammatories (Ibuprofen and Dexamethasone)—on template-switch mutagenesis. Studying FDA-approved drugs for their impact on TSM is important, as they are associated with diseases like hereditary angioneurotic edema, osteogenesis imperfecta, and biotinidase deficiency. The findings reveal no statistically significant effects on frequency of mutations after treatment with Gemcitabine hydrochloride, Ibuprofen, Dexamethasone, or Doxorubicin hydrochloride. However, CPT-11 treatment showed a notable decrease in TSM, suggesting a potential role in disrupting the template-switching process.

Keywords: quasi-palindrome; mutagenesis; DNA replication inhibitors; DNA repair

Corresponding author email: aswain@student.framingham.edu

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Introduction

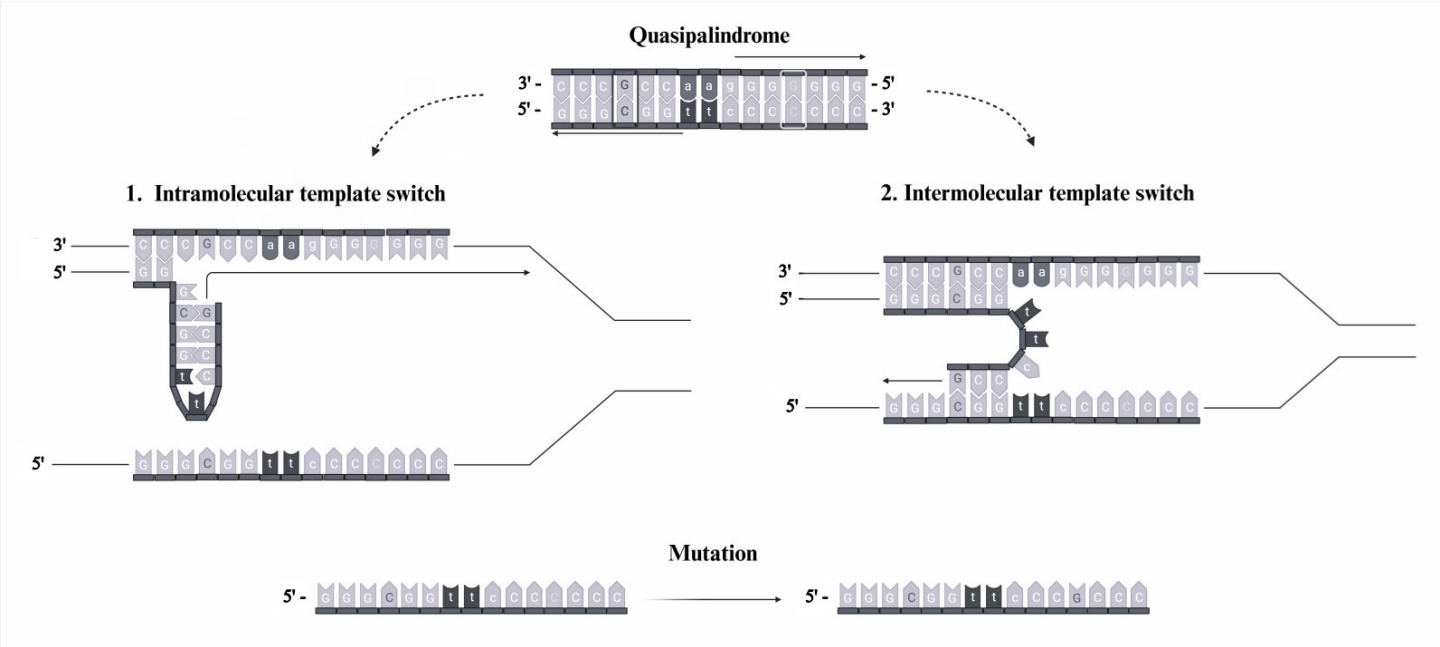
DNA mutations, changes that occur during DNA replication, can induce genetic instability. Among various mutation classes, this study investigates mutations caused by DNA secondary structures able to form quasi palindromes (QPs). QPs are imperfect inverted repeats of DNA that can give rise to hairpins and cruciform structures. DNA replication and gene expression are fundamental processes shared by all living organisms, including human cells. *E. coli* have simpler systems than human cells, making them ideal for preliminary studies. Their ease of genetic manipulation and rapid growth in the lab further enhance their utility.

The *thyA* hotspot in *E. coli* was the first natural QP hotspot studied for mutagenesis (1). These structures have been correlated with a specific type of mutation referred to as a template-switch mutation (TSM). TSMs are associated with several human diseases as reviewed (2), including hereditary angioneurotic edema (3), osteogenesis imperfecta (4), and biotinidase deficiency (5). Lynn Ripley was first to propose the two mechanisms for QP-induced TSM, intramolecular and intermolecular (Figure 1), both of which can involve a modification of the quasi-palindromic region into a perfected palindrome proposed to be after a momentary fork stalling event. (6)

While TSM occurs on both leading and lagging strands during DNA replication, the leading strand exhibits a bias towards TSM in both reversion (7) and TSM-specific assays (8). This bias is nullified in the absence of exonucleases (ExoI and ExoVII) (8), which are known to prevent TSM (1, 9) with ExoI playing the larger role (1). This suggests that both strands can undergo mutations,

Figure 1.

Quasi-palindrome Template-Switch Mutation Mechanisms



Two template-switch mutation models involving a temporary displacement of the nascent strand and relocation of the polymerase onto another strand different from the original template. In the intramolecular model, the nascent strand becomes the template, in the intermolecular model, the homologous strand becomes the template. Both mechanisms created a situation that can perfect an imperfect palindrome. (Created in https://BioRender.com)

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but the lagging strand is more adept at averting and correcting them (8). Additionally, research has demonstrated that exposure of QP sites to chemicals can influence the rate of TSM (10, 11). Azidothymidine (AZT), a thymidine analogue used as an antiretroviral medication to prevent and treat HIV/AIDS, has been seen to increase TSM rates (11). Other drugs found to increase rates of TSM include 5-Azacytidine (5-azaC), the antibiotic ciprofloxacin, and formaldehyde (10). 5-azaC interacts with cytosine methyltransferase (12) to cause DNA/protein crosslinks that stall replication (13), the antibiotic ciprofloxacin is a topoisomerase type II inhibitor, like DNA gyrase (14) and the chemical formaldehyde acts as a general mutagen, including creating DNA/Protein crosslinks in human cells (15). This study focuses on testing additional FDA-approved drugs, described below, for their impact on TSM frequency in *E. coli*.

A mutational reporter in the *lacZ* gene of *E. coli* has been used to identify the rate of quasi-palindrome mutations after exposure to various FDA-approved drugs. These mutations become apparent when *lacZ* alleles revert to Lac+, a phenomenon observed only if a template-switch event occurs. Additionally, since most studies on the influence of drug exposure on TSM have also used this mutational reporter in *E. coli*, continuing to use this system allows for consistent and fair comparisons across research.

All the FDA-approved therapeutics selected have at least one mechanism of action capable of causing replication fork stalling, a necessary condition for both intra- and intermolecular TSM. It is expected that these drugs are either inhibiting a process during DNA replication or disrupting DNA through intercalation and will lead to an increase in TSM frequency.

Gemcitabine Hydrochloride (dFdC)

Gemcitabine hydrochloride (dFdC) is an FDA-approved drug used in cancer treatment. Once inside cells, deoxycytidine kinase (dCK) phosphorylates dFdC, and subsequent phosphorylation steps lead to its active forms, dFdCDP and dFdTP. dFdCDP inhibits ribonucleotide reductase, reducing the pool of deoxynucleotides, mostly dCTPs, and enhancing the concentration of dFdCDP. This self-potentiation mechanism promotes masked chain termination that inhibits DNA synthesis and causes a fork stalling event (16). Although there are no previous studies focusing on gemcitabine's chain termination effects on TSM, studies conducted with the AZT chain terminator drug led to the hypothesis that gemcitabine might similarly be able to enhance TSM frequency (11).

Ibuprofen

Ibuprofen is an FDA-approved drug that treats inflammation and is used mostly to treat mild forms of pain and reduce fevers. A previous study found that the reactive oxygen species (ROS) generated by ibuprofen caused DNA strand scission and could cause DNA degradation (17). Furthermore, ibuprofen was identified to have intercalative binding with the DNA helix. This is where structures resembling base pairs will be stationed in the double helix rather than the appropriate base pairs and thereby distort the structure. In this sense, DNA replication is prevented from operating regularly. We have chosen to test ibuprofen due to the potential role it has in genetic instability.

Dexamethasone

Dexamethasone is an FDA-approved hormonal corticosteroid commonly prescribed to treat inflammation and swelling. Dexamethasone is comprised of multiple aromatics and hydroxy, methyl, and oxo groups, making it similar in structure to other corticosteroids such as hydrocortisone and prednisolone. Further studies examining the interaction between the dexamethasone-glucocorticoid receptor complex, and DNA have suggested the potential for dexamethasone to behave as an intercalating agent (18), giving it the potential to cause template switch mutations.

Doxorubicin Hydrochloride

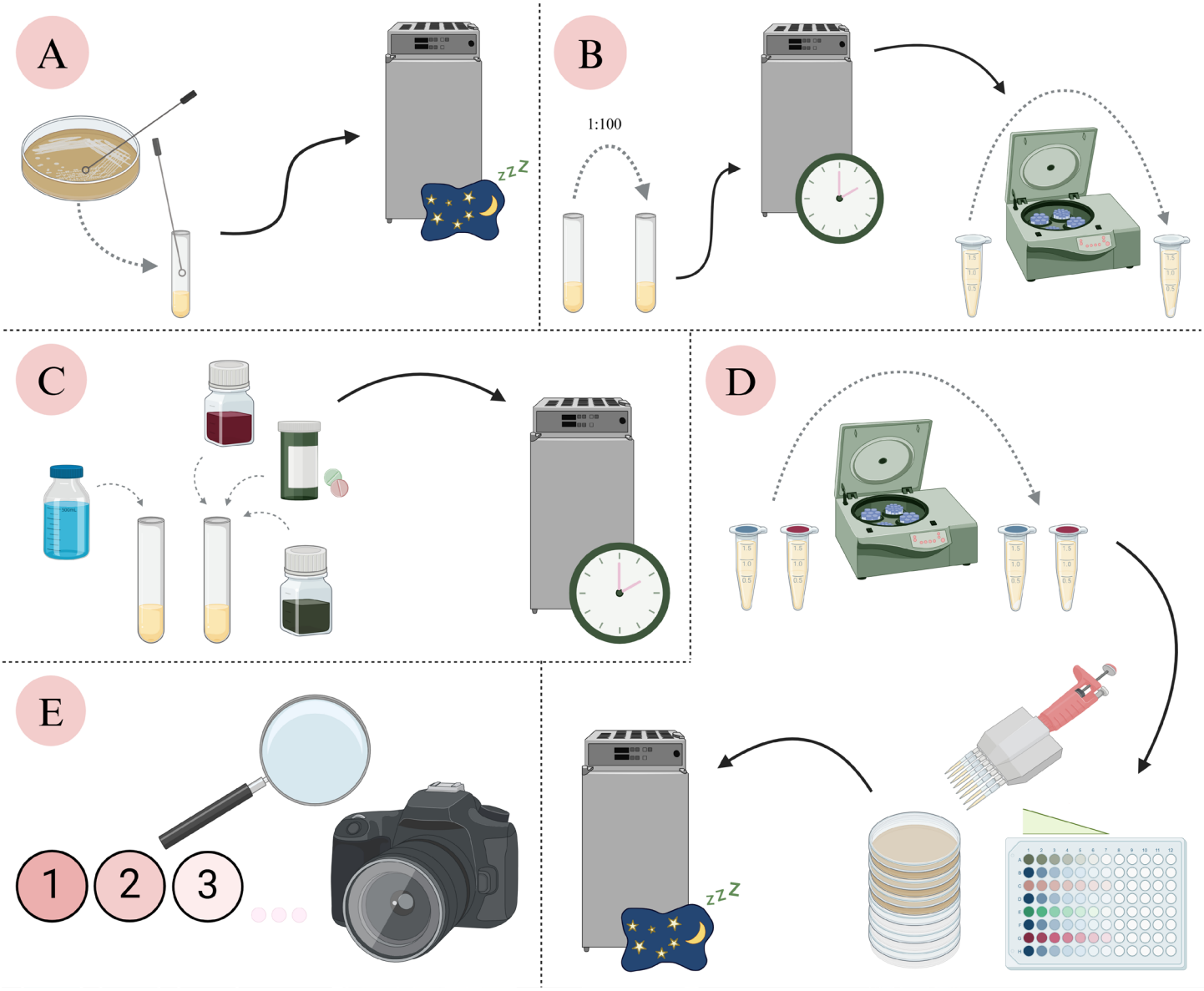
Doxorubicin hydrochloride (Doxo) is a chemical capable of inhibiting topoisomerase II (19), generating reactive oxygen species (20), and intercalating DNA (21). Here we evaluate the potential role of Doxo in TSM due to inhibiting topoisomerases and Doxo mediated DNA-protein crosslinks, similarly to how 5-azaC and ciprofloxacin have been shown to promote TSM by stalling replication (10) implicating the Dcm-DNA covalent complex trapped by 5-azaC as the initiator for mutagenesis. The leading strand of replication is more mutable than the lagging strand, which can be explained by blocks to the replicative helicase and/or fork regression. We find that template-switch mutagenesis induced by 5-azaC does not require double strand break repair via RecABCD; the ability to induce the SOS response is anti-mutagenic. Mutants in recB, but not recA, exhibit high constitutive rates of template-switching, and we suggest that RecBCD-mediated DNA degradation prevents template-switching associated with fork regression. A mutation in the DnaB fork helicase also promotes high levels of template-switching. We also find that other DPC-inducers, formaldehyde (a non-specific crosslinker.

