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Detection of Class I Integrons and Insertion Sites in Bacteriophage Genomes: Evidence of Shared Genetic Material with Multidrug-Resistant Klebsiella pneumoniae and Acinetobacter species

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Detection of Class I Integrons and Insertion Sites in Bacteriophage Genomes: Evidence of Shared Genetic Material with Multidrug-Resistant Klebsiella pneumoniae and Acinetobacter species

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Jerusalem-Palestine

1447/2025

Dedication

{رَبِّ أَوْزِعْنِي أَنْ أَشْكُرَ نِعْمَتَكَ الَّتِي أَنْعَمْتَ عَلَيَّ}

To the one who raised me and devoted her whole efforts throughout my entire life, my mother.

To my backbone, my father and my siblings for their continuous support and guidance.

To the most honorable of us all, the martyrs of Gaza and their never ending sacrifices.

I dedicate my work

Qais AmAli

Declaration

I hereby certify that the entirety of this thesis submitted for the degree of Masters' in Biochemistry and Molecular Biology is a result of my own devoted research unless where cited otherwise, and furthermore, this thesis is not to be submitted for a higher degree at any other universities or institutions.

Signature: Q. Amali

Qais Mohammed Saif El Din AmAli

Date: 30/8/2025

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Qais AmAli

Abstract

Ever since the emergence of antibiotic resistance, it has introduced a significant inevitable challenge in clinical administration and treatment of bacterial infections. During the year of 2021, health records have demonstrated a total of 4.71 million deaths due to antimicrobial resistance health consequences, with nearly 25% of them being directly attributable to resistant pathogenic bacteria. Data-based projections estimate the annual death toll to reach 8.2 million within 2025-2050. These concerning clinical records illustrate the necessity for continuous research efforts for developing approaches towards allocating this topic and limiting its health consequences. Ascribed to the latter, we introduce this innovative research, inspired by worldwide publications and implemented here in Palestine to address the yet to be allocated research gap. We designed a study approach that aims to investigate the significant incorporation of bacteriophages in mediating the transfer of antimicrobial resistance-related genetic material across different bacterial species. Furthermore, we focused on unravelling the molecular entities and constitutes involved in this integrational process characterized by Integrons, specific insertion sites and resistance-acquiring genes. In order to achieve this, we retrieved samples from clinically-relevant environments that represent a reservoir for such bacteriophages, samples were taken from both a sewage-water treating facility based in Al-Tireh, Ramallah & an oil mill located in Aroura village where the liquid by-product was collected. These samples exhibited a thorough well-structured process of laboratory work to eventually retrieve the extracted DNA material, two different specific primer sets were designed for an accurate investigation approach. The 1st pair being intCiF3a & intCiiiR3a which were utilized for the purpose of amplifying Class I Integrons that have been documented to harbor MDR genes, while the 2nd pair was designed for amplification of specific insertion sites where integration of resistance-affiliated genetic material takes place IS-F & IS-R. Eventually, we successfully managed to confirm and validate the presence of both Class I Integrons and insertion sites in our phage samples. Moreover, we further investigated the clinical relevance of our findings as we aligned them against already annotated genomes where we discovered a 100% similarity of a 200bp insertion site with MDR-resistant pathogenic Klebseilla pneumoniae. We also discovered another identical alignment of a 240bp sequence representing a Class I Integron with pathogen-inducing Acinetobacter harboring antibiotic-resistant characteristics. Finally, a 40bp identical alignment of few of our amplicons with E. coli plasmid “pV139-a” was highlighted. This highlights the incorporation of Class I Integrons, insertion sites and mobility-facilitating constituents taking part in transfer of MDR genes and the corresponding health consequences.

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Abbreviations

AMR	Anti-microbial resistance
ORFs	Open reading frames
PCR	Polymerase chain reaction
NGS	Next generation sequencing
MDR	Multi drug resistant
XDR	Extensive drug resistance
PDR	Pan drug resistance
LPS	Lipopolysaccharide
CALINs	Clusters of attC sites lacking integron-integrases
DDW	Double-distilled water
PFUs	Plaque forming units
WGS	Whole genome sequencing

Chapter one

Introduction:

1.1 Background

Ascribed to their host-dependency, viruses (bacteriophages in this context) are obligated to integrate their genomic material within the host genome to maintain their viability (Lauran, Kasman et al, 2022). Phages have developed a co-evolutionary relationship with their bacterial hosts which involves the exchange of genetic material between the two. This is demonstrated in bacterial species exhibiting anti-microbial resistance "AMR" traits against a broad spectrum of anti-biotics (Hong et al., 2022), in addition to various phages evolving retaliation strategies against bacterial-defense systems such as adopting anti-CRISPR genes which counteracts the activity of CRISPR systems (Camara et al,. 2023). Bacteriophages are considered mobile genetic constituents that take part in mediating horizontal-gene-transfer leading to evolutionary adaptations of host bacteria (Miguel, et al,. 2019). The process of integrating phage genetic material into the bacterial genome is attributed to Integrons, which are genetic elements responsible for capturing exogenous genes through site-specific recombination. This recombination occurs at complementary sites present on phages and bacteria, known as "attP" and "attB," respectively (Pfeifer, et al., 2022). The captured genes, referred to as gene cassettes, consist of open reading frames and recombination sites "attB." These cassettes lack a promoter region and therefore rely on integron-associated elements containing integrase enzymes to function (Qi et al,. 2023). This phenomenon of genetic exchange harbours pivotal potential of exploiting this natural occurring mechanism towards medical and genetic purposes.

Identification of the molecular process begins with determining the genetic material and components involved in integration. One approach is comparative genomic analysis to illustrate the incorporation of specific genes from phages into bacterial genomes (Marcu et al., 2019). Additional strategies include high-throughput genetic and molecular analyses of phage genomes (Qi et al,. 2023). These investigations involve characterizing the phage, including its morphology, type of genetic material, replication strategy (lytic or lysogenic), and ultimately unraveling its genomic identity using advanced sequencing technologies.

Because integration is mediated by integrons, assessing this process involves detecting genes such as integrase, recombination sites ("attP" and "attB"), and the integron-associated promoter "Pc." These represent the three critical components of a functional Integron (Bsharat, et al., 2023). In addition to analyzing the genetic sequences of these elements, identifying bacterial host genes harboring exogenous insertions provides a more comprehensive understanding of genomic adaptations.

An Integron can be defined as a versatile molecular entity commonly present in bacterial genomes. It functions as a gene acquisition system that facilitates genetic exchange, ultimately driving genomic complexity and evolutionary adaptation (Gillings, 2014). Due to their ability to capture and excise genes, Integrons play a dual role: they contribute to bacterial adaptation and promote the acquisition of exogenous genes from surrounding environments, influencing the current genomic state of bacterial species (T.M., et al., 2021).

Since their discovery, researchers have proposed that Integrons share an ancient evolutionary relationship with bacteria, with phages serving as a key component present in most bacterial genomes (Fonseca et al,. 2022). This conclusion is supported by evidence such as the structural compatibility between conserved Integron regions and complementary bacterial sequences, which enable integration and indicate a long-standing molecular interaction (Ghaly, et al., 2021). Furthermore, Integrons have been detected across multiple bacterial phyla from diverse environments, including thermophiles and non-pathogenic species. Their wide distribution, combined with their presence beyond clinical or pathogenic bacteria, strongly suggests that Integrons evolved early in bacterial history, establishing a co-evolutionary relationship that persists today (Escudero et al,. 2015).

Extensive research has examined the role of Integrons in mediating the horizontal transfer of antibiotic resistance genes among bacterial species. However, molecular studies also highlight their broader function in bacterial genome evolution by facilitating adaptive processes. This demonstrates that Integrons are not limited to mediating antibiotic resistance but play a fundamental role in shaping bacterial evolution and supporting the ancient evolutionary link between phages and bacteria. (Zhang et al,. 2018). When it comes to the structure of an Integron, we can't help but notice a modest yet exquisitely structured entity consisting of the required molecular constitutes enabling it to achieve its optimum prospective function. An Integron's structure is composed of three fundamental parts, first is the Integron integrase "IntI" gene which belongs to the tyrosine recombinase family. The function of this integrase is catalysis of the recombination that takes place between exogenous gene cassettes and the recombination site of the Integron "attI", which brings attention to the secondary constituent of an Integron. The Integron recombination site portrays the site where recombination of acquired genes takes place and is conserved for it to be later expressed when needed. The third and last core feature of an Integron is known as an Integron promoter region "Pc", this region is responsible for expression of the already acquired & well-conserved gene cassettes in response to environmental stress or stimuli (Ghaly, et al., 2021). The presence of a promoter region within the Integron provides a significant role, as these adopted exogenous cassettes consist of a recombination site that is complimentary to that in the Integron and an open reading frame "ORF" but lacking a bound promoter that can translate it (Ghaly et al., 2021). Therefore, the Integron's associated promoter region is responsible for translation and expression of the "ORF" found within the gene cassettes. This sophisticated Integron system provides advantageous features such as genomic flexibility since the newly adopted gene cassettes can be either expressed when needed or undergo excision and be released as free DNA material (Escudero et al., 2015). Moreover, the acquisition of the exogenous gene cassettes takes place in the specific recombination site mentioned above introducing no disassembly to previously conserved genes. Lastly, the bacterial cell harboring the Integron will eventually acquire beneficial phenotypic alterations providing it with longevity and adaptive evolutionary traits optimizing its characteristics (Engelstädter, et al., 2016).

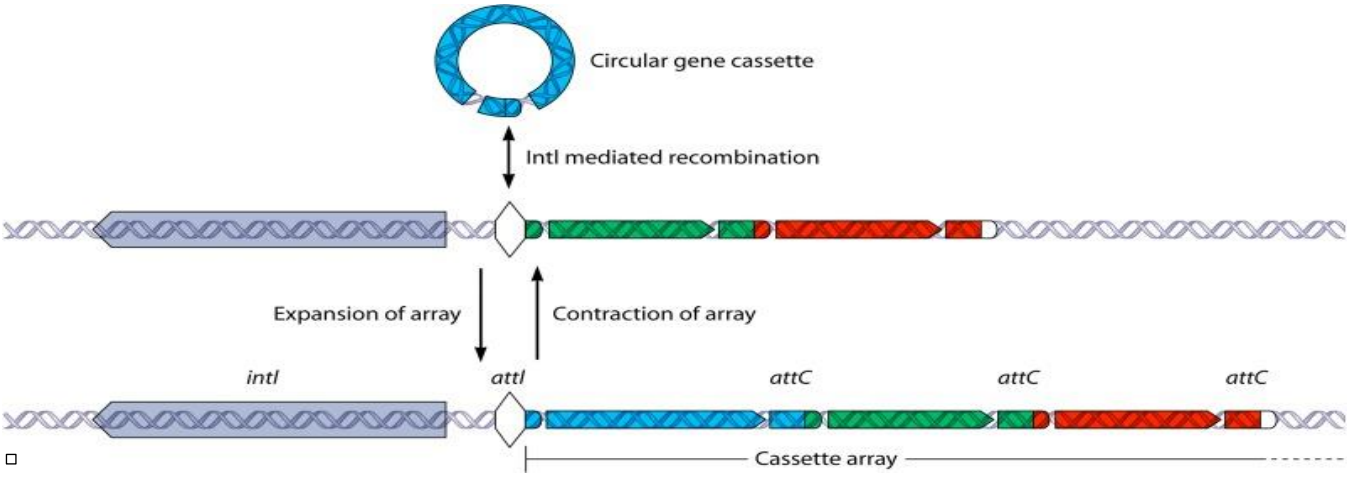


Figure 1.1. The structure of an Integron and its mechanism of act of acquisition and excision of gene cassettes

The typical structure of an Integron consists of the following: **1)** an integrase gene "intI" (in grey) with an **2)** integrated promoter "Pc" responsible for expression of organized gene cassettes (coloured), **3)** attachment sites "attI" where the acquisition of gene cassettes occurs as illustrated. The integration process occurs between the "attI" of the integron with the "attC" of the gene cassette. Already integrated gene cassettes can be also excised as circular DNA in a reverse process.

Reproduced from (Gillings, 2014). Integrons: past, present, and future. Microbiology and molecular biology reviews : MMBR, 78(2), 257–277.

https://doi.org/10.1128/MMBR.00056-13

Although Integrons have modest, simplified structures, they can still be distinguishable among each other due to varieties in molecular compositions. Furthermore, Integrons can be either mobile or chromosome-bound, mobile Integrons usually harbor gene cassettes associated which gain their hosts antibiotic resistance traits, and their mobility allows for integrating different phylas and species. The rationale underlying the mobility of some Integrons is the association of these Integrons with plasmids and transposons. On the other hand, stationary chromosome-bound Integrons "super-integrons" usually contain functional gene cassettes rather than antibiotic-resistance-acquiring ones, in addition to harboring high quantities of gene cassettes aiding the bacteria to adopt phenotypic adaptability and complexity in genome (Adeniji, et al., 2022). Moreover, efforts has been made by researchers to further distinguish these Integrons into classes depending on their characteristics, mechanism of act and most importantly their significance in antibiotic resistance gene transfer. Of the four different classes, Integrons of class I represent the most commonly dispersed Integrons within microorganisms and various environments.

Ascribed to their historical conjugation with plasmids and transposons, class I Integrons are mobile and they tend to prevail a cluster of gene cassettes which most often exhibit antibiotic resistance phenotypic traits. The presence of such characteristics within this class portrayed them as an important candidate for immunology & microbiology research. Therefore, multiple research studies and investigations have been implemented to associate these Integrons with their potential hazardous risk of transferring antibiotic resistance gene cassettes among different bacterial phyla and eventually being enlisted as the most commonly reported within pathogenic bacteria (Yang, et al., 2015). Harboring a sophisticated promoter region "Pc" that allows them to effectively translate numerous amounts of antibiotic resistance gene cassettes, in addition to exhibiting a well-organized site-specific recombination process with the complimentary attachment sites of gene cassettes provided a comprehensive platform that represents clinical hazardous potential. Multiple studies have been conducted on class I Integrons to investigate its incorporation and contribution to the broad spread of antibiotic resistance genes. Class I Integrons were considered a hotspot in molecular investigations performed on pathogenic bacteria to examine their prevalence and abundance. According to (Yang, et al., 2015), the clinical prevalence of class I Integrons within gram-negative pathogenic bacteria ranged from 22%- 59%, being dispersed among various species of gram-negative bacteria. Rendered as clinically relevant and highly affiliated with mediation of antibiotic resistance genes, varieties of gene cassettes were found integrated within these class I Integrons. A commonly found gene within these Integrons is the "aadA" gene which acquires bacteria resilience to spectinomycin and streptomycin, especially gram-negative bacteria (AC, Fluit; . Schmitz , F., 2004). Moreover, in the case of class I Integrons isolated from pathogenic human bacteria, Integrons tend to contain Beta lactam resistance illustrating evolutionary adaptation (Huang, et al., 2013).

