NANO-ENABLED ANTIBACTERIAL COMBINATION THERAPY (NeACT) AGAINST MULTI-DRUG RESISTANT BACTERIAL PATHOGEN ISOLATES OBTAINED FROM ANIMAL FARMS

By

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This thesis is dedicated to my late father Dr. Darshan Singh Brar, whose love and kind words kept me motivated throughout this degree. His journey from being a young boy in a small village in Punjab to being an outstanding scientist in his field and a mentor in an international institute will forever be an inspiration in my life. I am grateful that I got a chance to be his daughter.

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ABSTRACT

The emergence of multidrug resistant (MDR) bacterial pathogens in farm animals and their zoonotic spread is a concern to both animal agriculture and public health. Apart from antimicrobial resistance (AMR), bacterial pathogens from the genera of Salmonella and Staphylococci take refuge inside host cells. Intervention strategies that can eliminate intracellular MDR pathogens are warranted for resilience in animal husbandry. The thesis research focused on two main aspects: (1) determination of prevalent MDR and resistance mechanisms in *Salmonella* and *Staphylococcus* isolates obtained from animal farms in Quebec, and (2) design and development of nano-enabled antibacterial combination therapy to eliminate intracellular pathogenesis of MDR *Salmonella enterica* ser. Typhimurium.

In this study, clinical isolates of Salmonella (*S. enterica* ser. Typhimurium, *S. enterica* ser. Dublin, and *S. enterica* ser. Choleraesuis) and Staphylococcus (*S. aureus* M12, *S. aureus* M17, *S. hyicus* M43, and *S. hyicus* M48) from swine farms in Quebec were characterized for antibiotic (n=24) and heavy metal resistance and virulence characteristics. Resistance mechanisms (efflux pump activity and beta-lactamase enzyme activity), and virulence characteristics (biofilm formation and hemolysis) were investigated in the isolates. All the tested isolates were determined to be MDR, where *S. enterica* ser. Typhimurium had the highest resistance to the panel of antibiotics tested. *S. enterica* ser. Dublin and *S. aureus* M12 had the highest efflux pump activity, while *S. enterica* ser. Typhimurium had the highest beta-lactamase enzyme activity. *Salmonella* isolates were observed to be weak biofilm formers and caused partial hemolysis of sheep red blood cells (RBCs), while *Staphylococcus* isolates were stronger biofilm formers and caused complete hemolysis of sheep RBCs. MDR isolates *S. aureus* M12 and *S. enterica* ser. Typhimurium bacteria were subsequently

tested against combinations of antibiotics and potentiating adjuvants for improved antibacterial efficacy using checkerboard assay and their fractional inhibitory concentration index (FICI) was calculated. Combination of tetracycline (TET) and chlorpromazine (CPZ), which showed an additive effect, was then chosen for nanoparticle drug carrier encapsulation. To increase the potency of this combination even further, TET was encapsulated into chitosan nanoparticles (Ch-TET NPs) and CPZ was encapsulated into silica nanoparticles (Si-CPZ NPs), which were combined to form combination drug (CMD). The size of the particles ranged between 500-600 nm: 541.68±2.02 nm, 514.20±53.05 nm, and 577.00±35.83 nm for Ch-TET NPs, Si-CPZ NPs, and CMD respectively. The Zeta potential of CMD was determined to be +15.25±0.11 mV, and that of Ch-TET NPs and Si-CPZ NPs was determined to be +25.72±0.68 and -9.95±0.43 mV respectively. Encapsulation efficiency was spectrophotometrically determined and found to be 23.82±0.16% for TET and 44.77±0.12%. for CPZ. The CMD showed improved antibacterial efficacy, enhanced ability to reduce intracellular pathogens, reduced the efflux pump activity, and decreased the production of beta-lactamase enzyme and hydrogen sulphide in the CMD treated bacterial isolates. The results of the work shed light on the promising opportunity of nanoparticle-enabled combination therapy as a tool to combat multidrug-resistant pathogens encountered in animal agriculture.

RÉSUMÉ

L'émergence d'agents pathogènes bactériens multirésistants (MDR) chez les animaux d'élevage et leur propagation zoonotique constituent une préoccupation tant pour l'agriculture animale que pour la santé publique. Outre la résistance aux antimicrobiens (RAM), les agents pathogènes bactériens des genres *Salmonella* et *Staphylococci* se réfugient à l'intérieur des cellules hôtes. Les stratégies d'intervention qui peuvent éliminer les agents pathogènes MDR intracellulaires sont justifiées pour la résilience dans l'élevage des animaux. La recherche de la thèse s'est concentrée sur deux aspects principaux : (1) la détermination de la prévalence de la MDR et des mécanismes de résistance dans les isolats de *Salmonella* et de *Staphylococcus* obtenus dans les fermes d'élevage au Québec, et (2) la conception et le développement d'une thérapie antibactérienne combinée à base de nanotechnologies pour éliminer la pathogenèse intracellulaire de la MDR *Salmonella enterica* ser. Typhimurium.

Dans cette étude, des isolats cliniques de *Salmonella* (*S. enterica* ser. Typhimurium, *S. enterica* ser. Dublin et *S. enterica* ser. Choleraesuis) et de Staphylococcus (*S. aureus* M12, *S. aureus* M17, *S. hyicus* M43 et *S. hyicus* M48) provenant d'élevages porcins au Québec ont été caractérisés pour leur résistance aux antibiotiques (n=24) et aux métaux lourds ainsi que pour leurs caractéristiques de virulence. Les mécanismes de résistance (activité des pompes d'efflux et activité enzymatique de la bêta-lactamase) et les caractéristiques de virulence (formation de biofilms et hémolyse) ont été étudiés chez les isolats. Tous les isolats testés ont été déterminés comme étant multirésistants aux médicaments, *S. enterica* ser. Typhimurium présentait la plus grande résistance au panel d'antibiotiques testés. *S. enterica* ser. Dublin et *S. aureus* M12 présentaient la plus forte activité de pompe d'efflux, tandis que *S. enterica* ser. Typhimurium présentait l'activité enzymatique bêta-lactamase la plus élevée. On a observé que les isolats de *Salmonella* formaient faiblement des

biofilms et provoquaient une hémolyse partielle des globules rouges (GR) de mouton, tandis que les isolats de Staphylococcus formaient plus fortement des biofilms et provoquaient une hémolyse complète des GR de mouton. Les isolats MDR *S. aureus* M12 et *S. enterica* ser. Typhimurium ont ensuite été testés contre des combinaisons d'antibiotiques et d'adjuvants de potentialisation pour une meilleure efficacité antibactérienne en utilisant l'essai en damier et leur indice de concentration inhibitrice fractionnelle (ICIF) a été calculé. La combinaison de tétracycline (TET) et de chlorpromazine (CPZ), qui a montré un effet additif, a ensuite été choisie pour l'encapsulation de nanoparticules porteuses de médicament. Pour augmenter encore la puissance de cette combinaison, la TET a été encapsulée dans des nanoparticules de chitosan (Ch-TET NPs) et la CPZ a été encapsulée dans des nanoparticules de silice (Si-CPZ NPs), qui ont été combinées pour former une combinaison de médicaments (CMD). La taille des particules était comprise entre 500 et 600 nm : 541.68±2.02 nm, 514.20±53.05 nm et 577.00±35.83 nm pour les Ch-TET NPs, Si-CPZ NPs et le CMD respectivement. Le potentiel zêta du CMD a été déterminé à +15.25±0.11 mV, et celui des Ch-TET NPs et des Si-CPZ NPs a été déterminé à +25.72±0.68 et -9.95±0.43 mV respectivement. L'efficacité d'encapsulation a été déterminée par spectrophotométrie et s'est avérée être de 23.82±0.16% pour TET et 44.77±0.12% pour CPZ. Le CMD a montré une meilleure efficacité antibactérienne, a réduit l'activité de la pompe d'efflux, et a diminué la production d'enzyme bêta-lactamase et de sulfure d'hydrogène dans les isolats bactériens traités par le CMD. Les résultats de ce travail mettent en lumière l'opportunité prometteuse d'une thérapie combinée basée sur les nanoparticules comme outil pour combattre les pathogènes multirésistants rencontrés dans l'agriculture animale.

CONTRIBUTIONS OF AUTHORS

The following chapters are the manuscripts prepared for publication:

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The research work reported was carried out by Amarpreet Kaur Brar supervised by Dr. Saji George from the Department of Food Science and Agricultural Chemistry, McGill University.

Amarpreet Kaur Brar, as the MSc. candidate planned the experimental design and protocols, conducted most of the experiments, analyzed all the experimental results, and drafted the thesis and manuscripts for scientific publications.

Dr. Saji George, the MSc student's supervisor, guided the design, conduction of experiments, and correction and editing of manuscripts.

Mr. Satwik Majumder helped carry out assays for the determination of resistance mechanisms and effect of synthesized nanoparticles on resistance mechanisms.

Ms. Maria Zardon Navarro, from University of Montreal, Saint-Hyacinthe helped in identification and characterization of bacterial isolates.

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CHAPTER I: INTRODUCTION

Introduction

Antimicrobials are vital for use in animals as well as humans for preventing and treating dangerous infections. Various pathogenic bacteria infect food animals and are transmitted to humans as well through direct contact or food chain. These pathogens are responsible for enteric, septicaemic, respiratory, or skin infections in food animals and foodborne infections in humans that are difficult to treat (Martins et al., 2015). Infectious pathogens in food animals have a huge economic burden on the food industry as well on the human healthcare as there is enough evidence of transmission of pathogens from animals to humans (Martins et al., 2015; Park & Ronholm, 2021) and antimicrobials are vital to eliminating these pathogens. However, overuse of antimicrobials in food animals for growth promotion and to prevent infections is a powerful selector of antimicrobial resistant (AMR) pathogens (Van Boeckel et al., 2015). Eliminating the infectious AMR pathogens requires even higher doses of antibiotics or switching to antibiotics that are medically important and reserved for serious human infections. This in turn adds to the problem of unwise and excessive use of antibiotics (Martin et al., 2015; Park & Ronholm, 2021). To decelerate the growing AMR amongst pathogens, there is an urgent need to either reduce the amount of antibiotics being used, search for alternative therapeutics or preserve the antimicrobial efficacy of existing antibiotics.

Nanotechnology holds immense potential to revolutionize the field of drug delivery. Various properties of nanoparticles are beneficial in overcoming the limitations of traditional drug delivery methods as they have the ability to target specific sites for drug delivery, minimize cytotoxicity, improve bioavailability, release drugs in a controlled manner and simultaneously deliver more than one entity (Amreddy et al., 2018; Menina et al., 2019). Currently, 51 products based on

nanotechnology have already been approved by Food & Drug Administration (FDA) for clinical use out of which two are antifungal and aimed at reducing toxicity of the encapsulated drug (Caster et al., 2017). An important sphere of nanomedicine is the delivery of more than one entity simultaneously at a specific site. For instance, tetracycline and tretinoin were co-encapsulated in liposomes and their antibacterial efficacy was tested against acne causing *Staphylococcus aureus* and *Streptococcus epidermidis* strains, which showed enhanced antibacterial effect and low cytotoxicity (Eroğlu et al., 2020). Similarly, combinations of antibiotics and adjuvants which target specific resistance mechanisms can be co-delivered using nanoparticle-based drug carriers, which is an area of research that still requires additional exploration.

The main goal of this project was to investigate AMR prevalent in animal farms and resistance mechanisms employed by them so as to design and develop nanoparticle-based combination therapy to circumvent these resistance mechanisms to eliminate the pathogens. Seven bacterial isolates of *Salmonella* and *Staphylococcus* were obtained from animal farms in Quebec and their resistance mechanisms and virulence characteristics were determined phenotypically. Combinations of antibiotics-adjuvants were then tested for synergistic or additive effect against the multi-drug resistant (MDR) *Staphylococcus aureus* M12 and *Salmonella enterica* serovar Typhimurium. A combination of commonly used antibiotic tetracycline and adjuvant chlorpromazine, which is an efflux pump inhibitor was selected to further demonstrate the ability of nanoparticle encapsulation to enhance the antibacterial effect even further.

The two main objectives of the project were:

1. Investigating the AMR profile, the resistance mechanisms, and the virulence characteristics of seven bacterial isolates obtained from animal farms in Quebec.

2. Testing combination of antibiotics-adjuvants for possible synergistic or additive effects against MDR isolates, then selecting a suitable combination to synthesize nanoparticle-enabled combination therapy with superior antibacterial properties.

CHAPTER II: LITERATURE REVIEW

Antimicrobial resistance (AMR)

The discovery of penicillin by Alexander Flemming was a breakthrough discovery that changed the future of human medicine and led to the exploration of various antibiotics to treat various dangerous infections (Maurois & Fleming, 1959). The use of antibiotics was eventually translated to food industry as well. In 1946, Moore *et. al* found out that addition of antibiotics in the diet of chickens promoted growth and resulted in weight gain (Moore et al., 1946). Eventually in 1951, the United States Food and Drug Association (FDA) permitted the use of antibiotics as growth promoters in the feed of animals without any veterinary prescription (Jones & Ricke, 2003). Data from Food and Agricultural Organisation (FAO) suggests that 80% of the antimicrobials are used in food-producing animals (Van Boeckel et al., 2015). Use of antibiotics to treat various bacterial infections in humans and food animals has improved the health and welfare of humans and animals. However, overuse leads to emergence of AMR bacterial pathogens.

AMR refers to the ability of micro-organisms to avoid the killing or growth inhibitory activity of antimicrobials at a concentration at which they are normally susceptible (Schrader et al., 2020).

Introduction

Antimicrobials have been successfully utilized for treating and controlling various infectious diseases (Caniça et al., 2019; Farmer, 2013). Excessive and unwise use of antibiotics, in animal agriculture particularly, has given rise to the emergence of antimicrobial resistant (AMR) among bacterial pathogens in food animals. AMR in animal farms however spill over to humans through direct contact or through the food chain (Dadgostar, 2019). AMR has a huge impact and threatens

to set back the progress made by antimicrobials in controlling a wide range of infections (Moellering Jr, 2011). According to the World Bank, zoonotic infections costed more than USD 20 billion to livestock sector between 2000 and 2010 (Trench et al., 2012), besides being a source of foodborne diseases in humans These concerns demand alternative antimicrobial strategies which have the potential to control the infections caused by multi-drug resistant (MDR) pathogens.

Nanotechnology has recently advanced enormously and its application as a drug delivery system has impacted the healthcare sector significantly. Nanotechnology comprises the development of nanoscale devices, particles, or systems. Nanoparticles are developed within the size range of 0.2-100 nm and therefore have increased surface area to volume ratio (Rudramurthy et al., 2016). The stability and applicability of nanoparticles is determined by their surface charge, size, shape, the material used and interaction with chemical and biological surroundings. Therefore, nanoparticles can be designed and developed considering the requirements of application (Bhatia, 2016).

Nanoparticle-based drug delivery systems such as liposomes, polymeric nanoparticles, dendrimers etc. have been explored vastly. Drugs encapsulated in nanoparticles show improved bioavailability, stability and targeted delivery than the free drugs (Amreddy et al., 2018; Babos et al., 2018). In addition, encapsulating multiple drugs which have a synergistic effect is a research area that is being widely explored as well. Especially, to overcome AMR, encapsulation of an inhibitor of bacterial resistance mechanism and an antibiotic can revolutionize the food industry and healthcare sector by inhibiting the growth of AMR pathogens. In this review, the mechanisms of AMR in bacterial pathogens, the impact on the food industry, and the application of nanotechnology drug delivery are discussed.

Agricultural practices contributing to AMR in farms

Antimicrobial use to prevent and treat infections

Antimicrobials are used as therapeutics to eliminate or control pre-existing infections. Domesticated animals usually are treated individually. However, food animals are usually kept in large groups, which makes it difficult to administer antimicrobials individually. The method of administration of antimicrobials also depends on the size of animals. For instance, cattle are usually treated individually. However, in case of broilers which are kept in large groups of thousands, if one member shows any signs or symptoms of infection, it is anticipated that all the members will get infected too. Thus, the medication is applied to the entire group and is called **metaphylaxis.** This prevents the development of infection in other members of the group and save treatment costs. **Prophylaxis** is the administration of antimicrobials as a preventative measure, which can be either done individually to large groups. The timing of prophylaxis is also an important factor. For instance, in pigs and cattle, during weaning or when animals from different herds are mixed, there are increased chances of infection. Prophylaxis plays a key role in preventing various respiratory or enteric diseases in pigs and cattle and prevents the costs of large amounts of antibiotics required during metaphylaxis (Bousquet-Mélou et al., 2010). Although being essential in various animal farms, it invites a lot of criticism for being a significant factor in development of antimicrobial resistance (Dadgostar, 2019). The constant administration of antibiotics creates a selection pressure on pathogenic bacteria and leads to the development of resistance mechanisms against applied antibiotics.

Antibiotic use for growth promotion

Use of antibiotics as therapeutics was followed by their usage as growth promoters in industrial animal farms. Various growth-promoting antibiotics include bacitracin, salinomycin, efrotomycin, tylosin, etc. Often the same antibiotic is used for growth promotion as well as for infection treatment. The concentration used for growth promotion is less than that used for therapeutic purposes, which is referred to as subtherapeutic use (Landers et al., 2012). The introduction of antibiotics as growth promoters had improved the feed conversion, promoted growth, and decreased morbidity and mortality due to various infections. Around 4 to 8% average growth promotion was observed with 2 to 5% improvement in feed utilization (Butaye et al., 2003). The use of antibiotics as growth promoters has faced significant criticism and has been suggested as one of the primary causes of the development of AMR in food animals. Many antibiotics have been banned and to be used as a growth promoter, antibiotics must meet certain criteria for use as growth promoters, such as (i) the use should result in an economic difference in raising the animals (ii) they shouldn't be used as therapeutics in humans or animals, and (iii) they should not intervene with the therapeutic efficiency of other antimicrobials (Swann, 1969).

Heavy metal exposure

Apart from antimicrobials, heavy metals also contribute to selection of AMR bacteria. Heavy metal used in agriculture and aquaculture may lead to co-selection of resistant bacteria and thus promote spread of AMR. Metal-containing products or antibiotics are usually fed by farmers to fishes to prevent fouling and as precaution to limit the spread of infections (Burridge et al., 2010). The resistance to antibiotics on exposure to heavy metals is because resistance mechanisms against antibiotics and heavy metals are coupled either physiologically (cross-resistance) or genetically

(co-resistance). Cross-resistance is used to describe mechanisms that ascribe resistance to more than one antimicrobial. For instance, some multi-drug efflux pumps can extrude out both antibiotics and heavy metals (Chapman, 2003). Co-resistance occurs when resistance genes are present next to each other on mobile elements, and thus are transmitted together during horizontal gene transfer (Chapman, 2003).

Antimicrobials and mode of action

Antimicrobials used to treat infections are categorized according to the mechanism of action which include (1) interference of nucleic acid synthesis, (2) interference of protein synthesis, (3) interference with synthesis of the cell wall, and (4) inhibiting the metabolic pathways (5) Disruption of bacterial membrane

(1) **Disruption of nucleic acid synthesis** is one of the mechanisms used to exert an antibacterial effect on pathogens. Drugs such as fluoroquinolones disrupt the DNA synthesis by introducing double-stranded DNA breaks which are lethal. Trimethoprim and sulfonamide inhibit DNA synthesis by blocking folic acid synthesis (Petri, 2011).

(2) Macrolides, chloramphenicol, aminoglycosides, tetracyclines, and mupirocin act on bacterial infections by **interfering with the protein synthesis**. Antimicrobials selectively bind to prokaryotic ribosomes and inhibit the process of synthesis of protein. Tetracyclines, aminoglycosides, and macrolides bind to 30S subunit of ribosome while chloramphenicol binds preferably to 50S subunit of ribosome to intervene in the process of protein synthesis (Etebu & Arikekpar, 2016).

(3) β-lactam drugs like penicillin, cephalosporins, monobactams, carbapenems and, glycopeptides drugs such as vancomycin **inhibit the synthesis of cell walls** of the bacteria. β-lactam drugs obstruct cell wall synthesis by interfering with the enzyme that contributes to the synthesis of a peptidoglycan layer. Glycopeptides bind to the terminal D-alanine residues of nascent peptidoglycan chains and prevents synthesis of stable cell wall (Suarez & Gudiol, 2009).

(4) Some drugs act on bacteria by **inhibiting their metabolic pathways**. For instance, a combination of trimethoprim and sulfamethoxazole shows the antimicrobial action by obstructing two steps of the enzymatic pathway in the synthesis of tetrahydrofolic acid. Tetrahydrofolate is essential for one carbon transfer reactions. Trimethoprim prevents the conversion of dihydrofolate to tetrahydrofolate, and sulphonamide prevents the incorporation of para-amino benzoic acid (PABA) into folic acid (Petri, 2011).

