Review article

EVOLUTION OF TETRACYCLINE RESISTANCE IN BACTERIA

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ABSTRACT

Tetracyclines used to be potent broad-spectrum antibiotics, but the rampant use of these drugs in the clinic and agriculture has led to widespread resistance among bacterial populations. This systematic review integrates what is known about the molecular basis, ecological reservoirs and evolutionary dynamics regarding Tetracycline resistance as cited within peer reviewed literature from 1929 through 2025 and attempts to identify critical areas of knowledge to understand how these factors interact to produce differential dissemination patterns among bacterial taxa, most notably where enzymatic inactivation mechanisms such as *tet*(X) are concerned. Recent studies indicate that tetracycline resistance has evolved through multiple mechanisms including efflux pumps, ribosomal protection proteins, and enzymatic inactivation in diverse bacterial species. This review outlines the evolutionary foundation of tetracycline resistance by emphasizing the primary molecular mechanisms that comprise efflux pumps, ribosomal protection proteins and enzymatic inactivation. Efflux systems, programmed by heterogeneous *tet* genes, are prevalent in Gram-negative bacteria, while ribosomal protection proteins occur more frequently in Gram-positive strains. Enzymatic inactivation (EI), although less frequent, is gaining notoriety due to the increase in *tet*(X) variants. The rapid expansion over the last few years of *tet*(X) variants resulting in EI means the additional loss of efficacy of last resort tetracycline derivatives and evidence that EI is becoming an increasingly important mechanism from a clinical standpoint. The review also addresses the involvement of horizontal gene transfer via plasmids, transposons, and integrative elements in spreading resistance between clinical, veterinary, and environmental environments. Ecological surveys show that soil, water, animal, and plant-associated microbiomes are primary reservoirs of *tet* genes, which guarantee their persistence even without direct antibiotic pressure. Genomic and epidemiologic comparisons suggest that Gram-Negative Bacteria have a significantly higher rate of diversification and dissemination for Tetracycline Resistance Determinants than Gram-positives, highlighting their supremacy in resistance propagation. This information underscores the necessity for Coordinated One Health Initiatives that incorporate Antibiotic Stewardship in the Agricultural sector, Surveillance of Environmental Resistant Reservoirs and Development of Inhibitors against emerging Enzymatic Mechanisms such as *tet*(X).

Keywords: Tetracycline, efflux pumps, ribosomal protection, enzymatic inactivation, horizontal gene transfer, resistance evolution, ecology

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INTRODUCTION

Tetracyclines belong to the earliest and most commonly used classes of antibiotics. These have been used in popular human applications, agricultural and veterinary medicine, and have demonstrated broad activity against numerous bacteria (Chopra and Roberts, 2001; Nelson and Levy, 2011). While there has been a decrease in tetra- clinical use in regions around the world, the use of tetracycline continues to be high globally due largely to the continued high levels of use in livestock (Animal Health Institute, 2012) and across agricultural systems (Van Boeckel *et al*., 2015).

In general, the resistance seen with tetracycline is somewhat distinct as far as the other forms of antimicrobial resistance are concerned; in some ways, the history of tetracycline resistance goes back further than most other types of antimicrobial resistance. As such, it presents many characteristics of a successful and evolved form of bacterial resistance, and the mechanism of development and persistence of these phenotypes have continued to be well-distributed throughout the agriculture, clinical, and environmental ecosystems around the globe (D'Costa *et al*., 2011; Forsberg *et al*., 2012).

Current research predominantly investigates the mechanisms underlying tetracycline resistance as well as their effects in the clinic primarily at the molecular level. However, there is still a lack of understanding of how ecological and evolutionary processes allow for the persistence and spread of resistance genes over long periods of time. Evidence is accumulating to show that resistance mechanisms for tetracyclines have developed from a combination of selective pressures experienced in agricultural systems and environmental reservoirs as well as from the transfer of genes horizontally across the evolutionary timescale, not just from using antibiotics in medicine (Forsberg *et al*., 2012; Martínez, 2009; Roberts, 2019). This article reports the history of the development of tetracycline drugs, the mechanisms of tetracycline resistance, ecological reservoirs and horizontal gene transfer routes to develop a theoretical framework for the development of tetracycline resistance and establish major gaps in the current knowledge and areas where efforts for further studies are urgently needed.

A systematic literature search was carried out in order to identify pertinent studies regarding tetracycline resistance in bacteria through the use of reputable scientific databases such as Web of Science (WoS), Scopus, PubMed, and Google Scholar. The search employed a combination of the following key words in each database: “tetracycline resistance,” “*tet* genes,” “evolution of antibiotic resistance,” “efflux pumps,” “ribosomal protection proteins,” and “enzymatic inactivation.” Studies were further restricted to the time period between 1929 to 2025 and were limited to peer-reviewed articles. About 312 articles were retrieved from a combination of all the databases, where about 168 were retrieved through WoS and Scopus, while the rest were obtained through other databases. Articles were then selected after screening for duplicates, title, and abstract relevance according to certain criteria. These criteria include studies related to the mechanism, genetics, and evolution of tetracycline resistance in bacteria as well as articles with English full text available. Studies that were not related to tetracycline resistance, duplicate articles, non-peer-reviewed articles, and those with insufficient information in terms of methodology were eliminated. Finally, 113 articles were included in this study.

Discovery of antibiotics; a historical perspective: The discovery of antibiotics marked a major breakthrough in the control of infectious diseases and has saved millions of humans lives worldwide. Antibiotics are biologically active compounds produced by microorganisms that inhibit the growth of or kill other microbes. Despite their remarkable success, the effectiveness of antibiotics was soon compromised by the emergence of resistance among target pathogens. Penicillin, the first antibiotic, was discovered in 1928 (Fleming, 1929) and was later introduced into clinical use for the treatment of bacterial infections, including ophthalmia neonatorum. However, resistance to penicillin in *Staphylococcus aureus* was reported shortly thereafter (Rammelkamp and Maxon, 1942), and increasing cases of penicillin resistance were documented in subsequent years. Similarly, streptomycin, a broad-spectrum antibiotic discovered by Waksman *et al*. (1944), initially showed high efficacy against a wide range of Gram-positive and Gram-negative bacteria. Nonetheless, resistance to streptomycin rapidly emerged following its widespread clinical application (Crofton and Mitchison, 1948; Lederberg, 1951).

