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CHARACTERIZATION AND GENOMIC ANALYSIS OF TWO ESCHERICHIA COLI O157:H7 BACTERIOPHAGES ISOLATED FROM PIGEONS

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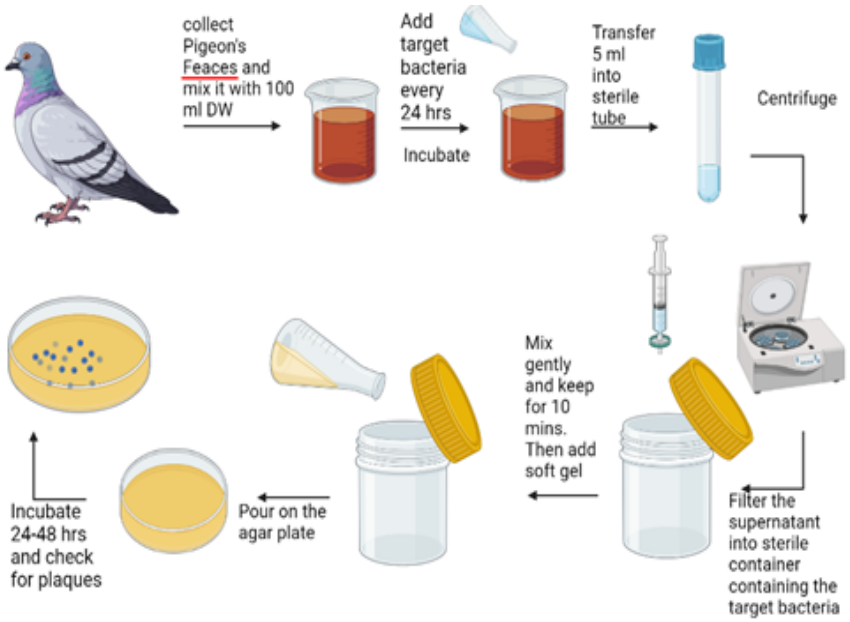

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This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Cellular and Molecular Biology

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Cover: Image related to Isolation of new bacteriophages from pigeons

(Photo: By Mohamad I Mohamad A Abdul Rahman Sultan Alolama)

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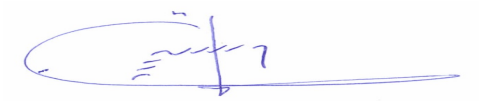
Declaration of Original Work

I, Mohamad I Mohamad A Abdul Rahman Sultan Alolama, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Characterization and Genomic Analysis* of *Two Escherichia Coli O157:H7 Bacteriophages Isolated from Pigeon*", hereby, solemnly declare that this is the original research work done by me under the supervision of Prof. Khaled A. El-Tarabily, in the College of Science at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this dissertation.

Student's Signature: 

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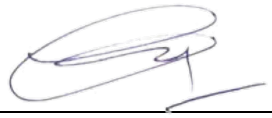
Signature 

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Abstract

Enterohemorrhagic *Escherichia coli*, also known as EHEC, is a subset of Shiga toxin-producing *E. coli* (STEC), and it has recently been identified as one of the principal foodborne pathogens. *E. coli* O157:H7 is the most important serotype of STEC for its role in causing foodborne illnesses. *E. coli* O157:H7 could cause various gastroenteritis symptoms such as diarrhea, hemolytic uremic syndrome, hemorrhagic colitis, and thrombotic thrombocytopenic purpura and may cause death. Elimination of *E. coli* O157:H7 during food processing and storage is a possible solution. Bacteriophages have a significant impact on bacterial populations in nature due to their ability to lyse their bacterial host. All *E. coli* O157:H7 phages that have been previously announced were found in ruminants or swine. Here, I studied the characterization and genomic analysis of two lytic *E. coli* O157:H7 bacteriophages isolated from feaces of wild pigeon; UAE MI-01 and Ec_MI-02. This is the first time to report *E. coli* O157:H7 phages from birds feaces. UAE_MI-01 belonged to the family Siphoviridae in order Caudovirales. UAE_MI-01 had a with a burst size of almost 100 plaque-forming units (PFU) per host cell after a latent period of 20 min and an adsorption rate constant (K) of 1.25×10^{-7} mL/min. UAE_MI-01 was found stable at a wide range of temperature, pH and some of the common laboratory disinfectants. The 44,281 bp-long genomes of the phage had an average GC content of 54.7%. On the other hand, Ec_MI-02 belonged to genus Tequatrovirus in the order Caudovirales. The K value of Ec_MI-02 was found to be 1.55×10^{-8} mL/min. The latent period was almost 50 min with burst size of almost 10 PFU /host cell. Ec_MI-02 was stable at a wide range of pH, and temperature and was resistant to ethanol 70%. Its genome was made of 266 genes with total 165454 bp and average GC content of 35.5%. It is worthy of note that wherever the phage is present, the host cell must be present. Thus, if the bacteriophage of *E. coli* O157:H7 is present in the feaces of a wild bird, then the *E. coli* O157:H7, is most probably present in wild birds. The current study provides additional evidence that wild birds could also be a good natural reservoir for bacteriophages and could be good candidates for phage therapy. Studying the genetic makeup of bacteriophages that infect human pathogens is crucial for ensuring their safe usage in the food industry.

Keywords: Bacteriophage, Bio-Preservative, Characterization, E. coli O157:H7, Lytic Phages, Phage Genome, Phage Therapy, Multiplicity of Infection.

Title and Abstract (in Arabic)

الخصائص الفيروسية والتحليل لإثنين من اللاقمات البكتيرية للإيشيريشية القولونية ذات النمط المصلي O157:H7 و المعزولة من الحمام

الملخص

تعتبر البكتيريا الإيشريشية القولونية ذات النمط المصلي "O157:H7" والمنتجة لسم الشيجا الزحارية هي المسؤولة عن تفشي معظم التسممات الغذائية عالميا والتي قد تؤدي إلى الوفاة. اللاقمات البكتيرية تعتبر الأعداء الطبيعيين للبكتيريا. جميع اللاقمات البكتيرية للعائل البكتيري الإيشريشية القولونية ذات النمط المصلي "O157:H7" المصرح عنها سابقا تم عزلها من المجترات والخنازير. هنا قمنا بدراسة الخصائص الفيروسية والتحليل الجيني لإثنين من اللاقمات البكتيرية المعزولة من براز الحمام الزاجل البري من عش واحد فقط و هما:UAE_MI-01 و Ec_MI-02. على حد علمنا، هذه هي المرة الأولى التي يتم فيها الإعلان عن لاقم بكتيري للإيشريشية القولونية ذات النمط المصلي "O157:H7" من ذرق الطيور. UAE_MI-01 كان ينتمى ينتمي لعائلة الفيروسات السيفاوية من رتبة الفيروسات الذنبية. و حتى يستنسخ الفيوس كان يحتاج الى فترة كمون تصل الى 20 دقيقة و تنتج حوالي 100 نسخة في كل خلية من خلايا العائل. و تشير نتائج هذا البحث إلى أن هذا اللاقم البكتيري مقاوم لمدى واسع من درجات الحرارة، معدل الحموضة والأس الهيدروجيني و بعض المعقمات الشائعة الإستخدام في المختبرات. دراسة التسلسل الجيني لهذا اللاقم البكتيري وضح أن مادته الوراثية تتكون من 44281 زوج قاعدي و كان معدل محتوى الغوانين السايتوسين (GC) 54.7%. أما اللاقم الثانى Ec_MI-02 فقد كان ينتمي إلى عائلة الفيروسات السيفاوية من رتبة الفيروسات الذنبية. إن دراسة دورة حياة اللاقم البكتيري Ec_MI-02 بينت أنه يستنسخ خلال فترة كمون تتراوح حتى 50 دقيقة و تنتج 10 نسخ في كل خلية من خلايا العائل. كما كشفت هذه الدراسة أن Ec_MI-02 يتميز بخصائص أهمها أنه مقاوم لمدى واسع من معدل الحموضة والأس الهيدروجيني ودرجات الحرارة. الجدير بالذكر أن Ec_MI-02 مقاوم للإيثانول 70%. ويحتوي على 266 جين ضمن مادته الوراثية المكونة من 165454 زوج قاعدي كما أن معدل محتوى الغوانين السايتوسين (GC) كان 35.5%. و يجدر بنا أن نتذكر أنه أينما وُجد اللاقم البكتيري فإن العائل البكتيري يفترض أن يكون موجودا. لذلك فإن وجود اللاقم البكتيري الخاص بالإيشيريشية القولونية "O157:H7" في براز الطيور البرية يدل غالبا على وجود الإيشيريشية القولونية "O157:H7" في تلك الطيور. من هذا المنطلق، من المهم جدا أن تفحص الطيور وخاصة الدواجن لمعرفة ما إذا كانت مصابة أو حاملة للإيشيريشية القولونية "O157:H7" ولاقمها البكتيري.

مفاهيم البحث الرئيسية: اللاقم البكتيري، العلاج باللاقمات البكتيرية، التحليل الجيني، الإيشريشية القولونية ذات النمط المصلي "O157:H7"، تعددية العدوى، الخصائص الفيروسية، اللاقمات البكتيرية المحللة لعائلها، المواد الحافظة الحيوية.

Author's Contribution

The contribution of Mohamad Alolama to the dissertation was as follows:

I. Actively participated in planning of the work.
II. Conducted extensive experimental research, collected relevant data in addition to analysis and interpretation of data in the dissertation.
III. Applied appropriate statistical method and qualitative analysis techniques to support accuracy of the results.
IV. Used the most recent bioinformatic tools to perform the genomic analysis.
V. Wrote and edited the dissertation and the scientific papers that were published.

Acknowledgements

I would like to thank my committee for their guidance, support, and assistance throughout my preparation of this dissertation.

Dedication

To my beloved parents and family

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List of Abbreviations

A/E	Attaching-Effacing
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BLASTn	Basic Local Alignment Search Tool (Nucleotide)
BLASTp	Basic Local Alignment Search Tool (Protein)
bp	Base Pair
CFU	Colony Forming Unit
CPA	Crossing Priming Amplification
CRISPR-Cas	Clustered Regularly Interspaced Short Palindromic Repeats and Its Associated Protein
DAEC	Diffusely Adherent *E. coli*
DNA	Deoxyribonucleic Acid
EAEC	Enteroaggregative *E. coli*
Ec_MI-02	2nd phage of *E. coli* O157:H7 Isolated from a Bird
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-Linked Immunosorbent Assay
EPEC	Enteropathogenic *E. coli*
ESBL	Extended-Spectrum Beta Lactamase
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal Pathogenic *E. coli*
g^{-1}	Per Gram
HCR	Hybridization Chain Reaction
I.M.	Interamuscular
IPEC	Intestinal Pathogenic *E. coli*
I.V.	Intravenous
K	The Adsorption Rate Constant
KPC	*Klebsiella pneumoniae* Carbapenemase-producing
LAMP	Loop-mediated Isothermal Amplification

LBA	Lauria Birtany Agar
LBB	Lauria Birtany Broth
LPS	lipopolysaccharide
LSD	Least Significant Difference
MHA	Mueller Hinton Agar
min^{-1}	Per minute
mL	Milliliter
mL^{-1}	Per Milliliter
NA	Nutrient Agar
NCTC	National Collection of Type Cultures
NGS	Next Generation Sequencing
nm	Nanometer
ORF	Open Reading Frame
PAS	Phage-Antibiotic Synergy
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
pH	Potential Hydrogen; A Measure of How Acidic/Basic Water Is.
PMA-dPCR	Propidium MonoAzido digital PCR
PMQR	Plasmid-Mediated Quinolone Resistance
QDs	Quantum Dots
qPCR	Quantitative Polymerase Chain Reactor
RCA	Rolling Circle Amplification
RAA	Recombinase-Aided Amplification
RAST	Rapid Annotation using Subsystem Technology
RI	Refractive Index
RNA	Ribonucleic Acid
SBA	SoyaBean Casein Digest Agar
SPR	Surface Plasma Resonance
TEM	Transmission Electron Microscopy
TerL	Terminase Large Subunit
TerS	Terminase Small Subunit

TLR4	Toll-like Receptor 4
UAE_MI-01	1st Phage of *E. coli* O157:H7 isolated from a Bird
µg	Microgram
µL	Microliter
WHO	World Health Organization

Chapter 1: Introduction

1.1 Overview

The battle between human and bacterial infection is well documented in all ancient cultures; Egypt, Greece and China (Sengupta et al., 2013; Ventola, 2015). *Escherichia coli* O157:H7 is a well-known foodborne pathogen that poses a serious threat to public health and economic property worldwide (Bai et al., 2022). However, antibiotics were discovered in 1928 by Alexander Fleming (Sengupta et al., 2013) and were first used as therapeutic agents to treat infectious diseases in 1940s (Ventola, 2015). Unfortunately, very soon, bacteria developed resistance to the antibiotics and antibiotic resistance was reported rapidly from different countries. The first report was penicillin-resistant *Staphylococcus* in 1940s and continued until now where super multidrug resistant bacteria are isolated from patients worldwide (Andersen, 2019). Antibiotic resistance are mainly due to; overuse (Nature, 2013; Read & Woods, 2014), incorrect prescription (Luyt et al., 2014), uncontrolled agricultural use (Bartlett et al., 2013).

On the other hand, the use of bacteriophage as an antibacterial agent has started one century ago (Fernández et al., 2019). Bacteriophages are used to treat different infections including; gastro-intestinal infections (McCallin et al., 2013), otitis (Wright et al., 2009), urinary tract infections (Khawaldeh et al., 2011) wounds (Servick, 2016), osteoarticular infections (Albee, 1933). Bacteriophages are administrated to patients through all modes of administration; per oral, spray, inter muscular (I.M.) (Górski et al., 2006; Oliveira et al., 2009), rectal administration (Górski et al., 2006), intravenous (I.V.) (Schultz & Neva, 1965) and through inhalation (Hraiech et al., 2015).

In addition to phage therapy, bacteriophages have different health application, for example; food biocontrol which is the ideal solution for *E. coli* O157:H7 to eliminate it during the food process before reaching human (Sultan-Alolama et al., 2022). Since bacteria has the ability to develop resistance to their phage as they have developed resistance to antibiotics. Therefore, in order to minimize the possibility of developing bacterial resistance to bacteriophages and subsequently escaping the treatment, cocktail phages are used, for example; in case of gastro-intestinal infections a cocktail of 18 bacteriophages of *E. coli* are used (McCallin et al., 2013). Consequently, it is very

important to isolate new phages to enrich the phage cocktails used in phage therapy and bacterial biocontrol strategy.

1.2 Statement of the problem

Phages' host-specificity is considered a major advantage and at the same time a disadvantage being a limiting factor in the antibacterial property of phages in phage therapy and bacterial biocontrol. It is very important to state here clearly, that similar to antibiotic resistance, bacteria have the ability to develop resistance to their specific bacteriophages (Örmälä & Jalasvuori, 2013; Doss et al., 2017).

Bacteria have developed different mechanisms and strategies to resist their parasites, these strategies include: preventing phage entry with different means (Cumby et al., 2015; Simmons et al., 2018; Rostøl & Marraffini, 2019); targeting bacteriophage nucleic acids (Restriction-modification systems (Tock & Dryden, 2005), CRISPR-Cas systems (Hille et al., 2018), prokaryotic argonautes (Hegge et al., 2018); abortive infection and toxin-antitoxin systems (Durmaz & Klaenhammer, 2007; Harms et al., 2018); bacteriophage assembly interference (Penadés & Christie, 2015) in addition to an under investigation newly discovered anti-phage mechanisms (Rostøl & Marraffini, 2019). Thus, bacterial resistance to their phage is a major challenge for various phage applications.

E. coli O157:H7 being the most famous verotoxin-producing *E. coli* and causing serious health problems which may lead to death (Thomas et al., 2015), has attracted so much attention. Since bacteriophages are considered to be a safe and natural alternative to antibiotics, virologists have been exploring ruminants and swine for any lytic phage of *E. coli* O157:H7. The major aim of the current thesis was to search for lytic phage for *E. coli* O157:H7 in the United Arab Emirates (UAE) from birds.

1.3 Relevant literature

E. coli, the most studied and used in research bacterium as it revealed by number of publications about *E. coli* in PubMed central which exceeded 730322 publications by Jan. 25th. 2023. *E. coli* was discovered in 1885 by a German-Austrian pediatrician Dr. Theodor Escherich (1857–1911) (Escherich, 1988). Theodor Escherich named it

"*Bacterium coli commune"* and revealed many of its features like size of 1–5 μm in length and 0.3–0.4 μm in width and its ability to ferment.

It was only in 1919 when Castellani and Chalmers suggested to change the name to *Escherichia coli* as a tribute to the discoverer (Erjavec, 2019). *E. coli* belongs into the family of *Enterobacteriaceae*. It is a Gram-negative rod-shaped bacterium, mainly characterized as being nonmotile or motile by peritrichous flagella, non-sporulating, producing acid from glucose, chemoorganotrophic, oxidase negative, catalase positive, mesophilic and facultative anaerobic (Erjavec, 2019) and it is one of the first commensal bacteria that colonize human intestine after the birth. However, many of them have acquired particular virulence factors and accordingly can cause a wide spectrum of diseases and thus called "pathotype" (Kaper et al., 2004). Commensal *E. coli* establishes the anaerobic environment of the intestine to allow other anaerobic bacteria to colonize and grow (Houghteling & Walker, 2015). Commensal *E. coli* in return of food and shelter, help the host by production of vitamin K and colonization resistance, in which colonized *E. coli* prevent pathogenic bacteria (including pathogenic *E. coli*) from colonization in the intestine (Stecher & Hardt, 2011).

It was only in the early 1940s when strains of *E. coli* were recognized as the etiological agent of diarrhea (Adams et al., 1995). Pathogenic *E. coli* are mainly divided into two major groups: the intestinal pathogenic *E. coli* (IPEC) and the extraintestinal pathogenic *E. coli* (ExPEC). IPEC associated with infections of the gastrointestinal tract, whereas ExPEC associated with infections of extraintestinal sites in the body (Kaper et al., 2004). Urinary tract infections (UTIs) and sepsis/meningitis are the most common pathotypes of ExPEC. The intestinal pathogenic *E. coli* (IPEC) includes six categories; enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro & Kaper, 1998). Here, I will focus on some features of EHEC.

1.3.1 EHEC antigens

Generally, bacteria are differentiated based on their biotypes. Therefore, *E. coli* were differentiated according to their ability to ferment a range of carbohydrates. Unfortunately, this method was not a proper for distinguishing strains of epidemiologic

impact. Thus, immunogenicity of surface structures of the bacterial cells was carefully regarded as a more effective method as it depends on the combination of three principal surface antigens O:H:K (DebRoy et al., 2011).

Gram negative bacteria, have two major antigens, the O polysaccharide and flagellin, also known as the O and H antigens respectively (Wang et al., 2003). Many pathotypes of *E. coli* usually are clonal groups and characterized by shared O and H antigens (Kaper et al., 2004) in addition to K antigen (Ørskov & Ørskov, 1992; Johnson et al., 1994). Since majority of laboratories were not able to type the K antigen, serotyping based only on the O and H antigens, became the 'gold standard' for *E. coli* (DebRoy et al., 2011).

O antigens which are located on lipopolysaccharide (LPS) of the outer membrane of *E. coli*, are of different varieties and specific for each O-serogroup (Bos et al., 2007). Flagellar H antigens which are carried on "flagellin", a protein subunit of the flagellum (Wang et al., 2003), define wither serogroups (O antigen only) or serotypes (O and H antigens) (Kaper et al., 2004). K antigen, capsular polysaccharides (CPSs) are the major surface antigens in *E. coli* with more than 80 serologically distinct K-antigens being identified in *E. coli* (Yang et al., 2018). In this thesis I will focus on O and H antigens only.

Theoretically, Both O and H can be used as targets for polyvalent vaccines. However, their wide diversity is a huge challenge, except if some common epitopes will be identified.

1.3.2 Enterohemorrhagic E. coli (EHEC)

Enterohemorrhagic *E. coli* (EHEC), being an etiological agent of diarrhea with possible life-threatening complications made them a serious public health problem globally (Welinder-Olsson & Kaijser, 2005). verocytotoxins of *E. coli* was first discovered by Konowalchuk et al. (1977), then it was purified and characterized by O'Brien et al. (1983) who found it similar to the toxin of *Shigella dysenteriae* type 1. Cattle are the main source of EHEC infection in human. EHEC are normal flora of many animals with asymptomatic colonization (Nataro & Kaper, 1998). However, under-cooked meat or unpasteurized dairy products, vegetables and fruits in addition to infected person are reported to be carriers and sources of infection (Welinder-Olsson & Kaijser, 2005).

Enterohemorrhagic *E. coli* have one of the lowest infectious dose, 1-100 colony forming units CFU (Paton & Paton, 1998) and characterized by high ability to adhere to epithelial cells and colonize the human intestine (Welinder-Olsson & Kaijser, 2005).

Pathogenicity of EHEC depends on their ability to cause attaching-effacing (A/E) lesions in the intestine (Melton-Celsa et al., 2011). The lesions occur when the EHEC bacterium attaches tenderly to the epithelial cells of the host intestine leading to loss of microvilli. Adherence of EHEC to the epithelial cells in the host's gut is mediated by intimin (outer membrane protein) and fimbriae (thread-like complex extending from bacterial outer membrane to adhere to the host cell) (Welinder-Olsson & Kaijser, 2005). On the other hand, EHEC have wide range of virulent factors including toxins, myriad of adhesion and many putative colonizing factors (Welinder-Olsson & Kaijser, 2005). EHEC includes many virulent strains and O157:H7 is the most clinically important serotype due to its ability to cause hemorrhagic colitis and hemorrhagic uremic syndrome. In this study, I will focus on O157:H7 serotype.

1.3.3 E. coli O157:H7

E. coli O157:H7 was first linked to a gastroenteritis outbreak in 1982, leading to initiation of the enterohemorrhagic *E. coli* (EHEC) (Riley et al., 1983; Rahal et al., 2012). *E. coli* O157:H7 is characterized by containing hemolysin encoding plasmid which causes A/E lesion (Levine, 1987), producing Shiga toxin either both 1 and 2 or only one of them (Rahal et al., 2012), ferments lactose but unlike majority of *E. coli* strains cannot ferment sorbitol (March & Ratnam, 1986) and cannot grow well in temperatures between 44-45.5°C (Raghubeer & Matches, 1990).

E. coli O157:H7 spread worldwide with outbreak reports from more than 30 countries (Lazar, 2003). CDC-USA estimates that *E. coli* O157:H7 is responsible for 36% of 265,000 STEC infections leading to 3,600 hospitalizations and 30 deaths each year in the USA (CDC, 2022a). As previously mentioned, the main animal reservoir of *E. coli* O157:H7 is cattle. Since cattle most probably do not have receptors for Shiga toxins. However, *E. coli* O157:H7 was isolated from many different animals like; dogs (Kataoka et al., 2010), horses (Lengacher et al., 2010), sheep (Urdahl et al., 2003), deer (Díaz et al., 2011) and goat (Mersha et al., 2010).

1.3.4 Detection methods of E. coli O157:H7

E. coli O157:H7 has a great impact on the public health and economy globally. Therefore, like any other pathogen, early detection is extremely important. Since the classic gold standard method for bacterial detection; culturing and counting colonies demand many days, more sophisticated methods are needed. These new methods must save time and efforts while providing better quality results with higher sensitivity. Subsequently, recently, many new detection strategies were developed to provide sensitive, specific and rapid detection of pathogenic bacteria in general and *E. coli* O157:H7 in particular (Bai et al., 2022). These methods cab be classified as:

1.3.4.1 Optical detection

Analyzing and detecting compounds using their optical properties is known as optical detection. Optical detection mostly entails the following for the detection of *E. coli* O157:H7 in foods and water; fiber optics, surface plasma resonance, chemiluminescence, fluorescence, colorimetry, and light scattering detection. Due to its non-contact nature and high degree of precision, optical detection has recently attracted a lot of academic attention. High background interference has always been a significant obstacle to the detection procedure, though. Optical detection methods can be:

1- Fluorescence detection

Fluorescence detection is the process of analyzing substances qualitatively and quantitatively by transforming the target's signal into a fluorescent signal using certain *in vitro* or *in vivo* fluorescent labels. To detect *E. coli* O157:H7, the fluorescent labeling complex absorbs outside energy and emits fluorescence at a specific intensity correlated with the concentration of the target bacteria. Due to its great sensitivity, this method is frequently used to *identify E. coli* O157:H7, and various additional, creative detection methods have been suggested based on this detection strategy such as:

A. Nucleic acid amplification

Nucleic acid amplification-based diagnostic techniques have been used to detect foodborne pathogens since the introduction of molecular biology technology. This method includes quantitative polymerase chain reaction (qPCR) (Deshmukh et al., 2019),

propidium monoazido digital PCR (PMA-dPCR) (Pan et al., 2020) and the cheaper alternatives to the expensive and complex PCR like; recombinase-aided amplification (RAA) (Mu et al., 2021), recombinase polymerase amplification (RPA) (Azinheiro et al., 2022), rolling circle amplification (RCA) (Jiang et al., 2019), crossing priming amplification (CPA) (Xu et al., 2020), hybridization chain reaction (HCR) (Duan et al., 2021), and loop-mediated isothermal amplification (LAMP) (Cao et al., 2022).

B. Fluorescence labeling

To reflect the concentration of the target microorganisms, the fluorescence labeling technique uses the fluorescence characteristics of fluorescent compounds that physically adsorb or covalently bind to certain chemical groups. A crucial luminous substance utilized in the detection of *E. coli* O157:H7, the membrane permeable fluorescent dye enables bacteria to release fluorescence that is easily seen under a fluorescent microscope (Wu et al., 2016; Bai et al., 2022). For example; quantum dots (QDs) and more specifically metal QDs have been employed to create fluorescent sensors for the detection of *E. coli* O157H7 (Wu et al., 2020).

2- Colorimetric

Colorimetric detection is a technique that compares or measures the color depth of a solution containing a colored s.ce to ascertain the quantity of the substance being tested. Enzyme reactions or modifications to nanomaterials cause color changes that are utilized for the colorimetric detection of *E. coli* O157:H7 (Bai et al., 2022). Enzyme-linked immunosorbent assay (ELISA) is a famous example of colorimetric technique, nanoparticles, chitosan, cellulose nanocrystals and glycerol, were found to increase the ELISA's sensitivity for detection of *E. coli* O157:H7 (Baraketi et al., 2020).

3- Chemiluminescence

Chemiluminescence detection is a unique and sensitive quantitative detection method that employs a chemiluminescent agent to produce chemiluminescence in the presence of specific catalysts or oxidants to detect *E. coli* O157:H7 (He et al., 2017; Bai et al., 2022). Because it does not require an excitation light source, it avoids the effects of excitation light source scattering and background fluorescence signal and allows for wide-

linear range supersensitive detection of target microorganisms. Electrochemiluminescent reaction system based on the electron transfer between receptor and the electron donor in the electrode surface causes continuous and stable luminescence (Liu et al., 2018). Bioluminescence is another example of chemiluminescence that relies on the luciferin-luciferase bioluminescence assay to detect *E. coli* O157:H7 (Zhang et al., 2017).