Irinotecan Hydrochloride (CPT-11)

Irinotecan hydrochloride (CPT-11), derived from camptothecin, is a chemotherapeutic drug that inhibits topoisomerase I (22, 23). The inhibition of topoisomerase I by CPT-11 results in the formation of highly cytotoxic topoisomerase I-DNA cleavage complexes, importantly triggering various cellular responses, including protein-linked DNA breaks [23] and replication fork arrest[24]. Due to the similarities of properties with other TSM causing drugs, we have included the evaluation of CPT-11.

Figure 2.

Mutational Assay Methods



A. Single colonies were selected, inoculated, and cultured overnight. B. Cultures were diluted and incubated for 2 hours before pelleting *via* centrifugation. C. Cultures were divided into treated and control tubes before adding drug or control at the provided concentrations. Cultures were then incubated for 2 hours. D. Cultures were pelleted *via* centrifugation and the cell pellets were washed before resuspending the cells and performing serial dilutions. Dilutions were plated on LB and minimum lactose plates and incubated overnight. E. Total colony forming units (CFUs)/mL were determined and percentage of reverting CFUs were calculated. (Created in https://BioRender.com)

Methods

Bacterial Strains, Growth Conditions, and Media

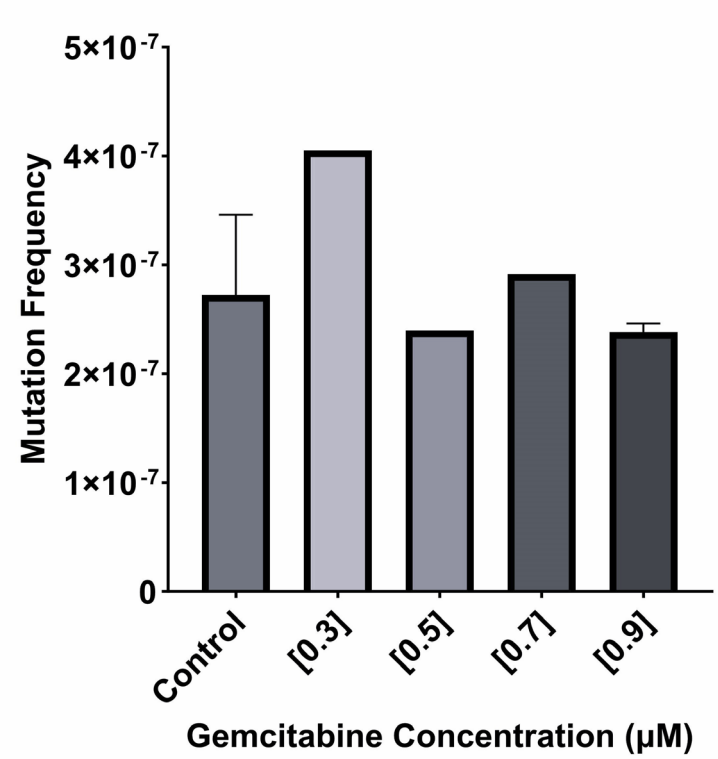
A *lacZ* mutational reporter strain of *Escherichia coli,* QP5, that reverts to Lac$^+$ when a 4 base pair deletion occurs due to a template switch mutation was acquired from Dr. Susan T. Lovett (used in Laranjo et al. 2017) [25]. Standard growth medium was Luria Broth (LB) medium, comprised of 1% Bacto-tryptone, 0.5% yeast extract, and 0.5% sodium chloride (Fisher Scientific, Waltham, MA, USA) and minimum lactose solid medium, comprised of 1x M9 salts, 0.2% lactose, 1mM Magnesium Sulfate, and 0.1mM Calcium Chloride (Alpha Teknova, Hollister, CA, USA). Plates were also comprised of 1.5% Bacto-agar.

Mutational Assay

Single colonies were inoculated and cultured in 1.5 mL of Luria Broth (LB) medium and grown overnight at 37°C with aeration (Figure 2A). The culture underwent a 1:100 dilution in fresh 1.5 mL LB medium, followed by a 2-hour incubation period at 37°C with aeration. Following incubation, the entire culture was pelleted by centrifugation at approximately 7000 rcf for two minutes so that the latent medium could be removed, and the pellet was resuspended in 150 µL of fresh LB (Figure 2B). The resuspended culture was divided evenly (75 µL in each) among two tubes followed

Figure 3.

Mutation frequency of Gemcitabine at varying concentrations



Gemcitabine was tested at 0.3 (n=1), 0.5 (n=1), 0.7 (n=1), and 0.9 µM (n=2) and mutation frequency were calculated. The fold increase of the samples was 0.67, 1.14, 0.93, and 1.14 respectively compared to the control showing no statistical difference. Error bars represent 95% CI based on the average mutation frequency.

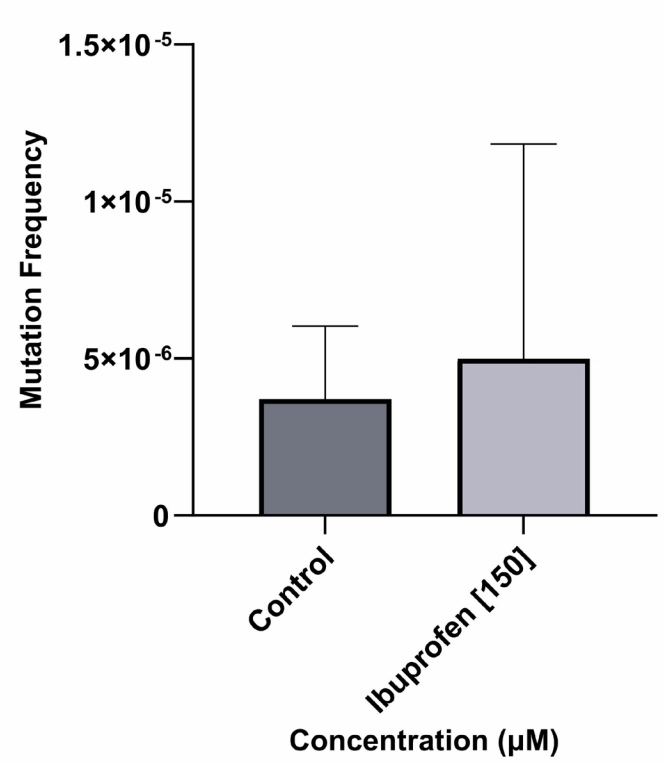
by an addition of 1.5 mL of fresh LB to each tube (Figure 2C). The specified concentration of the selected drug – CPT-11 (0.5 μM, 1 μM), Dexamethasone (60 μM), Ibuprofen (150 μM), Doxorubicin (0.025 μM, 0.4 μM, 0.5 μM) – was added to one tube while an equal amount of water was added to the other tube. Both tubes were placed back in the incubator for an additional 2 hours to grow at 37°C with aeration. Subsequently, both cultures were pelleted by centrifugation once more and the cell pellet was washed twice with 1 mL LB, followed by resuspension in 200, 400, or 600 μL LB. Cultures were then subject to serial dilutions in a 96-well plate to obtain 10-fold dilutions up to a 1:100,000 dilution, spotted onto LB and minimum lactose plates and grown overnight at 37°C to determine the CFU (colony forming units)/mL (Figure 2D).

Data Collection and Analysis

The percentage of reverting CFUs of the total was calculated for each drug by dividing the number of total CFU/mL on the LAC plates by the number of total CFU/mL on the LB plates (Figure 2E). All corresponding data points were averaged to obtain the average mutation frequency of each drug. Statistical analysis was done using an unpaired t-test that does not assume equal standard deviation among populations (Welch's t-test) using GraphPad Prism version 10.2.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

Figure 4.

Mutation frequency of Ibuprofen at 150 μM



Ibuprofen demonstrated a fold increase of 0.74 compared to the control (n=3). This was not statistically different than the control group. Error bars represent 95% CI based on the average mutation frequency.

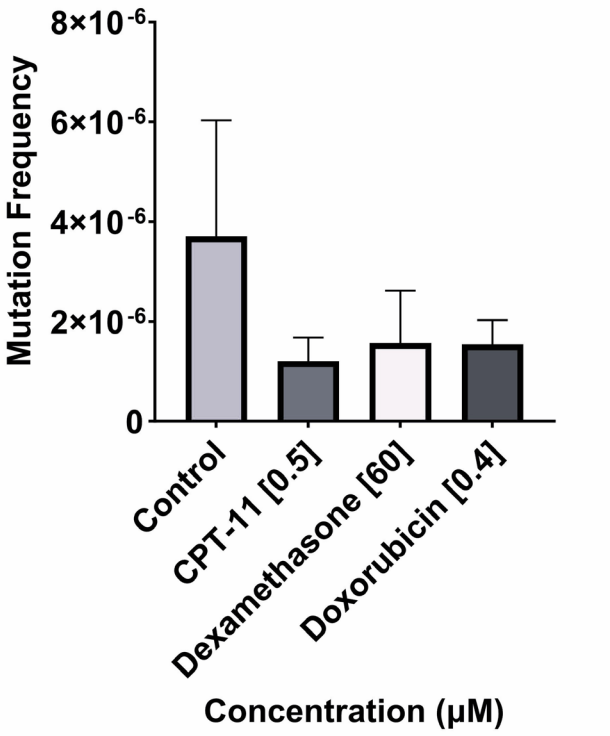
Results

Of the four concentrations of gemcitabine hydrochloride tested (0.3 μM, 0.5 μM, 0.7 μM, and 0.9 μM), the fold change was only between 0.67 and 1.14 (Figure 3) showing no statistical difference from the control group. Similarly, ibuprofen showed a fold change of 0.74 and no statistical difference from the control (Figure 4).

