IN-VITRO EFFICACY OF POLYHEXAMETHYLENE BIGUANIDE – GRAPHENE OXIDE NANOPARTICLES AGAINST *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* ISOLATES

BY

NUR FATIN NABILA BINTI HAMSAN

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF VETERINARY MEDICINE

FACULTY OF VETERINARY MEDICINE
UNIVERSITI MALAYSIA KELANTAN

2023

ORIGINAL LITERARY WORK DECLARATION

I hereby certify that the work embodied in this thesis is the result of the original research and has not been submitted for a higher degree to any other University or Institution.

OPEN ACCESS I agree that my thesis is to be made immediately available as hardcopy or online open access (full text).

EMBARGOES I agree that my thesis is to be made available as Hardcopy or online (full text) for a period approved by the Post Graduate Committee.
Dated from _____ until _____.

CONFIDENTAL (Contains confidential information under the Official Secret Act 1972)*

Restricted (Contains restricted information as specified by the organisation where research was done)*

I acknowledge that Universiti Malaysia Kelantan reserves the right as follows.

1.The thesis is the property of Universiti Malaysia Kelantan

2.The library of Universiti Malaysia Kelantan has the right to make copies for the purpose of research only.

3.The library has the right to make copies of the thesis for academic exchange.

_____ _____ _____

SIGNATURE OF CANDIDATE **SIGNATURE OF SUPERVISOR**

DR NOR FADHILAH KAMARUZZAMAN

_____ _____

NRIC/PASSPORT NO. **NAME OF SUPERVISOR**

DATE: **DATE:**

IN-VITRO EFFICACY OF POLYHEXAMETHYLENE BIGUANIDE (PHMB) – GRAPHENE OXIDE NANOPARTICLES AGAINST *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* ISOLATES

ABSTRACT

Caseous lymphadenitis (CLA) is a contagious and zoonotic disease caused by *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) affecting small ruminants all over the world, causing economic losses to farmers. The treatment has only been partially effective, in part, due to formation of thick encapsulated abscess that provided protective nature from the treatment and immune system, in the infected animal. Therefore an alternative therapy that could be formulated against the thick abscess would be needed. This research explores the potential of polyhexamethylene biguanide (PHMB) and graphene oxide (GO) nanoparticles as a potential treatment, by demonstrating their bactericidal effects against *C. pseudotuberculosis in-vitro* using the time kill assay. The result demonstrated that PHMB alone is effective in comparison to GO alone, or combination of PHMB+GO at three hours exposure time. This could be due to the interaction between GO and PHMB that hinders PHMB activities against the bacteria. In conclusion, this study demonstrates that PHMB alone is effective against *C. pseudotuberculosis in vitro* and has potential to be further tested *in vivo* before it can be developed for the CLA treatment.

Keyword: Caseous lymphadenitis, *Corynebacterium pseudotuberculosis,* Polyhexamethylene biguanide, Graphene Oxide

KEBERKESANAN *IN-VITRO* PARTIKEL NANO POLYHEXAMETHYLENE BIGUANIDE (PHMB) DAN GRAPHENE OXIDE TERHADAP *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* ISOLASI

ABSTRAK

Penyakit Bisul Nodus Limfa merupakan penyakit yang berjangkit dan zoonosis yang disebabkan oleh *Corynebacterium pseudotuberculosis (C. pseudotuberculosis)* yang menjangkiti binatang ternakan kecil di seluruh dunia, menyebabkan kerugian ekonomi kepada para petani. Rawatan yang ada hanya berkesan secara separa, sebahagiannya disebabkan oleh pembentukan abses tebal yang terkapsulasi, memberikan perlindungan dari rawatan dan sistem imun, pada haiwan yang dijangkiti. Oleh itu, terapi alternatif yang boleh diformulasikan untuk melawan abses yang tebal diperlukan. Kajian ini meneroka potensi Polyhexamethylene biguanide (PHMB) dan nanopartikel Graphene oxide (GO) sebagai rawatan berpotensi, dengan menunjukkan kesan bakterisida mereka terhadap *C. pseudotuberculosis* secara in-vitro menggunakan ujian pembunuhan masa. Hasil kajian menunjukkan bahawa PHMB secara tunggal berkesan berbanding dengan GO secara tunggal, atau kombinasi PHMB+GO pada masa pendedahan selama tiga jam. Ini mungkin disebabkan interaksi antara GO dan PHMB yang menghalang aktiviti PHMB terhadap bakteria. Kesimpulannya, kajian ini menunjukkan bahawa PHMB secara tunggal berkesan melawan *C. pseudotuberculosis* secara *in vitro* dan berpotensi untuk diuji lebih lanjut secara *in vivo* sebelum ia boleh dibangunkan untuk rawatan CLA.

Kata kunci: Penyakit Bisul Nodus Limfa, *Corynebacterium pseudotuberculosis,* Polyhexamethylene biguanide, Graphene Oxide

ACKNOWLEDGEMENTS

In the name of Allah s.w.t, the Most Gracious and Most Merciful. An utmost gratitude goes to Allah, with His blessing, I can finally complete my research paper. I want to express my sincerest gratitude to my supervisor Dr Noor Fadhilah Binti Kamaruzzaman. Her continuous advice, information, suggestion and knowledge are always inspiring. Deepest thanks to my co-supervisor Dr. Mohammad Sabri Bin Abdul Rahman. My appreciation also extends to all staff of the laboratory, Kak Ina, Kak Nani, Kak Salma and Kak Mimi. Thank you to my friends, Mai and Naiemah for their help and support. Last but not least, my precious gratitude goes to my beloved family, especially ibu and ayah for their boundless prayers, trusts, endless support and motivation.

DEDICATION

To my beloved parents Rogayah and Hamsan,

Your unwavering love, constant support, and heartfelt prayers have been the guiding light throughout my journey. Thank you for being the pillars of strength in my life, for every sacrifice made, and for shaping me into the person I am today.

To my dear sisters Kinah, Mira, and Nurin,

Your laughter, endless support, and silly jokes have been the antidote to my lowest moments. Thank you for being my source of cheer and comfort.

To my dearest Along,

Your companionship brought immeasurable happiness into my days. Your memory will forever be etched in my heart.

To Betty and Beto,

Your gentle purrs and comforting presence provided solace amidst life's chaos. Thank you for the companionship and tranquility you brought into my world.

I am forever grateful.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CLA	Caseous Lymphadenitis
C. pseudotuberculosis	*Corynebacterium pseudotuberculosis*
PLD	Phospholipase D
PHMB	Polyhexamethylene biguanide
GO	Graphene oxide
DNA	Deoxyribonucleic acid
ROS	Reactive oxygen species
PCR	Polymerase Chain Reaction
BA	Blood agar
MHB	Mueller-Hinton broth
NaCl	Sodium chloride
CFU	Colony forming unit
mL	Milimetre
µl	Microlitre

LIST OF SYMBOLS

%	Percentage
°C	Celcius

CHAPTER 1

INTRODUCTION

1.1 Introduction

Caseous lymphadenitis (CLA) or cheesy gland disease, which are contagious and zoonotic disease affects small ruminants all over the world, is caused by the gram-positive, non-capsulated, non-motile, non-spore-forming, pleomorphic, intracellular, and facultatively anaerobic microbe *Corynebacterium pseudotuberculosis (C. pseudotuberculosis)* (Dorella et al., 2005). There are two forms of CLA which are external (also known as cutaneous or superficial) and internal form. Internal form is characterised by abscessation of internal lymph nodes and other visceral organs, whereas external form is characterised by abscess development in externally palpable lymph nodes (Baird & Fontaine, 2007). CLA is prevalent in Australia, New Zealand, the Middle East, Asia, Africa and parts of North and South America (Karthik & Prabhu, 2023).

In Malaysia, the prevalence of CLA in small ruminants was found to be 30% (Komala et al., 2008) and has become one of the potential threats to the livestock industry (Jesse et al., 2016). Once the disease is established in a farm, it is hard to eradicate owing to the virulence factors of *C. pseudotuberculosis*; exotoxin phospholipase D (PLD) and mycolic acid that contribute to the survival of the organism in the environment together with the inefficacy of antimicrobial therapy and vaccination available.