4- Fiber-optic

The development of fiber optics and fiber optic communication technology led to the development of a novel technique known as fiber-optic detection. In this method, sensitive data is obtained by using light as a carrier and transmitted through fiber optics. The fundamentals of fiber-optical detection of *E. coli* O157:H7 or any microorganism based on the optical properties of the incident light, such as frequency, wavelength, intensity, and phase, will be altered because of the target bacteria adhering to the fiber surface. For the purpose of detection, the optical signal's alteration following modulation and demodulation is recorded (Bai et al., 2022). a U-bent polymer optical fiber (Rashid et al., 2020) and one-dimensional photonic crystal fiber (Hao et al., 2019) are 2 examples of fiber-optic detection method.

5- Surface plasmon resonance

The advanced biochemical detection method known as surface plasma resonance (SPR) detection is based on physical optics and allows for the measurement of the refractive index (RI) changes caused by molecular conjugation on the chip's surface, which are used to detect the binding process of numerous biomolecules (Bai et al., 2022). To detect *E. coli* serotype O157: H7, the chip surface's change in RI is linked to bacteria adsorbed on the metal. To monitor the binding process with *E. coli* O157:H7, the antibody is immobilized on the chip's metal film surface in the suspension. SPR signals are produced when the suspension's RI on the chip surface changes during the complex's formation or dissociation. By immobilizing various antibodies in channels, multiple bacteria can be detected simultaneously in multiple channels (Zhang et al., 2017).

6- Light scattering

When light travels through an inhomogeneous medium, the phenomenon known as light scattering occurs, in which some of the light departs from its original path. Due to the interaction with the analyte, light propagation alters the spatial distribution, polarization state, or frequency of light intensity. Using this guiding principle, a variety of light scattering techniques were investigated for the quantitative detection of *E. coli* O157:H7 (Bai et al., 2022). *E. coli* O157:H7 can be quantified using dynamic light scattering (DLS), which measures fluctuations in light intensity over time brought on by Brownian motion of particles. For example, DLS-based no-wash immunosensor which enhanced with gold nanoflowers was used for *E. coli* O157:H7 detection (Zhan et al., 2019).

1.3.4.2 Electrochemical

The correlational relationship between the target analytes' electrochemical properties and certain electrical parameters is the foundation for the qualitative and quantitative analysis method known as electrochemical detection. Due to its low cost, ease of integration, rapid detection rates, and suitability for on-site detection, electrochemical detection is frequently used to detect foodborne pathogens like *E. coli* O157:H7 (Bai et al., 2022). There are two ways to design an electrochemical detection:

1- Label-free electrochemical detection

Typically, the label-free electrochemical method involves modifying biorecognition elements and nanomaterials on the electrode surface to specifically adsorb *E. coli* O157:H7 or specific gene fragments and directly alter electrical parameters, allowing for quantitative detection of the target bacteria. To increase the detection sensitivity, electrochemical labels like enzymes, electroactive molecules, and nanomaterials were used to modify the electrode (Huang et al., 2016; Bai et al., 2022).

2- Label-dependent electrochemical

In order to improve, mediate, or even provide the measured electrical signal, the label-dependent electrochemical detection method typically relies on the labels of physicochemical or biological molecules on the target bacterial cells or target genes, such

as non-metallic nanoparticles, metallic nanoparticles (Bu et al., 2019), enzymes (Bu et al., 2019), and others (Wang et al., 2019).

1.3.5 Treatment options

Although the first clinical human HUS case occurred forty years ago in 1982, and the prevalence of HUS continues to rise, there is still no widely accepted or effective treatment for *E. coli* O157:H7-induced HUS (Mühlen & Dersch, 2020). Due to reports that antibiotics may increase the production of Shiga toxin, the use of antibiotics to treat the infection has long been controversial. Here we will explore the most promising treatment options for *E. coli* O157:H7 infections.

1.3.5.1 Antibody therapy

1- Shiga-toxin specific antibodies

Administration of Shiga-toxin (Stx)-specific antibodies soon after infection, can totally neutralize the cytotoxicity of the toxin *In Vitro* and shield animals from acquiring Stx-induced symptoms (Cheng et al., 2013). Rabbit serum, rich in Stx-antibodies, injection boosted animal survival by lowering the bacterial burden after 3-5 days but did not affect the initial colonization of mice with EHEC 86-24. To treat mice infected with *E. coli* O157:H7, preparations of bovine colostrum containing high titers of Stx1 and Stx2 antibodies were employed, which effectively prevented bacterial colonization (Funatogawa et al., 2002). But as Mice and piglets don't develop bloody diarrhea or HUS.

Results explaining the protective effect of Stx-specific antibodies
It cannot be readily transferrable to humans. Furthermore, the knowledge about the period between entrance of Stxs to the bloodstream and the time when the Concentrations of Stxs is high enough in blood and infected tissues are negligible (Mühlen & Dersch, 2020).

2- Eculizumab

Eculizumab is a recombinant HuMAb (monoclonal antibody encoding human heavy- and light-chain IgG genes which develops human antibodies in response to antigen) against the complementary component 5 (C5). Binding of the antibody at C5 inhibits the activation of the complements (Mühlen & Dersch, 2020). Eculizumab

was first tested in patients having severe STEC HUS at in an outbreak in Northern Europe in 2011, this trial revealed that Eculizumab has affected kidneys negatively (Buelli et al., 2019). However, positive effect of Eculizumab treatment in children and less severely infected patients was reported as well (Lapeyraque et al., 2011; Delmas et al., 2014).

1.3.5.2 Blocking Toxin Receptors

When the Stxs are released from the bacteria, they travel throughout the body and target cells (Lingwood et al., 1987) like renal glomerular cells that have the Gb3 receptor expressed on their cell surface as well as endothelial cells in the brain. The multivalent interaction of the five B-subunits with the trisaccharide moiety of Gb3 is what allows Stxs to bind to the Gb3 receptors. Therefore, a viable strategy to lessen Stx-mediated sickness involves interfering with receptor binding by utilizing receptor analogs to look for free toxin in the gut or the blood. A number of techniques have been used in the past few years to create either inhibitors of the Stx receptor Gb3 to stop binding and uptake of Stx by human cells and/or to neutralize Stx Gb3 analogs (MacConnachie & Todd, 2004; Serna IV & Boedeker, 2008; Rahal et al., 2012; Kavaliauskiene et al., 2017; Mühlen & Dersch, 2020). The following are examples of this treatment strategy:

1- Synsorb Pk

One of the earliest Stx receptor analogs was Synsorb Pk, which was tested in a significant multicenter trial involving 145 kids with STEC-induced HUS. Unfortunately, no therapeutic benefit was observed (Trachtman et al., 2003).

2- SUPER TWIG (1) and (2)

Carbosilane dendrimers with different numbers of terminal Gb3 moieties to block Shiga toxin while it is free in the bloodstream before it reaches target cells that express the Gb3 receptor. The SUPER TWIG Gb3 analogs attaches to Stx1 and Stx2 with high affinity to inhibit Stx absorption by host cells and promote macrophage phagocytosis of Stx. The SUPER TWIG Gb3 was able to protect mice from a lethal EHEC threat (Nishikawa et al., 2005).

3- Stx-Neutralizers developed by Phage-Display technology

The peptides; PC7-2, P12-26, and PC7-30 were generated using phage-display technology. They were found effective in binding to Stx and prevent. In addition, PC7-30 reduced Stx1-induced mortality in EHEC-infected rats, suggesting that this peptide may be helPFUl in preventing STEC-caused illnesses like HUS (Bernedo-Navarro et al., 2014).

4- Intracellular intervention

Stx can be targeted intracellularly before being released out of the bacterial cell. There are substances that can diffuse into the bacterial cell and aggregate in acidic compartments like chloroquine which can translocate StxA subunit into the cytosol and prevent stx-caused cytotoxicity (Dyve Lingelem et al., 2012; Kavaliauskiene et al., 2017).

1.3.5.3 Vaccines

Being one of the biggest advancements in public health during the past century, vaccines are thought to save between 2 and 3 million lives annually (Delany et al., 2014). Here I will discuss some of the promising approaches to develop vaccines for EHEC bacteria:

1- Toxin-based

Stx being the main virulence factor in STEC-mediated disease pathology, is a logic target for vaccine scheme (Mühlen & Dersch, 2020). Knowing the structure of the active site of Stx, made it easy to develop inactive forms of Stx. Various studies demonstrated that vaccinating animals with pure inactive Stx forms induced development of the neutralizing antibodies and protected animals (Kerner et al., 2015; Schmidt et al., 2018). Mice that received a hybrid toxin made of an inactive Stx2 A-subunit fused to the native Stx1 B-subunit were protected from the challenging subsequent of administrating either Stx1 or Stx2, or both toxins (Smith et al., 2006).

2- Bacterial cell-based vaccine

Non-pathogenic microorganisms or bacterial vaccine strains are used in numerous immunization strategies as delivery systems and to boost immunogenicity. Here I list two examples of vaccination approaches utilizing bacterial cell features:

a- Attenuated bacteria

By deleting the stx and the gene encoding the transcriptional regulator of the LEE (ler), a non-pathogenic version of the EHEC O157:H7 86-24 strain was produced.

In comparison to EHEC EDL933, these deletions fully eliminated cytotoxicity in vitro. This strain's descendant, which expresses Stx1 and Stx2 inactive versions from a plasmid, similarly displayed much reduced cytotoxicity in vitro. *E. coli* O157:H7 was less likely to colonize mice following infection when either the Stx1/Stx2-expressing strain or the stx/ler deletion mutant were injected. Additionally, mice that received an immunization during pregnancy transferred the immunity to their fetuses, who were shielded from *E. coli* O157:H7 infection (Liu et al., 2009).

b- Bacterial ghost

When bacteria are treated with viral E protein, bacterial ghosts (BGs) are left behind. This protein creates tubes across the cell's membrane, allowing the bacteria to expel their cytoplasm outside of the cell. Bacterial ghosts are all that is left, vacant membranes that nonetheless have the shape and cell surface features of bacteria. Bacterial ghosts are therefore extremely immunogenic. Bacterial ghosts can also be made from any desired strain including *E. coli* O157:H7 (Mühlen & Dersch, 2020). Mice that had received an oral vaccine, bacterial ghost, were protected from an EHEC strain challenge, since the immunization caused a particular immune response. When an oral booster vaccination was given on day 28 after an oral immunization, antibody production rose, improving survival even further (Mayr et al., 2005).

1.3.5.4 Antibacterial agents

A substance that kills or eliminates the growth of microorganisms including bacteria, fungus, and algae is referred to as an antimicrobial agent, these agents could be natural or synthetic (Burnett-Boothroyd & McCarthy, 2011). Antimicrobial agents that are used to treat *E. coli* O157:H7 can be divided into four main categories:

1- Antibiotics

The efficacy of antibiotics in *E. coli* O157:H7 infections is really controversial and conflicting. Early research looking at how antibiotics affect the treatment of STEC infections revealed that antibiotics trigger and increase the synthesis and release of Shiga toxin (Kimmitt et al., 2000) and subsequently increase the risk of developing HUS (Kakoullis et al., 2019). In contrast, others found ansamycins, chloramphenicol (Mühlen et al., 2020) and rifamycins (Fadlallah et al., 2015) effective. while some studies found that factors like: antibiotic class, antibiotic concentration, individual STEC strain, and Stx subtype all have a significant impact on how antibiotics affect stx expression (Walterspiel et al., 1992; Kimmitt et al., 2000; McGannon et al., 2010; Bielaszewska et al., 2012; Nassar et al., 2013) which explain the difference in the efficacy of antibiotics in treatment of STEC including *E. coli* O157:H7.

2- Probiotics

Recent research showed that the microbiota's composition, in the gut, influences how effectively intestinal pathogens colonize the various gut sections (Litvak et al., 2019). Probiotics' protective and advantageous properties have been described in several studies where they were used before an STEC infection *In Vitro* and in mice (Rund et al., 2013; Cordonnier et al., 2017; Giordano et al., 2019). Regardless of the positive effects that have been seen in animals, extrapolating the data to humans is very challenging, given that the probiotics' beneficial ratio to the pathogens ranged from 1:1 to $1:10^5$ CFU. Moreover, it's still not clear when and how to administer which type or strain of probiotics (Mühlen & Dersch, 2020).

3- Natural products

Various natural products were screened for their antibacterial activity against *E. coli* O157:H7, extracts of *Prosopsis alba* and *Ziziphus mistol* (Pellarin et al., 2013), green tea (Isogai et al., 1998), Ellagitannin extracted from *Quercus infectoria* (Voravuthikunchai et al., 2012), in addition to linoleic acid and lactic acid produces by bacteria (Pittman et al., 2012) had a positive outcome when tested on STEC-infected cells and animals.

4- Bacteriophages

A novel emerging potential strategy to prevent, control and treat bacterial infections. In this thesis I will discuss bacteriophage in detail.

1.3.6 Bacteriophage

1.3.6.1 Discovery

"Who discovered Bacteriophage?" Donna H. Duckworth in 1976 decided to investigate the illusions around Félix d'Herelle being honest in claiming independent discovery of bacteriophage (Duckworth, 1976). Since, Gunther Stent in his book "The Molecular Biology of Bacterial Viruses" says: "Gratia drew attention to Twort's forgotten or, rather, never noted" pointing to bacteriophage discovery (Stent, 1963). Stent also said somewhere else "Bacterial viruses were discovered in 1915 by the English microbiologist F. W. Twort, and two years later-perhaps independently, perhaps not-by the French-Canadian F. d'Herelle" (Stent, 1966). Duckworth concluded that as long as Frederick Twort's paper was not recognized before 1921 and there is no proof that d'Herelle had read it in 1971. Therefore, if Twort discovered bacteriophage in 1915, d'Herelle discovered it independently in 1917 or may be in 1910 and called it bacteriophage (bacteria eater) (Duckworth, 1976).

1.3.6.2 Biology and life cycle

Phages compose the biggest share of existing viruses which are believed to be the most abandoned creature in the planet (Fogg et al., 2014). Hendrix *et al*. in 1999 estimated that Bacteriophages are the most abundant creature on the earth, with population as high as 10^{31} particles (Hendrix et al., 1999). Mushegian, questioned the Hendrix's estimation of bacteriophages' population on the planet and decided to review the most recent statistics of bacterial and viruses' populations on earth. Mushegian, by the end of his study, concluded that Hendrix's estimate remains very close to best estimate of numbers of viral particles in our planet (Mushegian, 2020).

Bacteriophages are divided into three categories based on their life cycle; lytic cycle and lysogenic cycle and chronic release. In lytic cycle, phages adhere to particular receptor located on the surface of their bacterial host cell, which leads to an irreversible attachment.

The host range of a bacteriophage is largely determined by its capacity to recognize and bind to specific receptor which are present on the cell surface of specific bacterial strain, species or genus. Enzymatic tail degradation during cell wall penetration induces the phage's DNA insertion into the host's cytoplasm. After the phage genome enters the host cell, specialized enzymes encoded by the phage genome are produced to direct the host cell's DNA and protein synthesis toward the production of additional phage particles which will be assembled after the phage heads are filled with the freshly duplicated phage genomes. Then phage-encoded holins, which create pores in the cell membrane at a specific point of the phage lytic cycle, give phage-encoded peptidoglycan hydrolases (lysins) access to the peptidoglycan. This will destroy the bacterial cell to release of progeny phage which will infect nearby susceptible bacteria (Endersen et al., 2014).

Where the lysogenic cycle, starts once the phage genome entered the host cell and escaped the host resistance strategies, and decided to integrate in the host's genome. This decision depends on many factors, for example, genetic compatibility, host fitness and superinfections (Casjens & Hendrix, 2015; Howard-Varona et al., 2017), temperature, salinity, UV and environmental pollutants (Zhang et al., 2022). The integration can be random or on specific site, (attB) of both bacteria and phage (Fogg et al., 2014).

Prophage, in deed, has the ability to stay in the host cell in a free, plasmid-like state. In both forms, integrated to the host genome or free plasmid-like state, the prophage reproduce in harmony with host's DNA (Salmond & Fineran, 2015) without producing virions (Howard-Varona et al., 2017). Phage therapy and most applications of bacteriophage are interested in lytic phages which called virulent phages as well. However, lysogenic phages have great impacts on the cellular and ecosystem levels (Howard-Varona et al., 2017).

In chronic release, as in lytic cycle, escaping the host cell after assembly of the phage particles remain the main challenge. Unlike the majority of phages which achieve this by biologically terminating both the phage infection and the phage-infected bacteria by eroding the cell wall to the point where it fails. In addition to partially solubilizing the bacterium leading to formation of big pores in the host bacteria's cell membrane through which phage virions can scape to the surrounding environment. In the case of cell-wall-less bacteria, phages can release through budding as it happens with Acholeplasma phage

L2. However, filamentous phages, members of phage family *Inoviridae*, escape across intact cell-wall-containing cell envelopes and considered to be more common form of chronic release (Dennehy & Abedon, 2021).

1.3.6.3 Phages collective decision making

Similar to quorum sensing system in bacteria, in which bacteria has the ability to act collectively as if they are multi-cellular when taking decisions to perform group behaviors such as biofilm formation, production of virulence factor and light (Bioluminescence) (Mukherjee & Bassler, 2019). Bacteriophage also are able to make group decision. Erez et. al. (2017) when working on Bacillus, found that the phage creates a small molecule-communication peptide (made of six amino acids), that is released into the media during the early stage of infection of its Bacillus host cell. when the level of this peptide is high enough, phages will lysogenize. Furthermore, they discovered that several phages encode distinct variants of the communication peptide, indicating the existence of a peptide communication code for lysogeny decisions that is unique to each phage. The communication system was called the "arbitrium" system and it is encoded by three genes; *aimP, aimR and aimX* (Erez et al., 2017).

1.3.6.4 Bacteriophages application

Phage applications have become an important research option as a result of the recent increase of bacterial resistance to antibiotics. Phage particles are becoming widely used in a variety of biotechnological applications, including the treatment of harmful microorganisms and many other applied research areas. However, there are minimal issues with the use of phage-based remedies. Here, I will explore some of the most important applications of bacteriophages in food and food safety.

A. Phages application in food safety

1- Pre-harvest

Actual food safety starts on the farm during the raising of crops and livestock. In fact, the amount of antibiotics used in this industry is the highest proportion globally. for years, the agriculture environment has extensively relied on antibiotics for growth promotion, infection prevention, and control. As a result, due to the irresponsible and

excessive use of antibiotics in agriculture and livestock, multidrug resistance in bacteria emerged and spread, eliminating their effectiveness for controlling pathogens (Page & Gautier, 2012). Accordingly, This has led to an increased recognition of phages and their derivatives as practical alternative techniques for use in food safety at various phases of the production process (Endersen & Coffey, 2020). Several studies were conducted to confirm the efficacy of phage in combating bacterial infection in corps (Balogh et al., 2008, 2010; Jones et al., 2012).

Many phage products were approved by US-FDA to treat foods before marketing, for example, AgriPhage™, Intralytix, LISTEX and Listshield™ to treat Listeria sp. (Meaden & Koskella, 2013). Currently, farm animals are kept in huge numbers and frequently in cramped spaces, which facilitates the spread of infectious agents between cattle. Consequently, numerous animals serve as reservoirs for various zoonotic bacterial infections that infiltrate the food production processes and cause diseases and deaths in humans (Endersen & Coffey, 2020). In poultry, *Salmonella* and *Campylobacter* are the main pathogens. The use of phage cocktail made of three lytic phages of *S. enteritidis* significantly decreased the intestinal *S. enteritidis* counts in broilers. However, the researchers suggest that the result can be improved with multiple treatment (Vaz et al., 2020). At the same time, using phage cocktail against *Campylobacter coli* (Chinivasagam et al., 2020) *and Campylobacter jejuni* (Chinivasagam et al., 2020; Richards et al., 2019) induced significant decrease in bacterial count.

In cattle, controlling EHEC, specifically, *E. coli* O157:H7 by bacteriophage seems to be very challenging. Two recent studies were conducted to evaluate the efficacy of phage cocktails on reducing *E. coli* O157:H7 titer in cattle hides only (Tolen et al., 2018) and hide and carcass (Arthur et al., 2017). Unfortunately, results of both studies were not significant, suggesting the need for a wider host-range phage cocktail. In fertilizer, Pig slurry is an organic fertilizer that is widely applied to soil or crops. However, it increases the transfer of pathogenic bacterial like *Salmonella enteritidis.* Adding *Salmonella* phage "sall_v01" to pig slurry, reduced *Salmonella Enteritidis* counts by 3.8 log CFU/ml (Grygorcewicz et al., 2017).

2- During the food process

Currently, in order to remove microbes during food processing, standard pathogen decontamination processes in food processing predominantly use chemicals, irradiation and physical disruption techniques (Gómez-López, 2012). Pasteurization and high pressure processing are used to reduce numbers of pathogens in liquid food products (Vikram et al., 2021). Fortunately, various commercial effective phage cocktails are available for *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella, Shigella* (Vikram et al., 2021).

3- Post-harvest

Generally, foods are nutrient-rich habitats for microbes. Only the amount and type of preservatives used determine number and type of pathogenic bacteria that may survive. The use of well-chosen phages can lower the presence of specific dangerous diseases, according to the literature, which has extensively documented intervention strategies using phages to control pathogens in postharvest food products (Endersen & Coffey, 2020). Here I will look at the some of these studies which promotes the use of phages as bio-preservative agents. In meat, A recent study investigated the efficacy of an Intralytix phage cocktail EcoShield PXTM, designed for STEC, in eight types of food products (cantaloupe, salmon, cooked chicken, beef chuck roast, chicken breast, ground beef, cheese and romaine lettuce). The phage cocktail significantly reduced *E. coli* O157:H7 ($P<0.05$) in 97% of foods that were tested (Vikram et al., 2020). To evaluate the phages' efficacy in 4°C a phage cocktail made of 4 *Salmonella* phages that target five different *salmonella* serovars; *typhimurium*, *typhi*, *earatyphi* A, *enteritidis* and San Diego in chicken breast. The results revealed significant reduction ($P < 0.05$) in Salmonella counts during cold storage (Kim et al., 2020). A similar study was done in 8°C, using a five-phage cocktail, the experiment showed significant reduction ($P < 0.05$) in the numbers of *S. typhimurium* and *S. enteritidis* (Duc et al., 2018).

In processed foods, The necessity of safe food bio-preservative agents lead to development of FDA approved phage products against many food pathogens (de Melo et al., 2018). In a recent study, administration of anti-*Salmonella enteritidis* phage SE07 in different finished food products like fresh eggs, chicken and fruit juices, significantly

reduced numbers of the bacterium after 48 hours at 4°C (Thung et al., 2017). This study with many other studies (Abuladze et al., 2008; Zuber et al., 2008; Zhou et al., 2020) confirm the importance of phage as bio-preservative agent in processed food. In vegetables and fruits, several studies suggested the efficacy of phage in reduction of numbers of viable pathogenic bacteria in fresh cut fruits and fruit juices (Oliveira et al., 2014), lettuce and melon (Soffer et al., 2017) and cucumber (Bai et al., 2019). However, some studies concluded with some considerations. For example, Liu et. al., while trying to reduce numbers of Salmonella in lettuce and tofu, found that the reduction depends on the MOI (Liu et al., 2020). On the other hand, Wong *et. al*., studied the efficacy of a phage cocktail made of five anti-*S. enterica* phages in reducing different *S. enterica* serovars in romaine cantaloupe and lettuce leaves. The researchers observed the importance of phages' host range in eliminating pathogenic bacteria (Wong et al., 2020).

B. Phage display technology

Briefly, phage display is known as the method of expressing exogenous (poly)peptides on the surface of phage particles in order to bind to numerous target molecules (Newton & Deutscher, 2008). It has developed into a potent tool for discovering new drugs and finding and creating polypeptides with unique functions. The encoded foreign peptide is seen as a fusion to one of the coat proteins on the surface of the filamentous phage, as a result of insertion of the foreign DNA fragment into the genome of the phage. Phage display has become a standard way for choosing novel ligands with desired features because of the physical linkage of the displayed peptide on the viral particle and the idea of diverse peptide libraries. In 1985, the original idea of employing a filamentous bacteriophage as an expression vector was presented (Smith, 1985). Recombinant approaches are one of the many strategies that have been developed to broaden the library's diversity. On the other hand, libraries containing a range of biases can be developed for particular uses.

For example, by screening libraries made with minimal peptide mutagenesis, the affinity and selectivity of the peptide that binds the target can be improved when its sequence is known. With the specific ligands isolated from phage libraries, phage display technology has actively contributed to the development of vaccines, drugs, and therapeutic targets. Phage display has underwent major advancements that go above and beyond its

original concept, making it a potent tool for wide array of new applications in the future (Pande et al., 2010).

C. Phage therapy

Bacteriophages are viruses that attack bacteria, The direct administration of lytic phages to a patient with the goal of lysing the pathogenic bacteria that is producing a clinically significant infection is called “Phage therapy” (Viertel et al., 2014). Félix d’Herelle is considered to be the first scientist to introduce the concept of antibacterial use of bacteriophage and treated patients with various infectious diseases (Fernández et al., 2019). Since then, in former Soviet Union, bacteriophage therapy was advanced and treated millions of patients. In contrast, bacteriophage therapy was abandoned in the Western World due to the advent of more convenient broad spectrum antibiotics in the 1940s and the unreliability of bacteriophage preparations at the early stages (Debarbieux et al., 2016).

Why phage therapy?

Antibiotics are one of the most effective therapeutic approaches in the annals of medicine. It has prevented millions of deaths and contributed significantly to the advancement of numerous medical innovations, including as cancer chemotherapy and organ transplantation (Aminov, 2010). The world has been genuinely altered by antibiotics. A so-called "post antibiotic era" is rapidly approaching as a result of losing the efficacy of antibiotic therapy in medicine which would be disastrous (Alanis, 2005; Gordillo Altamirano & Barr, 2019). Both microbes and higher eukaryotes create a wide range of physiologically active compounds with antibacterial capabilities, some of which have been repurposed as contemporary antibiotics (Ganz & Lehrer, 1999). However, the levels of these chemicals in these natural settings are frequently lower than clinically meaningful thresholds, indicating that resistance does not only develop to counteract their toxic activity. It has been suggested that rather than acting as strictly antimicrobial agents, antibiotics and their interactions with antibiotic resistance mechanisms function in nature as a means of communication between the individuals who make up a microbial community. These substances have been shown to cause adaptable phenotypic and genotypic responses and influence the community's makeup (Aminov, 2009). It is not

surprising, when looking at antibiotics from a biological standpoint, that genes causing resistance to contemporary antibiotics have been found in old microbial populations, such as unpolluted arctic permafrost, indicating that antibiotic resistance exists even in the absence of anthropogenic impacts (Perron et al., 2015).