When compared to the control group, dexamethasone resulted in a lower average rate of template-switch mutations, though not statistically different from the control. Dexamethasone was tested at a concentration of 60 μM and resulted in a 2.37-fold change in mutation frequency, with the average rate of mutation 1.56E-06 compared to the control of 3.71E-06 (Figure 5). This was similar to the results of doxorubicin hydrochloride at a concentration of 0.4 μM which had a fold change of 2.41 and an average rate of mutation of 1.54E-06 (Figure 5). Testing done with CPT-11 showed a statistically significant (p=0.0383) change in reported template-switch mutation rate, producing a 3.08-fold decrease when tested at a concentration of 0.5 μM with an average rate of mutation of 1.20E-06 (Figure 5).

Figure 5.

Comparison of mutation frequency of CPT-11, Dexamethasone, and Doxorubicin



CPT-11 was tested at a concentration of 0.5 μM (n=17) and resulted in a statistically significant fold change of 3.08 compared to the control with a p-value of 0.0383. Dexamethasone, at a concentration of 60 μM (n=5), showed a statistically insignificant fold change of 2.37 compared to the control. Doxorubicin was also statistically insignificant, tested at a concentration of 0.4 μM (n=24) and displaying a fold change of 2.41 compared to the control. Error bars represent 95% CI based on the average mutation frequency.

Discussion

This study aims to expand our understanding of which FDA-approved drugs contribute to TSM. It is important to examine how these therapeutics, including some commonly used over-the-counter drugs, may affect mutation frequency, as TSM have been linked to the development of various diseases. There is still a significant gap in understanding TSM, their causes, and the mechanisms behind these mutations.

The expected outcomes for these experimental drugs were that they would either inhibit a process during DNA replication or act as intercalating agents to disrupt DNA, leading to an increase in TSM frequency. However, for most of the drugs tested (Ibuprofen, Dexamethasone, Doxorubicin hydrochloride, and Gemcitabine hydrochloride), no statistically significant change in mutation frequency was observed.

Our preliminary investigation into the effects of gemcitabine hydrochloride on TSM frequency suggests no significant impact on the leading strand. Contrary to the initial hypothesis, due to gemcitabine hydrochloride's chain termination effects during DNA replication, our results from limited trials do not support this notion. Instead, it appears that gemcitabine hydrochloride may have no effect on TSM frequency, aligning with its primary role in inhibiting DNA synthesis rather than causing replication fork stalling [26]. Similarly, although ibuprofen's potential to intercalate within DNA [17] could theoretically stall the replication machinery, our trials did not show a statistical increase in TSM frequency. Ibuprofen, hypothesized to act as an intercalating agent, likely failed to intercalate the DNA or caused excessive damage due to its relatively large size. This could explain the lack of change in mutation frequency, as the DNA either remained unchanged or was too damaged to synthesize properly.

Both dexamethasone and doxorubicin hydrochloride did not result in a statistically significant change in TSM rate when compared to the control group. Dexamethasone and doxorubicin hydrochloride, also predicted to intercalate DNA and stall the replication fork, may have caused enough damage to prevent successful replication, leading to no observed change in TSM frequency, though additional trials may provide further insights. In contrast, CPT-11 showed a statistically significant decrease in TSM frequency. This supports the hypothesis that CPT-11 inhibits topoisomerase I and induces DNA strand breaks. These breaks likely prevent the displacement of the nascent DNA strand and instead promote DNA damage repair through resection and homology search rather than Quasi-Palindrome hairpin formation, thereby reducing polymerase displacement onto the opposite strand. This highlights its potential role in mitigating TSM occurrence. We plan to further explore the potential impact of FDA-approved drugs on TSM using our extensive FDA-approved drug library. This ongoing investigation aims to investigate on the mechanisms underlying TSM, including both natural occurrences and drug-induced cases.

Additional research on the impact of drug exposure on TSM should be conducted in mammalian cells. While this experiment focused on *E. coli* and provided valuable insights, the effects on mammalian cells remain unknown. Testing these drugs in mammalian cells would allow for a more direct evaluation of the conclusions drawn from this study in a more complex system. This approach would also enable observations in cells that are susceptible to TSM-associated diseases. Such findings would be particularly compelling given that the drugs tested here are also used to treat other diseases not directly related to TSM. This could open discussions about the potential consequences of using these therapeutics, especially regarding their broader impact on mutation frequency.

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Distribution of Tetracycline-Resistant Bacteria Within the Fort Phantom Hill Reservoir Watershed

Leah M. Sanchez 0009-0008-3367-2475

Abilene Christian University
Abilene, TX USA

Jennifer A. Hennigan 0009-0009-2839-7998

Abilene Christian University
Abilene, TX USA

Abstract

Tetracycline resistance genes have been reported to be abundant in bacteria in environments impacted by agriculture. The Fort Phantom Hill reservoir watershed in Jones and Taylor counties in Texas includes feedlots, rangeland, and row crop agriculture with manure-treated soils. We hypothesized that tetracycline-resistant coliforms could be present throughout the watershed. To investigate our hypothesis, samples were taken from the sediment of seven sites within the watershed between June 2022-August 2023. Tetracycline-resistant coliforms and non-coliforms were isolated from six out of seven sites. The highest relative abundance of tetracycline-resistant isolates was found at a site at Cedar Creek. Additionally, the highest relative abundance of tetracycline resistance among lactose fermenters was at a site at Elm Creek. From a representative sample of tetracycline-resistant isolates across all sites, the *tetB* genotype was the most common. Additionally, four multi-drug resistant strains of *Escherichia* were identified: CCN-113, CCN-251, CCN-109, and ELM-161. A resistance plasmid was extracted from CCN-251 carrying *dhfr1* and *sulII* resistance genes. Monitoring of the spread of antimicrobial resistance genes through surface water in the Fort Phantom Hill Watershed provides data to support the need for antibiotic stewardship in this region.

Keywords: antimicrobial resistance (AMR); tetracycline; watershed; surface water

Corresponding author email: jah20c@acu.edu

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Introduction

The World Health Organization has declared the spread of antibiotic resistance a leading crisis in medicine (1). Agriculture and human-based activities in urban areas are primary factors in the rise of antimicrobial resistance genes (ARGs) in the environment. ARGs from wastewater treatment plants, agriculture runoff, and animal husbandry can contaminate surface waters, then spread through horizontal gene transfer in the natural environment (2). Application of animal manures to land significantly increases the level of and diversity of ARGs in the soil. Additionally, these ARGs can persist in manure-treated soils for up to 120 days (3). In livestock waste, the most frequently detected classes of ARGs include those that confer resistance to tetracyclines (TET) and sulfonamides (4). Of the medically important drug classes approved for use in food-producing animals in the United States, tetracyclines comprised the highest percentage of total kilograms of antimicrobials sold in 2022 (65%; ~4 million kg) (5). A study in Portugal found that TET resistance genes were present in all multi-drug resistant (MDR) *Enterobacteriaceae* isolated from poultry, swine, and cattle manures (6). Likewise, a study in Korea found that all commensal *E. coli* strains isolated from cattle farms were resistant to tetracyclines (7).

The Fort Phantom Hill reservoir watershed is a mixed-use watershed containing agricultural land and urban areas in Jones and Taylor counties in west Texas in the United States. The area is largely dominated by agribusiness, including feedlots, rangeland, and row crop agriculture with manure-treated soils. Additionally, the land is used by oil industries for exploration, refining, and drilling operations. Ten creeks, three reservoirs, and the Fort Phantom Hill Reservoir make up the watershed. All of these lies within the Brazos River drainage system. Because of topology and urban development, rain in the watershed often produces excessive, rapid runoff and flash floods. Surface water flows from south to north along creeks, ending in the Fort Phantom Hill reservoir. Therefore, nonpoint source pollution poses a concern for water quality. Row crop agriculture and the expansion of the city of Abilene increases the possibility of anthropogenic compounds entering streams and reservoirs through runoff (8). The goal of this study was to investigate the abundance of TET-resistant bacteria at seven sites throughout Fort Phantom Hill watershed (Figure 1). This study is unique in that it provides evidence of TET-resistance from multiple sites within a mixed-use watershed.

Materials and Methods

Sample collection

Two replicates of 50 grams of sediment and water were obtained from Kirby Lake (32.373,-99.728), Fort Phantom Hill reservoir (32.614, -99.676), Buck Creek (32.541, -99.709), Elm Creek (32.463, -99.777), Cedar Creek (32.471, -99.721 and 32.453, -99.721), and Lytle Creek (32.441, -99.715) using a previously described method (10). All sites were on public property. Samples were obtained at least five days after any rainfall event to reduce potential storm effects on the sites. Each site was sampled on at least two different days. Physical characteristics for sites were obtained using a Vernier LabQuest2 probe (Beavertown, OR), including pH, temperature, conductivity, turbidity, and dissolved oxygen. The metadata for each site was stored using the application Epicollect5 v 7.0.3 (Center for Genomic Pathogen Surveillance, University of Oxford)

Enrichment and selection of TET-resistant coliforms

Enrichment of the samples was completed in buffered peptone water as previously described (10). Serial dilutions were spread onto MacConkey agar (Sigma-Aldrich, USA) and MacConkey agar supplemented with 16 μg/mL tetracycline (IBI Scientific, Road Dubuque, IA) in accordance with the 2022 Clinical Laboratory Standards Institute concentration for tetracycline resistance for *Enterobacterales* (11). Plates were incubated at 37°C for 18-24 hours before colonies were counted. Isolates were streaked to isolation on

MacConkey agar with 16 µg/mL tetracycline, grown in tryptic soy broth (Neogen, Lansing, MI) supplemented with tetracycline, and stored in 50% glycerol at -80°C.

Metabolic profiling and antibiotic screening

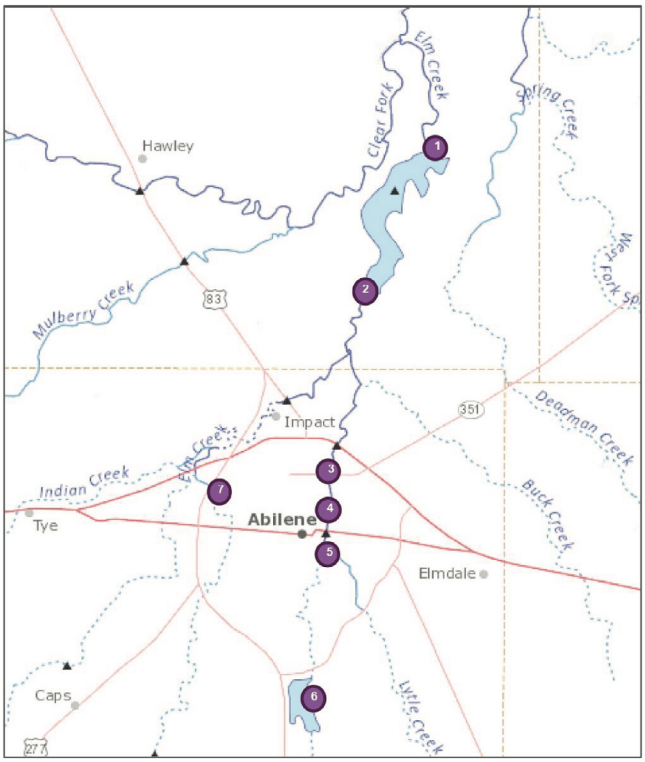
TET-resistant bacteria were inoculated in SIM deeps (HiMedia Laboratories, Kennett Square, PA) and citrate slants (Carolina Biological Supply, Burlington, NC), grown at 37°C for 18-24 hours before analyzing. Isolates were patched on tryptic soy agar (Neogen) supplemented with either 4 µg/mL cefotaxime (MP Biomedicals, Solon, OH) or µg/mL 32 nalidixic acid (Amresco, Solon, OH) and grown at 37°C for 18-24 hours.