Similar to class I Integrons, class II Integrons arises from the Tn7 transposon family sharing both the same recombination site and promoter region, eventually acquiring it with mobility (Thomas, et al., 2017). When it comes to comparing the integrase gene of class II Integrons to the integrase found in class I, significant differences can be found as they differ in more than 50% of amino acid sequence. Furthermore, integrase of class II Integrons is considered malfunctioning due to a missense mutation as a glutamic acid codon is inserted instead of a stop codon producing a defective integrase (Wang et al., 2021). Assumptions have been made to speculate the presence of such ineffective

integrase, one theory suggests the possible simultaneous harboring of two different classes of Integrons where the function of a class II integrase is not needed. Ascribed to the fact that both class I and II Integrons share similarities in gene cassettes composition, and excision takes place within class II Integrons, this can explain the presence of an ineffective integrase gene (Yu, et al., 2023). In comparison to Integrons of the first class, class II Integrons are considered significantly less clinically relevant in addition to displaying a low occurrence rate within pathogenic bacteria. Nevertheless, studies still illustrated the abundance of these class II Integrons within clinical isolates of gram-negative bacteria (Xu, et al, 2011). Lastly, class III Integrons share molecular similarities with class I Integrons, they contain an effective integrase gene that is capable of integrating gene cassettes efficiently unlike class II. Integration of gene cassettes occurs in their site-specific recombination sites, however they experience lower recombination frequencies when compared to class I Integrons. Additional similarities to class I Integrons can be observed in the molecular composition of the promoter region, and their capability of harboring gene cassettes conferring antibiotic resistance traits (Celejewski-Marciniak et al. , 2021). Due to their ability to mediate the transfer of antibiotic-resistant gene cassettes, thereby conferring a resistant phenotype to host bacteria, integrons are considered clinically significant. This was further illustrated in the multiple studies previously conducted where they prevailed a prevalence among gram-negative pathogenic bacterial isolates ranging from 1% to 10% harboring a wide range of gene cassettes (Pierette, et al, 2016).

1.2 Problem Statement and Research Objectives

While it comes to allocating this topic locally in Palestine, an evident observation was observable, only a limited number of studies have investigated Integrons and their clinical relevance in environmental settings or microbial populations. Especially, the significance of Integrons and their pronounced impact as a molecular constituent which harbors pivotal potential in mediating transfer of genes within numerous bacterial phyla gaining them evolutionary adaptations. When it comes to investigating Integrons and their clinical relevance here in Palestine, researches were only limited to studying these entities and their pathogenic significance in relation to pathogenic bacteria without addressing its incorporation and integration within bacteriophages. In this study, we aim to allocate this mentioned research gap by addressing the crucial roles of Class I Integrons & insertion sites as significant mediators of the integration process. Furthermore, we aim to highlight

the contribution of bacteriophages acting as reservoirs for MDR genes in environmental sources. This is achieved by unravelling the phage genome by employing sophisticated molecular approaches such as PCR and Next-generation-Sequencing “NGS”, as they provide us with a platform that deciphers the genetic composition. Moreover, this enables us to underlie the integration process occurring by highlighting each molecular constitute taking part such as the integrase gene, recombination sites, insertion sites or gene cassettes. In order to understand how our overall aim and objectives will be achieved, a well-structured, detailed procedure and protocol can be found in the methodology chapter below. Given the fact that it is a pioneering first of a kind research study here in Palestine, our study contributes to a scientific significance in the field of Immunology and Virology as an exploratory study that allocates a yet to be investigated research gap harboring encouraging potential benefits that could introduce inspirations and motivate innovative researchers of this field.

Chapter Two

Literature review

2.1 Emergence of Antibiotic resistance and Escalating Risks

The discovery of Penicillin by Sir Alexander Fleming in 1928 introduced a major breakthrough in medicine by imposing an effective therapeutic approach towards limiting the widespread of pathogenic bacteria (Dhingra, et al., 2020). This revolutionary discovery managed to increase the life expectancy of the patients which otherwise would succumb to their infection (Maugeri et al., 2019). Nevertheless, the emergence of Anti-microbial resistance "AMR" where bacteria began to develop resistant characteristics against natural antibiotics hindered the progress of disease prevention and treatment (Parsonage et al., 2017). Genetic exchange among different bacterial species, in addition to misuse of antibiotics by infected patients enabled the bacteria to acquire drug resistance towards antibiotics which imposed a global health risk and was eventually enlisted as the highest health risk in the 21st century (Ferri, et al, 2017).

"AMR" is further classified into three different groups depending on the bacteria's resistance to the wide options of antibiotics. For instance, bacteria classified as multi-drug-resistant "MDR" are resistant to one agent in three or more antibacterial groups while "XDR" extensively-drug-resistant can withstand at least one agent in all but two antibacterial groups and lastly those who are non-susceptible to all antibacterial groups are known as "PDR" pan-drug resistant (Bakhit, et al,. 2019). According to (Aslam et al,. 2018), infections caused by drug-resistant bacteria are considered one of the leading causes of mortalities worldwide leading to approximately 700,000 deaths annually. This emergence of non-susceptibility against antibacterial drugs coupled with the low scalable production process of antibiotics called for the incorporation of alternative therapeutic

applications. A rapid therapeutic intervention was needed due to the necessity of restricting bacterial infection that could exacerbate if not met with the appropriate preventive measures (Chaitali et al, 2019).

2.2 Discovery of Bacteriophages and its Promising Characteristics

Bacteriophages were first discovered by Frederick Twort in 1915 and have since demonstrated promising potential in treating bacterial infections, leading to the concept of phage therapy for medicinal purposes (Kilscher et al, 2019). However, the therapeutic application of phage therapy was later discontinued following the breakthrough discovery of Penicillin in 1928 and the subsequent development of a wide variety of antibiotics (Payasliyan, et al,. 2021). The recent emergence of antibiotic resistance among various bacterial species has renewed interest in phage therapy as an alternative therapeutic approach (Anderzej et al., 2020).

Phage therapy displayed effective advance in restriction of bacterial infections ascribed to their natural targeting of bacterial cells whilst being completely harmless towards eukaryotic cells (R.Y.K, et al., 2018). In addition to the latter, bacteriophages portray high host specificity by only replicating within the bacterial cells causing the infection, thus, causing no harm to the natural microbiota (Liu et al., 2022). Another advantage is that phage therapy provides an alternative for treating patients allergic to antibiotics.

A novel therapeutic strategy that exploits the co-evolutionary relationship between phages and bacterial host cells is known as “phage steering”. This approach is based on the mechanism of gene exchange that occurs between the two upon phage infection (Gurdey, et al., 2019). Interestingly, the strategy aims for the deliberate induction of phage resistance among the infected bacterial cell, however it simultaneously allows for reduced virulence and increased drug-sensitivity. Even though most approaches of phage therapy strive to eradicate the targeted pathogenic bacteria, “phage steering” aspires to produce antibiotic-susceptible and less virulent bacteria post therapy (Gurdey, et al., 2019). This results in vulnerable bacteria that can be eliminated by the typical immune-response, furthermore, this strategy can be combined with antibiotics for a thorough synergistic effect (Clara, 2018).

Due to the integral reliance of bacteria on surface factors as they mediate attachment, disease progression and display of antibiotic resistance mechanisms, the targeted binding of these factors by phages allows for a diminish of these features. For instance, LPS is

widely utilized by phages as attachment purposes, LPS is also associated with virulence effects by increasing cellular damage in infections by induction of Toll-like receptor 4 immune response. As demonstrated by (Javier et al., 2007), upon acquisition of phage resistance, a loss of O-polysaccharide in the LPS structure was observed which led to decreased virulence in Salmonella enteritidis infecting mice. Another approach is targeting of antibiotic-resistance mechanisms, such as in the case of Salmonella enterica which utilizes a ToIC efflux pump for resistance against ciprofloxacin. A phage which targets this pump as an attachment site was used and thus leading to increased sensitivity to ciprofloxacin (Ricci, Vito et al., 2010).

Phage infection initiates when viral proteins recognize and attach to membrane receptors on the host cell (Gummalla et al., 2023). Following attachment, bacteriophages can then either enter a lytic cycle in which viruses exploit host cell machinery for rapid synthesis of viral particles ultimately leading to bursting of the host cell which allows for release of virions, thus infecting intact neighbouring cells (Virulent phage) (Varona et al., 2017). On the other hand, (Temperate phages) are prone to initiating either a lytic cycle or a lysogenic cycle. In the latter, the phage enters a temporarily dormant phase where the viral genome is integrated into the bacterial genetic material (Chevallereau, et al., 2022). When surrounding conditions are unfavorable due to nutrition scarcity or environmental stress (physical and chemical), these temperate phages can switch to a lytic cycle to increase their survivability chance (Spriewald, et al., 2020). Since temperate phages adopt a dormant lifestyle where it is integrated within the bacterial host genome, they are neglected as an option for therapeutic approaches (Rodrigo, et al., 2019). Latent incorporation of the viral genome within the host genetic material represents a non-lethal episode that doesn't fulfill the requirements of phage therapy exhibited in rapid lysis and clearance of the target bacteria (Shroven, et al, 2021). Furthermore, as progenies containing viral genetic factors are produced, this can mediate horizontal gene transfer within bacterial species leading to acquired antibiotic and phage resistance (Borodovich et al., 2022). Hence, scientists perceived virulent phages as a more competent candidate in phage therapy as its lifecycle is characterized by rapid lysis of host cells (March et al., 2023).

2.3 Bacterial acquisition of gene cassettes by Integrons and adopting novel genetic features

Integrons are genetic structurers embedded within bacterial genome enabling these organisms to display new characteristics by adopting exogenous gene cassettes from surrounding environments (Ghaly, et al., 2021). Ever since the discovery of Integrons, it has been underlied that the presence of Integrons is a common feature among different bacterial species which have an old evolutionary background (Moura De Sousa,. et al., 2021). Integrons are characterized by several features enabling them to serve as a genetic repertoire for bacteria. For instance, Integrons can be found in nearly all environments and their genetic material can be exchanged among different bacterial phyla and species, this provides the species with a broad collection of genes over a long period of time (Ghaly et al., 2020). The structure of an integrin consists of three fundamental components, first is the integrin-associated recombination site “attI” followed by an integrin-integrase “IntI" and lastly an integron-associated promoter “Pc”. An explanation of the complementary interplay between the three components can be simplified as follows, the integrase “IntI” facilitates the recombination of “attI” with an “attc” of an exogenous gene cassette which is later expressed by the Integron promoter when needed (Cury et al., 2016). Modest genetic structures containing an open-reading-frame “ORF” in addition to a cassette-associated recombination site “attc” are known as gene cassettes (Jung, et al,. 2023). Integrons can acquire these gene cassettes from nearby environments and rearrange them into cassettes arrays with no unsettlement of the originally found genes. Furthermore, expression of those adopted gene cassettes is dependent on stimulating factors such as induction of SOS response triggering the upregulation of integrase activity and thus, increasing both the expression of gene cassettes in addition to their rearrangement (Jove et al., 2017).

2.4 Integron-mediated integration of phage genetic material into bacterial genome

The integration of phage DNA into the chromosomal unit of the bacteria initiates by recombination of the attachment site of the phage “attP” with the corresponding attachment site of the bacteria “attB”, phage integrase is responsible for the recombination process (Chiang et al., 2019). For instance, in lambda phages, the integrase facilitates the insertion of circular DNA as a linear sequence (Van Duyne, et al,. 2024). Furthermore, recombination reactions are reversible in the sense that integrated genetic material can be

excised as circular DNA representing a transition from a lysogenic to a lytic phase. This reaction is mediated by an integrase “intI’ in addition to another gene known as an excisionase “xis” (Shao, et al., 2017). Identifying the sequences of both “attP” and “attC” paves the way for underlying the integration interplay occurring which eventually results in acquisition of newly adopted genes.

(Williams, et al., 2013) analyzed the nucleotide sequence of the two possible sites of “attB” where the phage is inserted. In both sites, the integration occurred within genes that encode for ATPases, where one is responsible for encoding an ATP transporter and the other encodes for an ATPase flagella protein export apparatus. As described by (Williams, et al., 2013), the insertions were predicted to integrate within a gene region that encodes for a protein portraying a critical determinant of the ATPase functionality, thus, causing inactivation of the targeted genes. Illuminating the homology of phage genetic material with bacterial chromosomes is a crucial step towards breaking down the integration process occurring at molecular specific sites. Ever since the discovery of integrase-mediated integration of phage genetic material, comparative analytic studies have been conducted to evaluate genetic similarities among phage genomes and bacterial chromosomes (Anh Dieu et al, 2018). These analytic studies authenticate the integration of phage genomic material into the host DNA by illumination of the shared nucleotide sequences present in both the phage and the infected bacterial cell (Edwards, et al,. 2016). A study conducted by (Zhang et al., 2019) demonstrated the presence of a 10k genetic island shared among 20 different species of R. anatipestifer, in addition to deciphering the nucleotide sequence of an integrase involved in both incision and excision reactions within the bacterial species.

Genetic islands are often identified by harboring specific features, they are composed of huge DNA segments ranging from 10-200kb and have unique GC content distinct from other parts of the chromosome (Audrey et al, 2023). In addition to the latter, they are usually found in tRNA genes as they are inserted there, while being enclosed by nucelotidic sequences known as direct repeats such as “dL” and “dR” (Chan et al., 2019). Due to their integral role, integrases are commonly dispersed among the genetic material while also harboring mobile elements such as transposons which facilitate gene mobility (J, Liu et al, 2019). In the case of (Zhang et al., 2019)’s analysis, a 53b perfect DR sequence was found at the insertion site in addition to an integrase gene adjacent to the Arg-tRNA. This phenomena was further illustrated in (Wang, et al., 2022), as comparative

analysis was conducted between a 50k genomic island integrated within R. anatipestifer genome and genome of RAP44 phage which was suspected as the integration source. Homology between both genetic materials was detected, for instance, direct repeat sequences of “attL” and “attR” on opposite side of the genomic island sequence with an integrase gene present between the two. Moreover, the 50k genomic island was located downstream of the tRNA which indicates an occurring of integration as tRNA present vulnerable targets for exogenous genes since they share conserved sequences among different bacterial species. Ultimately, SOS-induction tests illustrated absent lytic transition which indicates the deletion of replication-necessary viral genes on the integrated genetic island. In addition to Integrons, clusters of “attC” lacking an integrin-integrase (CALINs) can be also found within phage genome, these clusters of gene cassettes can be then integrated in a bacterial recipient cells that harbors corresponding “attB” sites and an integrase gene (Qi et al., 2023).

