Isolation and Characterisation of Bacteriophages and Their Potential Use for the Control of Bacterial Infections in Poultry

By

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I, Kasweka Kakoma, declare that the dissertation hereby submitted by me for the Magister Scientiae degree at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty. I further more cede copyright of the dissertation in favour of the University of the Free State.

Kasweka Kakoma

May 2009

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES contd.

LIST OF FIGURES contd.

LIST OF FIGURES contd.

LIST OF TABLES

LIST OF TABLES contd.

LIST OF ABBREVIATIONS

°C	Degrees Celsius
µl	Microliter
µm	Micrometer
APEC	Avian Pathogenic *Escherichia coli*
bp	Base pair
Ca^{2+}	Calcium ions
CLED	Cystine Lactose Electrolyte Deficient
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ds	Double stranded
EDTA	Ethylene diamine tetra-acetic acid
EMB	Eosin Methylene Blue
EU	European Union
FDA	Food and Drug Administration
ICTV	International Committee on Taxonomy of Viruses
IPTG	Isopropyl-2-D-thiogalactopyranoside
kb	Kilobases
LB	Luria Bertani
Mg2+	Magnesium ions
$MgCl_2$	Magnesium Chloride
$mg.ml^{-1}$	Milligram per millilitre

LIST OF ABBREVIATIONS contd.

min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
M	Molar
nm	Nanometer
no	Number
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
RAPD	Random Amplified Polymorphic DNA
$rev.min^{-1}$	Revolutions per minute
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
s	Seconds
ss	Single stranded
TAE	Tris Acetate EDTA
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
UFS	University of the Free State

LIST OF ABBREVIATIONS contd.

US	United States
USDA	United States Department of Agriculture
UV	Ultraviolet
WHO	World Health Organisation
X-gal	5-Bromo-4-chloro-3-indolyl-A-D-galactopyranoside
XLD	Xylose lysine deoxycholate

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

The emergence of pathogenic bacteria resistant to most antibiotics is a worldwide concern (Bisht *et al*., 2009). Antibiotic resistance poses a threat to the public health and the agricultural industries where these type of antimicrobial agents have been extensively used for the treatment and prevention of bacterial infections. Additionally, antibiotic use in the agricultural sector at sub-minimal levels is administered for growth promotion purposes (Levy, 1998).

The poultry industry in South Africa is an important contributor to the agricultural sector, making up approximately 16% of the total gross agricultural value. In 2007 the poultry market was estimated at about R13.5 million and an increase of 10% has been predicted for the near future. This industry has experienced an upward trend from the early 1990s and the market is still growing (USDA Foreign Agricultural Service GAIN Report 2007). *Escherichia coli*, the causative agent of colibacillosis, is one of the bacterial pathogens that threaten the poultry industry worldwide. This is due to the significant economic losses incurred in production as a result of high mortality and morbidity rates as well as condemnations of carcasses at slaughter (Barnes & Gross, 1997).

Several antibiotics have been used to treat *E. coli* infections in the past. However, the increasing problem of antibiotic resistance, the ban of several antibiotics by the European Union and the United States Food and Drug Administration and furthermore, the decrease in antibiotic development by pharmaceutical companies have led to a growing interest and demand for alternatives to antibiotics (Casewell *et al.,* 2003; Spellberg *et al.,* 2004).

There are a number of possible substitutes available to circumvent the problem of resistance. These include bacteriocins, antimicrobial peptides and bacteriophages, which specifically infect and replicate in bacteria (Joerger, 2002). The potential use of bacteriophages (commonly referred to as phages) is of particular interest in this research project. Numerous reports have been published on the use of phages in treating bacterial infections from as early as 1920 (Alisky *et al.,* 1998). There are mounting concerns that humankind is on the verge of the "post-antibiotic" era and phages offer several advantages in comparison to antibiotics which makes them an attractive potential alternative.

1.2. BACTERIOPHAGES AND A BRIEF HISTORY OF THEIR DISCOVERY

Bacteriophages are ubiquitous in nature and are known to proliferate wherever their bacterial hosts exist (Hendrix *et al.,* 1999). Virion particles can exist independently outside the host, however all phages are obligate intracellular parasites and need their host to propagate (Jensen *et al.,* 1998). Several phages are highly specific for host cell surface receptors and any slight changes in structure results in little or no interaction between the phage and its host. Therefore, many phage typing schemes for the identification of bacterial species or subspecies are based on this specificity (Welkos *et al.,* 1974).

Frederick W. Twort and Felix d'Herelle are considered as the independent co-discoverers of bacteriophages. There has, however, been considerable controversy with regards to who actually discovered the bacterial viruses first. In 1896, British bacteriologist Ernest Hankin described his observations with regards to the presence of antibacterial activity against *Vibrio cholerae* in the Jumna and Ganges rivers of India. He proposed that an unidentified chemical substance was responsible for the decline in the spread of cholera. A few years later, other researchers made similar observations although they did not investigate their findings further (Sulakvelidze *et al*., 2001). Nearly 20 years after Hankin's report, Frederick W. Twort reported on a phenomenon referred to as the "glassy transformation" while working with *Vaccinia virus* which had been contaminated by micrococci. He speculated on the possibility that he had come across an ultra-microscopic virus and concluded that the glassy transformation was caused by an infectious agent that killed bacteria and multiplied itself in the process (Duckworth, 1976). In 1917, Felix d'Herelle independently

discovered ‘ultra viruses’ that resulted in the death of bacteria (Summers, 2001). He proposed the name ‘bacteriophage’ from ‘bacteria’ and ‘phagein’ (Greek word for to eat or devour) therefore implying that bacteriophages ‘eat’ or ‘devour’ bacteria. D’Herelle believed that a phage was an obligate parasite which is particulate, invisible, filterable, and self-reproducing in nature (Stent, 1963).

1.3. TAXONOMY

Bacteriophage characterisation has traditionally been based on host range, physical properties of the free virion such as capsid size, shape, resistance to organic solvents, structure, genome size and its nature (single- or double-stranded DNA or RNA). The International Committee for Taxonomy of Viruses (ICTV) requires phage particles to be observed by electron microscopy and capsid morphology to be established for their formal classification (Rohwer & Edwards, 2002). Genomic sizes range from 4 kb to 600 kb (Brüssow & Hendrix, 2002). Generally, viral families are described by the nature of nucleic acid and particle morphology whereas there are no set criteria for placement into genera and species (Ackermann, 2003).

According to the ICTV system, bacteriophages are currently classified into one order, *Caudovirales* which consists of three phylogenetically related families. In addition, there are 17 families or “floating genera” some of which presently await classification as indicated in table 1 below. Phage virions can be tailed, polyhedral, filamentous, and pleomorphic and most of them contain double-stranded DNA (table 1.1 & figure 1.1). About 5568 bacterial viruses have been examined by electron microscopy since 1959 when negative staining was introduced. At least 5360 (96.2%) of these are tailed and 208 (3.7%) are polyhedral, filamentous or pleomorphic (Ackermann, 2007).

Table 1.1: Phage families, their classification and basic properties

Shape	Nucleic acid	Virus group	Particulars	Example
Tailed	DNA, 2, L	*Myoviridae*	tail contractile	T4
		Siphoviridae	tail long, noncontractile	λ
		Podoviridae	tail short	T7
Polyhedral	DNA, 1, C	*Microviridae*	conspicuous capsomers	ϕX174
	2, C, S	*Corticoviridae*	complex capsid, lipids	PM2
	2, L	*Tectiviridae*	inner lipid vesicle, pseudotail	PRD1
	2, L	SHI, group*	inner lipid vesicle	SH1
	2, C	STV1 group*	turret-shaped protrusions	STIV
	RNA, 1, L	*Leviviridae*	poliovirus-like	MS2
	2, L, seg	*Cystoviridae*	envelope, lipids	ϕ6
Filamentous	DNA, 1, C	*Inoviridae*	a. long filaments	fd
			b. short rods	MVL1
	2, L	*Lipothrixviridae*	envelope, lipids	TTV1
	2, L	*Rudiviridae*	TMV-like	SIRV-1
Pleomorphic	DNA, 2, C, S	*Plasmaviridae*	envelope, lipids, no capsid	L2
	2, C, S	*Fuselloviridae*	same, lemon-shaped	SSV1
	2, L, S	*Salterprovirus*	same, lemon-shaped	His1
	2, C, S	*Guttaviridae*	droplet-shaped	SNDV
	2, L	*Ampullaviridae**	bottle-shaped	ABV
	2, C	*Bicaudaviridae**	two-tailed, growth cycle	ATV
	2, L	*Globuloviridae**	paramyxovirus-like	PSV

C Circular; *L* linear; *S* superhelical; *seg* segmented; *1* single-stranded; *2* double-stranded
*Awaiting classification

(Ackermann, 2007).

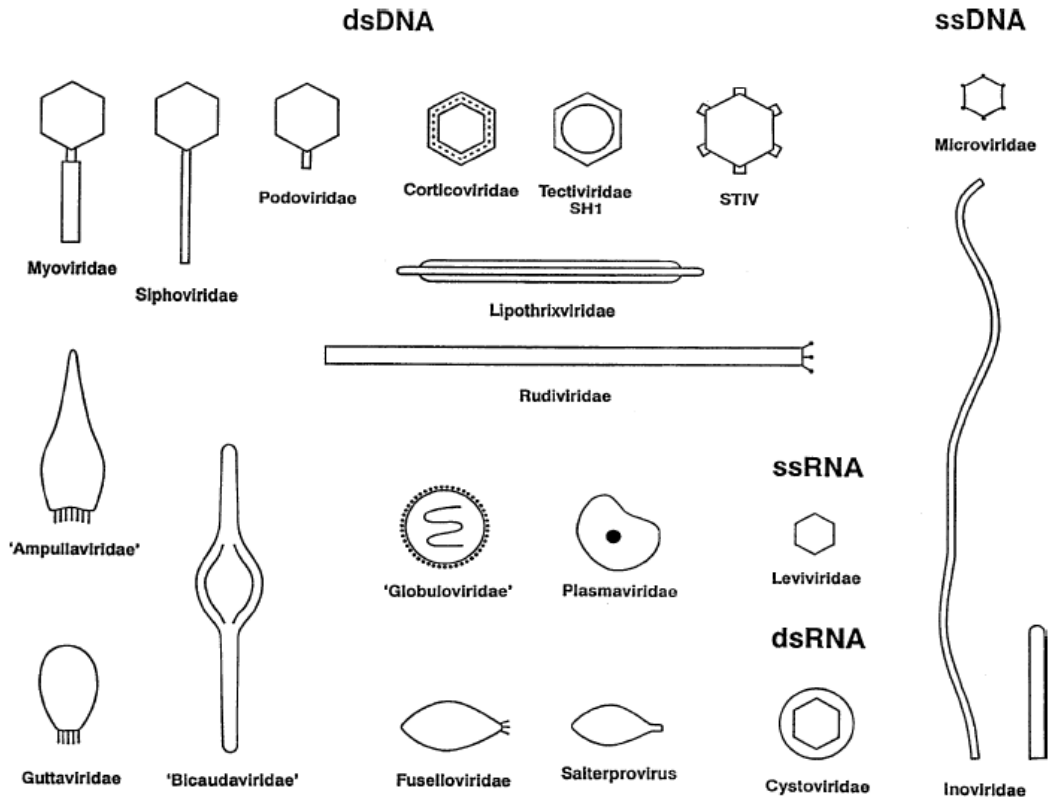


Figure 1.1: Basic bacteriophage morphotypes. (Ackermann, 2007).

It is estimated that the biosphere consists of approximately 10^{31} bacteriophages and archael viruses. Unfortunately less than one percent of microbial hosts have been cultured, and this makes identification procedures challenging. Moreover, there is no single gene which is conserved in all viral genomes (Edwards & Rohwer, 2005). In this scenario, culture-independent techniques are necessary to increase our understanding of the population in the microbial communities, the genetic diversity, and dynamics in microbial ecology. Culture-independent analyses are referred to as metagenomics, that is, the 'genomic analysis of microorganisms by direct extraction and cloning of DNA from their natural environment.' (Singh *et al.,* 2009). Since about 99% of microorganisms remain uncultured, there is an extremely high possibility of large numbers of yet unknown phages and many phage types whose identity is still to be elucidated in the future. Identity can be determined through sequence similarities with known phages whose sequences are available on GenBank. This literature review is focused on phages that have been cultured.

1.3.1. TAILED PHAGES

Tailed phages make up the largest group of bacterial viruses. Their particles possess a head (capsid) of cubic symmetry and helical tails. Heads are regular icosahedra or prolate. Capsid proteins are arranged into capsomeres of 5 or 6 protein units. Phage tails are helical or contain stacked disks and they often have terminal structures such as base plates, spikes or fibers (Ackermann, 2005). The tails have fixed dimensions, are proteinic in nature and are composed of subunits which form transverse striations (Ackermann, 2007).

The linear, double-stranded DNA composition of tailed phages is usually a reflection of their bacterial host although some DNAs, such as those of coliphage T4, contain unusual bases, for example, 5-hydroxymethylcytosine (Ackermann, 2003).

Tailed phages are considered the most diverse and widespread of all viral groups because their properties are highly wide-ranging. Some of these differences include DNA content and composition, host range, physiology, serology and the nature of constitutive proteins (Ackermann, 2005). Despite these differences, morphological, physiological and physiochemical properties reveal that this class of phages make up a monophyletic evolutionary group.

Three families, namely *Myoviridae, Siphoviridae*, and *Podoviridae* make up the tailed phages. This distribution is based on differences in tail structure. *Myoviridae* possess contractile tails consisting of a sheath and central tube. Myoviruses possess larger capsids and contain more DNA in comparison to their tailed counterparts. *Siphoviridae* make up about 61% of tailed phages and have long, noncontractile tails. Lastly, short, noncontractile tails are characteristic of the family *Podoviridae (*Ackermann, 2005).

1.3.2. POLYHEDRAL, FILAMENTOUS, AND PLEOMORPHIC PHAGES

Polyhedral, filamentous, and pleomorphic (PFP) phages include about 208 viruses, contributing to 3.7% of total examined population (Ackermann, 2007). This tailless group consists of families whose basic properties differ and appear to represent many independent lines of descent (Ackermann, 2003).

Microviridae (Polyhedral, ssDNA) have small, unenveloped virions containing circular ssDNA and are icosahedral in shape. DNA replication occurs via the 'rolling-circle' model as a double-stranded replicative form. Enterobacteria, *Bdellovibrio, Chlamydia* and *Spiroplasma* are some of the different hosts infected by *Microviridae* (Ackermann, 2005).

Some families of tailless phages have as few as one member which is fully characterised. An example is Corticoviridae (polyhedral, dsDNA), phages which possess a protein capsid with an internal phospholipoprotein. Maritime phage PM2, a lytic phage is the only member with a complete description in this family (Kivelä *et al.,* 2002) while little is known about two other similar phages isolated from sea water. Similarly, Fusselloviridae (pleomorphic, dsDNA) has one definite member, SSV1 which exists as a plasmid and an integrated prophage in *Sulfolobus shibate*. It can be induced by mytomycin C and UV light. Fusselloviruses are spindle-shaped and have short spikes at one end. The coat is composed of two hydrophobic proteins and lipids, and is sensitive to chloroform treatment. They are released from their host by extrusion (Ackermann, 2003).

Interesting and unique features are also found in other groups. Tectiviridae (Polyhedral, dsDNA) have a rigid protein capsid containing a thick, flexible, lipoprotein vesicle which has the ability to change into a tail-like tube of approximately 60 nm long. This tube serves the same function as tails of tailed phages in that it acts as a vehicle for nucleic acid ejection following the phages' adsorption to their respective host or treatment with chloroform (Ackermann, 2005). Some of the bacterial groups which form tectiviruses' hosts are enterobacteria, *Pseudomonas, Thermus, Vibrio, Bacillus, Acinetobacter*, and *Alicyclobacillus.* Tectiviruses of *Bacillus* have apical spikes (Ackermann, 2003).

The family Leviviridae (polyhedral, ssRNA) is divided into two genera based on serology and genome structure. Their genome is made up of four partially overlapping genes. In addition, their RNA serves as mRNA and is thus positive-stranded. Morphologically, these viruses are non-enveloped. Many of the known leviviruses are plasmid-specific coliphages that adsorb to F or sex pili (Ackermann, 2005).

The cystoviruses (polyhedral, dsRNA) have lipid-containing envelopes which enfold the icosahedral capsids. They contain a dodecahedral RNA polymerase complex and three molecules of dsRNA (Bamford *et al.,* 1993). During the infection process, the envelope is lost and the capsid passes through the spaces between the cell wall and the cytoplasmic membrane. Cystoviruses are highly host specific in that they only infect *Pseudomonas syringae* (Ackermann, 2003).

DNA replication in members of the Inoviridae family (filamentous, ssDNA) occurs via a rolling-circle mechanism in a double-stranded state. This group consists of two genera: *Inovirus* and *Plectovirus* which have different host ranges. Members of *Plectovirus* are short, straight rods and they only infect mycoplasmas. Viral particles of the *Inovirus* genus are long, rigid or flexible filaments whose length gives an indication of genome size (Day & Maniloff, 2000). Unlike plectoviruses, they infect a number of hosts namely, Clostridia, Propionibacteria, the genus *Thermus,* enterobacteria and their relatives. Progeny inoviruses are released forcibly from the host cells and no lysis takes place, thus phages may be produced for an indefinite period (Ackermann, 2003; 2005).

Progeny Lipothrixviruses (filamentous, dsDNA) are released by lysis. Virions of the Lipothrixviridae family have a rod-like shape, a lipoprotein envelope and a nucleosome-like core. Examples of their hosts are *Acidianus, Sulfolobus* and *Thermoproteus,* all of which are thermophilic archaebacteria. Peng *et al.* (2001) reported that this family as well as Rudiviridae (filamentous, dsDNA) share similarities in their genomes, which implies that they form a superfamily. Rudiviruses were isolated from the thermophile *Sulfolobus*. This family has two viruses whose

lengths differ. Their viral particles are straight, non-enveloped rigid rods with fixation structures on one end (Peng *et al.,* 2001)

Another mechanism which may be used to release progeny viruses is budding as is evident in the family Plasmaviridae (Pleomorphic, dsDNA). Virions possess an envelope and a thick nucleoprotein granule. The envelope fuses with the host membrane during the infection process before budding can follow. *Archoleplasma* virus MVL2 or L2 is the only definite member of this family (Ackermann, 2003).

Guttaviridae (pleomorphic, dsDNA) Virion particles are droplet-shaped with a distinct beehive-like structure and thin fibers at its pointed end. An example is SNDV (*Sulfolobus neozealandicus* droplet-shaped virus) which was found in a *Sulfolobus* isolate from New Zealand. Genome size is approximately 20 kbp and its DNA is only cleaved by a few restriction enzymes such as *Dpn*I. Host range is limited to a few *Sulfolobus* strains (Arnold *et al.,* 2000).

New phages of archaebacteria were recently isolated and they have yet to be classified by the ICTV.

Sulfolobus turreted icosahedral virus (STIV, polyhedral ssDNA) is an archael virus isolated from an acidic hot spring. It has apical turret-shaped protrusions on its capsid and is the only known member of this family. It has been speculated that the turret-like structures may function in host recognition and/or attachment. It has also been suggested that the nucleic acid might be transferred via the central channel in each turret (Rice *et al.,* 2004). *Sulfolobus solfataricus,* a hyperthermophilic archaeon is the only host (Khayat *et al.,* 2005).

SH1 (polyhedral, dsDNA) virions contain lipid components in their structure and a distinct proteinaceous outer layer (Porter *et al.,* 2005). The genome is linear, 31 kbp and is unique in that it does not share any similarities with any of the published sequences. They are lytic phages and infect halobacteria of the genera, *Halorubrum*

and *Haloarcula hispanica* (Bamford *et al.,* 2005). Another family which is also unique in that no significant matches are found between its genes and those in public databases is *Globuloviridae* (pleomorphic, dsDNA). Virions are about 100nm in diameter, spherical with an envelope and a nucleoprotein core (Häring *et al.,* 2004).

Ampullaviridae (Pleomorphic dsDNA) members possess a unique bottle-shaped structure and a funnel-shaped core. Its broad end has thin filaments and the pointed end possibly plays a role in adsorption and translocation of DNA into the host (Häring *et al.,* 2005a).

A distinctive attribute of independent morphological development outside the host has been observed in *Bicaudaviridae* (pleomorphic, dsDNA). Lemon-shaped viral particles develop long tails at each end after they are extruded from the host. The change occurs at 75-90°C, close to the temperature of the natural habitat in the absence of a host. This extracellular morphogenesis is thought to be a strategy for survival in harsh environments where host availability is also reduced (Häring *et al.,* 2005b).