Detection of tet resistance genes

Genomic DNA was isolated using a Wizard Genomic DNA purification kit (Promega, Madison, WI). Multiplex PCR of *tetA*, *tetB*, *tetC*, *tetD*, *tetM* and *tetO* genes was conducted using primers as previously described (12) with the following modifications. Briefly, reactions were completed for *tetA*, *tetM*, and *tetO* or *tetB*, *tetD*, and *tetC* genes using Platinum™ PCR Supermix (Thermo Fisher Scientific, USA) or Accuprime Supermix (Thermo Fisher Scientific) for 35 cycles of the following program: 94° for 5 min, 94° for 1 min, 55° for 1 min, 68° for 1:30 min, 68°C for 10 min. Rections were visualized on 1-1.5% agarose gels using a 100 bp standard ladder (NEB).

Figure 1.

Map of sampling sites in the Fort Phantom Hill watershed



Seven sites were selected from six different lakes and creeks within the Fort Phantom Hill watershed: Fort Phantom Hill Reservoir (1), Buck Creek (2), Cedar Creek (3, North; 4, Central) Lytle Creek (5), Kirby Lake (6) and Elm Creek (7). A creek map was generated using USGS Steamer (9). Solid blue indicated streams and dashed lines indicate intermittent streams. Red solid lines represent roadways. Black triangles show USGS stream gages.

16S PCR and Sanger sequencing

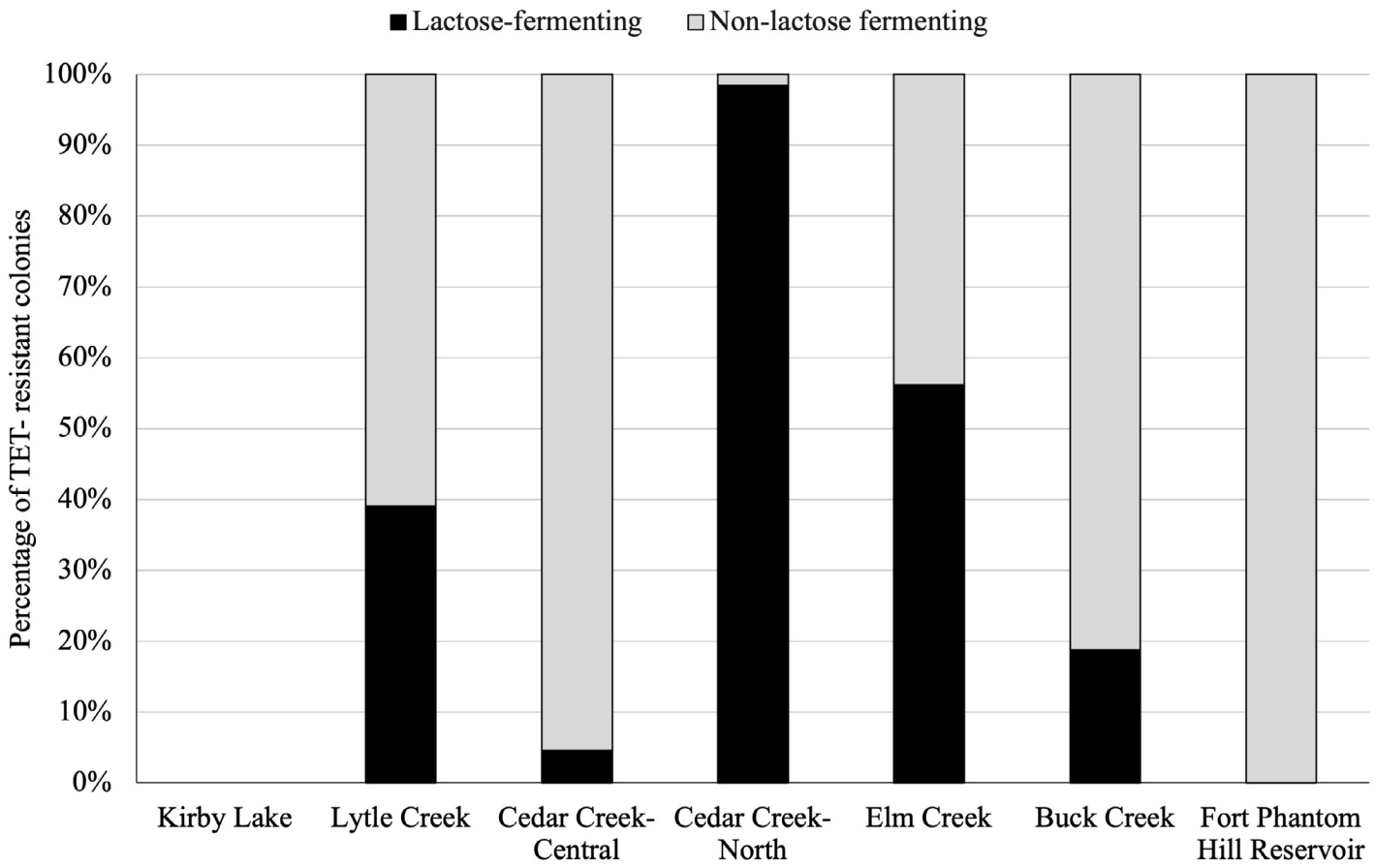
16*S* rDNA was amplified using 27F and 1492R primers as previously described (13). Amplicons were purified and concentrated using a Monarch® PCR & DNA Cleanup kit (NEB, Ipswich, MA) and submitted to MC BioLabs (San Francisco, CA) for Sanger sequencing. Sequences were analyzed and edited in Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI) and queried in the NCBI 16*S* ribosomal RNA sequence database using Megablast. Genus level identities were assigned to isolates with consistent percent identities of no less than 95%.

96 well plate antibiotic susceptibility testing

Environmental TET-resistant isolates and *Escherichia coli* ATCC 25922 were inoculated in Mueller-Hinton broth (Sigma-Aldrich) and grown overnight at 37°C. Cultures were standardized to 0.12-0.14 OD_{600} using a NanoDrop One (Thermo Scientific), equivalent to 0.5 McFarland Standard. In triplicate wells of a 96-well plate (CELLTREAT, USA), 100 uL of standardized cultures were added to 200 uL of Mueller-Hinton broth supplemented with antibiotics to produce the following final concentrations at 300 uL: 4 µg/mL cefotaxime, 16 µg/mL tetracycline, 32 µg/mL nalidixic acid, 64 µg/mL kanamycin (Alfa Aesar, Ward Hill, MA), 32

Figure 2.

Composition of the TET-resistant population per site



The proportion of TET-resistant lactose-fermenting (black) and non-lactose fermenting colonies (gray) was calculated as a percentage of total TET-resistant CFUs. The bars represent the average percentage of four samples for each site.

μg/mL ampicillin (Sigma Aldrich), and 16 μg/mL trimethoprim (Sigma Aldrich). Concentrations are consistent with the 2022 Clinical Laboratory Standards Institute concentration for resistances for *Enterobacterales* (Clinical and Laboratory Standards Institute (11). Plates were statically incubated at 37 °C for 24 ±2 hours of growth and read at OD_{630} using a SmartReader™ 96 (Accuris, Edison, NJ). OD_{630} readings ≥ 0.25 were classified as resistant.

Plasmid isolation and sequencing

The plasmids from CCN-251 and CCN-113 were isolated using a Monarch™ Plasmid Miniprep Kit (NEB) and visualized on a 1.5% gel using a 10 kB supercoiled DNA ladder (NEB). Whole plasmid sequencing was performed by Plasmidsaurus (Eugene, OR) using Oxford Nanopore Technology with custom analysis and annotation.

Results

To determine the relative abundance of TET resistance in the Fort Phantom Hill Reservoir watershed, sites were selected to represent the ten creeks and three reservoirs that comprise the watershed (Figure 1). Not all creeks could be sampled due to lack of accessibility and drought conditions. Sites at Indian Creek to the northwest and Catclaw Creek were dry, except immediately following rainfall. Because bacterial and ARG loads have been shown to increase with rainfall events, these sites were excluded from this study (14, 15). Of the seven sites investigated, TET-resistant isolates were found at six sites; however, lactose-fermenting TET-resistant isolates were only isolated at five sites (Figure 2).

No TET-resistant isolates were obtained from the southernmost site, Kirby Lake, even though the number of colony forming units (CFUs) on MacConkey agar plates for the same samples did not differ from other sites. Only non-lactose fermenting isolates were cultured from the Fort Phantom Hill Reservoir. TET-resistant lactose-fermenting bacteria composed the majority of the TET-resistant population at two sites, Cedar Creek-North (98.4%) and Elm Creek

Table 1.

Mean relative abundance of TET-resistance at each site

	TET-Resistance in Gram-negatives	TET-resistance in lactose-fermenters
Kirby Lake	0%	0%
Lytle Creek	0.00019%	0.0005%
Cedar Creek-Central	0.14676%	0.01829%
Cedar Creek-North	0.00347%	0.06531%
Elm Creek	0.04718%	3.38440%
Buck Creek	0.00275%	0.00879%
Fort PhantomHill Reservoir	0.00012%	0%

The mean relative abundance of TET-resistant lactose-fermenting and non-lactose fermenting colonies was calculated as a percentage of resistant CFUs to total CFUs on MacConkey agar lacking tetracycline for four samples for each site.

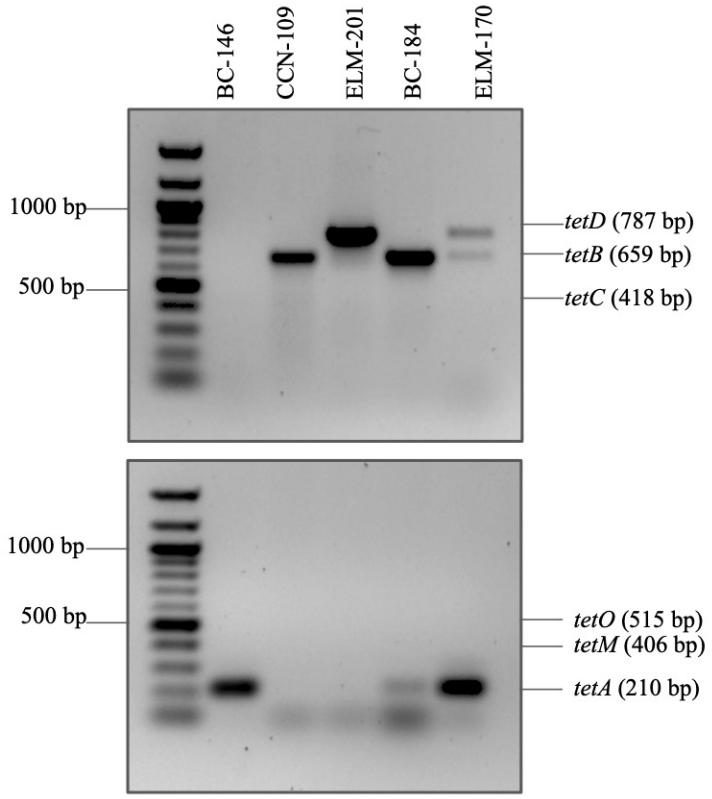
(56.1%) (Figure 2). The site with the highest overall TET-resistance was Cedar Creek-Central (0.146%), followed by Elm Creek (0.047%) (Table 1).

