IN VITRO ACTIVITY OF T4 BACTERIOPHAGE ON UROPATHOGENIC *E. COLI*

BY

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ABSTRACT

Bacteriophage is a virus that infects bacteria and can kill a bacterial cell or integrate its nucleic acid (DNA or RNA) into the host bacterial cell. One of the phage important medical applications is using it as an alternative approach to treatment of infections by resistant pathogenic bacteria. This research aims to find the susceptibility of drug resistant uropathogenic *E. coli* towards the T4 phage. The study involved collection of resistant uropathogenic *E. coli* (UPEC) toward (Ceftazidime, Gentamicin, Trimethoprim/Sulfamethoxazole, Ampicillin, Amoxicillin with Clavulanic Acid and Ciprofloxacin,) isolates from (Hospital Tengku Ampuan Afzan) HTAA, Kuantan, Pahang during a 4 months period (from 01/September/2015 to 31/December/2015). Re-identification of UPEC isolates was done by scientifically approved conventional diagnostic methods. To store isolates for further laboratory procedures, approved long term and short term bacterial storage methods were followed. All UPEC isolates were checked for antimicrobial susceptibility test by the Kirby & Bauer method and by the extended spectrum beta-lactamase (ESBL) screening test. The quantitation of T4 bacteriophage was done at first by the plaque assay on reference *E. coli* strain ATCC25922. In the plaque assay for UPEC, serial log dilutions of T4 phage (1×10^{-1}, 1×10^{-2}, 1×10^{-3} , 1×10^{-4} ,1×10^{-5} , 1×10^{-6} , 1×10^{-7} , 1×10^{-8} , 1×10^{-9} and 1×10^{-10}) were incubated with UPEC by using the double agar layer technique. Countable plaques were formed for all UPEC isolates in plates inoculated with phage dilutions of 1×10^{-6} and 1×10^{-7}. To determine the phage Minimal Inhibitory Concentration (phage MIC), the microbroth dilution method was performed. The phage MIC for the bacterial isolates was ~1×10^{5}/mL which is the lowest phage concentration or highest dilution which gave a clear broth (no bacterial growth). Serotyping of UPEC H & O antigens was done by standard agglutination method. The percentage distribution of UPEC serotypes was CAN55 (14%), MSHS94 (6%) and MSHS23a (10%) and unknown serotype (70%). In conclusion, the T4 phage concentration of 1×10^{5}/mL is regarded as the phage MIC for all the tested UPEC strains showing a lytic effect against UPEC.

خلاصة البحث

الفايروس العاثي هو الفايروس الذي يصيب البكتريا ويستطيع قتل الخليه البكتيريه اويكمل حامضه النووي في مضيف الخلية البكتيريه , احدى اهم تطبيقات الفايروس العاثي الطبيه هي استعماله كمشروع بديل للمضادات الحيويه ضد الجراثيم الممرضه المقاومه .هدفت هذا البحث لايجاد حساسيه الجراثيم الاشريشيه القولونيه البوليه المقاومه للفايروس العاثي T4 . شملت الدراسه جمع عزلات بكتريا الاشريشيه القولونيه البوليه المقاومه للمضادات الحيويه (السيفتازيديم، جنتاميسين، ترايميثوبريم / سلفاميثوكسازول، الأمبيسلين، أموكسيسيلين مع حمض كلافيولونك، سيبروفلوكساسين) من مستشفى HTAA في كوانتان، بهانك خلال فترة اربعه اشهر 2015/09/1 لغاية 2015/12/31. تم اعادة التشخيص المختبري لهذه العزلات بالطرق المتعارف عليها علميا. لحفظ العزلات للعمل المختبري تم اعتماد الخزن البكيتري الطويل والقصير الامد لحفظ العزلات . جميع العزلات اختبرت بأختبار الحساسية للمضادات الحيويه بطريقة Kirby & Bauer .وكذلك اختبار تمديد الطيف (ESBL).تم التحديد الكمي للفايروس العاثي T4 في البداية عن طريق حساب الوحدات المكونه للويحات وبأعتبار سلاله الاشريشريه القولونيه ATCC 25922. كمصدر . في اختبار اللويحه لجراثيم الاشريشيه القولنيه البوليه المقاومه تم تحضين تخافيف مختلفه من الفايروس العاثي T4 , 1×10^{-7} , 1×10^{-6} , 1×10^{-5} ,1×10^{-4} , 1×10^{-3}, 1×10^{-2}, 1×10^{-1} 1×10^{-10} 1×10^{-9} , 1×10^{-8} مع الجراثيم الاشريشيه القولنيه البوليه المقاومه باستخدام تقنيه الاكار ثنائي الطبقه. تكونت اللويحات القابله للعد لكل عزلات الاشريشيه القولونيه البوليه المقاومه في الاطباق المحضنه مع تخافيف الفايروس العاثي في 1×10^{-7}, 1×10^{-6}. لتحدد اقل تركيز مثبط للفايروس العاثي (MIC) تم اتباع طريقه الmicrobroth dilution . حفره الطبق الدقيق المحضنه1×10^5 لكل مل كانت اقل تركيز للفايروس العاثي والذي اعطي وسط مائي صافي بدون نمو بكتيري . تم تحديد الاختبارات المصليه لجراثيم الاشريشيه القولونيه البوليه باستخدام طريقه التلازن الجرثومي القياسيه اعتمادا على على كل من المولد المضاد H وال O , وكانت نسب سلالات لجراثيم الاشريشيه القولونيه البوليه كالتالي :- CAN55 (14%), MSHS94 (6%) and MSHS23a (10%) غير معروفه.(70%) استنتاجا, اعتبر تركيز الفايروس العاثي T4المحضن 1×10^5 لكل مل اقل تركيز مثبط لنمو جراثيم الاشريشيه القولونيه البوليه المقاومه وله قابليه تحليل جراثيم الاشريشيه القولونيه البوليه المقاومه.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Basic Medical Sciences