The limitations of early antibiotics led to the development of tetracyclines, with chlortetracycline (Aureomycin) introduced in 1948 by Lederle Laboratories as a broad-spectrum alternative (Nelson and Levy, 2011). Tetracyclines demonstrated enhanced activity against diverse bacterial pathogens and were rapidly adopted in clinical and agricultural settings. However, similar to earlier antibiotics, resistance to tetracyclines soon emerged, highlighting the persistent evolutionary capacity of bacteria to adapt under antibiotic selective pressure.

Several other antibiotics were discovered during this period, including cycloheximide (Whiffen *et al*., 1946; Whiffen, 1950), kanamycin (Umezawa *et al*., 1957), kasugamycin (Umezawa *et al*., 1965), gentamicin (Weinstein, 1963), oxalinic acid (Turner *et al*., 1967), and validamycin (Ogawa *et al*., 1983). The use of antibiotics like streptomycin and kasugamycin has proven to be effective in reducing the infection levels in plants (Adaskaveg *et al*., 2011; Moltmann, 1998; Lee *et al*., 2018). While these discoveries expanded the antimicrobial arsenal, the early and rapid emergence of resistance to tetracyclines positioned them as a key model for studying the mechanisms, evolution, and ecological persistence of antibiotic resistance.

Problem Statement and Scope: Tetracycline resistance is a long-standing and critical instance of antimicrobial-resistant development. Unlike the close temporal link between resistance mechanisms in newly developed classes of antibiotics and their respective antibiotics, the tetracycline resistance gene pool has existed for a longer period of time, has a much greater diversity of resistance mechanisms, and is continuously present in the environment and in environmental

reservoirs, either due to direct clinical selection or due to their inherent biological characteristics (D'Costa *et al*., 2011; Forsberg *et al*., 2015). The nature of these characteristics allows for long-term persistence and widespread dissemination.

While several studies have explored various aspects of tetracycline resistance mechanisms, no syntheses have been created that provide an overall understanding of the ecological and evolutionary determinants of tetracycline resistance. There are still many uncertainties regarding how environmental ecosystems maintain tetracycline resistance genes over long periods of time, which types of horizontal gene transfer mechanisms predominate under various types of selective pressures, and why the mechanisms of resistance in Gram-negative bacteria show greater diversity compared to those seen in Gram-positive bacteria (Roberts, 2019).

This review provides a comprehensive evaluation of tetracycline resistance from the molecular, ecological and evolutionary perspectives of antibiotic resistance and an opportunity to evaluate the dynamics of tetracycline resistance in interconnected ecosystems and to provide guidance on potential avenues for future research directions and strategies for the reduction of tetracycline resistance.

Use of antibiotics in agriculture: Antibiotics have been widely applied in the field of plant pathology, especially in the control of bacteria-caused infections like fire blight and rice blast (Zaumeyer, 1958; Ishiyama *et al*., 1965; Steiner, 2000). Because of tetracyclines' significant use in agriculture, this use has dramatically influenced the global ecology of tetracycline resistance; the primary factor motivating use was tetracyclines' broad-spectrum activity, low cost, and a lack of alternatives available to manage disease in intensive agricultural systems (Chopra and Roberts, 2001; McManus *et al*., 2002). Agricultural use also exerts a continuous selective pressure on environmental, animal, and plant-related microbial communities, thus enforcing the continued success and dissemination of tetracycline-resistant genes (Heuer *et al*., 2011; Forsberg *et al*., 2015). Tetracycline use patterns are highly variable depending on region and are more representative of the differences in regulatory policies, animal and plant disease pressure, and economic priorities. In some countries where tetracycline use has been restricted due to concerns about resistance, there are other countries where tetracyclines are actively permitted and/or used to address a pressing need for animal or plant health (Van Boeckel *et al*., 2015; Stockwell, 2012). Antibiotics are still used in agriculture to protect crops; however, much attention is paid to the development of sustainable alternatives to prevent resistance (Archer *et al*., 2020; Rauzan and Lorsbach, 2021).

Plant Protection: In plant agriculture, tetracyclines such as oxytetracycline and chlortetracycline have been used for many years to control bacterial diseases in crops for which there is no other effective chemical or durable disease resistance. The use of tetracyclines as a chemical control of plant pathogens began in the late 1940s when tetracyclines were shown to be effective in controlling diseases (Anderson and Gottlieb, 1952; Leben and Keitt, 1954). Later evaluations in the 1950s demonstrated that Oxytetracycline had a strong antifungal effect and enabled its use on a larger-scale through some crop-pathogen combinations (Koaze *et al*., 1956).

Of all tetracyclines, Oxytetracycline is by far the most widely used in agriculture due to both its efficacy and the lesser amounts of phytotoxicity compared to many of the other antibacterial compounds available for use (Valarmathi, 2020). Regulatory measures for using Oxytetracycline vary dramatically across different regions of the world. An example of this would be in the case of Huanglongbing (citrus greening disease), which has been given Emergency Exemptions in the US to allow the usage of Oxytetracycline in the fight against this economically devastating disease due to no viable alternative available (Hu *et al*, 2016; Hu *et al*, 2018; Dall, 2020). In contrast, many European nations have implemented more stringent regulations on the use of antibiotics for plant agriculture due to their concern over the evolution of bacterial resistance and contamination of the environment (Stockwell, 2012; Reininger *et al*., 2017).

Veterinary and Livestock Applications: Globally, the largest quantity of tetracyclines used for non-terrestrial purposes is within the Veterinary and Livestock industries. Tetracyclines are primarily used for medicinal, metaphyllactic and preventive application to many intensive animal production systems (Sarmah *et al*., 2006; Van Boeckel *et al*., 2015). Consumption patterns vary considerably by geographical area, with far greater consumption recorded for developing (low and middle income) countries, where governmental regulatory control may not be applied. (Van Boeckel *et al*., 2015). The routine administration of tetracycline has created a continuous selective pressure on animal gut microflora which favors the enrichment of tetracycline resistance genes within these bacterial populations. These genes have been exported into the surrounding ecosystem via the use of manure, the movement of contaminated water, and through aerosolization of contaminated particles, thereby allowing them to persist in local agricultural soils and water (Heuer *et al*., 2011; Forsberg *et al*., 2012). The contribution of tetracycline residues and the resistance determinants that occur in conjunction with them to the environment is greater than that which occurs via tetracyclines in conventional plant agriculture (Sarmah *et al*., 2006).