Nanotechnology is a rapidly evolving technology that has the potential to have a significant impact on veterinary medicinal applications. Graphene oxide (GO) and polyhexamethylene biguanide (PHMB) have been shown to be effective anti-inflammatory and antibacterial agents

(Lee et al., 2020; (Sowlati-Hashjin, Carbone and Karttunen, 2020). Because of the organism's virulence traits and intracellularity, most antibiotic therapies were unsuccessful. Nanoparticles of ultra-fine size allow for cellular penetration despite the thickness of the bacterial wall, causing damage where antimicrobial therapy has failed.

1.2 Problem statement

There is no specific treatment in treating caseous lymphadenitis despite the in vitro sensitivity of *C. pseudotuberculosis* to ceftiofur, florfenicol, penicillin, tulathromycin, oxytetracyclines and cephalosporins, treatment with these drugs is typically ineffective due to the protective nature of the capsule, organism's intracellularity and the formation of the thick encapsulated abscess (Simpson & Washburn, 2012). While drainage and daily flushing of the abscess is commonly practiced, it may result in environmental contamination and exposure to other naïve animals since the organism survives well in the environment. There is also currently no vaccine that induces 100% protection against the disease (Monther & Saleh, 2016). This is of concern to farmers because it results in significant economic losses due to reduction of wool, meat and milk yields, decreased reproductive efficiencies and condemnation of carcasses and skin (Paton et al., 1994; Arsenault et al., 2003). Alternatively, nanoparticles are one of potential approach in treatment and management of caseous lymphadenitis due to its higher surface to volume ratio together with antimicrobial and anti-inflammatory properties and ability to penetrate and induce cellular damage of bacterial cell (Hu, et al., 2010).

1.3 Research questions

What is the bactericidal concentration of graphene oxide and polyhexamethylene biguanide nanoparticles alone and in combination towards *C. pseudotuberculosis*?

1.4 Research hypothesis

Graphene oxide and polyhexamethylene biguanide nanoparticles alone and in combination are bactericidal towards *C. pseudotuberculosis*.

1.5 Research objectives

1. To determine the bactericidal activities of graphene oxide and polyhexamethylene biguanide alone against *C. pseudotuberculosis.*

2. To determine the bactericidal activities for combination of graphene oxide and polyhexamethylene biguanide against *C. pseudotuberculosis.*

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Caseous Lymphadenitis

Caseous lymphadenitis (CLA) affecting various animal species particularly sheep and goat is caused by an actinomycete *Corynebacterium pseudotuberculosis (C. pseudotuberculosis),* a Gram-positive facultative intracellular bacterium that has what is known as a "Chinese letter" or "picket fence" configuration under a microscope (Simpson & Washburn, 2012). *C. pseudotuberculosis* has earned the distinction of being “perfect parasite” (Baird & Fontaine, 2007) due to its remarkable ability to persist and multiply within host cells, including macrophages. This intracellularity is a key factor in the chronicity and persistence nature of CLA infections. Disease transmission in external form occur through direct contact or via fomites that are contaminated with the abscess while internal form is through aerosol.

The virulence factors contributing to the survival of this organism are exotoxin phospholipase D (PLD) and mycolic acid. Exotoxin PLD increases vascular endothelial membrane permeability following hydrolysis of sphingomyelin leading to leakage of plasma into surrounding tissues and into lymphatic drainage (Jolly, 1965; Carne & Onon, 1978). The waxy mycolic acid that coats the bacterial cell wall provide *C. pseudotuberculosis* with mechanical and biochemical protection from hydrolytic enzymes present within lysosomes which allow them to survive and escape phagocytosis and exist intracellularly and disseminate to other sites within macrophage (Williamson, 2001). Caseous lymphadenitis which is caused by the non-nitrate reducing biotype of *C. pseudotuberculosis* is a chronic suppurative condition in goats. The incubation period is

about three months, and it results in abscessation and enlargement of superficial or internal lymph nodes (Washburn et.al., 2023).

Caseous lymphadenitis stands out as an economically significant disease that impacts small ruminants, particularly sheep and goats. It leads to adverse effects on various aspects such as wool and leather quality, ill thrift, decreased meat and milk production, condemnation of carcasses and skins, and it also negatively influences the reproductive performance of flocks.

2.2 Treatment of Caseous lymphadenitis

Few treatment and control options are available for CLA, and this entails surgical excision of abscess, isolation of sick animals, culling of infected animals, intralesional antibiotic therapy using 10% formalin, and opening, flushing, draining open lesions. Culling of infected animals is the most practical method since infected animals serve as reservoirs of infection. Opening, flushing, and draining abscess when were done inappropriately will possess threat through contamination of the content to the environment.

Surgical treatment involves draining or removing the abscessed nodes followed by flushing the cavity with dilute disinfectant. This procedure must be done in a separate pen and the people performing this procedure should wear gloves. Proper disposal of collected pus through incineration is essential to prevent environmental contamination and potential sources of infection. The impacted goat should be isolated for an estimated duration of 30 days (Ashfaq and Campbell, 1980).

The prolonged use of antibiotics in the treatment regimen has yielded limited success. This is primarily attributed to the inherent characteristics of the bacteria—its encapsulated nature,

protection by a lipid membrane, and its ability to reside within cells, including the capacity to invade macrophages. However, the treatment had only been partially effective due to the nature of the bacteria.

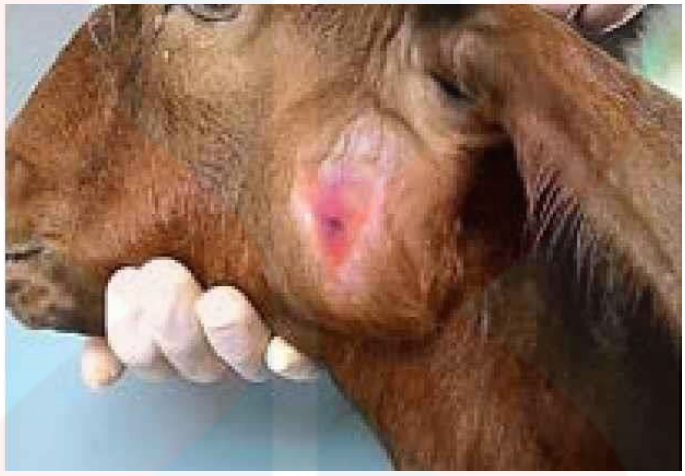

Figure 2.1: Non-movable solid mass of CLA-infected goat

Source: Department of Veterinary Services, 2017

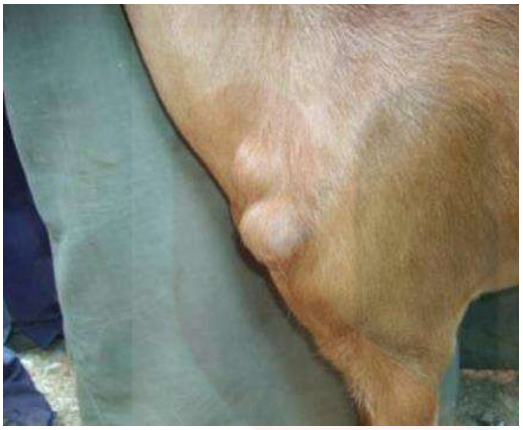

Figure 2.2: CLA infected goat showing affected closed parotid lymph node

Figure 2.3: CLA infected goat showing affected closed superficial cervical lymph node.

Source: (Al-Gaabary et al., 2009)

2.3 Pathogenesis of CLA

According to Muhammed Umer et al. (2017), the onset of *C. pseudotuberculosis* infection occurs as the organism gains entry into the body through minor breaches in the skin, mucous membranes, or superficial wounds, often caused during activities such as shearing, ear tagging, fighting, or contact with contaminated surfaces. Airborne transmission is also a potential route. Contaminated equipment or environments, via purulent discharge from abscesses or through coughed-up discharge, can also lead to animal infection. Subsequently, the bacteria localize in nearby lymph nodes and may disseminate through the lymphatic or vascular system to adjacent nodes or other organs.