(5) **Disruption of the bacterial membrane** is another mechanism of action against bacteria. Cyclic lipopeptide daptomycin kills the bacterial cell by depolarizing the cell membrane and polymyxins increase bacterial membrane permeability, causing leakage and eventually leading to cell death (Etebu & Arikekpar, 2016).

Class of antibiotics	**General mode of action**
Quinolones	Inhibition of DNA synthesis
Metronidazole	Inhibition of DNA synthesis
Macrolides	Inhibition of protein synthesis
Tetracyclines	Inhibition of protein synthesis
Chloramphenicol	Inhibition of protein synthesis
Penicillin	Inhibition of synthesis of cell wall
Carbapenems	Inhibition of synthesis of cell wall
Cephalosporin	Inhibition of synthesis of cell wall
Polypeptide antibiotics	Inhibition of synthesis of cell wall
Sulphonamides	Competitive inhibition
Rifamycin	Inhibition of synthesis of RNA
Lincosamides	Inhibition of protein synthesis
Aminoglycosides	Inhibition of protein synthesis

Table 2.1: *Classes of antibiotics and their general mode of action on bacterial cell*

Bacteria may use various mechanisms to resist the action of these antibiotics. Some bacteria are innately resistant to classes of antibiotics, which can be related to their general physiology or anatomy. In that case, every strain of that bacterial class is resistant to all members of those antibiotic classes. The cell membrane of the bacteria can be poorly permeable, bacteria may not contain the antimicrobial target, bacteria may use efflux pumps to extrude out the antimicrobial or it may inactivate the antimicrobial (Varela et al., 2021). For instance, gram-negative bacteria are resistant to macrolide antibiotics due to the outer membrane being impermeable to hydrophobic compounds. However, of greater concern is the acquired resistance in bacteria. Bacteria that are initially susceptible to antibiotics gain resistance against them, and under the selective pressure of the antibiotics, continue to grow and multiply. Eventually, the original population of bacteria susceptible to antibiotics is replaced by the resistant population (Rosenblatt-Farrell, 2009). The AMR genes can also be spread to other bacteria, due to which the bacteria no longer remain susceptible to that class of antibiotics.

Bacterial resistance mechanisms

a. Modification or elimination of the binding site on the target protein on which the antimicrobial binds and thus altering it - For instance, ribosomal resistance to streptomycin via modification of S12 protein. A change of a single amino acid at one of the two sites can lead to resistance. Modification of penicillin-binding protein leads to penicillin resistance in *Neisseria gonorrhoea*. Other instances include resistance of quinolones due to mutation of *gyrA, gyrC* or *gyrD* (Byarugaba, 2010).

b. Production of enzyme to inactivate the antimicrobial – β-lactamase production in bacteria contributes to resistance to β-lactam antibiotics in *Staphylococcus aureus, Neisseria gonorrhea, Moraxella catarrallis* and *Hemophilus influenza*. Other instance of inactivation of antimicrobial

using enzyme is resistance to aminoglycosides due to aminoglycoside modifying enzyme and resistance to chloramphenicol due to chloramphenicol acetyltransferase (Varela et al., 2021).

c. Prevention of entry of antimicrobials due to reduced membrane permeability – Susceptible *Enterobacter* allow entry of cephalosporin through porin F. However, cephalosporin resistant strains of *Enterobacter* have decreased porin F and increased porin C. Porin C does not let cephalosporin enter the cell readily. Some bacteria have impermeable cell walls to certain antibiotics such as in *Pseudomonas aeruginosa* (Chevalier et al., 2017).

d. Upregulation of the efflux pumps to extrude the antimicrobials out of the cell – Efflux pumps are vital to extrude out hydrophobic drugs that enter the cell through diffusion. The rate at which the drug enters the cell, it is expelled out of the cell at the same rate to avoid any interaction of the drug with the target. Tetracycline resistance is due to active efflux out of the cell (Byarugaba, 2010).

AMR can be acquired by mutation or by receiving genetic information encoding for resistance mechanisms. A spontaneous mutation can allow bacteria to survive the action of an antimicrobial, which otherwise would kill the bacteria. The newly resistant strains will eventually replace the susceptible strains. AMR because of selection due to chromosomal mutation is termed as *vertical evolution.* These mutations can prove to be beneficial to the bacteria and the bacteria can (a) modify or eliminate the binding site on target protein on which the antimicrobial binds and alter it, (b) inactivate the antimicrobial by production of an enzyme that is responsible of inactivation, (c) prevent the entry of antimicrobial into the cell due to reduced membrane permeability, or (d) upregulate the efflux pumps i.e. the pumps that extrude the antimicrobial out of the cell (Sommer et al., 2017). Bacteria can also develop MDR by acquiring genetic material from other resistant

bacteria and is called *horizontal evolution*. Horizontal transfer of genes may occur between the bacteria of same or different bacterial specie.

Gene transfer can occur through conjugation, transduction or transformation, and the transfer is facilitated by transposons. During conjugation, resistant genes are transferred from gram negative bacteria to another bacteria by a means of proteinaceous elongation called *pilus* which joins the two bacteria. In gram positive bacteria, conjugation occurs by production of pheromones by the bacteria, which leads to clumping of the recipient and the donor bacteria. In case of gene transfer with the aid of bacteriophage is called transduction. In transformation, bacteria acquire resistant gene containing DNA segments that have been released by other bacteria during cell lysis, due to which the initially susceptible bacteria become resistant (Tenover, 2006).

Acquisition of resistance due to mutation or gene transfer leaves the bacteria fitter to survive in the presence of antimicrobial. Even though a single mutation in the gene may only reduce susceptibility by a small degree, it contributes to the overall survival chances initially in an environment of antimicrobial. Eventually, the addition of mutations or resistant genes can make the bacterial strain resistant to a drug. In some cases, a single mutation might also lead to a completely resistant bacterial strain, for instance, fluoroquinolone resistance in *Campylobacter jejuni* (Bockstael & Van Aerschot, 2009).

Multi-drug resistant pathogens require higher doses of antimicrobials to be eliminated, which in turn creates an additional selection pressure for the development of AMR amongst pathogens, and thus the vicious cycle continues. The situation demands the use of alternative therapeutics to circumvent the MDR bacteria and minimize the use of medically important antibiotics. Nanotechnology holds the immense potential to revolutionize the field of medicine.

Impacts of AMR

Bacterial AMR has become a global issue in the field of both medicine and agriculture. Undeniably, antimicrobials play an important role in improving the health of animals and preventing life-threatening infections. However, AMR bacterial infections pose a threat to the health of animals, as well as impact the health of humans after being transmitted as foodborne contaminants (Moellering Jr, 2011). AMR directly or indirectly affects food safety, the economy and the health of humans working in close proximity to animals.

Food safety

The question of possible impact on human health has been raised since the advent of antimicrobial usage in food animals. There is enough evidence that suggests that antimicrobial use in food animals results in selection of AMR pathogens, which get transmitted to humans during consumption which has various adverse effects (Dadgostar, 2019; Park & Ronholm, 2021). An outbreak of resistant *Salmonella* infections in humans was found out to be related to use of antibiotics for therapeutics in sick calves. Following this, the Swann committee recommended using specific antibiotics for treating food animals and not using the same antibiotics as therapeutics in humans (Swann, 1969; Tollefson & Karp, 2004) In 1997, in a meeting in Berlin organized by World Health Organization (WHO) it was concluded that there was a direct relationship between the use of antibiotics and selection of AMR nontyphoidal *Salmonella* serotypes and through food and direct contact with animals, these bacteria get transmitted to humans (World Health Organization, 1997).

Natural microbiota of the intestine plays a vital role in prevention of infections by exogenous pathogens. Taking antibiotics for a treatment leads to reduction of this microbiota, which leaves

the patients more prone to infections from a foreign pathogen after a few days of taking antibiotics. According to various studies, the invasive pathogens are mostly AMR, rather than antimicrobial susceptible (Barza, 2002). On exposure to antibiotics, a person can become more prone to intestinal colonization by AMR pathogens, if the person consumes contaminated meat (Barza, 2002). Treating food animals with antimicrobials that closely resemble the antimicrobials used by humans to treat infections can lead to health burden on humans and make various pathogens infecting humans resistant to commonly used antibiotics. Thus, transfer of the pathogens through the food chain to humans can lead to colonization by these pathogens which cannot be treated by the same antimicrobial (McEwen & Fedorka-Cray, 2002).

The resistance genes are sometimes closely linked to virulence genes. AMR pathogens can transmit resistance characteristics through genes bacteria that are normally present in the body but have low pathogenicity and can lead to them becoming virulent and cause infections, especially in immune-compromised patients (Barza, 2002). AMR complicates the treatment process and limits the options; it requires either using higher doses of antibiotics or moving to antibiotics that are reserved for more serious infections.

Economic impact

AMR pathogen infections have huge financial implications for world healthcare and animal agriculture. In 2018, 14,000 deaths that occurred in Canada were reported to be associated with AMR. In addition, it costed the Canadian healthcare system around $1.4 billion in 2018 itself, which is predicted to increase up to around $13 billion per year by 2050 if the current rates of AMR persist (Finlay et al., 2019). The impact of infections by AMR pathogens also includes reduced food production, decreased food safety, environmental contamination, and subsequently

higher losses to farmers. Due to foodborne illnesses, a large economic impact occurs due to lost incomes and hospital charges. The AMR pathogens impact the food industry by infecting agricultural animals which become unfit for consumption and lead to huge losses to farm owners. In addition, outbreaks lead to product recalls and also discourage consumers from meat consumption. It was estimated by the United States Department of Agriculture (USDA) that foodborne illnesses cost the economy of the United States around $10-83 billion USD annually (Nyachuba, 2010). The economic loss due to AMR prevalent in food animals impacts the farm owners, food processing companies, and patients infected with foodborne pathogens.

Workplace hazard

Farmworkers work in close proximity to the animals and their job involves various tasks such as feeding and cleaning the animals, which involves direct contact with the animals. Animal farms involve usage of antimicrobials for treatment of infections, prophylaxis and as growth promoters. This leads to selection pressure and results in the emergence of AMR bacteria in animals which can infect the farmworkers being in close contact with animals. In a study, the commensal flora of pig farmers, who had not taken any antimicrobial for a month was assessed for the presence of AMR due to pig farming. Pathogens from Nasal, fecal and throat swabs were screened for presence of AMR pathogens and it was found out that resistance was higher in bacteria obtained from swabs of pig farmers than the bacteria obtained from swabs of healthy person, which indicated that there was significant association between practices involved in pig farming and presence of AMR commensal bacteria in farm workers (Aubry-Damon et al., 2004). A possible explanation for presence of AMR bacteria in farmers more than in non-farmers is that MDR bacteria get transferred from infected animals to workers due to direct contact during feeding, cleaning animal feces, or during the administration of antibiotics.

The concern is not just limited to pathogenic bacteria, but also commensal bacteria. Various commensal bacteria have both humans and animals as their hosts. Prolonged use of antibiotics in animals for infection treatment or for growth promotion results in these bacteria developing resistance to commonly used antibiotics. Transfer of these bacteria to humans might not directly cause infection, but these bacteria become reservoirs of AMR genes. The resistant commensal bacteria carry mobile elements such as R-plasmids and transposons which keep increasing and get transferred to pathogenic bacteria such as *Salmonella* or *Shigella,* making them MDR. In the event of infection by a pathogen, it may be difficult to eliminate the pathogens using commonly used antibiotics (Angulo et al., 2004; Verraes et al., 2013). There is a large number of health consequences of AMR in farms on workers. These include more frequent illnesses, which otherwise might not occur and the difficulty and complexity of treatment using common antibiotics.

What is nanotechnology?

Nanotechnology means technology, science or engineering conducted at nanoscale i.e., 1 to 100 nanometers. When materials are converted into their nanoparticles, the thermal, physical, chemical, magnetic and optical properties of the material change (Duncan, 2011). The extremely small size, large surface area to mass ratio and high reactivity can be used to overcome various limitations of traditional methods of food packaging, separation and purification of biological molecules and cells, antimicrobial agents, imaging, drug delivery etc. (Duncan, 2011; Herr et al., 2006). There is a vast contribution of nanotechnology in the fields of environmental science, physics, chemistry, medicine, and electrical and mechanical engineering. In the food industry, nanotechnology finds many applications as well. Encapsulation of compounds in nanomaterial is one way in which flavors, minerals, antimicrobials, probiotic micro-organisms etc. are being

explored. This is done in order to protect active ingredients from interacting, to mask certain odors or flavors, to deliver antimicrobials at specific sites and times, and to protect from chemical or biological degradation etc. (Chen et al., 2006; Seil & Webster, 2012).

Applications of nanotechnology in agri-food sector

One of the most rapidly increasing application is use of nanomaterials in food packaging. (Rashidi & Khosravi-Darani, 2011) Nanoparticle-based food packaging can have increased heat tolerance and mechanical resistance, and thus the food can have a longer shelf life. Natural materials can be formulated to make nanomaterials for food packaging as well. (Moraru et al., 2003). Apart from being used in food packaging, nanomaterials can be used in food processing as well. For instance, nano biosensors are used for detecting allergens and pathogens in food and thus can alert the food processing companies (Ye et al., 2005).

Nanotechnology has enormous potential for various applications in medicine. The use of nanomaterials for drug delivery provides for the tunable properties of solubility, immunogenicity, diffusivity, drug release rate and half life in blood circulation. The newly explored nanoparticle-based drugs may reduce toxicity, increase drug specificity, and increase the life of the drug (De Jong & Borm, 2008). In fact, various nanoparticle-based drugs have been approved and been used in the treatment of various infections and diseases, and the dominating ones are liposome-based and polymer-drug conjugates. Liposome IRIV vaccine for hepatitis A, micellular estradiol for menopausal therapy, and liposome daunorubicin for HIV-related Kaposi's sarcoma are some of the instances. Instances of polymer-drug conjugates include PEG-α-interferon 2a for hepatitis B and C and methoxy-PEG-poly (D,L-lactide) taxol for metastatic breast cancer (Zhang et al., 2008).

Nanotechnology in animal and human medicine

There is an increased interest in developing clinical treatments, drug delivery systems, vaccines, medical tools based on nanotechnology. Additionally, there are various fields of ongoing research which show a promising future, for instance development of nanoparticle-based drug delivery systems, nanoscale diagnosis tools, biosensors, nanorobots for elimination of pathogens etc. (Emerich & Thanos, 2003). In this review, the application of nanotechnology for advancement in drug delivery systems and as antimicrobials is discussed in detail.

Nanoparticles as antimicrobials

Antimicrobials are often rendered inefficient after the development of AMR pathogenic bacteria. There is always ongoing research to search for new, more efficient methods of eliminating bacterial infections. With the advancement in the field of nanotechnology, the development of antimicrobials using nanotechnology has been explored. Due to their high surface area to volume ratio the tunable physical and chemical properties, nanoparticles are promising antimicrobials. Antimicrobial properties of various materials have been explored such as zinc, copper, titanium, magnesium, etc. (Schabes-Retchkiman et al., 2006). The exact antimicrobial mechanism of metals is under investigation and there are two possible mechanisms. Dissolution of metals from the surface of the nanoparticles giving rise to free metal ion toxicity is one probable mechanism of antimicrobial activity. The generation of reactive oxygen species (ROS) by nanoparticles can lead to oxidative stress and thus hinder the growth of micro-organisms (Besinis et al., 2014). The antimicrobial effectiveness of metal and metal oxide nanoparticles depends on the material used, and the size of the nanoparticles. Positively charged nanoparticles bind easily to the negatively charged surface of bacteria (George et al., 2019). In addition, the smaller the size of the

nanoparticles, more effective the nanoparticles in inhibiting the growth of pathogens (Besinis et al., 2014; Seil & Webster, 2012).

Nanoparticles as drug delivery systems

One of the greatest applications of nanoparticles is their ability to encapsulate drugs and deliver them to specific locations inside the body. There are many nanoparticles that have high encapsulating efficiency. To be a suitable drug delivery system in the human body, the nanoparticle must be safe and biocompatible, provide more specific drug delivery, and have low toxicity with therapeutic effects (De Jong & Borm, 2008). The research for a suitable nanocarrier requires considering various aspects such as drug encapsulation capacity, drug release kinetics, immuno-compatibility, target specificity, shelf life, and stability (Gao et al., 2018). The residual material after drug release is another issue that needs to be kept in mind and therefore a biodegradable material is preferable.

The surface chemistry of the particles is important for successful targeting, attachment, and uptake of the nanoparticle-containing drug. A successful nanoparticle-based delivery system must target the desired location, have no opsonization, and prolonged circulation in the blood. This can be achieved by modifying the surface and coating it with polymers or synthesizing nanoparticles with desired co-polymers. For instance, hollow mesoporous silica nanoparticles covered with polyethylene glycol (PEG) are observed to be more stable and have increased life and circulation time in the blood (Zhu et al., 2011). Surface chemistry has also been observed to improve various properties of encapsulated drugs. In a study, titanium oxide (TiO_2) nanoparticles surface functionalized with -OH and -groups were shown to be more effective in killing cancer cells (Thevenot et al., 2008). This kind of improvisations can be used to enhance the killing effects of

nanoparticles by using the synergistic effect of loaded drugs and the surface chemistry of nanoparticles (George et al., 2019).

The surface can be functionalized with proteins, carbohydrates, antibodies, peptides, antibodies, and small molecules to actively target the nanoparticles to a specific site for drug delivery. PLGA nanoparticles surface functionalized with wheat germ agglutinin (WGA) is a strategy used in a study to modify the surface of nanoparticles for local drug delivery to the lungs. WGA, a lectin, has bio-adhesive properties that target the lectin receptors of the alveolar epithelium in the lungs (Sung et al., 2007).

Particle size is another factor that determines the drug loading, drug release, and cellular uptake of the nanoparticle. Control of particle size enables tuning the drug loading and drug release. A smaller nanoparticle has more surface area to volume ratio, therefore less drug is loaded but it stays closer to the cell. The drug release will be a smaller amount but faster rate. A larger nanoparticle on the other side has more volume and thus encapsulates more drugs. The drug released will be a larger amount but slower rate (Redhead et al., 2001).

Nanomaterials commonly used as drug delivery systems

Chitosan nanoparticles are known to be successful drug delivery carriers. Chitosan is obtained from chitin, which is found in the shells of crustaceans such as lobsters and shrimps. It is a natural carbohydrate polymer which is prepared by N-deacetylation of chitin. It consists of 2-deoxy-2-(acetylamino) glucose linked together by β-(1,4) glycosidic linkages. Chitosan nanoparticles (Ch NPs) can be prepared using four techniques including microemulsion, emulsification solvent diffusion, ionotropic gelation, or polyelectrolyte complex (Tiyaboonchai, 2013). Ch NPs are soluble in an aqueous solution of various acids, are biodegradable, have low toxicity, possess

reactive hydroxyl and amine groups, and are easily degraded in the human body Doxorubicin-loaded Ch NPs were prepared by masking the positive charge of doxorubicin by complexing it with dextran sulfate. The modified Ch NPs had double encapsulation efficiency and the loading efficiency was 4.0% doxorubicin (Janes et al., 2001).

Silica nanoparticles possess unique physical properties which makes them suitable for drug loading and targeted drug delivery. MSNs possess large pores which make a large volume available for drug loading. Other unique properties of MSNs are large surface area, biocompatibility, large pore volume, tunable shape and size, and thermal stability. The size of MSNs can be tuned to promote uptake by the cells (Wang et al., 2015). The large surface area and pore size allow for loading of appropriate amount of drug. MSNs also have higher mechanical strength and thus are more tolerant to heat, mechanical stress and pH. Another unique feature of MSNs is the availability of two surfaces, the internal and the external surface (Slowing et al., 2008). The uptake of MSNs into the cells takes place primarily through clathrin-coated endocytosis and some surface functionalized MSNs also are taken up by pinocytosis. Overall, MSNs are suitable for targeted drug delivery.

Liposomes – Liposomes are made of aqueous compartment surrounded by concentric single or multiple lipid bilayers. The size of liposomes can vary between 50-450 nm for use in the medicine. Lipids can be unilamellar i.e., have one layer. They have more aqueous volume and thus are suitable for hydrophilic drug delivery, while the multilamellar liposomes are more suitable for delivery of hydrophobic drug delivery (Immordino et al., 2006). Unilamellar liposomes due to greater volume than multilamellar liposome, can encapsulate larger amounts of drug. Therefore, the drug release of both is different; unilamellar drug release being faster than the multilamellar liposomes.