Implications for Human Medicine: The use of tetracycline in human medicine is infrequent as a first-line antibiotic in many high-income countries, however, the emergence of resistance as a result of the exposure of humans via treatment of agriculture with tetracycline implications should not be understated. Agricultural use of tetracycline creates long-term

reservoirs of resistance genes within an environment that can move and eventually infect humans due to horizontal gene transfer (Forsberg *et al*., 2015; Forsberg *et al*., 2012). The indirect transmission through the agricultural microflora and the vertical transmission of tetracycline resistance genes into bacteria that are part of our ecosystems create significant challenges to the ability to control resistance solely through clinical stewardship efforts (Roberts, 2019).

A major obstacle to improving human health is the disconnect between agricultural antibiotic policy and human health objectives. Tetracycline-type antibiotics have been increasingly regulated for clinical use; however, the agricultural use of tetracyclines, particularly for livestock production, as well as in the emergency management of plant diseases, still introduces selective pressure at ecosystem levels of tetracycline resistance (Van Boeckel *et al*., 2015; Heuer *et al*., 2011). Therefore, there is a pressing need for integrated regulatory strategies for agriculture, veterinary medicine, and human health, which recognize that regulatory efforts within each sector will affect each other (McManus *et al*., 2002).

Synthesis and Critical Perspective: Overall, the agricultural use of tetracyclines highlights both the economic necessity and the uncertainty due to inconsistent regulations and the long-term risk to public health that results from the use of tetracyclines. While the plant protection applications are intermittent and disease-driven, the veterinary use is ongoing and on a massive scale, and therefore produce separate but linked mechanisms of resistance development (Stockwell, 2012; Sarmah *et al*., 2006). The lack of standardized regulatory frameworks among geographic regions will likely accelerate the spread of resistance across geography and highlights the need for coordinated international policies that promote agricultural productivity while reducing the emergence of antimicrobial-resistant organisms (Van Boeckel *et al*.; 2015; Forsberg *et al*., 2015).

The emergence of resistance against tetracyclines; mechanism and distribution of the resistance determinants: It is interesting to think, how the bacteria evolved the resistance against these varieties of antibiotic compounds. The elegant scientific findings revealed that antibiotic resistance evolves in bacteria commonly through (i) by the acquisition of the "Antibiotic Resistance Gene (ARG)" from environment conferring the resistance by efflux or inactivation of antibiotics, (ii) mutating the target site protein, (iii) through producing the new target protein having insensitivity to the antibiotics (Blair *et al*., 2015; Munita and Arias, 2016; Nguyen *et al*., 2014). Which mechanism is adapted, it depends upon the antibiotics' mode of action (Sundin and Wang, 2018). Tetracyclines were isolated firstly from *Streptomyces aureofaciens* in the 1940s are broad-spectrum antibiotics effective against the gram-positive, gram-negative, spirochetes, protozoans' parasites and mollicutes like phytoplasmas.

The tetracycline inhibits the protein synthesis by preventing the association of the aminoacyl tRNA with the ribosome (Chopra and Roberts, 2001; Hash *et al*., 1964; Kester *et al*., 2011). So, tetracycline must have to traverse into the membrane(s) to interact with the target site of both gram-positive and gram-negative bacteria. Therefore, the uptake of the tetracycline and binding mechanism to ribosome are the point of interest for consideration. The tetracycline traverses the outer membrane of gram-negative bacteria as a Mg^{+2}-tetracycline complex through OmpF and OmpC porin channels (Chopra *et al*., 1992; Thaker *et al*., 2010). Due to Donnan's potential, the Mg^{+2}-tetracycline is attracted across the membrane and accumulates in the periplasm (Bahrami *et al*., 2012). The complex dissociates into uncharged tetracycline and diffuse lipid bilayer regions of cytoplasmic membranes. A similar mechanism is adopted in gram-positive bacteria. The traversing of tetracycline across the membranes is an energy-dependent process controlled by the changing pH-dependent proton motive force (Schnappinger and Hillen, 1996). The tetracycline in the cytoplasm is chelated because the metal ion concentration and the pH are much higher in the inner sider as compared to the outer side of the membrane. The association of tetracycline with the ribosome is reversible, provides the best explanation of bacteriostatic property(Chopra and Roberts, 2001; Grossman, 2016).

Soon after the clinical application of tetracycline worldwide, the resistance evolved and was reported in mid-1950s (Akiba *et al*., 1960). Till now, there are three mechanisms of resistance against tetracycline antibiotics have been discovered. The microbes confer resistance against the tetracyclines through i) antibiotic efflux (Thaker *et al*., 2010), ii) ribosomal protection (Chopra and Roberts, 2001), and (iii) enzymatic inactivation (Diaz-Torres *et al*., 2003; Forsberg *et al*., 2015). Efflux pumps, ribosomal protection proteins, and enzymatic deactivation work together to allow bacteria to survive exposure to tetracyclines. Many Gram-negative species show a prevalence of efflux-type resistance; this is due to their ecological specialization and evolutionary adaptation. Gram-positive species, on the other hand, frequently express ribosome-protecting mechanisms (Chopra and Roberts, 2001; Roberts, 2019).

(i) **Efflux mediated resistance:** Efflux-mediated resistance is the most common tetracycline resistance mechanism and functions by actively exporting the antibiotic out of the bacterial cell before it can reach its ribosomal target. To resist the tetracyclines, the microbes have developed the mechanism to export the antibiotic from the cell. For this, the microbes have evolved *tet* efflux gene encoding membrane-associated proteins that export the tetracycline from the cell and prevent it from reaching its target site (ribosome) (Grossman, 2016; Nguyen *et al*., 2014). Till now, 36 efflux genes

have been discovered in both gram-positive and gram-negative bacteria (http://faculty.washington.edu/marilynr/). Out of 36 efflux genes, 15 genes [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(Y), *tet*(30), *tet*(31), *tet*(57), *tet*(35), *tet*(41), *tet*(64)] have been only found in gram-negative species and 14 efflux genes [*tet*(V), *tet*(Z), *tet*(33), *tet*(38), *tet*(45), *tet*(58), *tet*(63), *tet*A(P), *tet*(40), *otr*(B), *otr*(C), tcr3, *tet*(43), *tet*AB(46)] are strict to gram-positive bacteria. The *tet*(39), *tet*(K), *tet*(L) and *tet*(42) genes are found both in gram-positive and gram-negative. The three tetracycline resistance genes i.e. *tet*(59), *tet*(62) and *tet*AB(60) have not been isolated from bacteria.