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DECLARATION

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To

The martyrs of Iraq, Who sacrificed themselves for us…..

My beloved family, the source of love, help and strength……

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LIST OF ABBREVATION

ATP	Adinosin triple phosphate
CFU	Cell forming unit
CPS	Capsular polysaccharide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EMB	Eosin -methylen blue
ESBL	Extended - spectrum Beta - lactamase
ExPEC	Extra-intestinal Pathogenic Escherichia coli
FDA	Food and drug administration
GIT	Gastrointestinal tract
gm	Gram
GRAS	Generally recognized as safe
HTAA	Hospital tengku ampuan afzain.
HUS	Hemolytic - uremic syndrome.
IMPR-Pa	Imipenem resistant Pseudomonas aeruginosa
ip	Inraperitoneal
LPS	Lipopolysaccharide
LTF	Long tail fibers
MIC	Minimum inhibitory concetration
MPSA	Methacillin resistant Staphylococcus aureus
nm	Nanometer

OMRI	Organic materials review institute
PFU	Plaque forming unite
Prs	P - Related sequences
STF	Short tail fibers
UPECs	Uropathogenic *E. Coli* strains
UTIs	Urinary tract infections
VRE	Vancomycin-resistant Enterococcus faecium
µl	Microliter
µm	Micrometer
µg	Microgram

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

The appearance of resistant pathogenic bacteria to most, if not all, currently available antimicrobial agents has become a serious problem in modern medicine, especially because of the accompanying increase in immunosuppressed patients. The concern that human being is returning to the “preantibiotics” era has become very real and the development of alternative anti infection patterns has become one of the highest priorities of modern medicine and biotechnology. Before to the discovery and widespread use of antibiotics, it was proposed that bacterial infections could be treated and/or prevented by the administration of phages. Although, the early clinical studies with bacteriophages were not widely pursued in the Western Europe and United States, bacteriophages were utilized in the former Soviet Union and Eastern Europe. "The results of clinical studies were extensively published in Russian, Georgian, and Polish journals, so were not easily available to the western scientific community" (Sulakvelidze et al., 2001).

1.2 RESERACH JUSTIFICATION

Urinary tract infections (UTIs) are one of the most common infections recorded in clinical study, mainly being related with different members of the family Enterobacteriaceae and among them *Escherichia coli* (*E. coli*) is the most predominant pathogen. 75–85% of UTIs are caused by uropathogenic strains of *Escherichia coli* (UPECs). In UPECs, there has been a progress toward antibiotic resistance, with

declining susceptibility to antimicrobial agents such as "nitrofurantoin, ampicillin, fluoroquinolones and sulphamethoxazole/trimethoprim (SXT)" (Nicolle, 2002).