Polymeric nanoparticles – These nanoparticles are biodegradable and are suitable as drug delivery devices. Although liposomes have been majorly used as drug delivery devices, there are inherent limitations of using liposomes as drug delivery devices such as low drug encapsulation, rapid drug release and poor stability (Gabizon, 2001). Depending on the method of synthesis, polymeric nanoparticles, nanosphere or nano capsules can be synthesized. Nanocapsules are nanosystems where the drug is entrapped and surrounded by a polymer, while nanosphere is matrix system in which the drug is dispersed uniformly. The polymeric nanomaterials are used for gene therapy, to deliver proteins, drugs, peptides as well as to target particular organs (Langer, 2000). The surface of nanoparticles is opsonized which are then recognized by the macrophages, which goes onto to remove the nanoparticles from circulation. In order to protect the nanoparticles from phagocytosis, they can be surface functionalized with poly(ethylene) glycol (PEG) (Owens III & Peppas, 2006).

Dendrimers – Dendrimers are made of repeating units; however, they differ from polymeric nanoparticles due to the fact that they are synthesized in a stepwise manner, rather than polymerization, in order to produce a highly predictable and reproducible structure. The dendrimers are synthesized by repetition of two consecutive steps due to which these layers are added to the outside. Dendrimers have various applications in the biomedical field such as nanoscale containers (Boas et al., 2001), imaging agents (Caravan et al., 1999), drug delivery systems and various other applications.

Inorganic nanoparticles – Inorganic nanoparticles offer various advantages since they are biocompatible, have lower toxicity, and have tunable size and drug release profile. Silver nanoparticles have been used as an antibacterial agent for many years and have been observed to have antibacterial properties against *E. coli, P. aeruginosa,* and *S. aureus* (Moyer et al., 1965).

Nitric oxide releasing nanoparticles (NO NPs) also possess antibacterial properties against various AMR bacteria. On reaching the target bacterial cell, the nitric oxide is able to alter the structure of bacterial cell membrane and modify essential proteins by producing reactive nitrogen species (RNS). It is found to be a successful antibacterial against *Enterococcus faecalis, P. aeruginosa* and *Klebsiella pneumoniae* (Friedman et al., 2011).

Metal nanoparticles have also been explored as possible drug delivery systems. **Gold nanoparticles** have benefits of tunable size, they can be surface functionalized and can be easily traced due to their visible light extinction behavior.

Combination therapy to overcome AMR pathogens

To combat AMR, the development of new strategies that divert from the overuse of antibiotics is required. Combinations of antibiotics or a combination of antibiotics with a suitable adjuvant can be employed to target resistance mechanisms and thus inhibit their growth. There can be three combination of drugs which include two drugs that inhibit the same target in two different ways, two drugs that inhibit two different targets in different pathways and two drugs that inhibit different steps in the same pathway (Cottarel & Wierzbowski, 2007). An important thing to consider while choosing the drugs for combination therapy is that the drugs should have synergistic or additive action and not antagonistic action *i.e.* the mechanism of action of the two drugs should not interfere with each other. Another important thing to consider is that the target cells should be susceptible state i.e. the cells should not be insensitive due to quiescence (Salehi et al., 2015).

Using a combination of an antimicrobial and a drug that can inhibit the resistance mechanisms of the bacteria is one of the most successful methods used in combination therapy. A combination of beta-lactam drug amoxicillin and beta-lactamase enzyme inhibitor clavulanate is one such

instance. The use of the combination of beta-lactam drug and beta-lactamase enzyme inhibitor in treating *Acinetobacter* and *Stenotrophomonas maltophilia* has shown promising results and warrants for exploration of more such combinations (Lee et al., 2003).

Combination therapy is readily used in treatment of cancer due to additive effect of various chemotherapeutic drugs. One of the biggest issues with using a single free chemotherapeutic drug is the toxicity of the drug to healthy cells surrounding the cancer cells. Due to synergistic effect, the concentration of drugs required in the combination is lower than when used individually, and this reduces the overall toxicity of the combination. In addition, (Mokhtari et al., 2013). Combination of trimethoprim and sulfamethoxazole is commercially sold under the name Bactrim© (Roche) where each of the drug inhibits the pathway of folic acid synthesis and also inhibits a specific step in the pathway of nucleotide synthesis (Cottarel & Wierzbowski, 2007). A significant and immensely promising advancement in the field of therapy is nanoparticle-based combination therapy.

AMR mechanisms in bacteria render various antimicrobials useless. A combination of antibiotic and inhibitors of bacterial resistance mechanism can be employed to overcome the problem of antimicrobial resistance. An ideal combination is where the antimicrobial is focused on killing the cell and the resistance mechanism inhibitor is focused on overcoming the resistance mechanism. The combination of antibiotic and efflux pump inhibitors for treatment where efflux pumps act as resistance mechanisms is an important method of overcoming the resistance to that antibiotic. For instance, bis benzimidazole (BBZ), which is known to have antibacterial activity against gram-negative and gram-positive bacteria is used in combination with Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which inhibits the action of efflux pump removing BBZ out of the cell, proves to be a more effective antibacterial agent, than BBZ individually (Sinha et al., 2017). The MIC of

ciprofloxacin-resistant *Acinetobacter* was reduced 2 to 64-fold when CCCP, an efflux pump inhibitor was used in combination with ciprofloxacin (Ardebili et al., 2014). Therefore, to circumvent various AMR mechanisms, combination therapy has shown to be promising and demands continuous exploration of unique and novel combinations.

However, there are some limitations to using oral or intravenous drug delivery methods for cancer treatment and other diseases as well such as limited bioavailability, low solubility, and low stability. Limited solubility poses a huge problem as most of the synthetic drugs or drugs obtained from plants being hydrophobic require formulations which can be toxic. Nanoparticle-based combination therapy is a field that has continuously shown promising results (Babos et al., 2018; Janes et al., 2001; Slowing et al., 2008). Nanoparticles can encapsulate drugs and protect them from the external environment to release the drugs in controlled manner.

Nanotechnology enabled combination therapy to overcome AMR mechanisms

There are various benefits of encapsulating combination of drugs in nanoparticles since nanoparticles provide improved solubility of insoluble or poorly soluble drugs, prolonged half-life due to reduced immunogenicity, targeted drug delivery, minimized toxicity to surrounding cells, and sustained and controlled drug release. Various nanoparticle systems have been approved to be used as therapeutic products. Encapsulation of drugs into nanoparticles aims at providing synergism of drugs in addition to the benefits of nanoparticles-based drug delivery (De Jong & Borm, 2008; Zhang et al., 2008).

The use of nanoparticles for delivery of a combination of cancer drugs to overcome drug resistance is being widely explored and many studies have shown the therapy to be successful. Commonly used nanoparticle systems include liposomes, polymeric nanoparticles, polymer-drug conjugates,

dendrimers, etc. Various cellular mutations enable the cancer cells to prevent the action of drugs, amongst which the efflux pumps and altered apoptosis are the main two mechanisms that cancer cells use to avoid the action of diverse types of drugs (Gottesman, 2002). Upregulation of efflux pumps that belong to ATP binding cassette superfamily (ABC) leads to extrusion of drugs actively out of the cell, leaving behind concentrations which are no longer affective in killing the cell.

Similarly, in bacteria efflux pumps lead to extrusion of the antimicrobial out of the cell, reducing its concentration intracellularly and thus protecting the target from the action of antimicrobial. The significance of efflux pumps in conferring multi-drug resistance in many bacteria has encouraged the exploration and development of efflux pump inhibitors (EPIs). Various EPIs have been designed based on the structure and mechanism of working of commonly present efflux pumps. Use of EPIs along with antimicrobials in expected to reverse the AMR against some useful drugs such as tetracyclines, fluoroquinolones and aminoglycosides, and thus preserve their antibacterial efficacy (Costa et al., 2013; Du et al., 2018).

In cancer cells, for instance, P-glycoprotein (P-gp), efflux pump driven by ATP, is responsible for extrusion of various drugs such doxorubicin, vinblastine, and paclitaxel (Li et al., 2008). Nanoparticles, on the other hand, can enter the cell through endocytosis; therefore, they can deliver the drug into the cell away from efflux pump spanning cell membrane (Mishra et al., 2018). The first attempt to encapsulate chemosensitizer and a chemotherapeutic was polyalkylcyanoacryl nanoparticle loaded with DOX and cyclosporin A (CyA), which is a P-gp pump inhibitor. The combination of CyA and DOX showed the most effective growth rate inhibition, as the combination showed synergistic effect. CyA effectively inhibits the efflux pump and thus increases sensitivity of the cell towards DOX (Soma et al., 2000).

Similarly, the efflux pump resistance mechanism is a vital resistance mechanism for bacterial pathogens to stay protected from various antimicrobials.

AMR bacterial pathogens pose a huge problem in the food industry as well as healthcare. Nanoparticle-based drug delivery system can revolutionize the drug delivery and eliminate the infection at the starting point in the food chain, i.e. the farm animals. Naturally obtained efflux pump inhibitors are also being explored and are able to reduce the MICs of drugs. For instance, in a wild strain of *S. aureus*, three compounds isolated from *Dalea* species, the 'smoke tree', namely arylbenzofuran aldehyde and isoflavone, were able to enhance the antimicrobial property of berberine against a wild-type strain of *S. aureus.* The MIC of berberine was seen to decrease 4 to 8-fold (Belofsky *et. al*,2006). Bacterial pathogens resistant to antimicrobials are a huge problem in the food industry and the healthcare system and employing a suitable antimicrobial-adjuvant combination encapsulated in nanoparticle drug delivery system is one answer to this issue.

Drug loaded nanoparticles can attack the normal cells and inflict injury. Therefore, cytotoxicity is one of the most common side-effects of drug delivery which can be overcome by targeting the nanoparticle to the target cells and making them target specific. Functionalizing the nanoparticles with cell-specific ligands can prevent an attack on the normal cells. To reduce cytotoxicity and improve target specificity, transferrin conjugated liposomes encapsulated with DOX and verapamil were developed. The formulations accumulated faster in drug-resistant leukemia cells (K562) in addition to reversing MDR (Wu et al., 2007).

Knowledge gaps

There are various benefits of nano-enabled combination therapy over traditional forms of drug delivery such as increased target specificity, controlled drug release, higher stability, and increased

bioavailability. The tunable size, material, structure, and chemistry of nanoparticles enable development of nanoparticle systems for the wide variety of drugs and target tissues (Bhatia, 2016; De Jong & Borm, 2008). To design and develop therapeutics against MDR bacterial pathogens, however, it is vital to understand the properties of target pathogens, and suitable combinations and nanoparticle materials. Specifically, more exploration is required to understand:

1. Prevalence and mechanism of AMR in animal farms: The first step is to understand the extend of spread of resistance to antibiotics, antibiotics to which high frequency of resistance is observed and the source of the AMR pathogens. Additionally, it's important to understand the resistance mechanisms employed by the AMR bacteria to overcome the action of antibiotics to focus on antibiotics against which there is a high frequency of resistance and the ones that are medically important to human healthcare. Recognition of resistance mechanisms allows for the design and development of treatment custom-designed to target the prevalence AMR mechanisms.
2. Virulence characteristics: Pathogenic bacteria can persist in the host by using various virulence characteristics. For example, the ability of AMR pathogenic *Salmonella enterica* to infect the host gut intracellularly, provides the bacteria the ability to derive nutrients from the host and proliferate (Bumann & Schothorst, 2017).
3. Combinations of antibiotics and adjuvants: To sensitize bacterial pathogens to existing antibiotics to which the bacteria have developed resistance, it is important to use adjuvants in addition to antibiotics. Adjuvants should be entities that can target the resistance mechanism, such as an efflux pump blocker, which can allow antibiotic to act on the cell.
4. Nano delivery platform: Traditional delivery methods leave the drug prone to harsh environments, and enzymatic actions inside the body (Yan Chan Edgar & Wang, 2017).

Nanoencapsulation of the drugs is vital to overcome these drawbacks. Appropriate material, which is non-toxic, biodegradable, and biocompatible must be chosen to synthesize nano delivery systems.

The application of nanoparticles in the field of medicine can advance and grow further, which requires deep understanding of the resistance mechanisms, the site of infection, and the host specificity of pathogens. To successfully overcome AMR, the nanoparticle designed must be able to reach the pathogen at the site of infection, target drug release at the pathogen, and the included drugs must be able to attack the resistance mechanism (Gao et al., 2018; Rudramurthy et al., 2016). Development of nanoparticle-based therapeutics to treat diseases and overcome AMR is promising. However, with all the benefits and possible uses, it still includes some possible risks. This requires focused studies and analysis of the potential risks of using new nanomaterials to humans and the environment. The next step would be to research about large scale production of complex nanoparticles and make it affordable, in addition to testing the possible long-term health implications and effectiveness.

Conclusion

The review discusses the issue of AMR in food animals, use of nanotechnology in medicine and the application of nanoparticle-based drug delivery in overcoming AMR. Antimicrobial use in farm animals, for disease treatment or growth, has contributed to the increased antimicrobial resistance amongst pathogens found in farms. Due to growing burden of AMR globally, the healthcare sector as well as the food industry requires the advent of a revolutionary treatment. Nanoparticles have been explored for long for their application as drug delivery systems and many studies have shown promising results. In addition, loading combination of drugs which have synergistic or additive affect is another method with favorable outcomes. Nanoparticle-based

combination therapy can also be applied to overcome drug resistance in bacterial pathogens. For instance, multidrug loaded nanoparticles possessing antimicrobials and resistance mechanism inhibitor seems to have a promising future in treating infections by MDR pathogens. Adequate amount of research and impact analysis of side effects of nanoparticles are required before their translation onto large scale for treatment of diseases.

CHAPTER III: UNDERSTANDING THE PREVALENCE AND MECHANISMS OF AMR AMONG SALMONELLA AND STAPHYLOCOCCUS ISOLATES FROM QUEBEC ANIMAL FARMS

Abstract

In this study, four *Staphylococcus* isolates (*Staphylococcus aureus* M12, *Staphylococcus aureus* M17, *Staphylococcus hyicus* M43, and *Staphylococcus hyicus* M48) and three *Salmonella* isolates *(Salmonella enterica* serovar Choleraesuis, *Salmonella enterica* serovar Typhimurium, and *Salmonella enterica* serovar *Dublin*) obtained from animal farms in Quebec were characterized for antibiotic (n=20) resistance, antimicrobial resistance (AMR) mechanisms, and virulence factors. Combinations of antibiotics and adjuvants consisting of efflux pump inhibitors (EPIs) such as chlorpromazine (CPZ), beta-lactamase inhibitor-tazobactam, and essential oils (EOs) were tested for possible synergistic effects against the most resistant isolates (*S. aureus* M12 and *S. enterica* ser. Typhimurium) using checkerboard assay. All isolates were resistant to more than one antibiotic, with the highest resistance being observed in *S. enterica* ser. Typhimurium. Efflux activity was highest among *S. enterica* ser. Dublin and *S. aureus* M12, whereas *S. enterica* ser. Typhimurium had high beta-lactamase activity. *Staphylococcus* isolates were strong biofilm formers and manifested complete hemolysis of the sheep red blood cells, whereas *Salmonella* isolates formed weak or no biofilm and caused partial hemolysis. The calculated fractional inhibitory concentration index (FICI) showed a synergism of ampicillin-tazobactam (FICI 0.14), ampicillin-cinnamon EO (FICI 0.38), and chloramphenicol-oregano EO (FICI 0.5) against *S. aureus* M12, while the combination of ampicillin-tazobactam (FICI 0.23) showed synergism against *S. enterica* ser. Typhimurium. Our study thus shows the potential of combination therapy to circumvent resistance mechanisms in multi-drug resistant pathogens prevalent in livestock farms.

Keywords: Antibiotic-adjuvant combination; Combination therapy; Antimicrobial resistance; *Staphylococcus; Salmonella*; Efflux activity; Beta-lactamase activity; Biofilm formation; Antimicrobial synergism; Animal farms

Introduction

The prevalence of pathogenic microorganisms in food animals and their possible zoonotic spread through direct contact or the food chain pose public health concerns (Martin et al., 2015; Martins et al., 2015). Common bacterial pathogens infecting food animals include *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp., *Clostridium perfringens*, and *Streptococcus suis* (Aarestrup et al., 2008). These bacteria cause enteric, respiratory, soft-tissue, and septicemic infections often requiring treatment with antibiotics. In addition, antibiotics are used as prophylactics and as antimicrobial growth promoters (AGPs); although, since 2018 the use of AGPs has been regulated in Canada (Canadian Pork Council, 2018). There is a clear relationship between antibiotic use in animals and the occurrence of antimicrobial-resistant (AMR) bacteria in human infections (T. Khanna et al., 2008; Martin et al., 2015; Park & Ronholm, 2021). For instance, zoonotic transmission of multidrug-resistant (MDR) *Salmonella enterica* serovar Typhimurium Definitive Type 104 (DT104) was reported in 1998 (Glynn et al., 1998). Transmission of methicillin-resistant *Staphylococcus aureus* (LA-MRSA) CC398 from swine to humans was first reported in the Netherlands in 2004 (Park & Ronholm, 2021). The emergence of AMR pathogens in animals and zoonotic potential necessitates the development of alternate therapeutics for disease management of MDR pathogens.

A combination therapy that involves concurrent treatment with two or more drugs having complementary or augment mechanisms of action to surpass the resistance mechanisms has been used for treating recalcitrant bacterial infections. While studies have shown a positive effect of combining antibiotics and adjuvants (MacNair et al., 2018; Yap et al., 2013) a rational approach would be to systematically combine antibiotics and adjuvants and build therapeutic solutions that are specific for certain AMR profiles of pathogenic bacteria. If commonly used antibiotics are

combined with suitable adjuvants that are custom tailored to target specific resistance mechanisms, the combinations can show improved antibacterial efficacy against MDR pathogenic bacterial isolates.

In this study, *Staphylococcus* and *Salmonella* isolates from Quebec-based farms were studied for AMR and AMR mechanisms. Resistance mechanisms such as efflux pump activity and beta-lactamase enzyme activity were investigated, in addition to heavy metal resistance and virulence factors such as biofilm formation, and hemolysis relevant for bacterial survival inside the host body. Bacterial isolates with high resistance to tested antibiotics (*Staphylococcus aureus* M12 and *Salmonella enterica* serovar Typhimurium) were selected to screen antibiotic-adjuvant combinations using a checkerboard assay. Results from our study show the effectiveness of specific combinations of antibiotics and adjuvants in eliminating MDR *S. aureus* M12 and *S. enterica* ser. Typhimurium.

Materials and Methods

Bacterial isolates. Seven pathogenic bacterial isolates from Quebec farm animals, submitted to the Complexe de diagnostic et d'épidémiosurveillance vétérinaires du Québec (CDEVQ) for diagnostic purposes with diseases such as exudative epidermitis, diarrhea, or septicemia were selected for this study. The isolates consisted of *Salmonella enterica* serovar Choleraesuis, *Salmonella enterica* serovar Dublin, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* M12, *Staphylococcus aureus* M17, *Staphylococcus hyicus* M43, and *Staphylococcus hyicus* M48. The cultures were maintained on tryptic soy agar (TSA) containing 5% sheep blood plates (Sigma Aldrich, Canada) and for each assay, isolates were grown in Mueller Hinton Broth (MHB).

S. aureus ATCC 25923 and *E. coli* ATCC 25922 (Oxoid, Thermo Fischer Scientific, Canada) were used as quality control (QC) strains.

Antimicrobial susceptibility determination. The susceptibility of bacterial isolates to 24 antibiotics (Oxoid, Thermo Fischer Scientific, Canada) (Supplementary information Table S3.1) was determined by the Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institutes Guidelines (Clinical Laboratory Standards Institute, 2021; Hudzicki, 2009) and QC strains were used as controls.

Determination of efflux activity. Efflux activity of the isolates was measured as described previously (Paixão et al., 2009). Briefly, bacterial cells cultured overnight at 37°C, were washed thrice in Phosphate buffered saline (PBS) (1X) and adjusted to McFarland 1.0. Ethidium bromide (EtBr) (3 µg/mL) was added to the bacterial suspension. Subsequently, EPIs - 3 µg/mL carbonyl cyanide m-chlorophenyl hydrazone (CCCP) for *Salmonella* and 30 µg/mL chlorpromazine (CPZ) for *Staphylococcus* isolates were added to allow maximum intracellular accumulation of EtBr under shaking at 25°C for 60 min. Cells were then resuspended in 1 mL of PBS and transferred to a 96-well plate. Glucose (0.4% v/v) was added to trigger the efflux of EtBr and fluorescence was measured continuously for 40 min at 37°C using a plate reader at 530-590 nm (excitation/emission) (SpectraMax i3, Molecular Devices, USA). Time ($T_{1/2}$) taken for the extrusion of half the amount of EtBr was measured. $T_{1/2}$ of isolates was compared based on which the efflux activity was determined. *S. aureus* 10812464, originally isolated from a case of bovine mastitis and known to have efflux activity as seen in our previous studies, and *S. enterica* ser. Typhimurium SL1344 was used as a positive control.