1.4. LIFE CYCLES

Bacteriophages can be categorized into virulent or temperate (also referred to as lysogenic) phages. This is determined by the events that follow injection of their nucleic acid into the host cell. Virulent phages always go through the lytic cycle which leads to the release of phage progeny once the host cell bursts (Engelkirk & Burton., 2006). Temperate phages have two life cycles. They have the ability to undergo lysis in their host cell, whereby their progeny are released into the environment. Moreover, they can establish a stable relationship with the host in which lytic genes are not expressed but rather their genome becomes integrated into the bacterial chromosome, and is replicated along with the host DNA (Little, 2005).

Bacteria have developed ways of avoiding or surviving viral attack at several stages of the phage life cycle. Interaction between the phage and the bacteria can be prevented by mutation of phage receptors or secretion of a capsule or slime layer. If

adsorption of phage to receptor is successful, transfer of nucleic acid can be blocked (Scandella & Arber, 1974, 1976; Elliot & Arber, 1978). The bacteria also employs certain survival mechanisms when phage nucleic acid transfer occurs; different phases of intracellular development can be aborted should the host be unable to meet the needs of the phage or the host restriction enzymes may destroy the DNA (Krüger & Bickle, 1983). Phages have also developed means to overcome the bacterial survival mechanisms. These include mutation which can change its adsorption specificity and specific host-controlled modifications which allow for improved propagation in cells of a particular strain while restricting multiplication in cells of a different strain (Luria, 1953).

1.4.1. LYTIC LIFE CYCLE

1.4.1.1. ADSORPTION

The life cycle of most phages involves a critical initial step, that is, the binding of the virion to a receptor in the host cell membrane followed by DNA transfer (Inamdar *et al*., 2006). Most phages are known to be species or strain-specific, while some can infect more than one bacterial species (Engelkirk & Burton, 2006).

Infection into the bacterial cell is initiated by binding to specific surface molecules or capsules using fibers or spikes which form the adsorption structures. Binding sites differ depending on the type of targeted bacteria, that is, gram positive or gram negative bacteria. Oligosaccharides, lipopolysaccharides and almost any of the proteins in gram negative bacteria can serve as receptors. The rate and efficiency of adsorption may differ for any particular phage-host system. These parameters are influenced by external factors and physiological condition of the host. Cofactors such as Ca^{2+}, Mg^{2+}, divalent cations or sugars may be required for successful binding to occur. For instance, the presence of maltose is crucial in order for the lambda phage receptor to be expressed (Guttman *et al.,* 2005).

1.4.1.2. PENETRATION

Following successful adsorption, phage genome transfer takes place. The mechanisms for this are specific for different phages. However, one common trait of

nearly all phages is that their capsids and tails remain outside the cell while only the genome is transferred to the cytoplasm. This is in contrast to what happens in the majority of eukaryotic viruses where the envelope fuses with the host plasma membrane when the genome is delivered (Letellier *et al*., 2004). Generally, the tail tip penetrates the peptidoglycan layer and the inner membrane to release DNA into the cell. Typical viral genome is approximately 10 µm long and its transfer from outside to inside the cell takes a variable amount of time, from seconds to minutes (Inamdar *et al.,* 2006). Specific examples will be cited to indicate some of the different strategies employed by different phages to transport DNA across the membrane.

Firstly, T4 phage: When it binds to the lipopolysaccharide (its receptor), the tail contracts and the tip of the internal tube is brought close to the membrane, thus allowing for 172 kbp of DNA to be injected into the cytoplasm in 30 seconds at 37°C. This is approximately 4000 base pairs per second, making it the highest rate for DNA transport (Lettelier *et al.,* 2004).

For phage T7, the genome is much smaller in comparison to T4 at approximately 40 kbp and it is transferred into the host in about 10 minutes at 30°C (Lettelier *et al.,* 2004).

Phage T5 has a genome size of 121 kbp which is translocated in two steps: 8% of the DNA first enters the cytoplasm. This is followed by a 4 minute pause at 37°C during which time protein synthesis occurs. Two of the newly synthesized proteins named A1 and A2, are essential for the remaining DNA to be transferred (Lanni, 1968). Bonhivers and Lettelier (1995) reported that at least 0.1 mM calcium is necessary for infection by T5 to take place. These authors noted that membrane permeability changes during DNA transfer is regulated by calcium levels and they proposed that the protein which forms the channel for DNA transport is affected by the presence of these same ions. Reduced calcium levels leave the channel open and this in turn leads to adverse conditions for synthesis of phage components and ultimately, the infection process is aborted.

The bacterial host has the potential to cleave phage DNA by the use of its exonucleases and restriction enzymes, hence protective measures are necessary. These include circularizing of phage DNA by sticky ends or terminal redundancies, inhibition of nucleases (T4, T7) or the presence of an odd nucleotide in the DNA, for example, hydroxymethyldoexyuridine (hmdU: SPO1) or hydroxymethyldeoxycytidine (hmdC: T4). In the coliphage N4 and Staphylococcal phage Sb-1, the genomes do not contain sites which can be recognised by the common host restriction enzymes as a result of selection over time (Guttman *et al.,* 2005).

1.4.1.3. BIOSYNTHESIS

This step in the lytic cycle involves the production of viral components (figure 1.2). This is accomplished using the host cell's enzymes such as DNA polymerase and RNA polymerase, amino acids, ribosomes, and nucleotides to synthesize viral nucleic acid and proteins (Engelkirk & Burton, 2006). The RNA polymerase of the host recognises strong phage promoters which results in the transcription of *immediate early* genes. A group of *middle genes* is then usually transcribed, and products of this are responsible for the synthesis of phage DNA. Subsequently, *late genes* that encode the different parts of the phage particle are produced. For the host cell to be reprogrammed to synthesize progeny phage, certain mechanisms are employed. These include the degradation of host DNA and inhibition of the translation of host mRNAs, some phages produce DNA-binding proteins to reprogram host RNA polymerase while others encode their own RNA polymerases which are much smaller and faster-moving than those belonging to bacteria (Guttman *et al.,* 2005).

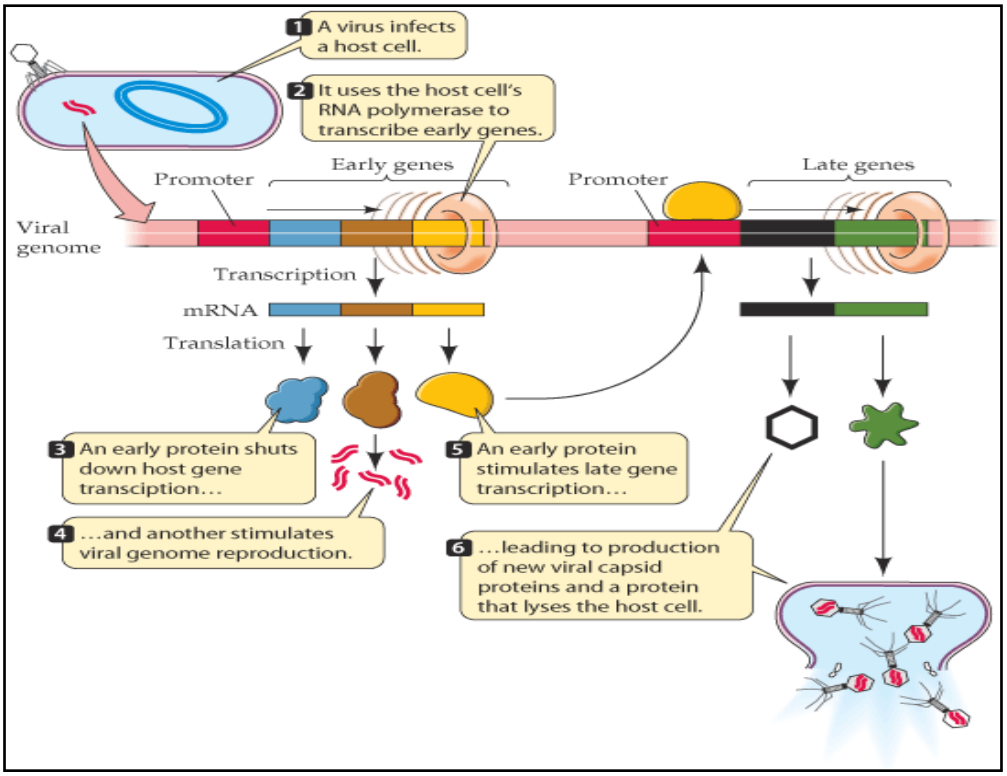


Figure 1.2 : Biosynthesis of new phages
http://bcs.whfreeman.com/thelifewire/default.asp?s=&n=&i=&v=&o=&ns=0&uid=0&rau=0

1.4.1.4. ASSEMBLY

Various phage components are assembled to produce entire virions. Nucleic acid is packaged into procapsids which are icosahedral protein shells (Guttman *et al.,* 2005). For tailed phages the initial head-like structure is a thick-shelled prohead around a protein scaffold. The scaffolding and the N-terminus of the main head proteins later undergo proteolytic cleavage. DNA is packaged into the head after which it is linked to preassembled tail structures to form infectious entities. The head expands and gains stability prior to or during DNA packaging, to accommodate the long DNA molecule. The stability also allows for swift exit of DNA when infection is initiated (Guttman *et al.,* 2005)

1.4.1.5. RELEASE

Bacterial lysis is the last stage in the lytic life cycle. Release of the progeny into the environment for most phages can only be accomplished once the host envelope is disrupted (figure 1.3). This is however, not the case for some phages, for instance, filamentous phages whose progeny are released by extrusion through the bacterial

membrane without destroying the host (Young *et al.,* 2000). Some differences do exist between lytic and non-lytic systems, the major one being the absence of a rigid capsid in filamentous phages. With the exception of phages with ssDNA and ssRNA, lytic phages encode and involve an enzyme, known as an endolysin which degrades the bacterial peptidoglycan (Blasi & Young, 1996). This enzyme is not the sole contributor to lysis. Endolysins need a second lysis factor, a phage-encoded membrane protein called holin. The holin-endolysin system is essential for host lysis (Young, 2002).

According to Young *et al.* (2000), most endolysins do not have a signal sequence and they require holins to gain entry into the murein as illustrated in figure 3. Therefore, holins serve as a timing mechanism for lysis and controls when this event takes place. Without this intrinsic secretory signal, endolysins simply accumulate in the cytoplasm (Young, 2002) and the membrane structure is kept intact while synthesis and assembly of progeny virions takes place (Wang, 2006). It is of great importance that the timing of lysis occurs under optimal conditions. That is, if lysis is triggered prematurely, virion assembly would suffer and in the event of late lysis, opportunities for infection of new hosts would be adversely affected.

In simple lytic phages such as those whose genome is ssRNA, phage genes encode for a single lysis protein which inhibits cell wall synthesis, weakening the cell wall and it eventually collapses. Muralytic enzyme activity has not been observed (Young *et* al., 2000; Engelkirk & Burton, 2006).

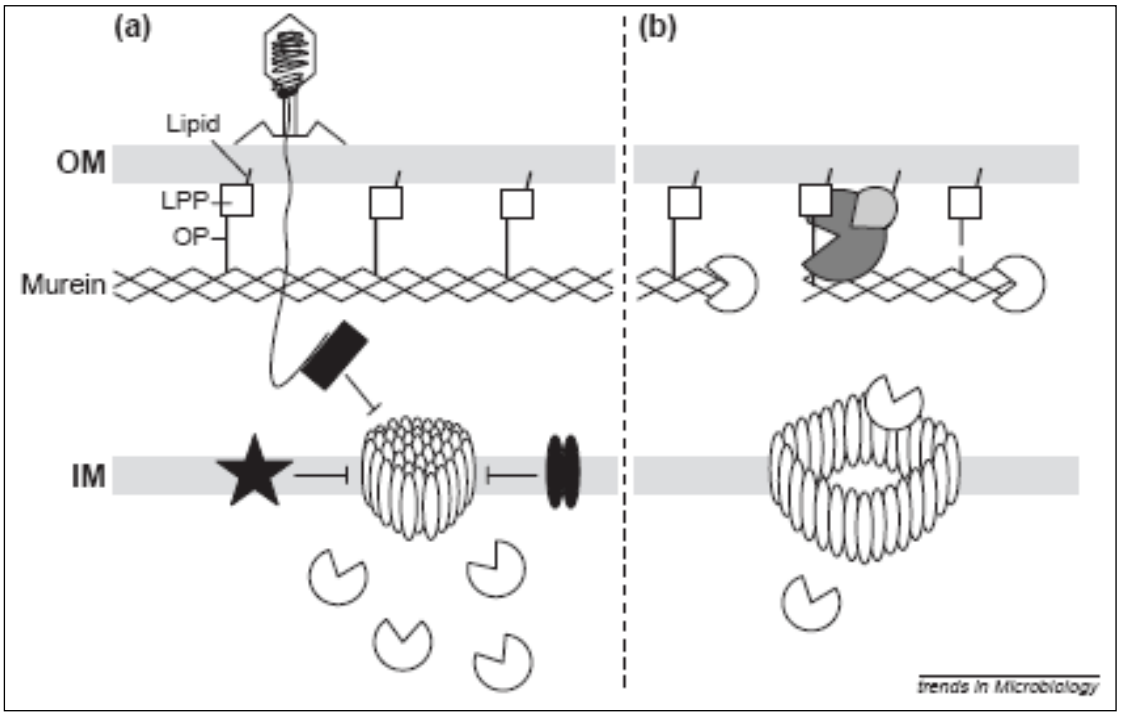


Figure 1.3: Degradation of the Gram-negative envelope during holin-endolysin lysis. The host envelope consists of the inner (IM), outer (OM) membrane and murein, linked to the OM by the oligopeptide (OP) links to the lipoprotein (LPP) tied to the OM with lipid moieties. (a) Pre-hole configuration. Holins (represented by clear ovals) accumulate in membrane aggregates and endolysin (serrated clear circles) builds up in the cytosol. Triggering of holin action can be inhibited by inhibitors. These can be membrane proteins orthologous (black ovals), or non-orthologous (star) to the holin, or a periplasmic protein (rectangle). Binding of periplasmic inhibitor to DNA is hypothetical and its proposal is used to explain that the signal for T4 lysis inhibition requires the injection process. (b) Hole configuration. A lesion is formed by the holin, allowing escape of endolysin, which attacks the murein. Adapted from Young *et al*. (2000).

1.4.2. LYSOGENIC LIFE CYCLE

In the lysogenic life cycle (figure 1.4), the viral genetic material is incorporated into the bacterial DNA. This is referred to as the prophage or proviral state. A virus can remain in this latent state and is replicated with the host DNA. Bacteria harbouring prophages are known as lysogens (Stansfield *et al*., 1996). Temperate phages do possess lytic genes but their expression is prevented by a repressor. This allows the viral genome to be replicated with the host DNA until the switch to the lytic stage is possible. The mechanisms by which this switch from the lysogenic to the lytic phase occurs is varies for different viruses (Little, 2005).

The best studied example is the bacteriophage λ and it is used to illustrate the lysis-lysogeny decision. The host regulatory system known as the SOS regulatory circuit plays a major role in the switching initiative. The decision to follow one life cycle or the other is made 10 to 15 minutes after infection. The physiological condition of the cell is thought to be influential in the outcome although the mechanisms for this are not known. The λ genome is organised into functional modules which have specific functions that they perform in the life cycle. The concentration of an activator called CII is a determining factor in the pathway followed after infection. When CII levels are high, the lambda repressor CI is expressed at high concentrations from P_{RE}, the promoter for the establishment of this repressor and the result is the lysogenic cycle. A different promoter, P_{RM} then maintains the lysogenic state. When CII is at low levels or absent, the lytic pathway ensues (Little, 2005).

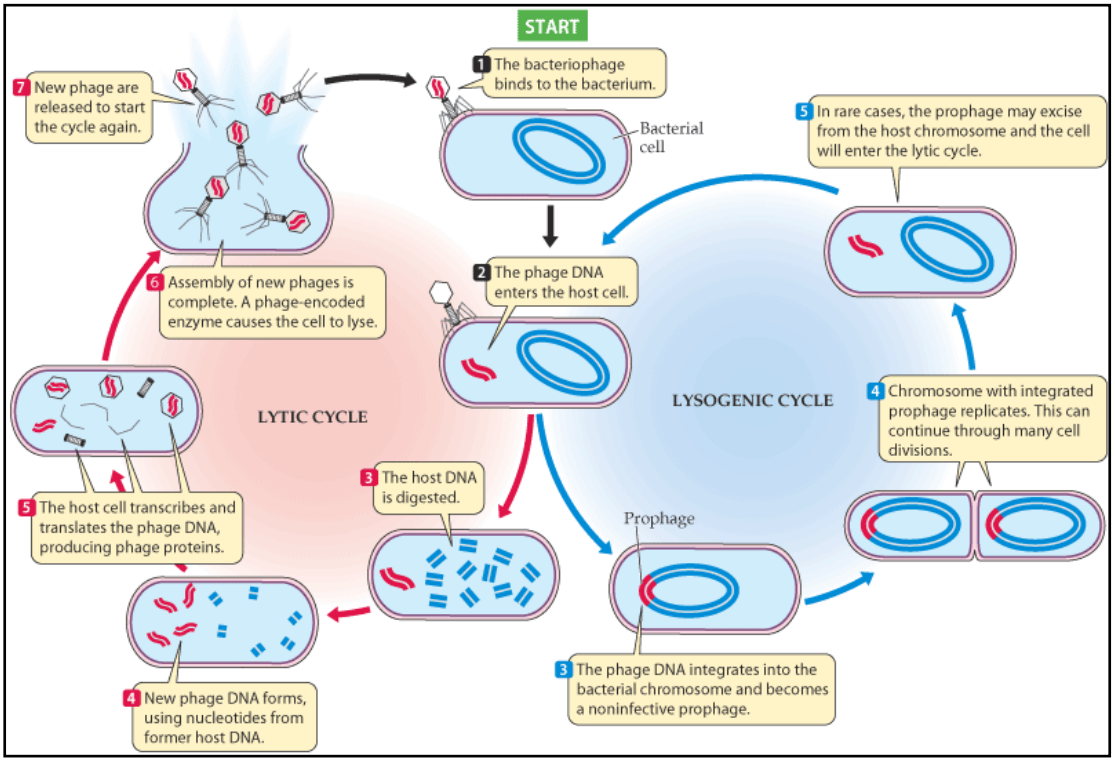


Figure 1.4: Life cycle of a temperate phage.
http://bcs.whfreeman.com/thelifewire/pages

1.5. BACTERIAL INFECTIONS IN POULTRY

Bacterial diseases in poultry are of economic importance to the industry worldwide due to the monetary losses incurred following infection. Bacteria may be primary pathogens, but more often than not, they are opportunistic pathogens or exist with other, mostly other viral other pathogens (Barnes *et al.,* 2003). There are several common bacterial diseases which are of major health concerns in the poultry industry. Examples are Salmonellosis, *Campylobacter* infections, Infectious coryza, mycoplasmosis, and colibacillosis. Some of the above mentioned diseases are important not only to poultry but to human beings as well.

1.5.1. COLIBACILLOSIS

Colibacillosis is a disease caused by avian pathogenic *Escherichia coli* (APEC), the majority of strains which exist as part of the normal microbiota on the intestinal tract and other mucosal surfaces of domestic poultry and wild birds (La Ragione & Woodward, 2002). In addition, pathogenic as well non-pathogenic serotypes may also be isolated from the bird's external environment. *E. coli* infections are characterised by colisepticemia, coligranuloma (Hjarre's disease), air sac disease (Chronic respiratory disease, CRD), avian cellulitis, swollen-head syndrome, egg peritonis, salpingitis, osteomyelitis/synovitis, panophthalmitis and yolk sac infection (Barnes *et al.,* 2003).

In normal healthy chickens, it has been reported that 10-15% of all intestinal coliforms are related to potentially pathogenic serotypes (Harry & Hemley, 1965). Embryo and chick mortality can be attributed to the presence of *E. coli* in yolk sacs. The most important source of infection is believed to be faecal contamination of the egg surface and penetration of the shell and membranes (Barnes & Gross, 1997).

Colibacillosis in poultry is usually a secondary system disease which occurs when the host immune system is compromised. This can be due to compromise of the mucosal barrier (wounds, lack of normal microbiota, mucosal damage from bacterial, viral, or parasitic infections), immunosuppression (e.g toxins or viral infections), exposure to adverse environmental conditions (poor ventilation, overcrowding, poor

litter conditions etc), stress extremes (minimal or severe) or damage of the mononuclear-phagocytic system (viral infections, nutritional deficiencies). Birds are most likely to suffer from colibacillosis following infection with infectious bronchitis virus (IBV) in chickens, hemorrhagic enteritis virus (in turkeys) and exposure to ammonia (avian species). Respiratory tract infection is the most common infection of poultry where IBV, *Mycoplasma* sp., and Newcastle disease have already established infection (Barnes & Gross, 1997).