Certain serotypes of *E. coli* are consistently associated with uropathogenicity and are listed as uropathogenic *E. coli* (UPEC). About 90% of all UTIs among ambulatory patients and up to 50% of all nosocomial UTIs are caused by UPEC strains(Jacobsen et al ., 2008) The concept of uropathogenic refers to certain *E. coli* strains that are selected from the faecal flora for their ability to colonize and infect the urinary tract. Uropathogenic *E. coli* strains are believed to show a variety of virulence factors that help them to colonize the mucosal surface of the host and avoid host defense to permit invasion of the normally sterile urinary tract. In 2014, the global report on surveillance from Worlds Health Organization (WHO) showed that the percentage of E coli resistant isolates was 77% in Western Pacific Region, 43% in European Region, 41% in Eastern Mediterranean Region and 16-68% in five different countries of South-East Asia Region (World Health Organization, 2014). In Malaysia, A report from Institute of Medical Research (IMR) displayed an increase in the percentage of *E coli* resistance to different types of antibiotics in 2013 and 2014. Another way to antimicrobial treatment would be a great importance to treat UTIs resistance (Chibeu et al., 2012).

1.3 THE PROBLEM STATEMENT

The emergence of antimicrobial resistant bacteria has become a serious problem in medicine, including the appearance of multidrug-resistant uropathogenic *E. coli* (UPEC).

1.4 AIM OF RESEARCH

1.4.1 GENERAL OBJECTIVES

- To find the susceptibility of resistant uropathogenic *E. coli* towards T4 phage.

1.4.2 SPECIFIC OBJECTIVES

- To identify resistant clinical isolates of uropathogenic *E. coli*.
- To serotype the identified UPEC.
- To determine the in vitro susceptibility of different UPEC strains to T4 phage.
- To determine the minimum inhibitory bactericidal concentration (MIC) of T4 phage (the concentration of phage that give complete inhibition/lysis of UPEC).

CHAPTER TWO

LITERATURE REVIEW

2.1 DEFENITION OF BACTERIOPHAGE

Bacteriophages are viruses that infect bacteria. They are obligate parasites and need bacterial cells to replicate. The name was formed from "bacteria" and "phagen" (to eat or devour in Greek), and was meant to imply that phages "eat or devour bacteria. Phages start infections by attaching to the surface of host bacterial cells and then inoculate their genomes into those cells (Clark & Richard, 2013).

Bacteriophages are widely distributed and exhibit both in water and soil. Phages are the most abundant living entities on earth- the estimate range from 10^{30} to 10^{32} in total-and have regulated the microbial balance in every ecosystem that has been explored (Kutter & Sulakvelidze, 2004).

2.2 HISTORY

Although bacteriophages are widely distributed in water and soil, they were not recognized for over 40 years after the beginning of bacteriology as a science in the 1880s. In 1917, Felix d'Herell, a French-Canadian microbiologist, described a "microbe" that was antagonistic to bacteria, lysed bacteria in watery cultures, and killed bacteria in discrete patches, that he termed plaques, on the agar surface spread with a bacterial film .D'Herelle considered these undetectable microbes as "ultraviruses" that infected bacteria and multiplied at their expense, and so d'Herell called them bacteriophages.

In Paris under the First World War conditions, Herelle worked at the Pasteur Institute when he was called to examine an outbreak of bacillary dysentery of French soldiers." D'Herelle examined the filtered dysentery specimens for invisible viruses that might alter the growth and pathogenicity of the bacteria from the dysentery patients. He noted lysis in liquid culture and the formation of clear spots in the confluent bacterial culture that covered the agar surface. He noted that the invisible agent multiplied and needed living cell in its multiplication. D'Herelle realized that the plaque count provided a way to enumerate these invisible agents. He believed that phages were responsible for much of the recovery from infectious diseases. Because he noted increasing titers of bacteriophage during the course of recovery from dysentery and typhoid, he concluded that the gradual adaptation of lytic phages to specific pathogens, their subsequent multiplication and lysis of the pathogen was the mechanism of recovery. He called phages exogenous agents of immunity "(Waldor et al., 2005).