Beta-lactamase assay. Bacterial isolates grown overnight were adjusted to McFarland 1.0. Ampicillin (50 µg/mL) was added to the cell suspension and incubated at 37°C for 3 h. Cells were then centrifuged at 9,000 x *g* for 10 min and washed in sodium phosphate buffer (pH-7.0). Pelleted cells were resuspended in buffer, sonicated on ice for 3 min, and centrifuged at 17,500 x *g* for 25 min. The cell-free extract was used for Nitrocefin assay (Majumder et al., 2021). Nitrocefin (abcam, Canada) solution of 10 µL was mixed with 10 µL of the cell-free extract, and the final volume was adjusted to 100 µL in wells of a 96 well plate. The absorbance was detected in kinetic mode for 10 min at 390 nm using a plate reader (supplementary information 2). The enzyme activity was calculated using the formula:

$$\text{beta-lactamase enzyme activity} = \frac{S_a}{Reaction\ time\ X\ S_v}$$

Where S_a is the amount of nitrocefin (in µM) hydrolyzed in the unknown sample between t_1 and t_2 of the standard curve; reaction time is the time difference of t_1 and t_2, and S_v is the sample volume (in mL). Beta-lactamase activity is reported as U/mL.

Biofilm formation. The ability of the bacterial isolates to form a biofilm was determined using a crystal violet assay (Xu et al., 2016). Briefly, bacterial cells were grown overnight at 37°C under shaking. The cell suspensions (0.5 McFarland) of 200 µL were added to the wells of two 96 well plates. One plate was incubated for 24 h whereas, the other for 48 h. The wells were washed with saline 3 X to remove any non-adherent cells and the adherent cells were fixed with 200 µL of 99% methanol. After 15 min, the methanol was removed, and the plates were air-dried. A 200 µL aliquot of crystal violet dye (0.2%) was added to the wells. After 40 mins, the wells were washed, resuspended with 200 µL of acetic acid (33%), and absorbance was measured at 570 nm. The ability of bacterial isolates to form a biofilm was categorized as strong, moderate, weak, or no

biofilm formation based on critical optical density (OD_c) compared to the optical density of the sample (OD_s) as described in Supplementary information 4.

Hemolysis of blood agar plates. Bacterial isolates were tested for the manifestation of hemolysins as detailed previously (Buxton, 2005).Briefly, bacterial isolates were grown overnight in MHB and streaked onto blood agar plates (TSA with 5% defibrinated sheep blood). The plates were incubated at 37°C for 24 h. The pattern of hemolysis was detected by visual inspection for the translucency around the bacterial colony (supplementary information 5).

Testing combinations of antibiotics and adjuvants. Ampicillin, tetracycline, and trimethoprim were tested for synergism as they are commonly used in food animals and humans (Agriculture & Horticulture Development Board (AHDB), 2020; World Health Organization, 2017). The use of chloramphenicol is restricted for treating livestock animals but is categorized as a highly important antimicrobial in human medicine (World Health Organization, 2017). The selected adjuvants consisted of EPI (CPZ), beta-lactamase inhibitor (tazobactam), and EOs (thymol EO, cinnamon EO, and oregano EO) with various modes of action reported. The composition of EOs has been determined (Bagheri et al., 2020).

MICs of the antibiotics and adjuvants were determined against *S. enterica* ser. Typhimurium and *S. aureus* M12 using the microtiter broth dilution method. Checkerboard assay was used for categorizing the type of antimicrobial effect exhibited by tested combinations (Orhan et al., 2005). Antibiotics were diluted column-wise, and the adjuvants were diluted row-wise in a 96-well plate. Each well contained a unique concentration of drug and adjuvant to which, 10 µL of cells (0.5 McFarland) were added. 96-well plates were incubated at 37°C overnight. Cell viability was checked using a resazurin assay (Supplementary material 3). The well with no visible growth of

bacteria (baseline reading for fluorescence) were considered for determining the MIC of the combination. Fractional inhibitory concentration index (FICI) was calculated according to the following formula.

$$FIC_A=\frac{A}{MIC_A};\ FIC_B=\frac{B}{MIC_B}$$

$$FICI= FIC_A+ FIC_B$$

Where *A* and *B* are the MICs of component A and B in combination respectively. FICI values <0.5, <1, <4 and >4 were categorized as synergistic, additive, indifferent and antagonistic, respectively.

Results

Antimicrobial susceptibility. All the seven bacterial isolates were resistant to more than one antibiotic, as shown in Figure 3.1. Resistance to tetracycline and spectinomycin was observed in all the isolates. Notably, all tested isolates were resistant to one or more beta-lactam drugs except *S. hyicus* M48. *S. enterica* ser. Typhimurium was the most resistant isolate, with a resistance to 75% of the tested antibiotics, followed by *S. enterica* ser. Dublin (58.3%). *S. hyicus* M48 resisted only 12.5% of tested antibiotics and was the most susceptible bacterial isolate.

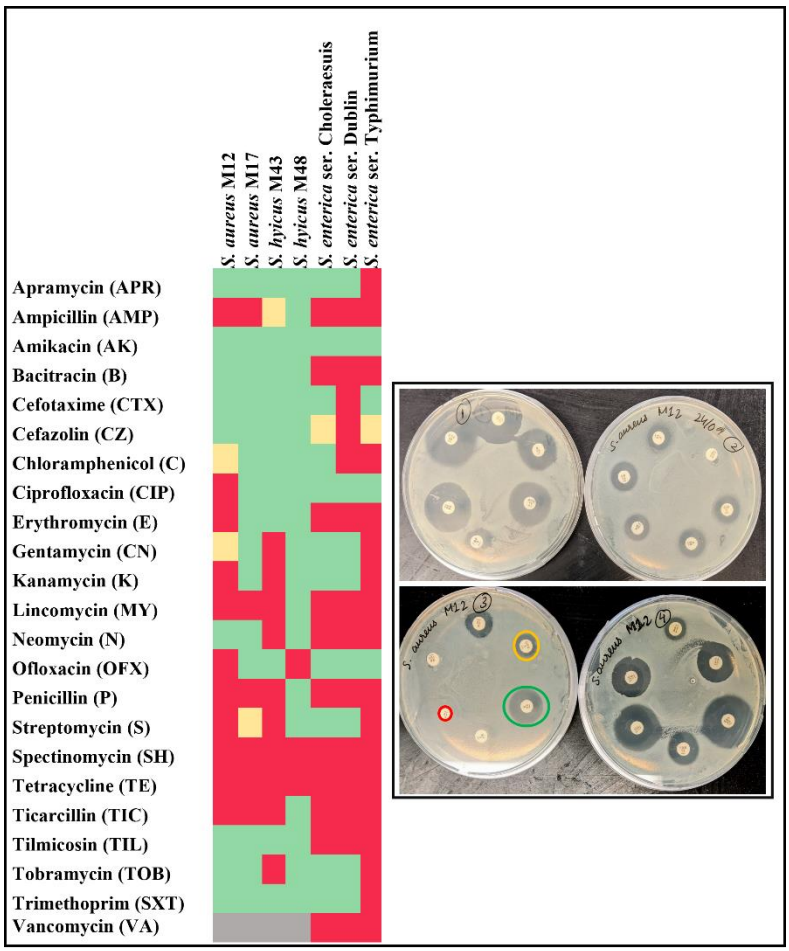


Figure 3.1. Antimicrobial susceptibility of bacterial isolates: Antimicrobial susceptibility of bacterial isolates was tested using Kirby-Bauer disk diffusion assay. Disks containing antibiotics were placed on the lawn of bacteria and incubated overnight at 37°C (as seen in the inlet). Zone of inhibition was measured, and isolates were categorized as resistant (red), intermediate (yellow), susceptible (green), and test not applicable (grey) according to CLSI guidelines. The heat map is based on susceptibility according to CLSI standards.

Interestingly, *S. enterica* ser. Typhimurium showed high resistance to ampicillin and survived concentrations of ampicillin up to 1000 µg/mL. Therefore, we monitored the growth curve of the bacteria by measuring OD at 600 nm continuously for 36 h at set intervals (Figure 3.2). It was observed that at higher concentrations of ampicillin (250, 500, and 1000 µg/mL), the cell population initially declined. Surprisingly, the survived cells resumed growth and even proliferated rapidly to reach cell densities higher than the cells with no ampicillin treatment (Supplementary information Figure S3.1).

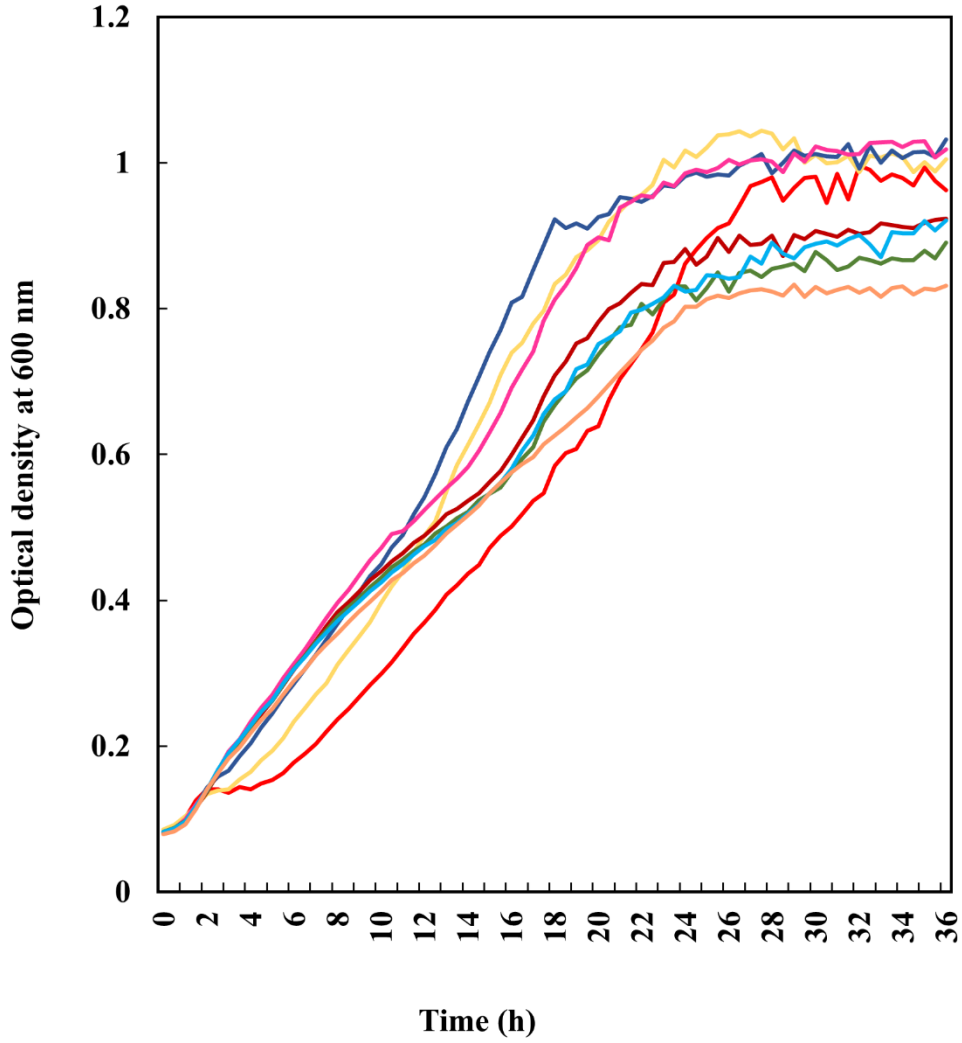


Figure 3.2. **Ampicillin induced cell proliferation in *S. enterica* ser. Typhimurium:** Growth curve of *S. enterica* ser. Typhimurium at extreme concentrations of ampicillin 1000 µg/ml (red) (1), 500 µg/ml (yellow) (2), 250 µg/ml (dark blue) (3), 125 µg/ml (pink) (4), 62.5 µg/ml (maroon) (5), 31.3 µg/ml (green) (6), 15.6 µg/ml (light blue) (7), and 0 µg/ml (orange) (8). Bacterial cells obtained from overnight culture were added with incremental concentrations of ampicillin and monitored at OD_{600} for 36 h.

Efflux activity. Amongst *Salmonella* isolates, *S. enterica* ser. Dublin showed the highest efflux activity, followed by *S. enterica* ser. Choleraesuis and *S. enterica* ser. Typhimurium (Figure 3.3(A)), with $T_{1/2}$ for EtBr extrusion as 241.56 s, 323.23 s, and 413.43 s respectively. Among *Staphylococcus* isolates, *S. aureus* M12 had the highest efflux activity, followed by *S. aureus* M17 and *S. hyicus* M43, with $T_{1/2}$ for EtBr extrusion as 240.4 s, 448.5 s, and 885.3 s, respectively. No efflux activity was observed for *S. hyicus* M48 (Figure 3.3(B)).

Beta-lactamase assay. All the tested isolates, except *S. hyicus* M48 showed beta-lactamase activity (Figure 3.3(C)). The highest beta-lactamase activity was observed in *S. enterica* ser. Typhimurium (84.73 U/mL), followed by *S. enterica* ser. Choleraesuis (72.62 U/mL) and by *S. enterica* ser. Dublin (67.52 U/mL). The least beta-lactamase activity was observed in *S. aureus* M17 (52.12 U/mL).

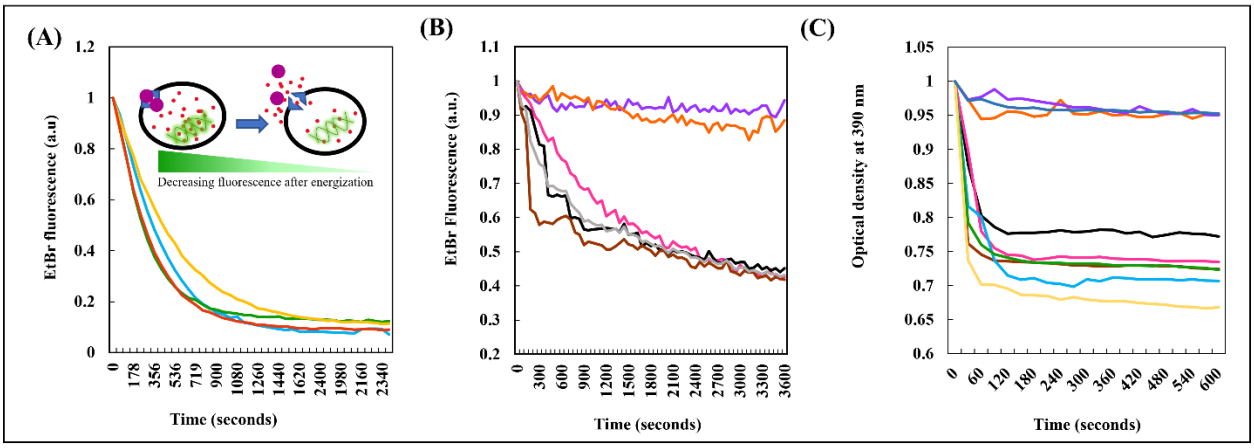


Figure 3.3. Efflux and beta-lactamase enzyme activity: Efflux pump activity in *Salmonella* (A) *and Staphylococcus* (B) isolates was assessed using EtBr as the substrate. Extrusion of EtBr was measured in kinetic mode by measuring the fluorescence at 530-590 nm (excitation/emission) after energizing the cells. (C) Beta-lactamase activity of *Staphylococcus* isolates (*S. aureus* M12 (67.10 U/mL), *S. aureus* M17 (52.12 U/mL), *S. hyicus* M43 (63.85 U/mL), *S. hyicus* M48 (no activity), *S. aureus* ATCC 25923 (no activity)), *Salmonella* isolates (*S. enterica* ser. Choleraesuis (72.62 U/mL), *S. enterica* ser. Dublin (67.52 U/mL), *S. enterica* ser. Typhimurium (84.73 U/mL)), and *E. coli* ATCC 25922 (no activity) was obtained by measuring nitrocefin hydrolysis. The cell-free extract was used as a source of beta-lactamase enzyme to determine time-dependent nitrocefin hydrolysis by measuring absorbance at 390 nm using a plate reader. *S. enterica* ser. Choleraesuis (light blue), *S. enterica* ser. Dublin (green), *S. enterica* ser. Typhimurium (yellow), *S. enterica* ser. Typhimurium SL1344 (red), *S. aureus* ATCC 25923 (purple), *S. hyicus* M48 (orange), *S. aureus* M43 (pink), *S. aureus* M17 (black), *S. aureus* M12 (brown), *S. aureus* 10812464 (grey), *E. coli* ATCC 25922 (dark blue).

Biofilm formation. The biofilm formation of bacterial isolates is compared in Figure 3.4. After 24 h, *S. enterica* ser. Choleraesuis, *S. enterica* ser. Dublin and *S. enterica* ser. Typhimurium SL1344 formed no biofilm while weak biofilm formation was observed for *S. aureus* M17, *S. hyicus* M48, and *S. enterica* ser. Typhimurium. *S. aureus* M12 and *S. hyicus* M43 formed moderate biofilm. After 48 h of incubation, *S. aureus* M12 and *S. hyicus* M43 formed stronger biofilm while there was no change in biofilm-forming ability for *S. hyicus* M48 and *S. enterica* ser. Typhimurium even after 48 h. *S. aureus* M17 formed weak biofilm, while no biofilm formation was observed in *S. enterica* ser. Choleraesuis and *S. enterica* ser. Dublin even after 48 h.

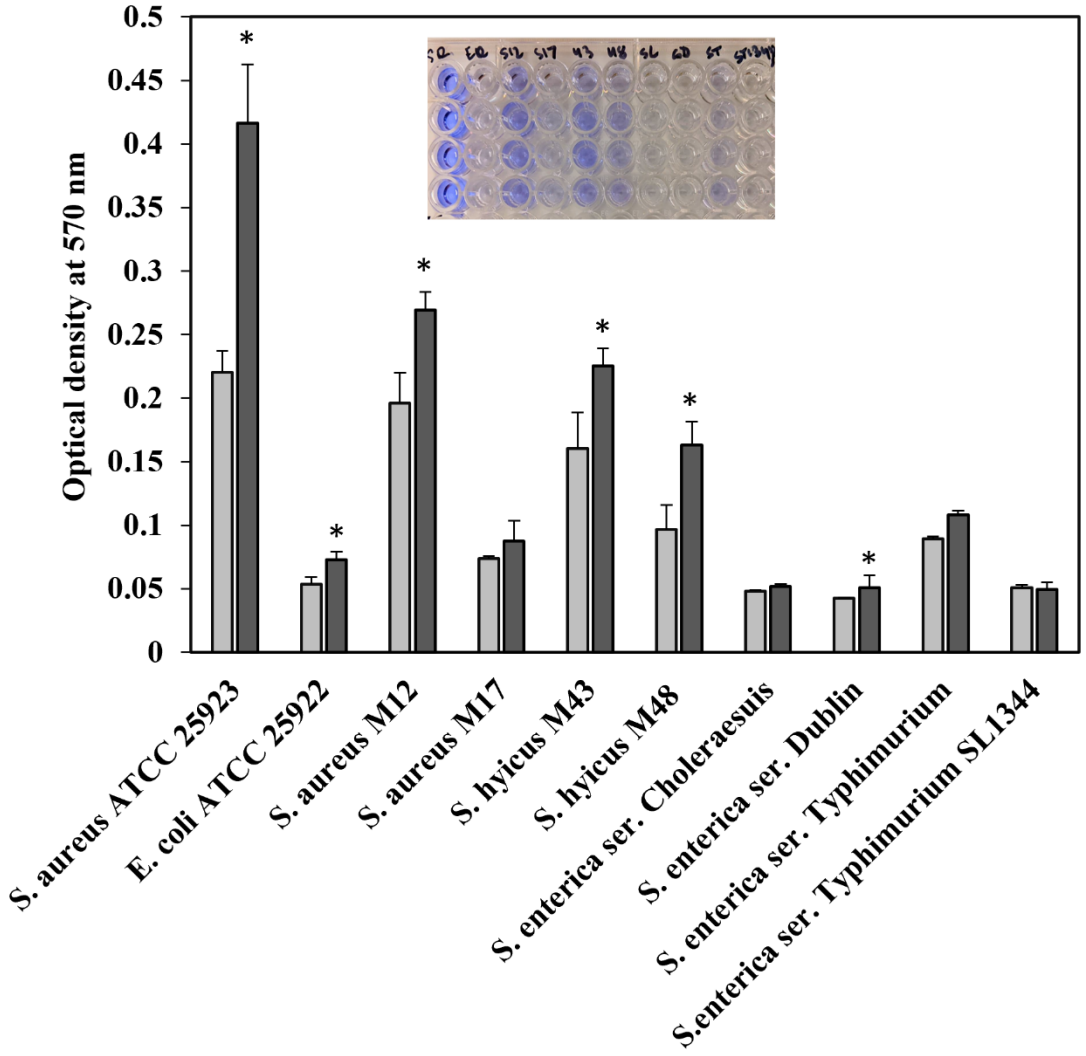


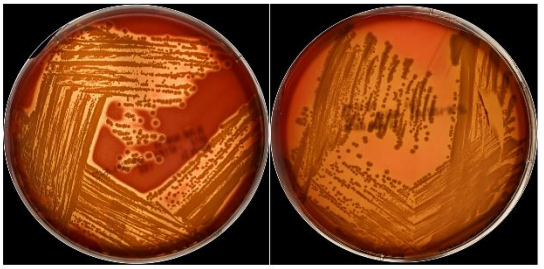
Figure 3.4. Biofilm formation assay: Biofilm formation of *Staphylococcus* and *Salmonella* isolates from animal farms measured using crystal violet assay after 24 h (light grey) and 48 h (dark grey). Inlet shows the image of one of the plates used for biofilm assay. A significant difference in biofilm formation ability ($p < 0.05$) is marked as '*'.