The organism's spread and persistence are facilitated by its two primary virulence factors: the mycolic acid cell wall and phospholipase D (PLD). PLD enhances vascular wall permeability, causing plasma leakage, while also offering protection against opsonization and cellular enzyme digestion. *C. pseudotuberculosis* demonstrates the ability to invade and multiply within monocytes and macrophages, aided by PLD, which releases them from phagosomes and induces macrophage death. The mycolic acid cell wall contributes to pathogenesis by providing mechanical protection, coated with cytotoxin, enabling prolonged survival of the organism in the environment. Incubation periods typically range from two to six months or more.

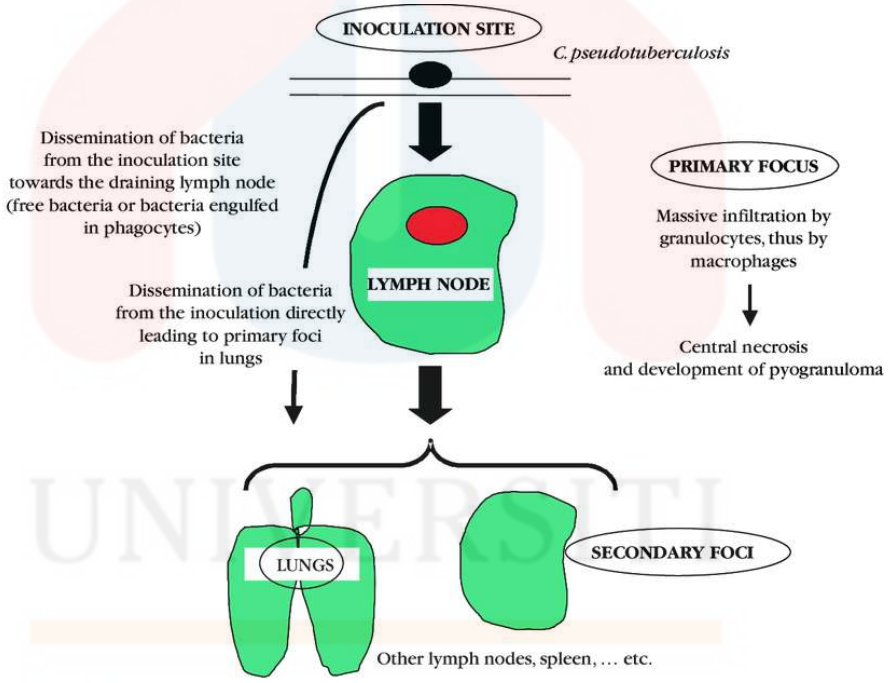

Figure 2.4: Schematic illustration of intracellular CLA

Source: (Lefevre et al., 2010)

2.4 Diagnosis of CLA

According to Burrel (1981), the presence of firm to slightly fluctuant subcutaneous enlargement of a lymph node and a herd history of CLA are indicative of assumed CLA. When a farm lacks a herd history, this can prompt further confirmatory laboratory examinations, such as bacterial isolation and identification. Culture samples can be procured by using a sterile needle to collect abscess material or via swab. Caution must be exercised to prevent leakage of contents, which could potentially contaminate the environment with CLA. Subsequently, bacterial culture on blood agar is conducted, incubating for approximately 48 hours at 37°C to observe colony growth. Colonies of *Corynebacterium pseudotuberculosis* typically appear as small, dry, whitish colonies surrounded by a narrow zone of hemolysis (Torky et al., 2023). Gram staining reveals gram-positive coccobacilli arranged in a Chinese pattern (Singh et al., 2017). The latest technological advancements involve employing ELISA tests to detect the presence of antibodies to the exotoxin (Pepin & Paton, 2010).

2.5 Zoonotic potential of CLA

Caseous lymphadenitis is a zoonotic disease. However, humans are rarely affected and the first human lymphadenitis case was recorded in 1966. Most reported cases of human lymphadenitis are associated with individuals having involvement or occupational exposure to animals. The prevalent types often involve axillary and inguinal lymphadenitis due to direct organism contact through the hands and legs, respectively (Peel et al., 1997). Nonetheless, an atypical case reported in the United States detailed patients experiencing eosinophilic pneumonia, a condition that resolved following treatment with erythromycin (Keslin et al., 1979).

In summary, clinical manifestations of human lymphadenitis caused by *C. pseudotuberculosis* are typically mild and sometimes absent. Treatment options include surgical incision, drainage, and antibiotic therapy.

2.6 Overview of Nanoparticles

Nanotechnology is a burgeoning subject that was founded in 1974 due to its potential impact on *in-vitro* and *in-vivo* medical applications and research. The term nano is derived from the Latin word nanus, which means "very small size" (1nm equals 10'9 m) (Boulaiz et al., 2011). The potential is owing to the high surface-to-volume ratio, stability, low toxicity, bioavailability, site-specific targeting, and stability. In the realm of pharmacology, nanoparticles are regarded as an effective medication delivery mechanism that not only protects animals from viral or bacterial infections, but also improves wound healing and can alleviate pain (Youssef et al., 2019).

2.7 Polyhexamethylene Biguanide (PHMB)

Gilbert and Moore (2005) characterize PHMB as a linear polymer featuring a hydrophobic core and multiple cationic groups interspersed with hexamethylene hydrocarbon chains. Despite its extensive use in human medicine, instances of resistance to PHMB have not been identified. The antimicrobial action of PHMB primarily operates through membrane disruption and interaction with DNA.

It's widely acknowledged that bacterial cells possess a negative charge, facilitating the binding of positively charged PHMB molecules to surface molecules on bacterial cells. Within the bacterial cytoplasmic membrane, PHMB also attaches to negatively charged acidic phospholipids (Gilbert and Moore, 2005). This interaction results in their integration into the bacterial cell

membrane, leading to disrupted growth, loss of function, leakage of cellular components, and rapid lysis, ultimately causing cell death (Broxton et al., 1984; Ikeda et al., 1984).

Moreover, PHMB initiates DNA repair pathways by interacting with the DNA backbone through its chain. These interactions disturb the DNA's overall structure, impede DNA replication, and activate DNA repair mechanisms (Shahin et al., 2020), leading to substantial disruption and/or precipitation of DNA function, culminating in cell death (Allen et al., 2006).

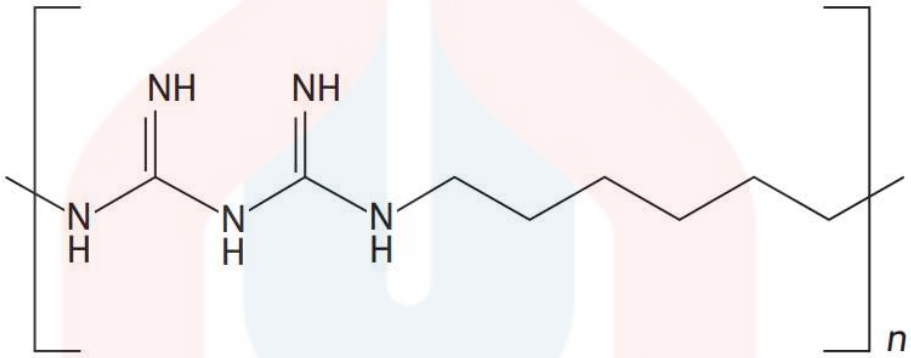

Figure 2.5 Structure of PHMB

Source: Kamaruzzaman et al., 2018

2.8 Graphene Oxide (GO)

Graphene oxide, originating from the acid oxidation of graphite initially conducted by B.C Brodie in 1859 (Brodie, 1859), represents a hydrophilic and negatively-charged single-layered material. Its structural resemblance to a graphene sheet includes oxygen-based functional groups like hydroxyl, carbonyl, carboxylic, and epoxy groups (Lerf et al., 1998; Ivey et al., 2008). This derivative has emerged as a promising nanoscale carbon-based particle distinct from graphene due to its advantageous attributes, including a high surface area-to-volume ratio and cost-effective production (Stankovich et al., 2006; Zhao et al., 2010). Graphene oxide exhibits properties associated with both antimicrobial effects and wound healing. The antimicrobial action involves a physicochemical interaction facilitated by the sharp edges of GO acting as nanoknives (Akhavan and Ghaderi, 2010; Liu S. et al., 2011). Additionally, it induces oxidative stress, potentially with the generation of reactive oxygen species (ROS), as part of its antimicrobial mechanisms (West and Marnett, 2006; Li et al., 2015).