Human also has a great role, antibiotic overuse, particularly in clinical and industrial settings, significantly worsens the issue of antimicrobial resistance. In agriculture and aquaculture, antibiotics are used to treat crop and fish diseases, as well as, of course, to treat infectious diseases in humans (Ventola, 2015). Actually, in the United States, up to 180 mg of an antibiotic agent are used to produce one kg of meat and it has been reported that other countries' agricultural use of antibiotics is even higher (Price et al., 2017). Millions of tons of antibiotics are released into water effluents and environmental reservoirs as a result of their agricultural use (15). The lack of adequate water treatment, the involvement of pharmaceutical waste, and the closer proximity of farms to cities all contribute to antibacterial resistance by increasing the amount of antibiotic release and persistence in the environment. The continued use of various antibiotics on environmental microbial communities has accelerated the evolution and spread the range of antibiotic resistance genes that are still present in natural reservoirs (Finley et al., 2013). Subsequently, the concomitant acquisition of antibiotic resistance traits led to the development of multidrug-resistant organisms (MDR), then extensively drug-resistant (XDR), and ultimately, pan-drug-resistant (PDR) bacteria (Magiorakos et al., 2012). Therefore, today, regardless of age, socioeconomic status, or country of residence, antibiotic resistance poses a serious threat to global health that could have an impact on anyone (Mendelson & Matsoso, 2015). To better understand the situation, according to a recent review, ten million lives will be lost annually due to antimicrobial resistance by 2050, costing the global economies US$100 trillion (Altamirano & Barr, 2019). Therefore, WHO responded with a global action plan with the following strategies: 1. Improve awareness, 2. Continu surveillance and research process, 3. Reduce incidence of the infection, 4. Use antimicrobials wisely, 5. Invest in countering antimicrobial resistance and discovery of new solutions. WHO, also, Initiated three initiatives to push for new solutions for the rising problem of antimicrobial resistance; 1. The Global Antimicrobial Resistance Surveillance System (GLASS), 2. Global Antibiotic Research

and Development Partnership (GARDP), 3. Interagency Coordination Group on Antimicrobial Resistance (IACG) (*Antibiotic Resistance*, n.d.). Therefore, according to above mentioned WHO responses and initiatives and the current situation, the world is seriously looking for new antibiotics and any alternatives such as phage therapy.

Understanding the challenges

The early employment of phage as a therapeutic agent had a great role in understanding the challenges of phage therapy. D'He'relle recognized the medicinal potential of phage as a cure for bacterial infections almost soon after his discovery. He employed it successfully in 1919, treating *Salmonella gallinarum*-infected poultry with a phage (d'Herelle & Smith, 1926). Based on his success in birds, In 1921, D'He'relle successfully treated five patients infected with *bacillary dysentery* using *Shigella dysenteriae* phage (Ho, 2001). Later on he treated cholera with phages, decreasing mortality rate from to 8.1% comparing to control group which had 62.8% mortality (d'Herelle et al., 1930).

D'He'relle's success encouraged other scientists to utilize the potential therapeutic activity of phages and exploring the advantages and challenges of phage therapy; 1. The first challenge to be addressed was the phages host-specificity, Beckerish and Hauduroy in 1923 were able to reduce the bacterial count in a patients' blood suffering from typhoid fever using phages (Hadley, 1928). However, next year, another scientist failed in treating a similar patient population with phages (Smith, 1924), the failure was due to the narrow host range of the phage that was used (Hadley, 1928). 2. The second challenge that was identified in early phage therapy practice was the purification of the phage lysate as a result of poor filtration techniques (Kortright et al., 2019). 3. Phages are washed quickly with spleen (Krestownikowa & Gubin, 1925). 4. Bacteria can develop resistance to phage (Luria & Delbrück, 1943). 5. *In Vitro* results not necessary match the *In Vivo* outcomes (Krueger & Scribner, 1941). Identification of these challenges was synchronized with the discovery of antibiotics, the fact that contributed to shift the attention from phage therapy to antibiotics. However, with the rapid rise of antibiotic resistance, phage therapy are reconsidered (Kortright et al., 2019).

Examples of human trials

In 1963, in Tbilisi, Georgia, 30 769 children (6 months to 7 years old), were recruited in a phage therapy where *Shigella* phages were administered orally once every week as a prophylaxis to prevent the bacterial dysentery. The children were divided into two groups, first group received the phage while the second group received placebo. Children were followed for a period of 109 days. Children who received the phage had 3.8-fold decrease in incidence of dysentery (Babalova et al., 1968). Between 1982–1983 in the Soviet Union, soldiers of the Red Army located in four different areas, were enrolled in an extensive double-blind phage treatment and prophylaxis trials. Comparing to the control group, soldiers in phage-treated group, reported 10-fold less incidence of dysentery (Alisky et al., 1998). On the other hand three clinical trials that were conducted independently in 2016 (Sarker et al., 2016), 2017 (Jault et al., 2019) and 2020 (Leitner et al., 2021) were stopped due to insufficient efficacy. However, according to Górski, et al. (2020) who reviewed those three clinical trials, the treatments were not successful because of the low titer phages-preparations that were used (Górski et al., 2020).

Phage-derived protein

In addition to the use of phage particles in phage therapy, phages secrete proteins which capable of lysing bacterial cells and accordingly killing the bacteria. These proteins are encoded in phage genomes. Phages in order to eject their genomes into the bacterial cell, need two groups of proteins: virion-associated peptidoglycan hydrolases (VAPGH) and polysaccharide depolymerases (Roach et al., 2017). VAPGH degrade the peptidoglycan layer to allow injection of the phage's genomes into the bacterial cell. While polysaccharide depolymerases are used to degrade polysaccharide components (e.g. lipopolysaccharide (LPS) in gram negative bacteria) in the cell wall of bacteria, allowing the phage to reach the secondary host receptors positioned within the cell wall (Maciejewska et al., 2018). Several studies were conducted to demonstrate the efficacy of the both proteins as potential antibacterial agent by various mechanisms like degrading bacterial capsules and harmonizing activities of the immune system, antibiotics and phages (Mushtaq et al., 2005; Born et al., 2014).

Furthermore, phages have two other enzymes which can be utilized as powerful antibacterial agents. These tow enzymes are essential for destroying the bacterial cell to

release the new phage particles. This happens as a result of careful coordination between the two enzymes: holins and endolysins. Holins accumulate in the bacterial cell until it reach a concentration that is enough to induce a hole in the cell membrane (Gründling et al., 2000). However, holin itself cannot lyse the bacterial cell (Roach et al., 2017). Endolysins utilize the holes that are made by holins to reach the polymer peptidoglycan in the bacterial cell wall and degrade it to release the new phage particles. However, in gram negative bacteria, spanins are required to facilitate the fusion of the inner and outer membranes, to complete the cell lysis (Young, 2013). Several studies demonstrated the efficacy of endolysins in treatment of *S. aureus* (Pastagia et al., 2011), *Streptococcus pneumoniae* (Loeffler et al., 2001) and *Streptococcus agalactiae* (Cheng et al., 2005) in mice.

Bacteriophage vs Antibiotics

Although both phage (lytic phage) and antibiotic are used to reach the same result; killing bacteria. They are different in many terms. The most obvious difference is that pahges are natural dynamic entities where antibiotics are chemicals with the ability of influencing the growth of the bacteria through specific mechanisms (Altamirano & Barr, 2019). However, at the same time, they share some similarities.

Phage-host specificity is considered the biggest advantage of phage therapy. Since phage targets the pathogenic bacteria, where antibiotics kill a wide range of microbiome. Therefore, phages do not cause side effects associated with disturbed microbiome (Langdon et al., 2016). However, this can be the biggest disadvantage of phage therapy as well. Since it requires identification of the pathogenic bacteria before starting the treatment, which is resource and time consuming (Caliendo et al., 2013).

Only lytic phages are used in phage therapy. Lytic phages are bactericidal, whereas antibiotics are either bactericidal or bacteriostatic. Therefore, the ability of bacteria to develop resistance to antibiotics are more than its ability to develop resistance to their specific lytic phage (Carlton, 1999; Stratton, 2003). Phages can target the antibiotic resistant-bacteria, since phages infect and kill bacteria using mechanisms that different from antibiotics' mechanism (Loc-Carrillo & Abedon, 2011).

Auto-Dosing, lytic phages which are used for phage therapy, kills the bacterial host at the end of its life cycle where it is replicated and increased in number. This is called

Auto-dosing, since the phage which were administered, will produce the future doses (Carlton, 1999). However, titer of the phages' dose should be adequate, since clinical studies with low phage titer have failed (Górski et al., 2020). On the other hand, very high titer should be avoided if it is associated with increased side effects (Loc-Carrillo & Abedon, 2011).

Bacteriophages, being protein, genetically are not toxic (Kutter et al., 2010). Phages may trigger immune response (Alisky et al., 1998). Therefore, it is extremely important to only use highly purified phage in phage therapy (Skurnik & Strauch, 2006) to avoid anaphylactic immune responses due to presence of bacterial residues, such as the endotoxins, in crude phage lysates (Skurnik & Strauch, 2006).

Phage production is relatively not costly, since it depends on two factors; growth of bacterial host and purification of the phages (Gill & Hyman, 2010). Host growth cost depends on the bacterial species, while the purification cost is getting cheaper as technology advances (Kramberger et al., 2010).

Phages, being made of nucleic acid and protein (Abedon & Thomas-Abedon, 2010) with narrow host range (Hyman & Abedon, 2010) have a very low environmental impact since unlike antibiotics (Ding & He, 2010) the discarded therapeutic waste will at worst react only with its host and can be inactivated by any environmental degrading factors like sunlight (Loc-Carrillo & Abedon, 2011).

Phages similar to antibiotics, are very flexible in terms of formulation and routes of administration. Phages can be formulated in almost all drug forms such as creams, impregnated into solids and liquids in order to be suitable for any routes of administration (Krylov, 2001; Kutateladze & Adamia, 2010; Kutter et al., 2010). Furthermore, bacteriophages can be prepared as cocktails to increase its antibacterial activity (Goodridge, 2010; Kutateladze & Adamia, 2010).

Phages are able to clear the biofilm. Antibiotic resistance in biofilms is typically much higher than that of planktonic bacteria. Phages, though, have the ability to break up at least certain biofilms. This may be because they actively enter biofilms and lyse bacterial layers, one layer at a time, or because they have exopolymer-degrading depolymerases that can break down biofilms (Loc-Carrillo & Abedon, 2011; Chan et al., 2013; Maciejewska et al., 2018).

Combination therapy, which means treatment of clinical condition with more than one medicine, is a common practice. For example in case of HIV and multiple-drug approach to treat tuberculosis (Worthington & Melander, 2013). Since it is expected that two sufficiently diverse selecting pressures will be more successful than one by itself (Torres-Barceló & Hochberg, 2016). Phage-antibiotic synergy (PAS) describes the advantage of using sublethal concentrations of antibiotics to increase the burst size of lytic phages which provides wider spread of phages (Comeau et al., 2007). The effectiveness of combination of phage with antibiotic was demonstrated in several studies (Altamirano & Barr, 2019). For example, combination of a single-dose of *P. aeruginosa* phage cocktail and ciprofloxacin antibiotic to treat experimental endocarditis in rats. Endocarditis is known to protect bacteria from host's immune response and antibiotics by formation of vegetations (Oechslin et al., 2017). In another study, synergism between a bactericidal agent and monophage therapy against *E. coli* infection in human urine, significantly decreased the emergence of resistant bacterial mutants (both singly and doubly) when used together (Valério et al., 2017). These findings suggest that phage therapy should not replace antibiotics, instead they both can be combined for better outcomes (Altamirano & Barr, 2019).

1.3.7 Phage genome analysis

DNA sequencing or in other words identifying the order of nucleic acid bases in the genome of the biological materials is an essential component of a wide range of researches. It is difficult to emphasize how crucial DNA sequencing is to the biological studies since, at its most basic, it allows us to gauge one of the key characteristics that allows different terrestrial life forms to be identified and distinguished from one another. As a result, over the past 50 years, numerous scholars from around the world have spent much time and money into creating and refining the technologies that support DNA sequencing. At the beginning of this field, researchers would laboriously spend years producing sequences that may range in length from a dozen to a hundred nucleotides, working mostly from accessible RNA targets. Sequencing technology has improved throughout time while also becoming more affordable, thanks to advances in molecular biology, automation, and sequencing methods. This has made it possible to read DNA with hundreds of base pairs in length and create gigabytes of data in a single run (Heather &

Chain, 2016). To obtain the complete genome of a bacteriophage, several processes must take place, here are the summary of the most important processes in phage genome sequencing, assembling and annotating:

1- Genome sequencing: It's crucial to know what the finished genome assembly will need to do before beginning any genome assembly. Distinct packaging techniques used by bacteriophages might lead to different genomic termini or circularly permuted genomes (Black, 1989). The amount of input genomic DNA (which is particularly high for long-read sequencing), the availability of service providers, the number of genomes to be sequenced, and the cost will all play a role in the selection of library preparation methods. For both short- and long-read sequencing, there are many distinct library preparation techniques (Shen & Millard, 2021). NEBNext® Ultra IIDNA Library Prep Kit is a very useful library preparation method for Illumina sequencing. DNA sequencing has gone through different stages starting with Watson and Crick who were the first to notice that the sequence of the bases in the DNA chain is irregular (Watson & Crick, 1953) which triggered the urge for sequencing, to shotgun sequencing by Staden in 1979 (Staden, 1979), to Third-Generation sequencing which started in 2010s (Giani et al., 2020) including Illumina Novaseq 6000 platform which produces up to 6Tb (Modi et al., 2021) and cost effective (Senabouth et al., 2020).

2- Quality control of reads: The quality control of the reads and comprehension of how these data have been created and/or provided by a sequencing provider are the first steps in the assembly of phage genomes (Shen & Millard, 2021). The use of FASTQC enables a summary of the reads that have been recovered, their quality statistics, along with number of the reads in each sample (Shen & Millard, 2021). Once the quality of the reads is assured, sequencing adapters and poor-quality bases can be removed. Several tools, like trim galore (Krueger, 2012), sickle (Joshi & Fass, 2011), and BBDuk can be used to trim reads before the assessment of the quantity of available data and randomly subsampled them into sets of 50-100 x coverage of the genome (Shen & Millard, 2021).

3- Assembly: Phage genomes can be assembled by many genome assemblers like: Geneious assembler in Geneious Prime v2022.2.2 (Adams et al., 2022), Megahit (Li et al., 2015), MetaVelvet (Namiki et al., 2011), SPAdes (Prjibelski et al., 2020) and Ray (Boisvert et al., 2010). At this point, It is crucial to check the assembled contigs to assess how well the assembly went and spot any mistakes (Shen & Millard, 2021). The assembled genome is called contig and it will be in *contig.fa* format. The assembly will most likely produce a single contig with high coverage, with the remaining contigs being very short and having low coverage. This indicates that the entire phage genome has been assembled. At this stage it is useful to find out the open reading frames (ORF) using and online Rapid Annotation using Subsystem Technology (RAST) and to cross-check the protein sequences using online BLASTP.

4- Bioinformatics: It is important to determine whether the putative phage genome resembles other sequenced phages and if the complete genome has been assembled. What comes next, depends on this step which can be done through different tools like INPHARED (Cook et al., 2021) and BLASTn. Complete genome of the closest relatives that were identified in the previous step can be furthermore compared using ProgressiveMauve (Darling et al., 2010). To identify the antibiotic resistant genes in phage genome, ABRicate can be used (Page & Seemann, 2019). There are several online tools for extra annotations, for example MEGA 11 and it's component MUSCLE to construct phylogenetic tree for any given gene to find out the closest relative gene (Tamura et al., 2021).

Chapter 2: Characterization and Genomic Analysis of *Escherichia coli* O157:H7 Phage UAE_MI-01 Isolated from Birds

2.1 Introduction

As defined by World Health Organization (WHO), food borne ailments are diseases of infectious or toxic nature that are caused by the consumption of food or water (Kadariya et al., 2014). Food borne diseases are considered a major health challenge worldwide with around 420,000 deaths reported annually (Oliver, 2019). Several microorganisms, including bacteria and fungi, are responsible for causing food borne diseases (Zhao et al., 2014). However, the majority of food borne disease outbreaks are often initiated by bacterial agents (Omer et al., 2018). In fact, several of the bacterial foodborne outbreaks reported between 1980-2015 were due to verotoxin-producing *Escherichia coli*. *Escherichia coli* O157:H7 instigated most of those outbreaks and claimed a number of lives (Omer et al., 2018).

Meat products (Authority et al., 2015) and leafy vegetables, mainly romaine lettuce (Coulombe et al., 2020), were the most common source of *E. coli* O157:H7 in the foodborne outbreaks reported. *E. coli* O157:H7 is a gram-negative bacterium that habitats the intestine of ruminant animals such as cattle and spread through their feces (Berg et al., 2004). *E. coli* O157:H7 infection could lead to hospitalization because of hemorrhagic colitis and, in severe cases, hemolytic uremic syndrome (Scallan et al., 2011; Sperandio & Nguyen, 2012; Pham-Khanh et al., 2019) and may even lead to death (Thomas et al., 2015). Antibiotics may not always be appropriate in *E. coli* O157:H7 infections (Bell et al., 1997; Neill, 1998) as it was found to be associated with serious outcomes (Carter et al., 1987; Pavia et al., 1990) and increased the risk of developing hemolytic–uremic syndrome in children (Wong et al., 2000). Therefore, eliminating *E. coli* O157:H7 during food processing remains the best solution. Many natural food preservatives or biocontrollers have been reported to eliminate bacterial food-borne diseases including ethanol, chitosan oligosaccharides, organic acids, lactoferrin, bacteriocins and bacteriophages (Lee et al., 2016).

Bacteriophages or “bacteria eaters” are natural killer of bacteria and were discovered by Félix d'Hérelle in 1917 (Fernández et al., 2019). Since then, bacteriophages’

antibacterial ability has been explored well (Abdelkader et al., 2019) alongside their other contributions to science (Janczuk-Richter et al., 2019). In fact, bacteriophages have several applications in food industry, drug delivery systems, diagnostics, and phage-display technology and in ecology (Wittebole et al., 2014; Janczuk-Richter et al., 2019).

Phages that replicate through the lytic cycle are called virulent phages whereas temperate phages may use either the lytic or lysogenic cycle to replicate (Pham-Khanh et al., 2019). The use of virulent phages in phage therapy, as an alternative antibacterial agent, and in food industry, as a food preservative, are well documented (Keen & Adhya, 2015; Soffer et al., 2016; Fernández et al., 2019). In 2006, the United States Food and Drug Administration (FDA) approved the use of phages as food preservative and several phage products were developed as natural bacterial biocontrol agents (Abuladze et al., 2008). Furthermore, phage-based therapy centers and clinical trials of therapeutic phage products have been rapidly increasing in many countries (Górski et al., 2020). Therefore, isolation and characterization of lytic phage that are capable of eliminating *E. coli* O157:H7 in food processing or as therapeutic agent is in high demand.

Previously, many *E. coli* O157:H7 phages were isolated from mammals like cattle (Litt & Jaroni, 2017), swine (Lee et al., 2016) and sheep (Raya et al., 2006). The current study explored different viral features and bioinformatics of an *E. coli* O157:H7 bacteriophage, designated as UAE_MI-01, isolated from a bird in the United Arab Emirates (UAE). This phage has potential to be used as a natural food preservative and adds to the arsenal of phages that could be used for therapy.

2.2 Materials and methods

2.2.1 Media

Luria-Bertani broth (LBB) pH 7.2 (HiMedia, Mumbai, India) was used in all the protocols in the current study. Bacterial dilutions from 18 h LBB cultures grown at 37°C was carried out in phosphate buffered saline (PBS; Oxoid, Basingstoke, UK). For performing the plaque assay, 'soft layer agar' which was LBB broth in Lambda-buffer [6 mmol L^{-1} tris pH 7.2; 10 mmol L^{-1} $Mg(SO_4)_2$. $7H_2O$; 50 mg L^{-1} gelatin (HiMedia)] and supplemented with 4 g L^{-1} agar (HiMedia) was used. To determine the optimum media, Luria-Bertani agar (LBA), nutrient agar (HiMedia), soyabean casein digest agar (HiMedia) and Mueller Hinton agar (Mast group, Bootle, UK) were used. All

bacteriophages were maintained and diluted in Lambda-buffer [6 mmol L^{-1} tris pH 7.2; 10 mmol L^{-1} Mg $(SO_4)_2.7H_2O$; 50 mg L^{-1} gelatin (Oxoid)] and were preserved at 4°C.

2.2.2 Cultivation of the propagation host E. coli O157:H7

Host cell bacterial strain: *E. coli* O157:H7 NCTC 12900, was used in this study. Cultures were stored at -20°C in 20% glycerol. Prior to investigation, a stock culture of the bacteria was maintained on an LBA plate. One loopful of *E. coli* O157:H7 was inoculated into a 15 mL sterile centrifuge tube with flat cup (ExtraGene, USA) containing 10 mL of LBB and incubated for 18 h at 37°C and 70 rpm in a shaker incubator (Innova 4000, New Brunswick Scientific, Connecticut, USA).

2.2.3 Isolation, purification and propagation of the phage against E. coli O157:H7

Slurry of feces samples from one nest was prepared in a beaker (250 mL). The slurry was seeded every 24 h with 18 h culture of *E. coli* O157:H7 NCTC 12900 for 96 h. The beaker was incubated in a shaker incubator at 37°C and 70 rpm. Ten mL of the slurry was transferred into 15 mL sterile centrifuge tube with flat cup and centrifuged at 12000 g for 10 min. The supernatant was filtered using sterile Millipore membrane syringe filter (Pore size 0.22 µm, Millipore Corporation, MA, USA) and then was diluted using ten-fold serial dilution technique in Lambda-buffer.

Each dilution was tested for the presence of the specific phage by a plate lysis procedure (Davis & Sinsheimer, 1963; Sambrook et al., 1989; Vidaver et al., 1973). Briefly, an aliquot (100 µL) of each dilution was mixed with 100 µL of an overnight LBB culture of *E. coli* O157:H7 NCTC 12900 in a sterile 1.5 mL Eppendorf micro-centrifuge tube (Greiner Bio-One) and was incubated for 15 min at 37°C to facilitate attachment of the bacteriophage to the host cells (Sambrook et al., 1989). The mixture was then transferred from the Eppendorf micro-centrifuge tube to a 10 mL Bijou bottle and then 2 mL of soft layer LBA (which had been melted and cooled to 40°C in a water bath) was added. The content of the bottle was gently mixed by swirling and poured over the surface of a plate of LBA and allowed to set for 15 min at room temperature before incubating for 18 h at 37°C (Davis & Sinsheimer, 1963).

The largest single clear plaque was aseptically removed with a scalpel and transferred into an Eppendorf micro-centrifuge tube containing 1 mL of Lambda-buffer

and mixed gently before filtering with sterile Millipore membrane syringe filter (0.22 µm). The filtrate was serially diluted and propagated as described above. The plates that showed almost confluent plaques was used to prepare a concentrated phage suspension by overlaying with 5 mL of Lambda buffer. Finally, chloroform was added to separate the bacteriophage from the bacterial cells (Sambrook et al., 1989) and filtered with sterile Millipore membrane syringe filter (0.22 µm). Titer of the phage stocks was calculated and then maintained in Lambda-buffer at 4°C for future investigations.

2.2.4 Determination of host range

To determine the ability of UAE_MI-01 to infect variety of host cells, the following bacterial strains were used: *E. coli* O157:H7 NTCC 12900, *E. coli* ATCC 25922, *E. coli* ATCC 8739, *E. coli* extended-spectrum beta lactamase-producing (ESBL) (patient isolate), *E. coli* ATCC 15223, *E. coli* ATCC 23227, *E. coli* ATCC 9637, *E. coli* ATCC 35218, *E. coli* ATCC 23224, *Bacillus subtilis* ATCC 6051, *Pseudomonas aeruginosa* ATCC 25668, *P. aeruginosa* ATCC 27853, methicillin-resistant *Staphylococcus aureus* (patient isolate), *S. aureus* ATCC 6358, *S. aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus saprophyticus* ATCC-BAA 750, *Streptococcus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 51299, *E. faecalis* (patient isolate), *Enterococcus casseliflavus* (patient isolate), *Enterobacter aerogenes* ATCC 13018, *Enterobacter hormaechei* (patient isolate), *Klebsiella pneumonia* ESBL- producing ATCC 700603, *K. pneumonia* KPC 2 +ve (patient isolate), *Haemophilus influenzae* ATCC 9007, *Stenotrophomonas maltophilia* ATCC 17666, *Salmonella enterica* ATCC 14028, *Salmonella* sp. (patient isolate), *Proteus vulgaris* ATCC 29905, and *Mycobacterium smegmatis* ATCC 607 (Table 1).

All bacterial hosts were grown on LBA plates and the bacterial cells were harvested by scraping the surface of the slant of 20 mL of sterile LBB containing 20% (v/v) glycerol and stored at -20°C.

The host range of the phage was studied by spotting 10 µL of UAE_MI-01 suspensions containing 10^{10} PFU mL^{-1} onto LBA plates, each previously seeded with a suspension (10^{10} CFU mL^{-1}) of one of the type strains tested. The phage suspension was added to the dried host seeded plates, 30 min after seeding the plates. The plates were then incubated at 37°C for 48 h and examined for lysis and plaque formation.

2.2.5 Plaque morphology and optimum media based on plaque size

To determine plaque morphology of the phage UAE_MI-01 (Williams et al., 1980) and the optimum media that will allow the phage to produce the largest plaque, UAE_MI-01 suspension containing 10^2 PFU mL^{-1} was used following plate lysis procedure (as described above) using different media; LBA, nutrient agar, soyabean casein digest agar, and Mueller Hinton agar.

2.2.6 Evaluation of phage stability in physical and chemical agents

The effect of the selected physical and chemical agents on viability and propagation of the phage UAE_MI-01 was evaluated according to the methods described by Brownell *et al.* (Brownell et al., 1967).

To determine the effect of physical conditions on UAE_MI-01 viability and propagation, LBB-grown preparations of each phage was diluted 1 to 10 in LB broth. Aliquots (0.1 mL) of the sample was mixed with 0.9 mL LBB, before applying the physical treatment. The phage numbers were enumerated after the application of the physical treatment and was represented as PFU mL^{-1}. The untreated LBB which was similarly diluted served as a control (Brownell et al., 1967).

The physical treatments tested were (i) temperatures: -20°C, 4°C and room temperature, for three months, (iii) heating the phages at 45°C, 65°C and 75°C for 15 min and 30 min, (iv) boiling at 100°C for 15 and 30 min, as recommended by Brownell *et al.* (Brownell et al., 1967).

To determine the effect of pH on the viability and propagation of the UAE_MI-01, different pH values (pH 3, 4, 7, 9 10) was used. The phage suspension (0.1 mL of 10^9 PFU mL^{-1}) was added to 0.9 mL distilled water and the tubes were incubated for 24 h at room temperature. At the end of the incubation period, the number of PFU were estimated as previously described by Brownell *et al.* (Brownell et al., 1967).