2.5 Investigating genetic disposition & Next-Generation-Sequencing of phage genome

One would imagine that considering the unique small size of phage genome, extensive molecular analytics have been conducted producing numerous complete phage genomes published in the NCBI database. Nevertheless, the latest records estimate around 8000 of phage genomes have been completely sequenced and published in the NCBI genome database (Alicia et al, 2019). Comparing the latter with the large abundance of bacteriophages represents a potential scientific significance that has not been addressed. However, phage researches and sequencing investigations have drastically increased over the last decade, where in 2012, the number of completely sequenced phages was only 800. This dramatic escalation in phage sequencing research represents that this field gained encouraging attention from virologists around the world allowing for more comprehensive investigations. As demonstrated in **figure 2** below, we can notice that within the first 15 years (2000-2015) the number of completely sequenced phage genomes was less than 2000 genomes, however in the past 10 years, the number drastically increased as until the beginning of 2025, more than 8000 phage genomes have been sequenced and published in the NCBI database (Alicia et al, 2019). Indicated by the graph curve, this number is predicted to continue its dramatic increase in the upcoming years.

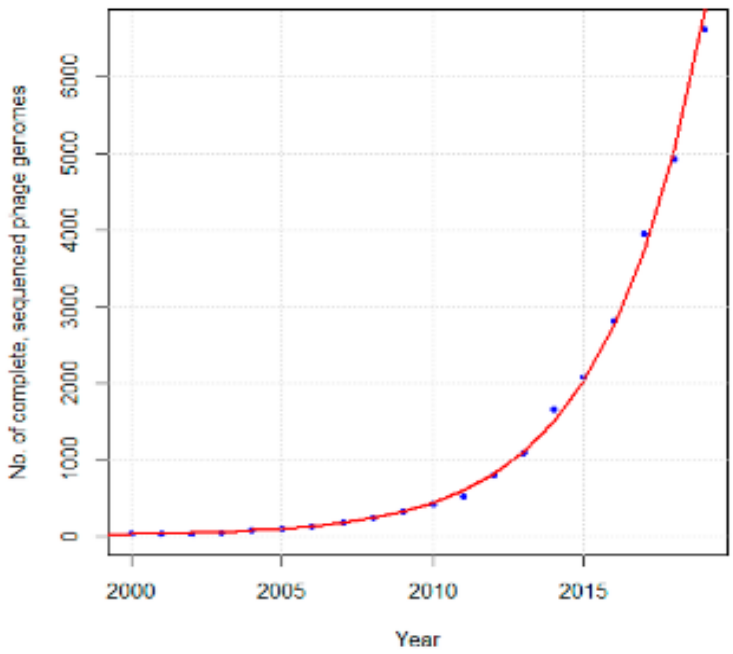


Figure 1.2: The cumulative number of complete phage genome sequences published in the NCBI genome data base.

. Cumulative number of completely sequenced phage genomes published in the NCBI database. The red curve represents an exponential curve fit to the data where (R^2 =0.989, p-value=0). Adopted from Alicia S et al. (2019). A Method for Improving the Accuracy and Efficiency of Bacteriophage Genome Annotation. International Journal of Molecular Sciences, 20(14), 3391 DOI:10.3390/ijms20143391

Moreover, the implementation of novel sophisticated & cutting-edge analytical instruments in the field of NGS introduced a more facilitated approach to molecular studies. This continuous development in the field of phage discovery research aided in bypassing obstacles and limitations that often represented hurdles to previous investigatory approaches. One of the primary limitations often faced by virologists is the host-dependent lifestyle of these entities as phages rely on host machinery for replication and growth. This represents a major set-back during the process of phage genomic material isolation and thus, requires a more extensive procedure in the phase of genomic purification to allocate the possibility of host material integration. Nevertheless, currently, this can be allocated utilizing bioinformatics following the generation of sequencing results. Moreover, a second obstacle arising due to the host-dependency lifestyle of phages is portrayed by the abundance of host contaminants and cell debris which calls for a more comprehensive purification and filtration process relying on nano-metric size differences.

A major difference between analytical studies of phages and genomes of other organisms is that generating a complete genomic sequence is often not required in the case of bacteria

or human genomes to further conduct investigations and come up with conclusions. On the other hand, in the case of small genomes such as phages, a fully complete genomic sequence represents a fundamental approach towards achieving a comprehensive deciphering of their biology. This includes unravelling the genetic disposition which aids in characterization of phage lifestyle (lytic or lysogenic), assessment of their potential of DNA transduction across bacterial species indicated by the presence of Integrons and other aiding cell-machinery which eventually provides an indication of their clinical relevance and potential in applications such as phage display or medical approaches.

Therefore, publications of phage genomes is both cost and time consuming which explains the hindering in this field. The genomes of phages are considered mosaic in general where even closely related phages can be extremely diverse in their genomic structure thus, portraying a significant limitation in generating reference mapping for phage genomics (Klump et al., 2012).

2.6 Novel approaches and methods implemented for NGS-based investigations of Integrons & MDR gene cassettes.

Appreciated innovative efforts have been conducted by researchers and virologists in this field to compensate for the limitations and set-backs hindering the scientific progress. Typical NGS-based approaches rely on production of large amounts of raw data which require further comprehensive analysis of the product sequences to obtain interpretative results. Following the assembly of the product raw data, the researcher can then conduct comparative analysis of these sequences with the online databases such as NCBI for referencing (Sell, et al., 2024).

The major limiting factor for researchers is the cost & time consumption of the data analysis process, as the bioinformatics-based analytic procedure requires intensive work. In a pioneering research study by (Guan-Jie, et al, 2018), they implemented a novel approach that aims to overcome the complexity and work intensity of data analysis within NGS. The introduced approach was based on computational calculations and algorithms that aim to identify Integrons and gene cassettes with no need for whole genome assembly. The research team investigated this proposed method and they successfully managed to identify the Integrons and gene cassettes found within A. baumannii, TYTH-1, introducing a more facilitated approach for investigations of Integrons and antibiotic resistant gene cassettes. In a recently published article, a research team managed to investigate and identify the presence of Integrons and associated gene cassettes in random environmental

samples (Veronica, et al., 2021). Utilizing NGS-based computational methods and bioinformatics analytical techniques, they succeeded in quality-filtering the output raw data and continue the de novo assembly process.

Furthermore, a website called IntegronFinder was utilized which is a program that implements methods of bioinformatics to screen input data sequences and detect Integrons, the program provides detailed results as they classify the detected into complete Integrons or partial constituents and elements of Integrons (Cury, et al., 2016). In order to investigate the incorporation of gene cassettes within these environmental samples, following the sequencing of the samples, comparative analysis was conducted with the CARD database to test for the presence of antibiotic resistant genes.

2.7 Phage-host interaction investigations by different approaches

The investigations conducted on bacteriophages and bacteria as their host exhibited alternating molecular analysis corresponding to the development and breakthrough of sophisticated tools and equipment. Ever since these research studies took place, virologists and researchers were scarce and limitations were hindering the progress in this field such as absence of interest or lack of funding (Turner, et al., 2024). The golden era of molecular biology was introduced primarily by Demerec and Fano in 1945 who implemented T-phages as their core of study where they investigated cross-resistance of phages between hundreds of mutants of E.coli (Demerec, 1954). The latter work coupled with the previously conducted research studies within the timeline of 1950-1970 represented this breakthrough era which was eventually labelled as the golden era. This period witnessed comprehensive research investigations which were mostly performed on T-phages and lambda phages as they resembled an eye-catching platform for molecular research. The mentioned conducted research unravelled essential principles underlying phage-host molecular interactions and dependency. These publications served as fundamentals for phage molecular biology in addition to introducing pivotal molecular approaches and instruments for advanced molecular research (Dion, et al., 2020)

Virology and phage research was later neglected as researchers and molecular biologists focused most of their efforts and initiative on animal models such as mice, drosophila and humans in required fields. That was until the emergence of the phenomena of antibiotic resistance, as interest in phage research resurfaced imminently to investigate the promising potential of phage therapy as a counteract to antibiotic resistance. Furthermore, the

breakthrough introduction of Next-generation-sequencing throughput instruments unravelled the broad abundance of these phages with their molecular significance which further intrigued researchers to conduct studies in this field (Kortright, et al., 2019).

These conducted studies unravelled the molecular complexity of the microenvironment and the incorporation of acquired antibiotic resistance and the utilization of phages as a mechanism of defense. Nevertheless, researchers faced limitations and obstacles hindering their scientific progress as screening of potential candidates was time and cost consuming due to the vast prevalence of these phages in addition to their genomic diversity. Initially, researchers investigated the significance of host receptors and the mechanism of penetration occurring between the phages and bacteria, this enabled characterization of phages according to their entry routes such as the Lipopolysaccharide "LPS" core acting as the receptor for phages in enterobacteria (Adler, et al., 2021).

2.8 Prophage incorporation and the arise of a new defense system

The typical defense system of bacteria against phages is portrayed by the CRISPR-Cas system which involves a group of specific enzymes that function in recognition of foreign genetic material, memory for sequence-specific immunity and eventually cut the DNA. This defense system harbors comprehensive characteristics as it enables adaptation by capturing a short sequence of the exogenous DNA as a memory storage in the case of later infections (Shabbir, et al., 2019).

The CRISPR-Cas system was thought to be the only defense mechanism of bacteria against phages, that was until the publishing of a breakthrough study revealing the integration of prophages within bacteria DNA acquiring them resistance against the adopted phage DNA (Bondy, et al., 2016). The integration of the inactivated prophages genetic material within the bacterial genome gives rise to lysogens that are resistant to no less than one phage due to receptor inactivation. In the mentioned breakthrough study, the researchers discovered a cross-resistance occurring between twenty different phages in a bacterial species of P. aeuroginosa.

2.9 Whole-genome phage sequencing studies and their scientific contribution

Research studies and molecular investigations conducted on phage for genomic discoveries introduce significant insight in this field, as being provided with the whole genome sequence of a phage or partial genomic sequences can facilitate further research in various approaches. For further clarifications, laying hands on a complete genomic sequence of an investigated phage allows for comparative sequence studies and evolutionary investigative studies. Moreover, following evaluations of the phage activity and lifestyle, a researcher can estimate the compatibility of this phage as a potential candidate in antibacterial approaches. Innovative inquisitive research can be conducted such as investigating the molecular integration occurring between phages and bacteria, demonstrating the incorporation of Integrons as aggravating mediators of antibiotic resistance.

In one of the latest pioneering research studies published, (Singh, et al., 2022) conducted an investigative study where they performed whole genome sequencing of an isolated phage that was sampled from a sewage water source. Initially, evaluation tests of phage activity against pathogenic and multi-drug resistant bacteria were performed to estimate its potential competency in phage therapy and identify its host range. The discovered phage genome was a linear double stranded DNA reaching up to 44.2 kb in size, harboring 72 ORFs, eventually only 30 of them were classified as functional proteins. Genes affiliated with pathogenic clinical relevance such as virulence, toxins and antibiotic resistance were absent in genomic screening tests which indicates its safe compatibility for therapeutic approaches.

In a similar approach, (Freidersdorff, et al., 2020) performed whole genome sequencing on five environmentally isolated phages from animal rumen. The initiative to isolate phages from rumen arises due to the modest interest this approach attracts as explained by the researchers. The isolated lytic phages were active when screened against ruminal bacteria as expected, however no sequence similarities were present between the phages and host genome indicating no sign of phage lysogeny. All five phages harbored a double stranded DNA while illustrating a lytic lifecycle, however speculations were observed on two of the phages to have a temperate phase due to genomic indications. The introduction of five whole phage genomes to the genomic database portrays a significant contribution especially that these phages arise from a source that is negligible research-wise, moreover,

this contribution also aids in comparative analysis studies as bacteriophages share molecular similarities.

Furthermore, a novel research study implemented by (Kumar, et al., 2021) contributed by unravelling the complete genomic sequence of a bacteriophage harboring significant potential. In this study, the researcher discovered the ability of the isolated phage of portraying lytic ability against Salmonella enteritidis, indicating its therapeutic potential due to the clinical relevance of this bacterial species. By utilizing the means of transmission electron microscopy, they managed to generate the structure of the phage illustrating its morphological characteristics. Moreover, promising observations were noted during lifestyle evaluation as the investigated phage demonstrated resistance to different pH conditions in addition to consistent viability when conditioned to broad temperatures (4 Celsius, 26 Celsius & 37 Celsius). Collectively, this introduces the investigated phage as a competent, promising candidate for phage therapy. Eventually, the complete phage genome was reassembled generating a proportionally large genome of 161.33 kb while illustrating partial similarity with other salmonella-lytic phages leading to its novel introduction in the SSE121 phage classification. Phage therapy research starts mainly by investigating potential phage candidates which harbor the required characteristics and properties. By conducting evaluation and screening tests on the phage to reveal its compatibility, a researcher can then decide whether to further continue its investigation and complete sequencing tests.

For instance, one of the most recent publications in this field conducted an investigative study on a phage isolated from pig fecal waste (Wan, et al., 2025). At first, the morphology of the phage was constructed by exploiting the use of transmission electron microscopy providing an insight into the structure and phage traits. Screening tests were performed against different bacterial species to determine the host range and phage lytic activity was assessed where it illustrated efficiency in destroying multidrug-resistant E. coli. The promising lytic activity of the isolated phage against antibiotic resistance bacteria encouraged further investigations in order to evaluate its incorporation in phage therap. Therefore, whole genome sequencing was performed revealing a double-stranded DNA consisting of 44.1kbp, 55 ORFs. Eventually this phage was considered a significant contribution as a reference in the database of phage therapy as it illustrated promising therapeutic traits with an absence in virulence, resilience or lysogenic genes.

Due to the broad dispersion of phage abundance, researchers should consider the various environmental sources available for phage inquiries. In the following research study conducted by (Liao, et al., 2023), a bacteriophage was isolated from a sewage water disposal in China that was capable of exhibiting lytic activity against P. acne. When screened on a double-layer agar plate, the phage was able of demonstrating a transparent plaque with a diameter range of 1.0-5.0mm suggesting a critical induction of lysis. Classification and grouping of the phage was done depending on the morphological visuals constructed through transmission electron microscopy. The bacteriophage illustrated favorable properties as the burst size was 26 PFU, it also managed to exhibit resilience against different pH levels and temperatures illustrating high tolerance and longevity. Whole-genome sequencing analysis was then performed revealing a linear double-stranded DNA with a length of 29,648 base pairs containing 45 ORFs. The bacteriophage was finally registered in the database serving as a competent candidate for therapeutic approaches against P. acne as well as providing a reference for further phage therapy investigations

Chapter three

Materials & Methods:

3.1 Sample collection

Ten different samples (250 ml each) of treated and untreated sewage water were collected where the samples were taken from the secondary stages of water treatment to avoid contamination from large debris, as bacteriophage sizes range from 20 nm to 600 nm. The samples were retrieved from a water-treatment facility located in Al-Tireh, Ramallah, which belongs to the Palestinian Water Authority.

Additionally, four samples were collected from olive-oil-treating wastewater, locally known as "Zaebar." These samples, each measuring 100 ml, were collected from Silwad and Aroura villages in Ramallah. All samples were collected using sterile 50 ml containers.