Among 36 efflux genes, *tet*(B) is a highly distributed gene, found in 35 gram-negative genera (Supplementary Table S1). Except for *tet*(B) gene, all other encoding proteins confer the resistance against the tetracyclines but not to the minocycline. The *tet*(B) coding protein confers resistance both for tetracyclines and minocycline (Chopra *et al*., 1992; Hatsu *et al*., 1992). The *tet*(L) have been identified from 25 gram-positive and 23 gram-negative bacteria. Each efflux gene codes approximately 46 -kDa protein and based on the amino acids sequence these are divided into six groups (McMurry and Levy, 2000). The proteins in each group share similarities from 41-78% of amino acids. The proteins of Group-1 have 12 predicted transmembrane a-helices having long cytoplasmic loop (Chopra and Roberts, 2001). The *tet*(Z) is the only one in this group found in the Gram-positive bacteria where the regulation is controlled by a repressor protein (Tauch *et al*., 2000). The efflux proteins reside in the lipid bilayer and have a hydrophilic loop protrude into the cytoplasmic region and exchange a proton for tetracycline-Mg^{+2} complex (Van Duijkeren *et al*., 2018).

The efflux genes of the Gram-negative bacteria are commonly regularized through the specific *tet* repressor gene. In the presence of tetracycline, the tetracycline-Mg^{+2} complex binds with *tet* repressor protein and the transcription starts while in the absence, the transcriptional process blocks (Hillen and Berens, 1994; Levy, 1984). However, this transcriptional regulation mechanism of efflux genes is not adopted in Gram-positive, the process is regularized the translational attenuation process (Chopra and Roberts, 2001; Van Duijkeren *et al*., 2018). The plasmidic *tet*(L) upstream region is translated into leader peptide with stem-loop mRNA structures and two binding sites, RBS-1 and RBS-2. When there is no tetracycline available in the cell, the ribosome binds the RBS-1, hence, a short leader is translated which ends before the RBS-2. In the presence of tetracycline, a second mRNA loops forms and the efflux protein is translated (Schwarz *et al*., 1992). The induction of chromosomal *tet*(L) gene is not like the plasmidic *tet*(L) where no unmasking of RBS involves (Stasinopoulos *et al*., 1998).

Likewise, novel efflux pump inhibitors have re-established tetracycline effectiveness against *E. coli* strains that are multidrug resistant, with exciting therapeutic potential (Pazra *et al*., 2023).

(ii) **Ribosomal protection proteins:** So far, 13 [*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32), *tet*(Q), *tet*(T), *tet*(36), *tet*(61), *otr*(A), *tet*B(P), *tet*, *tet*(44)] cytoplasmic ribosomal protection genes conferring resistance against tetracyclines have been discovered in bacterial species (http://faculty.washington.edu/marilynr/). Out of 13 ribosomal protection genes, only 5 genes [*tet*(32), *tet*(61), *otr*(A), *tet*B(P) and *tet*] have been only identified in gram-positive bacterial. No ribosomal protection gene has been identified which is strict to the gram-negative bacteria. Eight genes [*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Q), *tet*(T), *tet*(36) and *tet*(44)] have been identified both in gram-negative and gram-positive bacteria. Among 13 ribosomal protection genes, *tet*(M) is the highly distributed ribosomal protection gene found in 40 gram-positive and 42 gram-negative genera of bacteria. The *tet*(O) is the second most prevalent gene identified from 19 gram-negative and 19 gram-positive bacterial genera (Supplementary Table S1).

Comparing to the efflux proteins induced resistance, the ribosomal protection proteins conferred resistance is of the wide spectrum and of higher level. The ribosomal protection proteins protect the ribosomes from tetracycline, minocycline and doxycycline (Chopra and Roberts, 2001). The sequence analysis revealed that ribosomal protection proteins have the homology with ribosomal elongation factor EF-Tu and EF-G and are classified into supper family of GTPase (Connell *et al*., 2003; Sanchez-Pescador *et al*., 1988; Taylor and Chau, 1996; Woolley and Clark, 1989). The ribosomal protection proteins disrupt the tetracycline binding site resulting in the release of the tetracycline from the ribosome. Hence, the ribosome reinstates its initial state and starts normal functioning (Van Duijkeren *et al*., 2018). Among ribosomal protection genes, *tet*(M) and *tet*(O) have been most characterized. The *tet*(M) proteins coded by *tet*(M) gene releases tetracycline using the energy released from GTP hydrolysis allows the aminoacyl tRNA to bind with the acceptor site of the ribosome in the presence of tetracycline (Chopra and Roberts, 2001).

Indeed, yet only *tet*(M) and *tet*(O) have been experimentally examined, however, it is assumed that the other ribosomal protection proteins [*tet*(S), *tet*(T), *tet*(Q), *tet*B(P), *tet*(W), and *otr*(A)] confer the resistance in a similar mechanism because of the similarities in the amino acids. Based on amino acids sequence similarities, the ribosomal protection proteins have been classified into three groups. The 1st group includes *tet*(M), *tet*(O), *tet*(S) and *tet*(W) and 2nd group have *otr*(A) and *tet*B(P) and the 3rd one has *tet*(Q) and *tet*(M) ribosomal protection proteins (Connell *et al*., 2003; Roberts, 2005a). The induction of ribosomal protecting genes is regulated through translational attenuation similarly as has been described for efflux genes.

Ribosome-protecting proteins such as *tet*(M) and *tet*(O) occur at a wide range of frequencies in clinical, agricultural, and environmental environments, with mobile genetic elements (MGEs) allowing for horizontal transfer of these genes (Roberts, 2005a). These ribosomal protection genes have historically been found in Gram-positive species but have recently been identified within Gram-negative hosts, suggesting an increase in the ecological range of ribosomal protection genes and their evolution (Forsberg *et al*., 2015). However, evolutionary influences on long-term ribosomal protection gene persistence and fitness costs are still not well understood.

Ribosomal protection proteins such as *tet*(M) and *tet*(O) dominate in Gram-positive bacteria but are increasingly detected in Gram-negative hosts, reflecting expanding host range mediated by conjugative elements. Despite extensive documentation, comparative data on fitness costs and ecosystem-specific dissemination remain limited, representing a critical gap in understanding tetracycline resistance evolution (Roberts, 2019).