To determine the stability of UAE_MI-01 in the presence of chemical disinfectants (ethanol 70%, sodium hypochlorite 2%, commercial disinfectant 20% and liquid hand wash-soap), the phage in LBB was diluted in lambda buffer (1 to 10 dilutions). Phage samples (0.1 mL) was mixed with 0.9 mL of each chemical disinfectant at the concentration which were decided by the manufacturer in distilled water and incubated in the room temperature for 1, 2- and 3-min. At the end of the incubation period, the number

of PFU were estimated (PFU mL^{-1}). The control was similarly diluted but untreated LBB preparation as suggested by Brownell *et al.* (Brownell et al., 1967).

2.2.7 One-step-growth curve to determine the adsorption time, adsorption rate constant, latent period and burst size

To determine the adsorption time and the adsorption rate constant, unattached phages were enumerated as described by Dowding (Dowding, 1973). Briefly, conical flask containing 50 mL of LBB with a host suspension was incubated in shaking incubator for 3 h at 37°C and 100 rpm. The phage UAE_MI-01 was then added with multiplicity of infection of 0.1, and the flasks was incubated at 37°C. Samples were collected at different times, and filtered through membrane filters (0.22 um). Samples were then diluted and plated at 37°C and the numbers of PFU mL^{-1} was counted as recommended by Dowding (Dowding, 1973).

The equation $K = 2.3 / Bt \times \log_{10} (P_0/Pt)$ where B is the host concentration (CFU mL^{-1}); P_0 is the initial phage concentration (PFU mL^{-1}); Pt is the phage concentration at t min (PFU mL^{-1}); and t is the period of adsorption, was used to calculate the adsorption rate constant, K mL min^{-1} as described by Sykes *et al.*(Sykes et al., 1981).

One-step growth experiment was carried out as described by Dowding (Dowding, 1973) to investigate the latent period, rise period and the burst size for UAE_MI-02. Briefly, a suspension of the bacterial host *E. coli* O157:H7 NCTC 12900 (1×10^6 CFU mL^{-1}) was incubated for 3 h in LBB. The phage was then added in a low multiplicity of infection (0.1) and was incubated for 20 min. Sample of the attachment mixture (10 mL) was membrane-filtered to remove un-adsorbed phages from the infected host cells on the filter paper. The filter paper with the infected host cells was then transferred to a conical flask containing 50 mL of LBB at 37°C (first growth flask) and agitated to re-suspend the infected host cells in the broth. Furthermore, a 50-fold dilution was carried out in another flask held at 37°C (second growth flask), and both flasks were re-incubated at 37°C. Aliquots (1 mL) were collected from the first growth flask up to 35 min and then alternately from the second flasks up to 120 min. Samples were then membrane filtered and plated immediately. Numbers of PFU was counted after incubating the plates at 37°C for 48 h as demonstrated by Dowding (Dowding, 1973).

2.2.8 Phage examination using TEM

To determine the morphology of the phage, TEM was used with the negative staining technique; using uranyl acetate (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) as recommended by Ackermann and Heldal (Ackermann & Heldal, 2010) and Brum and Steward.

Briefly, the solution containing uranyl acetate was filtered using sterile Millipore membrane syringe filters (0.22 µm). The solution was then added into a 2 mL screw cap tube, and a drop of the phage suspension (10^{10} PFU mL^{-1}) was placed on 200-mesh copper grid with carbon-coated formvar films and the excess was drawn off and the grid left for almost 1 h to dry. The dry grid was stored in a desiccator until examination. Grids were examined using TEM (FEI Bio Twin Spirit G2 TEM, Netherlands).

2.2.9 Phage DNA isolation

One mL of the phage (10^{8}-10^{9} PFU mL^{-1}) was taken from phage stock kept at 4°C and phage DNA was isolated using the Phage DNA Isolation Kit (Norgen Biotek Corp, Thorold, Canada) following the manufacturer's recommended protocol. Briefly, 1 mL of phage suspension of 10^{8} PFU mL^{-1} yields 3-5 µg of DNA which is sufficient for next generation sequencing. Therefore, DNA isolation was done twice using 1 mL each time to yield 2 x (3-5 µg DNA) and was stored at -20°C for 48 h. The samples were sent to Macrogen (Seoul, South Korea) for sequencing.

2.2.10 Genome sequencing

DNA sequencing and assembly was performed as described previously (Sultan-Alolama et al., 2021).

2.2.11 Bioinformatics

A nucleotide BLAST (BLASTN) search was performed to identify phage genomes that were most similar to the genome sequence of UAE_MI-01. The top three whole genome sequences compared using progressiveMauve (Darling et al., 2010). A phylogenetic tree of terminase large subunit (TerL) protein, which is normally used as a genetic marker for the order Caudovirales, was constructed using MEGA11 (Tamura et al., 2021) with the neighbor joining method. For this, TerL sequence of UAE_MI-01 was

compared to sequences of the 15 most closely related TerL sequences identified from a protein BLAST (BLASTP) search. These were aligned using MUSCLE (https://www.megasoftware.net). Similarly, phylogenetic trees were also constructed using terminase small subunit, holin like class I & II proteins and lysin sequences identified using BLASTP searches. Map of the genome organization was created using Proksee (https://proksee.ca). Resfinder 4.0 (Bortolaia et al., 2020) was used to detect antibiotic resistance genes in UAE_MI-01 genome. The NCBI Conserved Domain Database (Lu et al., 2020) was searched to identify if the lysin protein annotated in the genome had the catalytic lysin domain. The holin sequences encoded by the genome were checked for the presence of transmembrane domains using DeepTMHMM (https://dtu.biolib.com/DeepTMHMM/).

2.2.12 Statistical analyses

All data were analyzed using analysis of variance (ANOVA) procedure of SAS Software version 9 (SAS Institute Inc., NC, USA). Mean values of treatments (four replicates) were compared using Fisher's protected least significant difference (LSD) test at P=0.05 levels.

2.3 Results

2.3.1 E. coli O157:H7 bacteriophage "UAE_MI-01"

E. coli O157:H7 bacteriophage "UAE_MI-01" was isolated from feces of wild pigeon in Abu Dhabi, UAE using *E. coli* O157:H7 NCTC12900 as the propagation host (Sultan-Alolama et al., 2021).

2.3.2 Host range

The host range of the bacteriophage UAE_MI-01 was investigated using different bacterial strains (Table 1). A wide range of gram positive and negative bacterial strains were used. Plaque with clear zone was considered positive and absence of the clear zone was considered negative. UAE_MI-01 was found to infect *E. coli* O157:H7 NTCC 12900, *E. coli* ATCC 8739, and *E. coli* ATCC 15223 producing a clear zone.

Table 1: Host range of the phage UAE_MI-01.

Bacteria	Sensitivity to phage UAE_MI-01
Escherichia coli O157:H7 NCTC 12900	+
Escherichia coli ATCC 25922	-
Escherichia coli ATCC 8739	+
Escherichia coli ESBL-producing (Patient isolate)	-
Escherichia coli ATCC 15223	+
Escherichia coli ATCC 23227	-
Escherichia coli ATCC 9637	-
Escherichia coli ATCC 35218	-
Escherichia coli ATCC 23224	-
Bacillus subtilis ATCC 6051	-
Pseudomonas aeruginosa ATCC 25668	-
Pseudomonas aeruginosa ATCC 27853	-
Methicillin-resistant Staphylococcus aureus (Patient isolate)	-
Staphylococcus aureus ATCC 6358	-
Staphylococcus aureus ATCC 29213	-
Staphylococcus aureus ATCC 6358	-
Staphylococcus aureus ATCC 29213	-
Staphylococcus epidermidis ATCC 12228	-
Staphylococcus saprophyticus ATCC-BAA 750	-
Streptococcus pyogenes ATCC 19615	-
Enterococcus faecalis ATCC 51299	-
Enterococcus faecalis (Patient isolate)	-
Enterococcus casseliflavus (Patient isolate)	-
Enterobacter aerogenes ATCC 13018	-
Enterobacter hormaechei (Patient isolate)	-

Table 1: Host range of the phage UAE_MI-01 (Continued).

Bacteria	Sensitivity to phage UAE_MI-01
Klebsiella pneumonia ESBL-producing ATCC 700603	-
Klebsiella pneumonia KPC 2 +ve (Patient isolate)	-
Haemophilus influenzae ATCC 9007	-
Stenotrophomonas maltophilia ATCC 17666	-
Salmonella enterica ATCC 14028	-
Salmonella sp. (Patient isolate)	-
Proteus vulgaris ATCC 29905	-
Mycobacterium smegmatis ATCC 607	-

NCTC; national collection of type cultures, ATCC; American type culture collection, ESBL; extended-spectrum beta lactamase.

2.3.3 Plaque's morphology and the determination of the optimum media for phage propagation based on plaque size

Bacteriophage suspension with 10^2 plaque forming units (PFU) mL^{-1} was used following the plate lysis procedure. When *E. coli* O157:H7 NCTC 12900 was used as the propagation host, the UAE_MI-01 phage produced the largest plaque size (5 mm) on soyabean casein digest agar and produced the smallest plaque size (2 mm) on Mueller Hinton agar (Figure 1). However, number of plaques was significantly ($P<0.05$) higher when nutrient agar medium was used, and this was followed by soyabean casein digest agar and Mueller Hinton agar (Table 2). UAE_MI-01 produced the lowest number of plaques on Luria-Bertani agar (Table 2). On all the different media, the phage formed uniform clear plaques which are characteristic of a lytic phage.

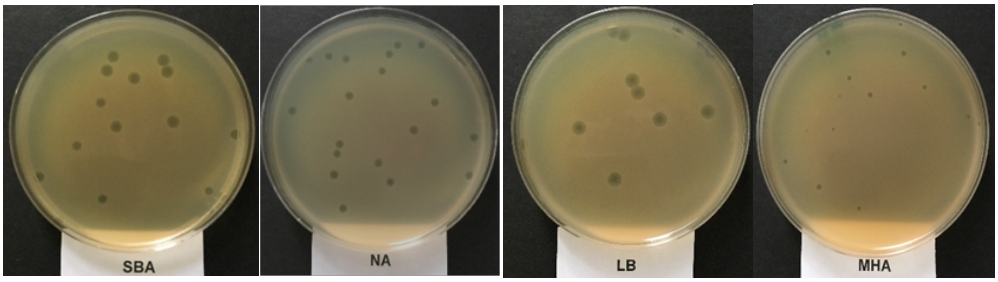

Figure 1: The formation of plaques of the phage UAE_MI-01 on different agar media

The formation of plaques of the phage UAE_MI-01 on different agar media using *Escherichia coli* O157:H7 NCTC 12900 as the propagation host (SBA; soyabean casein digest agar, NA; nutrient agar, LBA; Luria-Bertani agar, MHA; Muller Hinton agar).

Table 2: Effect of different complex media on the viability and propagation of the phage UAE_MI-01.

Type Of Medium	Soyabean Casein Digest Agar	Nutrient Agar	Luria-Bertani Agar	Muller Hinton Agar
(Log10 PFU mL^{-1})	6.59±0.21a	7.81±0.11b	5.53±0.39c	6.56±0.17a

Values of the number of phages (log_{10} PFU mL^{-1}) are means of four independent replicates ± standard deviation. Values with the same letters are not significantly (P>0.05) different within rows, according to Fisher's Protected LSD Test. PFU= plaque forming units. *Escherichia coli* O157:H7 NCTC 12900 was used as the propagation host.

2.3.4 Effects of temperature and pH on the phage UAE_MI-01

To assess the possibility of using UAE_MI-01 as a food preservative and biocontrol agent, its stability under various challenging conditions such as temperature, pH and chemical disinfectant was evaluated.

UAE_MI-01 was found to be stable at temperatures ranging from -20°C to 65°C (Table 3). The optimum temperature for long term storage was found to be 4°C (Table 4).

UAE_MI-01 was found to be stable at pH values ranging from 4-10, even though it was completely deactivated at pH 3 (Table 5).

Table 3: Stability of the phage UAE_MI-01 at different temperatures.

Temperature/ time	-20°C (log_{10} PFU mL^{-1})	4°C (log_{10} PFU mL^{-1})	25°C (log_{10} PFU mL^{-1})	45°C (log_{10} PFU mL^{-1})	55°C (log_{10} PFU mL^{-1})	65°C (log_{10} PFU mL^{-1})	75°C (log_{10} PFU mL^{-1})	100°C (log_{10} PFU mL^{-1})
15 Min	7.48±0.10Aa	7.51±0.16Aa	7.46±0.13Aa	7.49±0.14Aa	7.43±0.18Aa	7.47±0.19Aa	3.29±0.09Ab	0.00±0.00Ac
30 Min	7.49±0.15Aa	7.53±0.16Aa	7.42±0.15Aa	7.48±0.21Aa	7.46±0.12Aa	7.43±0.26Aa	0.00±0.00Bb	0.00±0.00Ab

Values of the number of phages (log_{10} PFU mL^{-1}) are means of four independent replicates ± standard deviation. Values with the same lower- and upper-case letters are not significantly (P>0.05) different within rows and columns, respectively, according to Fisher's Protected LSD Test. PFU= plaque forming units. *Escherichia coli* O157:H7 NCTC 12900 was used as the propagation host.

Table 4: Determination of optimum storage conditions of the phage UAE_MI-01.

Temperature	0:00 time (log_{10} PFU mL^{-1})	After 1 Month (log_{10} PFU mL^{-1})	After 5 Months (log_{10} PFU mL^{-1})
-20°C	7.49±0.07Aa	7.42±0.14Aa	7.10±0.15Ab
4°C	7.48±0.15Aa	7.39±0.15Aa	7.47±0.11Ba
25°C	7.41±0.18Aa	7.44±0.17Aa	6.79±0.28Cb

Values of the number of phages (log_{10} PFU mL^{-1}) are means of four independent replicates ± standard deviation. Values with the same lower- and upper-case letters are not significantly (P>0.05) different within rows and columns, respectively, according to Fisher's Protected LSD Test. PFU= plaque forming units. *Escherichia coli* O157:H7 NCTC 12900 was used as the propagation host.

Table 5: Stability of the phage UAE_MI-01 under different pH values.

pH value	3	4	7	9	10
(log_{10} PFU mL^{-1})	0.00 ± 0.00a	5.72 ± 0.12b	7.79 ± 0.11c	7.76 ± 0.15c	7.78 ± 0.16c

Values of the number of phages (log_{10} PFU mL^{-1}) are means of four independent replicates ± standard deviation. Values with the same letters are not significantly (P>0.05) different within rows, according to Fisher's Protected LSD Test. The initial titer was 8 x 107 PFU mL-1. PFU= plaque forming units. *Escherichia coli* O157:H7 NCTC 12900 was used as the propagation host.

2.3.5 Effects of chemical disinfectants on the phage UAE_MI-01

Sensitivity of UAE_MI-01 to common laboratory chemical disinfectants was studied. After exposing the phage to four different disinfectants for 3 min, the phage was found to be relatively resistant to commercial disinfectant 20% and 70% ethanol. However, it was completely abolished by sodium hypochlorite 2% and a commercial liquid hand wash after 3 min (Table 6).

Table 6: Effects of chemical disinfectants on the phage UAE_MI-01.

Disinfectant	Initial Titer (log_{10} PFU mL^{-1})	After 2 Min (log_{10} PFU mL^{-1})	After 3 Min (log_{10} PFU mL^{-1})	Percentage of Survivors (%)
Ethanol 70	7.48±0.11Aa	6.62±0.06Ab	4.57±0.11Ac	0.13% ±0.02A
Liquid hand wash	7.44±0.18Aa	7.11±0.07Bb	0.00±0.00Bc	0.00%±0.00B
Sodium hypochlorite 2%	7.51±0.15Aa	6.09±0.10Cb	0.00±0.00Bc	0.00%±0.00B
Commercial disinfectant 20%	7.45±0.10Aa	7.09±0.06Bb	7.06±0.13Cb	66%±0.21C

Values of the number of phages (log_{10} PFU mL^{-1}) are means of four independent replicates ± standard deviation. Values with the same lower- and upper-case letters are not significantly (P>0.05) different within rows and columns, respectively, according to Fisher's Protected LSD Test. PFU= plaque forming units. The initial titer was 8 x 10^7 PFU mL^{-1}. PFU= plaque forming units. *Escherichia coli* O157:H7 NCTC 12900 was used as the propagation host.

2.3.6 Determination of the adsorption time, adsorption rate constant, latent period and burst size of the phage UAE_MI-01

To determine the adsorption time, adsorption time constant, latent period and burst size, one-step growth curve was performed. The adsorption happened within 10 min (eclipse period) with 99.9% efficiency. The adsorption rate constant (K) of UAE_MI-01 was found to be 1.25 X 10^{-7} mL min^{-1}. The latent period was found to be approximately 20 min with burst size of more than 100 PFU $cell^{-1}$ in *E. coli* O157:H7 NCTC 12900 (Figure 2).

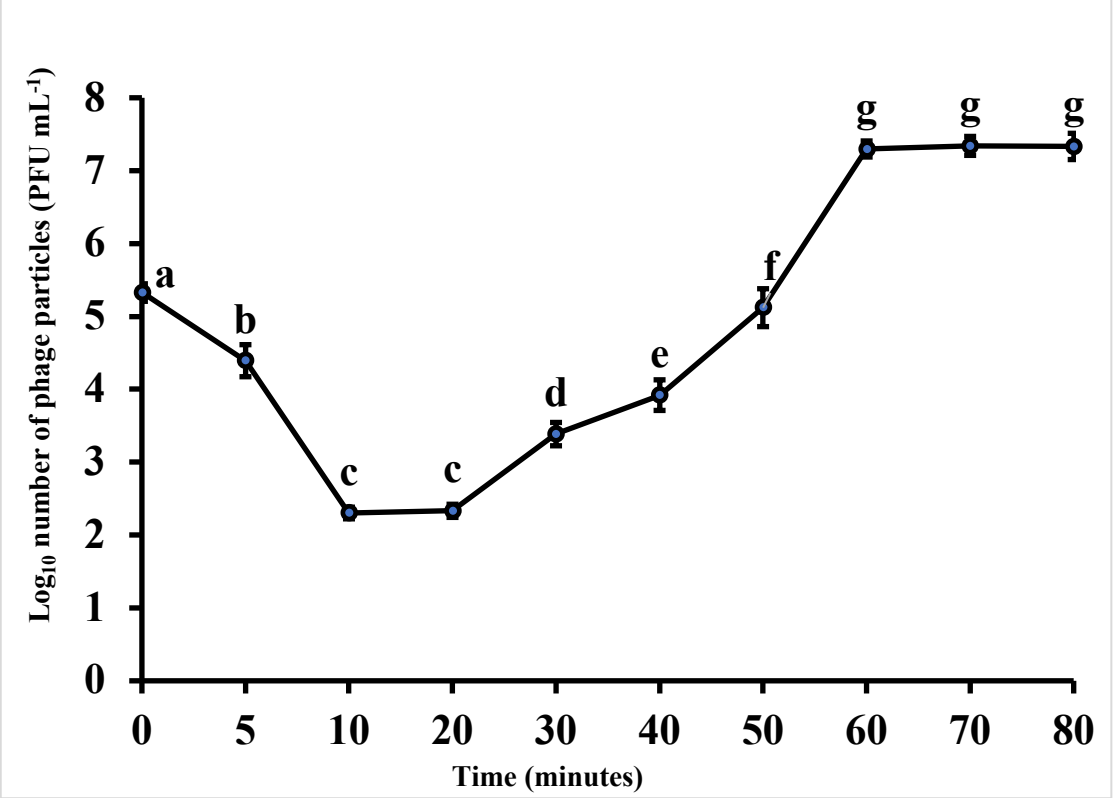

Figure 2: One step growth curve of phage UAE_MI-01

One *s*tep *g*rowth curve of phage UAE_MI-01 with *Escherichia coli* O157:H7 NCTC 12900 as the propagation host. Values are means ± standard deviation of four replicates for each timing. Mean values followed by different letters are significantly ($P<0.05$) different from each other according to Fisher's Protected LSD Test. Bars represent standard deviation. PFU= plaque forming units.

2.3.7 Electron microscopy

The morphological characterization of the phage UAE_MI-01 was studied by transmission electron microscopy (TEM), which revealed that the phage consisted of an icosahedral head, flexible non-contractile tail suggesting that UAE_MI-01 belongs to the family of Siphoviridae in the Caudovirales order. The icosahedral head dimension was approximately 50 nm while the tail dimension was almost 130 nm (Figure 3).

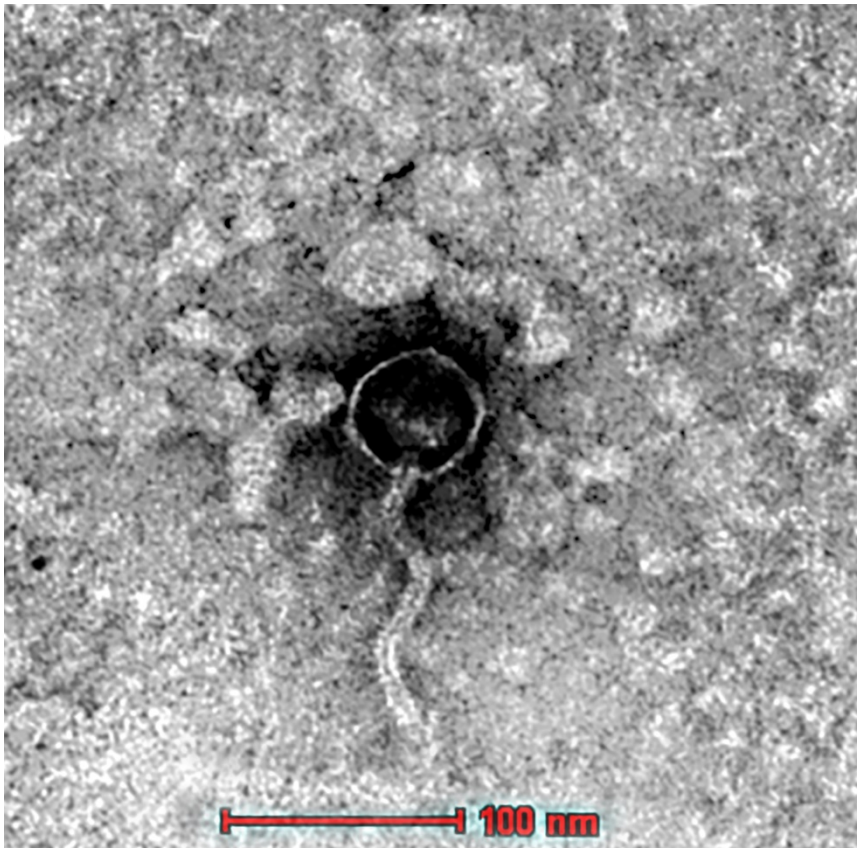

Figure 3: Transmission electron micrograph of the phage UAE_MI-01 phage.
Scale bar = 100 nm.

2.3.8 Genomic analysis and bioinformatics

The complete genome sequence of the phage UAE_MI-01 has been deposited with the accession number MW862804 in GenBank and announced previously (Sultan-Alolama et al., 2021). Annotation of UAE_MI-01 genome predicted 64 ORFs but no tRNA genes and no antimicrobial resistance genes. Its genome includes structural/assembly genes, including genes for HNH endonuclease, terminase, capsid protein, tail assembly proteins, tape measure proteins, and major and minor tail proteins (Figure 4). Replication/transcription-related genes included genes for helicase, helicase-primase and DNA polymerase I, endolysin and holin-like genes suggesting that UAE_MI-01 is a lytic phage and uses this mechanism to lyse the host cell membranes. Holin-like class I and class II genes and lysin, as depicted in Figure 4, are located adjacent to each other in the UAE_MI-01 genome. A search of the NCBI Conserved Domain Database confirmed the presence of an "endolysin and autolysin" conserved domain in the lysin sequence. Prediction of secondary structure using DeepTMHMM

(https://dtu.biolib.com/DeepTMHMM) indicated the presence of transmembrane (TM) helices characteristic of holin proteins. Proteins essential for lysogenic cycle like integrase, recombinase, excisionase and repressors were not encoded in the genome of UAE_MI-01. UAE_MI-01 was found to be free of any antibiotic resistant genes.

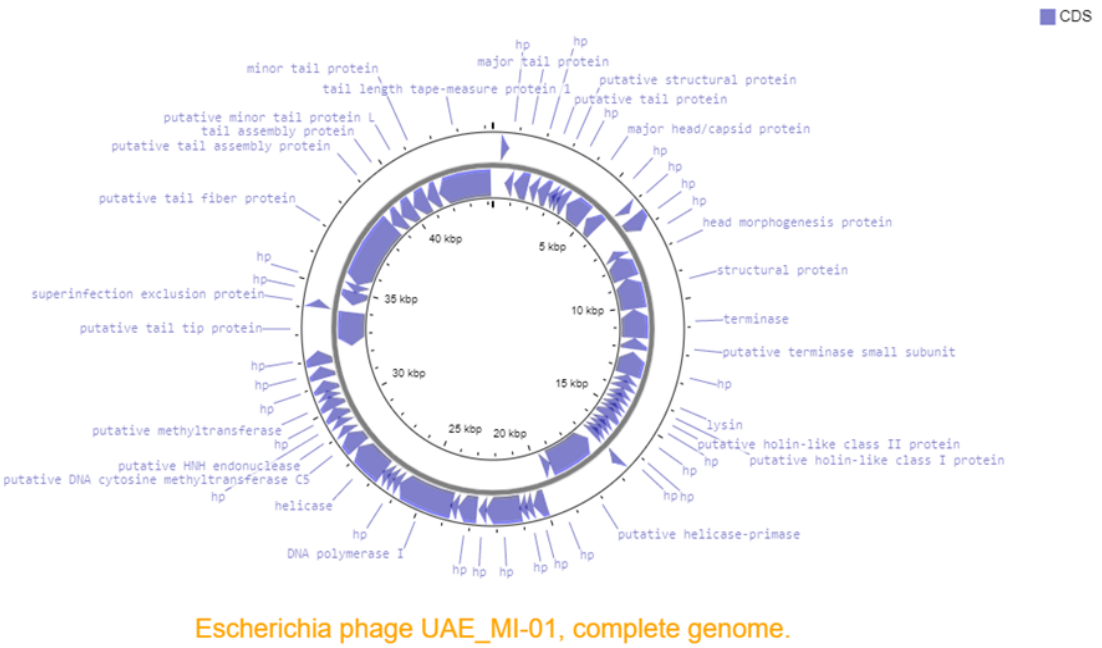

Figure 4: Genome map of the phage UAE_MI-01 drawn using Proksee.
Blue arrows show ORFs with predicted annotations. hp indicates hypothetical protein.