3.2 Sedimentation and filtration

The collected samples were centrifuged at 10,000 rpm for five minutes, and the supernatants were stored under refrigerated conditions. To separate bacteriophage particles from bacterial cells, the samples were filtered through a polymeric membrane with a pore size of 0.22 μm. The resulting purified bacteriophages were then co-cultured with host bacterial cells.

3.3 Phage plaque assay

The co-culture was initiated by mixing two different volumes of recovered phages (100 □l and 200 □l) with 200 □l of an overnight bacterial culture. To ensure a variety of host-phage interactions, different overnight cultures of various bacterial species were utilized.

The resulting mixture was gently agitated and then allowed to settle for 10 minutes. Subsequently, this mixture was combined with 5 ml of sterile top agar, which was maintained at 50 °C in a 15 ml tube. The entire contents of the tube were then immediately and evenly poured onto an agar plate. Following the solidification of the top agar, the plates were incubated overnight at 37 °C.

3.4 Serial dilutions of the liquid sample

In order to obtain single plaques, a 10-fold serial dilution of the phage solution was performed. Six 1.5 ml microcentrifuge tubes were prepared, each containing 90 µl of phage buffer and labeled according to the dilution factor (e.g., 10^{-1}, 10^{-2}, etc.). The dilution process began by adding 10 µl of the phage sample to the first tube. 10 µl was then serially transferred from this tube to the next, continuing up to the final desired dilution.

Each diluted sample was then mixed with an overnight bacterial culture in 5 ml of top agar and incubated overnight at 37°C. This method, as described previously, was used to achieve separated plaques.

3.5 Plaque picking

To isolate individual phages, only well-separated plaques displaying clear zones an indicator of lytic activity were selected. Each plaque was aseptically picked with a sterile toothpick or a 200 µl pipette tip. The picked plaques were then transferred to separate microcentrifuge tubes, each containing a TE buffer solution, to create a liquid phage mixture. This process ensured that each distinct plaque was isolated for further analysis

3.6 Phage DNA extraction

To extract the phage DNA, each isolated phage sample, which had been suspended in TE buffer within a 1.5 ml tube, was incubated in a water bath at 60 °C for 30 minutes. Following this incubation, the tubes were subjected to centrifugation at 15,000 rpm for five minutes. The supernatant containing the purified phage DNA was then carefully collected. This extracted DNA was used for subsequent analyses, such as PCR amplification, while any remaining isolated DNA was stored at 4 °C for future experiments

3.7 Primer design & Polymerase Chain Reaction (PCR)

In this study, two distinct PCR systems were employed, utilizing a total of four oligonucleotide primers, to amplify both Class 1 integrons and insertion sites. These integrons are a key genetic element known to be significantly associated with the acquisition and transfer of multi-drug resistance (MDR) genes among bacterial

populations. The primer sequences, as detailed in Table 1, were selected from previously published studies that have successfully used them for bacteriophage genome investigations (Ishikawa, 2011) (Zifan, et al,. 2025) .

Table 1.3: Primer design of the two different primer sets implemented.

Primer set number	Primer name	Nucleotide sequence	Expected band size
Primer set 1	intCiF3a	GTCAAGGAT(C/G)TGGATTTCGA	300bp
	intCiiiR3a	ACATGCGTGTA(A/G)ATCATCGT	300bp
Primer set 2	IS-F	GTTTGGTTGATAATGAACTGTGCTGATTAC	200bp
	IS-R	ATTCAATAATCGCATCCGATTGCAG	200bp

Nevertheless, modifications were made by our side to ensure optimum achieving of our research goals as the referenced article (Ishikawa, 2011) used several primer pairs for amplification of different conserved regions found within various classes of Integrons, (I,II,III), this was taken in consideration while designing our primers. As the objective of this study was to investigate the integration process taking place by Integrons harboring MDR gene. In addition to amplification of the conserved regions of class I Integrons by utilizing the above primer set, we also went further by testing the presence of inverted repeats. Therefore, our combined investigatory approach by testing for both class I Integrons & the commonly found inverted repeats found within the flanking regions providing a comprehensive molecular analysis which generates a throughput genetic model of the investigated phage. Our analytical approach was made possible by designing primer sets targeting target regions of MDR genes. These targeted MDR genes resembled the most commonly found genes present within these class I Integrons, which were highly affiliated with being responsible for bacteria acquiring novel MDR traits. Our design for these primers was inspired by a recent research study that aimed to detect and investigate insertion integration sites abundant in various environmental samples of different sources by designing novel primer sets targeting precise regions of such commonly found MDR genes (Zifan, et al,. 2025). The designed primers were responsible of amplifying a target region of the inverted flanking regions which acquires bacteria ability to undergo mutagenesis. The design of the mentioned primer set was as follows:

PCR amplification was performed according the manufacturer's instructions while considering the Tm of our designed primers to ensure efficiency and accuracy in results.

Traditional protocols of performing PCR includes the use of (dNTPs, enzyme, buffer & $MgCl_2$) where each is added separately into the mixture. In our procedure, we utilized a Taq enzyme mix 2x concentrated which resembles a combination of the four reagents mentioned. The stock primers were diluted to 20pmoles/ul by addition of 1ml of double-distilled water to each tube (forward & reverse) and gentle mixing via vortex. 1ul of each previously diluted primer tube was added to each reaction mix. The quantities and volumes of the ingredients required for reach PCR reaction mix where as follows:

Table 2.3: Quantities and volumes of each constituent in a single PCR reaction

Ingredient	**Volume in microliters**
Taq enzyme mix 2x	12.5 ul
Forward primer	1 ul
Reverse primer	1 ul
Double-distilled water	5.5 ul
DNA (added last)	5 ul
Total volume	**25 ul**

Since we were running 20 samples at the sample, a master mix mixture enough for the 20 samples was made in bulk then 25ul of the prepared mixture was aliquoted into each labeled PCR tube (each volume in the table was multiplied by 20) and an extra reaction was prepared to allocate & consider possible pipetting errors, positive and negative controls were prepared as well.

The constructed PCR program was as follows:

Initial denaturation step at 95 Celsius for 5 minutes, followed by repetitive 36 cycles of the following:

1- Denaturation at 95 Celsius for 30 seconds.

2- Annealing at 54 Celsius for 30 seconds.

3- Extension at 72 Celsius for 30 seconds

4- Final extension step at 72 Celsius for 10 minutes.

3.8 Agarose gel electrophoresis

Sample PCR products were analyzed by agarose gel electrophoresis in a 1.5% agarose gel consisting of the following: (1.5g agarose, 120ml 1x TAE buffer, 14ul Ethidium Bromide). The original 50x TAE buffer electrophoresis running buffer (242g Tris base, 57.1ml glacial acetic acid & 100ml 0.5M EDTA (pH 8.0)). The 100bp DNA ladder #SM0241 was used as a reference size marker for the PCR products.

3.9 NGS via High-throughput DNA deep sequencing utilizing Illumina MiSeq platform

In order to perform next-generation-sequencing, modifications must first take place such as pooling and clean-up of the product PCR samples from the previous step. This involves removal of excess primers, dNTPS & other reagents used in PCR by utilizing a well-performing magnetic beads purification kit (AMPure XP beads kit / Beckman coulter, USA) to ensure decontamination of the phage DNA and establish high quality purification and retrieval of the DNA for further sequencing.

Sample pooling and clean-up via AMPure XP magnetic beads

The first step in the process is pooling the samples. To do this, 15 µl from PCR product 1 (generated by primer set 1) is combined with 15 µl from PCR product 1 (generated by primer set 2), and this is repeated for the remaining samples. Next, 30 µl of AMPure XP magnetic beads are added to each pooled tube. After mixing well, the solution rests at room temperature for five minutes to allow the DNA to precipitate, a process facilitated by PEG and NaCl. The PCR strips are then placed on a magnetic 96-well plate stand for five minutes, allowing the beads to attach to the side of each tube and the solution to clear. The supernatant is carefully discarded, taking care not to disturb the attached beads. After removing the strips from the magnetic stand, 200 µl of 80% ethanol is added to each tube and left to rest for one minute. The strips are then returned to the magnetic stand, and the ethanol is cautiously removed. This ethanol wash is repeated a second time, followed by a five-minute rest period to allow the ethanol to fully evaporate. Finally, the PCR strips are removed from the stand, and 30 µl of double-distilled water (DDW) is added to elute the bound DNA. After resting for two to three minutes, the strips are placed back on the magnetic stand, and the eluted DNA is carefully transferred to a fresh PCR tube.

Index addition and library preparation "Second PCR"

This step involves addition of two indices (N7XX and S5XX) to each tube producing a distinct combination of indices ensuring facilitated referencing for when sequencing the pooled samples. Index addition will be performed by utilization of 2x master mix in a PCR program. The volumes of each ingredient will be as follows:

Table 3.3: Quantities and volumes of each constituent in a single PCR reaction.

Ingredient	**Volume in microliters**
Taq enzyme mix 2x	12.5 ul
N7XX index	2 ul
S5XX index	2 ul
DNA	8.5 ul
.Total volume	**25 ul**

For easier & time-effective mixing, the 8 tubes of N7XX indices were placed in a horizontal arrangement on a rack and the 10 tubes of S5XX indices were placed in a vertical arrangement on the rack. 2 ul of each index primer was then simultaneously added to the corresponding PCR tube already containing the 8.5 ul pooled and purified phage DNA.

Table 4.3: Reference representing the indices combination for the 60 phage samples.

Indices	**11R**	**1917R**	**1918R**	**21R**	**20R**	**18R**	**17R**	**16R**
N14D	S1	S2	S3	S4	S5	S6	S7	S8
N15D	S9	S10	S11	S12	S13	S14	S15	S16
N16D	S17	S18	S19	S20	S21	S22	S23	S24
N18D	S25	S26	S27	S28	S29	S30	S31	S32
N19D	S33	S34	S35	S36	S37	S38	S39	S40
N20D	S41	S42	S43	S44	S45	S46	S47	S48
N21D	S49	S50	S51	S52	S53	S54	S55	S56
N22D	S57	S58	S59	S60				

The samples are then ran in a PCR program as follows:

1- 1st step initiates by denaturation at 95 Celsius for 5 minutes.

8 repeated cycles of the following steps.

2- Denaturation at 95 Celsius for 30 seconds.

3- Annealing at 54 Celsius for 30 seconds.

4- Extension at 72 Celsius for 30 seconds.

5- Final extension step at 72 Celsius for 5 minutes.

Sample PCR products were analyzed by agarose gel electrophoresis.

Final preparation of pooling and indexing of PCR samples involves another round of sample purification via AMPure XP beads repeated as mentioned previously above. Following this last step of purification, pooling of samples takes place where 10 ul of each eluted sample is added into a single tube resembling the library and is ready to be sent for Next generation DNA sequencing at a professional NGS service company (sequencing was performed on a Miseq machine using 500 cycle kit from Illumina Co.).

3.10Bioinformatics & Data manipulation

The raw sequencing data from a MiSeq Illumina run was first uploaded to Galaxy.eu, where a sequential workflow was constructed for data processing and analysis.

1- The first step was fastq-join, which merged paired-end reads (forward and reverse) based on sequence compatibility. This process generated three file types: "matched" reads, "unmatched forward" reads, and "unmatched reverse" reads. With two different primer sets used, a total of six files were generated.

2- The files were then converted from fastq to fasta format, followed by conversion to a tabular format for easier data manipulation.

3- Primer Sequence Filtering: We performed a query selection to isolate only the nucleotide sequences corresponding to our primer sets. This step was crucial for excluding unwanted DNA and retaining only the amplified sequences for subsequent analysis.

4- Collapsing: A "collapse job" was used to reduce redundancy by grouping identical sequences. This step removed duplicate sequences while keeping a count of each sequence's occurrence. This was followed by trimming of poly-G tails.

5- Phage Identification: The top 10 most abundant sequences from each of the 360 sample files were submitted to NCBI BLASTn. This step was performed to identify the phage sequences, determine their identity and possible function, and annotate their similarity to other known and published sequences. For bacteriophages, this analysis also provides a prediction of their original bacterial host.

6- Final Data Organization and Visualization: The results were downloaded from the NCBI website in .XML format and converted to a tabular form to facilitate data organization and correlation with their original sources. Finally, figures and visualizations of the organized data were created for easier interpretation. The figure below provides a visual representation of this constructed workflow.

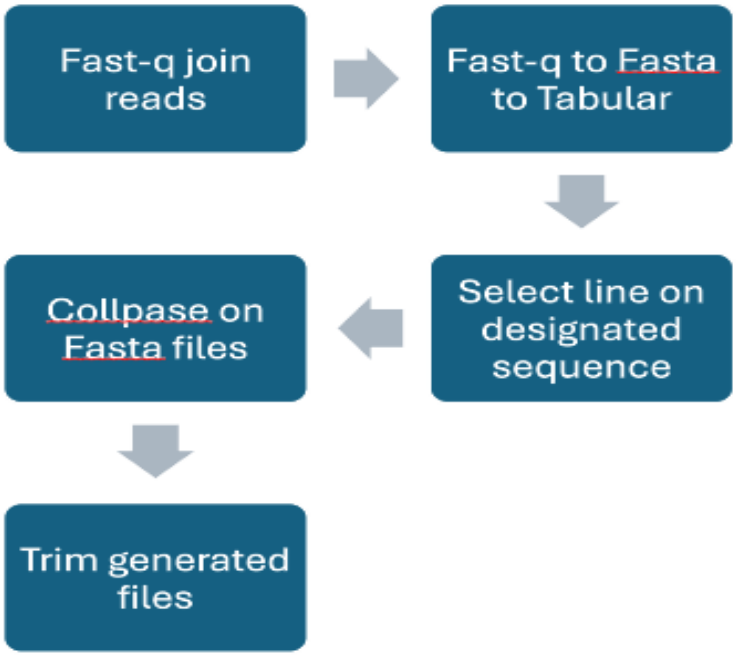


Figure 1.3: A summary of the workflow chart that was constructed on galaxy.eu & run in a sequential order as illustrated. Each step generated a specific output that was inserted as an input for the proceeding step.

Chapter Four

. Results

4.1 Phage plaque assay and culture.

Figure 4 demonstrates that following centrifugation and sedimentation of the sewage-water sample, the bacteriophage was isolated and cultured with an overnight culture of bacteria. In the figure above, 50 µl of the isolated bacteriophage was cultured with 200 µl of an overnight culture of E. coli through a method of top-agar. The image above illustrates the formation of the plaques across the plate, illustrating the wide distribution of the phage indicating the lytic activity of the phage against this bacterial species.