All ages may be affected by the disease; however, it is more widespread in young growing chickens and turkeys. Principal routes of invasion are the respiratory system and the gastrointestinal tract. The egg shell may be penetrated by bacteria before or during incubation while the navel (through the yolk sac) is also another potential area of entry which may result in omphalitis. Septicaemia is the most severe form of colibacillosis and affects chickens, ducks and turkeys of between 4-12 weeks old. Acute and extreme acute septicaemia leads to high mortalities, some without the usual lesions such as swelling of the liver, spleen, and kidneys, dehydration, fibrinous exudates in the air sac or on the surface of the heart, liver and lungs. The outcome of high mortality may also be due to omphalitis in newly hatched chicks and poults (Gross, 1991).

The poultry industry worldwide suffers significant economic losses as a result of *E. coli* infections which are responsible for morbidity, mortality and increased condemnations (Barnes & Gross, 1997). An example is the poultry industry in the United States of America which alone has an annual value of more than $50 billion and *E. coli* infections pose a major threat and millions of dollars may be lost. Economic losses of more than $80 million were incurred by the US poultry industry in 2002 due to *E. coli* infections in broilers (Kish, 2008).

APEC strains most often belong to the serogroups 01, 02, 05, 018, and 078 (Blanco *et al.,* 1997).

1.5.1.2. VIRULENCE FACTORS OF APEC

For pathogens to cause disease they must possess certain virulence factors which operate singly or in combination at different phases of infection (figure 1.5). Their actions may vary from direct interactions with host tissues to protective measures against the host's immune responses (Wu *et al.,* 2008*)*. The degree of pathogenicity varies between the different strains of *E. coli.* Many of these virulence determinants have been determined for APEC and they include: adhesins (type 1, curli and P fimbriae), aerobactin iron sequestering systems, K1 capsule, serum resistance, yersiniabactin uptake, colicin production, outer membrane proteins, temperature sensitive haemagglutinin, hemolysin E, flagella, toxins and cytotoxins, and iron response protein (Janβen *et al.,* 2001; La Ragione & Woodward, 2002). Although several virulence factors of APEC have been identified, understanding of the genes encoding them and their mechanisms are currently not well-known (La Ragione & Woodward, 2002).

Thus far, no specific virulence factor has been found which is wholly responsible for pathogenicity of APEC. Vidotto *et al. (*1990) found that a combination of certain virulence factors increased a strain's virulence and it is now generally accepted that the pathogenicity of *E. coli* is increased by a number of these characteristics in combination. Interestingly, many of the factors linked to virulence in APEC are also related with virulence in extraintestinal *E. coli* in different species. Therefore elucidation of the different functions of virulence genes in APEC may be useful for understanding colibacillosis in other species (Tivendale *et al.,* 2004) and is necessary for the advancement of preventive measures (Skyberg *et al.,* 2006).

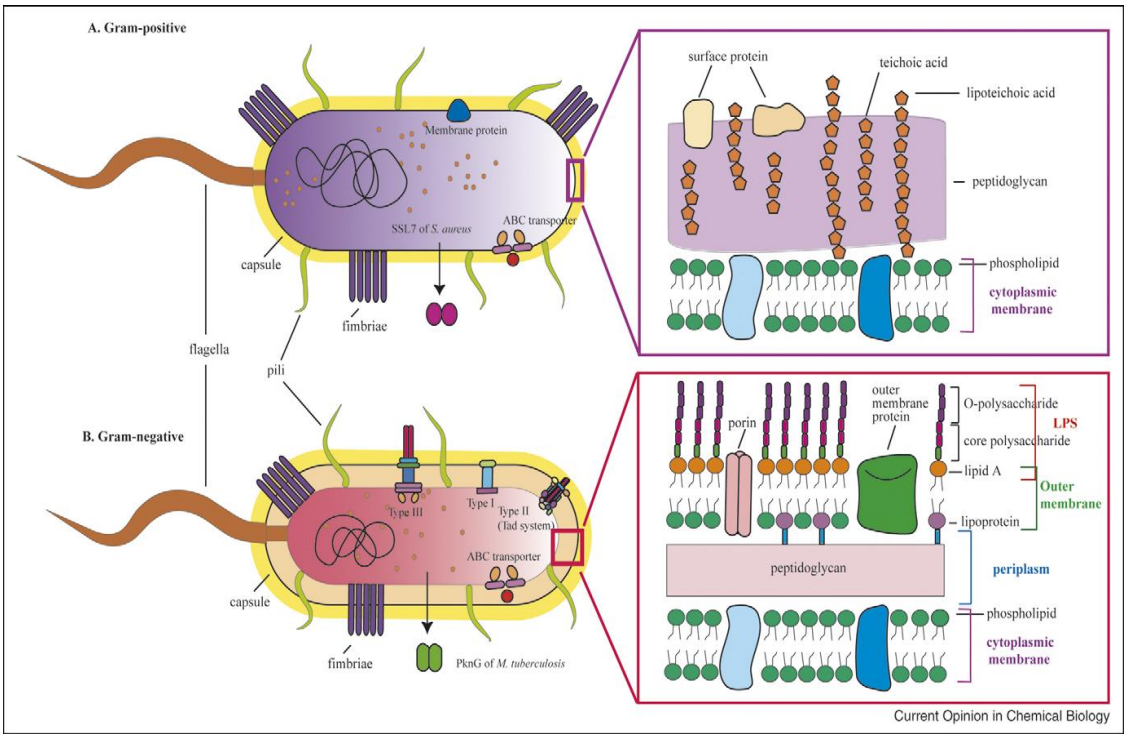


Figure 1.5: Main virulence factors of gram negative and gram positive pathogenic bacteria. (Wu *et al.* 2008).

For colonisation and subsequent pathogenesis to occur, the pathogen must first adhere to the host. This is achieved by the adhesins. Type 1 fimbriae mediate adhesion of *E.coli* to host epithelial cells of the pharynx and trachea. These adhesins have the ability to bind D-Mannose and several types of eukaryotic cells and were demonstrated through a specific pathogen free (SPF) chick model to be important in colonisation, invasion and persistence (La Ragione & Woodward 2000, La Ragione *et al.,* 2000). Type 1 fimbriae can agglutinate fowl or guinea pig erythrocytes but this agglutination is inhibited in the presence of mannose, hence the name mannose-sensitive haemagglutinating (MSHA) fimbriae (Vidotto *et al.,* 1997). It has also been proposed that type 1 fimbriae can offer *E. coli* protection from phagocytosis (Orndoff, 1994). P fimbriae are also thought to play a role in adherence to epithelial cells and the colonisation of systemic organs which later results in septicaemia (Pourbakhsh *et al.,* 1997). Curli fimbriae are found in most *E. coli* and are believed to be involved in the survival of the bacteria when it is outside the host, in early colonisation (Olsen *et al.,* 1994) and may contribute to the early stages of infection (Herwald *et al.,* 1998). Curli and P fimbriae confer mannose-resistant haemagglutination (MRHA).

Most APEC have flagella which aid in motility and promote penetration of intestinal mucus before adhesion to the epithelial cells can take place. In addition, La Ragione *et al*. (2000) demonstrated the importance of flagella in colonisation, invasion and persistence in an SPF chick model.

Temperature sensitive haemagglutinin may serve as an adhesin in the early phases of colonisation of the respiratory tract of chickens. Dozois *et al.* (2000) showed that the *tsh* gene, located on the col V plasmid and which can also be present on other plasmids (Stehling *et al.,* 2003), is important in the formation of lesions in the air sacs although it was not necessary for infection.

Serum resistance is an important virulence factor which Mellata *et al.* (2003) suggests could be a major contributor to pathogenesis of *E. coli* in poultry. This is because the bacteria are able to escape the bactericidal activity of the complement system. Studies by Ngeleka and colleagues (1996) on *E. coli* cellulitis in broilers suggested that resistance to serum is beneficial for the bacteria during production of cellulitis. All thirty-nine *E. coli* isolates from broilers with cellulitis survived in the presence of normal chicken serum. Recent studies by Mellata *et al.* (2003) also revealed a link between serum resistance and the continued presence of APEC in the body fluids and internal organs, thus effecting pathogenicity of the bacterial strains. The inclusion of serum sensitive mutants also supported these findings. The mutants did not infect internal organs.

Following invasion, host cells chelate iron in extracellular (e.g. trasferrin) or intracellular (ferritin) fluids as a defence mechanism against bacteria. Bacteria must find means to survive under such circumstances. Avian pathogenic *E. coli* can grow under iron-limiting conditions due to the presence of high affinity systems of iron acquisition and siderophores which can compete with transferrins for iron. The aerobactin system is encoded by plasmids or chromosomes and is a feature of virulent bacteria that elicits systemic infections and has been related to mortality. Dho and Lafont (1984) found a correlation between this characteristic and virulence in APEC for 1 day old chicks. Furthermore, aerobactin genes were present in virulent APEC and absent from most of the non-virulent strains (Lafont *et al.,* 1987).

Hemolysin activity is a mechanism which bacteria also use to survive the defence systems of hosts. Haemolysins lyse erythrocytes and release iron from haemoglobin, and therefore allow for the survival and prolifereation of invasive bacteria. Heamolysin have only been found in a few APEC (La Ragione & Woodward, 2002).

Toxins in APEC are uncommon. However, a few have been implicated in disease and may be lethal to host cells (Salvadori *et al.,* 2001). Blanco *et al.* (1997b) investigated the occurrence of toxins (enterotoxins, verotoxins and necrotoxins) in *E. coli* strains isolated from septicemic and healthy chickens. Only 7% of all the *E. coli* isolates from the study produced toxins.

Col V production is an important feature of virulence but it does not necessarily increase it. Col V and some R plasmids carry genes which are responsible for invasion and pathogenicity. It is suggested that these plasmids organise the outer membrane proteins which aid in serum resistance of *E. coli.* Col V plasmids also assist in improved absorption of Iron ions (Vidotto *et al.,* 1990).

The presence of K1 Capsular antigen is associated with infection and is thought to contribute to serum resistance. This is by possibly concealing structures on the surface of the cell wall to which complement elements might bind. Conflicting results from studies by different researchers exist on whether there is a correlation between K1 capsule and serum resistance. Wooley *et al.* (1993) found no relationship whatsoever while Stawski and co-workers (1990) found a link might be likely.

1.5.1.3. TREATMENT, PREVENTION AND CONTROL

Antimicrobial drugs have traditionally been used to treat and prevent *E. coli* infections among other bacterial infections. These include ampicillin, chloramphenicol, chlortetracycline, neomycin, nitrofurans, gentamicin, ormethiprim-sulfadimethoxine, nalidixic acid, oxytetracycline, polymyxin B, spectinomycin, streptomycin, sulfa drugs and fluoroquinolones such as enroflaxin and sarafloxacin (Barnes & Gross, 1997). Apart from treatment purposes, antibiotics have been used as supplements in animal feed for prophylaxis and to promote animal growth by

improving the feeding efficiency. Unfortunately, these practices have contributed to the development of antibiotic resistance. Endtz *et al.* (1991) noted an increase in *Campylobacter* spp. which were resistant to ciprofloxacin and decided to investigate whether there was a correlation between the use of quinolones in human and veterinary medicine. They did find a link between the two, and it was also concluded that resistance is probably largely as a result of the use of enroflaxin in poultry production. Al-Sam and colleagues (1993) observed that chicks given feed containing ampicillin at concentrations as low as 1.7 and 5 g/ton developed antibiotic resistance. A few years later, Manie *et al.* (1998) reported that Staphylococci, Enterobacteriaceae, *Salmonella a*nd other isolates on retail and abbatoir chicken in South Africa exhibited multiple antibiotic resistance to streptomycin, gentamicin, methicillin, and tetracycline. They attributed the occurrence of resistance to the inclusion of antibiotics at low doses which was referred to earlier. It is therefore imperative to screen all antibiotics to be used and select the ones to which *E. coli* strains are sensitive. However, with increasing concerns worldwide regarding antibiotic usage, alternatives to this method of treatment are currently being investigated.

A number of vaccines have been successfully used to provide protection against APEC in the past. However, these vaccines are no longer effective and by 2004 there was only one commercially available *E. coli* vaccine for use in broiler breeders, a subunit vaccine containing F11 fimbrial and flagellar toxin antigens (Vandekerchove *et al.,* 2004). Research into the development of a vaccine for APEC is currently underway by Dr. Curtiss and co-workers at Arizona State University (Kish, 2008).

The incidence of *E. coli* can be reduced by eliminating as many of the factors as possible that leave poultry susceptible to infection. For instance, raising *Mycoplasma*-free birds, proper ventilation and reduced exposure to viruses which cause respiratory disease are some of the factors which can prove useful in lowering the chances of infection occurring (Barnes & Gross, 1997). Other preventive and control measures include collecting eggs on a regular basis to decrease the levels of faecal contamination, disinfecting shell surfaces of eggs as soon as possible after collection, maintaining clean nest material, chlorination of drinking water, disposing

of broken eggs, and competitive exclusion from the intestines using normal microbiota from resistant chickens (Barnes & Gross, 1997; Weinack *et al.,* 1981).

1.6. THE SOUTH AFRICAN POULTRY INDUSTRY AND THE IMPACT OF DISEASE OUTBREAKS

The South African poultry industry had an annual value of R13.5 billion in the year 2007 and it was reported that production was increasing by about 7% per year, a trend which is expected to continue. The demand for poultry meat was greater in comparison to other sources of protein and this was attributed to a number of reasons. Health awareness, convenience, increased marketing by broiler producers, and competitive prices are some of the factors which have made poultry meat a favoured choice of protein (USDA GAIN report, 2007).

According to the US Department of Agriculture GAIN report (2007), the SA poultry industry makes up about 16% of the total gross value in the agricultural sector. A consistent growth in broiler production has been observed over the years. In 1990, 7.6 million broilers were produced weekly and by 2007, the number had increased to 13.8 million. This is equivalent to approximately 717 million birds per year. Despite the steady growth and increased demand, some risks which producers and investors are faced with are noteworthy and include: increase in corn prices, theft, imports of cheap poultry meat and disease outbreaks. The latter has an enormous economic impact. Viral and bacterial diseases such as Newcastle disease, Infectious Coryza (usually affects layers but can also be a problem in broiler breeders) and colibacillosis have the potential to result in massive monetary losses due to high mortality, and morbidity rates in the event of outbreak. Therefore, the need for effective disease control measures cannot be over emphasized.

1.7. ANTIBIOTIC RESISTANCE AND ALTERNATIVES TO ANTIBIOTICS

Antibiotics have been widely used in the healthcare sector since the discovery of penicillin completely revolutionised the way a wide variety of bacterial infections were treated. By 1996, more than 100 antibiotics had been produced by pharmaceutical companies. The main groups of antibacterial agents are β-lactams (penicillins,

cephalosporins, monobactams, carbapenems), aminoglycosides, tetracyclines, sulphonamides, macrolides (e.g. erythromycin), quinolones, and glycopeptides such as vancomycin (Chu *et al.,* 1996).

Antimicrobial agents have different mechanisms which they employ in their action against microorganisms. They include effective inhibition of the following processes: nucleic acid synthesis, and protein synthesis, disruption of the bacterial membrane structure, interference with cell wall synthesis and metabolic pathways. Bacteria may possess some degree of inherent resistance to at least one class of antimicrobial agents or they may acquire this trait through chromosomal mutation, gain resistance genes from other organisms (Tenover, 2006), exchange of new genetic material by transformation, conjugation or transduction. Antibiotics can be inactivated in three ways: inactivation of the antibiotic through modification or destruction, alteration of the target site of the antibiotic and lastly, inaccessibility to target (Neu, 1992).

The use of broad-spectrum antimicrobial drugs places selective pressure on bacterial strains and thus allows for resistance to develop. As early as 1944, reports of antibiotic resistance had surfaced. *Staphylococcus aureus* could destroy penicillin using penicillinase, also known as β-lactamase (Neu, 1992). This enzyme is also produced by *Bacillus subtilis, Bacillus antracis, E. coli, Klebsiella pneumonia, Proteus vulgaris* and *Shigella shigae* to name a few examples (Hare, 1967). Efforts were made to curb this challenge through the development of a semi-synthetic penicillin called methicillin. Unfortunately, by the 1980s *S. aureus* was also resistant to this new drug. A similar scenario of outbreaks of resistance to different antimicrobials was also noted in other bacteria such as *Streptococcus pneumoniae, Streptococcus pyogenes, Haemophilus influenzae, Neisseria* and enteric pathogens (Neu, 1992).

Diverse approaches can be taken to reduce incidence rates of resistance and they include screening the antibiotics to ascertain the one to which the pathogen is sensitive, increasing dosage concentrations to levels that almost, if not entirely destroy the infecting microorganisms, combination therapy whereby two unrelated antibiotics can be used (Hare, 1967).

Antibiotics have other applications apart from being a valuable means of disease treatment. They are used both in the plant and animal agriculture to treat or control disease, and in the food animal industry to promote growth. The use of antibiotics as growth promoters began in the 1940s when animals fed dried mycelia of *Streptomyces aureofaciens* supplemented with chlortetracycline residues were found to have improved growth (Castanon, 2007). Philips *et al.* (2004) describe 'growth promotion' as the use of antimicrobials for the improvement of average daily weight gain and feed efficiency in livestock. Knowledge on the mechanisms that bring about this effect is limited. In some cases the commensal intestinal microbiota may be changed and this leads to increased efficiency in digestion of feed and nutrient metabolism, while in other instances the immune system is stimulated and pathogens and disease are prevented.

Mellon and co-workers (2001) estimated that about 10.5 million pounds (approximately 4.8 million kilograms) of antibiotics in the US were used for poultry production alone, 10.3 million in swine and 3.7 million pounds in cattle production. About half of all antimicrobials which were utilised in the EU were administered to animals, with approximately 30% of this value for growth promotion. It is interesting to note that about 13.5 million pounds of antimicrobials banned in the EU are used in the US for "non-therapeutic purposes" in the agricultural sector on an annual basis according to Mellon *et al.* (2001). This gives an indication of the over-use of antibiotics. Unfortunately figures for antibiotic use for growth promotion in different countries are not well documented but one can get an indication of how common this practice is and the far reaching consequences. Other factors such as the inappropriate use (incorrect dosage or wrong duration) have contributed to selection for antibiotic resistance. Antibiotics used as growth promoters are often added to feed or water at subminimal levels (Barton, 2000).

Many of the antibiotic feed supplements usually belong to the same classes as the antibiotics used in the human medical field and their mode of action is often similar. Hence, one of the main concerns apart from the increase in the incidence of antibiotic resistance is the possibility that the resistance genes can be transferred to human microbiota through the consumption of animal products. This would in turn render antibiotic treatment for human bacterial infections useless. This is particularly

important in zoonotic bacteria such as species of *Campylobacter, Listeria, Salmonella* and enterohaemorrhagic *Escherichia coli* (Aarestrup & Wegener, 1999). It has been demonstrated that the potential threat of antibiotic residues in food products causing allergic or toxic reactions in humans is minor (Donogue, 2003).

The likelihood of dissemination of resistant genes from animals to humans and whether antibiotic use in animals compromises treatment in humans is an area that has been widely disputed. Nonetheless, the fact that widespread use of growth promoters has a negative impact in the long term on the use of antimicrobials for treatment cannot be ignored. To investigate this contentious topic, van den Bogaard *et al*. (2001) analysed the incidence of resistance in faecal *E. coli* in poultry, poultry farmers and slaughterers who had daily contact with the said animals. Their findings suggested that transmission of resistant bacteria from animals to humans does occur. Recently, Johnson and colleagues (2007) also made similar observations in a study conducted in Minnesota and Winsconsin between 2002 and 2004. Several other researchers have contributed significantly to our knowledge of the possibility of transmission of resistant bacteria from animals through the food supply to humans. As a result the World Health Organisation and the European Union (EU) resolved that feed additives were a public health issue (WHO, 1997; Economic and Social Committee of the European Union, 1998) and from early 2006, the EU authorised the withdrawal of antibiotics as growth promoters. In the US the FDA revealed that the use of Baytril (belongs to the fluoroquinolone class of antibiotics) in poultry production decreased the efficacy of Ciprofloxacin which is used to treat *Campylobacter* infections in humans. This subsequently led to the banning of Baytril (www.keepantibioticsworking.com) and other fluoroquinolone based products for use in poultry production.