Hemolysis of blood agar plates. Results of hemolysis are summarized in tabular data figure 3.5(A)**.** A clear zone of hemolysis (beta-hemolysis) was observed in S. *aureus* M12 (Figure 3.5(B)), *S. aureus* M17, S. *hyicu*s M43 while partial hemolysis or green discoloration (α-hemolysis) was observed in S. *hyicus* M48. *Salmonella* isolates showed green discoloration (Figure 3.5(B)) around the bacterial colonies, and thus categorized as hemolysin-producing isolates.

(A)

	α hemolysis	β hemolysis
S. aureus ATCC 25923	-	+
S. *aureus* M12	-	+
S. aureus M17	-	+
S. hyicus M43	-	+
S. hyicus M48	+	-
	Hemolysis	**No hemolysis**
S. enterica ser. Choleraesuis	+	-
S. enterica ser. Dublin	+	-
S. enterica scr. Typhimurium	+	-

(B)



***Staphylococcus aureus* M12** ***Salmonella enterica* ser.Typhimurium**

Figure 3.5. Hemolysis assay: Bacterial isolates were streaked onto tryptic soy agar plates containing 5% defibrinated sheep blood and incubated overnight. Hemolysis activity was assessed based on characteristic translucency around bacterial colonies. (A) Tabular data summarizing the type of hemolysis activity in tested isolates. (B) *S. aureus* M12 produced translucent halo around the cultured colonies – beta-hemolysis, *S. enterica* ser. Typhimurium showed hemolysis (green discoloration).

Checkerboard assay. Results of the checkerboard assay are summarized in Table 3.1. For *S. enterica* ser. Typhimurium, the ampicillin-tazobactam combination showed synergistic interaction (FICI 0.23) and tetracycline-cinnamon EO showed additive interaction (FICI<1). Chloramphenicol-CPZ, chloramphenicol-thymol, and tetracycline-CPZ combinations showed additive interaction in both *S. aureus* M12 and *S. enterica* ser. Typhimurium (FICI<1). In *S. aureus* M12, synergistic interaction was observed with ampicillin-tazobactam (FICI 0.14), ampicillin-cinnamon EO (FICI 0.38), and chloramphenicol-oregano EO (FICI 0.5). Ampicillin-thymol, ampicillin-oregano EO, and tetracycline-oregano EO combinations were observed to have additive interactions against *S. aureus* M12.

	S. enterica ser. Typhimurium					*S. aureus* M12				
	CPZ	TAZ	TML	CEO	OEO	CPZ	TAZ	TML	CEO	OEO
AMPICILLIN	IN	**SYN**	IN	IN	IN	IN	**SYN**	**ADD**	**SYN**	**ADD**
CHLORAMPHENICOL	**ADD**	IN	**ADD**	IN	IN	**ADD**	IN	**ADD**	IN	**SYN**
TETRACYCLINE	**ADD**	IN	IN	**ADD**	IN	**ADD**	IN	IN	IN	**ADD**
TRIMETHOPRIM	IN	IN	IN	IN	IN	IN	IN	IN	IN	IN

Table 3.1. Antibacterial effect of combinations of antibiotics and adjuvants

* Combinations of antibiotics (ampicillin, chloramphenicol, tetracycline, and trimethoprim) and adjuvants (efflux pump inhibitor chlorpromazine (CPZ), beta-lactamase inhibitor tazobactam (TAZ), essential oils – thymol (TML), cinnamon essential oil (CEO) and oregano essential oil (OEO)) were tested against *S. enterica* ser. Typhimurium and *S. aureus* M12 using checkerboard assay; synergistic (SYN), additive (ADD), indifferent (IN).

Discussion

Initially, seven isolates from animal farms in Quebec were characterized which gave an insight into the co-occurrence of antibiotic and metal resistance (supplementary information 3), mechanisms of resistance, and virulence factors in the bacterial isolates. The antimicrobial susceptibility profile showed that most of the pathogens were resistant to tetracycline, ampicillin, lincomycin, and penicillin, which include some of the most used antibiotics in agriculture (Rajić et al., 2006).

S. enterica ser. Typhimurium was observed to have extremely high resistance to ampicillin, up to a concentration of 1000 µg/mL. During incubation of bacterial cells in extremely high concentrations of antibiotic (500 µg/mL and 1000 µg/mL), the cell density decreased initially which subsequently regained and outgrew the bacteria grown in culture media alone. This observation concurred with *Salmonella* clinical isolates from humans, which survived extremely high ampicillin concentrations(Uddin & Ahn, 2018). The phenomenon of antibiotic-induced (doxycycline) cell growth has been reported in *E. coli* (Reding-Roman et al., 2017). The mechanism of antibiotic-induced cell proliferation is ascribed to mutations conferring bacteria with higher growth rate potential but at the expense of genes essential for stress protection (Reding-Roman et al., 2017). Further studies are warranted for exploring the differential expressions genes in *Salmonella* growing in extremely high concentrations of antibiotics.

MDR bacteria use multiple mechanisms to overcome the action of antimicrobials, such as active efflux or enzymatic degradation of the antimicrobial, mutation of an antimicrobial target site, and reduced cell wall permeability (Nishino et al., 2006; Shahid et al., 2009; Yap et al., 2013). In *Salmonella,* the most common efflux pump system, belonging to the RND family is the AcrAB-

TolC, which is responsible for resistance to various antibiotics (Uddin & Ahn, 2018). We observed efflux activity in the tested *Salmonella* isolates which might explain their strong resistance towards several tested antibiotics. Similarly, the action of chromosome-mediated efflux pumps such as Nor and plasmid-mediated Qac efflux pumps (Floyd et al., 2010) reported to extrude biocides and antibiotics could explain the efflux activity found in *Staphylococcus* isolates in our study.

Resistance to beta-lactam drugs is frequently observed in *Salmonella* and *Staphylococcus* isolates originated from retail meat and food animals in the United States and Canada (McDermott et al., 2016; Narvaez-Bravo et al., 2016). One of the major mechanisms of resistance is the action of the beta-lactamase enzyme which hydrolyzes the beta-lactam ring. Beta-lactamase enzyme in *Staphylococcus* strains is mediated by blaZ genes (Overesch et al., 2011), which in *Salmonella* is by genes such as bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV}, $bla_{\text{OXA-1}}$, $bla_{\text{CMY-2}}$ (Uddin & Ahn, 2018). Heavy use of ampicillin and penicillin in animal farms could be a contributing factor to the higher prevalence of beta-lactamase activity in the tested isolates (Rajić et al., 2006). In addition to AMR, pathogens also exhibit virulence factors that support invasion and survival in host organisms.

Hemolysis and biofilm formation were investigated because they are two key virulence factors promoting pathogen survival in animals (Dinges et al., 2000; George et al., 2010). Tested *Staphylococcus* isolates were strong biofilm formers, while *Salmonella* isolates formed very weak or no biofilms. This observation is on par with previous studies where the ability of *Staphylococcus* to form a biofilm, has been reported as being highly prevalent in MRSA in swine (Nicholson et al., 2013). Differential biofilm formation ability of *Staphylococcus* and *Salmonella* is reflected in the mode of infection in animals. Biofilm formation is more advantageous for skin infections, mostly caused by *Staphylococcus* than bacterial persistence as intracellular pathogens (MacKenzie et al., 2017).

In addition, *Staphylococcus* isolates mostly manifested complete hemolysis (beta-hemolysis) (Dinges et al., 2000), while *Salmonella* isolates showed hemolysis due to the production of HylE hemolysin protein, encoded by the *HylE* gene, which is a pore-forming protein that contributes to the systemic pathogenesis of *Salmonella* (Jajere, 2019). Hemolysin activity leads to membrane damage of neutrophils, macrophages to circumvent the host immune system (Dinges et al., 2000), and various mammalian erythrocytes to derive nutrients (Pishchany & Skaar, 2012).

Since we observed multiple mechanisms of AMR and virulence characteristics in *S. aureus* M12 and *S. enterica* ser. Typhimurium, we assessed whether combinations of antibiotics and adjuvants targeting resistance mechanisms would be advantageous for inhibiting bacterial growth. Synergism observed in ampicillin-tazobactam combination could be ascribed to the action of tazobactam on beta-lactamase enzyme which was highly prevalent in both isolates (Shahid et al., 2009). Similarly, the synergistic interaction of the ampicillin-cinnamon EO combination could be attributed to membrane disruption by the EO, allowing easy passage of ampicillin into the cells. In addition, ampicillin and cinnamon EO share a common mechanism of action and work together to disrupt the bacterial cell membrane (Yap et al., 2013). Yet another synergism was observed in the oregano EO-chloramphenicol combination against *S. aureus* M12. Oregano EO consists mainly of thymol and carvacrol, which affects the respiratory and energy metabolism in addition to membrane disruption (Yap et al., 2013). This can damage the pH homeostasis and further lead to disruption of LmrS efflux pumps, which confer resistance to chloramphenicol, thus increasing the susceptibility to chloramphenicol (Floyd et al., 2010). Combinations of chloramphenicol-CPZ and tetracycline-CPZ showed additive interaction. CPZ is an EPI of AcrB pumps in *Salmonella* (Grimsey et al., 2020a) and affects the potassium flux across the membrane in *Staphylococcus* and is hence able to disrupt the efflux activity of Tetk and NorA efflux pumps (Kaatz et al., 2003a).

Conclusion

In summary, the study highlights the possibility of eliminating MDR bacteria using combinations of antibiotics and adjuvants such as EOs and EPIs. Adjuvants that target specific resistance mechanisms when combined with antibiotics, can increase the efficacy of antibiotics. However, additional studies are required to understand the molecular-level interactions of combination therapy to design better therapeutics to circumvent AMR mechanisms.

Conflict of interest

Authors declare no conflicts of interest.

Supplementary Information

Antibiotic	Disk content
Apramycin (APR)	15 µg
Ampicillin (AMP)	10 µg
Amikacin (AK)	30 µg
Bacitracin (B)	10 iu
Cefotaxime (CTX)	30 µg
Cefazolin (CZ)	30 µg
Chloramphenicol (C)	30 µg
Ciprofloxacin (CIP)	5 µg
Colistin (CT)	10 µg
Erythromycin (E)	15 µg
Gentamycin (CN)	10 µg
Kanamycin (K)	30 µg
Lincomycin (MY)	2 µg
Neomycin (N)	30 µg
Ofloxacin (OFX)	5 µg
Penicillin (P)	10 iu
Streptomycin (SH)	100 µg
Spectinomycin (S)	10 µg
Tetracycline (TE)	30 µg
Ticarcillin (TIC)	75 µg
Tilmicosin (TIL)	15 µg
Tobramycin (TOB)	10 µg
Trimethoprim (SXT)	25 µg
Vancomycin (VA)	30 µg

Table S3.1. Antibiotics and their corresponding concentrations in the disks used in the determination of antimicrobial susceptibility of tested isolates. The antimicrobial susceptibility of the isolates was determined using Kirby-Bauer disk diffusion assay (Hudzicki, 2009). Isolates were cultured overnight at 37°C in MHB. The cells were maintained at McFarland 0.5, and 10 µL of the

inoculum was spread onto MH agar plates using a spreader. The disks containing the antibiotics were then placed on the lawn of bacteria using forceps and the plates were incubated for 18 h at 37°C. The diameter of the zone of inhibition was measured, and the isolates were categorized as resistant or susceptible according to CLSI guidelines (Clinical Laboratory Standards Institute, 2021).

S3.1. Growth curve of *S. enterica* ser. Typhimurium in high concentrations of ampicillin

S. enterica ser. Typhimurium showed extremely high resistance to ampicillin during initial trials. In order to investigate further, the growth curve of the isolate in different concentrations of ampicillin ranging from 1000 μg/mL to 15.6 μg/mL was determined for 36 h by measuring the optical density at 600 nm. In addition, hanging drop culture (Figure S3.1) of the isolates at the mentioned ampicillin concentration range was obtained at 18 h of the growth to confirm the growth of isolates at extremely high concentrations of ampicillin (Toh et al., 2017). It was observed that the number of cells in high concentration of ampicillin decline initially but survived cells proliferated faster than the cells treated with lower ampicillin concentrations or cultured in media alone. This observation suggested that mutations can occur in resistant bacteria to enable them to proliferate faster than their non-treated counterparts. These observations give an insight into the present challenge to inhibit the growth of resistant bacteria and the implications of overusing the antibiotics in animal farms.

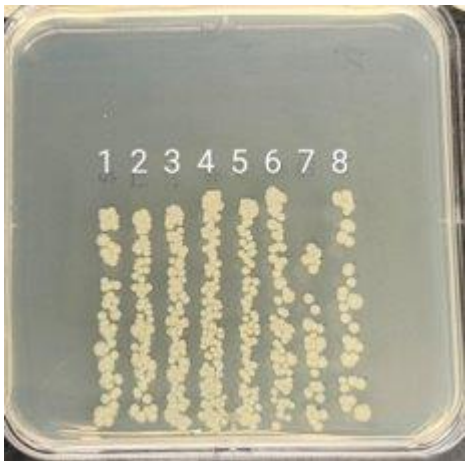


Figure S3.1. Hanging drop culture of *S. enterica* ser. Typhimurium representing the number of CFU/mL after 18 h of incubation at 37°C in ampicillin concentration of which were calculated as $1.40X10^{14}$(1), $1.97X10^{14}$ (2), $2.40X10^{14}$ (3), $2.48X10^{14}$ (4), $1.92X10^{14}$ (5), $1.70X10^{14}$ (6),$9.73X10^{13}$ (7) and $9.22X10^{13}$ (8). The concentration of ampicillin is 1000 µg/ml (1), 500 µg/ml (2), 250 µg/ml (3), 125 µg/ml (4), 62.5 µg/ml (5), 31.3 µg/ml (6), 15.6 µg/ml (7), and 0 µg/ml (8)

S3.2. Determination of beta-lactamase enzyme activity. Beta-lactamase enzyme production is resistance mechanism wherein the enzyme hydrolyses the lactam rings of beta-lactam drugs. To test the beta-lactamase enzyme activity, hydrolysis of nitrocefin, a chromogenic cephalosporin, was determined (Majumder et al., 2021). Briefly, nitrocefin (abcam, Canada), was dissolved in 5% DMSO and 95% buffer solution, which was used to make a stock of concentration 0.5 mg/mL; 10 µL of this solution was mixed with 10 µL of obtained cell-free extract, and the final volume was adjusted to 100 µL in wells of 96 well plate. The absorbance was detected in kinetic mode for 10 min at 390 nm using a plate-reader. Nitrocefin in buffer solution without cell-free extract was used as medium control. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used as negative controls. A nitrocefin standard curve with concentrations ranging from 125 µg/mL and 0.49 µg/mL was plotted and the enzyme activity was calculated using the formula:

$$\text{Beta-lactamase enzyme activity} = \frac{S_a}{\text{Reaction time X } S_v}$$

Where S_a is the amount of nitrocefin (in μM) hydrolyzed in the unknown sample well between t_1 and t_2 of the standard curve; reaction time is the time difference of t_1 and t_2, and S_v is the sample volume (in mL). Beta-lactamase activity is reported as U/mL. High nitrocefin enzyme activity indicates of the ability of pathogens to resist the action of drugs belonging to beta-lactam class, observed in most of the tested isolates.

S3.3. Heavy metal susceptibility. Minimum inhibitory concentration (MIC) of heavy metal ions, including zinc sulphate ($ZnSO_4$), cupric sulphate ($CuSO_4$), and silver nitrate ($AgNO_3$) was determined for bacterial isolates using the microtiter broth dilution method in a 96-well plate (Majumder et al. 2021). For this, the bacterial cells were adjusted to McFarland 0.5. Bacterial cells were then subjected to eight two-fold serial dilutions of the metal ions where the first working concentrations were 1250 μg/mL for $CuSO_4$ and $ZnSO_4$, and 200 μg/mL for $AgNO_3$. Assays were performed in quadruplicates and the plates were incubated at 37° C for 18 h in a shaking incubator. After incubation, resazurin was used to measure bacterial viability (Majumder et al. 2021). Briefly, 30 μL of 0.5% resazurin solution was added to each well, incubated for 2 h at 37°C, and read at 530/590 (excitation/emission) nm using a plate reader. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used as quality control strains to compare the MICs.

MIC of $CuSO_4$ for the isolates ranged from 635-1250 μg/mL; between 12.5-100 μg/mL for $AgNO_3$; and between 312.5-625 μg/mL for $ZnSO_4$. All the isolates were susceptible to copper, zinc, and silver, except *S. enterica* ser. Typhimurium. Precisely, when compared to *E. coli* ATCC 25922, a four-fold increase in the MIC of silver against *S. enterica* ser. Typhimurium was observed.

S3.4. Categorization of biofilm formation. OD_c was calculated using the arithmetic mean of the absorbance of negative control and three times the addition of standard deviation. The biofilm-forming ability was categorized as no biofilm formation ($OD_s < OD_c$), weak biofilm formation ($OD_c<OD_s\leq2OD_c$), moderate biofilm formation ($2OD_c<OD_s\leq4OD_c$), and strong biofilm formation ($4OD_c<OD_s$).

S3.5. Hemolysis detection using blood agar plates – detailed description of the method. Hemolysis is a key virulence factor of pathogens to invade host immune system. Bacterial isolates were tested for the presence of hemolysins (Buxton, 2005). Isolates were grown overnight in MHB and streaked onto blood agar plates (TSA with 5% defibrinated sheep blood). The plates were incubated overnight at 37°C for 24 h. The pattern of hemolysis was detected by visual inspection for the translucency around the bacterial colony. For *Staphylococcus* strains, the type of hemolysins was categorized based on clear halo around the colony (beta-hemolysis), green discoloration around the colony (α-hemolysis), and no clearing (γ-hemolysis) (Buxton, 2005). For *Salmonella* strains, the appearance of green discoloration indicated the presence of hemolysis ,and no discoloration was categorized as no hemolysis (Singh et al., 2004). *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used as controls.

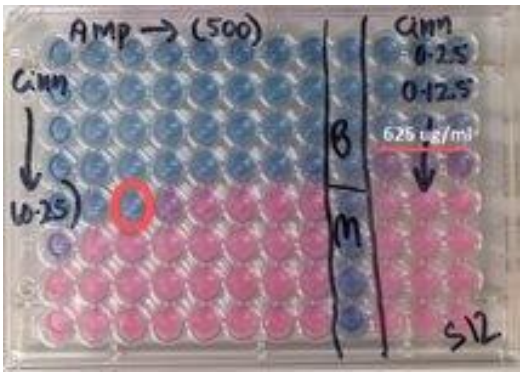


Figure S3.2. Combinations of antimicrobials ampicillin, chloramphenicol, tetracycline, and trimethoprim with adjuvants efflux pump inhibitor chlorpromazine (CPZ), beta-lactamase inhibitor tazobactam (TAZ), essential oils – thymol (TML), cinnamon essential oil (CEO) and oregano essential oil (OEO) were tested against *S. enterica* ser. Typhimurium and *S. aureus* M12 using checkerboard assay; synergistic (SYN), additive (ADD), indifferent (IN). Above is one of the plates showing synergism between antibiotic ampicillin and adjuvant cinnamon essential oil (CEO) against *S. aureus* M12. Minimum inhibitory concentration (MIC) of ampicillin decreased from 1000 µg/mL to 125 µg/mL, while MIC of CEO decreased from 625 µg/mL to 156.25 µg/mL. The FICI of the combination was calculated to be 0.38 and the combination was categorized to show synergistic activity (FICI < 0.5) (Orhan et al., 2005).