However, the site with the highest TET resistance in lactose-fermenters was Elm Creek (3.384%), followed by Cedar Creek-North (0.065%). Since the total number of TET-resistant potential coliform colonies for all sites was over 3000, every isolate could not be tested further. Instead, a random sample of up to ten well isolated colonies from each replicate was selected and metabolic profiles obtained for 148 TET-resistant isolates using the following criteria: nalidixic acid resistance, cefotaxime resistance, H_2S production, motility, indole production, lactose fermentation, and citrate utilization. Using these criteria, 23 different metabolic types were found throughout the watershed and 16*S* sequencing was performed to determine the genera of 37 representative strains for each metabolic type at each site (Table 2).

The most prominent genus identified throughout the watershed was *Escherichia*; however, TET-resistant strains of *Pseudomonas*, *Klebsiella*, *Providencia*, *Serratia*, *Enterobacter* and *Raoultella* were also identified. To determine the *tet* genotypes for the representative strains of each metabolic type, multiplex PCR was used to detect the presence of the *tetA*, *tetB*, *tetC*, *tetD*, *tetM*, and *tetO* genes. Of the 37 representative TET-resistant strains genotyped, isolates with the genotypes *tetA*, *tetB*, *tetD*, *tetA tetB*, and *tetA tetB tetD* were identified (Figure 3).

Figure 3.

tet genotypes represented in the Fort Phantom Hill Watershed



Multiplex PCRs for tet genes tetB, tetD, and tetC (top panel) and tetO, tetM, and tetA (bottom) panels are shown on a 1% agarose gel. The band sizes for each gene product are indicated. A 100 bp ladder (NEB) is included in the first lane for comparison.

Table 2.

Metabolic characteristics of representative TET-resistant strains from Fort Phantom Hill watershed sites.

Site	Genus	#of isolates	Rep Strain #	tetracycline resistance	nalidixic acid resistance	cefotaxime resistance	H_2S production	motility	Indole production	lactose fermentation	citrate utilization
Elm Creek (ELM)	*Klebsiella*	3	201	■	□	□	□	▒	□	■	■
	Klebsiella	10	218	■	□	□	□	□	□	■	■
	Escherichia	26	227	■	□	□	□	■	■	■	□
	Escherichia	1	237	■	□	□	□	■	■	■	■
	Escherichia	1	170	■	□	□	□	▒	■	■	□
	Enterobacter	1	152	■	□	□	□	▒	□	□	■
	Providencia	2	164	■	□	□	■	▒	□	□	□
	Escherichia	1	161	■	□	■	□	■	■	■	□
	Pseudomonas	1	215	■	■	■	□	□	□	□	■
Cedar Creek -Central (CCC)	*Escherichia*	2	112	■	□	□	□	■	■	■	□
	Klebsiella	3	268	■	□	□	□	■	□	□	■
	Providencia	1	272	■	■	□	□	■	□	□	■
	Providencia	4	ST-F4	■	□	□	□	□	■	□	▒
	Providencia	4	ST-F10	■	□	□	□	□	■	□	■
	Serratia	17	275	■	□	□	□	□	□	□	■
	Klebsiella	3	267	■	□	□	□	□	□	■	■
	Providencia	1	271	■	□	□	□	■	□	■	■

Positive characteristics are indicated by black squares, negative results are indicated by white squares, and ambiguous results are indicated by gray squares. Genus of representative strain for each metabolic type is listed. FP, Fort Phantom Hill Reservoir

Table 2 continues "Serratia" on page 3131.

Table 2 (Continued from page 30)

Site	Genus	#of isolates	Rep Strain #	tetracycline resistance	nalidixic acid resistance	cefotaxime resistance	H_2S production	motility	Indole production	lactose fermentation	citrate utilization
FP	*Serratia*	2	224	■				■			■
Cedar Creek-North (CCN)	*Escherichia*	11	113	■					■	■	
	Escherichia	14	251	■				■	■	■	
	Escherichia	1	140	■						■	
	Pseudomonas	1	142	■	■	■					■
	Escherichia	1	109	■	■	■			■	■	
Buck Creek (BC)	*Escherichia*	12	146	■				■	■	■	
	Escherichia	1	181	■					■	■	
	Raoultella	3	171	■						■	▒
	Raoultella	2	184	■				▒		■	
	Raoultella	1	178	■				■		■	▒
	Raoultella	1	179	■				▒			
	Raoultella	1	182	■							▒
	Pseudomonas	1	149	■	▒	■		▒			■
	Pseudomonas	3	263	■	■	■					■
Lytle Creek (LC)	*Escherichia*	1	110	■				■			
	Escherichia	1	189	■				■		■	
	Escherichia	9	145	■				■	■	■	
	Serratia	2	156	■				■			■
	Pseudomonas	1	225	■	■	■					■

Table 3.

tet genotypes identified at each site

	tetA	***tetB***	***tetD***	***tetA tetB***	***tetA tetB tetD***	**Undetermined**
Elm (n=9)	0.00%	22.22%	22.22%	33.33%	11.11%	11.11%
Cedar Creek- Central (n=8)	0.00%	100.00%	0.00%	0.00%	0.00%	0.00%
Fort Phantom Hill Reservoir (n=1)	0.00%	100.00%	0.00%	0.00%	0.00%	0.00%
Buck (n = 9)	33.33%	11.11%	0.00%	44.44%	11.11%	0.00%
Cedar Creek-North (n=5)	20.00%	80.00%	0.00%	0.00%	0.00%	0.00%
Lytle (n=5)	0.00%	20.00%	0.00%	80.00%	0.00%	0.00%
Total (n=37)	10.81%	45.95%	5.41%	29.73%	5.41%	2.70%

The percentage of *tet* genotypes for representatives of each metabolic group at each site are shown (n=37).

Genotypes including *tetC*, *tetM*, or *tetO* genes were not detected in any isolate. None of the six genes were detected in one isolate, ELM-164, indicating that it harbors a different *tet* gene. Of the represented group, the *tetB* genotype was the most abundant at 45.95% (17/37) and was present at all sites (Table 3).

The *tetA tetB* genotype was the second most abundant at 29.73% (11/37). Two isolates harboring *tetD* only were also detected at Elm Creek; *tetD* was found in combination with *tetA* and *tetB* at Elm Creek and Buck Creek. Altogether, 35.14% (13/37) of the representative isolates carried more than one *tet* resistance gene. The genotype found in the most different genera was *tetB*, being detected in *Escherichia*, *Enterobacter*, *Klebsiella*, *Providencia*, *Serratia*, and *Raoultella*. In contrast, the *tetD* genotype was only found in *Klebsiella*.

When the 148 TET-resistant isolates were screened for multi-drug resistance, seven isolates grew on MacConkey agar supplemented with cefotaxime and nalidixic acid: one *Escherichia* isolate (CCN-109) and six *Pseudomonas*. Additionally, one *Escherichia* isolate (ELM-161) showed resistance to cefotaxime only. The resistance profiles of these two isolates were further investigated using antibiotic sensitivity microdilution assays. Isolate CCN-109 shows additional phenotypic resistance to ampicillin, kanamycin, and trimethoprim (Figure 4).

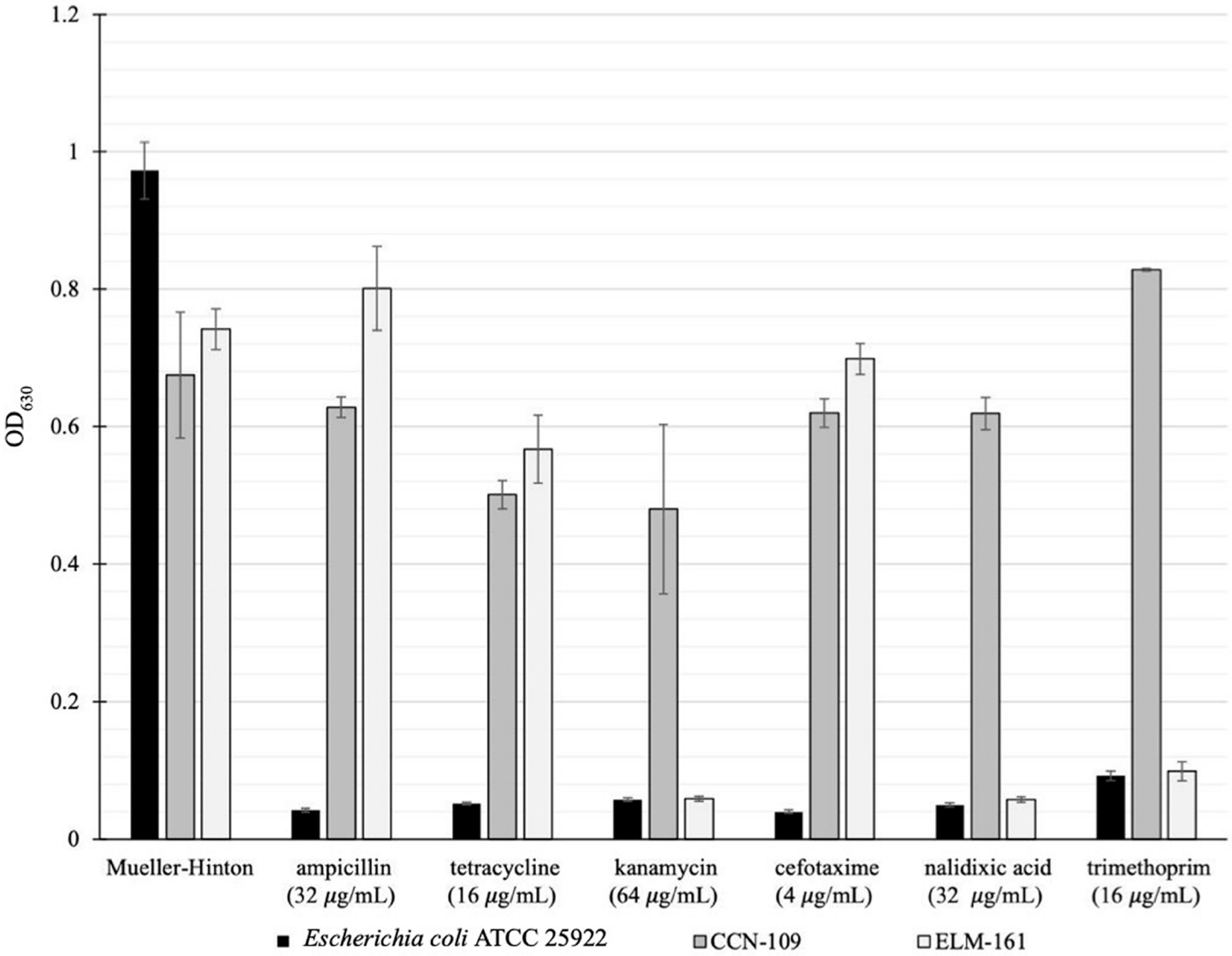
ELM-161 was further determined to be resistant to ampicillin only. Since CCN-109 appeared to have a unique resistance profile, other *Escherichia* isolates from the Cedar Creek-North site were further investigated to determine if others showed MDR phenotypes. CCN-251 showed additional resistances to ampicillin and trimethoprim, and CCN-113 showed an additional resistance to ampicillin only (Figure 5). Other *Escherichia* isolates were not phenotypically resistant to the other antibiotics tested.

Because ARGs can be transmitted horizontally, we investigated whether CCN-109, CCN-113, and CCN-251 strains harbored small plasmids that are responsible for these phenotypes. Using a plasmid isolation kit, we found that CCN-251 carried a 6.7 kb plasmid and CCN-113 carried a 4.6 kb plasmid (Figure 6A and B, respectively), but no plasmid was detected in CCN-109 using these methods.

The plasmid isolated from CCN-251 contains *sulII* and *dfr1* genes, which encode sulfonamide-resistant dihydropteroate synthase and

Figure 4.