An NCBI BLASTN search using the whole genome sequence of UAE_MI-01 revealed that it shared 92.6% sequence identity (98% coverage) with *Escherichia* phage SSL-2009a (NCBI GenBank accession FJ750948), 92.8% sequence identity (96% coverage) with *Escherichia* phage YD-2008.s (NCBI GenBank accession KM896878) and 91.38% sequence identity (92% coverage) with *Escherichia* phage Gluttony_ev152 (NCBI GenBank accession LR597646). However, it is unknown if these phages could specifically target *E. coli* O157:H7. A comparison of the whole genome of the above-mentioned phages were performed using progressiveMauve (Figure 5). A phylogenetic tree (Figure 6, Table 7) which was generated using the terminase large subunit (TerL) protein showed that *Escherichia* phage TheodorHerzl (GenBank accession MZ501107) and *Escherichia* phage vB_EcoS_Teewinot (GeneBank accession OK499993) have the most similar TerL with UAE_MI-01. *Escherichia* phages TheodorHerzl, KarlBarth and Envy were also found to have similar terminase large (TerL) and small (TerS) subunit proteins to

UAE_MI-01 as evident from the phylogenetic trees (Figure 6 and Figure S1, Table 7 and Table 8). *Escherichia* phage KarlBarth holin class I and lysin protein sequences also shared very high similarity with UAE_MI-01 and featured commonly in the respective phylogenetic trees (Figures 8 and 9, Tables 9 and 10), while *Escherichia* phages YD-2008.s and HK578 had holin class I and class II protein sequences similar to UAE_MI-01 and featured in the phylogenetic trees generated with these proteins (Figures 9 and 10, Tables 10 and 11).

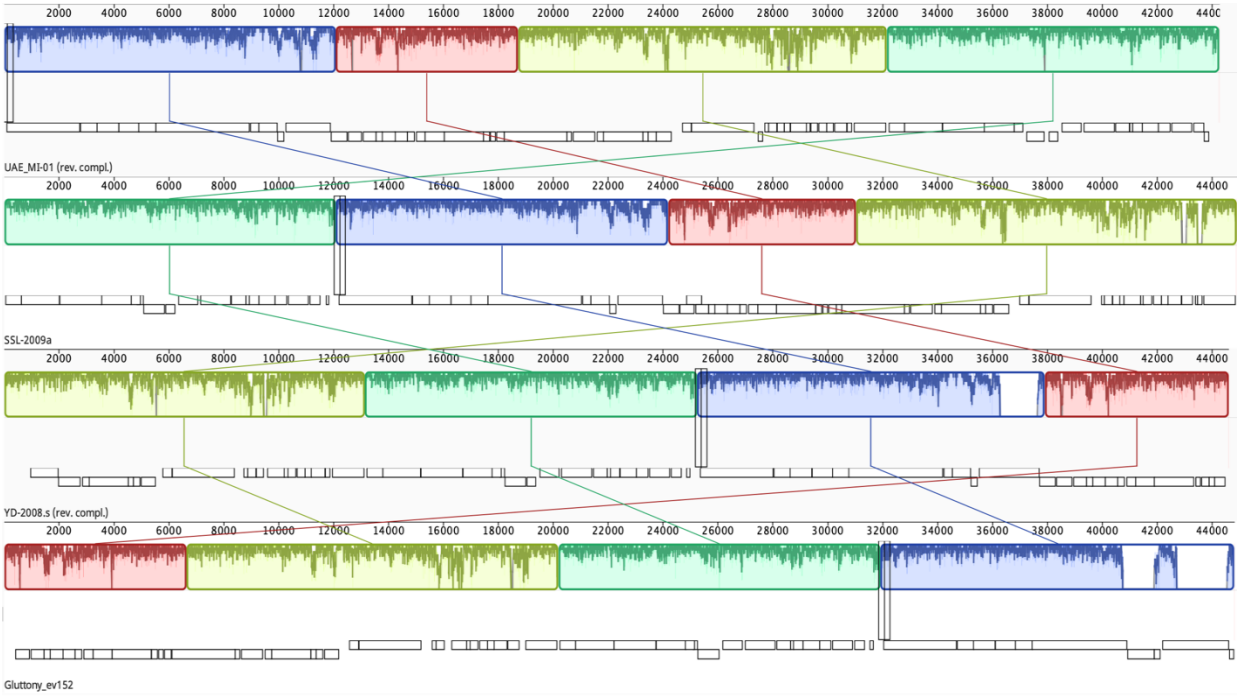

Figure 5: Whole genome alignment of the phage UAE_MI-01 and three phage genomes that were most similar to it as identified by a BLASTN search.

The reverse complement of the UAE_MI-01 and YD-2008.s sequences were used so that the terminase genes are on the forward strand. The alignment was generated using progressiveMauve. Colored blocks match regions in different genomes.

Table 7: Phage terminase large subunit sequences most identical to the phage UAE_MI-01 based on an NCBI BLASTP search.

Bacteriophage	Percentage Identity (%)	Sequence Length	Accession
Escherichia phage UAE_MI-01	100.00	461	QVD49023.1
Escherichia phage vB_EcoS_Teewinot	99.57	461	UGO51122.1
Escherichia phage welsh	99.35	461	QHR68122.1
Escherichia phage slur05	99.35	461	YP_009208118.1
Siphoviridae sp.	99.13	461	DAV90089.1
Escherichia phage bob	99.35	461	QHZ59646.1
Escherichia phage GeorgBuechner	99.13	461	QXV79450.1
Escherichia phage JLBYU60	99.13	461	UGO55258.1
Escherichia phage TheodorHerzl	99.13	461	QXV85009.1
Escherichia phage Envy	98.92	461	YP_009288166.1
Escherichia phage jat	98.92	461	QHR76385.1
Siphoviridae sp.	98.48	461	DAO67471.1
Escherichia phage KarlBarth	98.70	461	QXV81918.1
Escherichia phage vB_EcoS_Over9000	98.70	461	UGO49841.1
Siphoviridae sp.	98.92	461	DAO89130.1
Siphoviridae sp.	98.70	461	DAG74011.1

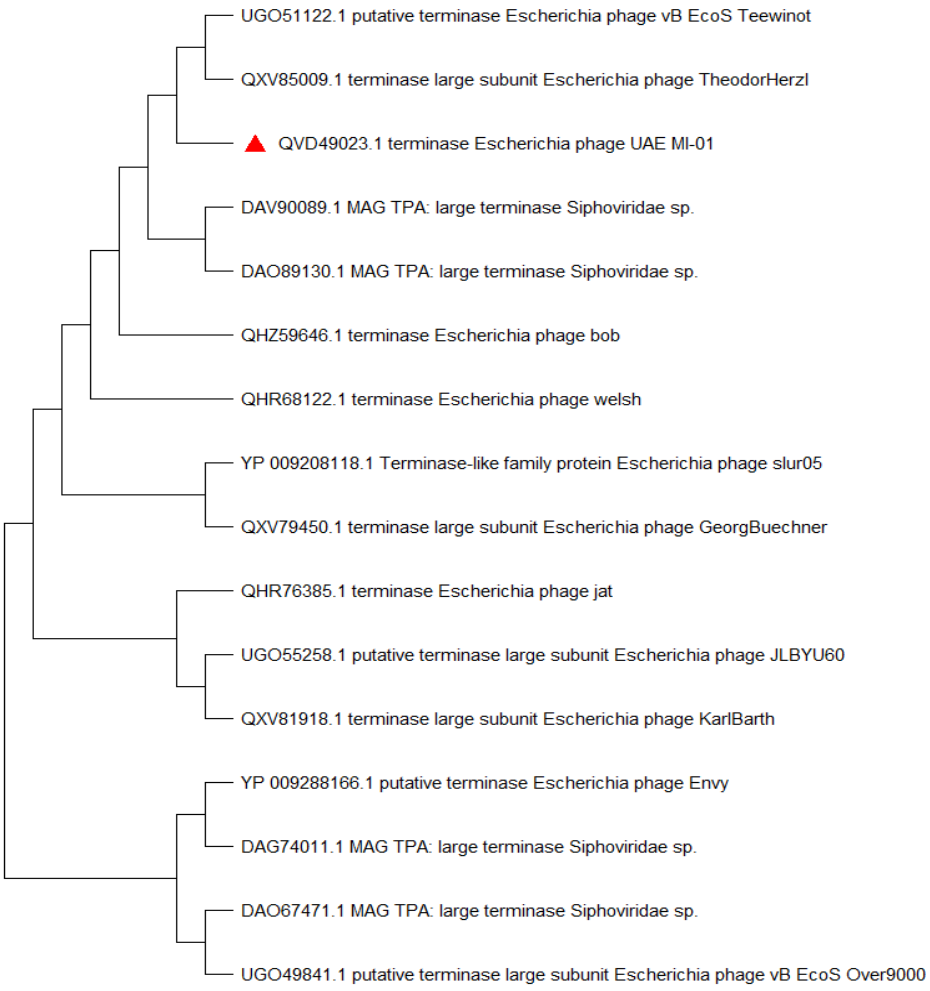

Figure 6: Phylogenetic tree showing position of the phage UAE_MI-01

Phylogenetic tree showing position of the phage UAE_MI-01 (red triangle) based on the amino acid sequence of the terminase large subunit (TerL) of the closest 15 sequences to UAE_MI_01 TerL identified using NCBI BLASTP. Sequences were aligned using MUSCLE in MEGA 11 (https://www.megasoftware.net) and the phylogenetic tree was generated using the neighbor joining method.

Table 8: Phage terminase small subunit sequences most identical to the phage UAE_MI-01 based on an NCBI BLASTP search.

Bacteriophage	Percentage Identity (%)	Accession Length	Accession
Escherichia phage vB_EcoS_Over9000	98.91	190	UGO49840.1
Escherichia phage Oekolampad	98.36	183	QXV82967.1
Escherichia phage TheodorHerzl	98.36	183	QXV85008.1
Siphoviridae sp.	98.36	190	DAH32446.1
Siphoviridae sp.	98.36	190	DAH38271.1
Escherichia phage EC115	98.36	190	UR083311.1
Escherichia phage EK99P-1	98.36	183	YP_009055279.1
Escherichia phage vB_EcoS_011D5	97.81	190	QMP82803.1
Sodalis phage SO1	97.81	190	YP_003344936.1
Escherichia phage Envy	97.81	183	YP_009288167.1
Siphoviridae sp.	97.27	190	DAW21243.1
Escherichia phage vB_EcoS_PNS1	97.27	183	AZF89826.1
Shigella phage EP23	97.27	190	YP_004957437.1
Escherichia phage KarlBarth	96.72	183	QXV81917.1
Escherichia phage PaulFeyerabend	97.27	183	QXV83304.1

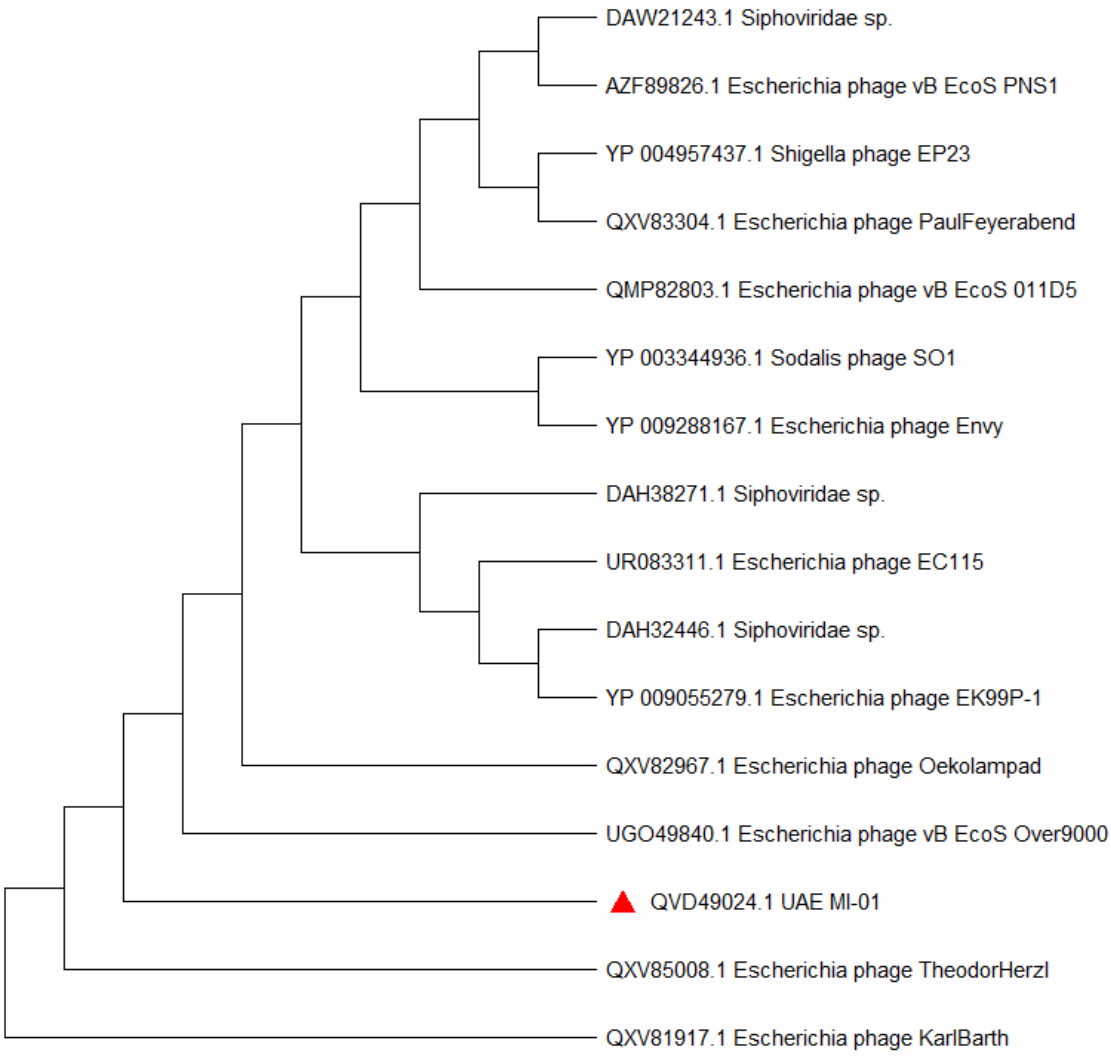

Figure 7: Phylogenetic tree constructed from terminase small subunit sequences listed in Table 8.

Table 9: Phage lysin sequences most identical to the phage UAE_MI-01 based on an NCBI BLASTP search.

Bacteriophage	Percentage Identity (%)	Accession Length	Accession
Escherichia phage vB_EcoS_PNS1	97.92	163	AZF89823.1
Escherichia phage bob	97.92	144	QHZ59650.1
Escherichia phage SECphi4	97.22	144	QJI52545.1
Escherichia phage PC2	97.92	163	URX65774.1
Escherichia phage vB_EcoS_WFI	97.22	144	QBQ80576.1
Dhillonvirus JL1	97.22	163	YP_006990393.1
Escherichia phage BF9	97.92	163	QXL91301.1
Escherichia phage vB_EcoS_L-h 1M	97.22	144	UNY42290.1
Escherichia phage vB_EcoS_WF5505	96.53	144	QBQ80509.1
Escherichia phage Oekolampad	97.22	163	QXV83029.1
Escherichia phage KarlBarth	96.53	163	QXV81978.1
Sodalis phage SO1	96.53	163	YP_003344991.1
Siphoviridae sp.	97.22	163	DAO89133.1
Shigella phage vB_SboS_StarDew	96.53	163	UGO46670.1
Shigella phage EP23	96.53	163	YP_004957490.1

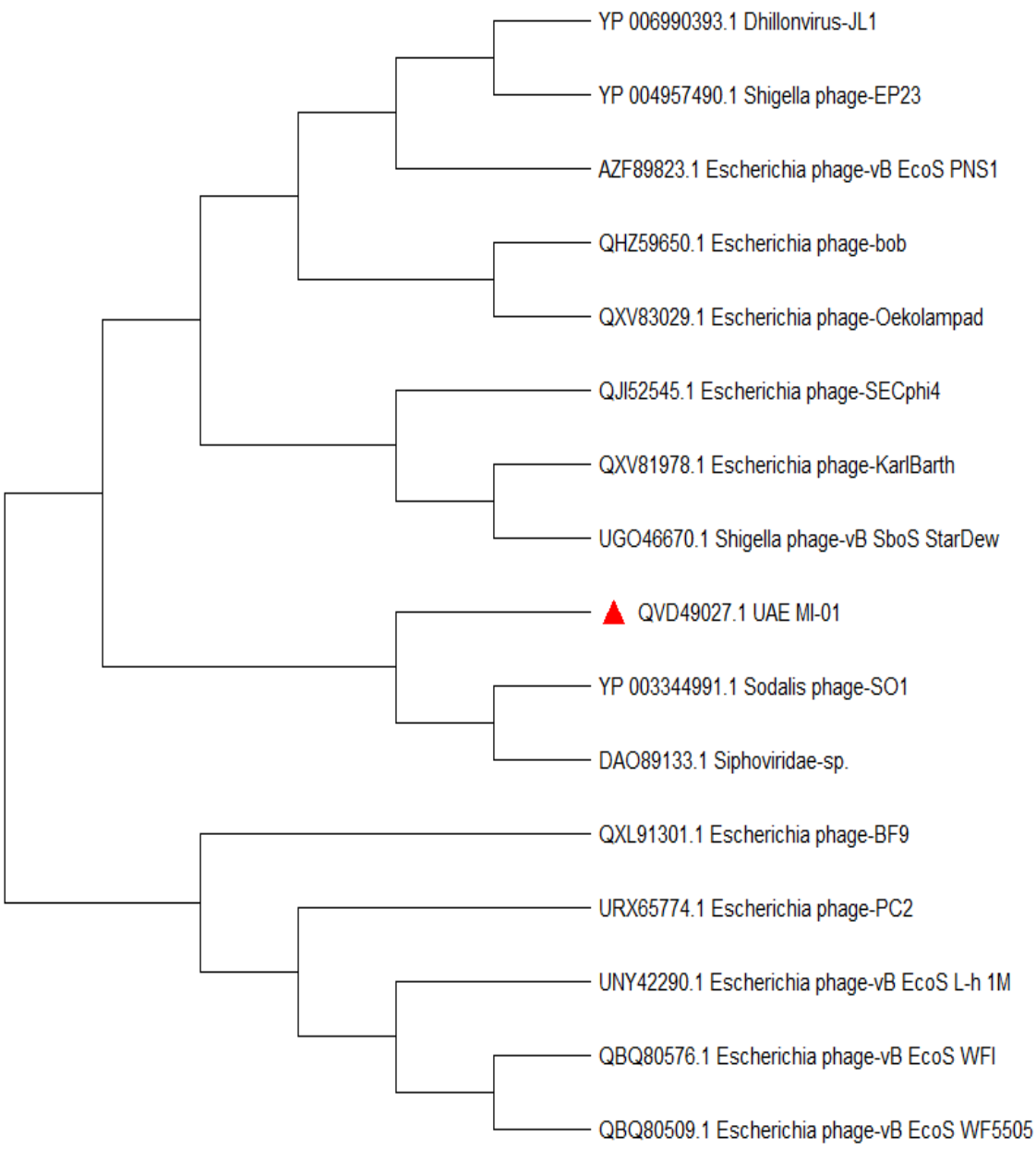

Figure 8: Phylogenetic tree constructed from lysin sequences listed in Table 9.

Table 10: Table S4. Phage holin class I sequences most identical to the phage UAE_MI-01 based on an NCBI BLASTP search.

Bacteriophage	Percentage Identity (%)	Accession Length	Accession
Bacteriophage sp.	98.77	81	UVX62569.1
Escherichia phage vB_EcoS_Opt212	97.53	81	UHS64789.1
Escherichia phage TheodorHerzl	96.30	81	QXV85068.1
Escherichia phage JLBYU60	96.30	81	UGO55315.1
Escherichia phage EK99P-1	95.06	81	YP_009055335.1
Escherichia phage vB_EcoS_PNS1	96.30	81	AZF89822.1
Escherichia phage vB_EcoS_Over9000	95.06	81	UGO49900.1
Escherichia phage YD-2008.s	96.30	81	YP_009152287.1
Dhillonvirus JL1	95.06	81	YP_006990394.1
Siphoviridae sp.	95.06	81	DAO89146.1
Escherichia phage HK578	96.30	81	YP_007112663.1
Siphoviridae sp.	95.06	81	DAQ64891.1
Escherichia phage JLBYU37	93.83	81	UGO56857.1
Escherichia phage PC2	95.06	81	URX65775.1
Escherichia phage KarlBarth	95.06	81	QXV81977.1

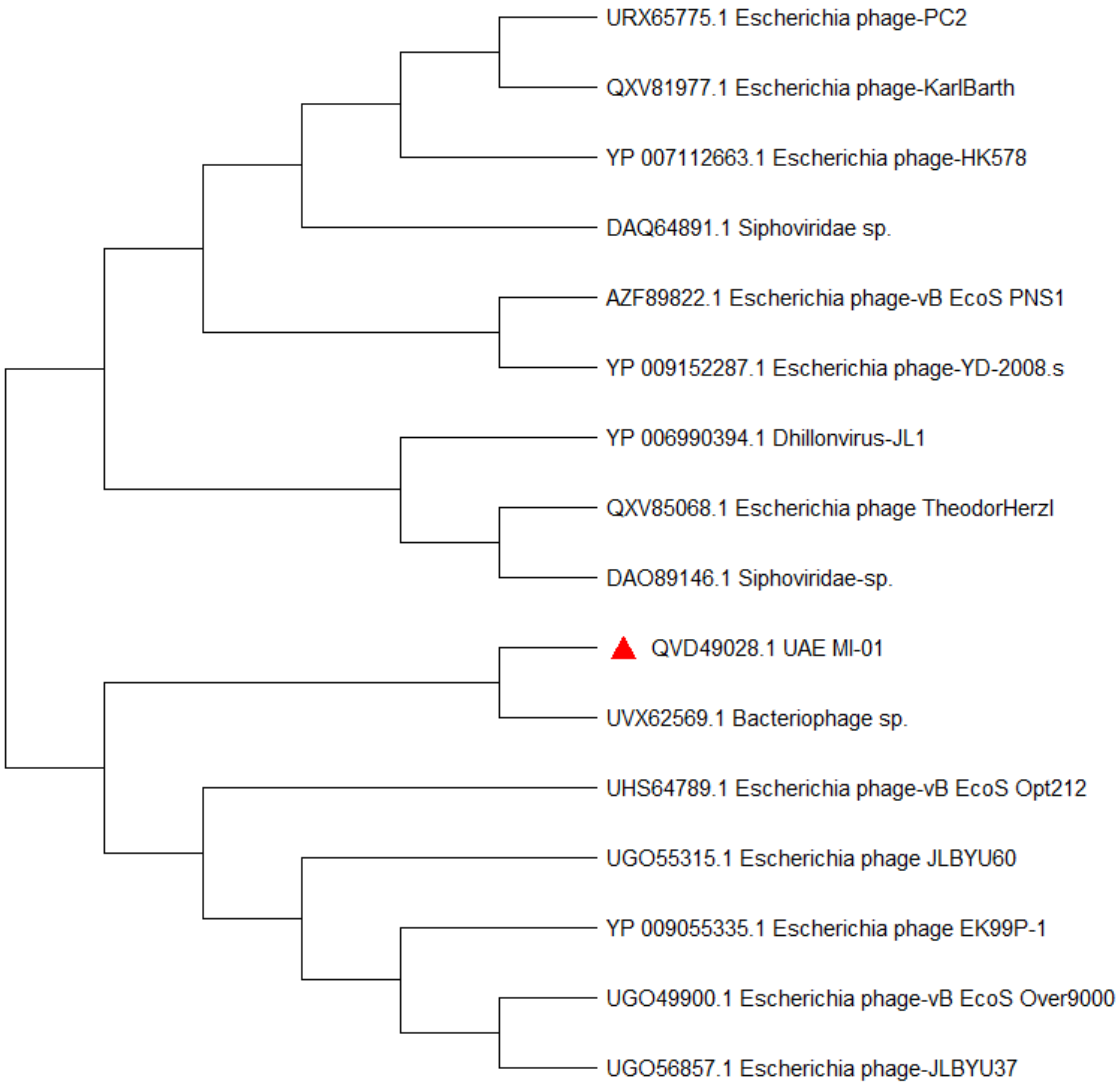

Figure 9: Phylogenetic tree constructed from holin class I sequences listed in Table 10.

Table 11: Phage holin class II sequences most identical to the phage UAE_MI-01 based on an NCBI BLASTP search.

Bacteriophage	Percentage Identity (%)	Accession Length	Accession
Escherichia phage ZCEC11	98.96	96	UJQ87858.1
Escherichia phage vB_EcoD_Pubbukkers	97.93	96	UGO50027.1
Bacteriophage sp.	97.92	96	DAG52103.1
Escherichia phage YD-2008.s	97.92	96	YP_009152288.1
Siphoviridae sp.	96.88	96	DAH32477.1
Shigella phage EP23	96.88	96	YP_004957488.1
Escherichia phage SECphi4	95.83	96	QJI52543.1
Escherichia phage vb_EcoS_bov11C2	95.83	96	QNR53577.1
Siphoviridae sp.	95.83	96	DAW21288.1
Escherichia phage PEC14	96.88	96	UVD33129.1
Escherichia phage vB_EcoS_PNS1	95.83	96	AZF89821.1
Escherichia phage HK578	94.79	96	YP_007112662.1
Bacteriophage sp.	94.79	96	UVX62568.1
Escherichia phage JLBYU27	94.79	96	UGO55330.1
Escherichia phage vB_EcoS_Teewinot	95.83	96	UGO51174.1

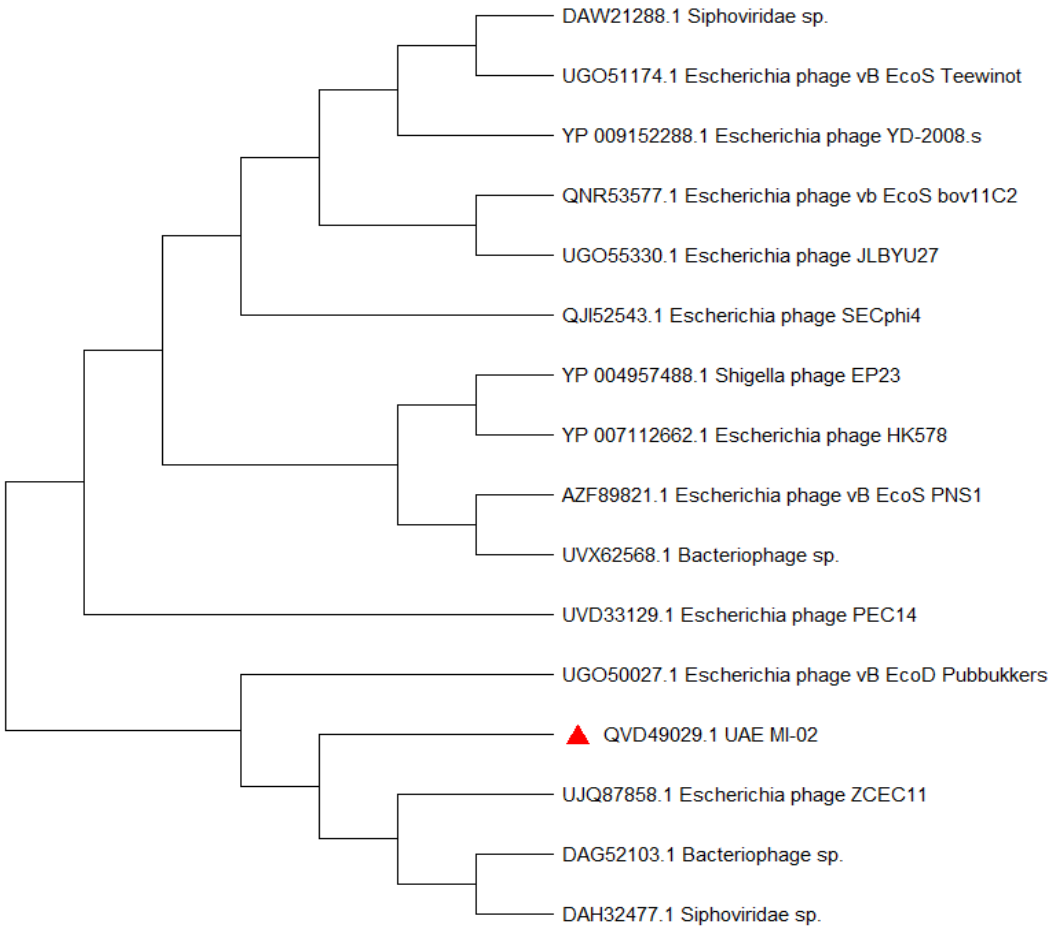

Figure 10: Phylogenetic tree constructed from holin class II sequences listed in Table 11.