1.8. PHAGE THERAPY

The emergence of antibiotic-resistant bacteria, as well as the ban on fluoroquinolones and impending future bans on other antimicrobials for use in animal production has prompted interest in alternative forms of treatment for bacterial infections. Interestingly, the potential for phage therapy was recognised and pioneer work in this field was applied from the time phages were discovered and before the first antibiotic, penicillin was introduced (Alisky *et al.,* 1998). This form of therapy was

discontinued in the Western world in the 1940s when antibiotics were discovered. A general lack of understanding and knowledge of phage biology also strongly discouraged further research in the West. Carlton (1999) cites the use of “highly inadequate scientific methodologies” as a major shortcoming of early phage therapy. This includes the lack of placebo-controlled studies, failure to demonstrate scientific proof of efficacy through reliable and reproducible results, and the presence of bacterial debris in therapeutic phage preparations. The former Soviet Union and the Eastern Europe, however, continued actively using phages to combat bacterial diseases despite the advent of the antibiotic era (Sulakvelidze *et al.,* 2001).

There are numerous reports of success stories in the Eastern Europe. These mostly involve treatment and prophylaxis of bacterial infections in humans. Bacterial dysentery, salmonellosis, lung and pleural infections, suppurative skin infections, peritonitis, osteomyelitis, lung abscesses, cerebrospinal meningitis and postoperative wound infections in cancer patients are some of the human infections which were successfully treated or prevented with phages (Babalova *et al.,* 1968; Cislo *et al.,* 1987; Meladze *et al.,* 1982; Sakandevelidze & Meipariani, 1974; Kochetkova *et al.,* 1989).

1.8.1. ADVANTAGES OF PHAGE THERAPY OVER ANTIBIOTICS

Lytic phages are the most suitable candidates for phage therapy. A number of advantages make it an attractive alternative to antibiotics: (1) bacteriophages are highly specific for their target pathogens. This is in contrast to antibiotics which usually have a broad spectrum of activity and destroy both the pathogens of interest and affect the normal microbiota which may make the subject susceptible to secondary infections. The narrow host range of phages is a feature that would prove useful in prophylaxis of infections caused by enteric bacteria (Barrow & Soothill, 1997); (2) phages use mechanisms different to antibiotics to destroy bacteria and thus remain highly effective even against multiple drug resistant bacteria; (3) cheaper to develop new phage systems than new antibiotics; (4) quick response to phage resistance because phages can also mutate in time with evolving bacteria; (5) side effects or allergic reactions are rare; (6) phages increase exponentially within their host and are consequently released at the site of infection where they are required (Carlton, 1999; Matsuzaki *et al.,* 2005).

In practical terms, the high host specificity of phages can also be a limitation to phage therapy and phage cocktails rather than single phage preparations are more desirable. Administration can be in liquid form or in a lyophilised state. The latter form is easier to use as pills, is more stable and greatly reduced in bulk size (Brussow, 2005). Lyophilised phages can be added to drinking water for animals, for disease prevention or treatment.

1.8.2. PHAGE THERAPY IN ANIMALS

In the Western world, Smith and Huggins (1983) successfully demonstrated the use of bacteriophages in treating *E. coli* infections in calves, piglets and lambs. In previous investigations by the same authors (1982) where mice were challenged with *E. coli,* these researchers showed that a single dose of phage is more effective than multiple injections of chloramphenicol, tetracycline, ampicilin, or trimethoprim with sulfafurazole. These were some of the studies that encouraged other scientists in the West to revisit the potential use of bacterial viruses, especially in the midst of the challenging times of antibiotic resistant bacteria. Several years later, Soothill (1992; 1994) used phage therapy to control *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infection in burn wounds so that skin grafts would not be rejected.

Huff and co-authors (2002; 2003; 2005; 2006) investigated the use of phages to treat *E. coli* infection particularly in broiler chickens and found the great potential for disease treatment as well as prevention in poultry production. They established that significant decrease in mortality from severe respiratory infection was possible with intramuscular phage injection after birds were challenged with *E. coli* (Huff *et al*., 2003). Furthermore, broilers obtained considerable protection when a bacteriophage specific for a poultry pathogenic *E. coli* isolate was administered as an aerosol spray at a phage concentration of 10^7 plaque forming units (pfu) (Huff *et al.,* 2002). Mortality rates were reduced by 50%. Barrow *et al.* (1998) found that a lytic phage could prevent septicaemia and meningitis-like infection in chickens, and delay the appearance of signs of *E. coli* bacteremia in colostrum-deprived calves.

Research on the use of phages to control *Salmonella* infections has also been carried out and encouraging data was obtained. A marked decrease in *Salmonella* on poultry products was observed (Higgins *et al.,* 2005). Similar results were also shown by Fiorentin and colleagues (2005b) when they experimentally contaminated chicken parts with *Salmonella* Enteriditis and treated them with phages. *Salmonella* is one of the main contaminants of poultry and its reduction is extremely important in preventing its introduction to the food chain. Thus, the likelihood of the occurrence of food poisoning in humans is lessened (Fiorentin *et al.,* 2005b). A cocktail of phages administered orally to broilers reduced the levels of *Salmonella* Enteriditis PT4 in caecal contents, therefore confirming the importance and value of phages for controlling bacterial infections (Fiorentin *et al.,* 2005a). Competitive exclusion has also been used singly and in combination with phages to decrease *Salmonella* colonisation from infected chickens (Toro *et al.,* 2005*)*. No definite synergistic effects were clear, although favourable results were observed for phages and competitive exclusion separately. This combination might prove useful in the future, however, further research is still necessary.

Campylobacter jejuni is a food-borne pathogen and commensal organism in mammals and poultry, among other examples of birds. It is of importance to the poultry industry and man due to the infections in poultry which may subsequently be transmitted to humans. *Camplylobacter* contamination can be reduced by phages (Atterbury *et al*., 2005; Connerton *et al*., 2004; Goode *et al*., 2003) and the poultry and medical field can benefit from this result, in terms of economic losses and overall well-being of consumers.

In the same way that antibiotic resistance developed over a period of time, resistance to phages is inevitable at some point. This is usually due to changes in the phage-receptor molecules in gram-negative bacteria (Matsuzaki *et al.,* 2005). The rate at which this may occur can be reduced by using a cocktail of phages instead of only one. Phages have the ability to co-evolve with bacteria and would thus be able to once again adsorb to their respective hosts. This is an important factor to note which proves how valuable phages are for therapy. Selecting and screening for a new effective phage in the event of phage resistance is a relatively

fast process. Development of novel antibiotics in comparison can take up to several years (Sulakvelidze *et al.,* 2001).

In spite of all the benefits outlined for phage therapy, there are concerns from the general public with regard to safety. Will the use of phages in animal or human treatment have a detrimental effect on humans? No serious side-effects have been reported in literature. To attempt to answer that question, a small study was recently conducted with 15 human volunteers to test the safety of phage therapy (Bruttin & Brüssow, 2005). Each volunteer received *E. coli* T4 phage to a final concentration of 10^8 pfu orally. No adverse effects were observed and the concentrations of two liver transaminases in serum indicative of liver damage remained within the normal range (Bruttin & Brüssow, 2005). Safety trials will be an integral feature of any phage preparation which has potential for therapy before it is released for commercial use.

In 2006, the US FDA made history when it approved the use of bacteriophages, active against *Listeria monocytogenes* in meat and poultry products (Peek & Reddy, 2006). The bacteriophage preparation is produced by Intralytix and consists of 6 purified phages (LMP-102). This is the first time a phage preparation has been allowed as a food additive by the FDA and this decision clearly indicates that the West is no longer a skeptic of phage therapy but fully recognises the invaluable contribution that can be made through this method.

There has been an increase in the level of acceptance of phage therapy in the West. More work is still necessary in different parts of the world as well in terms of consumer acceptance. In the veterinary field, studies by authors such as Smith and Huggins (1982) to cite a few examples were instrumental in pioneering work which not only demonstrated the great potential of phages to reduce bacterial infections but included correct scientific methods (e.g. use of controls, placebos). The earlier work done in Eastern Europe was not readily accepted by the West for reasons previously stated.

Phage therapy can be used as the sole choice of treatment for bacteria that are completely resistant to antibiotics or in combination with antibiotics in cases where antibiotic therapy is still effective to thwart the development of strains resistant to either form of treatment.

1.9. CONCLUSIONS

Bacterial infections such as those caused by *E. coli* are of great significance to poultry industry worldwide. Colibacillosis results in large economic losses due to mortality, morbidity and condemnations. Antibiotic use is no longer as effective as it once was for disease control due to the development of antibiotic resistance. Several bans imposed on antibiotic use for non-therapeutic purposes in animal production in the European Union and United States and the reduction in the development of new drugs demonstrate that there is a desperate need for alternatives to the use of antibiotics.

The development and subsequent dissemination of multidrug-resistant genes in bacteria has created a window of opportunity for phage therapy to be reappraised. This method offers numerous advantages compared to antibiotics which makes it an attractive potential substitute. In financial terms, phage therapy represents potential to avert economic disaster for the poultry industry worldwide.

1.10. AIMS OF THE STUDY

The potential of phages for therapeutic purposes was recognised soon after their discovery. However, factors such as poor scientific procedures, lysogeny and most importantly, the discovery of antibiotics led to a declined interest especially in the Western world. Unfortunately, the use and abuse of antibiotics, especially for growth promotion purposes has resulted in the development and prevalence of antibiotic resistance in the agricultural industry. Furthermore, there are concerns that the antibiotic resistance genes may be passed on to humans through the consumption of animal products. This is due to the fact that the structures of antibiotic growth promoters are similar to antibiotics used to treat human diseases. There is a great need for substitutes to antibiotic use and lytic bacteriophages as alternatives for treatment and prophylaxis of bacterial infections offer several advantages over antibiotics, as outlined in the literature review. Therefore, the principal objective of this study was to investigate the potential use of bacteriophages for the control of colibacillosis in poultry. This involved the isolation of phages from sewage samples and chicken faecal matter and subsequently determining their lytic patterns on ten *E. coli* strains which had been isolated from diseased chickens (Poultry Reference Laboratory, University of Pretoria) and are presumed to be pathogenic. *E. coli* K12, a laboratory strain was also included in the study to represent the positive control and non-pathogenic strain. A number of molecular techniques, namely conventional polymerase chain reaction (PCR), random amplified polymorphic DNA (RAPD) PCR, restriction fragment length polymorphism (RFLP) and pulse field gel electrophoresis were employed to identify and establish molecular identification tools for the phage isolates. A few isolates were selected for this purpose. Finally, morphological characterisation by transmission electron microscopy was performed on purified sewage sample, specific phage isolates (from sewage samples) as well as whole purified chicken faecal matter samples. The main purpose for this procedure was to allow for classification of the phages into families.

CHAPTER 2

ISOLATION AND CHARACTERISATION OF *ESCHERICHIA COLI* BACTERIOPHAGES BY THEIR LYTIC SPECTRA

2.1. INTRODUCTION

Escherichia coli is a gram negative, non-spore forming bacterium which falls in the family Enterobacteriaceae. This bacterium forms part of the normal intestinal microbiota of animals such as poultry, humans and other mammals. As a result, *E. coli* in drinking water sources is an indicator of faecal pollution. In poultry, it is the causative agent of colibacillosis, an opportunistic disease which usually occurs when the host is susceptible to infection due to a weakened immune system (Barnes *et al.,* 2003).

Outbreaks of poultry diseases such as colibacillosis are a major cause of mortalities, morbidity and condemnations at slaughter with substantial monetary losses to the poultry industry worldwide. Antibiotics such as ampicillin, streptomycin and chloramphenicol have been used to treat this disease for decades (Barnes & Gross, 1997). Unfortunately, antibiotic resistance is a major threat to effective disease control and no vaccine is currently available to protect flocks against colibacillosis. There is a great demand for alternatives to antibiotics and bacteriophages are among the potential candidates.

Lytic phages have been used to treat a variety of bacterial infections from the time of their discovery. However, this was discontinued in the West with the advent of chemotherapy although research in this field continued in Eastern Europe and the Soviet Union. The emergence and prevalence of antibiotic resistance has led to a renewed interest in the application of phages for the treatment and prevention of diseases (Carlton, 1999).

Bacteriophages are ubiquitous in nature and can be found in their host's natural habitat. There are approximately 10^{31} viruses in nature; the majority are phages which makes them the most abundant living entities on earth (Brüssow & Hendrix, 2002). Coliphages (viruses that infect *E. coli*) are frequently isolated from sewage, stool and other environmental samples. The presence of coliphages in sewage and stools implies that these viruses are part of the intestinal tract system of animals.

The first aim of this part of the study was to isolate phages from sewage samples and chicken faecal matter. The second aim was to determine their host strain range specificity through their lytic profiles on the different *E. coli* strains.

2.2. MATERIALS AND METHODS

2.2.1. BACTERIAL STRAINS AND GROWTH CONDITIONS

A collection of *Escherichia coli* strains were used in this study. This collection included ten which were obtained from the Poultry Reference Laboratory of the Faculty of Veterinary Science of the University of Pretoria. They were isolated from different organs of chickens suffering from colibacillosis, as indicated in table 2.1. *E. coli* K12 was used as a positive control. This strain is frequently used as a positive control because it can be grown easily, is safe to work with and has been well studied. For this research, *E.coli* K12 represents a non-pathogenic strain.

Bacterial strains were grown on Tryptone Soy Agar (TSA) or Luria Bertani (LB) agar and incubated at 37°C overnight. Single colonies were picked and several conventional identification tests were performed which included gram stain, oxidase, catalase, motility, oxidative/fermentative and glucose utilisation tests.

Table 2.1: Table showing *E. coli* isolates and the organs they were isolated from

E. coli strain	Source
34/2000	Poultry
1323/99	Poultry air sac
B98/07	Poultry
B841	Poultry joint
31/2000	Poultry joint
B1634	Poultry
1304/99	Poultry air sac
76/00	Poultry lung
B771/07	Poultry
1080/99	Poultry

2.2.2. GRAM STAIN REACTION

A bacterial smear was prepared on a clean microscope slide and heat fixed. The smear was entirely covered with Crystal Violet solution (Merck) and allowed to stand for one min. The slide was rinsed with water and flooded with Iodine (Merck). After one min, the slide was rinsed with water. Acetone Alcohol was flooded onto the slide and rinsed with water after 15 s. Safranin (Merck) was added to the slide and washed off after a minute. The slide was gently blotted dry and viewed under the microscope. Purple coloured cells are interpreted as gram positive and red or pink coloured cells are interpreted as gram negative bacteria.

2.2.3. OXIDASE TEST

Impregnated Bactident® oxidase test strips were used (Merck). Fresh bacterial growth was removed from a TSA or nutrient agar plate with a sterile inoculation loop and gently rubbed onto the strip. Observations were recorded after about 20 s. A blue-violet colour was interpreted as a positive reaction.

2.2.4. CATALASE TEST

A colony from an overnight culture was picked with a sterile tooth pick and placed onto a clean microscope slide. A drop of 3% hydrogen peroxide solution (Merck) was added and the slide was examined for bubbling within 10 s.

2.2.5. MOTILITY TEST

Motility media (Sigma) was inoculated with bacteria and the test tubes were incubated at 37°C for approximately 18 hours. Growth along the line of inoculation is considered negative for motility while growth throughout the media indicates that the bacteria are motile.

2.2.6. OXIDASE/FERMENTATIVE TEST

Oxidative/Fermentative test media (Fluka Biochemika) were inoculated with bacteria with an inoculation needle. Media in one test tube contained a layer of mineral oil on top to create an anaerobic environment while the media in the other tubes did not. Tubes were incubated overnight at 37°C.

2.2.7. GLUCOSE UTILISATION TEST

Loopfuls of overnight bacterial cultures were inoculated into test tubes containing media and durham tubes specific for the determination of the ability of bacteria to utilise glucose and gas production. Samples were incubated at 37°C overnight. Media before inoculation is purple in colour. A change from purple to yellow is a positive indicator that the bacteria utilises glucose.

2.2.8. GROWTH ON SELECTIVE MEDIA

Single colonies from overnight cultures were streaked onto the following selective media: Eosin Methylene Blue (Merck), MacConkey (Fluka Biochemika), Brilliant green (Fluka Analytical), Cystine-Lactose-Electrolyte Deficient (Merck), and Xylose Lysine Deoxycholate agar (Merck). Cultures were incubated at 37°C overnight.

2.3. EXTRACTION OF BACTERIAL DNA

Genomic DNA was extracted from bacterial cultures using the QIAamp® DNA Mini Kit (QIAGEN) according to the manufacturers' instructions for Protocol C and the tissue protocol with slight modifications.

Bacterial cultures were grown in TS broth overnight at 37°C, and 1 ml was added to a 1.5 ml microcentrifuge tube. The suspension was centrifuged for 5 min at 5 000 *g*, supernatant was removed and buffer ATL (supplied with kit) was added to the pellet to a final volume of 180 µl. Proteinase K (20 µl) was added, the sample mixed by vortexing and incubated at 56°C for 3 hours. The sample was vortexed at least 3 times per hour during this period to ensure efficient lysis. After incubation, the microcentrifuge tube was briefly centrifuged, 200 µl of buffer AL (supplied with kit) was added to the sample, mixed by pulse vortexing for 15 s and incubated at 70°C for 10 min. The microcentrifuge tube was briefly centrifuged to remove drops from inside the lid after which 200 µl ethanol (99.5%) was added, mixed by pulse vortexing for 15 s and briefly centrifuged to ensure thorough mixing. The sample was then carefully applied to a QIAamp Spin Column (in a collection tube) and centrifuged at 6 000 *g* for 1 min. A volume of 500 µl buffer AW1 (supplied with kit) was carefully added to the spin column, centrifuged at 6 000 *g* for 1 min and the filtrate was discarded. The spin column was placed in a clean collection tube, 500 µl buffer AW2 (supplied with kit) was added and the column was centrifuged at 20 000 *g* for 3 min. In order to reduce the chances of buffer carryover, the column was placed in a new collection tube and an additional centrifugation step was performed at 20 000 *g* for 1 min. Collection tube with filtrate was discarded and the column was then placed in a clean 1.5 ml microcentrifuge tube, 200 µl buffer AE (supplied with kit) was added and incubated at room temperature for 5 min. The spin column was centrifuged at 6000 *g* for 1 min and the DNA was stored at -20°C until required.

2.3.1. *E. coli* PCR

To confirm the identity of the different *E. coli* strains, a polymerase chain reaction (PCR) was performed. The primer pair (table below) used amplifies a 147 bp amino coding region of *uidA* gene of *E. coli* (Bej *et al.,* 1991). The reaction mixture consisted of 1 µl dNTPs (100 mM each), 0.5 µl of each primer (100 µM), 5 µl

extracted bacterial DNA, 5 µl buffer (10X concentration), 1 µl 25 mM $MgCl_2$, 0.3 µl SuperTherm DNA polymerase and 36.7 µl Milli-Q water.

Table 2.2: Primer information

Primer	Sequence	Position
UAL-754	5'-AAAACGGCAAGAAAAAGCAG-3'	754-773
UAR-900	5'-ACGCGTGGTTACAGTCTTGCG-3'	880-900

A 2720 thermo cycler (Applied Biosystems) was used for the PCR reactions. The reactions were slightly modified from those outlined by Bej *et al.* 1991. Denaturation was carried out at 95°C for 3 min, 25 cycles of 94°C for 1 min, annealing at 50°C and extension at 72°C for 10 min. Amplified PCR products were electrophoresed on a 1.5% agarose gel in 1 X TAE buffer stained with gold view.

2.4. BACTERIOPHAGE ISOLATION AND PURIFICATION

Bacteriophages were isolated from sewage collected from Bloemspruit purification plant in Bloemfontein and from chicken faecal matter collected from chicken pens in the Animal unit at the University of the Free State. The sewage samples were centrifuged at 4000 *g* for 20 mins and filtered with a 0.22 µm membrane filter. For the chicken faecal matter, a volume of 100 ml distilled water was added after which the samples were centrifuged and filtered as described for the sewage samples. The presence of phages was observed by plaque assay (details of procedure can be seen below) with all the different bacterial hosts. Specific plaques were selected from a few agar plates with different hosts and these were then used for further analyses to determine host strain specificity, phage morphology and molecular characterisation studies.

Phages were detected by the plaque assay in the following manner: an overnight bacterial culture and phage sample (1 ml respectively) were mixed in 0.8% soft TSA agar (at a temperature of 45°C) and poured over TSA plates. Plates were incubated overnight at 37°C and examined for plaques the following day. For a negative

control, phage alone was added to the molten agar. Plaque assay was performed in triplicate.