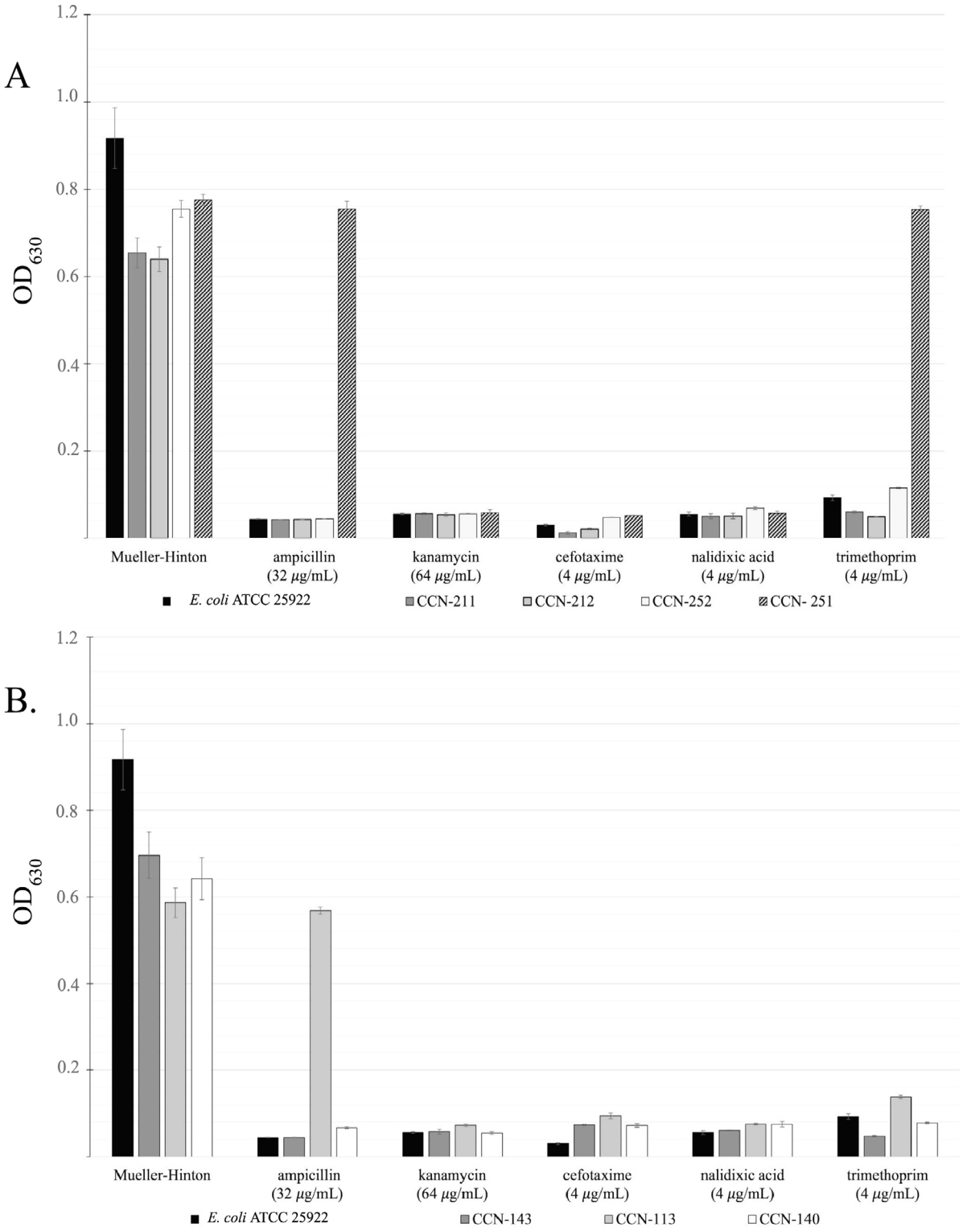
Antibiotic susceptibility of CCN-109 and ELM-161



OD_{630} readings are shown for *E. coli* ATCC 25922, CCN-109, and ELM-161 grown in Mueller-Hinton with and without antibiotics at concentrations in accordance with CLSI breakpoints for *Enterobacterales* (11). Standard deviations for three replicates are indicated.

Figure 5.

Antibiotic susceptibility of Escherichia isolates from Cedar Creek-North



OD_{630} readings are shown for *E. coli* ATCC 25922 and *Escherichia* isolates from Cedar Creek- North. All were grown in Mueller-Hinton broth with or without antibiotics at concentrations in accordance with CLSI break-points for *Enterobacterales* (11). Standard deviations for three replicates are indicated.

trimethoprim-resistant dihydrofolate reductase enzymes, respectively. The *aphE* gene on the plasmid is incomplete, but encodes an aminoglycoside phosphotransferase which would inactivate aminoglycoside antibiotics like kanamycin. The plasmid from CCN-113 harbors the *fepE* gene, but no ARGs.

Conclusions

Our investigation of the Fort Phantom Hill Reservoir watershed found a low level of TET resistance throughout, consistent with our hypothesis. The highest relative percentage of TET-resistant lactose-fermenters was 3.38% at a central location where Elm Creek flows through a neighborhood. A correlation was not observed between greater TET-resistance and the direction of waterflow in the watershed (Figure 2). Reported frequencies of TET resistance in environmental surface water samples varies; for example, in *E. coli* from the water and sediments of two rivers in Austria, the frequency was between 1% and 11% of isolates (17). Therefore, TET-resistance in the Fort Phantom Hill watershed was similar to or less than that found in other locations. While no sites were on active farmland, all sites were no more than 8 km downstream of land zoned as agriculture-open space by the city of Abilene. Future studies comparing TET-resistance in sediments from ponds on nearby ranchland would provide a valuable comparison for our data set.

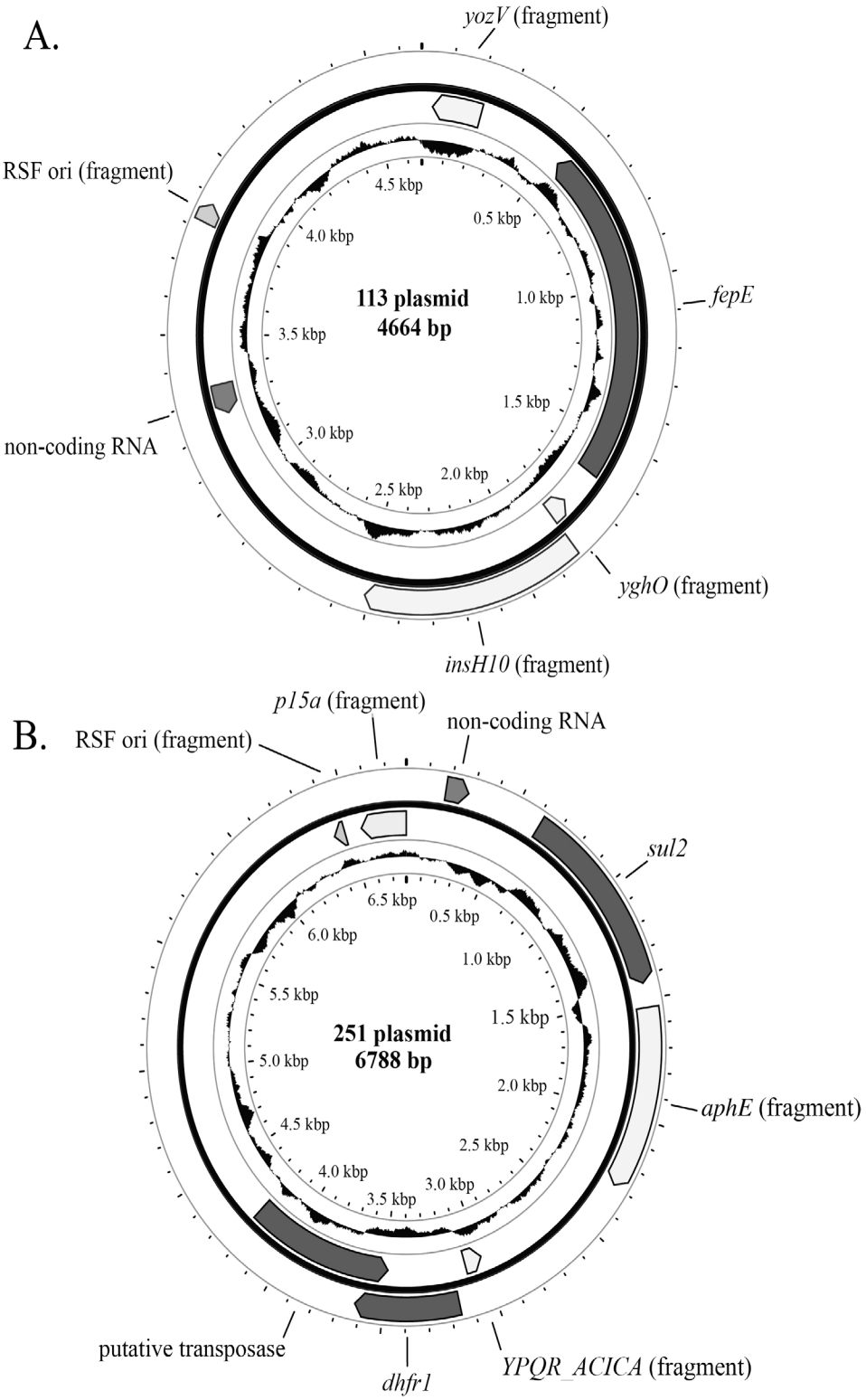
Most of the representative TET-resistant isolates that were genotyped in this study carried *tetA* and/or *tetB* genes (Table 3), which is consistent with a previous report that found *tetB* was the most frequent resistance gene in non-clinical *E. coli* isolates from animal and human sources (63%), and *tetA* was the second most frequent (35%) (18). Other studies also found that *tetA* and *tetB* were the most common *tet* resistance genes in commensal *E. coli* isolates from cattle (7) and *E. coli* from meat and meat products (19). The genotypes *tetA*, *tetB*, *tetC*, *tetO*, *tetW*, and *tetM* have all been detected in hospital wastewater collected from wastewater treatment plants, and *tetA*, *tetQ,* and *tetW* have been detected in municipal wastewater (2). Other studies show the average abundance of *tetM* to be higher in manure and wastewater samples than *tetA* or *tetB* (20). Perez-Valera and colleagues found that the treatment of soils with manure increased the abundance of *tetM*, and, moreover, *tetM* most likely originated from the manure (21). In a study that compared the presence of genes in the outlets of manure applied catchments, *tetM* was significantly higher than non-manure catchments (22). The *tetM* gene was not detected in any isolate in our study.

Of the isolates that were genotyped in this study, 35.14% (n=13) carried more than one *tet* gene (Table 3). In contrast, another study found that only 8.7% of TET-resistant *E. coli* cattle commensals carried more than one *tet* gene. The presence of more than one gene for TET resistance was thought to indicate selective pressure due to the high level of tetracycline in an environment (7). Further investigation is needed to determine concentration of tetracycline in the Fort Phantom Hill surface water and if the isolates with more than one *tet* gene are more resistant to tetracycline; these isolates were not challenged with tetracycline concentrations greater than 16 μg/mL. Additionally, since the methods in this study only tested viable isolates that were phenotypically TET-resistant, it is possible that additional non-functional TET genes in the populations were missed.