As per Zou et al. (2016), the nanoknives mechanism entails the penetration of the microbial cell membrane, causing cytoplasmic leakage and eventual cell death. Pham et al. (2015) suggested that graphene oxide (GO) may induce pore formation in the bacterial cell membrane, disrupting osmotic balance and culminating in cell death. Furthermore, as outlined by Zhao et al. (2014), GO-generated reactive oxygen species (ROS) can trigger mitochondrial dysfunction and DNA damage, leading to the oxidation of lipids, nucleic acids and proteins, resulting in cell membrane destruction inhibiting bacterial growth due to oxidative stress.

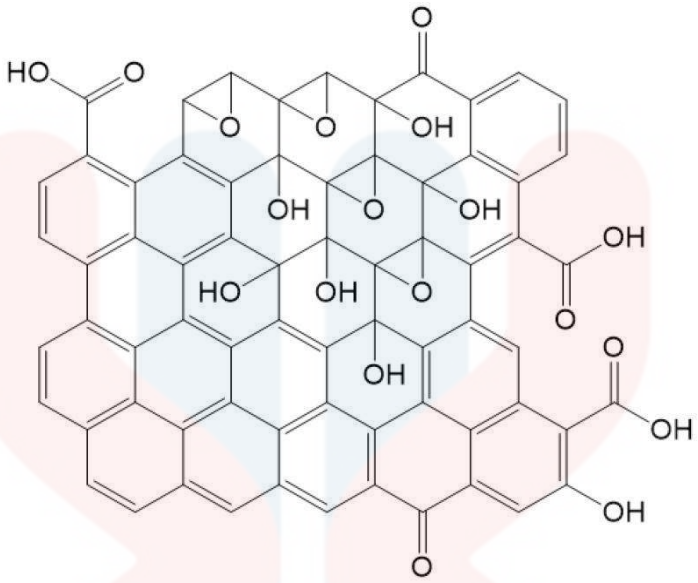

Figure 2.6 Structure of GO

Source: Jiříčková et al., 2022

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Sample

A clinical sample previously confirmed positive for CLA was employed in the study. This sample was stored in a tube containing glycerol stock at -80°C. The addition of glycerol serves to stabilize the frozen bacteria, safeguarding cell membranes from damage while maintaining their viability. Before storage, the sample underwent molecular detection using polymerase chain reaction (PCR) and sequencing to confirm the presence of *C. pseudotuberculosis*. Prior to utilization, the sample was thawed.

3.2 Isolation and Identification of *Corynebacterium pseudotuberculosis*

Corynebacterium pseudotuberculosis was isolated from thawed stocks and cultured in both blood agar (BA) plates and 10 mL Mueller-Hinton broth (MHB). Optimal growth occurred with BA solid media inoculation. Bacteria were scraped from stocks and streaked onto BA plates or added directly into MHB with gentle stirring to promote dissolution into the broth. Following overnight incubation for 48-72 hours at 37°C (with MHB in a shaker incubator), the cultures were assessed for characteristic *C. pseudotuberculosis* colony and cell morphology.

3.3 PHMB-GO Nanoparticles drugs formulation

3.3.1 Determination and calculation of formula for PHMB and GO nanoparticles

Polyhexamethylene biguanide (PHMB) and Graphene oxide nanoparticles are formulated at various concentrations using a 1:50 ratio. The required final volumes for PHMB and GO are 0.5ml each, to be prepared in a 1.5ml Eppendorf tube.

Table 3.1: Concentration of PHMB and GO nanoparticles

Concentration	**Final Concentration (µg/ml) (in 5ml)**		**Final Concentration (µg/ml) (in 0.5ml)**	
	GO	PHMB	GO	PHMB
1	100	2	1000	20
2	200	4	2000	40
3	300	6	3000	60

3.3.2 Preparation of PHMB and GO solution from stock solution

Based on the chosen concentrations mentioned earlier, the drug formulation of PHMB-GO nanoparticles is created using stocks of 0.16mg/ml and 3mg/ml, respectively. Therefore, dilution is required by adding distilled water to attain the final desired concentration.

Table 3.2: GO nanoparticles preparation

Concentration	Final concentration (mg/ml) (0.5ml)	Volume of GO (µl)	Volume of distilled water (µl)
1	1	167	333
2	2	333	167
3	3	500	-

Table 3.3: PHMB nanoparticles preparation

Concentration	Final concentration (mg/ml) (0.5ml)	Volume of PHMB (µl)	Volume of distilled water (µl)
1	0.02	62.5	437.5
2	0.04	125	375
3	0.06	187.5	312.5

To prepare the combination drug solutions, PHMB and GO nanoparticles were combined at three different concentrations (1, 2 and 3). The nanoparticles were mixed by pipetting up and down to produce a final volume of 1 mL containing both PHMB and GO.

For the individual drug solutions, PHMB or GO were prepared separately at the specified concentrations. Distilled water was then added to reach a final volume of 1 mL containing only the single nanoparticle type.

3.4 *In-vitro* bactericidal activity of PHMB-GO nanoparticles towards *C. pseudotuberculosis*

3.4.1 Time kill assay for PHMB and GO alone and in combination

Time kill assay utilized to evaluate and compare the bactericidal efficacy of polyhexamethylene biguanide (PHMB) and graphene oxide (GO) nanoparticles, both individually and in combination against *C. pseudotuberculosis* following the guidelines outlined in the Clinical and Laboratory Standards Institute (CLSI), 1999. Test tubes containing 1 mL of bacterial suspension (105 CFU/mL) in Mueller-Hinton broth was inoculated with 1 mL treatment solutions of either PHMB-GO combination, PHMB alone, or GO alone nanoparticles prepared at a range of concentrations. The tubes were incubated at 37°C while being vortexed at 200 rpm to prevent bacterial adhesion to the tube's walls. After inoculation, 50µl samples were withdrawn at 0, 3, and 6-hour intervals and serially diluted in 450 microliters of saline solution (0.9% NaCl) to achieve a 10-fold dilution. Subsequently, 10 microliters from each dilution was pipetted onto nutrient agar plates. These plates were then undergoing incubation for 48 hours at 37°C, followed by the enumeration of viable cells (CFU/mL) (Balouiri et al., 2016). This process was repeated for two remaining concentrations and a control group that does not receive treatment. Colony forming unit per ml was calculated using the formula below:

$$\frac{Number\ of\ colonies\ on\ plate\ \times\ Dilution\ factor}{Volume\ of\ cultured\ plate\ in\ ml}$$

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Recultivation of *C. pseudotuberculosis*

C. pseudotuberculosis was successfully cultivated and displayed distinct morphology after 24-hours incubation. On blood agar, the morphological traits of *C. pseudotuberculosis* included small, opaque, round, whitish colonies with narrow zones displaying hemolysis as shown in Figure 4.1. Additionally, Gram staining was conducted, revealing Gram-positive short coccobacilli grouped in pairs, as well as individually arranged resembling Chinese letters (Figure 4.2).