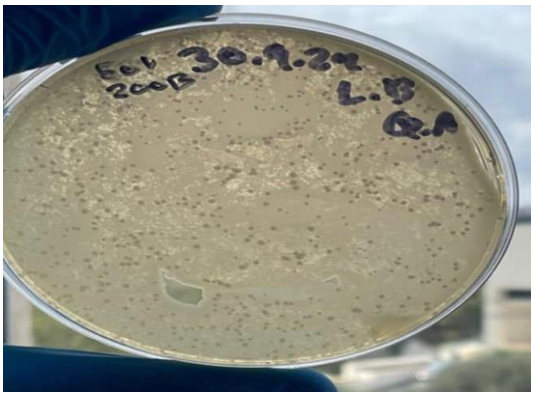


Figure 1.4: A plate of 50 µl of sewage-water isolated incubated phage with 200 µl of E. coli. This plate illustrates the phage plaque assay by enrichment with E.coli

4.2 Phage screening against different bacterial species and host range determination.

As observed in figure 5 above, the same isolate of sewage-water bacteriophage was incubated with 200ul of an overnight culture Klebsiella pneumoniae "**5A**", to determine the phage host range and lytic activity against this bacterial species and it demonstrated successful infection in addition to illustrating phage lytic activity where the formation of plaques can be observed clearly above. "**5B**" showcases the successful penetration exhibited by the sewage-water isolated bacteriophage against the Bacillus subtillus bacterial species. 50 µl of the isolated phage was incubated with an overnight culture of 200 ul of Bacillus subtillus and managed to demonstrate webbed-like formation of plaques across the plate illustrating successful lytic activity and broader host compatibility. The webbed-like plaque formation indicates a somehow temperate life style or a slower induction of lysis against this bacterial species. For further demonstration of the broad host range of the sewage-water isolated bacteriophage, 50 µl of the mentioned phage was incubated with an overnight culture of 200 µl of Pseudomonas aeruginosa "**5C**". The phage managed to induce a dense plaque formation within the plate indicating highly rapid phage lytic activity and a short latency period of the phage life cycle. As observed in the procedure of host determination and phage lytic activity evaluation, we reach a conclusion that the sewage-water isolated bacteriophage had a broad host range against various bacterial species (E. coli, Klebsiella pneumoniae , Bacillus subtillus & Pseudomonas aeruginosa). When the same volume of the same-sourced phage was screened against 200 µl of different bacterial species in an overnight culture manner, the phage illustrated success in infecting the bacteria in addition to demonstrating critical lytic activity as indicated by the plaque formation across the diverse plates.

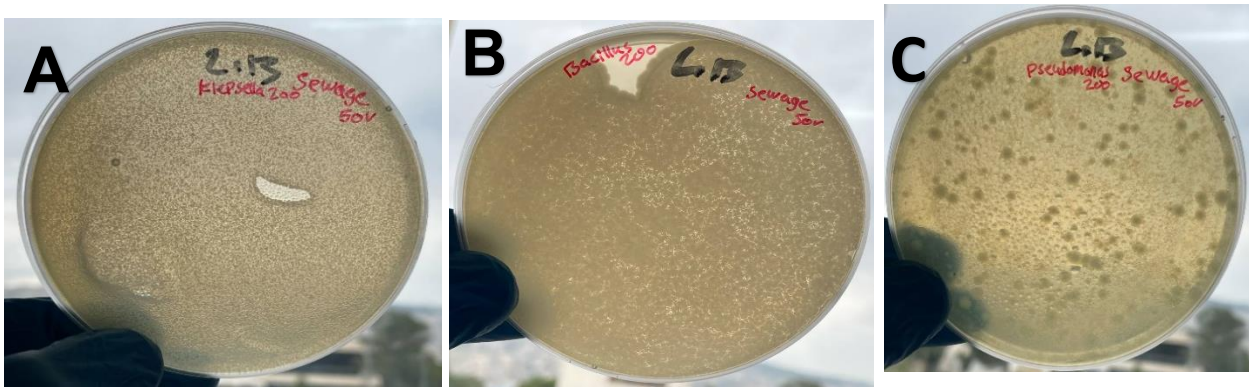


Figure 2.4: A plate of 50 µl of sewage-water isolated incubated phage with 200 µl of different bacteria species. The bacterial species are A) Klebsiella, B) Bacillus, C) Pseudomonas.

As we observe figure 6 above, investigating the second source of bacteriophages, a phage isolated from an oil mill located in Aroura was screened against various bacterial species for host determination and evaluation of phage lytic activity. 50 µl of the mentioned phage was incubated with 200 µl of an overnight culture of Pseudomonas aeruginosa, the phage managed to infect the bacteria successfully and illustrated significant lytic activity against this bacterial species as clearly observed by the formation of highly-dense plaques. The same oil-mill isolated phage was incubated with Bacillus subtillus **"6B"** where 50 µl of the phage was incubated with a 200 µl of an overnight culture. The phage portrayed an effective infection of the said bacteria and an additional reliable lysis as observed by the plaque formation. A must mention observation is the formation of the webbed-like plaque across the plate which is similar to the one seen in figure "**6B"**. For further clarifications, in figure "6B", the same volume of a different sourced phage was incubated with 200 µl of the same Bacillus subtillus species and a similarly webbed-like plaque formation emerged. This raises attention to a significant observation, the Bacillus species used for incubation in both cases of the two different phages probably harbors a defense mechanism such as CRISPR or other restrictive enzymes leading to defective lytic activity of the phages. The latter characteristic of the bacteria leads to partial success in phage lytic activity and leads to a non-uniformal lysis giving rise to the emerging of the webbed-like plaque formation observed in both phage screening. For further evaluation of the oil-mill isolated bacteriophage host range, 50 µl of the phage was incubated with an overnight culture of 200 µl of Klebsiella **"6C"**. The phage successfully managed to infect the bacterial host and induced lytic activity which can be significantly observed by the formation of plaques across the incubated plate.

To summarize the host range and lytic activity of the investigated oil-mill isolated phage, we can conclude by mentioning the successful infection and lytic activity elicited by the phage against three different bacterial species (Pseudomonas aeruginosa, Bacillus subtillus & Klebseilla pneumoniae). This was demonstrated by the formation of plaques when screened against both Pseudomonas aeruginosa and Klebsiella pneumoniae. In regards to the incubation occurring with Bacillus subtillus, the webbed-like plaque formation which was common with the same-host & differently-sourced phage was rationed by the potential defense mechanism of the Bacillus bacteria.

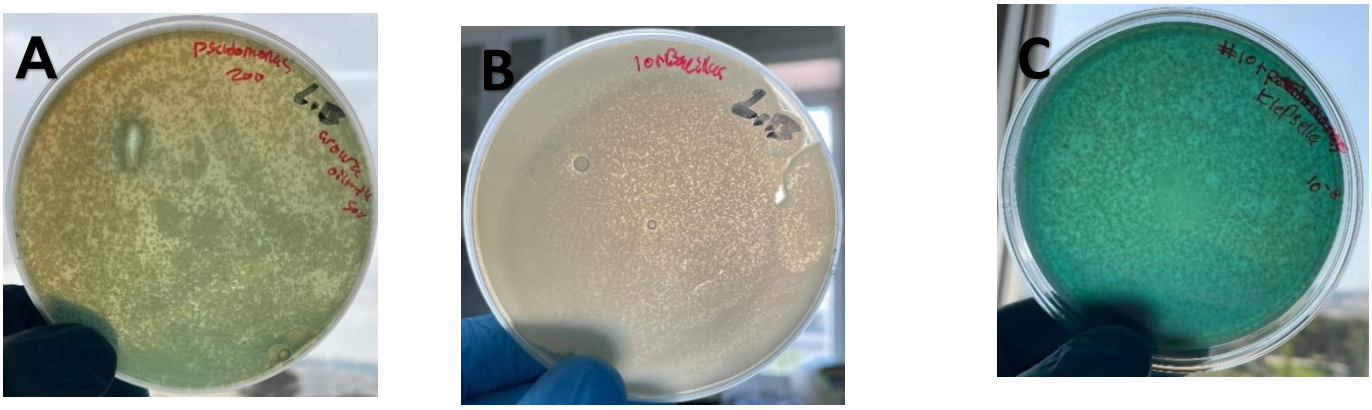


Figure 3.4: An oil-mill isolated phage incubated with 200 µl of different bacterial species. Where the bacterial species are A) Pseudomonas, B) Bacillus, C) Klebsiella,

4.3 Phage titer and serial dilutions.

Serial dilution of the original phage titer was performed by mixing with a 10^{-3} ratio volume of phage buffer and screened against a bacterial host. The significance of the serial dilution lies in determining phage titer and evaluate its potency. As expected, the first serial dilution illustrates a dense plaque formation with uncountable plaque forming units "PFUs" which is clearly portrayed in the figure above **"7A".** Proceeding serial dilutions will demonstrate less dense and broader plaque formations. The following 10^{-5} serial dilution **"7B"** represents a less-dense plaque formation as clearly observed by the wide-ranged distances between the plaques. The evaluation of the PFUs is more manageable with an estimation of 500 PFUs and indicates high phage potency as the 10^{-5} diluted phage aliquoted still managed to induce infectious lytic activity. As illustrated by the figure above **"7C",** a 10^{-7} serial dilution produces the least dense plaque formation with an easily manageable PFU estimation, which can be estimated by 200-300 PFUs indicating a successful serial dilution. Moreover, as clearly observed, the phage still managed to introduce an infectious lytic activity even when diluted illustrated high phage potency and compatibility. This enables us to conduct a successful plaque picking in the following procedure allowing for a more facilitated DNA extraction.

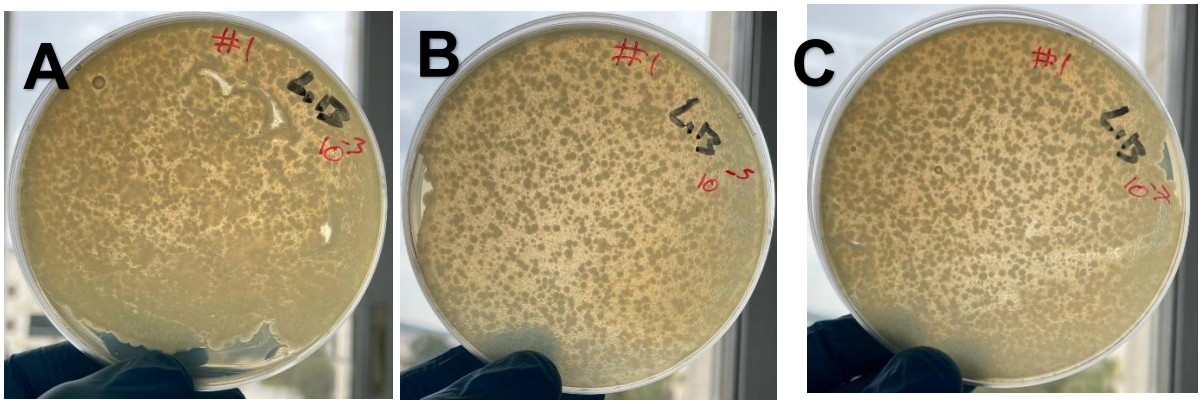


Figure 4.4: Serial dilutions of the original phage aliquot with phage buffer. A) indicates a 10^{-3} dilution, B) 10^{-5} dilution, C) 10^{-7} dilution.

4.4 Plaque picking and elution

The procedure of plaque picking relies on random picking of a densely populated phage plaques using a micropipette tip which is later eluted into a tube containing phage buffer. As an illustration, in the figure above we can see the plate that was used for plaque picking of the 37th sample. The plate was clearly labelled for aiding as a reference for later procedures. The labelling indicates that source of the phage which is sewage water in this case. Klebsiella was used as the bacterial host for this plate as clearly observed by the label. As represented by figure 8B above, sample 29 was picked from a plate of an oil-mill sample (indicated by #10 tag corresponding to previous lab notes) incubated with S. aureus. Both figures 8A and 8B represent the diversity of the plaques that were picked from the different plates, all the 60 samples were picked from combinations of the two different phage sources (sewage-water and oil mill) with the 5 different bacterial strains (E. coli, pseudomonas aeruginosa, Bacillus subtillus, staphylococcus auerus & klebsiella pneumoniae) totalling up to around 60 plates that represented sources for sample collection.

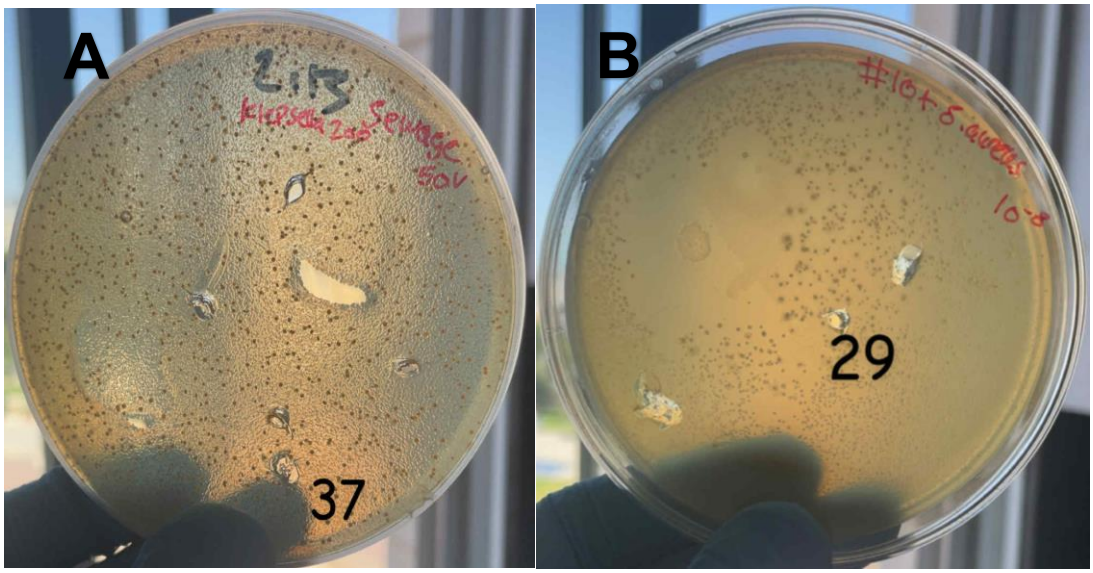


Figure 5.4: Random plaques picked from different plate sources. A) Represents the 37th sample picked from a sewage water-sourced phage incubated with Klebisella. B) The 29th sample picked from an oil-mill-sourced phage incubated with S. aureus.

4.5 PCR amplification and gel electrophoresis

Oligonucleotides were designed in the objective of amplifying class I Integrons that are significantly associated with harboring & transfer of MDR genes among bacteria. Therefore, the following primer pair intCiF3a & intCiiiR3a represent the first primer set and were designed as they aligned with our prospective investigatory goal.