In addition to TET resistance, MDR resistant *E. coli* were also recovered, including two isolates that carried small non-conjugative plasmids (Figure 6). The *dhfr* gene encoded in the plasmid recovered from CCN-251 provides an explanation for its phenotypic trimethoprim resistance (Figures 5 & 6). Likewise, the presence of *sulII* would be expected to confer resistance to other sulfonamides but must be tested experimentally. Plasmids encoding both *sul* and *dhfr* genes have been readily isolated from sulfamethoxazole and trimethoprim-resistant *E. coli* in stream water. Moreover, these *E. coli* isolates were found to harbor multiple *sul* and *dhfr* genes, which is likely due to the influence of sub-inhibitory concentrations of trimethoprim and sulfamethoxazole

Figure 6.

Plasmid maps of plasmids isolated from Escherichia isolates



Plasmid maps generated from plasmids isolated from *Escherichia* isolates 113 (A) and 251 (B). GC content is mapped in black, genes in dark gray, gene fragments in white, and origins of replication in gray. Plasmid maps were generated using PlasMapper 3.0 (16).

found in the water (23). The association of *sul* and *dhfr* genes on mobile elements, such as the CCN-251 plasmid isolated in this study, is highly relevant to the spread of resistance in aquatic environments like the Fort Phantom Hill Watershed and has a potential impact on human health and agriculture.

In contrast, sequencing of the plasmid recovered from CCN-113 did not reveal any ARGs, only one full-length coding sequence for *fepE*. In pathogenic *E. coli* O157:H7, the FepE protein (also called Wzz_{FepE}) is responsible for very long O-antigen chain lengths in lipopolysaccharide (>80 repeat units) (24). In both *Salmonella typhimurium and Shigella flexneri,* Wzz_{fepE} and homologue cld_{pHS-2}, respectively, were found to be essential for serum resistance (25, 26). Importantly, FepE was found to be positively selected in uropathogenic *E. coli* (UPEC) clinical isolates (27). More investigation of CCN-113 is necessary to determine the contribution of the plasmid-borne *fepE* to its potential virulence and to conduct core genome multilocus sequence typing analysis. MIC values for all isolates with the antimicrobial agents tested must be determined to follow-up on MDR isolates, particularly CCN-109 and ELM-161.

Of notable interest is the mechanism of cefotaxime resistance in these isolates since resistance is often due to the production of extended-spectrum beta-lactamases (ESBLs). Certain ESBLs, such as bla_{CTX-M}, bla_{TEM} and bla_{SHV} are associated with clinical infections caused by Enterobacterales, with bla_{CTX-M} being the most prevalent type. The highly successful mobilization of ESBL genes on conjugative plasmids has led to the rapid spread of ESBL-producing Enterobacterales globally over the last ten years (28). ESBL-producing Enterobacterales are not limited to the clinic, but have been detected in livestock, wildlife, companion animals, wastewater, environmental waters, and healthy carriers. Considering the data on the presence of ESBLs in aquatic environments are lacking in North America, more research should be conducted to examine the prevalence of ESBL-producing Enterobacterales in surface waters (29). We recommend additional studies be completed on the prevalence of ESBLs within the Lake Fort Phantom Hill Reservoir watershed, particularly in areas impacted by agriculture, to further elucidate their impact on the region.

Acknowledgements

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Stepping into the Unknown: A Graduate's Guide to the Real World

Abigail Smith
B2S Life Sciences, Franklin, IN USA

Madison Wheeler
B2S Life Sciences, Franklin, IN USA

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1. Introductions

Author Smith: When you are young and the question of what you want to do when you grow up is posed, for some people, one answer comes to mind and it is as sure as the seasons change. As a former student convinced of my future, the path to realizing my actual interests has been winding. The year I turned 13, an under-calculated jump off a trampoline landed me a hospital bed with a broken ankle, a visit during which I questioned each nurse and doctor that was assigned to me about their job. It was then, sitting in the hospital, that I realized that helping to ease people's pain was exactly what I wanted to do. As my high school years passed, I began to focus more on the challenging science classes and academic experiences that would provide access to any program or degree I pursued in university. I can vividly remember conversations with my mom about different types of doctors and the commitments I would have to make to become one, and we both discovered the magical field of Neuroscience. Within a week of researching and visiting hospitals to bug the doctors about their professions, I was completely convinced neurosurgery was going to be my career. When it was time to apply to college, I chose a small school that had a reputable Neuroscience program and began eagerly moving through my classes, taking on extracurriculars and classes that medical schools value. After three semesters of slowly coming to the conclusion that a small school was not the best fit for me, I transferred to a larger school in hopes of finding research experience that would propel me further into my medical school dreams. It was not until I stumbled upon an opening in a graduate student's lab conducting research in the adaptability of demographics to robotic surgery tools that I allowed myself to consider anything other than my "dream" career. Being a part of a whole different branch of research opened my eyes to the possibility that I didn't have to go to medical school to have an interesting and fulfilling career. Desperate for this new discovery to not sway my goals, I fought the excitement and growing interest in the idea of pursuing research. It was not until senior year of college that I confronted the reality of my interest in research and made the decision that I would take some time off after graduation to work and gain research experience before applying to a PhD in Neuroscience research. This decision led me to become a scientist at B2S Life Sciences, learning the tactical skills that accompany the theoretical ideas I had been taught in school. The different knowledge and hands-on training I have learned in my industry job has given me confidence in my own ability to learn and perform at high levels, giving me the last push I needed to submit applications for graduate schools. As those application dates are steadily moving closer and I have given myself permission to explore careers in the research world, I have become increasingly ecstatic about the idea of being on the cutting edge of discovery of the brain and its incomprehensible abilities. I am grateful that I trusted myself to follow that small grain of curiosity, as it ultimately led me to confidently pursue my interests and find a career that engages and excites me.

Author Wheeler: My journey started in Franklin, Indiana in a high school chemistry class. At first, it seemed like my teacher was much too excited about chemistry than anyone in their right mind should be. However, as the school year wore on, I found his enthusiastic energy rubbing off on me and awakening my interest in the logical ways that science and math weaved together to explain how even the tiniest bits of the universe works. It just made sense to me.

Fast forward 2 years: I stepped onto Memorial Mall at Purdue University, marching excitedly to the first day of Biology I. Over the course of my four years at Purdue, I pursued courses that interested me, even if they were not part of the typical biology degree path. One of these was a graduate-level ecology lab typically reserved for environmental engineers and ecology majors, and it ended up being my favorite class by far. We went on field trips to Indiana Dunes to collect data on succession and walked from campus to the nearby wildlife preserve to investigate migration of milkweed seeds. Although I participated in various activities that were related to my major such as medical scribing, biology club, and career

seminars, my favorite parts of my education were the most unexpected: the out-of-the-box courses, mentorship programs, Purdue Old Masters, and exchange student club. The commonality among all of these favorite memories is that they allowed me to step outside of my comfort zone and learn about myself through the different lenses these unique experiences offered.

As I continue to pursue a path of lifelong learning, I keep challenging myself to actively contribute to both my career and community. I am a scientist at B2S Life Sciences, a biotherapeutic company that develops quantitative assays and creates custom reagents to aid research teams in the drug development process. Outside of work, I am a Court-Appointed Special Advocate (CASA) volunteer advocating for children that have experienced neglect or abuse. I identified a need for improving my programming and statistics skillset, so I am also enrolled in a Data Science Master's program. In my free time, I thrift, mend, and resell vintage clothes. In essence, even if I'm not sure what my next career step may be at a given moment, I'm learning more about myself and my interests each day with the confidence that this knowledge will guide me in the right direction.

2. Post-Grad Transition

Transitioning from student life to the workforce is one of the most complicated and tumultuous life adjustments that society leaves us exceptionally unequipped for. Over and over during this period, I expressed my worries to other adults, with their only attempt at reassurement being a simple and unencouraging "Welcome to the real world." College sends students off with a resume stuffed full of technical skills and awards; however, there is no blueprint for what comes next. Until this point, the paths of academics had been carefully laid out, starting on the first day of elementary school and ending with university graduation.

In order to start navigating the shift from school to work, it's important to first highlight the differences between the two. As every job is different, it can be hard to get a clear picture of the day-to-day until you actually begin working; nevertheless, there are a few common challenges detailed in the following sections about transitioning into the workforce that apply to most professions. In addition to outlining these obstacles, we have compiled our recommendations for overcoming them based on our personal experiences.

A. Becoming Adjusted to Working Life:

The time you spend for yourself is so crucial to a healthy work-life balance, and it tends to be one of the largest differences between school and a job. Most college experiences have structured class schedules, but you are completely free to fill the time between and after classes however you choose. The abundance of student activities on campuses make occupying free time an easy choice between interests and friends who share them. However, in the setting of a typical adult working life, clubs and organizations are less accessible. While finding groups that share your interests is not quite as easy as it is in undergraduate programs, the exciting thing about being a true adult is having complete control over your free time and deciding how to best use it without limitations of campus resources and with more of an access to personal funding.

Similarly, there are a few more barriers to making and spending time with friends after college. During undergraduate school, if you take courses and join clubs that interest you, then by default you are surrounded by others with similar interests. This dynamic makes for easy connection and camaraderie; you share the same struggles, interests, and schedules. After college, a general sense of loneliness emerged as my friends scattered across the country and everyone was suddenly living according to starkly different timelines. Some of my friends are mid-way through professional school, some of them are traveling the world, some of them are married with kids, and some of them are completely changing their career path. Although this may sound scary, in reality it is such an exciting time in which you are free to write your own story, learn who you are, and cultivate intentional and meaningful friendships.

Our Solution: Finding friendships as an adult can feel daunting, but the reality is that it just takes a new approach. You would be surprised how almost all post-graduate adults feel the exact same way! All it takes is a bit of intentionality and bravery to take the first step, like reaching out to an acquaintance on social media or talking to the other new person in your yoga class. Essentially, I found it the most helpful to put myself in environments where I was guaranteed to meet people with shared interests. For example, if you like to read, go to a library event, join a book club, or visit your local independent bookstore. If you like to workout, join a workout class, go every week, and get to know the other people in the class. Just like a muscle, it's hard to push yourself outside of your comfort zone at first, but after each repetition, it gets easier and easier until it's second nature.

B. Individualism versus Being a Team Player:

While directly opposite to the individualistic mindset in school, a productive and hireable employee needs to be a team player and have a sense of community effort in the work that they do. When working in a collaborative environment, it is important to understand that your work reflects your peers and supervisors and that doing your best boosts yourself and everyone around you. In school, oftentimes, the work you submit is solely yours and it is a direct correlation with the information you have learned, meaning your grades only reflect on you.

There is instant and constant gratification of your work in academia that acts as a direct gauge for your success. In a work environment, there is much less chance for recognition or gratification in your work. In most work environments and depending very much on the career, group recognition is much more common than individual appreciation, as the company is wholly dependent on the productivity of the team. For a recent graduate entering the workplace, it can be disheartening when your contributions are not acknowledged and instead lumped into the whole. Learning to change expectations of acknowledgment can be very difficult for some. Immediate, measurable feedback for every single assignment and project gives a sense of linearity and achievement to students; recognition in the workplace is more subtle and less personal, often only occurring for major milestones. However, many people find this type of recognition, like being selected to lead a major project or receiving encouragement from a boss, to be more meaningful and fulfilling as it is tied to real world impact and is a direct result of your deliberate efforts.

Our Solution: When beginning to work for a common goal among peers, as is the case for most careers, your small successes are often lost to the bigger picture. Because of this transition, it is very common to feel overlooked and undervalued unless your individual role is singled out and praised. I have found that celebrating your own victories and work can change your outlook on how valuable you are to your company. It is important to begin with clearly outlined personal goals and make a point to celebrate them once you achieve them. Many direct supervisors will congratulate and encourage you, but being your own cheerleader helps you ultimately happier with the effort you put forth.

C. Input versus Output:

Yet another key difference between the learning stage and career stage of life is the type of work expected. During school, a student is asked to assimilate loads of information and absorb the bigger pictures and connections between the concepts they are learning. In a working environment, one is expected to produce by means of information, data generation, reports, presentations, etc. For young adults coming from a phase of life that expects constant intake, it can be overwhelming to consistently yield productivity.