CHAPTER IV: NANOENABLED ANTIBACTERIAL COMBINATION THERAPY TO ELIMINATE MULTI-DRUG RESISTANT SALMONELLA AND STAPHYLOCOCCUS ISOLATES OBTAINED FROM ANIMAL FARMS

Abstract

Rise of multidrug resistance (MDR) among infectious pathogens has made it necessary to explore alternative therapeutics or enhance the effectiveness of existing antibiotics. In this study, commonly used antibiotic, tetracycline (TET) was encapsulated into chitosan nanoparticles and an efflux pump inhibitor, chlorpromazine (CPZ) was encapsulated into silica nanoparticles, which were further combined to obtain the benefits of their additive antibacterial effect. It was observed that the combination drug (CMD) had improved antibacterial efficacy and hampered AMR mechanisms such as the production of hydrogen sulfide (H_2S), and activities of beta-lactamase enzyme and efflux pumps. CMD showed improved antibacterial effect against *Staphylococcus aureus* M12 and *Salmonella* enterica serovar Typhimurium. In addition, it was significantly able to reduce the intracellular *S. enterica* ser. Typhimurium in Caco-2 cells in comparison to the free TET and CPZ at the effective concentration encapsulated into the nanoparticles. Enhanced antibacterial effect of CMD could be attributed to target specificity, improved bioavailability, tissue penetration and controlled drug release. Thus, nanoparticle enabled combination therapy is a novel and a promising approach towards combatting the intracellular MDR pathogens.

Keywords: Antimicrobial resistance, tetracycline, chlorpromazine, combination drug, nanoparticle, hydrogen sulfide, beta-lactamase enzyme, efflux pumps

Introduction

Prevalence of multi-drug resistant (MDR) bacterial pathogens in animal farms has been recognized as a major burden to food industry and well as the healthcare system. MDR bacterial isolates cause stubborn infections in food animals, treatment of which requires expensive antibiotic treatments, decreased output and heightened mortality and morbidity in animals, which in turn causes economic pressure on animal husbandry (Grace et al., 2012). In addition, emergence of antimicrobial resistant (AMR) pathogens in animals and their transmission to humans through food chain or direct contact have direct implications on economy of public health sector (Grace et al., 2012; Martins et al., 2015). Various bacterial pathogens such as *Staphylococcus aureus*, *Salmonella* species, *Campylobacter* species and *L. monocytogenes* and *Escherichia coli* are responsible for foodborne illnesses globally, mostly due to improper handling and consumption of meat products (Abebe et al., 2020). Evidently, some of these pathogens invade the host cells and survive intracellularly. For instance, *Salmonella* infects the gut epithelium and attach to M cells in ileal Peyer's patches. Subsequently bacteria enter macrophages and survive intracellularly (Elbi et al., 2017). These internalized bacteria are shielded from external antibiotics.

In addition to usage of antibiotics for infection treatment, antibiotics are also used as prophylactics and as growth promoters in food animals (Mathew et al., 2007). Overuse of antibiotics in livestock is a major contributing factor in the emergence of antimicrobial resistant (AMR) bacterial pathogens (Dadgostar, 2019; T Khanna et al., 2008). This necessitates the development of alternative therapeutics to treat bacterial infections caused by multi-drug resistant pathogens to reduce the amount of antibiotics required to eliminate infections.

Combining antibiotics with potentiating adjuvants is one such alternative that can enhance the antibacterial properties of antibiotics or increase sensitization of bacteria to antibiotics. For instance, essential oils have shown to facilitate easy penetration of antibiotics into the bacterial cells (El Atki et al., 2019). Similarly, adjuvants such as efflux pump inhibitors have shown to subdue the resistance mechanisms of bacterial cells and promote the action of antibiotics (Ferrer-Espada et al., 2019). This treatment modality of using combination of drugs named as 'combination therapy' is successful in boosting the effectiveness of antibiotics. Combination therapy in present form lacks target specificity, controlled drug delivery, and simultaneous or consecutive drug delivery (Prausnitz et al., 2004; Raza et al., 2019). These drawbacks highlight the urgent need to device alternate modes of drug delivery.

Recently, development in nanoparticle-based drug delivery has provided numerous promising opportunities. Nanoparticle based drug delivery systems have been observed to have longer circulation time, controlled drug release, ability to target specific sites as well as deliver more than one drug at the same time (Amreddy et al., 2018; Babos et al., 2018; Menina et al., 2019). In addition, nanoparticle-based drug delivery systems can be custom designed to meet desired properties. For instance, chitosan nanoparticles are mucoadhesive, biodegradable, positively charged, and easily surface modifiable to target specific sites inside the body, thus making chitosan nanoparticles (Ch NPs) a promising candidate for drug delivery (Coutinho et al., 2020; Dudhani & Kosaraju, 2010; Li et al., 2018). In addition, silica nanoparticles (Si NPs) have various advantageous features such as high drug loading capacity, biocompatibility, and ability to surface modify (Castillo et al., 2017; Tang et al., 2012).

In previous study, seven bacterial isolates obtained from animal farms were tested for antimicrobial resistance against 24 antibiotics and their resistance mechanisms were investigated.

It was determined that all the tested isolates were MDR. *S. enterica* ser. Typhimurium and *S. aureus* M12 were resistant to most antibiotics and were chosen for subsequently testing combinations of antibiotics and adjuvants for their additive or synergistic antibacterial effect. Various combinations showed additive or synergistic effect, and combination of tetracycline (TET) and chlorpromazine (CPZ) was chosen for further nanoparticle encapsulation, as it had additive antibacterial effect. TET and CPZ combined and encapsulated into nanoparticles can show enhanced antibacterial properties against MDR *S. aureus* M12 and *S. enterica* ser. Typhimurium.

TET is classified as Category III antibiotics and is not the preferred option for treatment of serious human infections (Health Canada, 2009). TET is permitted for use in food animals, but due to its overuse, the frequency of AMR pathogens has increased lately (Varga et al., 2019). To increase the antimicrobial efficacy of TET, it was combined with chlorpromazine (CPZ) which was encapsulated in mesoporous silica NPs. CPZ is an efflux pump inhibitor of AcrB efflux pumps and is also known to have effect on TetK efflux pumps, both of which have TET as substrate. The loaded nanoparticles were combined in 1:1 to obtain the combination drug (CMD). Therefore, this study aimed to determine the antibacterial effect of combination drug (CMD) prepared by combining TET loaded chitosan nanoparticles (Ch-TET NPs) and CPZ loaded Si NPs (Si-CPZ NPs), against multi-drug resistant isolates of *Salmonella enterica* ser. Typhimurium and *S. aureus* M12.

Materials and Methods

Resazurin sodium salt, mesostructured silica, and ethidium bromide (EtBr) was purchased from Sigma-Aldrich, Canada. 85% deacylated chitosan was obtained from Alfa Aesar, MA, USA. TET and CPZ were purchased from Bio Basic, ON, Canada. Nitrocefin dye was purchased from Abcam,

Canada. The quality control strains *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923 were purchased from Oxoid company, Canada. Multi-drug resistant *Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus* M12 isolates were obtained from swine in Quebec-based farm and obtained from Complexe de diagnostic et d'épidémiosurveillance vétérinaires du Québec (CDEVQ).

Preparation of nanoparticles

Synthesis of chitosan nanoparticles

Ch NPs were prepared according to ion-gelation method, with some modifications (Anitha et al., 2009). Chitosan (50 mg) was dissolved in 25 mL of 1% (v/v) acetic acid and allowed to stir for 48 hours. The pH was adjusted to 4.5-4.6 using 1.0 N NaOH and sonicated for 10 min. Tri poly phosphate (TPP) was dissolved in de-ionized (DI) water at a concentration of 10 mg/mL. TPP was added dropwise to the chitosan solution at 3:1 of chitosan:TPP under constant stirring. The resulting cloudy solution was allowed to stir for 30 min, followed by 10 min sonication. The solution was allowed to stir for another hour. The resulting Ch NPs were centrifuged at 11,000 X *g* for 12 min and the supernatant was removed. The particles were washed twice and then resuspended in DI water.

Synthesis of drug loaded nanoparticles and combination drug (CMD)

TET loaded Ch NPs were synthesized using ionic gelation method using TPP as described earlier, with some modification (Anitha et al., 2009). The pH of the solution was changed to 4.5-4.6 using 1.0 NaOH. 2.5 mL of the 1% (w/v) TET solution was added dropwise to the chitosan solution under constant stirring. TPP was added dropwise, and the resulting suspension was sonicated for

10 min, and allowed to stir for another hour to obtain TET loaded Ch NPs (Ch-TET NPs). The resulting particles were washed similarly as described in 2.2.1.

For synthesis of CPZ loaded Si NPs (Si-CPZ NPs) 40 mg of mesoporous Si NPs were dispersed in 10 mL of DI water. CPZ was dissolved in DI water to obtain 1% (w/v) solution, 4 mL of which was added dropwise to the Si NPs suspension. The suspension mixture of CPZ and Si NPs was allowed to stir for 24 h and then freeze dried to obtain a white powder, containing Si-CPZ NPs. Finally, CMD was obtained by combining Ch-TET NPs and Si-CPZ NPs in 1:1 ratio.

Physiochemical characterization

Determination of size and surface charge

Dynamic light scattering (DLS) (NanoBrook Omni instrument, Brookhaven's, USA) was used to measure the surface charge and hydrodynamic size of particles dispersed in DI water (50 µg/mL). The particle suspension was loaded into a pre-rinsed folded capillary cell and a voltage 100 V was applied for measurement of zeta potential.

Scanning electron microscopy (SEM) observation

Particle morphology was examined by SEM (FEI Quanta 450 Field Emission Scanning Electron Microscope). Nanoparticle samples were prepared by dehydration in serially diluted absolute ethanol (30%, 50%, 70%, and 90%) for 10 min each and resuspended in 100% ethanol at concentration of 50 µg/mL. A small amount of sample was dropped onto copper grip and allowed to dry for a few minutes. The particles were then coated with platinum before taking SEM images.

FTIR spectra

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) (ALPHA-P, Bruker, Billerica, MA, USA) was used to analyze the surface functional groups of the particles. 5 µL of the nanoparticle suspension was dropped on the ATR probe and allowed to dry. FTIR spectra was obtained using wavelength range of 600-4000 cm^{-1}, with a resolution of 4 cm^{-1}, and 32 scans. The obtained data was analyzed using OMNIC 8.2.0.387 software.

Determination of encapsulation efficiency (EE)

Antibiotic-loaded-nanoparticles were dispersed in DI water (1 mg/mL). The suspensions were sonicated for 30 min, followed by centrifugation at 40,000 x *g* for 40 min to remove loaded antibiotics from respective nanoparticle carriers. The supernatant was used to quantify the amount of TET and CPZ loaded by measuring the absorbance using a spectrophotometer (Genessys^{TM} 10S, Thermo Fisher, USA) at wavelength 285 nm and 305 nm respectively (R. Price, 2001). The measurement of the EE was done using a standard curve.

Determination of release behavior

The release behavior was studied by preparing samples of loaded nanoparticles in PBS of pH 4.3 representing the gastric pH 6.3 representing the intestinal pH of swine. The samples were centrifuged at 2000 x *g* for 3 min and the supernatant was removed every 24 h for seven days. The absorbance of the supernatant was measured as a function of time using a spectrophotometer (Genessys^{TM} 10S, Thermo Fisher, USA) (285 nm and 305 nm respectively for TET and CPZ) to determine the amount of TET and CPZ released.

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Antibacterial activity

Antibacterial efficiency of Ch-TET NPs, Si-CPZ NPs and CMD was determined against *S. enterica* ser. Typhimurium and *S. aureus* M12. Bacterial cultures were grown overnight in Mueller-Hinton Broth (MHB) overnight at 37°C under shaking. In 96-well plates, samples were serially diluted in a two-fold manner. Bacterial cultures (10 µL) maintained at 0.5 McFarland standard were added to the wells. The plates were incubated at 37°C for 18 hours under mild shaking.

After 18 hours, bacterial viability was monitored using Resazurin assay (Majumder et al., 2021). 30 µL of 0.5% resazurin solution was added to each well of 96-well plate and then incubated for 2 h at 37°C. The plate was then read at 530/590 (excitation/emission) nm using a plate reader (SpectraMax i3, Molecular Devices, USA). *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as quality control strains.

Effect on bacterial resistance mechanisms

Beta-lactamase inhibition assay

The beta-lactamase inhibition ability of the synthesized particles was tested as reported previously (Majumder et al., 2021). Briefly, the bacterial isolates were maintained at 1.0 McFarland standard, introduced to sub-lethal concentrations of samples (MIC X $\frac{1}{3}$), and further incubated for 18 h at 37°C. After 18 h, the cells were again adjusted to McFarland 1.0, centrifuged at 5,000 X *g* for 10 min and washed with sodium phosphate buffer (pH 7.0). The cells were then resuspended in the same buffer and sonicated for 5 min on ice. Cell-free extract was obtained by centrifuging the cells at 16,000 X *g* for 30 min and collecting the supernatant, which was used as the source of beta-

lactamase enzyme. Stock solution (0.5 mg/mL) of nitrocefin (a chromogenic cephalosporin) was made by dissolving in 5% Dimethylsulfoxide (DMSO). 15 µL of the nitrocefin solution was mixed with 30 µL of the cell-free extract and the volume was adjusted to 100 µL using buffer solution in a 96-well plate. The absorbance was measured using the plate reader (SpectraMax i3, Molecular Devices, USA) in kinetic mode at 490 nm for 15 min.

H_2S production inhibition assay

Ability of the samples to inhibit the production of H_2S was measured as reported previously (Basic et al., 2015). Bacterial isolates were adjusted to McFarland 0.5 and introduced to sub-lethal concentrations of samples and ampicillin (MIC X $\frac{1}{3}$), and then incubated for 18 h in MHB media with 20 mM L-cysteine. The bacterial cells were then centrifuged at 5000 X *g* for 10 min and washed twice with MHB + L-cysteine media. The cells were adjusted to 0.5 McFarland and left to incubate for 30 min. 40 µL of the cell suspensions were added to 80 µL of the distilled water (pH 9.6 +0.1 mM diethylenetriaminepentaacetic acid) in a microtiter well. 40 µL of the solution (17.1 mM *N, N*-dimethyl-*p*-phenylenediamine sulfate salt, and 37.0 mM $FeCl_3$ in 6 M HCl) was added immediately. The MB method measures the H_2S produced during bacterial growth in the broth. The absorbance was immediately detected in kinetic mode at 668 nm for 45 min using the plate-reader.

Efflux inhibition assay

The ability to inhibit the efflux activity of the bacterial isolates was measured as mentioned previously with minor modifications (Paixão et al., 2009). Bacterial cultures were incubated for 16 h in MHB broth at 37°C. Bacterial cells were then centrifuged, washed twice in phosphate

buffer saline (PBS) (1X), and adjusted to McFarland 1.0. The cells were treated with sub-lethal concentrations of the samples and CPZ (MIC X $\frac{1}{3}$), vortexed and incubated for 30 min. Sub-lethal concentration of EtBr (MIC X $\frac{1}{3}$) was added to the bacterial samples and 150 µl of the suspension was added to 96 well plates, and accumulation was determined by monitoring fluorescence (530/590 nm) for 60 min at 25°C using a plate reader. Subsequently, the cells were washed with PBS, and 10 µL of glucose was added to 140 µL of the cell suspension to a 0.04% w/v final concentration in a 96 well plate. Efflux activity was then determined by monitoring fluorescence (530/590 nm) for 60 min using a plate reader.

Determination of nanoparticle-enabled combination drug against intracellular *S. enterica* ser. Typhimurium

Antimicrobial effect of nanoparticle treatments against intracellular pathogen was determined as previously described (Xie et al., 2017). Caco-2 was selected as the *in vitro* model of intestinal epithelium to determine the antimicrobial effect of nanoparticle treatment against intracellular pathogen. Caco-2 cells (ATCC, Virginia, USA) were cultured in a 96-well plate ($2X10^4$ cells per well) until confluent and then were infected with overnight culture of *S. enterica* ser. Typhimurium (Multiplicity of index =1) for 1 h. Next the media containing the bacteria was removed and the cells were washed 2 times with PBS at 4°C. The cells were then incubated with 100 µL of 200 µg/mL of gentamicin to kill all the extracellular bacteria for 30 min. Gentamicin was removed and the cells were washed with PBS at 4°C three times. The cells were incubated with Gibco Dulbecco's Modified Eagle Medium (DMEM) (Thermofisher) medium for 4 h to establish the intracellular infection model. Next, the nanoparticle treatments were added to the cultures and incubated for 12 h and 24 h. At determined time points, the cells were washed three times with

PBS at 4°C and the extracellular bacteria was removed. The cells were lysed using 100 µL of 0.5% Triton-X and the lysate was diluted to plate on Mueller Hinton agar plates. Infected cells with no treatment were used as negative control and infected cells treated with ciprofloxacin (20 µg/mL) were used as positive control. The number of colonies were counted after incubation for 24 h at 37°C to determine viable *Salmonella* counts (CFU/mL).

Statistical analysis

Experiments were performed in triplicates and repeated at least twice. The data is expressed as the mean value ± standard deviation (SD). T-test was performed and one way *P* value ≤0.05 was considered significantly different.

Results

Physiochemical Characterization of the particles

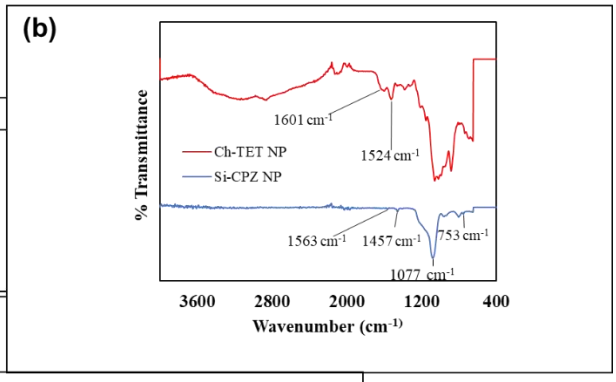
The hydrodynamic size and the zeta-potential of the synthesized particles were measured using DLS, as summarized in tabular data (figure 4.1a). Zeta potential and the mean size of synthesized Ch NPs was observed to be 26.55±4.73 mV and 536.30±10.61 nm, respectively. On addition of TET to Ch NPs, the zeta potential and mean size didn't change significantly and were 25.72±0.68 mV and 541.68±2.02 nm respectively. Size of Si NPs encapsulating CPZ was determined to be 514.20±53.05 nm, and the zeta potential changed significantly from -24.18±0.08 mV to -9.95±0.43 mV after addition of CPZ. The size of Si NPs was observed to be 778.01±8.16 nm before encapsulation mainly due to presence of aggregation in Si NPs (Bałdyga et al., 2019). In CMD, the average particle size and zeta potential was observed to be 577.00±35.83 nm, and 15.25±0.11 mV respectively. Through SEM imaging, the size of Si-CPZ NPs was ranged between 315.5 nm

to 416.5 nm. The size of Ch-TET NPs appeared to be smaller in SEM (33.49-59.17 nm). This is mostly because most of the particles exist as aggregates around the size observed in DLS. CMD also appeared to be a dumbbell shaped cluster with individual particles around 457.1-650.7 nm (Figure S4.1).

The FTIR spectra (Figure S4.2) of Si NPs showed broad peaks in the region from 1000-1250 cm^{-1}, which is attributed to Si-O vibrations (Datt et al., 2012). The characteristic peaks of CPZ (1563 cm^{-1}, 1457 cm^{-1}, and 753 cm^{-1}) become visible in Si-CPZ NPs, indicating the presence of CPZ in the synthesized particle (Govender et al., 2015). Peaks at 1637 cm^{-1} and 1540 cm^{-1} in Ch NPs shifted to 1601 cm^{-1} and 1524 cm^{-1} respectively in Ch-TET NP. The shift in the IR spectrum peaks confirm the formation of new bonds and encapsulation of TET into Ch NPs (El-Alfy et al., 2020).

(a)

Sample	Zeta-potential (mV)	Size (nm)
Chitosan nanoparticles (Ch NP)	+26.55±4.73	528.80±10.61
Tetracycline loaded chitosan nanoparticles (Ch-TET NP)	+25.72±0.68	541.68±2.02
Silica nanoparticles (Si NP)	-24.18±0.08	778.01±8.16
Chlorpromazine loaded silica nanoparticles (Si-CPZ NP)	-22.51±0.94	514.20±53.05
Combination drug (CMD)	+15.25±.11	577.00±35.83



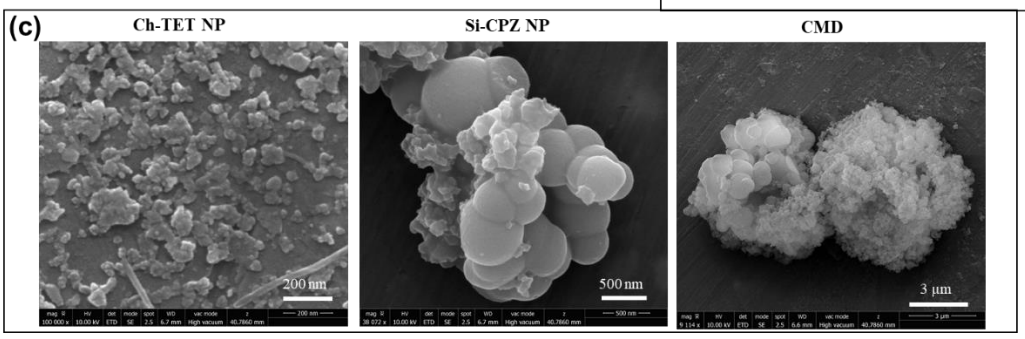


Figure 4.1. Characterization of particles. (a) Tabular data showing zeta-potential, and size of the particles measured using dynamic light scattering (DLS). (b) FTIR spectrum of Ch-TET NP and Si-CPZ NP. (c) Nanoparticles were platinum coated and imaged using Scanning Electron Microscopy (SEM).