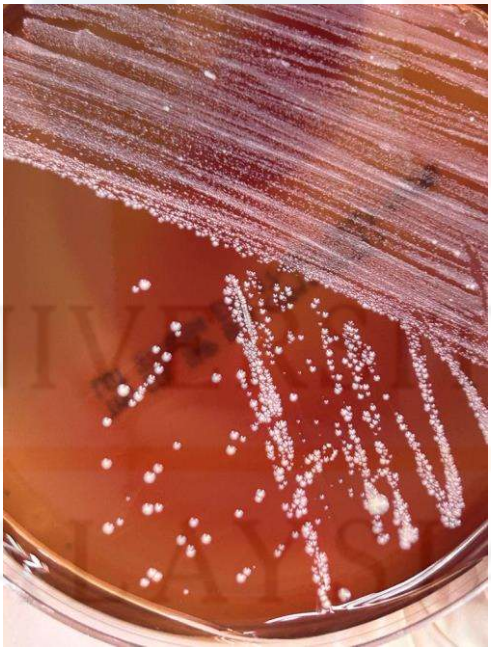

Figure 4.1: Morphology of C. pseudotuberculosis on blood agar

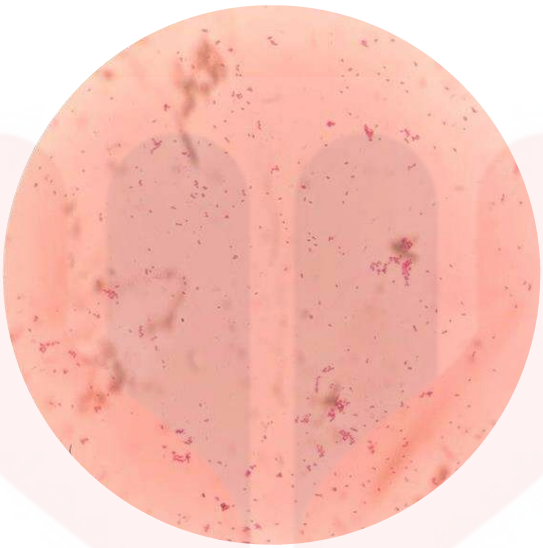

Figure 4.2: Gram staining of C. pseudotuberculosis

4.1.2 Bactericidal activity of PHMB and GO alone against *C. pseudotuberculosis*

To determine the bactericidal activity of PHMB and GO alone, time kill assay was performed using different concentrations of PHMB and GO against *C. pseudotuberculosis* by calculating the colony forming units per ml (CFU/mL).

Referring to Table 4.1 and Table 4.2, at 0 hours, both PHMB and GO alone recorded 10^7 CFU/mL. At three hours, PHMB demonstrated 6 x 10^6, 11 x 10^4 and 7 x 10^3 CFU/mL, reduction in colony forming units at 2, 4 and 6 µg/ml concentration respectively as in Figure 4.3.

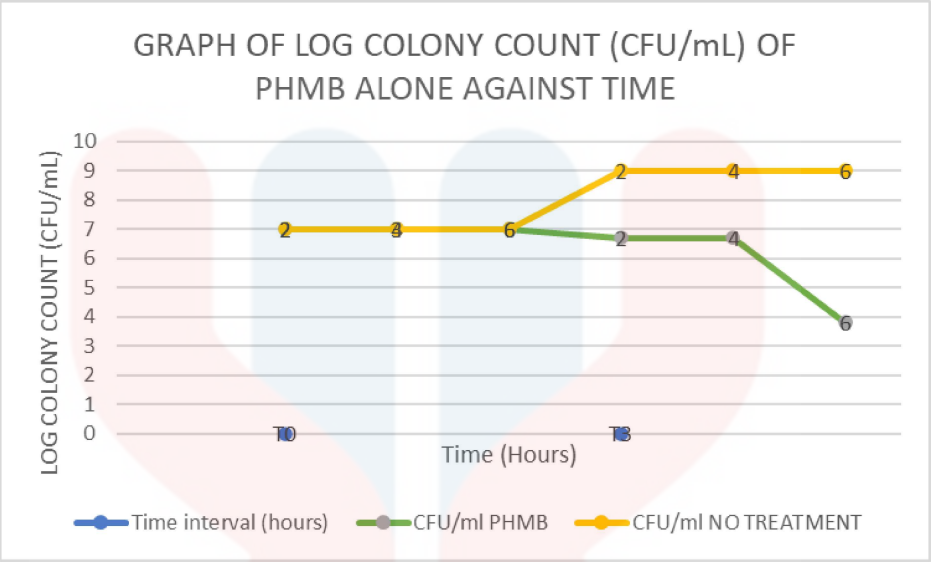

Figure 4.3: Graph of Colony Count (CFU/mL) of PHMB alone Against Time

Table 4.1: Bactericidal activity of single drug PHMB nanoparticles

Time interval (hours)	**Final concentration of PHMB (µg/ml)**	**CFU/mL**
T_0	2	10^7
	4	10^7
	6	10^7
T_3	2	$6x10^6$
	4	$11x10^4$
	6	$7x10^3$

Meanwhile for GO, at three hours, it recorded a steady pattern in CFU/ml with 10^7 at both 100 µg/ml and 200 µg/ml concentrations before recording a reading of 6 x 10^6 at 300 µg/ml as

shown in Figure 4.4 . Hence, by the definition mentioned earlier, only PHMB possesses bactericidal activity as it shows reduction of at least 99.9% of the total count of CFU/mL.

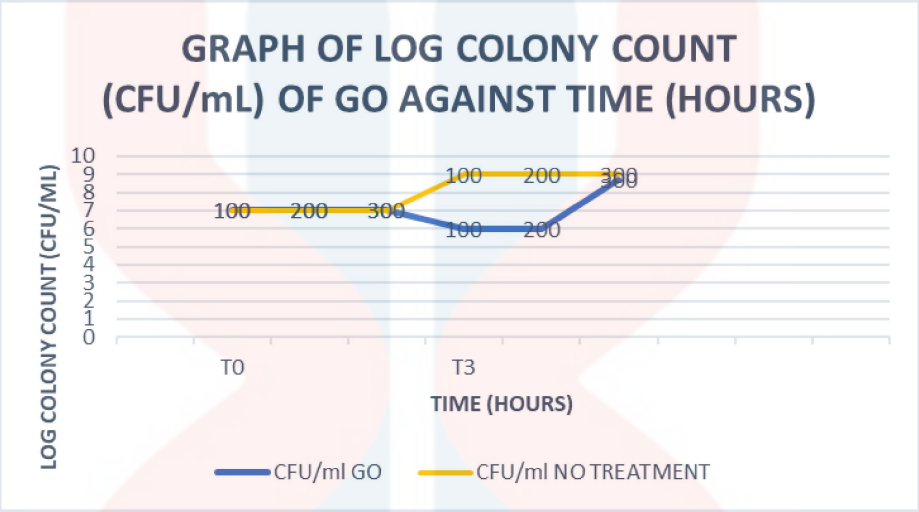

Figure 4.4: Graph of Colony Count (CFU/mL) of GO alone Against Time

Table 4.2: Bactericidal activity of single drug GO nanoparticles

Time interval (hours)	Final concentration of GO (µg/ml)	CFU/mL
T_0	100	10^7
	200	10^7
	300	10^7
T_3	100	10^6
	200	10^6
	300	$6x10^8$

4.1.3 Bactericidal activity of PHMB and GO in combination against *C. pseudotuberculosis*

Based on Figure 4.5 for the combination therapy, at 0 hours, it recorded 10^7 CFU/mL for all three concentrations. At three hours, the combination shows an increase in CFU/mL reading with 10^8 for all of the concentration.