For purification purposes, well-isolated plaques were serially propagated until a single phage type was obtained. Purification was performed as follows: plaques of specific sizes were individually picked, placed in 2 ml Tryptone soy broth (TSB) containing at least 3 drops of chloroform. This was gently shaken, centrifuged at 14 500 *g* for 10 min and top part of the sample was carefully removed to avoid the chloroform at the bottom and filtered with a 0.20 µm pore size filter. A volume of 100 µl phage sample was mixed with 100 µl bacterial culture in 5 ml 0.8% TSA (at 45°C) and poured onto TSA plates which were incubated at 37°C once the molten agar had set. This procedure was repeated until only one phage type based on plaque size was obtained.

2.5. BACTERIOPHAGE AMPLIFICATION

A volume of 2 ml of pure phage sample was mixed with 2 ml of the bacterial host from which the plaque had originally been picked in a 20 ml TSB. This was incubated at 37°C on an orbital shaker at 150 rev.min^{-1} overnight. The sample was centrifuged at 4000 *g* for 20 min and filtered with a 0.20 µm filter. Thereafter, 1 ml of the phage sample was inoculated into 100 ml TSB with 1 ml of the appropriate host and incubated overnight at 37°C on an orbital shaker at 150 rev.min^{-1}. Centrifugation and filtration steps were repeated. The amplified phage sample was stored at 4°C or in 50% glycerol at -20°C for longer periods.

2.6. HOST STRAIN RANGE DETERMINATION OF PHAGES

The isolated phages were tested for host strain range specificity against all the bacterial strains used in the study. Briefly, 100 µl of overnight bacterial cultures (grown at 37°C) and 100 µl specific phage samples were added to 5 ml 0.8% TSA (at 45°C), mixed and poured onto TSA plates. The plates were inverted, incubated at 37°C overnight after which they were examined for lysis. Experiment was done in triplicate.

2.7. RESULTS AND DISCUSSION

The identity of all the *E. coli* strains used in this study had to be confirmed before any further studies could be carried out. All the identification tests including the PCR test gave results that indicated that the isolates were indeed *E. coli.*

E. coli strains used in this study have flagella which aids in motility. This is however not the case for all *E. coli* strains. Gram negative rods were observed and the strains were also able to utilise glucose. This is in accordance with literature (Barnes *et al.,* 2003) and in addition *E. coli* is able to utilise other carbohydrates namely maltose, mannitol, xylose and arabinose with the production of acid and gas.

2.7.1. Selective media

All bacterial strains gave results characteristic of *E. coli*. On EMB agar, black colonies with a metallic green sheen were observed; Pink colonies were seen MacConkey agar; Green colonies on Brilliant green agar; Yellow colonies on CLED agar; Yellow colonies were produced on XLD agar.

Table 2.3: *E. coli* identification tests results

E. coli strain	Gram Rxn	Catalase	Oxidase	Motility	O/F	Glucose utilisation
31/2000	-ve, rods	+ve	-ve	+ve	F	+ve
34/2000	-ve, rods	+ve	-ve	+ve	F	+ve
1323/99	-ve, rods	+ve	-ve	+ve	F	+ve
1304/99	-ve, rods	+ve	-ve	+ve	F	+ve
B98/07	-ve, rods	+ve	-ve	+ve	F	+ve
B841	-ve, rods	+ve	-ve	+ve	F	+ve
B771/07	-ve, rods	+ve	-ve	+ve	F	+ve
B1634	-ve, rods	+ve	-ve	+ve	F	+ve
1080/99	-ve, rods	+ve	-ve	+ve	F	+ve
76/00	-ve, rods	+ve	-ve	+ve	F	+ve
K12	-ve, rods	+ve	-ve	+ve	F	+ve

-ve Negative; +ve Positive; F- fermentative

2.7.2. POLYMERASE CHAIN REACTION (PCR)

The PCR results were in agreement with the identification tests performed in that they confirmed that the bacteria samples were indeed *E. coli* since the expected band size of approximately 147 bp was obtained (figure 2.1).

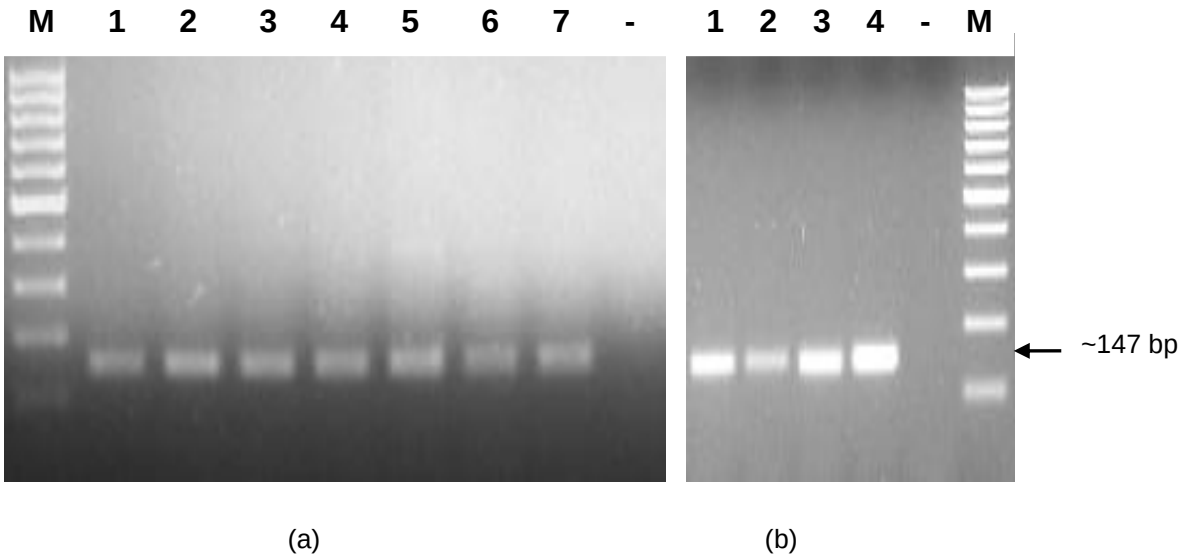


Figure 2.1: 1.5% agarose gel electrophoresis of PCR-amplified *uidA* gene fragments from *E.coli* isolates**.** Lanes 1-7 in (a) and lanes 1-4 in (b) represent the PCR products. Amplicon is approximately 147 bp. Lane M represents the 100 bp DNA which was used as the molecular weight marker. Lane – is the negative control.

2.7.3. BACTERIOPHAGE ISOLATION

2.7.3.1. PHAGES ISOLATED FROM SEWAGE SAMPLE

Lytic bacteriophages were successfully isolated by plaque assay using the double agar overlay. This was evident from the clear zones (plaques) on bacterial lawns formed after hours of incubation of agar plates. Differences in plaque morphology, clear or turbid and sizes were observed. Variation in plaque morphology indicated the presence of several strains of phages because this feature is specific for different phages. According to Stent (1963) the diameter of the plaque is influenced by bacterial host, the nature of the phage, plating and incubation conditions. The size of virions is also said to be important determinant in plaque size, small particles spread through solid media more rapidly than larger ones. One viral particle triggers the formation of each plaque and this can consist of up to 10^9 virions (Luria *et al.,* 1978).

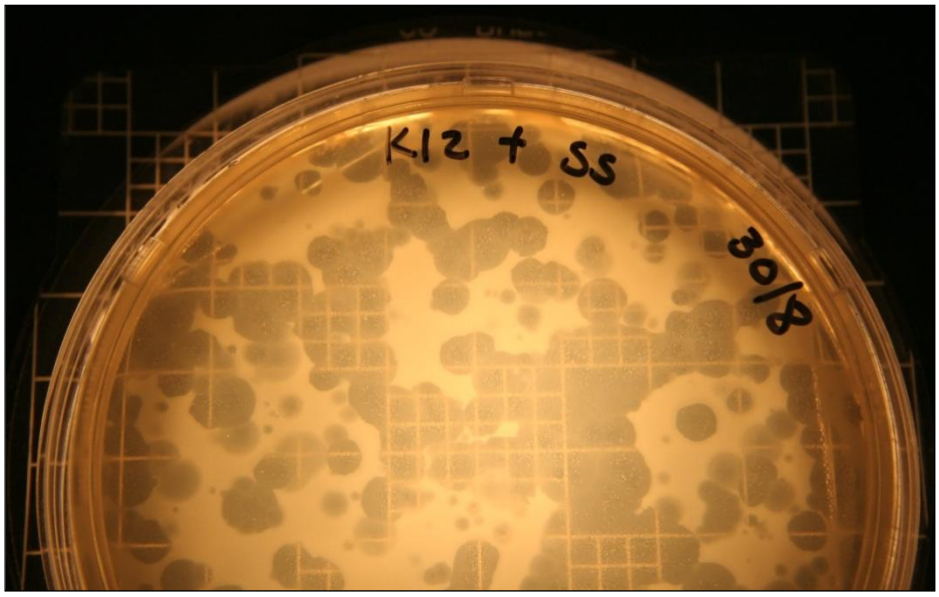


Figure 2.2: TSA plate showing plaques on *E.coli* K12 (sewage sample)

E. coli K12 was used as the positive control to investigate for the presence of phages from the sewage and chicken excreta samples. This strain consistently showed the greatest degree of lysis in comparison to the other bacteria. Chibani-Chennoufi *et al.* (2004b) used *E. coli* K803, a strain similar to K12 in which prophage lambda is absent, in their studies as the laboratory strain among a set of pathogenic *E. coli.* The lack of prophage lambda in the K803 strain aids in the isolation of temperate phages such as lambda phages. It was noted that the K12 derivative is sensitive to a majority of the T4-like phages in the Evergreen collection (at the Evergreen State College in Washington). Bacteriophages mainly from the *Myoviridae* family were isolated from sewage, environmental water and stool samples on K803.

Therefore, it can be assumed that *E. coli* K12 is highly sensitive to lysis because it possesses receptors which are easily recognisable by phages. Some T-even bacteriophages recognise the *OmpA* protein on the outer membrane of *E. coli* as receptors. This then allows for binding between the phage and bacteria and the lytic cyle ensues. Morona and co-workers (1985) showed that mutations on the OmpA protein result in bacteria becoming resistant to phages. It was also noted, however that the T-even phages do not identify with the same structure on the outer membrane protein and as a result, different resistance types may be observed.

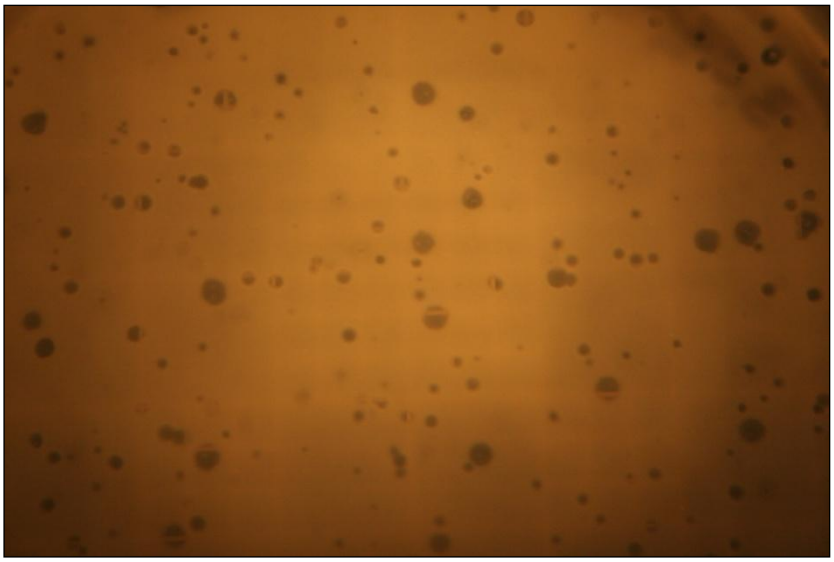


Figure 2.3: TSA plate showing plaques of different sizes on a representative of *E. coli* isolated from diseased poultry (sewage sample).

A heterogenous mixture of plaques was evident on the different bacterial strains which were susceptible to lysis as shown in figure 2.3. This indicated the presence of different types of phages in the samples from which they were isolated. Aside from differences in diameter, the clarity of the plaques also differed in some instances. Some plaques were clear zones while others were turbid. A number of specific plaques were selected for further studies (table 2.4) and they showed varying degrees of lysis for different strains as indicated in table 2.4. The differences in lysis observed are probably due to the presence or absence of specific receptors on the bacterial cell wall to which the phage must bind. In addition, raw sewage sample contained different types of phages as indicated by the different plaque sizes. As a result, different phages may not recognise the same receptors on the bacterial cell wall and this in turn would account for the varying degrees of lysis mentioned earlier.

Various phages bind to different parts of the bacterial cell membrane. For example, some bind to membrane proteins, others to lipopolysaccharide apparatus and still others may possess adhesins that recognise either the membrane proteins or the lipopolysaccharide. Several phages of gram negative bacteria have adhesins which can bind to a number of outer membrane proteins. Moreover, a specific adhesin can identify various kinds of the receptors. For example, T4 can bind to the outer membrane protein OmpC on K strains of *E. coli* but does not bind to B strains. On the other hand, T4 can also be attached to the glucose of the *E. coli* B strain

lipopolysaccharide (Kutter *et al.,* 2005). This is one of the reasons why different strains and even different species of bacteria do not exhibit the same lysis pattern.

Table 2.4: Table showing information on phage isolates from sewage samples

Name of phage isolate	Original host bacteria	Plaque size (mm)
BS4	B1634	4
SK1	K12	±1
SK2	K12	2
SK3	K12	3
SK4	K12	4
SK5	K12	5
E13/5	1323/99	5
E13/2	1323/99	2
31-5	31/2000	5

The names of the phage isolates included part of the host strain designation and the source of the phages in some instances. The numbers at the end of the name of the phage isolate correspond to the plaque size. For example, in naming BS4, the letter B is from the host strain *E. coli* B1634, the letter S stands for sewage (source of phage) and the number 4 represents the plaque size. In the case of E13/5, the E13 is from the *E. coli* host strain 1323/99, the slash / is used to separate the strain name which consists of numerals from the plaque size of 5 mm.

Table 2.5: Lytic activity of phages in sewage samples to different *E. coli* strains

E. coli strain	Lytic activity	plaque forming unit (pfu)
31/2000	+++	201
34/2000	+++	55
1304/99	+++	34
1323/99	++	41
1080/99	+	14
76/00	++	51
B98/07	+	3
B841	+	6
B771/07	+	40
B1634	+	8
K12	+++	CL

+ 20 plaques or less; ++ more than 20 plaques; +++ more than 200 plaques; CL Confluent lysis
Results shown are the mean values of triplicate independent experiments.

The mixture of phages in the sewage sample revealed varied lytic patterns in the collection of bacteria. *E. coli* K12 was by far the most sensitive to destruction and confluent lysis was consistently seen on all plates (experiment was done in triplicate). Among the pathogenic bacteria, 31/2000 was the most sensitive to lysis, followed by 34/2000, 76/00 and B98/07 showed the least sensitivity.

A different project is currently underway in the Veterinary Biotechnology Research group to investigate the presence of virulence genes on different *E. coli* strains including pathogenic strains. The results from that study will be used to determine whether there is a correlation between the strains which possess certain virulence genes and the lyic patterns observed from this study.

Table 2.6: Sensitivity of *E. coli* isolates to phages isolated from sewage samples

	Bacteriophage isolates								
E. coli strain	BS4	SK1	SK2	SK3	SK4	SK5	E13/5	E13/2	31-5
31/2000	+++	-	-	-	-	-	+++	+++	-
34/2000	-	-	-	-	-	-	+++	+++	-
1304/99	+++	-	-	+++	-	-	+++	++	-
1323/99	-	-	-	-	-	-	-	+++	+++
1080/99	-	-	-	-	-	-	-	+++	-
76/00	+++	-	+++	++	+++	+++	-	+	+++
B98/07	-	-	-	-	-	-	-	-	++
B841	-	-	-	-	-	-	-	++	+++
B771/07	-	-	-	-	-	-	-	-	-
B1634	+++	+	+	-	-	+	+++	+++	+
K12	-	+++	+++	-	+++	+++	-	-	-

- No lysis; + 20 plaques or less; ++ more than 20 plaques; +++ more than 200 plaques

Nine phages were selected to determine the host specificity spectrum, and the results revealed varied lysis patterns (table 2.6). Most of the phages with the exception of E13/2 and 31-5 were highly strain specific, lysing four strains or less out of the entire collection of 11 strains. Based on their similarities of susceptibility to phage activity, SK2 and SK5 can be categorised into one group. As was expected, the phage isolates lysed the bacterial hosts from which they were isolated. Isolate 31-5 was unique in that its host was resistant to lysis. Similarly, isolates SK3 and E13/5 did not lyse their original hosts. These experiments were performed in triplicate and similar results were obtained. Therefore, the most likely explanation for this observation is that these phages may have entered the lysogenic phage after isolation or even that some mutation may have occurred. Lysogeny involves the integration of phage nucleic acid into the bacterial chromosome and therefore becomes inactive as the lytic genes are suppressed by a repressor. This inactive state confers resistance on the bacterium because infection by similar phages is prevented. Other factors which generally contribute to resistance include failure of virus to attach to bacteria due to a lack of suitable receptors or binding sites or as a

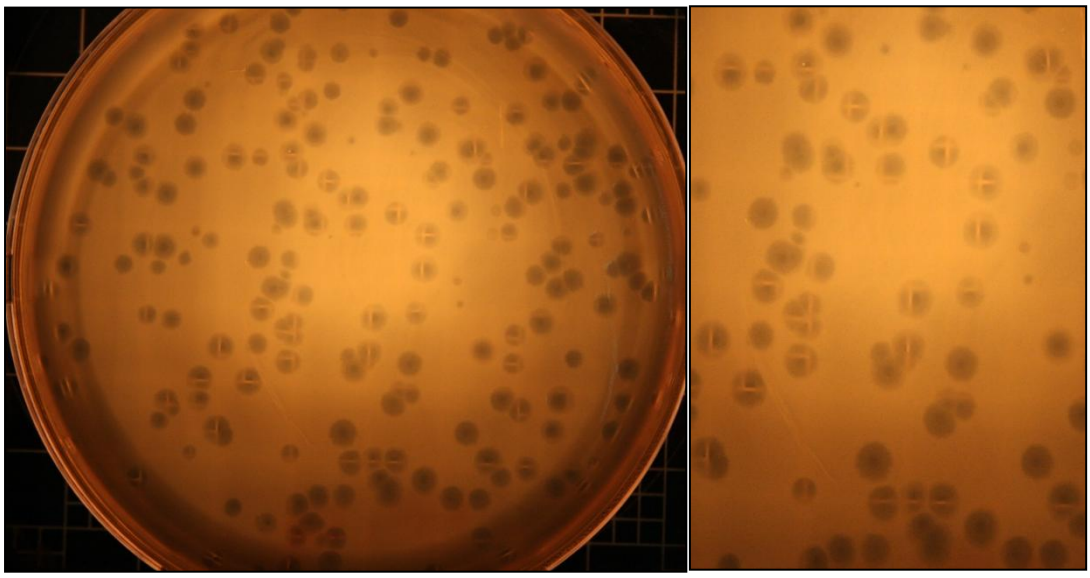
result of mutations on the phage or bacteria which can alter the way receptors are arranged and prevent adsorption (Adams, 1959).

Kim and co-workers (2008) also found phage resistance with some of the *Listeria monocyogenes* used in their study and in addition to the above-mentioned factors abortive infections, restriction systems and prevention of phage DNA transfer into the host are cited as additional reasons for phage resistance to be conferred. Abortive infections occur when attachment and genome transfer has taken place but the host is thereafter unable to meet the requirements for intracellular phage development at different stages (Krüger & Bickle, 1983). The host may also utilise restriction enzymes to degrade phage DNA once genome transfer is successful. Abortive infections and restriction systems are some of the measures taken by bacteria to survive or thwart viral multiplication. In some cases, phage attachment to bacteria is possible, but penetration of DNA is blocked (Elliot & Arber, 1978).

2.7.3.2 PHAGES ISOLATED FROM CHICKEN FAECAL MATTER

A mixture of mostly turbid plaques was observed on lawns of bacterial growth for most of the strains. In contrast, the majority of plaques observed from phage isolation using sewage sample were clear. Adams (1959) suggested that clear plaques were indicative of virulent phages and broad host range while lysogenic phages apparently could be identified by turbid plaques and a narrow host range. This is however, not a generally accepted concept. Strauch and colleagues (2001) demonstrated that some lysogenic phages can have broad lytic spectra. Several factors have been identified which determine plaque morphology in terms of the clarity of the plaques. These are: the physiological state of the host, temperature and pH of media (Sulakvelidze & Kutter, 2005). However, Ashelford *et al.* (1999) reported that turbid plaques observed over bacterial lawn by of a number of phage isolates were indeed temperate phages while the phage isolate which produced clear plaques did not produce lysogens. Therefore, Adam's concept of a relationship between plaque morphology and the type of phage (lytic or temperate) is not a rule of thumb for all phages.