2.4 Discussion

The aim of this investigation was to identify novel phages against the verotoxin producing *E. coli* O157:H7 which is a food borne pathogen reported as the main cause of many outbreaks worldwide. Many pathological symptoms have been associated with such bacteria including abdominal cramps, diarrhea, hemolytic colitis, hemolytic uremic syndrome (HUS), kidney failure. These effects are often serious enough and may lead to death in some cases (Thomas et al., 2015). Due to their ability to recognize and eliminate specific bacterium, bacteriophages are considered a potent biocontrol agent since their discovery in 1917.

UAE_MI-01, a lytic bacteriophage of *E. coli* O157:H7, was isolated from pigeon feces. To the best of our knowledge, this is the first report of a *E. coli* O157:H7 bacteriophage from a bird. All previously reported *E. coli* O157:H7's bacteriophages were

isolated from ruminants (Callaway et al., 2008; Kropinski et al., 2013) and swine (Morita et al., 2002) and environmental samples (Y.-D. Lee & Park, 2015; Park et al., 2012). The host range investigation and one-step-curve showed that UAE_MI-01 targets *E. coli* O157:H7 strain effectively. However, many of the reported *E. coli* O157:H7's bacteriophages had wider host range (Park et al., 2012; Kim et al., 2021). This makes UAE_MI-01 an ideal biocontrol agent in food, veterinary and agriculture industry.

UAE_MI-01 showed media preference, since its largest plaque was formed on soyabean casein digest agar and produced the smallest plaque size (2 mm) on Mueller Hinton agar. However, the number of plaques was significantly ($P<0.05$) higher when nutrient agar medium was used, and this was followed by soyabean casein digest agar and Mueller Hinton agar (Table 2). In addition, the phage UAE_MI-01 produced the lowest number of plaques on Luria-Bertani agar. The stability of the phage UAE_MI-01 in different environmental conditions such as temperature was as high as 65°C , pH values between 4-10, similar to HY01 bacteriophage (H. Lee et al., 2016), in addition to its stability in many chemical disinfectants, emphasizes its applications in food, veterinary and agriculture industries.

The latent period of UAE_MI-01 was observed to be 20 min. This is longer than the latent period of bacteriophages vB_Eco4M-7 and ECML-117, which was 10 min (Necel et al., 2020) for HY01, 15 min (H. Lee et al., 2016)**,** and in contrast to SFP10 which was 25 min (Park et al., 2012, p. 10). However, the average burst size of almost 100 PFU cell^{-1} for UAE_MI-01 was similar to both vB_Eco4M-7 and ECML-117 which is four times greater than burst size of HY01, which was 25 PFU cell^{-1}, but half of the burst size of phage SFP10, which was more than 200 PFU cell^{-1}. This is another important factor that support the suitability of UAE_MI-01 for the intended applications.

The current study supports the use of phage as an alternative rapid tool for detection of bacteria in a sample. Since detection of phage in any animal or environmental sample indicates the presence of the host cell (bacteria) in that animal or environment.

The presence of endolysin and holin-like genes suggests that UAE_MI-01 is a lytic phage. This is also evident from the morphology of the plaques (Figure 1) and one-step-growth (Figure 2). Most tailed phages make use of the archetypical holin-endolysis system to effect the lysis of the host cell and the release of virions (Li et al., 2021; Wang et al.,

2000; Wu et al., 2021). Holin-like class I and class II genes and lysin were identified indicating lytic activity. The completeness of the catalytic domain of lysin was confirmed by a matching hit in the NCBI Conserved Domain Database. Furthermore, the holin proteins were also predicted to possess TM helices characteristic of the role of holin proteins in the membrane. Additionally, no lysogenic components, including integrase, recombinase, excisionase and repressors that are hallmarks of lysogenic cycle, were identified in the genome (Li et al., 2010).

A whole genome BLAST search identified phages with genome sequences that are similar to UAE_MI-01. The alignment revealed that the genomic arrangements were largely preserved in all the compared phages. Phage SSL-2009a was isolated from an engineered *E. coli* culture, while phages YD-2008.s and Gluttony_ev152 were isolated from goat and human feces, respectively (Li et al., 2010). While phage SSL-2009a was reported to infect several *E. coli* strains, its ability to infect *E. coli* O157:H7 was not considered (Li et al., 2010).

Being a small phage with a genome size of 44,281 bp that is free of any antimicrobial resistance genes, it posses advantages from the perspective of phage therapy and as a food bio-preservative.

As many phages are being marketed as natural food preservatives with the approval of US FDA, UAE_MI-01, with the above-mentioned capabilities, could be an ideal candidate for natural biocontrol as well as in phage therapy.

Chapter 3: Comparative Genomic Analysis and Characterization of Bacteriophage Ec_MI-02 Infecting *Escherichia coli* O157:H7

3.1 Introduction

Escherichia coli was first discovered in 1885 by Theodor Escherich (Escherich, 1989). *E. coli* O157:H7 is the most important serotype of Shiga-toxigenic *E. coli* (STEC) for its role in causing foodborne illnesses. *E. coli* O157:H7 has a low infectious dose of 50–100 CFU g^{-1} of solid materials or 50–100 CFU mL^{-1} of liquid materials (Puligundla & Lim, 2022) due to its stress resistance mechanisms and surviving in low pH environments such as acidic food. *E. coli* O157:H7 could cause various gastroenteritis symptoms such as diarrhea (Slutsker et al., 1997), hemolytic uremic syndrome (Boyce et al., 1995), hemorrhagic colitis (Honish et al., 2005) and thrombotic thrombocytopenic purpura (Kovacs et al., 1990) and may even cause death (Thomas et al., 2015). The main sources of *E. coli* O157:H7 infection are livestock, poultry and their products (Li et al., 2021) and polluted water (Iwu et al., 2022).

Furthermore, multidrug resistant *E. coli* is a global health challenge in human and veterinary medicine (Poirel et al., 2018). Different pathogenic and commensal *E. coli* have successfully developed antibacterial resistance by acquiring, for example, genes that encode for carbapenemases (becoming resistant to carbapenems), plasmid-mediated quinolone resistance (PMQR) (becoming resistant to [fluoro]quinolones), extended-spectrum β-lactamases (becoming resistant to cephalosporins), mobile colistin resistance (becoming resistant to polymyxins), and 16S rRNA methylases (becoming resistant to aminoglycosides) (Poirel et al., 2018).

Phage therapy, the use of bacteriophages as a natural antibacterial agent, was introduced soon after the discovery of bacteriophage by Frederick Twort in 1915 (Twort, 1915) and Félix d'Hérelle in 1917 (d'Herelle, 1917). Bacteriophages, in addition to their use as alternative natural antibiotic, have many applications in different fields such as phage-display technology (Goracci et al., 2020; Hess & Jewell, 2020; Zhao et al., 2021), drug delivery systems (Sioud, 2019), diagnostics (Machera et al., 2020), food industry and in environmental sciences (Wittebole et al., 2014; Janczuk-Richter et al., 2019).

Phages are mainly divided into two categories, virulent phages that replicate by the lytic cycle, and temperate phages that replicate by either the lytic cycle or lysogenic cycle (Pham-Khanh et al., 2019). Virulent phages are widely used in phage therapy, as alternative antibacterial agent, and in food industry, as a food preservative. Both these applications are well documented (Keen & Adhya, 2015; Soffer et al., 2016; Fernández et al., 2019).

The United States Food and Drug Administration (FDA) approved the use of phages as food preservative since 2006. Subsequently, several phage products were developed as safe bacterial biocontrol agents (Abuladze et al., 2008). Despite several advantages of phage therapy over antibiotics (Pirisi, 2000; Loc-Carrillo & Abedon, 2011), phage therapy also has some challenges like the ability of the bacterial host to develop resistance to its phage (Örmälä & Jalasvuori, 2013; Doss et al., 2017) through many mechanisms and strategies such as preventing phage entry (Cumby et al., 2015; Simmons et al., 2018; Rostøl & Marraffini, 2019), restriction-modification systems (Tock & Dryden, 2005), interference with the assembly of bacteriophages (Penadés & Christie, 2015), CRISPR-Cas systems (Hille et al., 2018), abortive infection and toxin-antitoxin systems (Durmaz & Klaenhammer, 2007; Harms et al., 2018) and prokaryotic argonauts (Hegge et al., 2018) that are developed by bacteria to eliminate their attackers. Host specificity of phages or in other words, narrow host range of phages is another challenge that limits the efficacy of phages to combat the target bacteria (Durmaz & Klaenhammer, 2007; Harms et al., 2018).

There has been an increase in clinical trials of therapeutic phage products as well as the increase of globally distributed phage-based therapy centers (Górski et al., 2020). Therefore, the search for new sources for bacteriophages and isolation and characterization of virulent phages that are capable of eliminating undesired pathogenic bacteria as well as food contaminating bacteria is in high demand and an urgent necessity (Tock & Dryden, 2005; Keen & Adhya, 2015; Pham-Khanh et al., 2019).

Human and animals harbor a wide range of bacteria and consequently bacteriophages. This current study suggests that wild animals, specifically wild birds can be a very good source for bacteriophages. In the current study we explored wild pigeons and isolated the bacteriophage Ec_MI-02 from its feces. Ec_MI-02 was characterized and

its genome was sequenced and compared to other *E. coli* phages. The characterization was performed using *E. coli* O157:H7 as the propagation host.

Ec_MI-02 is the second *E. coli* O157:H7 phage isolated from the same bird. Previously, we reported UAE_MI-01 (Sultan-Alolama et al., 2022), another *E. coli* O157:H7 phage from birds. Both phages were characterized to emphasize the diversity of phages within a single source. Thus, this study enriches the collection of phages that could be used to develop cocktails for better food preservation and therapeutic applications.

3.2 Materials and methods

3.2.1 Propagation media

Luria–Bertani broth (LBB; tryptone 10 g L^{-1}, yeast extract 5 g L^{-1} and sodium chloride 10 g L^{-1} pH 7.2 (HiMedia, Mumbai, India) was used in all the protocols in the current study. Bacterial dilutions from 18 h LBB cultures grown at 37 °C were carried out in phosphate-buffered saline (PBS; Oxoid, Basingstoke, UK).

For performing the plaque assay, the soft layer agar which consisted of LBB broth in Lambda-buffer (6 mmol L^{-1} tris pH 7.2; 10 mmol L^{-1} $Mg(SO_4)_2 \cdot 7H_2O$; 50 mg L^{-1} gelatin (HiMedia)) and supplemented with 4 g L^{-1} agar (HiMedia) was used. To determine the morphology of the plaques, Mueller Hinton agar (Mast group, Bootle, UK), tryptone soyabean agar (HiMedia), Luria-Bertani agar (HiMedia), and nutrient agar (HiMedia), were used. Ec_MI-02 was maintained and diluted in Lambda-buffer (6 mmol /L Tris pH 7.2; 10 mmol/L Mg $(SO_4)_2.7H_2O$; 50 mg/L gelatin (Oxoid)) and stored at 4°C.

3.2.2 Cultivation of host bacterium E. coli O157:H7

E. coli O157:H7 NCTC 12900 was used as the propagation host to isolate the phage Ec_MI-02 and also it was used for all the characterization study outlined below.

As previously described (Sultan-Alolama et al., 2022), cultures were stored at 20°C in LBB with 20% glycerol. Before investigation, a stock culture of the propagation host was maintained on Luria–Bertani agar plates.

One loopful of *E. coli* O157:H7 was used to inoculate a 15 mL sterile centrifuge tube with a flat cup (ExtraGene, Taichung City, Taiwan) containing 10 mL of LBB. The tube was then placed in a shaker incubator set to 37°C and 70 revolutions per minute (rpm) (Innova 4000, New Brunswick Scientific, Edison, New Jersey, USA).

The slurry of the bird's excrement was seeded with this bacterial stock solution as described below.

3.2.3 Isolation, purification, and propagation of the phage Ec_MI-02

As described previously (Sultan-Alolama et al., 2022), pigeon feces of a single nest was collected from Abu Dhabi, United Arab Emirates, and transferred to a sterile beaker (250 mL). The slurry was prepared with sterile distilled deionized water. Cultures of *E. coli* O157:H7 NCTC 12900 (18 h) was used to seed the slurry every 24 h for 96 h. The beaker was incubated in shaker incubator at 37°C and 70 rpm. After 96 h, 10 mL of the slurry was transferred into 15 mL sterile centrifuge tube with flat cup and centrifuged for 10 min at 12,000 rpm. Millipore membrane syringe filter (Pore size 0.22 µm, Millipore Corporation, New Bedford, MA, USA) was used to filter the supernatant. The filtrate was then diluted in Lambda-buffer using the ten-fold serial dilution technique (Page & Seemann, 2019).

A plate lysis procedure (Davis & Sinsheimer, 1963; Sambrook et al., 1989; Vidaver et al., 1973) was used to test all the dilutions for the presence of the phage. Briefly, an aliquot (100 µL) of each dilution was added to 100 µL of 24 h culture of *E. coli* O157:H7 NCTC 12900 (in LBB) in a sterile 1.5 mL Eppendorf micro-centrifuge tube (Greiner Bio-One GmbH, Frickenhausen, Germany). The tubes were gently mixed and was then incubated at 37°C for 10 min to facilitate bacteriophage-host cell attachment (Sambrook et al., 1989). The mixture was then transferred from the Eppendorf micro-centrifuge tube to a 10 mL Bijou bottle, and 2 ml of previously melted soft layer Luria–Bertani agar was then added. The content of the bottle was gently mixed by swirling and pouring over the surface of a plate of Luria–Bertani agar and allowed to set for 25 min at room temperature before incubating for 24 h at 37°C.

Only the single clear plaque was aseptically picked with a scalpel, transferred to an Eppendorf microcentrifuge tube containing 1 mL of Lambda buffer and mixed gently before filtration through a sterile Millipore membrane syringe filter (0.22 µm). The filtrate was serially diluted and propagated as mentioned above. Plates with nearly confluent plaques were used to prepare enriched phage suspensions by overlaying with 5 mL lambda buffer. Finally, chloroform was added to separate the bacteriophage from the host cells (Sambrook et al., 1989). Titer of the phage stocks in Lambda buffer was calculated using

Miles and Misra technique (Miles et al., 1938). The phage stock then was stored at 4°C for future experiments.

3.2.4 Determination of the host range of the phage Ec_MI-02

The following bacterial strains were used to determine the ability of Ec_MI-02 to infect various gram negative and positive bacteria including: *E. coli* O157:H7 NTCC 12900, *E. coli* ATCC 8739, *E. coli* ATCC 25922, *E. coli* ATCC 35218, *E. coli* ATCC NCTC 13841, *E. coli* ATCC 15223, *E. coli* ATCC 23227, *E. coli* ATCC 9637, *E. coli* ATCC 23224, *E. coli* (Patient isolate-urine), *E. coli* extended-spectrum beta lactamase-producing (ESBL) (patient isolate), *Bacillus subtilis* ATCC 6051, *Pseudomonas aeruginosa* ATCC 25668, *P. aeruginosa* ATCC 27853, methicillin-resistant *Staphylococcus aureus* (patient isolate), *S. aureus* ATCC 6358, *S. aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus saprophyticus* ATCC-BAA 750, *Streptococcus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 51299, *E. faecalis* (patient isolate), *Enterococcus casseliflavus* (patient isolate), *Enterobacter aerogenes* ATCC 13018, *Enterobacter hormaechei* (patient isolate), *Klebsiella pneumonia* ESBL-producing ATCC 700603, *K. pneumonia* KPC 2 +ve (patient isolate), *Haemophilus influenzae* ATCC 9007, *Stenotrophomonas maltophilia* ATCC 17666, *Salmonella enterica* ATCC 14028, *Salmonella* sp. (patient isolate), *Proteus vulgaris* ATCC 29905, and *Mycobacterium smegmatis* ATCC 607 (Table 1).

Phage host range was determined by spotting 10 µL of Ec_MI-02 suspension containing 10^{10} PFU mL^{-1} onto dry Luria–Bertani agar plates inoculated with all bacterial strains described above. Plates were incubated for 48 h at 37°C and examined for the formation of plaques (Williams et al., 1980).

3.2.5 Plaque morphology of the phage Ec_MI-02

To investigate the ability of the phage Ec_MI-02 to produce uniform clear plaques on different media, Ec_MI-02 suspension containing 10^2 PFU mL^{-1} was used following the plate lysis procedure described above. Four different media were used Mueller Hinton agar (Mast group), tryptone soyabean agar (HiMedia), Luria-Bertani agar (HiMedia), and nutrient agar (HiMedia). Ec_MI-02 was grown on all four media to determine which type of these common bacteriological media Ec_MI-02 prefers,. This could ultimately

determine how much it will cost to commercially produce the phage for any future application.

3.2.6 Effect of physical and chemical agents on the phage Ec_MI-02 stability and viability

The impact of a few selected physical and chemical factors on the survival and proliferation of the phage Ec_MI-02 was investigated according to Dowding (Dowding, 1973).

To determine the effects of the selected physical treatments on phage propagation and viability, LBB-grown Ec_MI-02 phage was diluted 1 to 10 in LBB. Sample (0.1 mL) was added to 0.9 mL of LBB, and the physical treatment was applied according to the desired concentration. Viable phage numbers were estimated in the selected time points after the treatment, and it was expressed as PFU mL^{-1}. The control used was a similarly diluted Ec_MI-02 phage but untreated with the selected physical treatments (Brownell et al., 1967).

The physical treatments tested were pH and temperature. To determine the effect of pH on the viability and propagation of the phage Ec_MI-02, different pH values (pH 3, 4, 7, 9, 10) were used. Aliquots (0.1 mL) of phage suspension (10^9 PFU mL^{-1}) was added to 0.9 mL of each pH value in an Eppendorf micro-centrifuge tube and was incubated for 24 h at room temperature. After 24 h, viable phages were enumerated and the number of PFU was determined (Brownell et al., 1967).

With regards to temperature, the following temperatures were used: (i) temperatures: 25°C, (iii) heating the phage at 45°C, 55°C, 65°C, and 75°C for 15 min and 30 min, (iv) boiling at 100 °C for 15 and 30 min (Brownell et al., 1967). The viable phages were enumerated and the number of PFU mL^{-1} was determined (Brownell et al., 1967).

The effect of common laboratory disinfectants (ethanol 70%, commercial disinfectant (liquid hand wash soap 20%), and sodium hypochlorite 2%, on the stability of the phage Ec_MI-02 was determined using LBB grown preparations of Ec_MI-02. Aliquots (0.1 mL) phage (10^7 mL^{-1}) was added to 0.9 mL of each disinfectant at the specified concentration in distilled water. After 30-, 60- and 120-seconds incubation at room temperature, the mixtures were assayed for viable phage enumeration (PFU mL^{-1}). The control used was a similarly diluted Ec_MI-02 phage but untreated with the selected chemical treatments (Brownell et al., 1967).

3.2.7 Adsorption time, adsorption rate constant, latent period and burst size of the phage Ec_MI-02

The adsorption time and the adsorption rate constant of the phage Ec_MI-02 was determined by measuring residual plaque-forming ability in membrane-filtered samples of an attachment mixture as described by Dowding (Dowding, 1973). Briefly, a 250 mL Erlenmeyer flask containing 50 mL of LBB was inoculated with a host suspension (10^7 CFU mL^{-1}) and was incubated with shaking at 100 rpm for 3 h at 37°C. The phage was added at multiplicity of infection (0.1), and the flasks were incubated at 37°C. At various times, samples (1 mL) were removed, membrane filtered, diluted and plated and the numbers of PFU mL^{-1} were counted (Dowding, 1973). The ratio of the number of phages to the host was termed the multiplicity of infection and values much less than one are commonly used in kinetic studies on phage growth in order to ensure that each cell is infected by a single virus (Sykes et al., 1981).

The adsorption rate constant, K mL min^{-1} was calculated as described by Sykes *et al.* (Sykes et al., 1981) by using the equation $K = 2.3 / Bt \times \log_{10} (P_0/Pt)$ where B is the host concentration (CFU/ml); P_0 is the initial phage concentration (PFU mL^{-1}); Pt is the phage concentration at t min (PFU mL^{-1}); and t is the period of adsorption.

In order to determine the latent period, rise period and the burst size for the phage Ec_MI-02, a one-step growth experiment was carried out as described by Dowding (Dowding, 1973). A suspension (1×10^6 CFU mL^{-1}) of the propagation bacterial host was incubated in LBB for 3 h. A predetermined quantity of the phage was then added to give a low multiplicity of infection (approximately 0.1) and incubated for 20 min. Aliquots (10 mL) sample of the attachment mixture was removed and membrane filtered. Unadsorbed phages were removed from the infected host cells by passing the 10 mL of the broth through the filter paper to wash them. The filter paper was then transferred to a flask containing 50 mL of LBB at 37°C (first growth flask) and the infected cells were re-suspended by agitating the flask. A 50-fold dilution was made to another two flasks held at 37 °C (second and third growth flask) and the two flasks were re-incubated at 37°C. Samples (1 mL) were taken (from the first growth flask until time 60 min and then alternately from the second growth flask until 120 min and from the third flask until 180 min), filtered immediately, diluted and plated and the numbers of PFU were determined

after incubating the plates at 37°C for 48 h (Dowding, 1973). The one-step growth curve for each phage was plotted between PFU mL^{-1} and time in minutes.

3.2.8 Electron microscopy of the phage Ec_MI-02

To determine phage Ec_MI-02 morphology**,** TEM was used with a negative staining method. Staining with uranyl acetate (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was recommended by Ackermann and Heldal (Ackermann & Heldal, 2010) and Brum and Steward (Brum and Steward, 2010). Briefly, solutions containing uranyl acetate were filtered using sterile Millipore membrane syringe filters (0.22 µm). This solution was then placed in a 2 mL screw-cap tube. A drop of the phage suspension (10^{10} PFU mL^{-1}) was placed on a 200-mesh copper grid with carbon-coated Formvar film and the excess was removed. A drop of the filtered stain was placed on the grid which was loaded by the phage Ec_MI-02. After 5 min the excess stain was removed and allowed to dry for approximately 1 h**.** Dry grids were stored in a desiccator until inspection. Grids were investigated using a TEM (FEI Bio Twin Spirit G2 TEM, Eindhoven, The Netherlands).

3.2.9 Isolation of phage DNA

Ec_MI-02 was propagated overnight in LBB to reach titer of (10^{8}-10^{9} PFU mL^{-1}) as described previously. One mL of the suspension was used for DNA isolation using phage DNA isolation kit (Norgen Biotek Corp, Thorold, Canada), following the manufacturer's protocol. The extracted DNA was stored in -20°C for 48 h.

3.2.10 Genome sequencing and assembly

Samples were sent to Novagen (25 Pandan Crescent #05-15 TIC Centre, Singapore 128477) for whole genome sequencing. The DNA sequencing library was prepared using NEBNext® Ultra IIDNA Library Prep Kit and sequenced on the Illumina Novaseq 6000 platform. A total of 15,456,744 paired-end reads of 150 bp length were obtained. FastQC v0.11.5 was used to evaluate the quality of the reads (Darling, 2010; Russell, 2018). BBDuk v38.84 was used to remove adapters and low-quality reads with a minimum quality scores of Q30. Furthermore, reads less than 50 bp in length were discarded. As a large number of reads were generated, it was randomly subsampled into three sets of 200,000 paired-end reads each (Russell, 2018).

Geneious assembler in Geneious Prime v2022.2.2 (Geneious Prime 2022.2.2, https://www.geneious.com) was used to independently assemble these *de novo*. Rapid Annotation using Subsystem Technology (RAST) (https://rast.nmpdr.org; Accessed on 2 November 2022), the online prokaryotic genome annotation service, was used to identify open reading frames (ORFs) and to annotate the genome. Protein sequences were manually cross-checked with BLASTP searches (https://blast.ncbi.nlm.nih.gov; Accessed on 4 November 2022).

3.2.11 Bioinformatics analysis

To identify phages with most similar genomes to the genome sequence of Ec_MI-02, nucleotide BLAST (BLASTN) search was performed. ProgressiveMauve was used to compare the whole genome of Ec_MI-02 with the three most identical phages genomes identified in the above step (Darling et al., 2010). Terminase large subunit (*TerL*) is commonly used as a genetic marker for the order Caudovirales. *TerL* gene sequence of Ec_MI-02 was compared to sequences of the 15 most identical *TerL* sequences identified by BLASTN. The 16 sequences were aligned using MUSCLE in MEGA 11 (Tamura et al., 2021) (https://www.megasoftware.net; Accessed on 27 October 2022). Subsequently, a phylogenetic tree was constructed using these sequences with the maximum likelihood method and 100 bootstraps.

ABRicate (https://github.com/tseemann/abricate, Accessed on 10 January 2023) (A. J. Page & Seemann, 2019), which employs multiple databases, was used to detect the presence of antibiotic resistance and virulence genes in the Ec_MI-02 genome. HostPhinder (Villarroel et al., 2016) was used to predict the host range of Ec_MI-02. Proksee (https://proksee.ca/; Accessed on 5 January 2023) was used to visualize the genome map.

3.2.12 Statistical analyses

All data were analyzed using the analysis of variance (ANOVA) method in SAS Software version 9 (SAS Institute Inc., NC, USA). Mean values of treatments (four replicates) were compared using Fisher's protected least significant difference (LSD) test at $p = 0.05$ levels.

3.3 Results

3.3.1 Isolation and host range of the phage Ec_MI-02 active against E. coli O157:H7

Ec_MI-02 bacteriophage was isolated from a wild pigeon feces from a single nest using *E. coli* O157:H7 NCTC 12900 as the propagation host. The host range of the phage Ec_MI-02 was studied using stock bacterial culture collection listed in Table 1. Ec_MI-02 was tested on a wide range of bacterial strains ranging from closely related *E. coli* and other gram-negative bacteria. Although coliphages are not thought to be able to infect gram-positive bacteria, various species of some gram-positive bacteria, such as *Staphylococcus*, *Streptococcus*, *Mycobacterium*, *Bacillus*, and *Enterococcus*, were also investigated as potential hosts for Ec_MI-02 (Table 1). The presence of plaques on the bacterial lawn was regarded as positive, while the absence of plaques was regarded as negative.