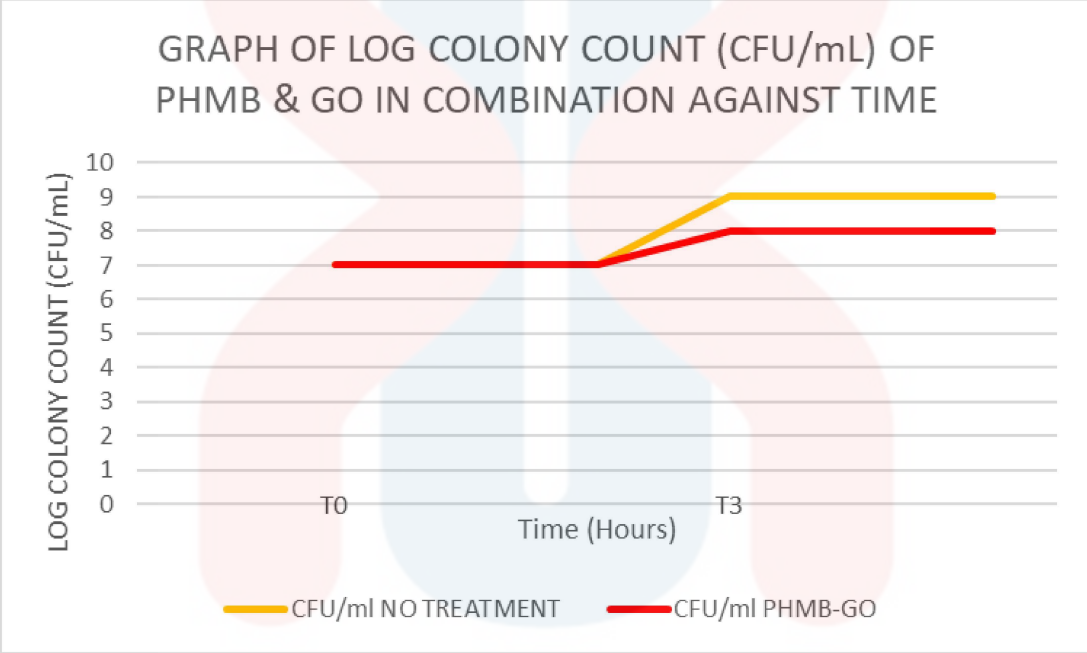

Figure 4.5: Graph of Colony Count (CFU/mL) of PHMB and GO in Combination Against Time

Table 4.3: Bactericidal activity of combination drugs (PHMB-GO) nanoparticles

Time interval (hours)	**Final concentration of PHMB-GO (µg/ml)**		**CFU/ml**
	GO	**PHMB**	
T_0	100	2	10^7
	200	4	10^7

	300	6	10^7
T_3	100	2	10^8
	200	4	10^8
	300	6	10^8

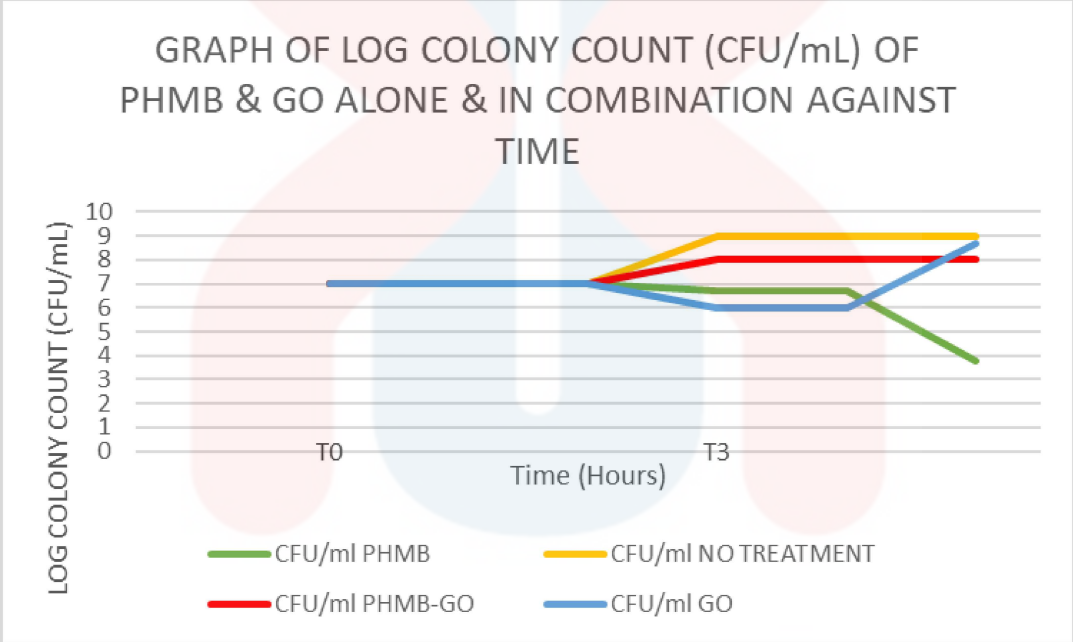

Figure 4.6: Graph of Colony Count (CFU/mL) of GO alone and In Combination Against Time

4.2 Discussion

4.2.1 Cell wall composition of *C. pseudotuberculosis* that affect the penetration of PHMB and GO nanoparticles

The experimental findings reveal varying levels of bactericidal impact when using PHMB and GO nanoparticles against *C. pseudotuberculosis*. Treatment with PHMB demonstrates the most substantial bactericidal effect, while GO exhibits no bactericidal effect on eliminating *C. pseudotuberculosis*. Surprisingly, the combined use of PHMB and GO shows no bactericidal effects, with colony forming units per ml increasing over time. The causes behind these outcomes could be due to several factors, which include the physiochemical cell wall of *c. pseudotuberculosis* that affect the penetration of PHMB and GO nanoparticles. The complex cell wall structure of *C. pseudotuberculosis* contributes significantly to its innate tolerance and resistance against many standard antibiotics, as evidenced by recent comparative assessments of PHMB and GO nanoparticles. At the foundation, *C. pseudotuberculosis* has a dense peptidoglycan layer that is cross-linked to arabinogalactan polysaccharides (Burkovski, 2013). Attached to this heteropolymer matrix is an outer membrane composed of interlinked mycolic acid chains, creating an impermeable lipid barrier functionally akin to the Gram-negative outer membrane (Figure 4.4) (Damien et al., 2004; Jesse et al., 2013). The robust architecture of the mycolic acid membrane in particular renders the overall cell envelope impenetrable to many external antimicrobial assaults (Mohammed Naji Odhah et al, 2022). Together, these specialized cell wall elements enable innate tolerance mechanisms against conventional antibiotics and likely underlie the varying effectiveness observed between PHMB and GO nanoparticles. Further investigations characterizing the molecular properties and interactions enabling *C. pseudotuberculosis* resistance

against different compound classes will provide crucial insight guiding future development of targeted antimicrobials against this recalcitrant pathogen.

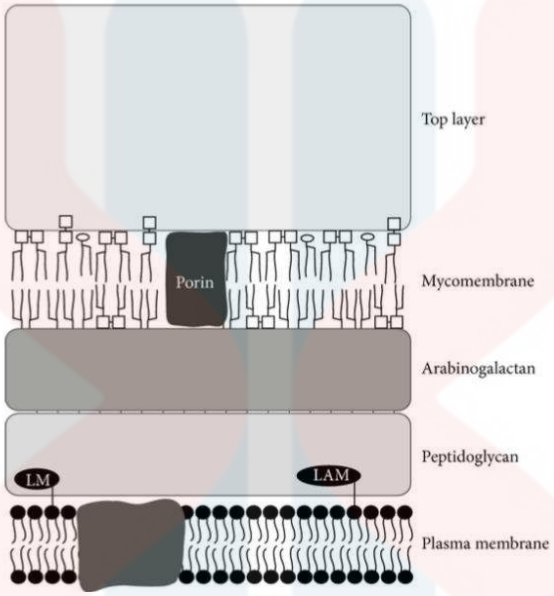

Figure 4.7: A corynebacterial cell envelope

Source: Burkovski, 2013

4.2.2 Antagonism interaction between PHMB and GO nanoparticles

Antimicrobial agents can interact through different mechanisms when combined, including synergism, antagonism or indifference. Synergism occurs when the combined effect is greater than the sum of individual effects, while antagonism denotes an inferior combined effect compared to the individual agents (Giguere et al., 2006). The anticipated synergy between polyhexamethylene biguanide (PHMB) and graphene oxide (GO) nanoparticles based on their reported antimicrobial and anti-inflammatory properties was unexpectedly not observed. Instead, an antagonistic interaction was revealed, wherein the bactericidal efficacy against *C. pseudotuberculosis* declined for the combined nanoparticles over time.