Interestingly, halos were evident around some of the turbid plaques. A similar observation was made with one of the phage isolates from sewage, E13/2. These zones are a result of secondary lysis in which endolysins are produced (Luria *et al.,* 1978). Endolysins are muralytic enzymes produced by phages in infected bacterial hosts and they degrade the peptidoglycan of the cell wall. These enzymes have a concerted activity involving transglycosidases and lysozymes which break down the glycosidic bonds between amino-sugars as well as degradation of amide and peptide bonds by amidases and endopeptidases (Young, 1992). Endolysins hold potential for therapeutic applications apart from the use of whole phage isolates.



(a) (b)

Figure 2.4: Plaques formed on *E. coli* K12 by phages from chicken faecal matter samples. Distinct zones of secondary lysis caused by endolysins can be seen around the centre of some of the plaques.

Plaques formed on some of the bacteria such as 1323/99 were very distinct in that they were tiny in size and turbid (figure 2.5). Diameters were less than 1mm. Such small plaques were not recorded on any of the *E. coli* host strains when attempting to isolate phages from human sewage samples.

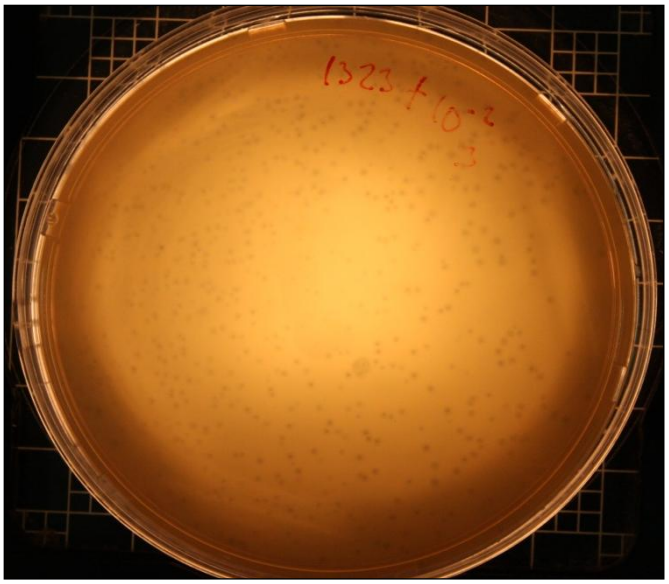


Figure 2.5: Tiny and distinct plaques obtained when phages from chicken faecal matter were grown on *E. coli* host strain 1323/99

Plaques of different sizes were obtained from plates on which different bacterial hosts were grown. This is indicated in table 2.7 below.

Table 2.7: Table showing information on phage isolates from chicken excreta

Name of phage isolate	Original host bacteria	Plaque size (mm)
βP2	B1634	2
βP5	B1634	5
βP6	B1634	6
K3	K12	3
CP4	1304/99	4

Phage isolates were arbitrarily named in some instances; however, the number in the phage 'name' represents the plaque size. For example, K3 has a plaque size of 3 mm and was isolated from *E. coli* K12 while the name CP4 has no relation to the host bacteria, *E. coli* 1304 from which it was isolated.

The host range of the phages in the chicken faecal matter samples was investigated and differences in lytic activity were observed. With regards to the chicken faecal excreta samples, 31/2000, 34/2000, 1304/99, B98/07 and B1634 were the most susceptible host bacterial strains.

In comparison, some of the *E. coli* strains which exhibited some level of resistance to lysis were highly susceptible to the phages in these faecal excreta samples. For example, *E. coli* strains B1634 and B98/07 which were among the least sensitive to lysis when host specicifity tests were carried out with the sewage sample (table 2.5) were extremely susceptible to destruction with the chicken faecal excreta sample (see table 2.8). As mentioned previously, the presence or absence of receptors on the bacterial membrane plays a crucial role in determining whether the lytic pathway will take place. For the lytic cycle to be initiated, binding between the phage and the potential host bacteria must occur. This is made possible through the presence of receptors which usually include oligosaccharides, lipopolysaccharides and a majority of any of the proteins in gram negative bacteria. Receptors vary depending on the type of bacteria being infected, that is, gram positive or gram negative bacteria. Furthermore, phages are highly host or species-specific (Engelkirk & Burton, 2006). If the bacteria have many receptors which can be recognised by the phages, they can be easily destroyed and a greater number of plaques will be observed on the agar plates. Therefore, it may be concluded that this was possibly the reason why different lytic patterns were observed between the different bacterial strains. A number of strains which were completely resistant were also noted, for example *E. coli* 1080/99 and 76/00 (table 2.8).

Table 2.8: Lytic activity of phages from chicken faecal matter to different *E. coli* strains

E. coli strain	Lytic activity	pfu/ml
31/2000	+++	TNTC
34/2000	+++	TNTC
1304/99	+++	TNTC
1323/99	++	30
1080/99	-	0
76/00	-	0
B98/07	++ +	TNTC
B841	-	0
B771/07	-	0
B1634	+++	CL
K12	+++	CL

+ 20 plaques or less; ++ more than 20 plaques; +++ more than 200 plaques; CL Confluent lysis
TNTC- Plaque forming units greater than 300
Results shown are the mean values of triplicate independent experiments.

According to Carlton (1999), a 'parent' phage can produce about 200 'daughters' per lytic cycle. By the third successive cycle whereby each of the daughters destroys the bacterial host, the end result will be 8 million progeny. These figures are largely dependent on the bacterial species and the conditions; however, they do give an indication of how rapidly lytic phages reproduce and why they are the candidates of choice for treatment of bacterial infections.

Bacteriophages were successfully isolated from chicken faecal matter. The lytic activity of the phages in the whole sample is shown in table 2.8 while table 2.9 indicates the degree of bacterial lysis by the specific phage isolates. Oliveira *et al.* (2009) reported on the isolation of 5 phages from poultry faeces. These researchers tested the lytic spectra of each of the phage isolates against 148 avian pathogenic *E. coli* strains which had been isolated from the organs of birds affected by colibacillosis. They found that 2 of the isolates, phi258E and phiF61E lysed

approximately 44.6% and 48.0% of the bacteria respectively. In comparison, 4 of the 5 phages isolated from chicken faecal matter in this study lysed only 18.2% (2 out of 11 *E. coli* strains) of the bacteria.

Bacteriophages are found in their hosts' natural environment. Golomidova et al. (2007) isolated coliphages from horses' faeces while Klieve and Bauchop (1988) discovered 26 different types of phages from the ruminal fluid of sheep and cattle. Chicken faecal matter has not only been the source of coliphages but of *Campylobacter jejuni* phages as well. *C. jejuni* is a food-borne pathogen which causes enteric diseases in humans (Connerton *et al.,* 2004).

Table 2.9: Sensitivity of *E. coli* strains to phages isolated from chicken faecal matter

	Bacteriophage isolates				
E. coli strain	βP2	βP5	βP6	K3	CP4
31/2000	+	-	+	-	-
34/2000	-	-	-	-	-
1304/99	-	-	-	-	+++
1323/99	-	-	-	-	-
1080/99	-	-	-	-	-
76/00	-	+	-	-	-
B98/07	-	-	-	-	-
B841	-	-	-	-	-
B771/07	-	-	-	-	-
B1634	+++	+++	+++	+	-
K12	-	-	-	++	-

+ 20 plaques or less; ++ more than 20 plaques; +++ more than 200 plaques

The study relating to phage isolates from chicken faecal excreta revealed high degrees of specificity to lysis in comparison to the isolates from sewage. The

maximum number of sensitive strains was two as shown in table 2.9 while the majority were resistant. Phages βP2 and βP6 have similar lytic patterns in that 31/2000 and B1634 are susceptible to their activity and thus, can be placed into one group. The narrowest host range is shown by phage isolate CP4 which is only lytic towards the bacteria strain from which it was isolated while the rest of the strains are resistant. One advantage of these phage isolates which is particularly noteworthy is the fact that they did not lyse *E. coli* K12, which is a representative of the non-pathogenic *E. coli.* This would prove useful in a therapeutic setting since one of the major considerations for therapy is the maintenance of the normal microbiota while pathogenic strains are destroyed. Reasons for phage resistance have already been covered in the previous section. These isolates may prove valuable in phage typing although further research in this regard would be required before this can be exploited.

Phage typing refers to the use of phages to differentiate between strains of bacteria. The absence or presence of receptors determines whether the virus is able to bind to the potential bacterial host. This technique has been successfully employed in bacterial species such as *Salmonella* Enteritidis (Ward *et al.,* 1987). The phage typing would also be particularly interesting to investigate with the isolates from the sewage samples whose host range was broad compared to the poultry faecal samples. For members of the *Listeria* genus, it is reported that a significant improvement from 70% to over 90% was seen in the phage typing system when virulent phages with a broad host range were used (Loessner & Busse, 1990).

There are some biological factors that can be investigated to determine a phage's ability to infect and proliferate in a given host. These factors are observed in a single step growth curve of a phage and are represented by the eclipse and latent period and the burst size (Carlson, 2005). The eclipse period is the time after infection when new phage particles form within infected bacteria while the latent period refers to the period during which the initial infective phage particles are released from the host (Guttman *et al.*, 2005). These phage characteristics give valuable insight into the phage-host interactions and should be considered for future studies of the isolates.

Research by Smith *et al*. (1982, 1983) has indicated successful treatment of experimental *E. coli* infection in mice, piglets, lambs and calves with phages. For phage treatment to be successful it is vitally important that the correct phage titer and rate at which the dosage is given is well established. Barrow and co-workers (1998) also showed that a lytic phage isolate could treat and prevent septicaemia and cerebritis or meningitis in chickens. Additionally, these researchers demonstrated that development of signs of disease in *E. coli* bacteremia was delayed in colostrum-deprived calves. A notable decrease in mortality of poultry suffering from colibacillosis was found when phages specific to *E. coli* serotype 02 (pathogenic to poultry) were administered (Huff *et al.,* 2002, 2005).

Chibanni-Chennoufi and colleagues (2004b) isolated T4-like phages from stool samples which were found to exhibit broad host spectrum towards *E. coli* serotypes that cause diarrhoea in humans. The phages were administered orally in drinking water and adverse effects to *E. coli* of the normal microbiota were minimal. This is an important aspect of safety that needs to be tested for every phage to be potentially used for treatment.

Phage solates βP6, K3 (from chicken faecal matter) and E13/2 (from sewage sample) produced endolysins. As stated previously, lysins can also serve as a possible alternative for therapy. They share similarities with phages in their advantages over antibiotics because they are encoded for by phages themselves. Narrow host range and reduced chances of development of resistance are some of the advantages. In addition, their activity against bacteria despite their sensitivity to antibiotics is also beneficial (Borysowski *et al.,* 2005).

Different *E. coli* strains possess a variety of virulence factors which work in combination to bring about varying degrees of pathogenicity in the affected hosts. A narrow host range for the chicken excreta and a broader spectrum for the sewage samples were observed. This may necessitate knowledge of which *E. coli* strains are responsible for disease in a particular instance so that the correct phage preparation may be administered. If not, treatment efforts would be ineffective. In addition, an investigation into the common strains that cause disease in a given region can be

carried out so that these can be included in a phage cocktail. For treatment or prophylactic purposes, it is preferable to use a mixture of phages (phage cocktails) as opposed to a single phage preparation. This aids in reducing the likelihood of phage resistance developing (Sulakvelidze & Kutter, 2005). With regards to the results obtained in this study, the ideal candidates to include in a phage cocktail would be E13/2, E13/5, BS4 and 31-5. All these isolates have a broad host strain range of high lytic activity towards at least four of the presumed pathogenic bacteria. Studies by Tanji and co-workers (2005) into the use of phage cocktails for controlling gastrointestinal *E. coli* 0157:H7 in mice showed favourable results for phage cocktails consisting of 3 phages. When one or two phage preparations were used on batch cultures of *E. coli* cells, reduction in cells was observed although it was not complete. However, a phage mixture of 3 phages completely eliminated cells and the appearance of resistant bacteria was delayed.

Enrichment of environmental samples to be used for phage isolation increases the chances of the isolation of broad host phage isolates. This is done by inoculating a mixture of bacterial strains with the phage sample and performing phage isolation techniques (Carlson, 2005).

2.8. Conclusions

The sewage and chicken faecal matter were found to be rich in phages as seen from the plaque assays. *E. coli* is an intestinal habitant of animals and its presence in these samples is not surprising. A total of 14 coliphages were isolated from sewage and chicken faecal matter samples using a basic method of isolation. Each isolate was purified and profiles of the lytic spectrum were determined. Different spectra were noted for the majority of the isolates with βP2 and βP6 from the chicken faecal matter samples as well as SK2 and SK5 from sewage samples exhibiting similar patterns in their respective groups. Based on this, phage isolate βP2 and βP6 were placed in one group. Similarly, isolates SK2 and SK5 were also allocated to a separate group from the other isolates. To the best of our knowledge from literature, this is one of the few studies where phages which lyse avian pathogenic *E. coli* have been successfully isolated from chicken faeces. Oliveira and co-workers (2009) recently reported on the isolation and characterisation of phages from poultry sewage.

The isolation of phage isolates which can destroy poultry pathogenic *E. coli* is the first step in realising the potential use of these isolates for treatment of colibacillosis and its prevention. Further work is still necessary to fully characterise the phages and optimise the conditions which enable complete destruction of bacterial *E. coli* cells. Once this has been established, the potential of the phage isolates can be investigated through *in vivo* trials in poultry. Gaining full understanding of phage and host interactions *in vivo* and taking measures to minimise the development of resistance which may render phage therapy ineffective in due time is also crucial.

In addition to therapy, it is proposed that some of the isolates could possibly be employed in phage typing due to their high levels of specificity.

CHAPTER 3

MOLECULAR CHARACTERISATION OF ISOLATED *ESCHERICHIA COLI* BACTERIOPHAGES

3.1. INTRODUCTION

Bacteriophages are a diverse group and are found in a variety of environments which include oceans, soil, drinking water, and food (Kutter & Sulakvelidze, 2005) The biosphere is especially rich in the tailed, dsDNA phages (Breitbart *et al*., 2004) of the order *Caudovirales* which is comprised of the families *Myoviridae, Siphoviridae,* and *Podoviridae.*

It is estimated that the biosphere consists of approximately 10^{31} bacteriophages and archael viruses. However, about 99% of microbial hosts have not been cultured, and this makes identification of phages particularly challenging. This implies that a large number of phages remain unidentified. It also means that GenBank is limited in the number of sequences available which are used for comparison in order to allow for identification of unknown phages. Polymerase chain reaction is one of the easiest methods used for identification purposes. However, unlike bacteria where universal primers such as those that amplify the 16s mRNA are available, there is no single gene which is conserved in all viral genomes (Edwards & Rohwer, 2005). Therefore, for culturable viruses prior knowledge of the phage to be identified such as morphology can be useful in selecting the specific primers to use for PCR. For unculturable viruses, metagenomics which involves direct extraction and cloning of genetic material from the natural environment can be employed (Singh *et al.,* 2009).

Characterisation of phages usually involves DNA analysis (procedures that involve the use of genetic material extracted from phages), biological parameters of phage isolates, analysis of proteins and electron microscopy. Current approaches include: pulse field gel electrophoreses and restriction digest studies which allow for the initial

classification while PCR, shotgun cloning and sequence analysis produce more comprehensive information (Carlson, 2005).

The principal objective of this part of the project was to establish molecular techniques to aid in the identification of coliphages isolated from sewage sample and which have previously been reported on in chapter 2 of this dissertation. For this purpose, phage isolates SK4, SK5, E13/5, 31-5 from sewage sample and whole purified chicken excreta sample were used. The information from the results obtained would allow for initial classification to be made.

3.2. MATERIALS AND METHODS

A few of the different bacteriophage isolates were used in this section and their information is presented in table 3.1 to facilitate clarity.

Table 3.1: Table showing information on phage isolates

Name of phage isolate	Original host bacteria	Plaque size (mm)
SK4	K12	4
SK5	K12	5
E13/5	1323/99	5
31-5	31/2000	5
Chk	N/A	N/A

The names of the phage isolates included part of the host strain designation and the source of the phages in some instances. The numbers at the end of the name of the phage isolate correspond to the plaque size. For example, in naming SK4, the letter K is taken from the host strain *E. coli* K12, the letter S stands for sewage (source of phage) and the number 4 represents the plaque size. In the case of 31-5, the 31 is from the *E. coli* host strain 31/2000, the dash sign – is used to separate the strain name which also consists of numerals from the plaque size 5. Chk represents part of the phage community in the chicken faecal matter sample. DNA was extracted from whole purified raw chicken faecal matter. N/A – Not applicable.

3.2.1: VIRAL DNA EXTRACTION

Viral DNA was extracted using the blood and body fluid spin protocol in the QIAamp® DNA Mini Kit (QIAGEN). Manufacturer's instructions in the booklet were followed with slight modifications. These methods were briefly as follows:

Proteinase K (20 µl) was added to a 1.5 ml microcentrifuge tube after which 200 µl purified phage sample was also added to the tube. The sample was then mixed with 200 µl Buffer AL (supplied in the kit) by pulse-vortexing for 15 s and incubated at 56°C for 10 min. The tube was briefly centrifuged and then 200 µl ethanol (99.5%) was added, mixed and the sample was centrifuged briefly. The sample was carefully applied to the QIAamp Spin Column (in a collection tube) and centrifuged at 6000 *g* for 1 min. The filtrate was discarded with the collection tube and the column was placed in a clean tube. A volume of 500 µl Buffer AW1 (supplied in the kit) was added, centrifuged at 6000 *g* for 1 min and the filtrate was discarded. The column was placed in a clean collection tube and 500 µl Buffer AW2 (supplied in the kit) was added. The mixture was centrifuged at 20 000 *g* for 3 min. To eliminate any possible chance of Buffer AW2 carryover the filtrate was discarded, column placed in a new collection tube and centrifuged at 20 000 *g* for 1 min. The collection tube containing filtrate was discarded and a clean 1.5 ml microcentrifuge tube was provided for the column. Buffer AE (supplied in the kit) in a volume of 60 µl was added, incubated at room temperature for 30 min and centrifuged at 6000 *g* for 1 min. Extracted DNA was stored at -20°C until required for use.

3.2.2. RESTRICTION FRAGMENT LENGTH POLYMORPHISM

The extracted DNA was digested with restriction enzymes, *Eco*RI, *Alu*I, *Acc*651, *Bg*III, *Hae*III, *Hind*III, *PstI*, XbaI, *Sa*lI, *Bam*HI, *HhaI* and *Pot*I (Fermentas). Manufacturer's instructions were followed. Briefly, 5 µl DNA, 0.5 µl *Eco*RI, 1 µl *Eco*RI buffer and made up to a final volume of 10 µl with sterile water. Buffers were varied depending on the enzyme being used. However, the volumes of the reaction mixtures remained the same. Samples were incubated at 37°C for 1-2 hours and visualised on a 1% agarose gel containing gold view.

3.2.3. AMPLIFICATION OF PHAGES BY PCR

All the primers used in amplification reactions are presented in table 3.2.

RAPD degenerate primer (Comeau *et al.,* 2004) was used to establish the relatedness of the isolated phages. Each reaction consisted of the following reagents: 5 µl extracted viral DNA, 1 µl 2 mM $MgCl_2$, 1 µl R10D primer, 1 µl dNTP (10 mM each), 5 µl 10x PCR buffer and 0.3 µl SuperTherm DNA polymerase made up to a final volume of 50 µl with Milli-Q water. Negative control contained reagents and Milli-Q water without any template DNA. The following cycle parameters were used: Initial denaturation at 95°C for 1.5 min, 40 cycles of denaturation at 95°C for 45 s, annealing at 40°C for 3 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min and a final cooling step at 4°C for an indefinite period. Amplified fragments (10 µl) were electrophoresed in 1.5% agarose in TAE buffer. Gel was stained with gold view and visualised under ultra violet light with 100 bp ladder (Fermentas).

Degenerate gp23 primers MZIA1bis and MZIA6 which amplify the g23 capsid protein of T4 phages was also used to identify the isolated phages (Filée *et al.,* 2005). This primer set amplifies the amino acid sequence from 111 to 304 in the coliphage T4. PCR mixture contained: 5 µl viral DNA, 1 µl 2mM $MgCl_2$, 0.5 µl forward primer, 0.5 µl reverse primer, 1 µl dNTP (10 mM each), 5 µl 10x PCR buffer and 0.3 µl SuperTherm DNA polymerase made up to a final volume of 50 µl with Milli-Q water. No DNA template was added to the negative control. PCR parameters were as follows: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min, extension at 72°C for 45 s and a final extension at 72°C for 10 min. PCR products were electrophoresed in 1% agarose gel stained with gold view and viewed under UV illumination. O'Gene DNA ladder (Fermentas) mix was used as the molecular weight marker.