Ec_MI-02 was found to infect *E. coli* O157:H7 NCTC 12900 and *E. coli* ATCC 8739 producing plaques with clear zones. On the other hand, Ec_MI-02 produced less clear plaques on lawns of *E. coli* ATCC 35218, *E. coli* NCTC 13841, *E. coli* ATCC 15223, *E. coli* ATCC 23227, *E. coli* ATCC 9637, *E. coli* ATCC 23224, and an *E. coli* strain isolated from a patient with urinary tract infection. This suggests that a virulent phage of a specific strain, can act as a prophage in other related strains. These results may explain failure of some phages in phage therapy. Therefore, *E. coli* O157:H7 NCTC 12900 was used as the propagation host in this study for the characterization experiments. Additionally, based on the genome sequence, HostPhinder, a tool to predict the host cell of a given phage (Villarroel et al., 2016), predicted Ec_MI-02 as a *E. coli* phage.

It is noteworthy that the phage Ec_MI-02 was not able to infect the other gram-positive and gram-negative bacteria tested including *E. coli* ATCC 25922 and an extended-spectrum beta-lactamase-producing *E. coli* from patient blood (Table 12).

Table 12: Host range of the phage Ec_MI-02.

Bacterial strains	Sensitivity to phage Ec_MI-02
Escherichia coli O157:H7 NCTC 12900	++
Escherichia coli ATCC 8739	++
Escherichia coli ATCC 25922	−
Escherichia coli ATCC 35218	+
Escherichia coli ATCC NCTC 13841	+
Escherichia coli ATCC 15223	+
Escherichia coli ATCC 23227	+
Escherichia coli ATCC 9637	+
Escherichia coli ATCC 23224	+
Escherichia coli (Patient isolate-urine)	+
Escherichia coli ESBL-producing (Patient isolate-blood)	−
Bacillus subtilis ATCC 6051	−
Pseudomonas aeruginosa ATCC 25668	−
Pseudomonas aeruginosa ATCC 27853	−
Methicillin-resistant *Staphylococcus aureus* (Patient isolate)	−
Staphylococcus aureus ATCC 6358	−
Staphylococcus aureus ATCC 29213	−
Staphylococcus epidermidis ATCC 12228	−
Staphylococcus saprophyticus ATCC-BAA 750	−
Streptococcus pyogenes ATCC 19615	−
Enterococcus faecalis ATCC 51299	−
Enterococcus faecalis (Patient isolate)	−
Enterococcus casseliflavus (Patient isolate)	−
Enterobacter aerogenes ATCC 13018	−
Enterobacter hormaechei (Patient isolate)	−
Klebsiella pneumonia ESBL-producing ATCC 700603	−
Klebsiella pneumonia KPC 2 +ve (Patient isolate)	−
Haemophilus influenzae ATCC 9007	−

Table 12: Host range of the phage Ec_MI-02 (continued).

Bacterial strain	Sensitivity to phage UAE_MI-01
Stenotrophomonas maltophilia ATCC 17666	–
Salmonella enterica ATCC 14028	–
Salmonella sp. (Patient isolate)	–
Proteus vulgaris ATCC 29905	–
Mycobacterium smegmatis ATCC 607	–

NCTC: national collection of type cultures; ATCC: American type culture collection; ESBL: extended-spectrum beta-lactamase. (++) = host species susceptible to phage lysis with clear plaque, (+) = host species susceptible to phage lysis with less clear plaque, and (–) indicates no plaque. *Escherichia coli* O157:H7 NCTC 12900 was used as the propagation host.

3.3.2 Plaque morphology of the phage Ec_MI-02

This experiment was performed on four common bacteriological media with a wide range of prices to determine the potential cost of production for any future application. Ec_MI-02 suspensions with 10^2 plaque forming units (PFU) mL^{-1} was used in the plate lysis assay. When *E. coli* O157:H7 NCTC 12900 was used as the propagation host, Ec_MI-02 phage produced uniform plaques that were characteristic of a virulent phage on the following four bacteriological media - Mueller–Hinton agar, tryptone soy bean agar, Luria–Bertani agar, and nutrient agar. There were no significant ($p > 0.05$) differences among the plaque sizes when these four media were used.

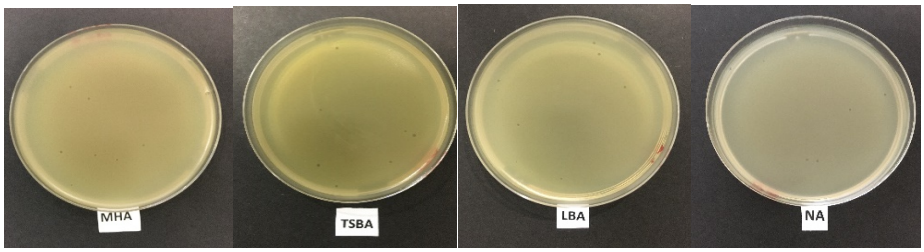

Figure 11: Plaque morphology of Ec_MI-02 on different agar media
Plaque morphology of Ec_MI-02 on different agar media using *E. coli* O157:H7 NCTC 12900 as the propagation host (MHA: Muller Hinton agar, TSBA: tryptone soy bean agar, LBA: Luria-Bertani agar; and NA: nutrient agar).

3.3.3 Effect of pH and temperature on the activity of the phage Ec_MI-02

The pH level is a factor that is extremely significant in all kinds of food. In addition, the stability of the phage in varied pH environments is an important consideration in phage therapy, particularly when the phage is administered orally. Similarly, many kinds of food have a range of temperature requirements for the various stages of processing and preserving. Therefore, stability of Ec_MI-02 at various pH and temperatures was investigated.

Ec_MI-02 was stable at pH values ranging from 3-10 (Table 13); there were no significant ($p > 0.05$) variations in the $\log_{10}$ PFU mL^{-1} across all the pH that were tested (3-10).

Ec_MI-02's stability in temperatures ranging from 25°C to 100°C was tested and the phage was found to be stable at 65 °C (Table 14). It was completely disabled by being heated for 15 and 30 min at 75°C and 100°C. There were significant ($p < 0.05$) differences in the $\log_{10}$ PFU mL^{-1} in all the tested temperature ranges (Table 14). When the temperature was raised from 25°C to 65°C, there was a significant ($p < 0.05$) drop in the $\log_{10}$ PFU mL^{-1} count that occurred when the phage Ec_MI-02 was exposed to these temperatures for either 15 min or 30 min (Table 14).

Table 13: Stability of the phage Ec_MI-02 at different pH values.

pH	3	4	7	9	10
($\log_{10}$ PFU mL^{-1})	5.93 ± 0.048 a	5.96 ± 0.047 a	5.91 ± 0.10 a	5.89 ± 0.10 a	5.92 ± 0.13 a

Values of the number of phages ($\log_{10}$ PFU mL^{-1}) are means of four independent replicates ± standard deviation. Values with the same letters are not significantly ($p > 0.05$) different within rows, according to Fisher's Protected LSD Test. PFU = plaque forming units. *Escherichia coli* O157:H7 NCTC 12900 was used as the propagation host.

Table 14: Stability of the phage Ec_MI-02 at different temperatures.

Temp. /Time	25°C (log_{10} PFU mL^{-1})	45°C (log_{10} PFU mL^{-1})	55°C (log_{10} PFU mL^{-1})	65°C (log_{10} PFU mL^{-1})	75°C (log_{10} PFU mL^{-1})	100°C (log_{10} PFU mL^{-1})
15 min	5.71 ± 0.078 Aa	5.46 ± 0.055 Ab	5.23 ± 0.087 Ac	4.91 ± 0.080 Ad	0.00 ± 0.00 Ae	0.00 ± 0.00 Ae
30 min	5.57 ± 0.055 Ba	5.43 ± 0.032 Ab	5.19 ± 0.114 Ac	4.85 ± 0.067 Ad	0.00 ± 0.00 Ae	0.00 ± 0.00 Ae

Values of the number of phages (log_{10} PFU mL^{-1}) are means of four independent replicates ± standard deviation. Values with the same lower- and upper-case letters are not significantly ($p > 0.05$) different within rows and columns, respectively, according to Fisher's Protected LSD Test. PFU = plaque forming units. *Escherichia coli* O157:H7 NCTC 12900 was used as the propagation host.

3.3.4 Effects of chemical disinfectants on the activity of the phage Ec_MI-02

In order to determine the most effective method for inhibiting or inactivating the phage in the laboratory, the sensitivity of Ec_MI-02 to typical disinfectants used in laboratories was evaluated after the phage was subjected to three distinct disinfectants for a total of 2 min.

Ec_MI-02 was found to be relatively resistant to 70% ethanol. There were significant ($p < 0.05$) differences in the log_{10} PFU mL^{-1} when the phage was exposed to ethanol for 60 sec compared to 30 sec (Table 15). However, there was no significant ($p > 0.05$) differences when the time of exposure increased from 0 to 30 sec.

When the phage Ec_MI-02 was exposed to ethanol for 60 sec as opposed to 30 sec, there were significant ($p < 0.05$) variations in the log_{10} PFU mL^{-1} (Table 15). However, when the period of exposure was prolonged from 60 to 120 sec, there were not any significant ($p > 0.05$) differences (Table 15).

When the commercial disinfectant (liquid hand wash) at 20% was employed, there were significant variations ($p < 0.05$) in the log_{10} PFU mL^{-1} when the length of exposure increased from 30 to 60 to 120 sec (Table 15). The commercial liquid hand wash caused one log reduction after 60 sec and two log reduction after 120 sec.

On the other hand, the phage Ec_MI-02 was completely inactivated by sodium hypochlorite 2% after 30, 60 and 120 sec. According to these findings, sodium hypochlorite 2% was noticeably more effective than ethanol at a concentration of 70%, which is typically used in laboratories.

Table 15: Effects of chemical disinfectants on the phage Ec_MI-02.

Disinfectant	Initial Titer (log_{10} PFU mL^{-1})	After 30 Seconds (log_{10} PFU mL^{-1})	After 60 Seconds (log_{10} PFU mL^{-1})	After 120 Seconds (log_{10} PFU mL^{-1})
Ethanol 70%	6.19 ± 0.028 Aa	6.16 ± 0.060 Aa	5.96 ± 0.134 Ab	5.91 ± 0.155 Ab
Commercial disinfectant 20%	6.20 ± 0.057 Aa	5.44 ± 0.121 Bb	4.75 ± 0.149 Bb	3.14 ± 0.240 Bd
Sodium hypochlorite 2%	6.18 ± 0.075 Aa	0.00 ± 0.00 Cb	0.00% ± 0.00 Cb	0.00% ± 0.00 Cb

Values of the number of phages (log_{10} PFU mL^{-1}) are means of four independent replicates ± standard deviation. Values with the same lower- and upper-case letters are not significantly ($p > 0.05$) different within rows and columns, respectively, according to Fisher's Protected LSD Test. PFU = plaque forming units. *Escherichia coli* O157:H7 NCTC 12900 was used as the propagation host.

3.3.5 Adsorption time, adsorption rate constant, latent period and burst size of the phage Ec_MI-02

The phage Ec_MI-02 was propagated in the host cell *E. coli* O157:H7 NCTC 12900 in order to determine the adsorption time, adsorption rate constant, latent period and the burst size. Adsorption was observed within 5 min (eclipse period) with 99% efficiency. The adsorption rate constant (K) of Ec_MI-02 was found to be 1.55×10^{-8} ml min^{-1}. The latent period was almost 50 min with burst size of almost 10 PFU ml^{-1} (Figure 12). Thus, this phage potentially be commercially produced as a biopreservative as well as for phage therapy.

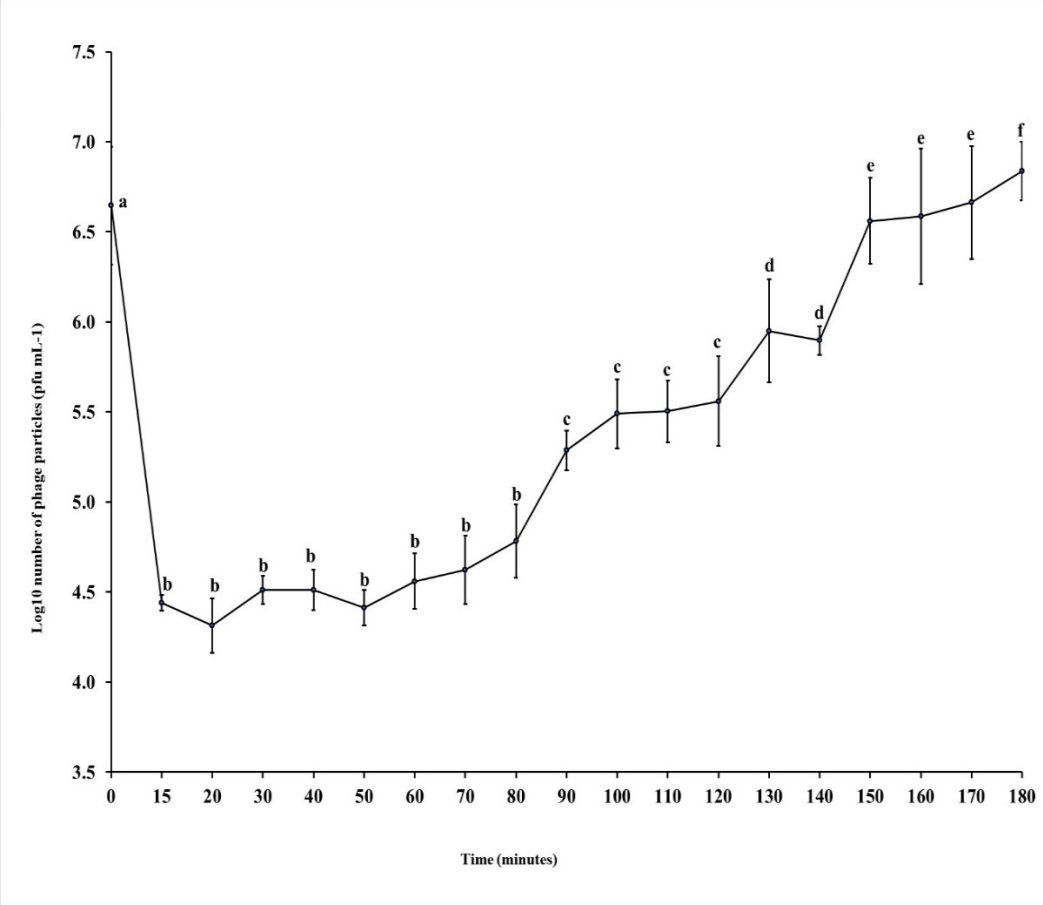

Figure 12: One-step growth curve of the phage EC_MI-02

One-step growth curve of the phage EC_MI-02 with *Escherichia coli* O157:H7 NCTC 12900 as the propagation host. Values are means ± standard deviation of four replicates for each timing. Mean values followed by different letters are significantly ($p < 0.05$) different from each other according to Fisher's Protected LSD Test. Bars represent standard deviation. PFU = plaque forming units.

3.3.6 Electron microscopy of the phage Ec_MI-02

The morphological characterization of Ec_MI-02 was studied by TEM, which along with genomic analysis, indicated that Ec_MI-02 bacteriophage belongs to the Tequatrovirus genus in the order Caudovirales. The phage was approximately 220 nm long and 80 nm wide with a 100 x 80 nm head and 120 x 20 nm contractile tail (Figure 13).

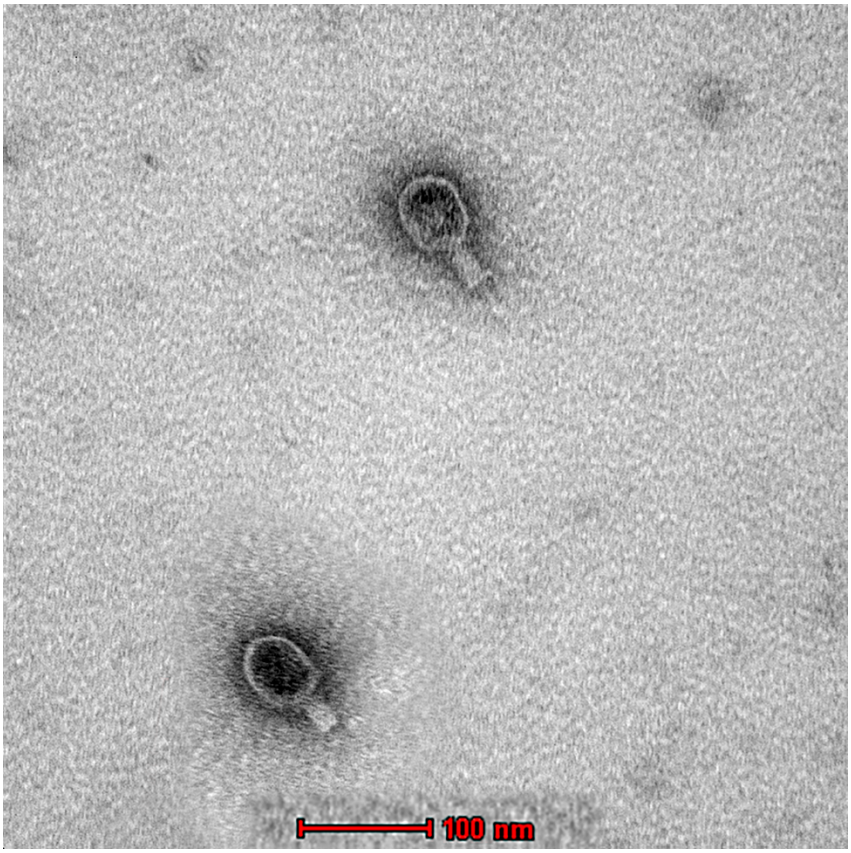

Figure 13: Transmission electron micrograph of the phage EC_MI-02. Scale bar = 100 nm.

3.3.7 Genomic analysis and bioinformatics of the phage Ec_MI-02

Genome assembly using the three subsampled reads produced the same assembled genome sequence of 165,454 bp length and GC content of 35.5%. The complete genome sequence of Ec_MI-02 has been deposited in NCBI GenBank with accession OP856590. Annotation of the genome predicted 266 protein coding genes and 9 tRNAs, which covered 94.7% of the genome (Figure 14). The genome encodes structural/assembly genes, including genes for terminase large and small subunits, capsid proteins, assembly proteins, and tail fiber proteins.

Replication/transcription-related genes included DNA helicase, DNA helicase loader, DNA polymerase, DNA topoisomerase, and RNA polymerase. Analysis with ABRicate (Page & Seemann, 2019) did not identify any antimicrobial resistance or virulence genes. The presence of holin along with glycoside hydrolase family protein (lysozyme/endolysins) suggests that Ec_MI-02 could be a virulent bacteriophage. No lysogeny-associated genes, like integrase, excisionase, transposase, etc., were identified in the Ec_MI-02 genome.

NCBI BLASTN search (Table 17) revealed that Ec_MI-02 shared 98.56% sequence identity with *Shigella* phage pSs-1 (97% coverage) with GenBank accession NC_025829.1, 98.44% sequence identity with *Shigella* phage Sfk20 (96% coverage) with GenBank accession MW341595.1 and 97.57% sequence identity with *Escherichia* phage HY01(96% coverage) with GenBank accession KF925357.1. Whole genome alignment of the four bateriophages (Ec_MI-02, pSs-1, Sfk20 and HY01) with progressiveMauve indicated the conservation of order of the genomic blocks (Figure 15).

The terminase large subunit (*TerL*) is generally used as a genetic marker for phages of the order Caudovirales. Here, *TerL* sequences most similar to that of Ec_MI-02 were identified using a nucleotide BLAST (BLASTN) search. Subsequently, a phylogenetic tree was constructed, which placed Ec_MI-02's *TerL* close to that of Shigella phages KNP5 and pSs-1 (Table 16, Figure 16). While Terminase Small subunit of Ec_MI-02 was found to be 100% identical to *Escherichia* phage T4 with accession number NP_049775.1 (Table 19, Figure 18). Glycoside hydrolase family protein of Ec_MI-02 is 100% identical to glycoside hydrolase family protein of *Enterobacteria* phage RB51 with accession number YP_002854084.1 (Table 20, Figure 19) and holin of Ec_MI-02 is 100% identical to holin of *Escherichia* phage ime09 with accession number YP_007004625.1 (Table 18, Figure 17).

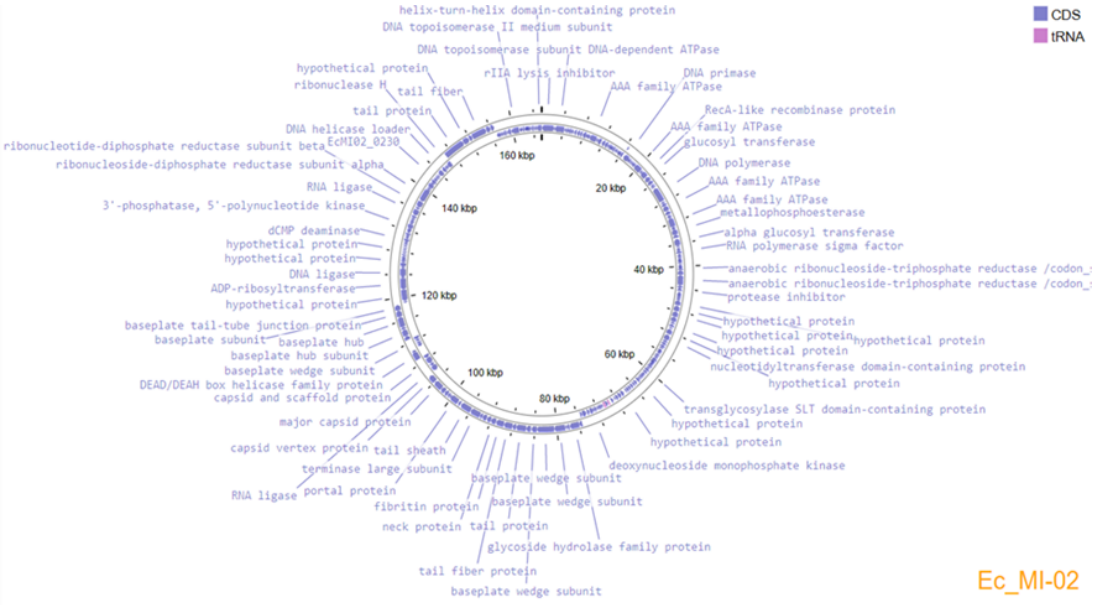

Figure 14: Genome organization map of Ec_MI-02 created with Proksee, showing annotated ORFs

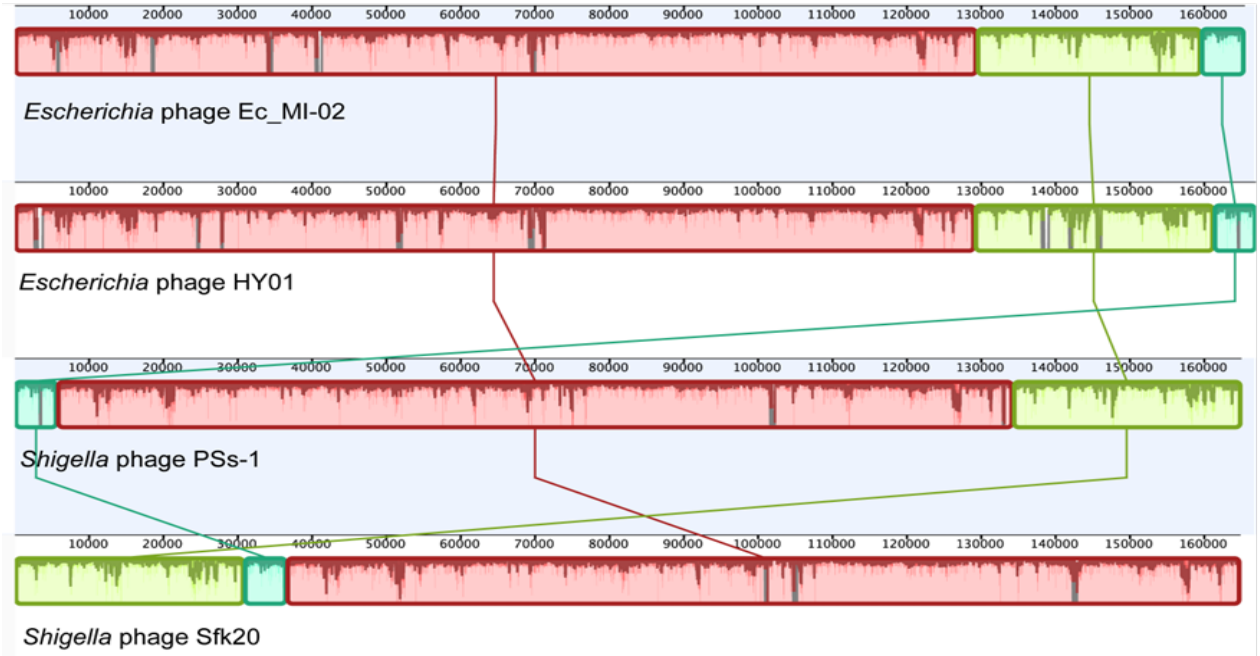

Figure 15: Whole genome alignment of the bacteriophage Ec_MI-02 and the three phages. Whole genome alignment of the bacteriophage Ec_MI-02 and the three phages with genome sequence most identical to the genome of Ec_MI-02 through a BLASTN search. The alignment was generated using progressiveMauve. Colored blocks match regions in different genomes.

Table 16: Terminase large subunit protein sequences of 15 bacteriophages most similar to EC_MI-02 based on an NCBI BLASTP search (Accessed on 15 December 2022).