(iii) **Enzymatic inactivation:** Initially, it was believed that effluxing and ribosomal protection are the two mechanisms that bacteria have evolved to resist against the tetracyclines. Recently, another mechanism "enzymatic inactivation" has been observed in many genera of bacteria including gram-positive and gram-negative. Till now, 13 enzymatic inactivation [*tet*(X), *tet*(37), *tet*(34), *tet*(47), *tet*(48), *tet*(49), *tet*(50), *tet*(51), *tet*(52), *tet*(53), *tet*(54), *tet*(55) and *tet*(56)] genes have been identified to confer resistance against tetracycline in prokaryotes (http://faculty.washington.edu/marilynr/). The *tet*(X) has been isolated from 25-gram negative genera and one gram-positive genera (Supplementary Table S1). The gene was the first described that modifies or inactivates tetracycline in the presence of oxygen and was firstly isolated from a strict anaerobe "*Bacteroides*" (Roberts, 2005b). The gene encodes for NADP-requiring oxidoreductase which confers resistance in a bacterial cell by inactivating the tetracycline molecule (Van Duijkeren *et al*., 2018).

A weak resistance has been observed in bacteria against the tigecycline, however, the resistance is enhanced by the substitution of four amino acids. Hence, it may be assumed a serious threat in near future (Linkevicius *et al*., 2016). The second gene *tet*(37) has not been found in bacteria yet and was identified in humans' oral cavity (Diaz-Torres *et al*., 2003). The *tet*(34) is similar to xanthine-guanine phosphoribosyl transferase gene and is found in four gram-negative genera (Nonaka and Suzuki, 2002). The *tet*(56) has been recently described and isolated from one gram-negative genus. The *tet*(47), *tet*(48), *tet*(49), *tet*(50), *tet*(51), *tet*(52), *tet*(53), *tet*(54) and *tet*(55) has been isolated from agricultural soils and grasslands (Forsberg *et al*., 2015). The *tet* family confers enzymatic degradation of tetracyclines and has gained renewed attention due to its potential to compromise last-resort derivatives, highlighting its evolutionary significance beyond classical tetracycline resistance (Pei *et al*., 2025).

Enzymatic deactivation has become an important mechanism of resistance to tetracycline and is gaining prominence in the clinical world. The flavin-dependent monooxygenase *tet*(X) confers resistance to a number of tetracycline derivatives, including tigecycline, which raises concerns regarding the efficacy of last-line therapies (Yang *et al*., 2019). Several *tet* variants exist in human-associated microbiomes, but their evolutionary origins and roles in resistance dissemination are still under investigation (Roberts, 2019).

Evolution of tetracycline resistance in bacteria; a quest for ecological fitness: To persist in an ecosystem, a specie must rapidly adapt the environmental biotic and a-biotic changes. Failing to do the prompt response, a specie may lose its existence. The tetracycline was isolated from *Streptomyces aureofaciens* and was clinically applied in the late 1940s commercially (Nelson and Levy, 2011). Since the time of commercial availability and targeting the microbes, striking questions come in our mind that i) how the microbes evolved themselves to resist against this chemical for their survival; ii) how long ago the resistance evolved in bacteria against the antibiotics; iii) were these resistant genes present before the discovery of antibiotics; iv) what is the rate of evolution after the discovery of tetracyclines, and v) how these genes diverged to each other and what are the mechanisms and vehicles for their distribution in different bacterial genera.

Natural selection continuously exerts pressure on microbes over time to modify the genetic makeup for survival in an ecosystem. The emergence of the *tet* resistance genes is prehistoric and co-evolved billions of years ago with the antibiotics producing bacteria. For example, several *tet* resistance genes i.e., *otr*A, *otr*B, and *otr*C have been discovered in *Streptomyces remosus* (tetracycline producer) and are hypothesized to have the protection mechanism against the antibiotic produced by the bacteria (McMurry and Levy, 1998; Ohnuki *et al*., 1985). The incidence of ARGs has been observed in metagenome samples of 30,000 (D'Costa *et al*., 2011) and 5,000 (Petrova *et al*., 2009) years old permafrost. Furthermore, these genes have also been found in the gut microbiome of 900 years old mummy (Santiago-Rodriguez *et al*., 2015). The tetracycline resistance genes have been found in a very diverse environment like in human gut (Scott *et al*., 2000; Van Schaik, 2015), soils (Kang *et al*., 2018; Nogrado *et al*., 2021; Wang *et al*., 2017), air microflora (Li *et al*., 2020; Song *et al*., 2020), freshwaters (Ekundayo and Okoh, 2020), agriculture farms (Jauregi *et al*., 2021; Lu *et al*., 2020), animals (Di Francesco *et al*., 2021; Schwarz *et al*., 1998), and in feces of birds (Islam *et al*., 2021; Lee *et al*., 2021).

Since the discovery of antibiotics, millions of tons of tetracyclines have been deployed on humans and agriculture (McManus and Stockwell, 2000) and significantly contributed to the genomic drift and expanded the divergence and incidence of resistance genes in inter and intra ecosystems. An interesting study was conducted to measure the ARGs frequency by collecting the soil samples from 1940 to 2008. The results revealed that 15 folds more tetracycline ARGs were observed in 2008 as compared to 1970 (Knapp *et al*., 2010). Another significant study was conducted by collecting the Enterobacteriaceae from 1917 to 1954. Only 24% had the conjugative plasmids and among them, 2% were resistant to tetracycline. Interestingly, all the tetracycline resistant isolates were from *Proteus* genera and no isolate from *Escherichia, Klebsiella, Shigella* and *Salmonella* were resistant. However, in the mid-1950s, tetracycline resistance was observed in *Escherichia coli* and *Shigella* (Hughes and Datta, 1983). The soil resistome consists of various genetic elements, while the phylogenetics of bacteria greatly affects the prevalence of the resistance genes (Forsberg *et al*., 2014).

Cousin Jr *et al*. (2003) screened 76 isolates of *Neisseria gonorrhoeae* collected between 1940 to 1987 and seven *Neisseria meningitidis* taken between 1963 to 1987 for the presence of *mef*(A), *erm*(B), *erm*(C), and *erm*(F) genes. The *mef*(A), *erm*(B) and *erm*(F) were identified from *Neisseria gonorrhoeae* in 1955 and *erm*(C) was identified in 1963. In case of *Neisseria meningitidis*, only *mef*(A) and *erm*(F) genes were found in 1963, however, all four genes were identified in later isolates of both species. So, these studies indicate that the ARGs incidence has exponentially expanded with time by the application of antibiotics on humans and agriculture.