In the Second World War, phage therapy was widely used to treat the war-wounded as well as the many soldiers suffering from dysentery. Bacteriophage therapy continued to be researched extensively with military support after the war and became part of general standard treatment in some countries, particularly in Republic of Georgia (Sulakvelidze et al., 2001).

2.3 CLASSIFICATION AND STRUCTURE OF T4 BACTERIOPHAGE

Enterobacteria phage T4 is a bacteriophage that infects *Escherichia coli* bacteria. It is a member of the T- even phages, which are a group including enterobacteriophages T2 and T6.

Table 2.1 Classification of T4 pahge

Group	Group 1 (dsDNA).
Order	Caudoviales.
Family	Myoviridae.
Genus	T4 like viruses.
Species	Enterobacteria phage T4.

T4 phage is a large phage at approximately 90 nm wide and 200 nm long. Like all viruses, T4 phage contains nucleic acid (double-stranded DNA) coated by capsid (a protein coat). The DNA genome is encased in an icosahedral shaped head. Bacteriophages are not enveloped, unlike some plant and animal viruses.

The structure of T4 phage includes a head, tail, and baseplate (Fig. 2.1). The head contains double-stranded viral DNA that is ejected into bacterial cell to propagate the viral infection. The phage tail connects to the baseplate, which attaches to long and short tail fibers responsible for recognizing bacterial cell and then anchoring the phage to the bacterial cell. Also the phage tail consists of retracting cell-puncturing device which is a tool used for injecting the phage DNA into the bacterial cell (Adams, 2009)

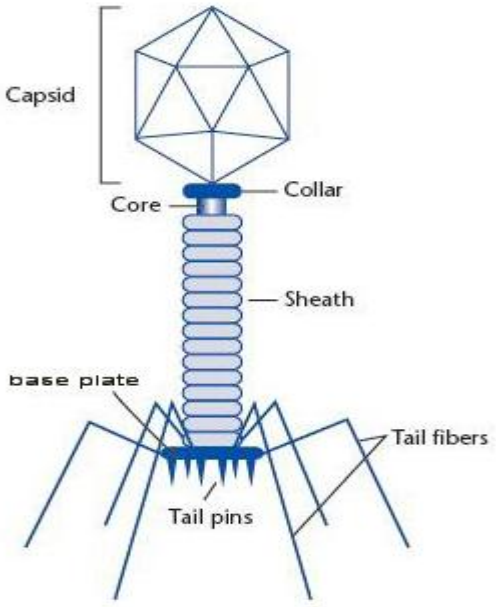

Figure 2.1 The structure of T4

2.4 THE LIFECYCLE OF T4 BACTERIOPHAGE

The T4 bacteriophage Long tail fibers (LTF) identify surface receptors of *E. coli* thereby starting the infective process. LTFs send a recognition signal to the base plate. The short tail fibers (STF) are then unraveled thus binding irreversibly to the surface of the *E. coli* cell. The changes of base plate which results in contraction of tail sheath causing GP5 present at the end of the tail tube to perforation the outer cellular membrane. The periplasmic peptidoglycan layer is degraded by the activated lysozyme domain of GP5. When remaining part of the membrane is also degraded then DNA from the phage's head enters the *E. coli* by traveling through the tail tube as shown in Figure 2.2 (Tarahovsky et al., 1994).

In 30 minutes, the life cycle of T4 bacteriophage will be completed. From entering a bacterium to its destruction (at 37 °C), phage life cycle composed of:

1. Adsorption and penetration (starting immediately)
2. Arrest of host gene expression (starting immediately)
3. Enzyme synthesis (starting after 5 minutes)
4. DNA replication (starting after 10 minutes)
5. Formation of new virus particles (starting after 12 minutes)

After the completion of lytic lifecycle the host cell bursts open and releases the newly built viruses leading to destroy the host cell. "The burst size of T4 bacteriophage is about 100-150 viral particles per infected host. T4 bacteriophage infects a host cell by their information afterwards blowing up the host cell thus propagating their progeny and increasing themselves" (Taj et al., 2014)