Encapsulation efficiency and release behaviour

The EE was measured spectrophotometrically by measuring the absorbance of the supernatant at 285 nm for TET and 305 nm for CPZ. EE of TET in Ch NPs was determined to be 23.82±0.16% and EE of CPZ in mesoporous Si NPs was 44.77±0.12%. The release of TET from Ch NPs and of CPZ was measured by monitoring the absorbance of the supernatant over seven days (Figure 4.2). It was found out the release was faster for both TET and CPZ in pH 4.4. On day 1, 67.48±1.57% of TET was release from Ch NPs and 81.49±0.29% of CPZ was released from Si NPs at pH 4.4. In pH 6.3, 52.31±0.12% of TET and 61.95±0.91% of CPZ was released on day 1. Following the initial burst release, the release was relatively slower in the next six days.

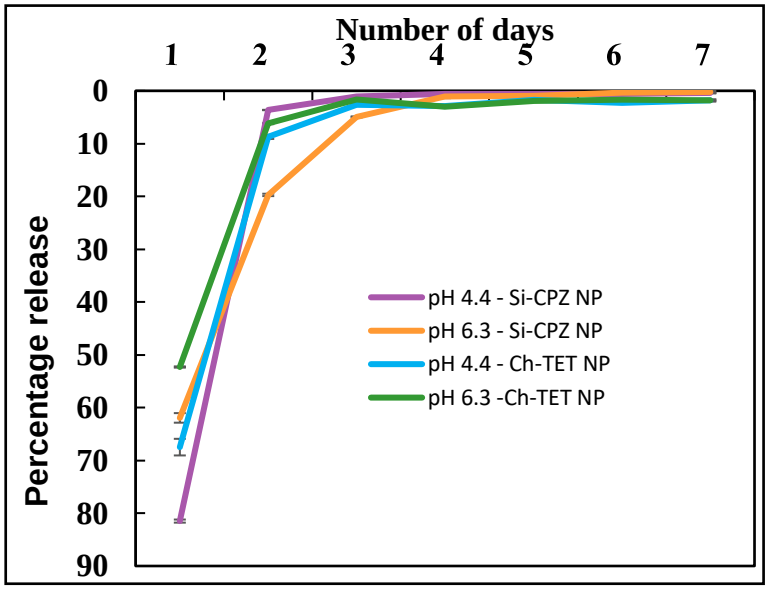


Figure 4.2. Release kinetics of drugs from nanoparticles. Release kinetics of tetracycline (λ=285 nm) and chlorpromazine (λ=305 nm) from chitosan nanoparticles and silica nanoparticles measured respectively using spectrophotometer.

Antibacterial efficacy

The MIC of the synthesized particles and the CMD was determined against *S. enterica* ser. Typhimurium and *S. aureus* M12, summarized in table 4.1. For *S. enterica* ser. Typhimurium, the MIC of Ch-TET NP was determined to 1000 µg/mL, and the MIC of Si-CPZ NP was measured to be 500 µg/mL. On combining the loaded particles in 1:1 ratio, the MIC of CMD was determined to be 250 µg/mL. A decrease in MIC was also observed against *S. aureus* M12 upon combination of the loaded particles. The MIC of the Ch-TET NP was determined to 500 µg/mL, and the MIC of Si-CPZ NP was measured to be 250 µg/mL. The MIC of CMD against *S. aureus* was observed to be 125 µg/mL. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as quality control strains.

	Si-CPZ NP (µg/mL)	**Ch-TET NP (µg/mL)**	**CMD (µg/mL)**
***E. coli* ATCC 25922**	62.5	7.82	3.90
***S. aureus* ATCC 25923**	125	3.90	1.95
***S. enterica* ser. Typhimurium**	500	1000	250
***S. aureus* M12**	250	500	125

Table 4.1. Minimum inhibitory concentration (µg/mL) of Ch-TET NP, Si-CPZ NP and CMD

Beta-lactamase enzyme inhibition assay

The ability of the nanoparticle treatments to inhibit the production of beta-lactamase enzyme was determined, as represented (figure 4.3 a). It was observed that Si-CPZ NP inhibited the production of beta-lactamase enzyme the most in both *S. enterica* ser. Typhimurium and *S. aureus* M12. CMD also successfully inhibited the production of beta-lactamase enzyme more than Ch-TET NP. Bacteria cells treated with ampicillin without any particle treatment were used as positive control. Ampicillin treated *E. coli* ATCC 25922, and ampicillin treated *S. aureus* ATCC 25923 were used as negative control.

H_2S production inhibition assay

H_2S production by bacterial isolates after treatment with particles was obtained by measuring the absorbance at 668 nm and measuring the H_2S production using a standard curve (Figure 4.3 b). It was observed that in *S. enterica* ser. Typhimurium, H_2S production was inhibited the most by CMD followed by Si-CPZ NP and then Ch-TET NP. In case of *S. aureus* M12, H_2S production was inhibited the most by Ch-TET NP, followed by CMD. H_2S production inhibition was lowest in Si-CPZ NP. *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as negative controls, where no H_2S production was observed.

Efflux pump activity inhibition

The differential ability of the particle treatments to block the efflux pumps present in bacterial isolates, and thus cause accumulation of EtBr and then inhibit the efflux of EtBr was measured. The accumulation of EtBr in bacterial cells is represented in Figure 4.3 c. It was observed that in *S. enterica* ser. Typhimurium, fastest accumulation occurred when cells were treated with Si-CPZ

NP with t_2^1 of 1398 sec. CMD also led to fast accumulation of EtBr with t_2^1 of 1771 sec. Ch-TET NP treated cells showed very slow accumulation with t_2^1 of >3600 sec. *S. enterica* ser. Typhimurium treated with CPZ was used as a positive control. In case of *S. aureus* M12, Si-CPZ NP treated bacterial cells showed fastest accumulation of EtBr (t_2^1 of 1793 sec). CMD also successfully led to accumulation of EtBr (t_2^1 of 2176 sec). Extremely slow accumulation of EtBr was observed for bacterial cells treated with Ch-TET NP (t_2^1 > 3600 sec). *S. aureus* M12 treated with CPZ was used as positive control.

Following accumulation, the cells were washed and treated with glucose to measure active efflux of EtBr from cells treated with particles (Supplementary information figure S4.3). It was observed, Ch-TET NP treated bacterial cells of both *S. aureus* M12, and *S. enterica* ser. Typhimurium had t_2^1 >3600 sec for extrusion of EtBr, which can be explained by the extremely low accumulation of EtBr to begin with. Si-CPZ NP treatment led to slowest extrusion of EtBr with t_2^1 of 728.5 sec and 684.7 sec for *S. enterica* ser. Typhimurium and *S. aureus* M12 respectively. CMD also successfully inhibited the efflux of EtBr with t_2^1 of 319.3 sec and 584.2 sec for *S. enterica* ser. Typhimurium and *S. aureus* M12 respectively. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used as negative control.

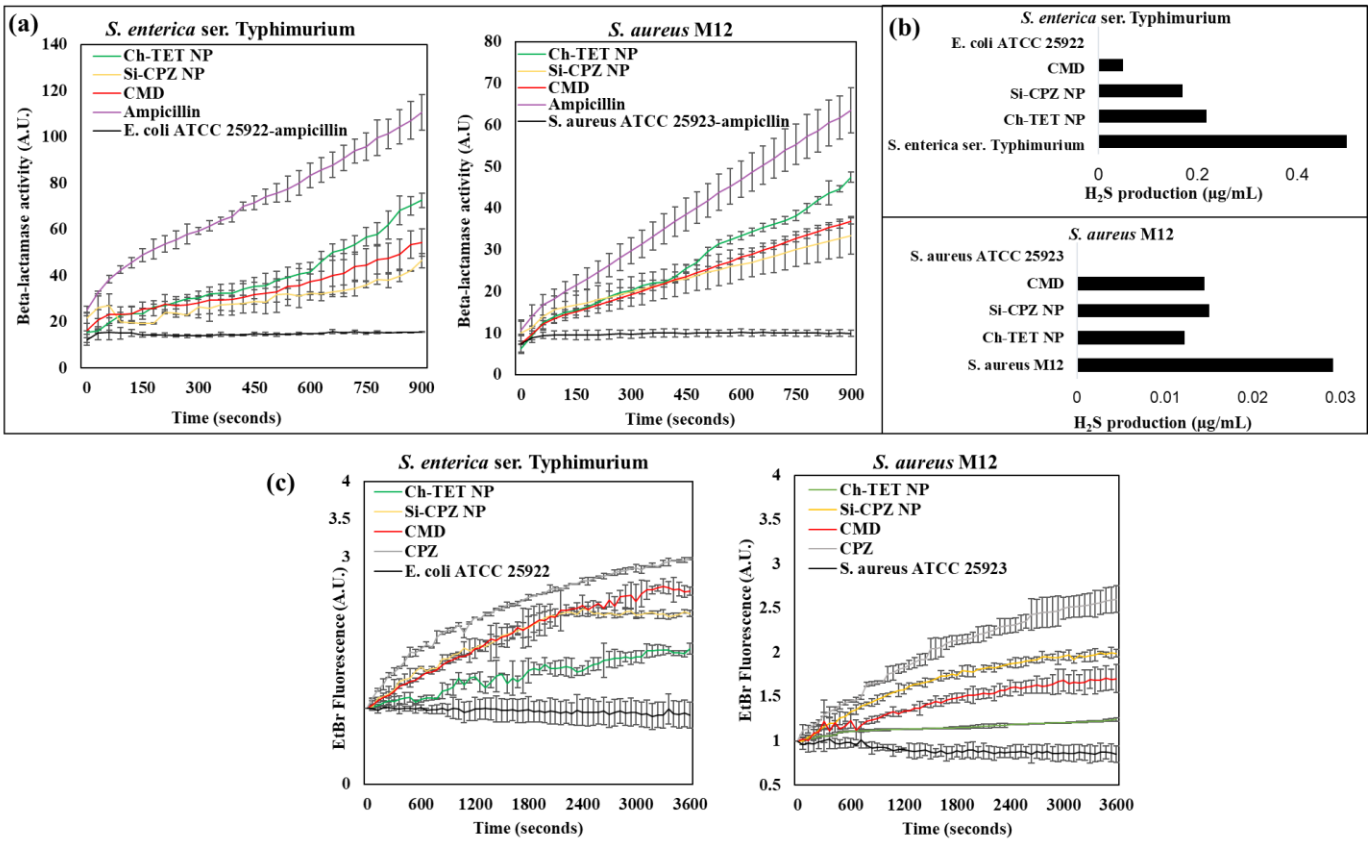


Figure 4.3. **Effect of synthesized nanoparticles on resistance mechanisms.** (a) Inhibition of beta-lactamase enzyme in bacteria treated with synthesized nanoparticles measured using nitrocefin assay, (b) H_2S production of bacterial strains was measured after treatment with synthesized particles, (c) EtBr accumulation inside bacterial cells after treatment with nanoparticle treatments determined by measuring fluorescence.

Antibacterial efficacy against intracellular *S. enterica* ser. Typhimurium

Antibacterial efficacy of the synthesized particles against intracellular *S. enterica* ser. Typhimurium in Caco-2 cell model was tested and the colony forming units (CFU) of the intracellular bacteria were determined (figure 4.4) and log reduction for the treatments was calculated. It was determined that CMD (250 ppm) had significantly higher log reduction (0.77±0.26), which was 83.02±14.35% compared to other treatments. Log reduction for individual Ch-TET NP and Si-CPZ NP was lower and was 0.01±0.05 and 0.13±0.03 respectively, which is just 2.64±11.21% and 25.85±5.60% reduction. Even the pristine TET had lower log reduction of 0.17±0.13. CPZ had a negative log reduction value which essentially means there was no reduction in the bacterial colonies.

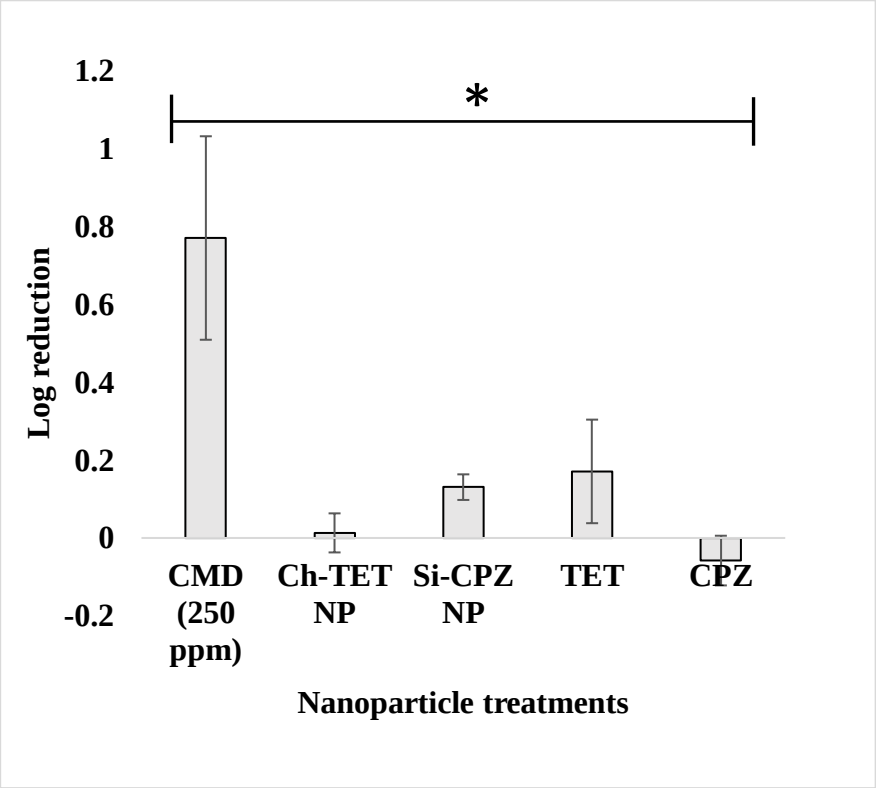


Figure 4.4. Effect of nanoparticle treatments on intracellular bacteria. Infected Caco-2 cells were treated with nanoparticle samples for 24 h. The cells were lysed, and the lysate was used to count the intracellular bacteria that survived. Log reduction was calculated. T-test was performed and $p<0.05$ was considered significantly different (represented by *).

Discussion

In this study, Ch NPs encapsulating TET and Si NPs encapsulating CPZ were combined in 1:1 ratio to achieve the additive and enhanced antibacterial effect against multidrug resistant *S. enterica* ser. Typhimurium and *S. aureus* M12 isolated from animal farms. The combination showed improved antibacterial effect and successfully inhibited the H_2S production, efflux activity and beta-lactamase enzyme activity in treated bacterial cells, with low toxicity against *C. elegans.*

The CMD, which was Ch-TET NP and Si-CPZ NP combined in 1:1, successfully inhibited the growth of *S. enterica* ser. Typhimurium and *S. aureus* M12 with MIC of 250 µg/mL and 125 µg/mL respectively. The EE% of TET into Ch NPs was 23.82±0.16%, which equates to the effective concentration of pristine TET drug present in the combination as 19.05±0.16 µg/mL against *S. enterica* ser. Typhimurium and is 14.89±0.10 µg/mL against *S. aureus* M12. Similarly, the EE% of CPZ is 44.77±12%, which indicates that effective pristine CPZ present in the CMD was 55.96±0.15 µg/mL against *S. enterica* ser. Typhimurium and 27.98±0.07 µg/mL against *S. aureus* M12. These concentrations show improved efficacy of components due to combinatorial effect and nanoparticle encapsulation as the MIC of pristine TET was 250 µg/mL and 62.5 µg/mL against *S. enterica* ser. Typhimurium and *S. aureus* M12 respectively; and that of pristine CPZ was 125 µg/mL and 62.5 µg/mL against *S. enterica* ser. Typhimurium and *S. aureus* M12 respectively.

The enhanced antibacterial efficacy of the drugs encapsulated into nanoparticles and combined can be attributed to two main factors: the additive effect between TET and CPZ, as observed in previous experiments, and the improved delivery due to nanoparticle encapsulation. CPZ interferes with substrate binding and thus inhibits AcrB-mediated efflux in *Salmonella* (Grimsey et al.,

2020b). In *Staphylococcus*, CPZ impacts the potassium flux across the membrane, and thus disrupts the TetK efflux pumps, responsible for extrusion of TET (Kaatz et al., 2003b). Therefore, CPZ holds the ability to sensitize bacterial cells towards TET and thus increase its effectiveness. In addition, encapsulation in nanoparticle drug carriers offer various added advantages, as discussed below.

The therapeutic efficacy of systemically or orally administered pristine antibiotics can be hindered due to various obstacles; limited bioavailability, poor target specificity, low absorption and possible side-effects on surrounding un-infected cells (Patra et al., 2018). Nanoparticle-based drug delivery systems offer the opportunity of enhancing the efficacy of encapsulated antibiotics, while minimizing the side effects. CMD had an overall positive surface charge due to presence of Ch NPs, which improved binding onto the negatively charged bacterial surface. CPZ was also shown to have effect in *blaZ* gene, which is responsible for encoding beta-lactamase enzyme, which explains the reduced beta-lactamase enzyme production on treatment with particles (Kong et al., 2016). Therefore, overall due to additive effect of TET and CPZ, various mechanisms of action of CPZ, and encapsulation of drugs in nanoparticle drug carrier, the CMD showed enhanced antibacterial efficacy.

The ability of CMD to significantly reduce the amount of intracellular MDR *S. enterica* ser. Typhimurium points towards the superior properties of nanoparticle drug carriers which can enable targeting bacteria taking refuge inside the cells. CMD had surface positive charge, which is beneficial for oral administration of drugs as the positive charge of CMD could penetrate the mucous layer on the intestinal epithelial cells, the site of intracellular bacteria causing gastrointestinal diseases (Van der Lubben et al., 2001). In addition, Ch NPs can overcome cellular barriers and reversibly open the tight junctions in epithelial cells enabling delivery of drugs into

systemic circulation (Van der Lubben et al., 2001). Once delivered at the site of infection, the interaction of positively charged nanoparticles with cell membrane of gram-negative bacteria can also assist in disruption of bacterial cell (Rabea et al., 2003).

Most importantly, the ability of CMD to successfully reduce intracellular pathogens significantly sheds light on the scope of usage of nanoparticle enabled antibacterial combination therapy to preserve and enhance the antibacterial efficacy of various commonly used antibiotics in food animals, to which various pathogens have developed resistance. This can extend the usability and effectiveness of an antibiotic which is permitted to use in food animals, slowing down the need to move to other antibiotics which are of higher importance in human medicine and are last resort antibiotics for various harmful infections.

Various antibiotics are considered to have lost their effectiveness due to AMR, however, are readily available and economical. Encapsulation of these antibiotics inside nanoparticle-based drug carriers that are synthesized from biocompatible, economical, non-toxic, and scalable material to preserve and enhance the effectiveness, provides the basis of ideal therapeutics for treatment of AMR infections in food animals. This will decrease the overall mortality and morbidity of food animals due to AMR infections, and thus reduce the burden on food industry and animal farm owners. In addition, this will lead to administration of minimal amounts of antibiotics in food animals, which makes it safer to consume than animals administered with high doses of antibiotics.

In conclusion, nanoparticle-based combination therapy is a promising tool to combat the grave issue of AMR pathogens. Combining drugs that are custom designed based on expected additive or synergistic effect and encapsulating them in nanoparticles can reduce the overall amount of

antibiotics required to treat infections, overuse of which is a major cause of ever-increasing frequency of AMR pathogens (Dadgostar, 2019). In this study, combination of TET and CPZ was employed to show the improved effectiveness of nanoparticle-based combination therapy in targeting and eliminating intracellular bacteria. Similarly, various combinations of antibiotics and adjuvants can be encapsulated in nanoparticles of desired properties and customized according to the infection causing pathogen, host organism, mode of delivery etc.

Conflict of interest

Authors declare no conflicts of interest.