Our Solution: Continue to prioritize learning after graduation. If there are opportunities at work to learn something new, seize it and ask as many questions as you can. If there aren't, identify a need or gaps in knowledge and consider asking your boss for an opportunity to learn about it through a class or conference. It's also been

incredibly invigorating to explore my passions and hobbies after work hours. In the year since we graduated college, we attended events with our local bookstore where we learned to bedazzle books; we visited a yarn store downtown for a beginners knitting class; we started learning the native languages of countries that we are soon traveling to. In the transition from the mass intake of information to constant output and productivity, it's been vital to continue to foster opportunities for learning, both professionally and in our personal lives.

D. Communication:

In college, professional communication is somewhat limited and rarely formal; however, at work, efficient and effective communication is single-handedly the most valuable skill. Emails, meetings, newsletters, and direct messages are all frequently used throughout a workday to ensure work is being done correctly. However, during school, the most formal means of communication are occasional emails to professors or fellow students. At our job, if there is a simple miscommunication between the Principal Investigator (PI) and Scientist regarding experiment setup resulting in assay failure, it can cost thousands of dollars to re-run it, and our company is forced to absorb that cost. Additionally, being able to summarize data analysis from the day's experiment and relay it effectively to the PI is imperative to guarantee that everyone involved is on the same page. Similarly, if a Scientist does not accurately record and convey reagent requirements for a project, it can cause a weeks-long setback and completely disrupt timelines. It becomes absolutely vital to communicate every single detail, no matter how miniscule.

Our Solution: This shift in expectations presents quite the learning curve. Thus, it is important to be proactive and hone these skills throughout school as you prepare for the transition from academics to work. To improve your ability to present complex information in a straightforward manner, take that presentation course you've been avoiding because you hate talking in front of people. To learn to break down information quickly and effectively, try teaching or tutoring your favorite subject for practice. While you have access to your university's career center, work on a resume and cover letter template and ask for feedback or schedule a mock interview. You'll have a head start after graduation if you commit time and energy to refine your communication skills as a student. When you arrive at your first job, ask as many questions as you can come up with (especially the "dumb" ones), don't be afraid to repeatedly clarify expectations or plans, and be meticulous in both your written and verbal communication.

3. Navigating the Job Market

After graduation, embarking on the job search journey can be intimidating. Imposter syndrome, determined confidence, and enthusiasm of a first job can coalesce to create an uncomfortable mix of worry and excitement. Before starting a job search, it is important to clarify which features of a job are the most important to you. After giving an overview of our journeys through this process, we hope to provide some actionable tips to help prepare for navigating the job market.

Author Smith: As graduation grew nearer and the daunting task of applying to jobs and entering the workforce became more than a distant thought, I sought advice from those around me who had been through the process. A few of my older friends who had recently graduated from my Purdue School of Science program were more than happy to share their experiences while job hunting. Their advice was to be patient and to be diligent about following up. While applying is the first step of finding your spot in your chosen industry, remembering to check back in is often overlooked. It's important to show employers that you are eager and intentional throughout the entire process. While this seems like a small detail, companies look for committed workers who are willing to go out of their way to achieve their goals. When asking my father what he would look for in a hirable candidate for his company, he described that good communicators and team players are always moved to the front of the line as potential employees. Keeping these tips in mind as I began to put out applications and sit for interviews, I found that oftentimes I was complimented on these very aspects after meeting with several companies. I am a strong believer that an attractive personality, an eagerness to be involved and learn any way you can, and the ability to take direction and communicate well will be key to any person participating in the difficult endeavor that is job hunting.

Author Wheeler: Upon graduation, I began the tedious process of finding my first "big girl" job. Initially, I was overwhelmed by the sheer volume of job listings requiring a PhD or 10+ years of experience. It felt as though everything I had worked so hard for over the past 4 years had amounted to nothing. The strong sense of pride I felt during graduation was in sharp contrast to the lack of confidence I felt immediately upon embarking on my job search.

I sought advice from anyone that I thought could relate– peers grappling with similar emotions, family that constantly encouraged me, and older friends that had experienced the exact same challenges. In retrospect, I recognize that I was not seeking advice, but reassurance. I desperately wanted reassurance that I was on the right path, that I could truly offer value to an employer, and that the right job opportunity would eventually come. I realized that I needed a major shift in my mindset. Rather than simply viewing it as asking a company to give me a job, I began to see all of the ways my skillset would benefit an organization. In place of worrying whether my qualifications and experience was enough to impress an employer, I shifted my focus to how the role could help me grow and develop. In this time of stress and worry, it took a great deal of intentionality to elucidate exactly what I wanted from a job and transform both my mindset and my confidence.

With this newfound perspective, I was able to approach my job search in a new light, with a sense of purpose and self-assurance. Ultimately, this shift in mindset not only helped me find a job that aligned with my needs and goals but also set me on a path of continual professional and personal growth, with the confidence that the right opportunities would present themselves when the time was right.

The most important tip, albeit cliche, is to be patient. From submitting an application and awaiting a response to completing various rounds of interviews and discussing pay and benefits, the process of securing a job is often lengthy and sluggish. As many companies receive hundreds of applications each day, it may take a while to hear back at all. This is the case regardless of your

experience or qualifications; it is simply a requisite element of the process. Nonetheless, if there is a position you are especially excited about, it can be helpful to reach out to the hiring team again to check in and restate your interest in the position.

Before ever browsing Indeed or LinkedIn, it is imperative that you have a concrete list of your non-negotiables and needs that you are looking for in a job. You are interviewing the company just as they are interviewing you. Ensure that you know exactly what you need from an employer by investigating the aspects of a job that are the most important to you. For example, if you know you enjoy travel, make sure that the company's PTO policy will accommodate your needs. In addition to technical aspects such as salary, PTO, and benefits, it is important to seek work environments and teams that best suit your personality. Some companies greatly value teamwork, while others reward individual accomplishment. Some people thrive in competitive environments while others flourish in more collaborative workspaces. While interviewing, inquire about things like the work-life balance, the flexibility of work hours, the possibility of hybrid/remote work, relocation needs or opportunities, and any other aspect that would help you reach your individual life goals. While it is common to discuss salary and pay before committing to a job, many recent graduates are not taught that they have the ability to negotiate. It is perfectly acceptable to make a counter offer that would better meet your needs with regard to salary or PTO, for example. There will be times when an employer is unable to meet a request for negotiation outside the offer they have already made, at which time, you will need to examine if you are happy accepting the job as is, or if you feel that your experience and time would be better suited in another place. A crucial part of finding the best fitting job for you is to advocate for yourself and your needs. If you are fulfilled and find your company and work community rewarding, it will be much easier for you to be productive and in the mindset to learn and participate.

4. Final Reflections

Ultimately, the transition from college to the workforce is challenging, but it is also rewarding and fulfilling to see the culmination of years of work. Although we dove into various tips and solutions that may help in the job search process, there is no need to overcomplicate it. You've done the work and are deserving of the job you are looking for! In times of doubt and worry, it is vital to lean on your friends or peers who are experiencing the same lengthy process as you, find comfort and encouragement that most job-hunting journeys take time. We sincerely hope that our experience provides the helpful groundwork needed to succeed in transitioning from university to the workforce, as the advice included here are the exact tips we desperately wished we had during that period of change.

After securing your first job in the industry, prioritize a clear boundary between work and the rest of your life. The habits developed at the start of a new job lay the foundation for your entire tenure at that company. Fulfilment at work is equally influenced by your life outside of work as by your work itself. The freedom and opportunities that working life provides are unparalleled– take advantage of it! Embrace a well-rounded life, explore new hobbies, and be purposeful with the time you spend with those you care about.

A Universal Tongue?

Kaikeyi Paxton
Ball State University, Muncie, IN USA

Did you know that, as of 2023, there are more than 7,000 known spoken languages in the world (Eberhard et al., 2024)? Did you know that the population of the world is more than 7 billion, and increases every day? Did you know that out of that 7 billion, only about 1.5 billion speak English (Statistics and Data, 2024)? To most, those facts are common knowledge. To everyone, those facts are only a Google search away. However, here is a fact you may not know-English is widely considered to be the language of science. This means that nearly all journals with national or international impact accept submissions in English only (Malayil, 2024). This initially may make sense, but I would like to take some time to dig deeper and fully understand some of the history behind this, the implications of it, and how we as a community might work to create a more informed, inclusive environment.

The English language began in Europe with the Angles, Saxons, and Jutes, who ultimately settled in Britain. Over time, the language underwent modifications due to common differences in dialect and, ultimately, became what we know it to be today. However, beginning in the 16th century, English spread throughout the world through British colonialism (Encyclopaedia Britannica, 2021). The British established occupation of North America and the Indies and brought with them their language and culture. Deeming their ways "superior" over the culture of the native inhabitants of these regions, the British imposed these ways upon the native people of their colonies. Soon, English became the common tongue in most of North America, the Indies, and in some places in every time zone of the world (Crystal & Potter, 2025). This process happened repeatedly, until the British Empire reached its

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end in the 1960s (Encyclopaedia Britannica, 2020).

The 1960s were not all that long ago, and our world today still sees the effects of the spread of the British Empire. In most schools across the world, English is taught in addition to the native language. I am from India, and I can say that anyone who went to school there even briefly had to learn English while they were in school. Many Indians who stayed in school for a while are fluent in English, while those who did not get the privilege to finish school either do not speak English at all or are nowhere near fluent. This is the case in many non-Western countries around the world.

It is in this light that I would like to challenge the thought that English should be the common language. It is true that it is convenient to have some kind of common spoken language in case of travel, but does it make sense? When people come to America or go to London, they are generally expected to be at least semi-fluent in English. Non-English speakers are constantly required to accommodate English speakers, so why do we not accommodate them? However, let's look even deeper into this, and examine it from within the light of our community of scientists.

If a scientist wanted to publish their research in an accredited journal, it would need to be submitted in English. The only exception to this would be a regional journal, where only regionally relevant research could be published. For example, a botanist in Kashmir could find something in the agricultural system there that affects the growth of Kashmiri chilies, they could publish their research in Hindi in a regional journal that would only reach farmers in Kashmir. This makes sense.

However, what happens if that same botanist found a chemical in Kashmiri chilies that could be a component in a cure for a worldwide illness? What if that research was so important that pharmacologists and doctors everywhere needed to know that information to effectively treat their patients? What if that botanist didn't speak English? How would that research get published? Would we then expect them to learn English just to publish their paper? Why can only English-speaking scientists get their work out there?

Science and research are two fields in which diversity is vital to our mission. We cannot grow in knowledge and understand the world around us if we remain a uniform community. This is something we know and can understand well. In our constant fight for diversity and open access to information, language should not be a barrier we are unwilling to overcome. Restricting the language of information to one means that the amount of information out there is reduced and restricted.

In today's world, we have access to translators for almost every language. We certainly have access to translators for all the most common languages. If a paper is submitted in a different language, what's the harm in hiring translators for that paper? If journals are worried about mistranslation or misleading information being inserted, then a process similar to peer reviewing could be used, where multiple translators work on the translation and check each other's work.

In our beautifully diverse world, there is so much learning that can happen. As scientists, our mission and our purpose is to find as much information as possible about the world around us, and language should not be a barrier to sharing this information with each other. While a common tongue can be helpful and convenient in some situations, sticking exclusively to that convenience is not always the right way to do things, and is not at all conducive to our mission. A person does not need to speak English to have accurate information or to share their research. If research is accurate, tested, original, and includes all the aspects we look for in articles that are read or published, it shouldn't matter where the person is from or what language they speak.

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