From these studies, another fact to ponder is that is there any direct relation between tetracycline application on the emergence of resistance in the targeted pathogen? The recent studies indicated that the continuous application of tetracycline didn't emerge the resistance in *Erwinia amylovora* and *Xanthomonas arboricola* pv. *pruni* isolates recovered from apple and peach orchards (Sundin and Wang, 2018). In another study examining the effect of tetracycline and gentamycin application on the coriander plants grown in the field, Rodríguez-Sánchez *et al*. (2008) found no bacterial isolate having resistance to these two antibiotics.

Bacteria adapt the resistance against the antibiotics i) through a chromosomal mutation that may alter the protein properties or prevent its expression (Roberts, 2019); or (ii) through acquiring the ARGs from the environment by the Horizontal Gene Transfer (HGT) (Li *et al*., 2019; Lopatkin *et al*., 2016). The HGT is a widely adopted mechanism of acquiring the ARGs in bacteria. The ARGs may be acquired (i) through transformation and integrated into the host DNA creating the mosaic genes, and (ii) through the acquisition of ARGs on mobile elements (Transposons, plasmids and integrons) mostly by conjugation but maybe by the transformation (Roberts, 2019). Some bacteria have receptors that recognize the specific DNA and integrate it into its genome where it is expressed. Furthermore, some bacterial species acquire the ARGs by the transduction where the foreign DNA reaches the targeted bacteria through bacteriophages (Di Luca *et al*., 2010).

Mostly, the bacteria attain resistance against the antibiotics by adopting the new resistance genes associated with the mobile elements. In the HGT mode of acquisition of ARGs, conjugation is the most common way the bacteria use to attain and disseminate the resistance against the tetracyclines. The conjugative gene exchange has the main role in the tetracycline resistance evolution and rapid spread of the genes within the ecosystem or between the different ecosystems. Mostly the *tet* genes are associated with nonconjugative, conjugative and mobile plasmids, transposons and conjugative transposons (CTns) but not to the integrons. The mobile elements are responsible for the adaptability and dissemination of a particular *tet* gene(s) (Roberts, 2019).

The first tetracycline-resistant bacterium carrying a conjugative plasmid with multiple resistance genes was identified from Japan. The plasmid was firstly described in the 1950s as circular DNA and replicates independently from the chromosome (Watanabe, 1963). It may have integrons or transposons carrying many different ARGs genes and moves from one bacterium to the other as one unit (Grundmann *et al*., 2006). The *tet* plasmids vary in size. The *tet*(K) associated with pT181 small plasmid (4.45 kb) (Roberts, 2017) has been identified in 4 gram-negative and 14 gram-positive genera (Supplementary Table S1). Many large *tet* plasmids are conjugative and have the ability of mobilization (Bennett, 2008). However, the *tet*(E) carrying plasmids have not the tendency mobilization (Roberts, 2019).

Despite this, *tet*(E) has been recognized in 13 gram-negative genera. How the *tet*(E) has spread in 13 genera is still a mystery. The CTns have the ability of self-transmissible integration (Salyers *et al*., 2004) and lack the incompatibility exclusion systems (Hurst, 2017) thus having fewer restrictions of moving from one bacterium to another through cell-to-cell contact (Bellanger *et al*., 2014). So, CTns have a wider spread than plasmid associated *tet* genes (Roberts, 2019). Due to lacking the incompatibility exclusion system, multiple copies of the same CTns or related CTns may be found in plasmid or chromosome of a single bacterium (Norgren and Scott, 1991). The presence of CTns associated *tet* genes in plasmid or chromosome determine the flexibility and stability of these genes. The insertion of new transposons in chromosomes may result in gene loss or modification in gene expression. The deleterious mutation is eliminated from the environment and non-lethal mutations sustains and expands. Till now, 63 *tet* genes and 11 mosaic genes have been discovered in 88 gram-negative and 58 gram-positive bacteria (Supplementary Table S2). As discussed

earlier, so far, bacteria have evolved 36 efflux genes, 13 ribosomal protection genes, 13 enzymatic inactivation genes, one gene that has an unknown function and 11 mosaic genes. Among these genes, *tet*(M) conferring the resistance through ribosomal protection has been identified in 42 gram-negative and 40 gram-positive bacteria (Supplementary Table S1). The widest host range of *tet*(M) is thought to be due to association with Tn*916* CTns (Bonomo and Tolmasky, 2007).

The *tet*(B) associated with Tn*10* CTn confers the tetracycline resistance in 35 gram-negative bacterial genera through efflux mechanism. The *tet* carrying conjugative elements are in continuous evolution and have acquired new ARGs and heavy metals resistance genes (Li *et al*., 2021). The *tet*(W) associated with CTns has been identified in 13 genera of gram-positive and 21 genera of gram-negative bacteria including aerobes and anaerobes. The tetracycline promotes the conjugal transfer of CTns which also mobilize the co-non CTns, plasmids and unlinked non-replicating units (Facinelli *et al*., 1993; Wood and Gardner, 2015). Wood and Gardner (2015) revealed that tetracycline stimulates the excision and transfer of CTnDOT and mobilizes the co-resident non CTns. The 65 kb CTnDOT carrying *tet*(Q) gene normally linked to erm(f) gene coding for rRNA methylase is responsible for conferring resistance against macrolides and erythromycin are found in both gram-negative and gram-positive genera particularly in more than 80% *Bacteroides* spp.

Over the past 70 years, an increase in tetracycline resistance has been recorded in pathogenic, commensal and opportunistic bacteria. Significant evolutionary adaptations for the development of resistance against tetracycline in bacteria have been observed over time. To date, *tet* genes have been identified in 88 gram-negative and 58 gram-positive bacterial genera. Comparative analyses of resistance gene diversity and host range indicate that Gram-negative bacteria harbor a greater number of *tet* genes and exhibit broader mechanistic diversity than Gram-positive bacteria, likely due to higher rates of horizontal gene transfer and plasmid mobility (Knapp *et al*., 2010; Roberts, 2019; Sundin and Wang, 2018). So far, fourteen gram-negative bacterial genera have been identified that have adapted all the three resistance mechanisms while the in case of gram-positive, these are found only in "*Clostridium*" genus (Supplementary Table S1). From these findings, we may assume that gram-negative bacteria may exhibit greater adaptability due to higher rates of horizontal gene transfer in the environment against the tetracycline than the gram-positive. Detailed gene-by-genus distributions are provided in Supplementary Tables S1 and S2. The distribution of resistance mechanisms across bacterial genera is summarized in Tables 1 and 2. The ecological circulation and evolutionary persistence of tetracycline resistance determinants across clinical, agricultural and environmental settings are summarized schematically in Figure 1.