Supplementary information

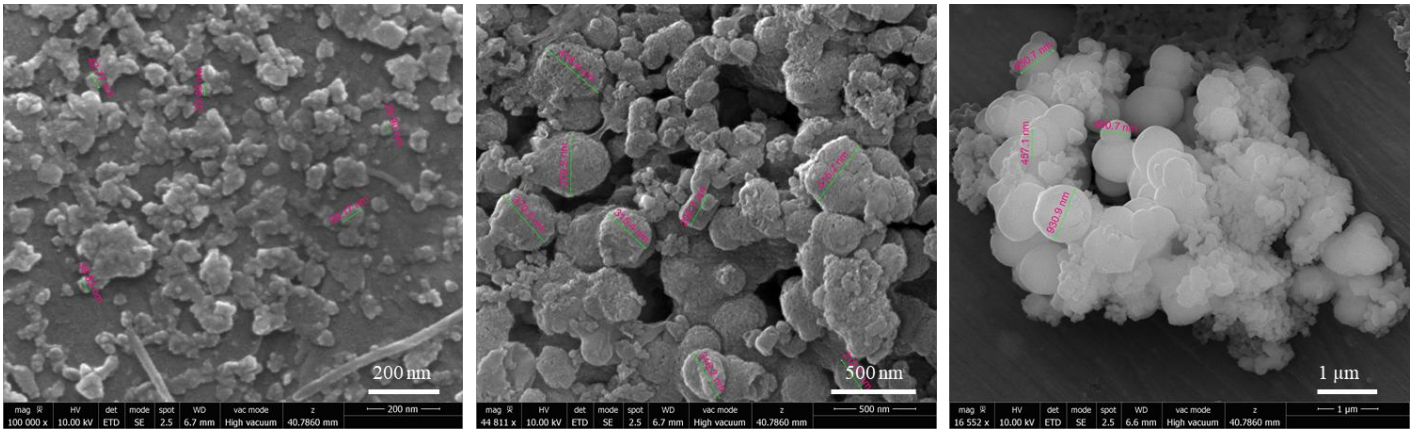


Figure S4.1 SEM images of Ch-TET NPs, Si-CPZ NPs and CMD with marked dimensions.

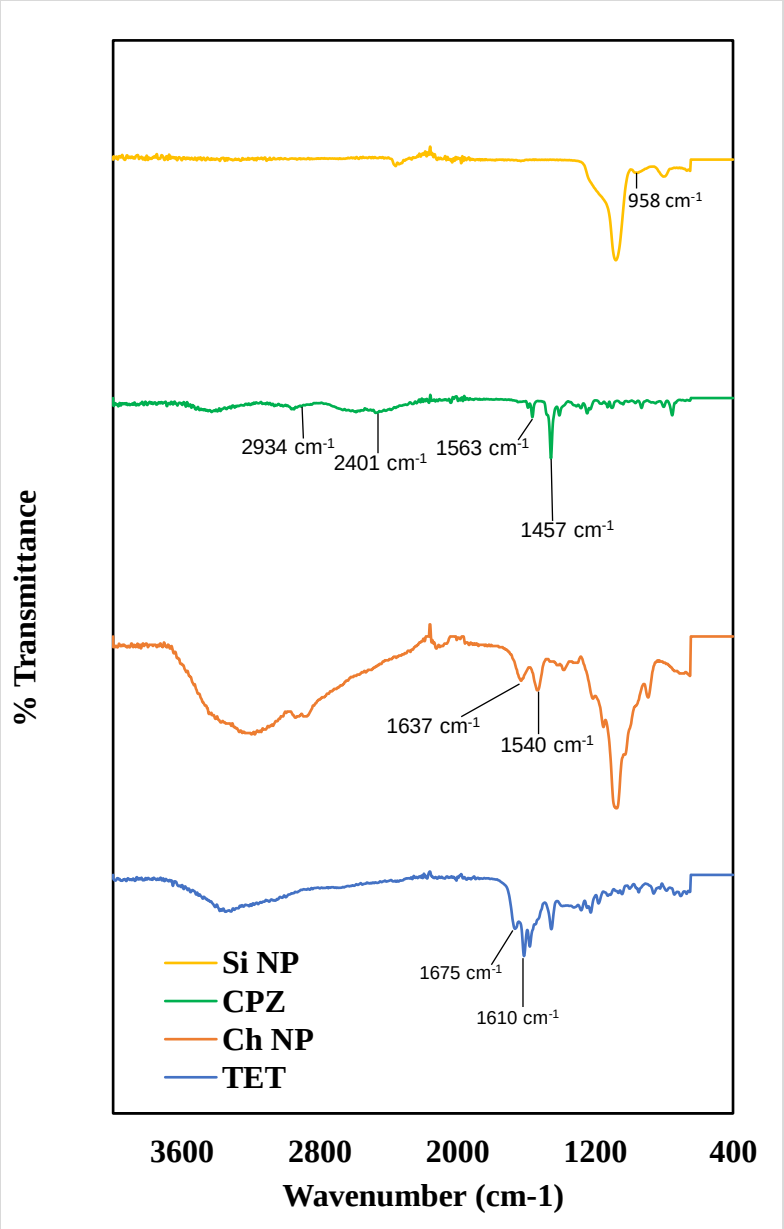


Figure S4.2. FTIR spectra of Si NP, CPZ, Ch NP, and TET.

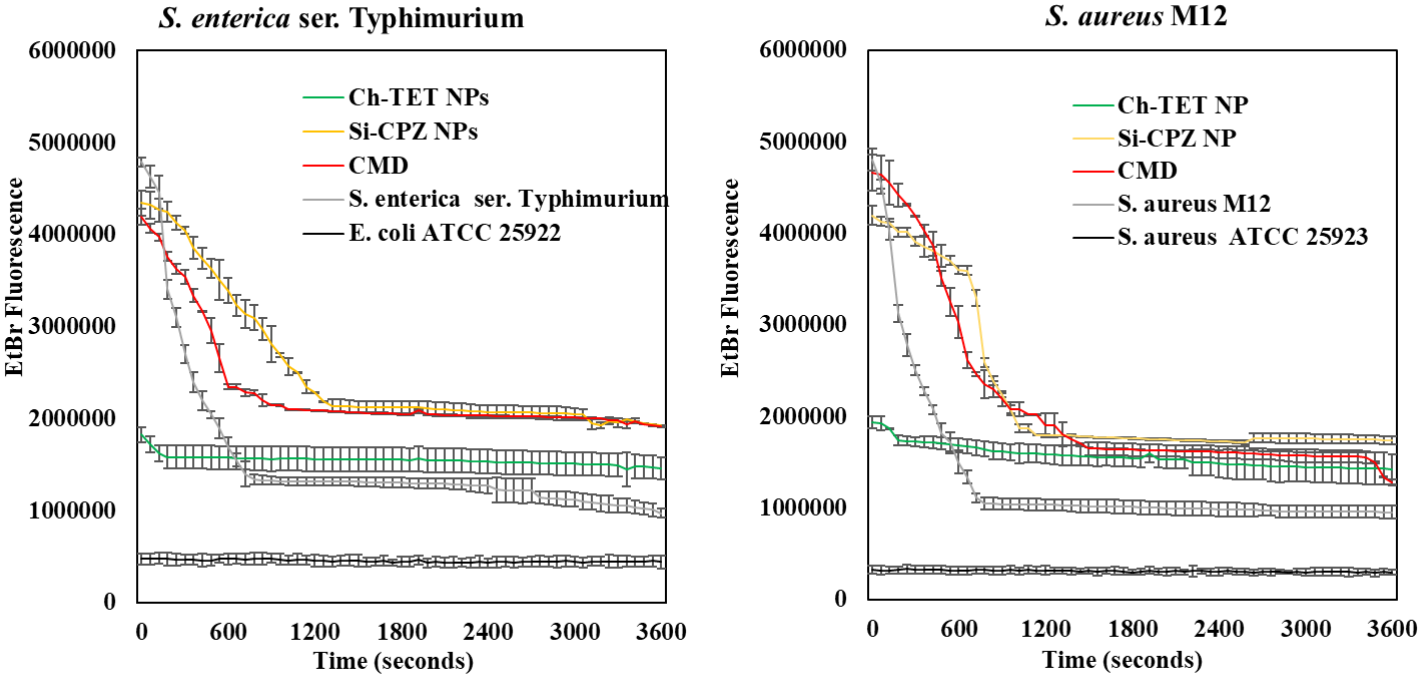


Figure S4.3. Efflux of EtBr from *S. enterica* ser. Typhimurium (a) and *S. aureus* M12 (b) treated with synthesized nanoparticles determined by measuring fluorescence (530/590) nm.

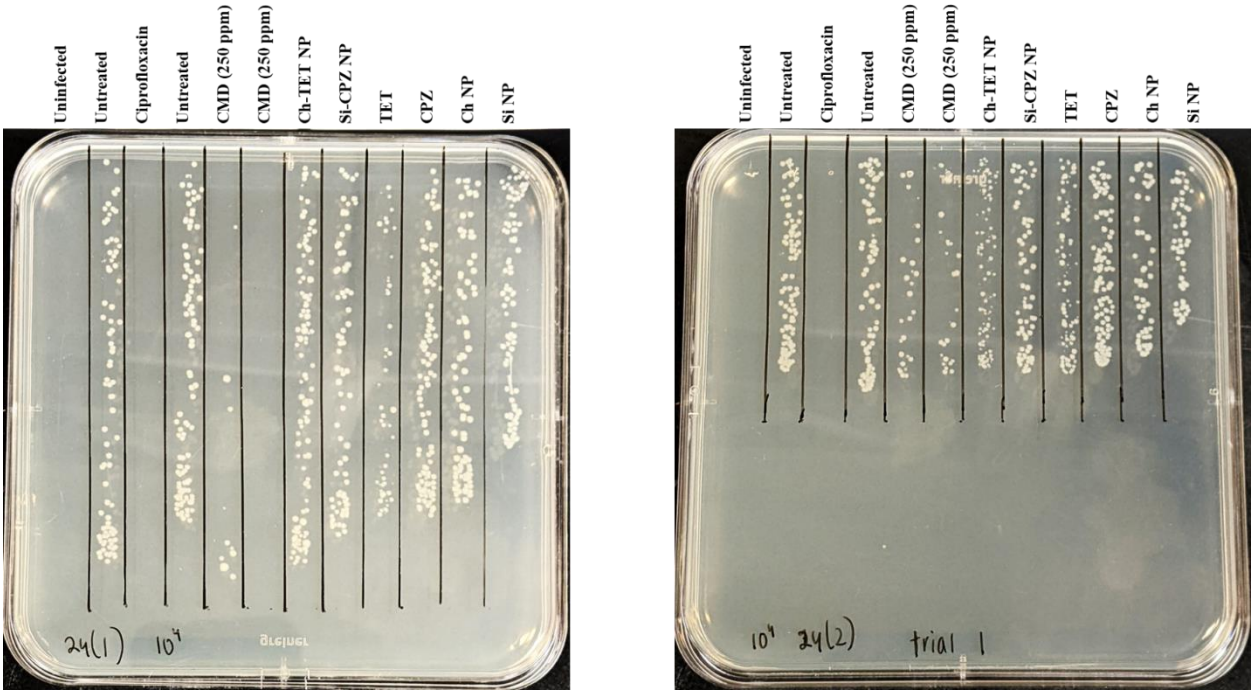


Figure S4.4. Image of plates used to determine CFU/mL of lysates of infected Caco-2 cells treated with nanoparticle samples.

***In vivo* toxicity studies**

Method: The *in vivo* toxicity of the synthesized Ch-TET NPs and Si-CPZ NPs was tested using *C. elegans* models (Stokes et al., 2015). The *C. elegans* CF512 were first synchronized using alkaline bleach solution, and grown to L4 stage on lawns of *E. coli* OP50 on solid nematode growth medium (NGM) for 48 h at 21°C. The worms were then washed in M9 buffer and transferred to S-basal media.

To assess the toxicity of the synthesized drug-loaded nanoparticles, five well series of two-fold serial dilutions of the nanoparticles was performed in 24-well plate in S-basal media. 40-50 worms were added the each well, and the plate was incubated at 25°C. *C. elegans* in media without addition of any drug were considered as the control. The plate was monitored for 24 h and 48 h for worm survival using a dissection microscope (Wild Heerbrugg, Switzerland).

Results: For determining the toxicity of the Ch-TET NPs, Si-CPZ NPs and the CMD, nematodes were treated with different concentrations of the particles, and their survivability was monitored for 24 h and 48 h periods (Supplementary information Figure S4.5). The nematodes seen to be moving and maintaining a sinusoidal shape were scored as living, and nematodes with straight, rigid and rod shape were scored as dead. When treated with Ch-TET nanoparticles, after 24 h, 94.84±0.56% and 97.22±3.93% of worms survived at 250 µg/mL and 125 µg/mL. The % survivability remained the same after 48 h. It was determined that Si-CPZ NPs were relatively toxic above concentration of 62.5 µg/mL. On treatment with Si-CPZ NPs, after 24 h exposure, 85.32±10.66% worms survived at 250 µg/mL and 81.89±2.68% of worms survived at 125 µg/mL. After 48 h, only 54.76±16.84% and 54.73±6.69% of worms survived at 250 µg/mL and 125 µg/mL respectively. When the worms were treated with the CMD, 83.10±8.74% and 91.11±3.74% of

worms survived after 24 h exposure with 250 µg/mL and 125 µg/mL of particles respectively. After 48 h exposure, 55.36±7.58% and 81.01±0.34% of worms survived at 250 µg/mL and 125 µg/mL. CMD treatment showed lower toxicity than Silica-CPZ nanoparticles at concentrations that were previously determined to be effective against *S. enterica* ser. Typhimurium and *S. aureus* M12. Ch-TET NPs were observed to be less toxic towards worms, however ineffective against tested bacteria at concentrations of 250 µg/mL and 125 µg/mL. Therefore, considering the toxicity as well as the antibacterial efficacy, CMD performed better than Si-CPZ NPs and Ch-TET NPs individually.

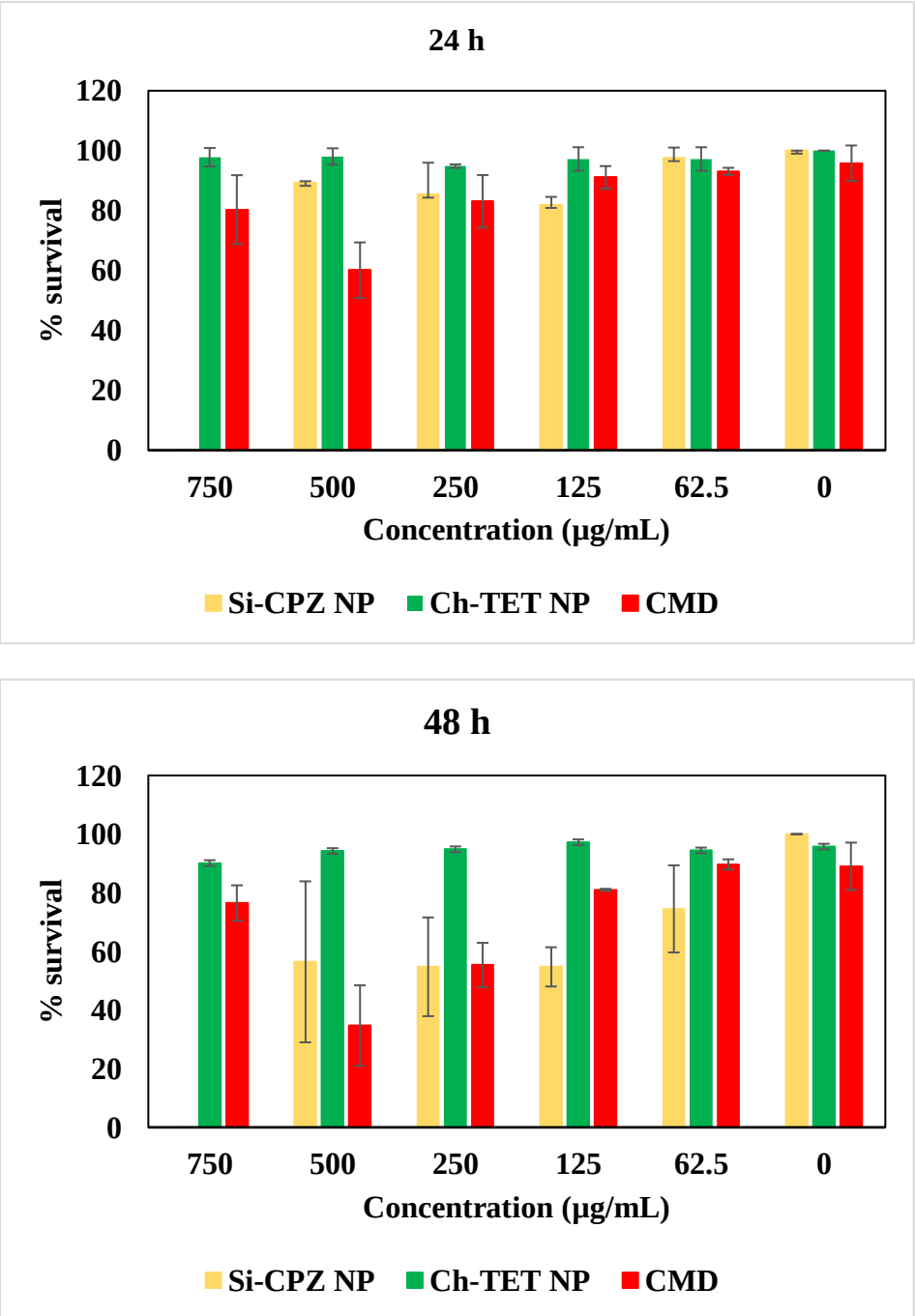


Figure S4.5. *In-vivo* studies using *C. elegans*. Toxicity of particle treatments on *C. elegans* observed for 24 h and 48 h intervals measured as % survival of *C. elegans.*

CHAPTER V: GENERAL CONCLUSION AND RECOMMENDATIONS FOR FUTURE

General Conclusion

This research focused on design and development of nano-enabled antibacterial combination therapy to inhibit the growth of MDR bacterial pathogens obtained from animal farms across Quebec. Seven bacterial isolates were characterized for resistance to commonly used antibiotics (n=24 antibiotics), and their resistance mechanisms and virulence characteristics were investigated. It was determined that all seven isolates were MDR, out of which majority employed resistance mechanism (efflux pump activity and beta lactamase enzyme activity), and some were biofilm formers and caused hemolysis of sheep RBCs. *S. enterica* ser. Typhimurium and *S. aureus* M12 were resistant to majority of tested antibiotics. Therefore, combinations of antibiotics and adjuvants were tested against them to determine the antibacterial efficacy, out of which many combinations showed synergistic or additive antibacterial effect. These results give a glimpse of the prevalent MDR in livestock related bacterial pathogens, and the resistance mechanisms employed to resist antimicrobial action. However, various combinations are capable of targeting the resistance mechanisms, enhancing the effect of antimicrobials and inhibiting the growth of MDR bacteria.

Out of the tested combinations, the combination of TET and CPZ was preferred for nanoparticle encapsulation as the antibiotic TET is commonly used in both animals and humans and is not of high importance in human medicine. TET and CPZ were encapsulated in Ch NPs and Si NPs respectively, and then combined together (1:1) to form CMD and its antibacterial efficacy and effect on bacterial resistance mechanisms were tested. In addition, CMD successfully reduced the intracellular *S. enterica* ser. Typhimurium in Caco2 cells and performed better than the pristine drugs at the effective concentration encapsulated and even the individual Ch-TET NPs and Si-CPZ

NPs. In addition, CMD was able to circumvent the resistance mechanisms previously identified in *S. aureus* M12 and *S. enterica* ser. Typhimurium and reduced the beta-lactamase enzyme activity and efflux activity. The enhanced antibacterial properties of CMD can be imputed to the encapsulation of drugs into nano-carriers and then the additive effect of the encapsulated entities. Nano-encapsulation provides the benefits of targeted drug release, improved bioavailability, ability to target bacterial cells taking refuge intracellularly, and protection from harsh environment inside the host.

This research provides an insight into the prevailing issue of AMR in animal farms and the high potential of nano encapsulated combinations of antibiotics and adjuvants in circumventing resistance mechanisms in MDR bacterial pathogens. This research contributes to the vast knowledge required to increase application of NeACT in food animals for treatment of various MDR infections and to reduce the huge amounts of antibiotics being used.

Recommendations for future

- *In-vivo* studies are recommended to evaluate the translation of NeACT into animals as it gives a finer understanding of the fate of drug under varying pH, enzymes, host metabolism, and antibacterial effectiveness.
- Additional testing must be done of combinations of commonly used antibiotics and suitable adjuvants to be encapsulated into nano-carriers to preserve and enhance the antibacterial activity of existing antibiotics.

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