The potential reason for this could be attributed to the molecular structures of both PHMB and GO. PHMB is described as a cationic polymer consisting of repeating hexamethylene biguanide groups (Kamaruzzaman et al., 2018), while graphene oxide features a layered carbon structure with various oxygen-containing functional groups (=O, -OH, -O-, -COOH) attached to its layers and edges. Conversely, the peptidoglycan cell wall of gram-positive bacteria carries a net negative electrostatic charge primarily due to the presence of phosphoryl groups in substituent teichoic acid and teichuronic acid, as well as lipopolysaccharide residues (Li et al., 2019; Beveridge, 1988).

This observation might be explained by competitive binding, inhibition of bactericidal activity, inhibition of cell membrane permeability, electrostatic interactions, complex formation, and surface modification occurring either between drugs or between drugs and bacteria (Giguere et,al, 2006). The positively charged PHMB and negatively charged GO nanoparticles might compete for binding sites on the bacterial surface, or they could interact to form drug complexes due to the electrostatic interaction. Due to the nature of graphene oxide to attach to the surface of positively-charged PHMB via the functional group -COOH present on the surface of GO renders the ability of PHMB to capture the negatively-charged *C. pseudotuberculosis* bacteria. Additionally, this binding activity may alter the surface properties of both drugs and bacteria. As a result of these mechanisms, the drugs' capability to effectively bind and capture the bacteria could be compromised. This explains the observed trend of increasing colony forming units per ml over time in the results.

4.2.3 Differences in bactericidal effect between PHMB and GO

The molecular characteristics of nanoparticles substantially influence their interactions and antibacterial effects against *C. pseudotuberculosis*. Several factors may contribute to the

differences seen between polyhexamethylene biguanide (PHMB) and graphene oxide (GO) nanoparticles. A key difference lies in their mechanism of action. Positively-charged PHMB readily binds to the negatively-charged phospholipid bilayer of bacterial cell walls, enabling formation of salt bridges between the biguanide groups of PHMB and phosphate head groups of phospholipids (Sowlati-Hashjin et al., 2020). These charge interactions facilitate insertion and translocation of PHMB across the cell membrane (Broxton et al., 1984; Ikeda et al., 1984), causing disruption of membrane structural integrity, permeation, and influx into the bacterial cell. In contrast, GO nanoparticles inflict membrane damage predominantly through physical piercing effects and nanoparticle-driven pore formation from their knife-like edges (Ravikumar et al., 2022). This results in leakage of intracellular components. However, the complex lipid layers of the *C. pseudotuberculosis* cell wall may prove less prone to such physical piercing mechanisms. Thus, the broader spectrum binding and membrane disruption mechanism of cationic PHMB nanoparticles to anionic phospholipid bilayers may be more effective against *C. pseudotuberculosis* compared to the physical membrane slicing mode-of-action of graphene oxide.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

In summary, only PHMB exhibits bactericidal effects against *C. pseudotuberculosis* but not GO. These results can be interpreted as antagonistic in their drug interactions as antagonism denotes an inferior combined effect compared to the individual agents. However, further investigation is necessary to understand the interaction of these drugs with the bacteria in vitro to ensure their effectiveness. This may involve increasing the drug concentration, as it's possible that PHMB and GO nanoparticles might require higher doses to reach the minimum effective concentration threshold and extend the exposure duration. This consideration and exposure time are critical due to the categorization of drugs based on their time or concentration-dependent activity. Time-dependent drugs maintain a consistent rate and extent of microorganism elimination regardless of the antimicrobial concentration, while concentration-dependent drugs show varying rates and extents of microorganism elimination, increasing with higher concentrations.

The absence of bactericidal effects when the drugs are combined requires further investigation, as some instances of antagonism in antimicrobial combinations might stem more from laboratory artifacts than actual clinical scenarios.

Since this study marks the initial exploration of the bactericidal impact of polyhexamethylene biguanide and graphene oxide nanoparticles on *C. pseudotuberculosis*, numerous shortcomings in the findings could be enhanced. The aim is for this research to potentially aid and establish itself as a point of reference for future studies.

Throughout the process of experimentation, several obstacles have been encountered notably the potential for result contamination originating from equipment, materials or

environmental sources. Therefore, maintaining proper disinfection protocols and working meticulously in a sterile manner are essential measures to minimize and forestall any contamination that could potentially impact the overall results.

REFERENCES

Simpson, K. M., & Washburn, K. E. (2012). Caseous lymphadenitis: Realities in treatment and prevention. The Bovine Practitioner, 46(2), 94–100 https://doi.org/10.21423/bovine-vol46no2p94-100

Baird GJ, Fontaine MC. (2007). Corynebacterium pseudotuberculosis and its role in ovine caseous lymphadenitis. J Comp Pathol. 2007 Nov;137(4):179-210. https://doi.org/10.1016/j.jcpa.2007.07.002

Dorella, F.A., Pacheco, L.G., Oliveira, S.C., Miyoshi, A., & Azevedo, V.A. (2006). Corynebacterium pseudotuberculosis: microbiology, biochemical properties, pathogenesis and molecular studies of virulence. Veterinary research, 37 2, 201-18. https://doi.org/10.1051/VETRES%3A2005056

Karthik, K., & Prabhu, M. (2021). Bacterial Diseases of Goat and Its Preventive Measures. In *www.intechopen.com*. IntechOpen. https://www.intechopen.com/chapters/76276

Komala, T.S. *et al.* (2008) 'A survey of caseous lymphadenitis in small ruminant farms from two districts in Perak, Malaysia -- Kinta and Hilir Perak', *Tropical Biomedicine*, 25(3), pp. 196–201. https://pubmed.ncbi.nlm.nih.gov/19287357/ (Accessed: 29 February 2024).

Jesse, F.F.A. *et al.* (2016) 'Strategic Management of a Clinical Case of Recurrent Caseaous Lymphadenitis in a Goat Farm', *Research Journal for Veterinary Practitioners*, 4(3), pp. 42–46. Available at: https://doi.org/10.14737/journal.rjvp/2016/4.3.42.46.

Sowlati-Hashjin, S., Carbone, P. and Karttunen, M. (2020) 'Insights into the Polyhexamethylene Biguanide (PHMB) Mechanism of Action on Bacterial Membrane and DNA: A Molecular Dynamics Study', *The Journal of Physical*

Chemistry B, 124(22), pp. 4487–4497. Available at: https://doi.org/10.1021/acs.jpcb.0c02609.

Monther, W., & Saleh, M. (2016). *UNIVERSITI PUTRA MALAYSIA EFFICACY OF A PROTOTYPE VACCINE FOR CASEOUS LYMPHADENITIS DISEASE IN GOATS*. Retrieved February 29, 2024, from http://psasir.upm.edu.my/id/eprint/75406/1/FPV%202016%2038%20-%20IR.pdf

PATON, M., ROSE, I., HART, R., SUTHERLAND, S., MERCY, A., ELLIS, T., & DHALIWAL, J. (1994). New infection with Corynebacterium pseudotuberculosis reduces wool production. *Australian Veterinary Journal*, *71*(2), 47–49. https://doi.org/10.1111/j.1751-0813.1994.tb06152.x

Arsenault, J., Girard, C., Dubreuil, P., Daignault, D., Galarneau, J.-R., Boisclair, J., Simard, C., & Bélanger, D. (2003). Prevalence of and carcass condemnation from maedi–visna, paratuberculosis and caseous lymphadenitis in culled sheep from Quebec, Canada. *Preventive Veterinary Medicine*, *59*(1-2), 67–81. https://doi.org/10.1016/s0167-5877(03)00060-6