Bacteriophage	Percentage Identity (%)	Sequence Length (Number of amino acids)	NCBI Accession
Shigella phage Shfl2	100	610	YP_004415059.1
Escherichia phage slur02	99.84	610	YP_009210351.1
Escherichia phage 132	99.84	610	QWY90533.1
Escherichia phage slur14	99.84	610	YP_009180666.1
Shigella phage ESh24	99.84	610	URY12904.1
Escherichia virus KFS-EC3	99.84	610	QVW54379.1
Shigella phage ESh30	99.84	610	URY14677.1
Escherichia phage vB_EcoM-RPN226	99.84	610	ULA51715.1
Enterobacteria phage RB51	99.84	610	YP_002854122.1
Escherichia phage vB_EcoM_ACG-C40	99.84	610	YP_006986718.1
Escherichia phage vB_EcoM-UFV09	99.84	610	QWT76877.1
Enterobacteria phage vB_EcoM_IME340	99.67	610	YP_010066488.1
Bacteriophage sp.	99.84	610	UVM85381.1
Enterobacteria phage RB18	99.84	610	YP_010067047.1
Shigella phage CM8	99.67	610	YP_010075972.1

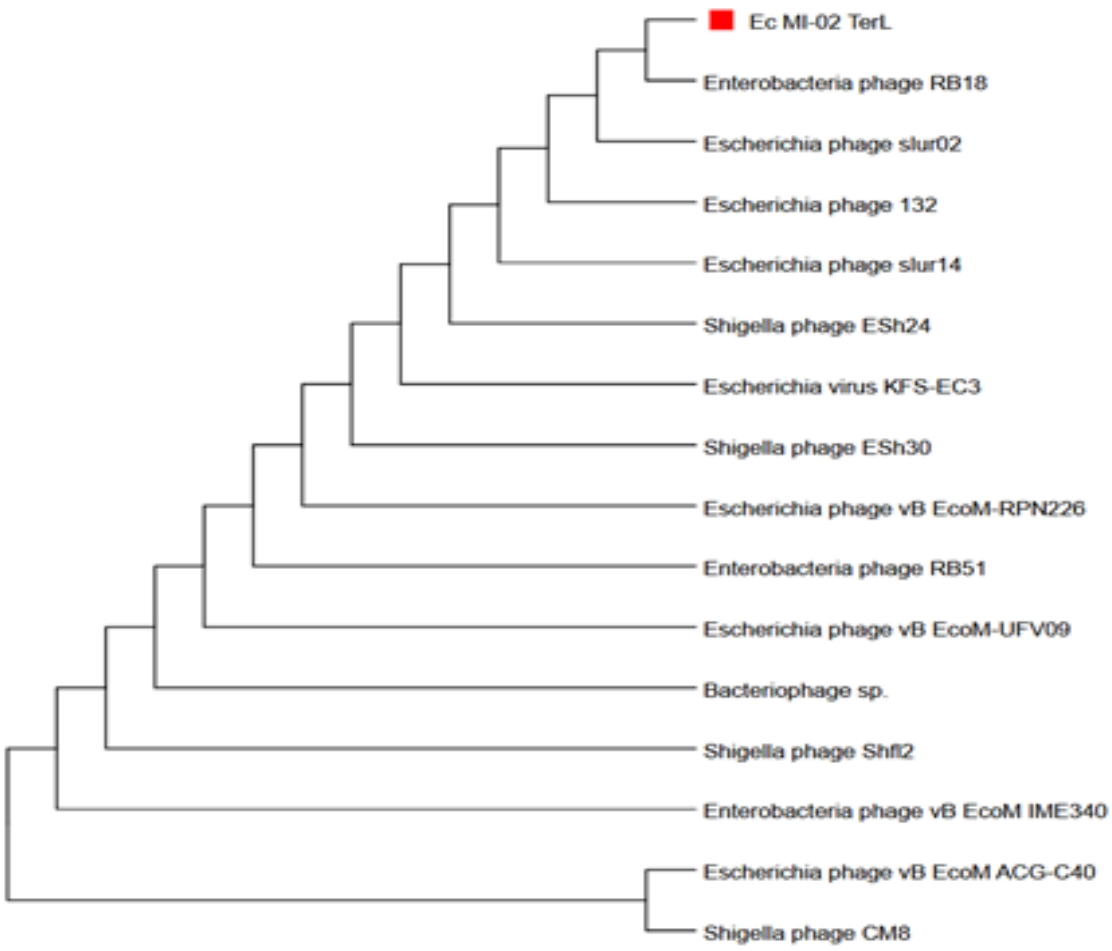

Figure 16: Phylogenetic tree constructed using the terminase large (TerL) subunit protein sequences of Ec_MI-02 phage (red square).

The phylogenetic tree constructed using the terminase large (TerL) subunit protein sequences of Ec_MI-02 phage (red square) and 15 closest sequences identified using an NCBI BLASTP search (Table 16). MUSCLE in MEGA 11 was used to align the sequences and the phylogenetic tree was generated using the neighbor-joining method.

Table 17: Ten phage genomes most identical to the genome of the phage Ec_MI-02 as identified by an NCBI BLASTN search.

Bacteriophage	Percentage Identity (%)	Genome Size (bp)	NCBI Accession
Shigella phage pSs-1	98.56	164,999	NC_025829.1
Shigella phage Sfk20	98.44	164,878	MW341595.1
Shigella phage KNP5	98.27	193,624	NC_055713.1
Escherichia phage PEC04	97.57	167,552	KR233165.1
Escherichia phage HY01	97.57	166,977	KF925357.1
Escherichia phage HY03	97.38	170,770	KR269718.1
Escherichia phage vB_EcoM_IME537	97.18	168,642	NC_054921.1
Escherichia phage vB_vPM_PD112	97.07	168,084	NC_054928.1
Escherichia phage vB_Eco_NR1	96.95	167,153	LR990704.1
Escherichia phage vB_EcoM_CE1	96.93	167,955	ON229909.1

Table 18: Holin protein of the 15 bacteriophages most similar to Ec_MI-02 based on an NCBI BLASTP search (Accessed on 15 December 2022).

Bacteriophage	Percentage Identity (%)	Accession Length	Accession
Escherichia phage ime09	100.00	218	YP_007004625.1
Escherichia phage vB_EcoM_G4507	99.54	218	YP_010070632.1
Salmonella phage Lv5cm	99.54	218	QVW09059.1
Escherichia phage MLF4	99.08	218	YP_010099940.1
Yersinia phage vB_YepM_ZN18	99.54	218	YP_010077484.1
Escherichia phage vB_EcoM_IME537	99.54	218	YP_010071019.1
Escherichia phage vB_EcoM_G2133	99.08	218	QBO60988.1
Escherichia phage vB_EcoM_ACG-C40	99.54	218	YP_006986808.1
Enterobacteria phage RB18	99.08	218	YP_010067132.1
Shigella phage Sf21	98.62	218	YP_009619057.1
Citrobacter phage PhiZZ23	98.26	218	YP_010065891.1
Enterobacteria phage RB51	99.08	218	YP_002854205.1
Escherichia phage EC121	99.08	218	YP_010067932.1
Escherichia phage vB_EcoM_G4498	99.08	218	YP_010070353.1
Shigella phage JK38	99.08	218	QEG06096.1

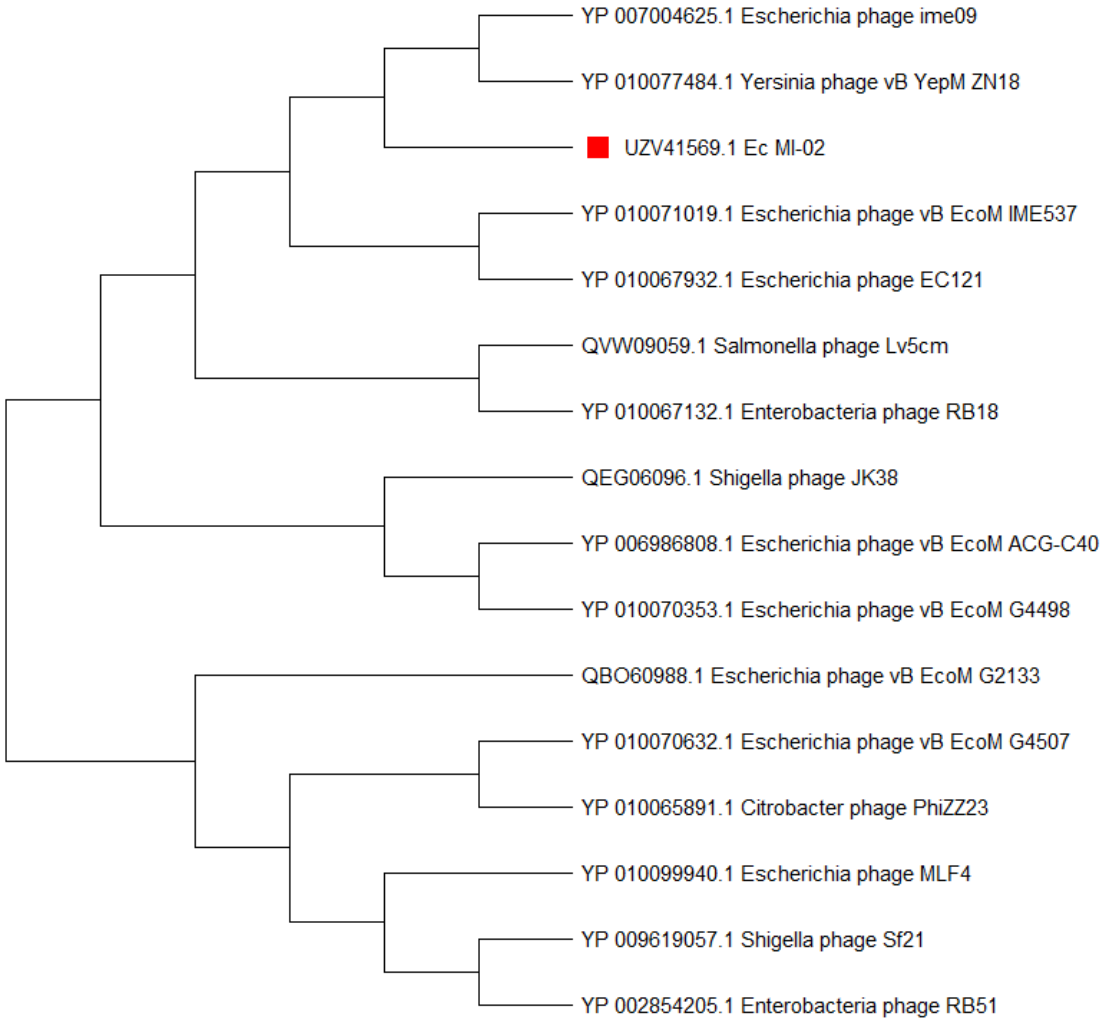

Figure 17: Phylogenetic tree of holin protein of the 15 bacteriophages most similar to Ec_MI-02 (Table 18).

Table 19: Terminase small subunit protein of the 15 bacteriophages most similar to Ec_MI-02 based on an NCBI BLASTP search (Accessed on 18 December 2022).

Bacteriophage	Percentage Identity (%)	Accession Length	Accession
Escherichia phage T4	100.00	164	NP_049775.1
Shigella phage Sf24	99.39	164	YP_009619098.1
Escherichia phage T2	99.39	164	YP_010073814.1
Escherichia phage vB_EcoM_F1	99.39	164	YP_010068655.1
Shigella phage Shfl2	99.39	164	YP_004415058.1
Escherichia phage RB3	99.39	164	YP_009098548.1
Escherichia phage slur14	99.78	164	YP_009180665.1
Shigella phage CM8	99.39	164	YP_010075971.1
Escherichia virus KFS-EC3	99.39	164	QVW54380.1
Escherichia phage AndreasVesalius	99.39	164	QXV75855.1
Escherichia phage vB_EcoM_G4507	99.39	164	YP_010070544.1
Escherichia phage RB69	90.24	164	NP_861868.1
Escherichia phage vB_EcoM_KAW3E185	91.36	164	QBQ78726.1
Escherichia phage OLB35	90.24	164	AYR04083.1
Escherichia phage p000v	90.80	164	YP_010100013.1

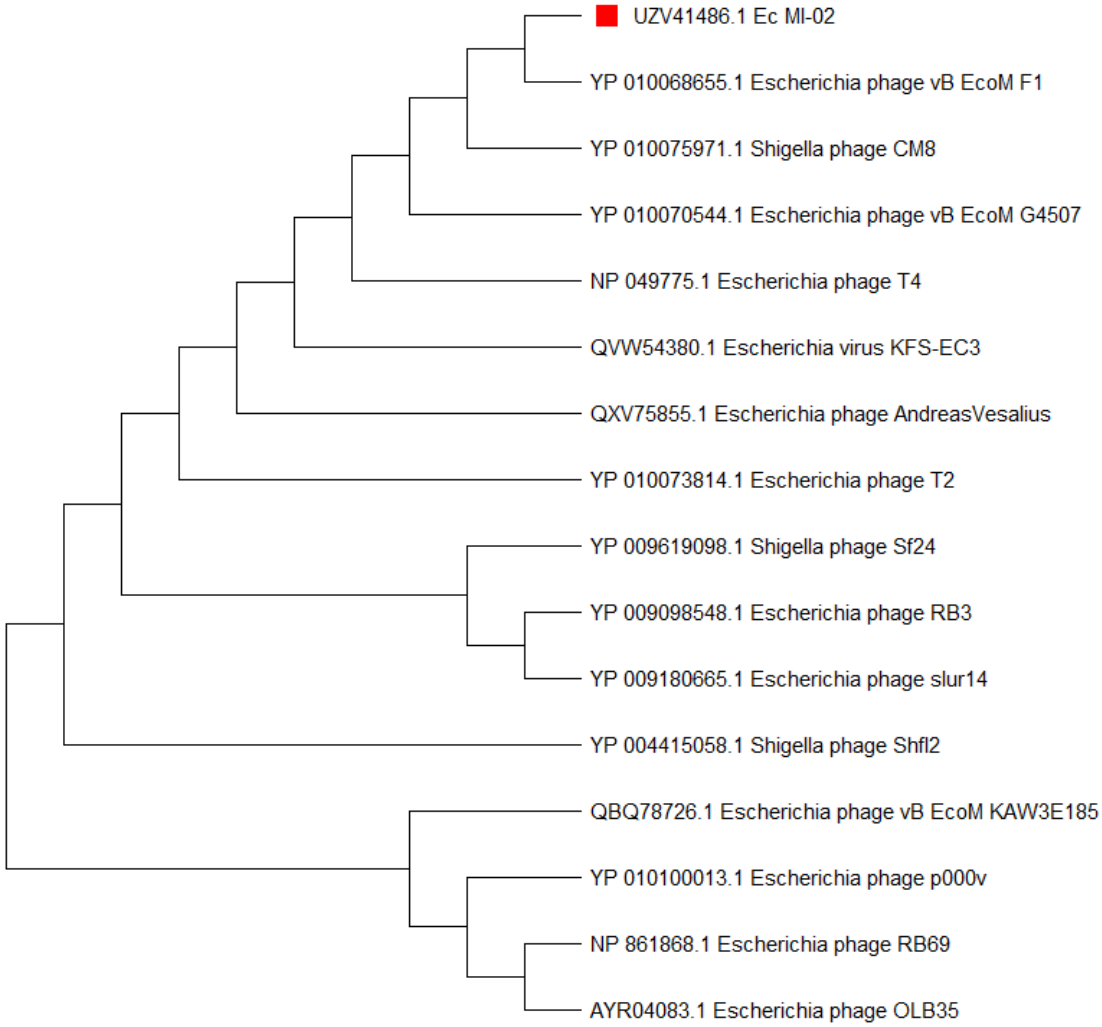

Figure 18: Phylogenetic tree of terminase small subunit protein of the 15 bacteriophages most similar to Ec_MI-02 (Table 19).

Table 20: Glycoside hydrolase family protein (lysozyme / endolysin) protein of the 15 bacteriophages most similar to Ec_MI-02 based on an NCBI BLASTP search (Accessed on 3 January 2023).

Bacteriophage	Percentage Identity (%)	Accession Length	Accession
Enterobacteria phage RB51	100	164	YP_002854084.1
Bacteriophage sp.	99.39	164	UVX66474.1
Escherichia phage vB_EcoM_FT	99.39	164	QLF81126.1
Enterobacteria phage RB27	99.39	164	YP_009102332.1
Enterobacteria phage phiC600P9	99.39	164	QNI20213.1
Citrobacter phage PhiZZ6	99.39	164	YP_010065496.1
Escherichia phage UFV-AREG1	99.39	164	YP_009281462.1
Escherichia phage teqsoen	99.39	164	QHR63974.1
Escherichia phage MLP2	99.39	164	UEN68765.1
Escherichia phage VEc74	99.39	164	QOI68307.1
Yersinia phage PYps5T	99.39	164	QOI70630.1
Bacteriophage sp.	99.39	164	UVM85343.1
Escherichia phage vB_EcoM_NBG2	99.39	164	YP_010072141.1
Escherichia phage RB14	99.39	164	YP_002854463.1
Shigella phage SHBML-50-1	99.39	164	YP_009288492.1

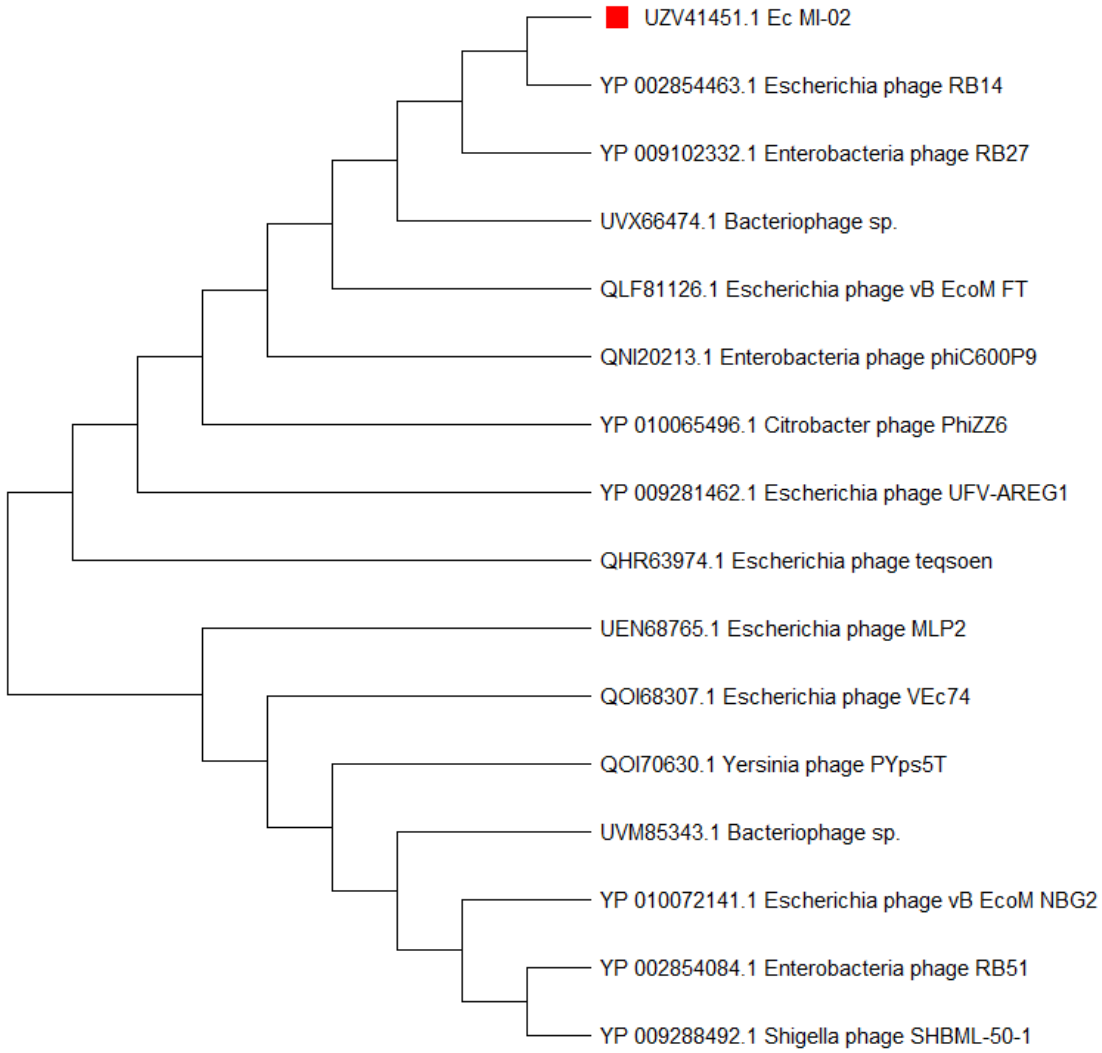

Figure 19: Phylogenetic tree of glycoside hydrolase family protein of the 15 bacteriophages most similar to Ec_MI-02 (Table 20).

3.4 Discussion

As the prevalence of antibiotic resistant bacteria increases, the demand for new bacteriophages also increases. In order to diversify the range of available phages, new sources should be explored. Wild animals and birds are excellent sources of phages that are free of drug resistant genes as they are not normally exposed to antibiotics. In the present study, we examined birds' feces for phages of the verotoxin producing *E. coli* O157:H7, an important food borne pathogen that was responsible for many outbreaks worldwide (Thomas et al., 2015). *E. coli* O157:H7 infection could cause many pathological symptoms such as abdominal cramps, diarrhea, hemolytic colitis, hemolytic uremic syndrome and kidney failure, which are often very serious and may lead to death

(Thomas et al., 2015). In fact, very recently Centers for Disease Control and Prevention (CDC) of the United States of America had reported two *E. coli* O157:H7 outbreaks in September, 2022; one outbreak was linked to ground beef (CDC, 2022b) and the other outbreak with unknown food source (CDC, 2022a).

Bacteriophages or bacteria eaters can recognize their bacterial hosts and eliminate them. Therefore, bacteriophages are potent biocontrol agent (Keen & Adhya, 2015). In the present study Ec_MI-02 lytic phage of *E. coli* O157:H7 was isolated from pigeon feces. The host range investigation and one-step-curve showed that Ec_MI-02 targets *E. coli* O157:H7 strain effectively in addition to a few other *E. coli* strains. This was also supported by the prediction of the in silico HostPhinder tool which showed that Ec_MI-02 is an *E. coli* bacteriophage (Villarroel et al., 2016). Several previously reported bacteriophages of *E. coli* O157:H7 showed wider or narrower host range. This makes Ec_MI-02 an excellent candidate as a biocontrol agent in veterinary, food, and agriculture industry. Ec_MI-02 was propagated on four different media; tryptone soy bean agar, Luria-Birtani agar, nutrient agar and Mueller Hinton agar using *E. coli* O157:H7 NCTC 12900 as the propagation host. Ec_MI-02 produced clear plaques with clear zone on all of the four media tested.

To identify phage genomes that are close in sequence to Ec_MI-02, NCBI nucleotide BLAST (BLASTN) was employed (Table 17). Only three of the closest ten phages have been classified - *Shigella* phage pSs-1, isolated from environmental water in South Korea (Jun et al., 2016), *Shigella* phage Sfk20, isolated from water bodies of a diarrheal outbreak location in India (Mallick et al., 2021, p. 2) and *Escherichia* phage HY01 (H. Lee et al., 2016, p. 01). Ec_MI-02 is similar in size to pSs-1; 218 x 98 nm, but larger than Sfk20; 190 X 62 nm and HY01; 200 nm long. Ec_MI-02 showed higher temperature stability, since it was stable at temperatures up to 75 °C compared to pSs-1 which was stable at temperatures up to 50 °C; Sfk20 was found to be deactivated at a temperature of around 50 °C and HY01 could survive in temperatures up to 65 °C. Ec_MI-02 was also found to be stable in the pH range 4-10, which is similar to HY01 but more stable than pSs-1 with a pH range of 5-9 and Sfk20 with a range of 7-9. Interestingly, ethanol 70% was not able to cause one log reduction of Ec_MI-02 within 120 sec. Sodium

hypochlorite 2% showed significant antiviral activity against Ec_MI-02 since it caused six log reduction within 30 sec.

Ec_MI-02 has a latent period of almost 50 minutes which is longer than the closest bacteriophages which had latent periods of 15 min (HY01), 20 min (Sfk20), 25 min (pSs-1). Burst size of HY01, pSs-1 and Sfk20 were 100, 97 and 123 PFU host cell^{-1}, respectively. While burst size of Ec_MI-02 was found to be almost 10 PFU host cell^{-1}.

However, large T4 phages and their relatives are known for a unique type of phage-encoded plasticity that is known as lysis inhibition (Abedon, 1999). Bull et. al. (Bull et al., 2004) stated that not all phages lyse their hosts, for example, fd, f1 and M13 are *E. coli* phages that are released through the host's cellular membrane without destroying the host cell (Bull et al., 2004). Annotation of Ec_MI-0e genome revealed that it has genes encoding for rI, rIIA and rIII lysis inhibition proteins. rI and rIII are found to have direct effect on lysis inhibition. rII has an indirect control in the lysis inhibition process (Paddison et al., 1998). The presence of the genes rI, rIIA and rIII and their direct and indirect roles in delaying cell lysis, could explain the pattern of the one-step-growth curve.

Multiplicity of infection (MOI) 0.1 is not suitable for phages with latent period of >40 min in case of bacterial host with relatively short life cycle of 30-40 min (for example: *E. coli*), especially if the phage has small burst size, since the uninfected bacteria will double in number in 40 min. This explains why the titer of Ec_MI-02 after one lytic cycle was less than the initial titer of phages. In fact, titer of the phage at any given time (after the absorbance period) reflects number of the 1% of the total number of phage particles which were not able to attach to the host bacteria since the attachment efficiency of Ec_MI-02 was 99%. Since there are more than 10 host cells for each phage particle, therefore, we suggest MOI (0.5) for phages with latent period longer than 40 min and burst size less than 100 PFU host cell^{-1}.

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List of Publications

1. Sultan-Alolama, M. I., Amin, A., Vijayan, R., & El-Tarabily, K. A. (2023). Isolation, characterization, and comparative genomic analysis of bacteriophage Ec_MI-02 from pigeon feces infecting Escherichia coli O157:H7. *International Journal of Molecular Sciences*, *24*, 9506. https://doi.org/10.3390/ijms24119506
2. Sultan-Alolama, M. I., Amin, A., El-Tarabily, K. A., & Vijayan, R. (2022). Characterization and genomic analysis of Escherichia coli O157: H7 phage UAE_MI-01 isolated from birds. *International Journal of Molecular Sciences*, *23*, 14846. https://doi.org/10.3390/ijms232314846
3. Sultan-Alolama, M. I., El-Tarabily, K. A., & Vijayan, R. (2021). Complete genome sequence of Escherichia coli O157: H7 phage UAE_MI-01, isolated from bird feces. *Microbiology Resource Announcements, 10*(28), e00348-21. https://doi.org/10.1128/MRA.00348-21
4. Saad, A. M., Sitohy, M. Z., Sultan-Alolama, M. I., El-Tarabily, K. A., & El-Saadony, M. T. (2022). Green nanotechnology for controlling bacterial load and heavy metal accumulation in Nile tilapia fish using biological selenium nanoparticles biosynthesized by Bacillus subtilis AS12. *Frontiers in Microbiology*, *13*, 1015613. https://doi.org/10.3389/fmicb.2022.1015613

Conference Publication:

1- Sultan-Alolama, M. I., and El-Tarabily, K.A., (2021). "*Escherichia coli* O157:H7 phage from bird faeces and its potential application as a therapeutic agent and as an industrial food preservative". International Applied Microbiology Conference hosted by Society for Applied Microbiology from 7-6-2021 up to 11-6-2021, UK.

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UAE UNIVERSITY DOCTORATE DISSERTATION NO. 2023: 23

This dissertation provides comprehensive information about *Escherichia coli* O157:H7, which is the most significant Shiga-toxigenic *E. coli* serotype (STEC) for its role in causing foodborne illness and the use of bacteriophages in phage therapy and food bio- preservatives to control pathogenic bacteria. This is the first time to isolate lytic Bacteriophage of *E. coli* O157:H7 from birds. The current thesis provides additional evidence that wild birds could also be a good natural reservoir for bacteriophages that do not carry antibiotic resistance genes and could be good candidates for phage therapy. In addition, studying the genetic makeup of bacteriophages that infect human pathogens is crucial for ensuring their safe usage

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