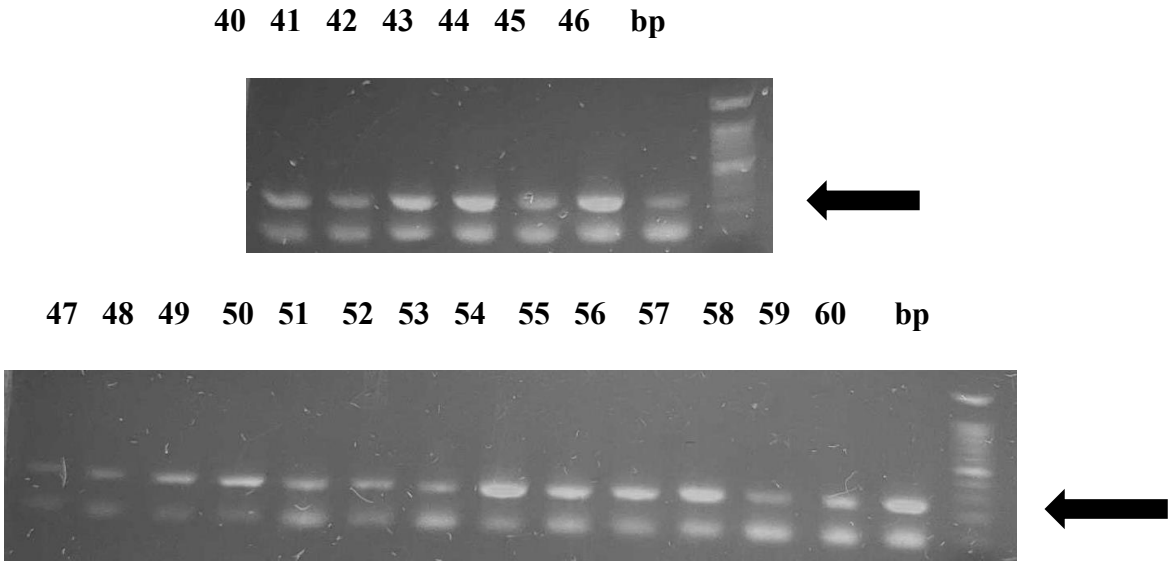


Figure 6.4: A representative gel illustrating the gel electrophoresis of the PCR products of the 1st primer set. The PCR products shown in the figure above represent the samples 40-61. Each arrow placed indicates the band marker size 300bp.

The above gel represents an accurate PCR amplification where the product bands represent an estimated size of 300-400bp according to the DNA size marker. Precise purity and integrity in PCR amplification is illustrated by the clear DNA band visualization and accurate running of the gel is indicated as well.

The designed primers IS-F & IS-R were responsible of amplifying a target region of the inverted flanking regions which acquires bacteria ability to undergo mutagenesis. The design of the mentioned primer set was as follows:

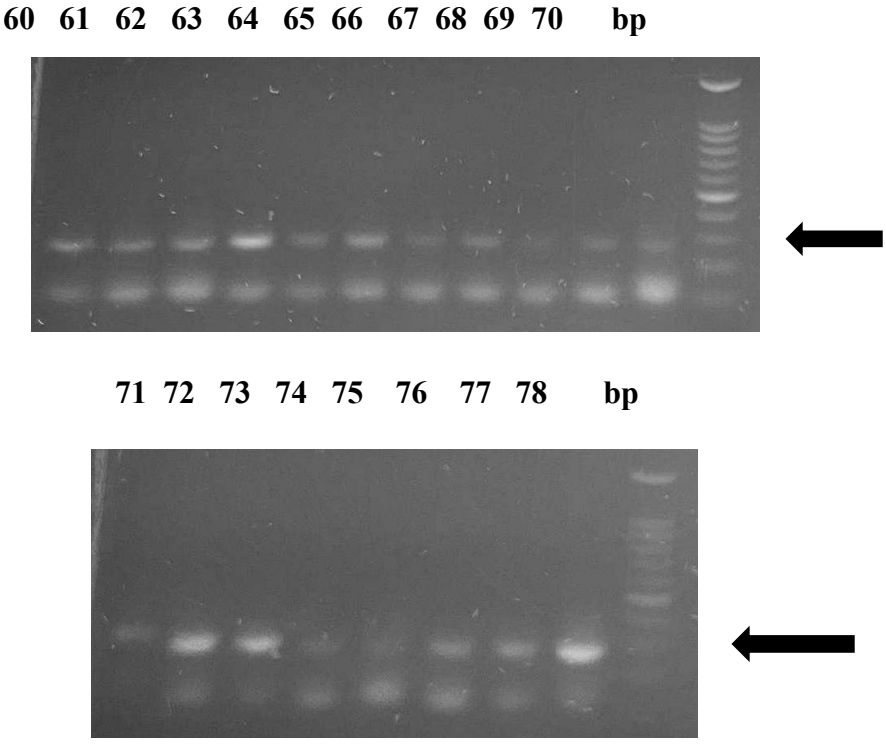


Figure 7.4: A representative gel illustrating the gel electrophoresis of the PCR products of the 2nd primer set. The PCR products shown above represent the samples 60-79. The inserted arrow represents the band marker size 200bp.

The above figure represents adequate amplification of the targeted DNA regions in a few samples (60-79) which portray the expected DNA band size of 200-300. This illustrates accurate generation of the targeted gene amplification, while ensuring accurate DNA purity. Visualization of the gel seems well observed and clear indicating an accurate and precise running of the gel and adequate performance.

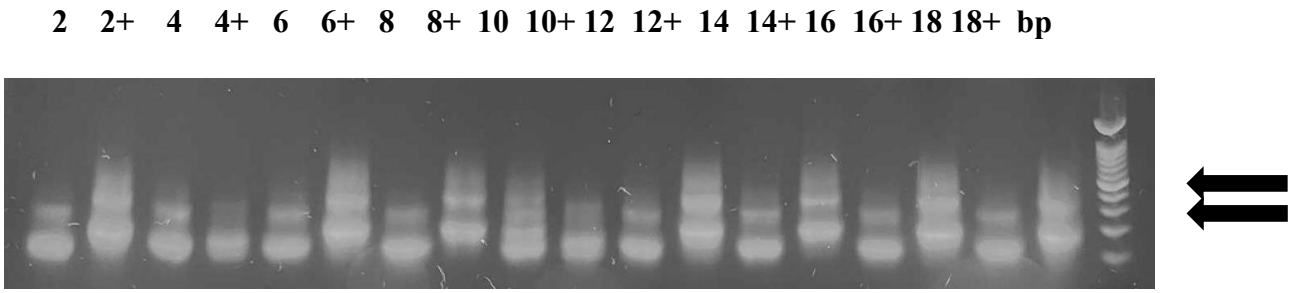


Figure 8.4: A gel representing the comparison of DNA size between PCR samples before and after indices addition. When comparing each two samples together (-index, +index) an increase in DNA size can be observed representing the "shift". The placed arrow indicates the marker sizes (top arrow represents 400bp, bottom arrow represents 200bp.

For further validation of the indices addition process, we conducted a gel electrophoresis of the same samples before the index addition and after. For instance, even numbered samples from 2 till 18 were used in this procedure, (i.g: sample #2 represents the product sample following PCR with the primer set while #2+ represents the same latter sample with a further step of index addition). As observed in the figure, the DNA products generated by primer PCR amplification generate a size of 200-300bp as referenced by the DNA ladder on the right. On the other hand, those samples marked by "+", which underwent index addition experienced a small addition in DNA size estimated at around 80-150bp which emerges as a result of adding the index primer sequences. This slight shift in DNA size confirms & validates the accurate procedure of indices addition "2nd PCR".

4.6 Miseq DNA library analysis and quality control

Following the process of sample pooling and validation of the indices addition, quality control check of the returned data must be performed for assurance. Therefore, a Tapstation electrophoresis analysis was conducted (Agilent Technologies, CA, USA) which utilizes a SYBR green I fluorescence detection system in the aims of performing a qualitative and a quantitative quality check of the obtained data, the results are illustrated in the below figure.

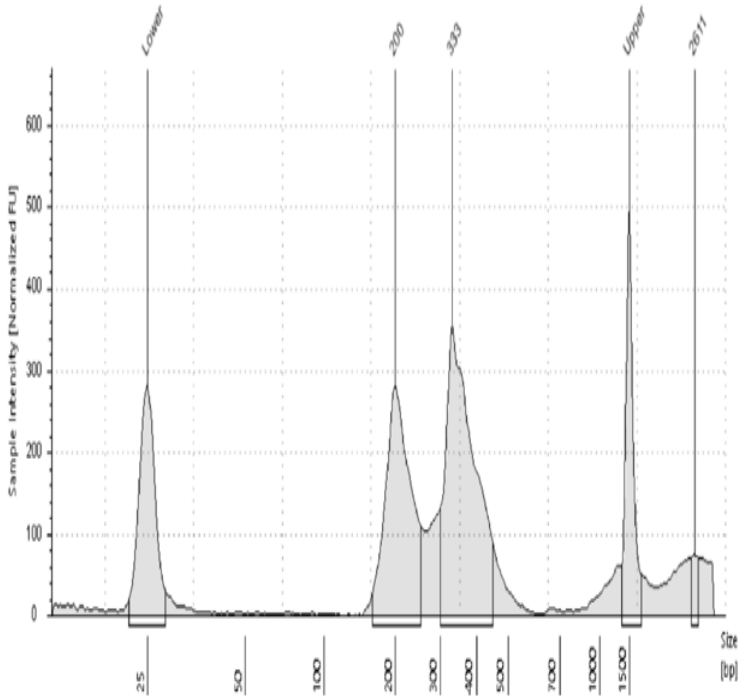


Figure 9.4: Results of the Miseq library quality control analysis utilizing a sensitive fluorescent DNA SYBR green-based analysis conducted on a Tapstation electrophoresis system. There are two clearly distinct bands found at (200bp) representing the NGS primers and (300-400bp) indicating accurate PCR amplicons of the targeted regions.

4.7 NGS interpretation of phage amplicons and alignment with pathogenic bacteria genome

Based on our NGS analysis, we managed to extract significant data regarding the top hit repetition for our different PCR amplicons with regards to their environmental source (sewage-water or oil mill), original host culture bacteria and primer set used. The tables below numbered 5-8 represent the top hit alignment found within the different bacterial species. The numerical value found indicates a quantitative measurement for the read counts obtained from the PCR amplification.

Table 1.4: “Int-unmatched R2” amplicon top hit repetition of sewage & oil mill sourced samples.

Sewage Phage	**Bartonella krasnovii**	**Enterobacteriaceae bacterium**	**MAG: CrAssphage**	**Moraxellaceae bacterium**	**Staphylococcus aureus**	**Streptococcus parasuis**	**Vibrio phage**
E. coli	65816	124218	27396	52743	9618	22	33936
Bacillus	0	0	0	0	0	18058	0
Pseudomonas	22382	23920	0	0	0	22046	0
Klebsiella	0	0	22435	24203	8455	88102	13916
S.aureus	-	-	-	-	-	-	-
Oil mill phage	**Bartonella krasnovii**	**Enterobacteriaceae bacterium**	**MAG: CrAssphage**	**Moraxellaceae bacterium**	**Staphylococcus aureus**	**Streptococcus parasuis**	**Vibrio phage**
E. coli	-	-	-	-	-	-	-
Bacillus	0	0	27094	0	8913	0	0
Pseudomonas	0	0	0	26397	0	0	16225
Klebsiella	0	42318	0	67442	0	17344	39268
S.aureus	57346	38285	12753	0	4687	0	0

Table 2.4: “IS-unmatched R2” amplicon top hit repetition of sewage & oil mill sourced samples.

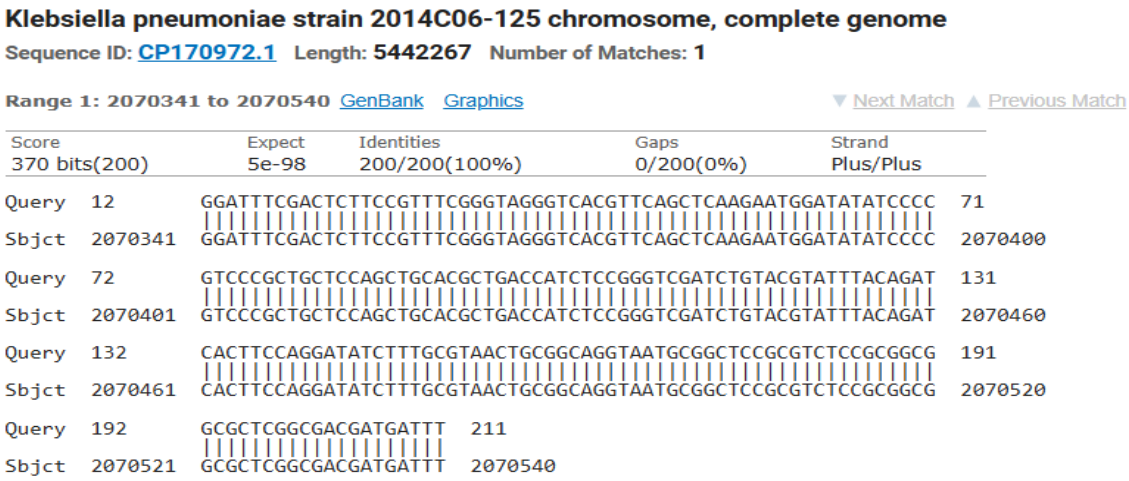
Sewage phage	**Bartonella**	**Chromobacterium**	**E. coli**	**Klebsiella pneumoniae**
E. coli	61273	1586	101231	3497
Bacillus	9585	276	0	130
Pseudomonas	21205	457	0	680
Klebsiella	10720	1431	17701	1743
S.aureus	-	-	-	-
Oil Mill phage	**Bartonella**	**Chromobacterium**	**E. coli**	**Klebsiella pneumoniae**
E.coli	-	-	-	-
Bacillus	0	0	0	454
Pseudomonas	0	245	35388	0
Klebsiella	0	1529	62943	1535
S.aureus	61436	0	0	1169

Table 3.4: "Int-paired" amplicon top hit repetition of sewage & oil-mill sourced samples.

Sewage phage	**Acinetobacter lwoffii**
E.coli	70
Bacillus	2
Pseudomonas	18
Klebsiella	19
S.aureus	-
Oil mill phage	**Acinetobacter lwoffii**
E.coli	-
Bacillus	1
Pseudomonas	1
Klebsiella	25
S.aureus	12

Table 4.4: "IS-paired" amplicon top hit repetition of sewage & oil-mill sourced samples.