Degenerate primers, HECTORPol29F (Breitbart *et al.,* 2004) and HECTORPol500R (Mabizela NB, PhD thesis, unpublished data) that amplify the DNA polymerase of T7-like podophages were also used. PCR mixture consisted of the following: 5 µl viral DNA, 1 µl 25 mM $MgCl_2$, 0.5 µl forward primer, 0.5 µl reverse primer, 1 µl dNTP

(10 mM each), 5 µl 10x PCR buffer and 0.3 µl SuperTherm DNA polymerase made up to a final volume of 50 µl with Milli-Q water. No DNA template was added to the negative control. PCR parameters were as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were electrophoresed in 1% agarose gel stained with gold view and viewed under UV illumination.

All PCR reactions were thermocycled in a 2720 Thermal cycler (Applied Biosystems).

Table 3.2: Table showing primers used for the amplification of random and specific regions in the phage genome, their sequences and sizes

PRIMER	SEQUENCE	SIZE (bp)
R10D	5'-GTCASSWSSW-3'	10
MZIA1bis	5'-GATATTTGIGGIGTTCAGCCIATGA- 3'	25
MZIA6	5'- CGCGGTTGATTTCCAGCATGATTTC-3'	25
HECTOR29F	5'-GCAAGCAACTTTACTGTGG-3'	19
HECTOR500R	5'-GAATGATCTACACTCTTTGCCATACGGTG -3'	29

3.2.4. PURIFICATION OF DNA

DNA was purified using the illustra GFX PCR DNA and Gel Band Purification kit and the manufacturer's protocols were followed. DNA was purified either from PCR product in solution or from the TAE agarose gel.

For purification from solution, 500 µl capture buffer type 2 was added to 40 µl PCR product and mixed thoroughly. A GFX MicroSpin column was placed in one collection tube for each individual preparation. The capture buffer type 2-sample mix was briefly centrifuged and loaded onto the GFX MicroSpin column and collection tube. This was spun at 16 000 *g* for 30 s. The flow through was discarded from the collection tube and the column was placed back into the same tube. Wash buffer type 1 (500 µl) was added to the sample in the column and centrifuged at 16 000 *g* for 30 s. Wash and dry step was repeated for samples which would be used in ligation but not for those that would be sequenced directly. The collection tube was discarded and the column was placed in a clean DNase-free 1.5 ml microcentrifuge tube. Elution buffer type 4 or type 6 (supplied in kit) in 50 µl amount was added to the centre of the membrane in the column. Elution buffer type 4 (supplied with the kit) was used for samples to be used in cloning and for long-term storage while elution buffer type 6 (supplied with the kit) for fragments to be sequenced only.

The column was incubated at room temperature for 1 min and centrifuged at 16 000 *g* for 1 min. The purified DNA was stored at -20°C.

For purification of PCR products from TAE agarose gels this protocol was as follows: firstly, a 1.5 ml DNase-free microcentrifuge tube was weighed and the weight was recorded. Agarose band containing the amplicon of interest was cut using a clean scalpel and its weight determined. Capture buffer (10 µl for each 10mg of slice) was added and the agarose was incubated at 60°C until it had dissolved completely. The sample was centrifuged briefly and placed in an assembled GFX MicroSpin and collection tube, incubated at room temperature for 1 min after which it was centrifuged at 16 000 *g* for 30 s. The flow through was discarded and the column was placed back into the collection tube. A volume of 500 µl Wash buffer type 1 was added to the column and centrifuged at 16 000 *g* for 30 s and the column was placed in a clean microcentrifuge tube. Elution buffer in 50 µl was added to the center of the column and incubated at room temperature for 1 min. The column was centrifuged at 16 000 *g* for 1 min to recover the purified DNA which was then stored at -20°C.

3.2.5. TRANSFORMATION OF COMPETENT CELLS

Purified PCR products were ligated into pGEM® T Easy Vector System I (Promega) in a reaction consisting of 5 µl 2x ligation buffer, 1 µl pGEM® T Easy, 1 µl T4 DNA ligase and 3 µl insert DNA. The ligation mixture was incubated at 4°C overnight to obtain the maximum number of transformants.

For the transformation reaction, 200 µl of TOP 10 competent cells were thawed on ice and the entire contents of the ligation mixture (10 µl) were added to it. No plasmid was added to the negative control while 1 µl of puc19 was added to competent cells as positive control. The cells were incubated on ice for 30 min, heat shocked at 42°C for 40 s and placed on ice for a further 2 min. Pre-warmed LB broth (800 µl) supplemented with 1M glucose and 2M Mg^{2+} was added to all the samples and they were incubated at 37°C on an orbital shaker for 1 hour. Cells were centrifuged at full speed for 30 s and approximately 900 µl was discarded. The pellet was resuspended in the remaining supernatant and plated out onto pre-warmed LB plates supplemented with ampicillin, X-gal (5-Bromo-4-chloro-3-indolyl-A-D-galactopyranoside) and IPTG (Isopropyl-2-D-thiogalactopyranoside). The plates were incubated overnight for ~20 hours at 37°C. Blue-white selection was performed following overnight growth.

3.2.6. ANALYSIS OF TRANSFORMANTS

Single white colonies were inoculated into respective 5 ml LB broth containing 10 $mg.ml^{-1}$ ampicillin (50 µl) and grown for ~17 hours at 37°C on an orbital shaker.

Small scale plasmid isolation and purification was performed using the Zyppy™ Plasmid Miniprep Kit (ZYMO RESEARCH) according to the manufacturer's instructions booklet with slight modifications. Bacterial culture of 5ml was centrifuged in 1.5 ml aliquots at maximum speed for 30 s and the supernatant was discarded. This was repeated until the entire volume of bacteria had been centrifuged. The pellet was completely resuspended in 600 µl sterile water, 100 µl 7X lysis buffer was added and the mixture was mixed by inverting the tube 6 times. Cold neutralization buffer (350 µl) was added and mixed thoroughly. The sample was inverted an additional 3 times to allow for complete neutralisation and centrifuged at 16 000 *g* for 4 min. Approximately 900 µl of the supernatant was carefully added to a Zymo-

Spin™ column without disturbing the cell pellet and centrifuged for 15 s at 16 000 *g*. The filtrate was discarded, 200 µl of Endo-Wash Buffer was added to the column and centrifuged for 15 s at 16 000 *g*. A volume of 400 µl Zyppy™ Wash Buffer was added to the column and centrifuged for 30 s. The column was placed in a clean 1.5 ml microcentrifuge tube, 30 µl of Zyppy™ Elution Buffer was added and allowed to stand at room temperature for 30 min. Thereafter, the column was centrifuged for 15 s and the eluted plasmid DNA was stored at -20°C.

Plasmid DNA was confirmed to contain inserts by restriction digest with *Eco*RI. Reaction consisted of 3 µl plasmid DNA, 0.5 µl *Eco*RI, 1 µl *Eco*RI buffer and made up to a final volume of 10 µl with sterile water. The samples were incubated at 37°C for 2 hours. Restriction profiles were observed under UV light on a 1% agarose gel stained with gold view.

3.2.7. DNA SEQUENCING OF GENOMIC FRAGMENTS

Purified PCR products were sequenced by Inqaba Biotechnical Industries or in house at the Department of Microbial, Biochemical and Food Biotechnology. Sequencing runs performed in the department required a sequencing PCR to be performed on all the samples. HITACHI 3130*xl* Genetic Analyzer (Applied Biosystems) was the sequencer used for DNA sequencing within the department. The Big Dye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) was used for this purpose of preparing the samples for sequencing. Reaction mixtures consisted of the following reagents: 0.25 µl premix, 1 µl of either SP6 or M13 primers (3.2 pmol. μl^{-1}), 1-4 µl of template plasmid DNA, 2 µl dilution buffer and made up to a volume of 10 µl. Thermal cycling parameters were: initial denaturation 96°C for 1 min, 25 cycles of 96°C for 10s, 50°C for 5 s, 60°C for 4 min and a final cooling step of 4°C. Post reaction clean up was performed using the EDTA/Ethanol precipitation protocol. The sequencing reaction volume was adjusted to 20 µl with Milli-Q water and placed in a 1.5 ml eppendorf tube containing 5 µl 125 mM EDTA and 60 µl absolute ethanol. Samples were vortexed briefly for 5 s, precipitated at room temperature for 15 min and centrifuged at 20 000 *g* for 15 mins. The supernatant was completely aspirated, 60 µl 70% ethanol was added to the tubes and centrifuged at 20 000 *g* for 5 min at 4°C. The supernatant was aspirated and pellet was dried in a Speed-Vac for 5 min. Samples were ready for sequencing and stored in the dark at 4°C.

3.2.8. PHYLOGENETIC ANALYSIS OF SEQUENCES

Sequences obtained were assembled and edited with the VECTOR NTI® software (Invitrogen™). Consensus sequences were compared with genomes available on GenBank with the nucleotide-nucleotide BLAST tool (http://www.ncbi.nlm.nih.gov). Multiple alignments were done with CLUSTAL in MEGA version 4 and a phylogenetic tree constructed using the results obtained (Tamura *et al.,* 2007).

3.3. RESULTS AND DISCUSSIONS

3.3.1. CONVENTIONAL PCR FOR PHAGE ISOLATES FROM SEWAGE SAMPLES AND CHICKEN FAECAL MATTER

It has been reported that most phages consist of dsDNA (Ackermann, 2007), therefore the first attempts at nucleic acid extraction focused on DNA methods. Once this was successful with the evidence of visible bands on agarose gels after extraction, further applications such as PCR were performed with the extracted DNA. If the gel electrophoresis had yielded a negative result (no bands visualised), one of the possible reasons could be the presence of RNA and not DNA phages and the appropriate method of extraction would have been used. PCR results were positive for amplification of the *g*23 capsid protein for one isolate from the sewage sample (lane 2, figure 3.1) and DNA polymerase of T7-like podophages from DNA extracted from phages in chicken faecal matter (figure 3.2, lanes 5-6). DNA fingerprinting analysis was also possible for some of the isolates through the use of a degenerate primer.

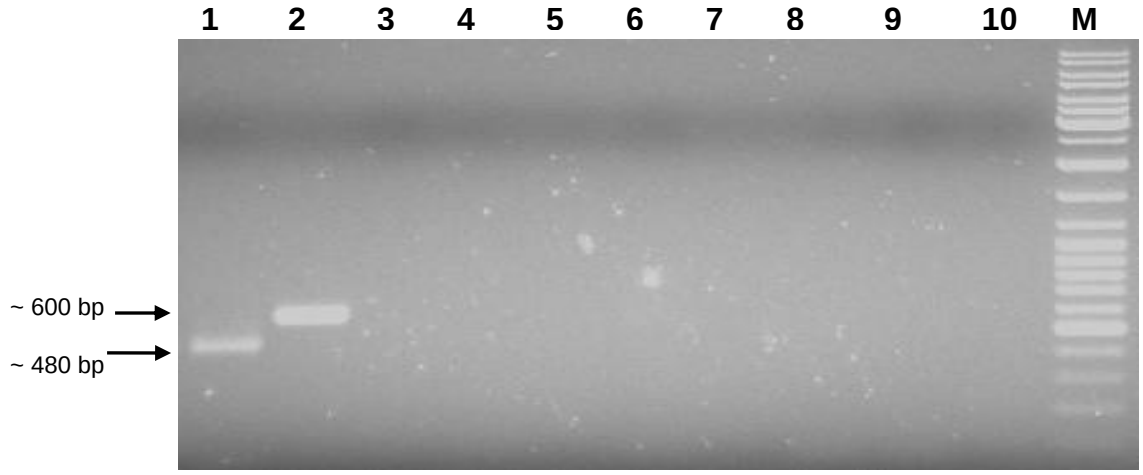


Figure 3.1: A 1 % agarose gel of PCR products for phage isolates from sewage and Chicken faecal matter samples. Primers which amplify the g23 capsid protein of T4 phages were used. Amplicon is approximately 600 bp and the positive control is approximately 480 bp in size. Positive control is specific for Exo T-even phages.

Coliphages are abundant in nature and efficiently destroy *E. coli*. Bacteriophage T4 is part of a superfamily which initially consisted of T-evens, Pseudo T-evens, Schizo T-even and Exo T-even groups. Among all these groups, T-evens are the closest relatives to T4 and Exo T-even are the most distant. This superfamily has grown and includes other T4-like phages which have recently been reported and infect hosts other than members of enterobacteria and its relatives (Comeau & Krisch, 2008). The *g*23 capsid gene of T4 and T4-like phages is useful for analysis of diversity within the T4 superfamily. Our PCR results revealed the presence of only one T-even phage isolate. This result is hardly surprising considering the abundance of coliphages found in sewage. The electron micrograph in chapter 4 (figure 4.3) is in agreement with the PCR result for this particular isolate. Various studies have indicated the presence of T4-type phages in water contaminated with sewage, paddy fields (Fujii *et al.,* 2007) and even different marine environments (Filée *et al*., 2005). Other isolates belonged to viral groups other than the T4 and T4-like phages.

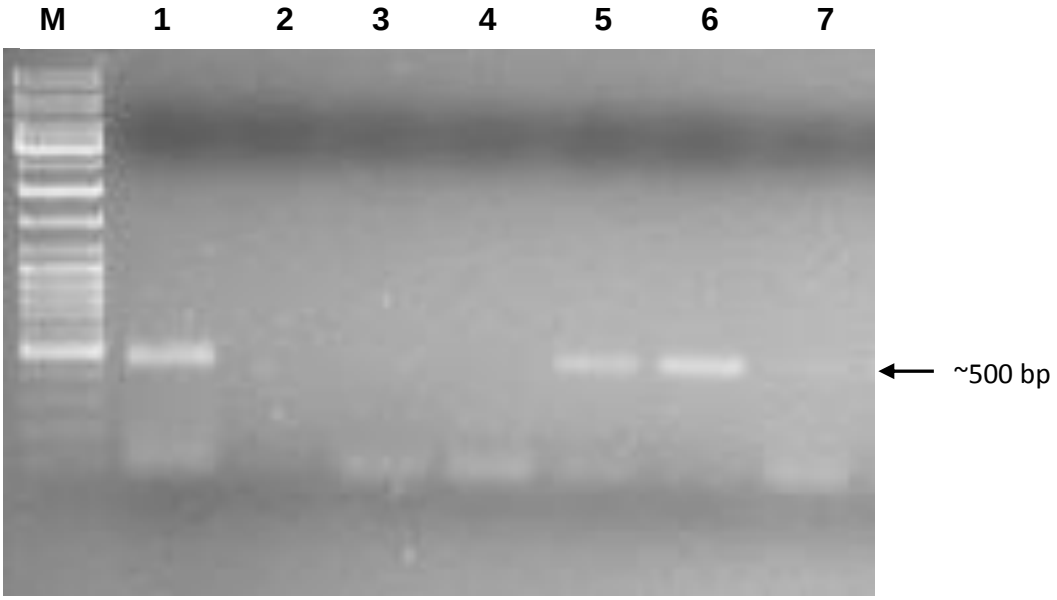


Figure 3.2: A 1% agarose gel of PCR products for phages isolated from sewage sample and chicken faecal matter samples. Primers used were specific for T7-like podophages. Lane M represents the O'Gene DNA Ladder mix which served as the molecular weight marker. Lane 1 is the positive control. Lanes 2 – 4 represent the samples where no amplification occurred. Lanes 5 – 6 represent phages from chicken faecal matter samples obtained from different chicken pens. Lane 7 is the negative control. Amplicons are approximately 500 bp in size.

Positive results were obtained for the PCR where degenerate primers specific for conserved regions of the DNA polymerase in T7-like podophages were obtained for DNA samples from filtered samples of the chicken faecal matter. The two amplicons both represent phages from chicken faecal matter samples collected from two different pens in which the chickens were housed. Whole samples for chicken faecal matter which had previously been purified by centrifugation and filtered were used in DNA extractions and molecular reaction carried. This was done in an attempt to determine whether any of the phages present in the sample were podoviruses. These results do not however, represent the entire phage population in the samples. Our results are similar to those found by Breitbart *et al.* (2004) in a study on the phage diversity in marine, freshwater, sediment, terrestrial and other extreme environments (Breitbart *et al.,* 2004) where they found a high level of homology between the sequences of HECTOR DNA polymerases of T7-like podophages in different environments. It is generally accepted that phages are found in environments where their hosts exist. However, the results from the above-mentioned study suggest that the DNA polymerase sequences of T7-like

podophages are transferred across different environments since minimal differences of about 3 base pairs over a 533 bp fragment were observed. The movement of these DNA sequences is believed to have occurred in recent evolutionary time. No podoviruses were found in our investigations from the sewage samples.

Virus taxonomy is challenging in that universal primers cannot be used to assess genetic diversity. This is because no single gene is known to exist in all viral genomes. Therefore, this leads to trial and error methods using different sets of primers specific for different viruses. This can be a cumbersome procedure. Morphological information can be useful in this regard as a possible identity of an isolate can be made from EM micrographs. This can make the search for a particular set of primers to use easier. For example, figure 4.3 from chapter 4 shows an electron micrograph of a phage isolate that resembles T4-like phages. Therefore, one can use primers specific for these phage types. However, if that information is not readily available, relatedness of viral isolates can be inferred by methods such restriction fragment length polymorphism (RFLP), pulse field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD). An RFLP analysis is labour intensive and time consuming compared to RAPD which is simple and inexpensive (Bardaski, 2001). Pulse field gel electrophoresis was performed with the aim of determining the genomic sizes of the phages but results obtained were consistently unfavourable for any useful information to be obtained. The electrophoretic run was for 22 hours and only smears were observed at the end of the run. However, PFGE remains one of the favoured techniques to determine genome size and once all the parameters have been optimised for the phage isolates, it will prove to be useful. Connerton *et al.* (2004) determined the genomic size of *Campylobacter jejuni* bacteriophages by PFGE by running the gel for 18 hours.

3.3.2. RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) PCR

DNA fingerprinting was then investigated by RAPD PCR technique. Prior information with regards to the genome of the sample to be analysed is not necessary. Short and arbitrary primers of random sequences are used to amplify genomic DNA at low annealing temperatures (Bardacki, 2001). Different banding patterns were observed for some of the isolates while others were similar. Similarities were seen with phage

isolates SK4 and SK5 (isolated from sewage sample) and this suggests that they could belong to the same family. Different patterns were observed for phage SK2 (isolated from sewage sample) and the chicken phage samples and other bands were too faint for any conclusions to be made.

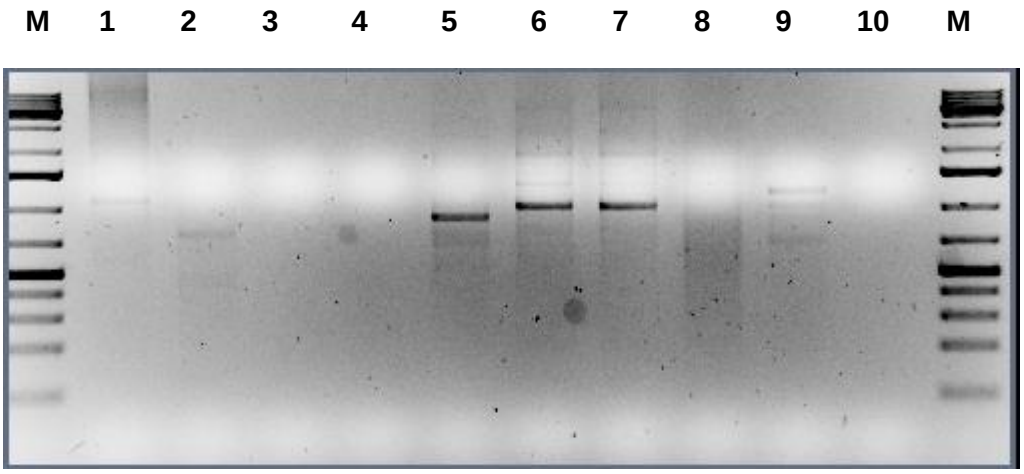


Figure 3.3: A 1.5% agarose gel depicting RAPD fingerprinting for phage isolates from sewage and chicken faecal matter samples. Lane 1: λ; lanes 2-4: phages from chicken faecal matter; lane 5: SK2; lane 6: SK4; lane 7: SK5; lanes 8-9: purified whole sewage samples; lane 10: Negative control. A 1kb plus ladder was used as the molecular weight marker represented by lanes M.