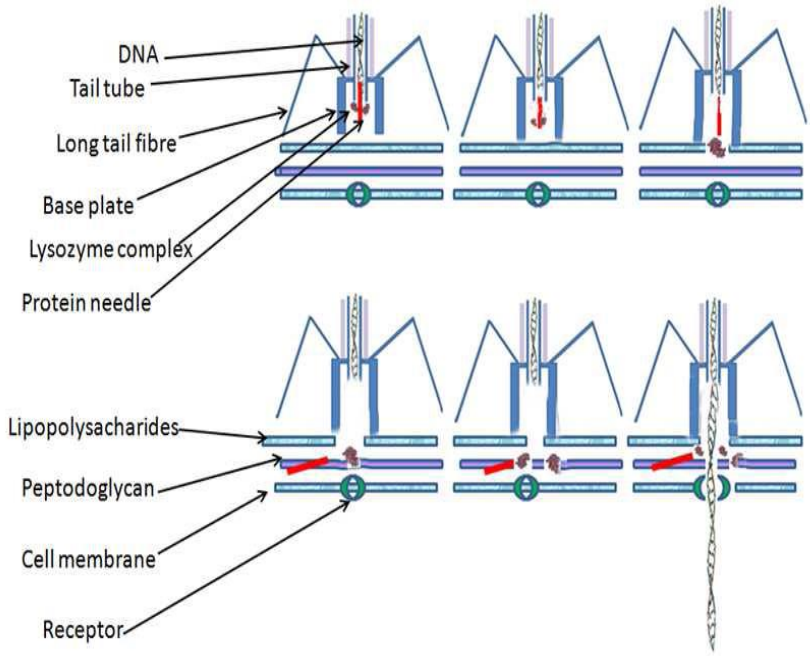

Figure 2.2 Injection process of T4 bacteriophage DNA into a bacterial cell

2.5 PHAGE APPLICATION

For more than 50 years, clinicians and researchers have been depending primarily on antimicrobial agents to treat infectious diseases caused by pathogenic bacteria. On the other hand, the appearance of bacterial resistance to antimicrobial agents following widespread clinical, agricultural, and veterinary application has made antibiotics lower effective (Perisien et al., 2008; Fischetti, 2008). Nowadays, researchers are facing the intimidation of superbugs" i.e. pathogenic bacteria active against to most or all available antibiotics "(Livemore, 2004; Fischetti, 2006). Since 1975 no new classes of antibiotics have been created, even with the use of biotechnology like genetic engineering. The development of new generation of antibiotic products derived from the known classes of antibiotics by Pharmaceutical companies regards as major concern. Therefore, searching for other approaches to develop antibiotics products is also a worthwhile task, and re- investigation the potential of promising older techniques might be of value. (Carlton, 1999; Sulakvelidze et al., 2001) using of

phages as anti-agents is one of the possible replacements for antibiotics (Shasha et al., 2004; Vinodkumar et al., 2008). Phage therapy includes the use of lytic phages for treatment of bacterial infections, in particular, those caused by antibiotic resistant bacteria. Generally, there are two major kinds of bacteriphages, "lysogenic and lytic". The lytic bacteriophages "(known as virulent phages)" are a better option for developing therapeutic bacteriophage preparations. The activity of bactericidal bacteriophages has been used to treat infections in human for many years as an alternative or supportive mode to "antibiotic therapy". Bacteriophages are used in the treatments of bacterial infections in human beings, mammals, birds, fishes, plants, food industries as well as biofilm eradication (Chhibber & Kumari, 2012).

2.5.1 Animal Trials

In the United Kingdom, Huggins and Smith (1982, 1983) conducted a series of well-controlled experiments on the use of bacteriophages in infected mice by "systemic *E. coli*" and then in gastrointestinal tract diseases in pigs and young calves. Other researchers examined the effects of specific bacteriophage therapy in white mice infected by intraperitoneal injection of *"K. pneumoniae* K25053" (Bogovazova et al., 1991). Chhibber & Kumari tested the bacteriophage ability in experimentally infected wounds of guinea pigs to prevent the skin grafts rejection. Their results revealed "that the -treated grafts by phage were protected in six out of seven cases, however untreated grafts failed uniformly". They suggested that bacteriophage therapy might be useful for the prevention of "*P. aeruginosa* infections" in patients with burn wounds. Bacteriophage therapy has been successfully used to eradicate "*E. coli* 0157:H7" from cattle (Chhibber & Kumari, 2012).