Sewage phage	**Klebsiella Penumoniae**
E.coli	25566
Bacillus	972
Pseudomonas	3327
Klebsiella	16842
S.aureus	-
Oil mill phage	**Klebsiella Penumoniae**
E.coli	-
Bacillus	1110
Pseudomonas	1270
Klebsiella	8872
S.aureus	4284



Klebsiella pneumoniae strain 2014C06-125 chromosome, complete genome

Sequence ID: CP170972.1 Length: 5442267 Number of Matches: 1

Range 1: 2070341 to 2070540 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
370 bits(200)	5e-98	200/200(100%)	0/200(0%)	Plus/Plus

```
Query  12       GGATTTCGACTCTTCCGTTTCGGGTAGGGTCACGTTCAGCTCAAGAATGGATATATCCCC  71
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2070341  GGATTTCGACTCTTCCGTTTCGGGTAGGGTCACGTTCAGCTCAAGAATGGATATATCCCC  2070400

Query  72       GTCCCGCTGCTCCAGCTGCACGCTGACCATCTCCGGGTCGATCTGTACGTATTTACAGAT  131
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2070401  GTCCCGCTGCTCCAGCTGCACGCTGACCATCTCCGGGTCGATCTGTACGTATTTACAGAT  2070460

Query  132      CACTTCCAGGATATCTTTGCGTAACTGCGGCAGGTAATGCGGCTCCGCGTCTCCGCGGCG  191
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2070461  CACTTCCAGGATATCTTTGCGTAACTGCGGCAGGTAATGCGGCTCCGCGTCTCCGCGGCG  2070520

Query  192      GCGCTCGGCGACGATGATTT  211
                ||||||||||||||||||||
Sbjct  2070521  GCGCTCGGCGACGATGATTT  2070540
```

Figure 10.4: "nblast" Alignment of phage amplicon "IS" with specific regions of Klebsiella pneumoniae strain "2014C06-15" chromosome.

https://www.ncbi.nlm.nih.gov/nuccore/CP170972.1?report=graph&rid=7R9KZNDK015[CP170972.1]&tracks=[key:sequence_track,name:Sequence,display_name:Sequence,id:STD1,category:Sequence,annots:Sequence,ShowLabel:true][key:gene_model_track,CDSProductFeats:false][key:alignment_track,name:other%20alignments,annots:NG%20Alignments|Refseq%20Alignments|Gnomon%20Alignments|Unnamed,shown:false]&v=2070332:2070549&appname=ncbiblast&link_loc=fromSubj

The above alignment illustrated in figure 13 was shared among the top five PCR amplicon repetitions which originate from different bacterial hosts (E. coli, Bacillus, Pseudomonas, Klebsiella, S. aureus). This brings up a significant observation, our targeted & amplified "IS" region identified a commonly shared flanked-insertion sequence representing a hotspot for exogenous molecular integration which may introduce Integrons, prophages, MDR genes or any form of potential horizontal gene transfer. For further clarifications, sample 14 which was picked from a sewage-water source originally incubated with E. coli demonstrated an amplicon hit repetition of 6055. When the PCR product sequence was inserted into NCBI nblast for alignment detection with previously published genome references, it unravelled a perfect match with a unique 200bp region found within this Klebsiella species suggesting high conservation and specificity. Following review of the GenBank entry, the aligned region represents a conserved mobile genetic element which could represent either an integrase or a transposase, direct repeats were also found flanking the insertion region indicating the typical characteristic of prophage insertion attachment sites "attc". The presence of such a unique insertion site in this region indicates its

significance as an incorporation site for molecular constituents such as MDR genes, prophages or partial genomes. Furthermore, when other samples from different bacterial species (Bacillus, Pseudomonas, Klebsiella, s. aureus) with the highest amplicon hit repetitions where inserted into NCBI nblast, they all shared the same alignment with the same Klebsiella species as illustrated in figure 13 above, this indicates the unique conservation of this highly specific 200bp insertion region among broad range bacteria originating from Klebsiella bacterial species that harbor significant clinical relevance and pathogenicity. This further suggests the high potential of horizontal gene transfer occurrence between a broad range of bacterial species especially that this region was flanked by integrase genes and attachment sites "attc".

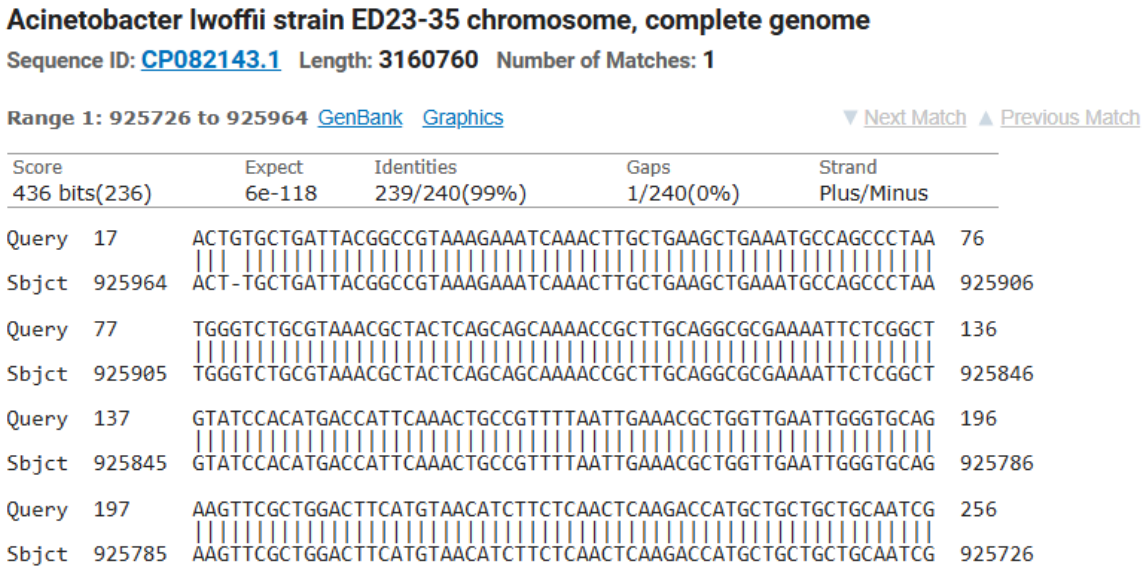


Figure 11.4: "nblast" Alignment of phage amplicon "IntC" with specific regions of Acinetobacter lwoffii strain ED23-35 chromosome.

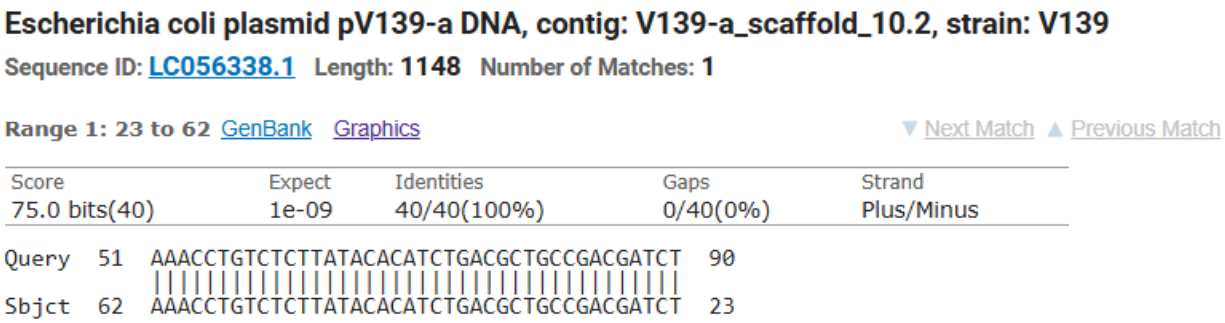
https://blast.ncbi.nlm.nih.gov/Blast.cgi

The above illustrated figure demonstrates the alignment observed within the top 3 PCR amplicons with the most repetitions originating from three different bacterial species (E. coli, Pseudomonas & Klebsiella) while the samples incubated with both S. aureus & Bacillus illustrated absent amplification. This indicates a highly conserved Class I Integron region that is shared within several diverse infection-inducing bacterial species commonly known to harbour antimicrobial-resistant features which is mostly common among gram negative bacteria such as E. coli, Pseudomonas & Acinetobacter. Furthermore, this suggests the presence of a Class I Integron which is known to harbor MDR genes among

our samples which shares the same nucleotidic sequence with the referenced Acinetobacter lwoffi strain ED23-35 chromosome that belongs to a clinically significant genus.

Figure 12.4: "nblast" Alignment of phage amplicon "IS-unmatched-R2" with specific regions of an E. coli plasmid pV139-a DNA.

https://www.ncbi.nlm.nih.gov/nuccore/LC056338.1?report=graph



When blasting the PCR top hit amplicons of the "IS-unmatched-R2" sequences to a reference data base, we found an alignment of 40bp that is 100% identical to an E. coli plasmid "PV139-a" belonging to the V139 strain. This confirms the presence of a partial DNA-derived plasmid that was inserted through either through IS or Integron molecular regions, however no confirmation of the full plasmid integration or insertion can be concluded unless a WGS analysis is performed.

Chapter Five

Discussion

Antibiotic resistance has always resembled a critical health risk hazard, the possibility of genetic transfer between diverse species of bacteria imposed severe health consequences. This phenomena of antibiotic resistance allows previously non-adaptive bacteria to gain newly adopted characteristics allowing for evolutionary adaptations that gain them increased survivability and tolerance against bactericidal agents such as antibiotics. Several factors led to the rapid emergence of antibiotic resistance, some can be classified as manageable such as misuse of antibiotics by patients. On the other hand, an inevitable natural phenomena also contributed significantly to its escalation, horizontal gene transfer and molecular transfer occurring between bacteria species of diverse environments. The synergistic effect of both the inevitable and manageable factors aggravating antibiotic resistance eventually led to it being enlisted as the highest health risk of the 21st century, implying its critical health risk and affiliated complications (Ferri, et al, 2017).

When it comes to antibiotic resistance among bacteria, several classifications have been implemented by scientists and researchers to group bacteria according to their resistance range span and clinical relevance. Bacteria classified as “AMR” are those which recently gained resistance to antibiotics that were previously effective against their corresponding infections. “MDR” bacteria are those which exhibit resistance against at least one agent in three or more antibacterial groups. While “XDR” bacteria are resistant to a broad range of antibiotics exhibiting resistance to all but one antibacterial categories. In rare critical cases,

“PDR” bacteria can be found which portray resistance against all agents of antibacterial groups including those which are used as last-resort. (Dhingra, et al., 2020).

Horizontal gene transfer and evolutionary adaptations occurring in the microenvironment portrays a significant platform for acquisition of novel characteristics and features increasing the survivability, tolerance and stress-response of the recipient bacteria. Several different molecular mechanisms can be involved to give rise for genetic transfer, 1) Conjugation which involves the transfer of DNA usually via mobile plasmids through direct physical contact, 2) Free transfer where bacteria can take up surrounding circulating DNA found within the environment, and lastly 3) Transduction which is mediated by bacteriophages which have the ability to package certain molecular constituents from one bacterial species to another (Chevallereau, et al., 2022). In all the mentioned cases, bacteria eventually adopts exogenous molecular material allowing it to portray novel survival-increasing characteristics.

The molecular mechanism that has attracted attention from researchers and virologists is the bacteriophage-mediated transduction, due to its high region-specific integration and associated clinical relevance, in addition to the fact that viruses portray the highest prevalent entity indicating its high incorporation in horizontal genetic transfer. Several key molecular constituents take part in the integration process occurring between the bacteriophages and the bacteria, an Integron resembles a plasmid-like genetic structure harboring the Integrase gene which is responsible for the insertion and excision of the gene cassettes resembling the second component, lastly a promoter region is found that is necessary for expression of the adopted gene cassettes. (Escudero et al., 2015).

The insertion of these exogenous DNA structures usually take place at specific regions known as insertion sites, these sites share common features despite the molecular diversity. These insertion sites resemble conserved genomic regions which portray stability and no disturbance upon introduction of inserted DNA. Following mass investigatory research of the structure of insertion sites and their surrounding genetic environment, researchers have come up with a confident approach towards identification of newly explored insertion sites. According to (Wai Yin, et al, 2025), insertion sites are often resembled by unique specific short sequences which are conserved among the whole genome, these insertion sites are often flanked by specific genes such as tRNAs, integrases, ribosomal clusters, transposases and insertion sequences.

Moreover, researchers have also investigated the spanning region of those conserved insertion sites and identified certain functional genes that can aid in recognition of those sites. The latter genes usually include those which function in increasing bacterial survivability, tolerance and defense mechanisms, mostly found genes are those that function in cell wall synthesis and alter membrane permeability as a coping mechanism for bactericidal agents such as antibiotics. Gene clusters such as ACFS06_10250–10255 function in build-up of N-acetylglucosamine which resembles a pivotal component of the cell wall structure, it has been identified in several previous publications (Kyu, et al,. 2020) and (Yang, et al,. 2023) where they demonstrated the flanking of highly conserved insertion sites by the gene clusters involved in build-up of N-acetylglucosamine. Other usually found genes which gain bacteria evolutionary adaptations such as antibiotic resistance were found such as FadD gene which portrays a significant role in membrane-lipid alterations eventually altering its permeability and charge attraction to antibiotics. (Kaimin, et al., 2022) demonstrated how the FadD gene functions in introducing membrane alterations upon stress-stimuli corresponding to decreased membrane permeability and thus, reducing the attraction of yet to be bound antibiotics.

Therefore, the collective information presented by researchers have implemented a successful and confident approach for recognition of insertion sites, by identifying the specificity and uniqueness of the sequence within the genome and its high conservation among different bacterial species. Furthermore, identifying the commonly found flanking genes such as housekeeping essential genes and genes that function in bacterial survivability and antibiotic resistance often marks the presence of an integration hotspot. A unique characteristic of those insertion sites is the high conservation of the specific sequences among the different bacterial species, this aids virologists and researchers in tracing genetic transfer occurring horizontally providing chronological tracking of the antibiotic resistance genes being transferred and its origins. The latter provides significant contribution towards allocating the development of pathogenic bacteria and the corresponding clinical risks. (Mogro et al., 2021) introduced a computational system called IS-compare which functions in identifying the mobilization of conserved regions among broad ranges of bacterial species, it provided a sophisticated, straightforward approach for easier recognition.

Pathogenic bacteria such as Klebsiella pneumoniae resemble high health hazard and clinical relevance due to their associated health consequences. K. pneumonaie contributes

as a global priority pathogen and has been extensively affiliated with cases of severe hospitalizing infections which if not treated properly, patients succumbed to their disease (Rim, et al., 2024). The mentioned bacteria is abundant in diverse environmental habitants ranging from soil to hospital sewage water, resembling a critical health risk due to its wide prevalence. Moreover, K. pneumonaie is in a constant armor race of antibiotic resistance through developing and adopting novel mechanisms for evading antibiotics and optimizing its defense line for maximum survival and tolerance.

Due to its extensive development of antibiotic resistance and evolutionary adaptations, they resemble a reservoir for similarly-linked bacterial species such as E. coli. This was resembled in many cases such as (Savelieva et al., 2025), where they demonstrated how the frequency of conjugation occurring between the two was constant while conjugation between K. pneumonaie and other bacterial species was intolerable implying incompatibility. The historical genetic exchange occurring between K. pneumonaie and E. coli suggests a significant clinical hazard due to the wide incorporation of both bacterial species in broad environments, in addition to their pathogenicity and clinical relevance due to their impactful arsenal of virulence factors that introduce critical health consequences with high mortality rates. E. coli, one of the most abundant bacteria, found in diverse environmental settings ranging from food products, sewage-contaminated water sources, fecal wastes to hospital settings suggest a critical health risk due its broad incorporation. The harboring of adopted antibiotic resistance genes from different bacterial pathogens such as K. pneumoniae combined with its prevalence in accessible settings introduces a clinical health risk that must be allocated to avoid its consequences.