Hu, H., Wang, Z., Pan, L., Zhao, S., & Zhu, S. (2010). Ag-Coated Fe_3O_4@SiO_2 Three-Ply Composite Microspheres: Synthesis, Characterization, and Application in Detecting Melamine with Their Surface-Enhanced Raman Scattering. *The Journal of Physical Chemistry C*, *114*(17), 7738–7742. https://doi.org/10.1021/jp100141c

Jolly, R. D. (1965). The pathogenic action of the exotoxin of Corynebacterium ovis. *Journal of Comparative Pathology*, *75*(4), 417–431. https://doi.org/10.1016/0021-9975(65)90022-8

Carne, H. R., & Onon, E. O. (1982). The exotoxins ofCorynebacterium ulcerans. *Journal of Hygiene*, *88*(2), 173–191. https://doi.org/10.1017/s0022172400070054

Williamson, L. H. (2001). Caseous Lymphadenitis in Small Ruminants. *Veterinary Clinics of North America: Food Animal Practice*, *17*(2), 359–371. https://doi.org/10.1016/s0749-0720(15)30033-5

Washburn, K. E., Fajt, V. R., Lawhon, S. D., Adams, L. G., Tell, L. A., & Bissett, W. T. (2013). Caprine Abscess Model of Tulathromycin Concentrations in Interstitial Fluid from Tissue Chambers Inoculated with Corynebacterium pseudotuberculosis following Subcutaneous or Intrachamber Administration. *Antimicrobial Agents and Chemotherapy*, *57*(12), 6295–6304. https://doi.org/10.1128/aac.00936-13

Al-Gaabary, M. H., Osman, S. A., & Oreiby, A. F. (2009). Caseous lymphadenitis in sheep and goats: Clinical, epidemiological and preventive studies. *Small Ruminant Research*, *87*(1-3), 116–121. https://doi.org/10.1016/j.smallrumres.2009.10.008

Burrell, D. H. (1981). CASEOUS LYMPHADENITIS IN GOATS. *Australian Veterinary Journal*, *57*(3), 105–110. https://doi.org/10.1111/j.1751-0813.1981.tb00468.x

Boulaiz, H., Alvarez, P. J., Ramirez, A., Marchal, J. A., Prados, J., Rodríguez-Serrano, F., Perán, M., Melguizo, C., & Aranega, A. (2011). Nanomedicine: Application Areas and Development Prospects. *International Journal of Molecular Sciences*, *12*(5), 3303–3321. https://doi.org/10.3390/ijms12053303

Osman AY, Nordin ML, Kadir AA, Saharee AA (2018) The Epidemiology and Pathophysiology of Caseous Lymphadenitis: A Review. J Vet Med Res 5(3): 1129. https://www.jscimedcentral.com/jounal-article-info/Journal-of-Veterinary-Medicine-and-Research/The-Epidemiology-and--Pathophysiology-of-Caseous--Lymphadenitis%3A-A-Review-9816#

Allen, M. J., White, G. F., & Morby, A. P. (2006). The response of Escherichia coli to exposure to the biocide polyhexamethylene biguanide. Microbiology (Reading, England), 152(Pt 4), 989–1000. https://doi.org/10.1099/mic.0.28643-0

Sowlati-Hashjin, S., Carbone, P., & Karttunen, M. (2020). Insights into the Polyhexamethylene Biguanide (PHMB) Mechanism of Action on Bacterial Membrane and DNA: A Molecular Dynamics Study. The journal of physical chemistry. B, 124(22), 4487–4497. https://doi.org/10.1021/acs.jpcb.0c02609

V, J, Mohanraj., Y, Chen. (2007). Nanoparticles - A Review. Tropical Journal of Pharmaceutical Research, 5(1):561-573. https://www.doi.org/10.4314/TJPR.V5I1.14634

Gupta, D.K., Rajaura, R.S., & Sharma, K. (2015). Synthesis and Characterization of Graphene Oxide Nanoparticles and their Antibacterial Activity. https://www.gyanvihar.org/researchjournals/3.pdf

Ridongia, K., Tan, A. TL., & Huang, J. (2014). Graphene Oxide: Some New Insights into an Old Material. In S. Iijima, & K. Tanaka (Eds.), Carbon Nanotubes and Graphene (2 ed.). Elsevier. http://dx.doi.org/10.1016/B978-0-08-098232-8.00014-0

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. Journal of pharmaceutical analysis, 6(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Borra, R. C., Lotufo, M. A., Gagioti, S. M., Barros, F.deM., & Andrade, P. M. (2009). A simple method to measure cell viability in proliferation and cytotoxicity assays. Brazilian oral research, 23(3), 255–262. https://doi.org/10.1590/s1806-83242009000300006

Warner, D. R., Sakai, D., and Sandell, L. L. 2015. Mammalian cell culture. Curr. Protoc. Essen. Lab. Tech. 10: 4.3.1- 4.3.33. https://doi.org/10.1002/9780470089941.et0403s10

Torky, H. A., Saad, H. M., Khaliel, S. A., Kassih, A. T., Sabatier, J., Batiha, G. E., Hetta, H. F., Elghazaly, E. M., & De Waard, M. (2023, January 14). *Isolation and Molecular Characterization of Corynebacterium pseudotuberculosis: Association*

with Proinflammatory Cytokines in Caseous Lymphadenitis Pyogranulomas. Animals. https://doi.org/10.3390/ani13020296

Singh, R., Kumari, S., Yadav, J. P., & Kumar, S. (2017, September 29). *Suppurative Pneumonia and Lymphadenitis in a Goat Associated with Infection by Corynebacterium. . .* ResearchGate. https://www.researchgate.net/publication/320324745_Suppurative_Pneumonia_and_Lymphadenitis_in_a_Goat_Associated_with_Infection_by_Corynebacterium_Pseudotuberculosis_A_Case_Study

Pepin, M., & Paton, M. (2010, January 1). *Caseous lymphadenitis in sheep and goats*. ResearchGate. https://www.researchgate.net/publication/292514560_Caseous_lymphadenitis_in_sheep_and_goats

Burkovski, A. (2013, January 21). *Cell Envelope of Corynebacteria: Structure and Influence on Pathogenicity*. ISRN Microbiology (Print). https://doi.org/10.1155/2013/935736

Ravikumar, V., Mijaković, I., & Pandit, S. (2022, December 1). *Antimicrobial Activity of Graphene Oxide Contributes to Alteration of Key Stress-Related and Membrane Bound Proteins*. International Journal of Nanomedicine. https://doi.org/10.2147/ijn.s387590

Gautam, C., & Saha, L. (2008, February 21). *Fixed dose drug combinations (FDCs): rational or irrational: a view point*. British Journal of Clinical Pharmacology. https://doi.org/10.1111/j.1365-2125.2007.03089.x

Li, Z., Ma, J., Ruan, J., & Zhuang, X. (2019, June 4). *Using Positively Charged Magnetic Nanoparticles to Capture Bacteria at Ultralow Concentration*. Nanoscale Research Letters. https://doi.org/10.1186/s11671-019-3005-z

Wilson, W. W., Wade, M. M., Holman, S. C., & Champlin, F. R. (2001, January 1). *Status of methods for assessing bacterial cell surface charge properties based on zeta*

potential measurements. Journal of Microbiological Methods. https://doi.org/10.1016/s0167-7012(00)00224-4

Kamaruzzaman, N. F., De Fátima Pina, M., Chivu, A., & Good, L. (2018, May 12). *Polyhexamethylene Biguanide and Nadifloxacin Self-Assembled Nanoparticles: Antimicrobial Effects against Intracellular Methicillin-Resistant Staphylococcus aureus*. Polymers. https://doi.org/10.3390/polym10050521

Bijnsdorp, I. V., Giovannetti, E., & Peters, G. J. (2011, January 1). *Analysis of Drug Interactions*. Methods in Molecular Biology. https://doi.org/10.1007/978-1-61779-080-5_34

Mohammed Naji Odhah et al.,*View of Current Review on Mycolic Acid Immunogen of Corynebacterium pseudotuberculosis*. https://www.advetresearch.com/index.php/AVR/article/view/876/517