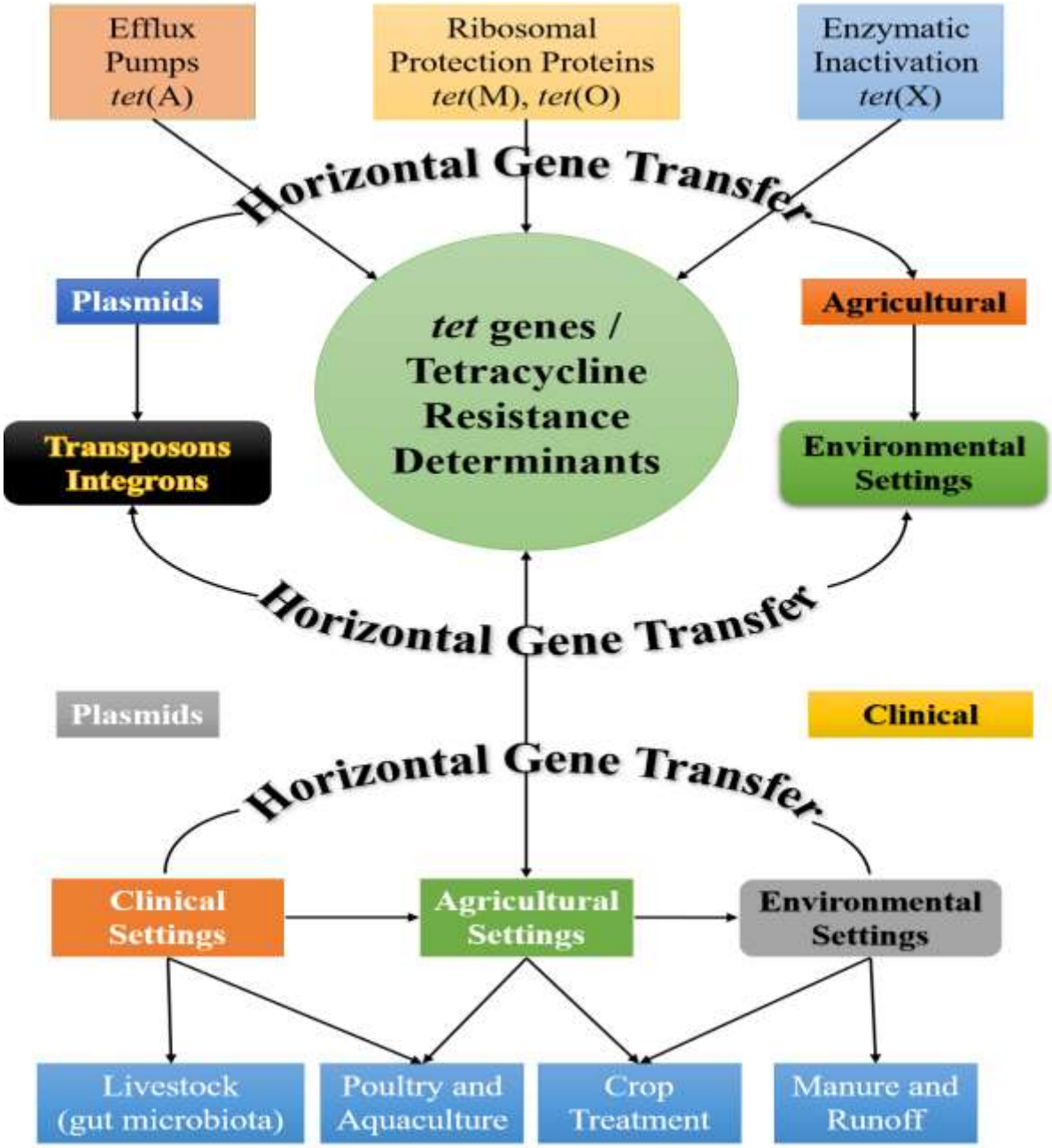

Figure 2: Schematic representation of the evolution and dissemination of tetracycline resistance across interconnected clinical, agricultural, and environmental ecosystems. The figure illustrates the major selective pressures driving resistance development, including antibiotic use in human medicine, livestock production, and crop protection. Tetracycline resistance genes (*tet* genes) are shown circulating among bacteria via horizontal gene transfer mechanisms such as plasmids, transposons and integrative conjugative elements. Environmental reservoirs, including soil, water bodies, plant-associated microbiomes and animal gut microbiota, facilitate long-term persistence and amplification of resistance determinants. The schematic highlights key resistance mechanisms (efflux pumps, ribosomal protection proteins and enzymatic inactivation) and emphasizes the bidirectional flow of resistance genes between environmental, commensal, and pathogenic bacterial populations.

Indeed, millions of tons of tetracycline have been deployed on humans, animals and plants since its discovery. The prevalence of *tet* genes in different ecosystems has exponentially increased over time. The incidence of different tetracycline genes *i.e. tet*(A), *tet*(B), *tet*(C) *and tet*(G) has been recorded in epiphytic bacteria where the tetracycline has

never been applied before (Schnabel and Jones, 1999). Recent studies revealed that the continuous exposure of tetracycline has not induced resistance in targeted *Erwinia amylovora* causing fire blight of apple and *X. arboricola* pv. *pruni* causing bacterial canker of peach. However, tetracycline resistance has been observed in *Pseudomonas syringae* (47, 105) and *Agrobacterium tumefaciens*. Furthermore, the *tet* homologs *tet*(A) and *tet*(M) have been identified in many plants' pathogenic bacteria (N. Wang unpublished data). The *tet*(A) gene conferring resistance through efflux mechanism has been identified in *E. piriflorinigrans*, *X. citri*, *X. phaseoli*, *X. perforans*, *X. campestris*, *P. syringae*, *P. aeruginosa* and *R. solanacearum* (Sundin and Wang, 2018).