When the nearby flanking regions were annotated in GenBank, genes representing the MinCDE system were found from the left side, genes of this system usually function in regulating bacterial cell division. The presence of the aligned amplified region indicates its high conservation as it is found within essential genes without disrupting their regulatory functions. A ribonuclease D “rnd” gene was also found flanking the region which is involved in tRNA processing playing a role as a housekeeping gene which was supported by the findings of (Stasiewicz, et al,. 2021) where they demonstrated the insertion of two prophages within clusters of ribosomes.

As illustrated by (Zhang, et al., 2025), the introduction of such prophage insertion sites within tRNA precursors and ribosomal clusters is strongly affiliated with stress-response

gene expression as the inserted genes can be expressed upon environmental stress aiding in bacterial survival and adaptations. This further supports our findings that the aligned amplified region represents an insertion site of prophage genomes as the insertion of phages usually takes place within highly conserved regions with no introduction of disturbances occurring such as tRNAs, ribosomal clusters such as in the case of the MinCDE system or regulatory housekeeping genes which were observed in our case (Campbell A, 2003). Furthermore, within the 2kb spanning of the aligned region, genes such as ACFS06_10250 were found flanking the region. The latter gene functions within a gene cluster associated with the catabolic pathway of N-acetylglucosamine "GlcNAc" which portrays a significant component of the bacterial cell wall. The bacterial cell wall represents a primary line of defense for bacteria against exogenous bactericidal agents such as antibiotics or any chemical agents which further supports our findings of the significance of this insertion site, its high & unique conservation among different bacterial species.

This was previously illustrated in the findings of (Bednarek et al, 2022) where they demonstrated that GlcNAc portrays a crucial role in cell wall synthesis and certain modifications within this layer leads to reduced antibiotic attraction and permeability . The region being flanked with tRNA precursors, ribosomal clusters and other regulatory genes in addition to genes functioning in bacterial defense systems such as the cell wall suggests the significance of this insertion site and its affiliation with horizontal gene transfer occurring between the bacterial species eventually leading to acquisition of exogenous genes which further contributes to its survival and evolutionary adaptations. FadD gene was also found to be flanking the insertion region, FadD is known to be involved in b-oxidation of long-chain fatty acid chains which take part in synthesis of membrane lipids, changes in membrane compositions translates into alterations in permeability of molecules such antibiotics (Lu et al., 2023). In the latter study, lipid metabolism alterations revealed altered membrane permeability especially to antibiotics, this was illustrated in other studies such as (Dandan, et al., 2025) where lipid modifications reduced the negative charge of LPS in Klebsiella cell membrane making it less attracted to Colistin which is usually administered as a last resort antibiotic.

This illustrates the health hazard affiliated with horizontal gene transfer occurring in conserved insertion sites often flanked by such MDR-acquiring characteristics and survivability. Colistin resistance in Klebsiella acquired due to lipid membrane alterations

has been demonstrated worldwide, it has also been discovered in plasmid-transferred mobilised Colistin resistance genes “mcr-1” often found in Enterobacteriaeceae and especially E. coli (Yi-Yun, et al., 2016). The alignment of our findings with previously published articles especially in confirming the incorporation of LPS-related alterations leading to Colistin resistance in Klebsiella and plasmid-transferred Colistin resistance genes into E. coli indicates the significance of our study results and how the presence of such conserved insertion sites with the flanking MDR-associated genes within a broad range of bacterial species and especially Klebsiella and E. coli facilitates horizontal gene transfer leading to acquisition of MDR genes, increasing resistance of clinically pathogenic bacteria to last resort antibiotics which further aggravates antibiotic resistance health risks.

When investigating the presence and incorporation of Integrons and more precisely Class I Integrons due to their significant affiliation with MDR-related genetic features, we observed the depicted alignment in figure 14 above. This alignment with the clinically relevant Acinetobacter lwoffi which was common among three different phage samples incubated with diverse bacterial species (E. coli, Pseudomonas & Klebsiella) raises critical significance as the alignment was between 240bp which suggests no coincedence. As A. lwoffi is a clinically relevant gram-negative bacteria that has been previously investigated and proven to be associated with pathogenic consequences due to their evolutionary adaptations and eventual health risks (Elizabeth, et al., 2024). This confirms the presence of a Class I Integrons among our phage samples suggesting the occurrence of horizontal gene transfer among diverse bacterial species which could be reasoned by the acquisition of this Integron by a shared source or transferred to one another via either of the various molecular transfer mechanisms.

Two of the phages samples showcasing the alignment were retrieved from a sewage-water source while the third one was taken from an oil-mill by-product water waste, this demonstrates the abundance and wide incorporation of those phages in diverse environmental settings in the investigated areas and the clinically hazard potential they might harbor if not addressed properly. Moreover, this illustrates the critical role of such phages which partake their role as mediators of MDR genes and other clinically related genetic material across different bacteria eventually contributing to the reservoir of MDR genes.

For further investigations of the amplified region, we went on to discover the genes spanning this region and came upon intriguing findings. For instance, several regulatory proteins such as “PepSY-associated TM Helix Protein” were found, this protein functions in protecting the cell from self-degradation by inhibiting peptidases, it is often activated as a response to stress stimulators. The presence of such a significant protein that is stimulated in stress conditions facilitating bacterial survival and protection suggests its possible co-mobilization with other resistance genes due to its spanning of the amplified Class I Integron region. A TPR domain was also found which often functions as a mediator of protein-protein interactions, thus, altering enzymatic activity or complex assembly such as in the case of functional clusters. Moreover, TPR domains are also activated upon presence of stress stimuli further suggesting the association of the amplified Class I Integron with mobilization of resistance genes.

Furthermore, key regulatory proteins of the methylation reactions were found such as Adenosylhomocysteinase, these critical proteins resemble significance in bacterial physiology as their activity plays a role in bacterial cells homeostasis when exposed to external stress. Furthermore, inhibitors of the mentioned enzyme have been implemented as anti-microbials & anti-virals ascribed to the enzyme’s critical metabolic role and the eventual consequences. Furthermore, Class I Integrons are not restricted to only harboring MDR genes, however, they can often carry essential metabolic genes which aid in the survival and resistance of the bacteria, especially in the presence of stress-stimuli. This was showcased in the work of (Hosam, et al., 2014) as they managed to validate the packagaing of essential functional genes in addition to the already packaged MDR genes within the Integron due to their neccessity in such heavily stressed environments.

Our findings also aligned with further publication such as the one published by (Zhang, et al., 2015) where they demonstrated the presence of essential genes transferred to foreign bacterial species in a big genomic island that was inserted as exogenous genetic material to acquire the recepient bacteria advantageous features. A. lwoffi has been extensively researched due to it being characterized as prominent antibiotic-resistant species where it exhibited no susceptibility to a wide range of antibiotics eventually being labelled as MDR resistant with emerging development of resistance towards colisin which is a last resort antibiotic (Uwingabiye, et al., 2016).

Furthermore, a study conducted on Acinetobacter species retrieved from clinical environments in Iran revealed its pathogenic induction potential due to its wide range resistance in addition to its molecular pattern of harboring Class I Integrons that mediate their transfer. The researchers of this study revealed that even last resort antibiotics didn't exhibit 100% success in eliminating all Acinetobacter species, moreover, Class I Integrons represented a 50% prevalence among the bacterial samples illustrating its role as a facilitator (Japni, et al., 2011). Therefore, when observing the significant clinical relevance of Acinetobacter species and its tendency of harboring Class I Integrons that mediate MDR genes transfer, we can reach a consensus that the presence of an alignment of around 240bp of our phage samples with A. lwoffi suggests potential hazardous health risks given that the samples were retrieved from different environments "sewage-water & oil mill". The latter represents the wide distribution of antibiotic resistance emergence in Palestine as phage samples were taken from two far-distanced locations "Al-Tireh & Aroura village".

Figure 12.4 represents the 40bp shared alignment that was found between our phage sample #26 of the unmatched R2 IS reads with a previously published E. coli plasmid "pV139-a". Even though the alignment was somehow short, it is still considered a significant finding or a hint that we can later build on to fully unravel the molecular basis lying underneath. Furthermore, the insertion of plasmids occurs at either IS which facilitate the adoption of exogenous molecular constitutes or Integron regions harboring MDR genes acquiring the recipient bacteria with increased resistance and survival. Nevertheless, a follow-up sequence annotation and comparative mapping are required for conclusive validations.

When investigating the molecular relevance of the aligned counterpart "pV139-a", no full annotation or genetic record was available online, however, the familial history and characteristics suggest strong clinical relevance. For instance, other plasmids from similar E. coli strains have been extensively documented and annotated for their affiliation in pathogenic activity and acquisition of MDR genes in addition to IS and Integron insertion regions. Moreover, species-related plasmids such as pEK499 & pUMNK88 have been well documented to be significant contributors to the spread of antibiotic resistance. The pEK499 strain was revealed to harbor genes that confer resistance to eight different antibiotic classes, the study also confirmed the presence of insertion sequences, lastly, a

Class I Integron was also found harboring genes that exhibit resistance to multiple classes of antibiotics (Woodford, et al., 2009).

Furthermore, another study illustrated the presence of antibiotic-resistance genes found within the pUMNK88 plasmid in addition to a Class I Integron involved the horizontal genetic transfer of these MDR genes (Castillo, et al., 2013). The pV139-a plasmid itself is also known to be a contributor to antibiotic resistance as it was documented to possess gene conferring resistance to several antibiotics classes, primarily b-lactam (Anthony, et al., 2020). Therefore, the previous studies associate the importance of our alignment findings and the significant molecular & clinical relevance it harbors.

Conclusion and recommendations

The findings of this research represent the first step towards allocating the significant health concerns of the spread of antibiotic resistance and the underlying facilitating mediators, bacteriophages and their utilization of Integrons and insertion sites. The primary aim of this study was confidently fulfilled, as we managed to conduct a successful amplification of the Class I Integrons found within these phages in addition to the insertion sites and eventually demonstrate their affiliation with the transfer of MDR genes and shared genomic content with pathogenic bacteria such as Klebsiella pneumoniae & Acinetobacter lwoffi. Furthermore, this unravels the imposed health consequences underlying the prevalence of these phages embedded in our environments that are capable of integrating into diverse bacterial genomes and mediate genetic transfer. Nevertheless, we shall address certain limitations that restricted a thorough investigation as conducting a whole genome sequencing “WGS” analysis would have unravelled the incorporation of the different MDR genes and molecular constituents involved in this integration process. Lastly, we sincerely hope that our work and findings encourage other aspiring researchers to further investigate this important topic of phage-mediated antibiotic resistance due to the corressponding health consequences affiliated with it.

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الكشف عن الإنتغرونات من الصنف الأول ومواقع الإدماج في جينوم العاثيات البكتيرية وتسليط الضوء على المادة الوراثية المشتركة مع البكتيريا الممرضة متعددة المقاومة Klebsiella pneumoniae و Acinetobacter

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ملخص

منذ ظهور مقاومة المضادات الحيوية، أصبح هذا التحدي يمثل عقبة جوهرية في الإدارة السريرية ومعالجة العدوى البكتيرية. تشير السجلات الصحية لعام 2021 إلى تسجيل ما يقارب 4.71 مليون حالة وفاة مرتبطة بعواقب مقاومة الميكروبات، حيث يعزى نحو 25% منها مباشرة إلى البكتيريا الممرضة المقاومة. وتُظهر التقديرات المستندة إلى البيانات أن عدد الوفيات السنوي قد يرتفع ليصل إلى 8.2 مليون خلال الفترة ما بين 2025 و2050. تعكس هذه الإحصاءات السريرية المقلقة الحاجة الملحة إلى تكثيف الجهود البحثية المستمرة لتطوير استراتيجيات فعّالة للحد من هذه الظاهرة وآثارها الصحية. انطلاقاً من ذلك، نقدم في هذا البحث دراسة مبتكرة مستوحاة من الأدبيات العلمية العالمية، تم تطبيقها محلياً في فلسطين لسد فجوة بحثية لم يتم تناولها سابقاً.

يهدف هذا البحث إلى استقصاء الدور الحيوي للعاثيات البكتيرية (البكتيريوفاج) في نقل المادة الوراثية المرتبطة بمقاومة المضادات الحيوية بين الأنواع البكتيرية المختلفة. كما ركزنا على تحديد الكيانات الجزيئية والعناصر المشاركة في هذه العملية التكاملية، بما في ذلك الإنتغرونات(Integrons) ، ومواقع الإدماج الخاصة، والجينات المسؤولة عن اكتساب المقاومة.

ولتحقيق هذا الهدف، جُمعت عينات من بيئات ذات صلة سريرية تُعد خزانات لهذه العاثيات، حيث تم الحصول على عينات من محطة معالجة مياه الصرف الصحي في حي الطيرة بمدينة رام الله، وأخرى من معصرة زيتون في قرية عارورة من المخلفات السائلة الناتجة عن المعصرة. خضعت العينات لعمليات مخبرية دقيقة لاستخلاص المادة الوراثية(DNA) ، مع تصميم مجموعتين محددتين من البادئات (Primers) لضمان دقة الاستقصاء. استُخدمت المجموعة الأولى المكونة من البادئين intCiF3a وintCiiiR3a لتضخيم الإنتغرونات من الصنف الأول (Class I Integrons)

المعروفة باحتضانها لجينات متعددة المقاومة، بينما استُخدمت المجموعة الثانية لتضخيم مواقع الإدماج الخاصة، عبر البادئين IS-F و.IS-R

أظهرت النتائج نجاحنا في تأكيد وجود الإنتغرونات من الصنف الأول ومواقع الإدماج في عينات العاثيات. علاوة على ذلك، قمنا بمواءمة هذه النتائج مع جينومات مشروحة مسبقاً، حيث كشف التحليل عن تطابق كامل بنسبة 100% لموقع إدماج بطول 200 زوج قاعدي مع بكتيريا Klebsiella pneumoniaeالمتعددة المقاومة، بالإضافة إلى تطابق لتسلسل بطول 240 زوج قاعدي يمثل إنتغروناً من الصنف الأول مع بكتيريا Acinetobacter الحاملة لصفات مقاومة للمضادات الحيوية. كما تم الكشف عن تطابق بطول 40 زوجاً قاعدياً مع بلازميد E. coli المعروف باسم."pV139-a"

تؤكد هذه النتائج الدور المحوري للإنتغرونات من الصنف الأول، ومواقع الإدماج، والعناصر المساعدة على الحركة في عملية نقل الجينات متعددة المقاومة، وما يترتب على ذلك من تداعيات صحية بالغة الخطورة