Environmental ecosystems play a central role in the persistence and dissemination of tetracycline resistance genes. Soils, aquatic systems, animal microbiomes, and plant-associated bacteria act as long-term reservoirs where resistance determinants are maintained through low-level antibiotic exposure, co-selection with heavy metals, and dense microbial interactions. These environments facilitate horizontal gene transfer via mobile genetic elements, enabling *tet* genes to circulate between environmental, commensal, and pathogenic bacteria even in the absence of direct therapeutic antibiotic use (Martínez, 2009; Wellington *et al*., 2013; Forsberg *et al*., 2012; Sundin and Wang, 2018). Evidence from studies of ancient and modern DNA, metagenomics and modern treatments together indicate that increasing tetracycline resistance is a multi-step development from ancient sources (gene reservoirs) through increases in environmental abundance (ecological amplification) into the current use of horizontal gene transfer (HGT). To anticipate future resistance patterns and develop effective interventions, an understanding of the relationship between these stages is required.

Table 1: Major tetracycline resistance mechanisms and their ecological distribution

Resistance mechanism	Representative *tet* genes	Dominant bacterial groups	Primary ecosystems	References
Efflux pumps	*tet*(A), *tet*(B), *tet*(C), *tet*(D)	Gram-negative bacteria (*Escherichia*, *Salmonella*, *Acinetobacter*)	Clinical, livestock, wastewater	(Chopra and Roberts, 2001; Roberts, 2019)
Ribosomal protection proteins	*tet*(M), *tet*(O), *tet*(Q), *tet*(S)	Primarily Gram-positive; increasing Gram-negative hosts	Clinical, agricultural, soil	(Roberts, 2005a; Forsberg *et al*., 2015)
Enzymatic inactivation	*tet*(X), *tet*(X3–X6)	Diverse Gram-negative bacteria	Environmental, animal, clinical	(Yang *et al*., 2019; Roberts, 2019)
Uncharacterized/novel *tet* genes	Multiple metagenomic variants	Uncultured bacteria	Soil, aquatic systems	(D'Costa *et al*., 2011; Forsberg *et al*., 2012)

Gram-negative bacteria largely display efflux-based resistance mechanisms in clinical and livestock-associated environments, which is consistent with increased selective pressure due to frequent exposure to tetracycline. Whereas ribosomal protection proteins have a broader ecological distribution and a greater persistence in the evolutionary record, this is likely due to their lower fitness costs and association with MGEs. These protease-mediated enzymatic inactivation mechanisms (e.g., by *tet*(X) variants), which represent an emerging clinical threat because they represent the connection between environmental reservoirs and last-resort antibiotic resistance, highlight the current gaps in understanding the global tetracycline resistome given that uncharacterized *tet* genes are present in environmental metagenomes.

Table 2: Comparative distribution of tetracycline resistance mechanisms across bacterial groups.

Bacterial group	Dominant resistance mechanisms	Mechanistic diversity	Evolutionary implication	References
Gram-negative bacteria	Efflux + enzymatic inactivation	High	Rapid adaptation, multidrug resistance	(Chopra and Roberts, 2001; Roberts, 2019)
Gram-positive bacteria	Ribosomal protection	Moderate	Stable long-term persistence	(Roberts, 2005a)
Environmental bacteria	Ribosomal protection +	Very high	Reservoir for novel resistance genes	(D'Costa *et al*., 2011; Forsberg *et*

	enzymatic inactivation			*al*., 2012)
Clinical pathogens	Efflux + ribosomal protection	Moderate–high	Selection-driven amplification	(Forsberg *et al*., 2015)

Gram-negative bacteria exhibit a greater diversity of mechanisms of resistance, as they frequently establish efflux systems that complement their enzymatic-inactivation mechanisms, thus allowing them to adapt to high levels of antibiotic pressure. In contrast, Gram-positive bacteria largely rely on ribosomal protection proteins that confer long-term stability, rather than rapid adaptability. Environmental bacteria likely represent the greatest diversity of tetracycline-resistance determinants, supporting the proposal that many clinically relevant resistance determinants originated from environmental (i.e., non-healthcare-related) sources. These results also demonstrate that there is a lack of balance in evolutionary strategies and the role of environmental reservoirs in developing resistance.

Future Perspectives: Notwithstanding decades of inquiry, many gaps in the understanding of tetracycline resistance evolution in environmental microbiomes, the functional capabilities of resistance-defining genes with poorly understood mechanisms of action (e.g., *tet*(U)), and the effects of low-level antibiotic contamination on resistance selection persist. Synthesis of high-resolution metagenomic monitoring with worldwide One Health surveillance networks will be paramount. Promising developments are:

- Construction of efflux pump inhibitors to re-establish tetracycline activity.
- CRISPR-based antimicrobials could be designed to selectively target and disrupt prevalent *tet* genes (e.g., *tet*(M), *tet*(X)) in bacterial populations, thereby reducing resistance reservoirs without broadly disrupting beneficial microbiota.
- Environmental management tactics like bio sorbents and microbial consortia to minimize ARG burdens in agricultural runoff.

However, the ecological risks, off-target effects, and ethical implications of deploying gene-editing technologies in open environments must be carefully evaluated before large-scale implementation. While multiple types of inhibitors and CRISPR-based strategies for treatment of tetracycline resistance have been proposed, practical applications of either are minimal at present. Clinical translation of efflux inhibitors has been hampered by both toxicity and lack of selectivity, while CRISPR approaches have significant limitations concerning their delivery, stability and environmental safety in heterogeneous environments. Current available environmental remediation techniques (bio sorbents and microbial consortia) are primarily experimental at this time, emphasizing the continued need for evidence-based integrated stewardship approaches.

Conclusion: In conclusion, tetracycline resistance exemplifies the remarkable adaptive capacity of bacterial populations under sustained selective pressure. Efflux systems, ribosomal protection proteins, and enzymatic inactivation, coupled with extensive horizontal gene transfer, have enabled resistance determinants to permeate clinical, agricultural, and environmental ecosystems. The accelerated diversification of resistance mechanisms in Gram-negative bacteria further complicates control efforts. Addressing this challenge requires coordinated One Health strategies, strengthened surveillance across ecosystems, judicious antibiotic stewardship, and focused research on emerging mechanisms such as *tet*(X). Without integrated and proactive interventions, tetracycline resistance will continue to undermine both public health and agricultural productivity.

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