In order to determine the identity of some of the isolates, bands were purified, ligated into pGEM® TEasy vector and transformed. Positive clones were sequenced. Sequences obtained were edited using VECTOR NTI software and the consensus sequences were further analysed and compared to sequences available on GenBank by using the BLAST tool on the NCBI website. The RAPD PCR was a fairly simple procedure to perform as indicated from literature and it proved to be useful in providing valuable information on the relatedness of the phages especially as the identity of the phages which were isolated in Chapter 2 were unknown viruses. Comeau *et al.* (2004) used degenerate primers in RAPD PCR for algal viruses and bacteriophages and their studies showed distinct but closely related bands for *Micromonas pusilla* Virus while a distinction was made between these viruses and two *Chlorella* viruses. These results were in agreement with the authors' prior knowledge from literature on molecular techniques which involved genomic hybridisation and DNA polymerase gene phylogeny. Furthermore, three coliphage isolates from sewage contaminated seawaters were also differentiated genetically

through this technique using ten different primers (Fattouh *et al.,* 2002). Different banding patterns were obtained for amplicons and it was concluded from that study that the coliphage isolates were possibly different species. One of the challenges to note with RAPD PCR is that results at times can be irreproducible; nonetheless it remains an important tool. It is important to ensure that the DNA to be amplified is free from contaminants because the single primer used in RAPD is arbitrary thus, DNA from other sources may also be amplified (Bardacki, 2001)

3.3.3. RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

Restriction endonuclease analysis gave negative results with several restriction enzymes. *Eco*RI, *Alu*I, *Acc*651, *Bam*HI, *Bgl*II, *Hae*III, *Hind*III, *Pst*I, XbaI, *Sal*I and *Pot*I are some of the enzymes which were used and none could cleave the phage DNA (figure 3.3). Possible reason for this is that the nucleic acid is methylated and therefore, the coliphages remained insensitive to enzyme activity. To eliminate the possibility that the sample had bacterial DNA contamination, a 16s rRNA PCR was performed and no amplification occurred. Therefore, it was certain that the DNA which had been extracted was indeed viral.

Many strains of *E. coli* used to produce plasmid DNA contain methyltransferases (*dam, dcm, EcoKI* or *EcoBI*) which add a methyl group to either cytosine or adenine of the DNA with the ultimate result of altering recognition sequences of restriction enzymes. DNA methylation allows the cell to store information other than that found in the nucleotide sequences and this could be functionally significant by means of modified protein-DNA interactions. There are 3 major DNA methyltransferases of *E. coli* K12: *EcoK, dam,* and *dcm (*Krüger *et al.,* 1985). The *dam* methylase alters the GATC sequence; *dcm* methylates the C5 position of an internal cytosine CC(A/T)GG sequence while *EcoK* modifies the AAC(N_6)GTGC or GCAC(N_6)GTT recognition sequences (Hattman *et al.,* 1978; Geir & Modrich, 1979; Marinus & Morris, 1973; May & Hattman, 1975). The T7 genome has been found to possess restriction sites for *EcoK, dam* and *dcm* (Krüger *et al.,* 1985). It has also been reported that T2 and T4 sequences have restriction sites for *dam.* The only difference in the two phages with regards to *dam* restriction sites is 3 amino acids (Schlagman & Hattman, 1989). Most of the phages which were isolated in this study were found to belong to the T-even group, which includes T2, T4 and T6 phages. The presence of restriction sites

for methyltransferases in T2, T4 and even T7 is a possible explanation for the lack of cleavage which was observed. The majority of restriction enzymes are affected by DNA methylation. The effects of an overlap between the enzyme target site and the methylation site are: no effect, partial inhibition or a complete block. It is therefore important to consider whether the restriction enzymes to be used are sensitive to methylation or not (www.fermentas.com/techinfo/re/restrsensmeth.htm).

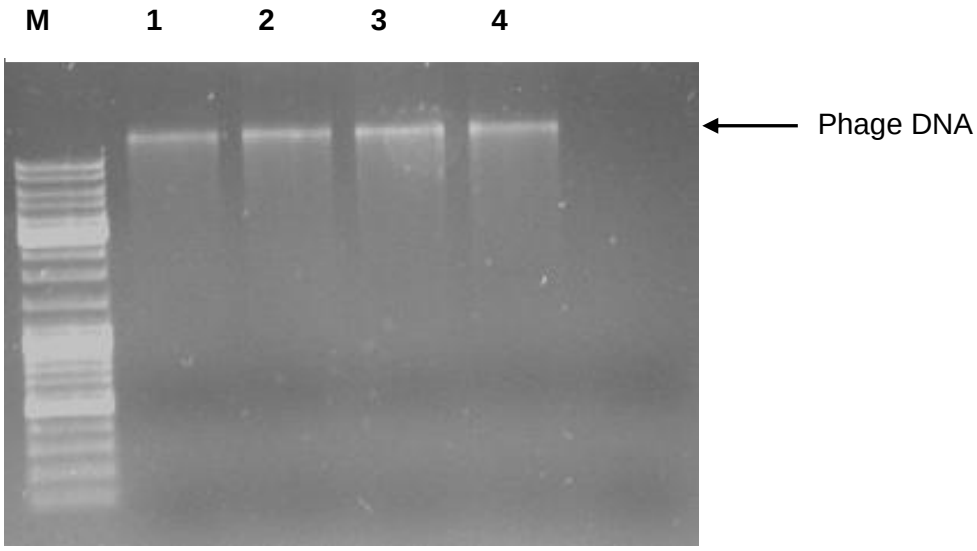


Figure 3.4: 1% agarose gel representative of restriction analysis on phages. Phage DNA was insensitive to enzyme activity.

Jensen *et al.* (1998) also found that the DNA of phages (SN-1, SN-2 and SN-X) isolated from sewage treatment plants used in their study were insensitive to restriction enzyme activity by all the enzymes tested. Some of the restriction enzymes they used are similar to the ones used in this study, namely, *Eco*RI, *Bam*HI, *Bgl*II and *Hind*III. Jensen and co-workers (1998) speculated that the insensitivity to cleavage was probably due to a modification of the viral DNA and not merely constraints on the activity of the restriction enzymes. Earlier studies on the DNA of SN-1 (Winston & Thompson, 1979) and SN-2 revealed that the nucleic acid was modified.

Bacteria have a number of defense mechanisms to prevent adsorption, penetration and other stages of the phage life cycle. These include mutation of the receptors in the cell wall and restriction enzymes (Krüger & Bickle, 1983). However, once successful adsorption of the phage into the bacteria has occurred and the nucleic

acid had been transferred into the cell, the phage must ensure the successful completion of the life cycle and this may involve evasion of host restriction enzymes. Modification of the viral DNA is one of the protective means employed by phages against the restriction enzymes. Apart from methylation which has already been mentioned above, viral DNA can also be modified by glucosylation. The DNA of T-even phages, that is, T2, T4 and T6 has been glucosylated and this is useful in either preventing or minimising cleavage. Some of the electron micrographs in chapter 4 show T-even phages (figs. 4.3, 4.4 & 4.6) and most likely glucosylation is also one of the reasons for the insensitivity which was observed in this case. Additionally, some phages, for example, T3 and T7 survive enzyme activity by encoding proteins that inhibit the enzymes (Madigan *et al.,* 2003).

3.3.4. PHYLOGENETIC ANALYSIS OF SEQUENCES

By 2008, complete genome sequences for seventy-three tailed phages whose bacterial hosts fall in the Enterobacteriaceae family had been established. Only six of the genera within this family are infected by these phages, that is, *Escherichia, Salmonella, Shigella, Erwinia, Klebsiella* and *Yersinia* (Casjens, 2008). The nucleotide-nucleotide BLAST results showed varying degrees of homology with 78% for *Erwinia* phage phiEa21-4 being the least and 99% for podoviruses from the phages in the chicken excreta samples as the highest. All the BLAST results were homologous to known phages in GenBank (refer to table 3.3 below). The phage isolated from sewage samples which was tentatively identified as SK4, showed the highest homology of 95% to *Enterobacteria* Felix 01 followed by 78% homology with *Erwinia* phage phiEa21-4. These phages contain dsDNA and both belong to the family *Myoviridae* of the order *Caudovirales*. This bacteriophage is lytic towards a few enteric bacteria and the majority of all *Salmonella* strains. The high homology is possible since *E. coli* strains such as those used in this study are closely related to *Salmonella* strains and they all fall under the group of enteric microorganisms. The receptor for coliphage T4 has been found in *Erwinia carotovora* (Pirhonen & Palva, 1988). Interestingly, Villegas and co-workers (2009) found a high resemblance between the whole genome and proteome of *E. coli* 0157:H7 phage, Felix 01 and *Erwinia amylovora* phage phiEa21-4. The high degree of similarity was observed in their tail fibre proteins. According to Villegas *et al.* (2009), sequence similarity between fibre proteins of related bacterial viruses is usually exhibited at the N-termini. That is where the link between the protein and the tail plate occurs. As a

result of these findings and comprehensive analysis, it was suggested that the *E. coli* 0157:H7 phage (named wV8) be placed in the newly created genus Felix 01-like viruses.

Nucleotide-nucleotide BLAST results are presented in table 3.3 below. Phages tentatively termed SK4, SK5, 31-5, E13/5 are phage isolates from sewage (see chapter 2). Phage Chk is a tentatively named representative of phages from chicken faeces (DNA was extracted from whole sample).

Table 3.3: Table indicating nucleotide-nucleotide BLAST results for phage isolates

SEQUENCE	DESCRIPTION	ACCESSION NO	HOMOLOGY
SK4	Enterobacteria phage Felix 01	AF320576.1	95%
	Erwinia phage phiEa21-4, complete genome	EU710883.1	78%
SK5	Bacteriophage AR1 putative prohead protease (g21) gene, partial cds	AF022930.1	98%
	Uncultured Myoviridae clone 3757 major capsid protein (gp23) gene	DQ105881.1	96%
	Enterobacteria phage KEP10 23 gene for major capsid protein	AB326953.1	96%
	Bacteriophage JSD5 gp23-like protein gene	AY744076.1	94%
Chk	Uncultured T7-like podovirus clone 115 DNA polymerase gene, partial cds	AY600059.1	99%
	Uncultured T7-like podovirus clone 114 DNA polymerase gene	AY600058.1	99%
	Uncultured T7-like podovirus clone 113 DNA polymerase gene	AY600057.1	99%
E13/5	Bacteriophage CEV partial sequence	AY331985.1	97%
	Uncultured Myoviridae clone 3751 major capsid protein (gp23) gene,	DQ105883.1	96%
	Uncultured Myoviridae clone 48519 major capsid protein (gp23) gene, partial cds	DQ105897.1	95%
	Bacteriophage TuIa major capsid protein (g23) gene, partial cds	AF221994.1	93%
	Uncultured Myoviridae clone 48510 major capsid protein (gp23) gene, partial cds	DQ105896.1	91%
31-5	Enterobacteria phage KEP10 23 gene for major capsid protein, partial cds	AB326953.1	92%
	Bacteriophage AR1 putative prohead protease (g21) gene	AF022930.1	88%
	Uncultured Myoviridae clone 3757 major capsid protein (gp23) gene	DQ105881.1	87%
	Bacteriophage JSD5 gp23-like protein gene	AY744076.1	87%

Phage isolate SK5 shares the highest homology with bacteriophage AR1, a T4-like phage which has a broad host range among *E. coli* strains. The morphological features of bacteriophage AR1 place it among myoviruses and this was also found to be the case in the electron micrographs of phage SK5 (see figure 4.3). Goodridge and colleagues (2003) also observed similarities between the phage they had isolated AR1, and T4 through host ranges, genome size and southern hybridisations, thus supporting these findings.

For the representative of some phages in the chicken faecal matter community, Chk, all the hits obtained matched to T7-like podoviruses. These are virulent phages with short tails. Their host range generally includes enterobacteria. Podoviruses are the least frequent among the tailed phages. They have also been isolated from extreme environments such as marine and terrestrial biomes (Breitbart *et al.,* 2004). Phage E13/5 (isolated from sewage sample), on the other hand, is closely related to T-even phages, CEV1, a phage that was isolated from sheep which lyses *E. coli* 0157:H7 (Raya *et al.,* 2006). Isolate E13/5 is highly similar to T4-like bacteriophage CEV1, which was originally isolated from ewes. A high homology with uncultured myoviruses was also observed. Bacteriophages CEV1 and AR1 (for 31-5 isolate) are closely related according to phylogenentic analysis performed by Raya *et al.* (2006). This was also observed from the multiple alignments and phylogenetic tree (figures 3.5 and 3.6 below).

The majority of phage isolates from this study shared high homologies with several T4-like phages such as bacteriophage CEV1 and bacteriophage Tula. Isolate 31-5 and E13/5 also share similarities in their lytic patterns (table 2.6, Chapter 2). They both did not lyse *E. coli* K12. Isolates SK4 and SK5's lytic spectra were also similar to one another in that they both destroyed *E. coli* 76/00 and K12.

```
SK4          1  ------------------------------------------------------------
31-5         1  CACTATAGGGCGATTGGGCCCGACGTCGCATGCTCCCGGCCTCCATGGCGGCCGCGGGAA
E13/5        1  CACTATAGGGCGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAA
Chk          1  ------------------------------------------------------------
SK5          1  ------------------------------------------------------------

SK4          1  ----------------------------------------------------TAATCTCACC
31-5        61  TTCGATTGATATTTGAGGGGTTCAGCCGATGAACAACCCGAATGGTCAGGTATTCGCACT
E13/5       61  TTCGATTGATATTTGGGGGGTTCAGCCGATGAACAGCCCTACCGGTCAGGTATTTGCTCT
Chk          1  ----------------------------------------------GTGAGTCATACGGCCT
SK5          1  ----------------------------------------------------TATTCGCACT

SK4         11  -TAAACCATAACCACCATAAGTCAGCCTGTTCAAATCTCCG---AAAGACTCTGGGAAAG
31-5       121  GCGCGCAGTATATGGTAAAGACCCAATCGCTTCCGGCGCTTTC-AAGCATTCCACCCACT
E13/5      121  CCGTGCAGCATATGGTAAAGACCCTATCGCTGCTGGCGCTAAAGAAGCTTTCCATCCGAT
Chk         17  GT-TACAGCAC-CGTTGGTGATAACTCTGCCGTGTACCCTG-----CCATCTTTCTCGTG
SK5         11  GCGCGCAGTATATGGTAAAGACCCAATCGCTTCCGGCGCTAAAGAAGCATTCCACCCAAT

SK4         67  GAATCAATGGAATCTGACCACGATTCTTCATTGCCTCAAAACCATCTGCGAAGTTTTTGA
31-5       180  GTATGGTCAAGATGCTATGTTCCCAGGTCTTGGTGCTGCTAACAAATTCCCTGCTTTGTC
E13/5      181  GTATGCCCCGGACGCGATGTTCTCTGGTCAGGGTGCTGCTAAGAAATTCCCAGCTCTGGC
Chk         70  GGTTAGCCAAGAGTCAATCTGTGCTGCTCTCTTCTGCAACATCAGGTACTCGTATATCGT
SK5         71  GTATGGTCCAGATGCAATGTTCTCTGGTCAGGGTGCTGCTAAGAAATTCGCTGCTTTGAA

SK4        127  TACCAGCAGGGCAGT--AACGAGGTGCATTGTAGATACGCTGCTTAAATCCTTCCAGAAC
31-5       240  AGCGGGTGATACTCTTGAACTCTGAACTATTTA-GCTCACTTCTTCCCTAA-ACTGGTAC
E13/5      241  TGCTGACACCACTACCGTTGTAGGTGATATCTATACTCACTTCTTCCAAGAAACTGGTAC
Chk        130  CTTAGCCTCTGGTAT----GTCAATGCCTTCTAACACCTTCTSGTTGACAATGACAGCGC
SK5        131  AGCAAGTGATACTCTTGAAGTTGGAACTATTTACACCCACTTCTTCCAGGACACTGGTAC

SK4        185  TGTATCTTT-CTCTTTATAGAACTTTGTCCACC--ATTCATTGAGGTCTTTTACACCTT-
31-5       298  AGTATATCTGCAAGCTACAGAAGTTAAACGAATTGATTCAGGCGCATCTGACGCAGACAA
E13/5      301  TGTATATCTGCAAGCTTCTGCTGTCGTAACACTTGATTCTGGTGCAACTGATGCAGCTAA
Chk        186  CCT-TMTCAGTTTTCTGTTTGAACTTAACGCCTACACCTACCAGCCTCTC-TGCAAT---
SK5        191  TGTGTATCTGCAAGCTACAGAAGTTAAGCAAATTGATACTAGTGCAACTGACGCAGCTAA
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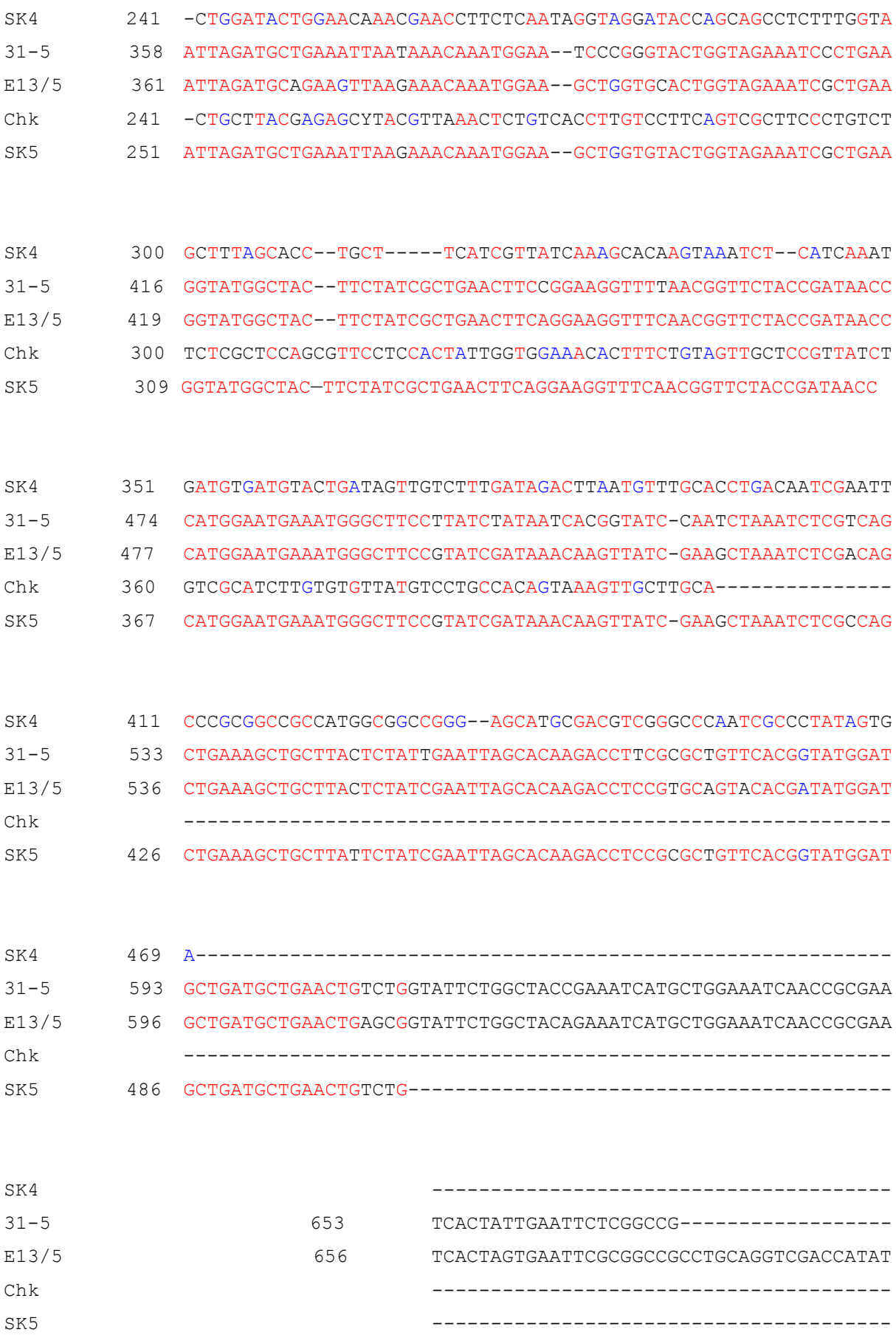


Figure 3.5: Multiple alignments of selected phage isolates showing varying levels of homology in the nucleotide sequences.

The multiple alignments of the phage isolates showed high levels of homology, particularly for 31-5, SK5 and E13/5. However, the first two were the most similar to each other. This implies that 31-5 and SK5 are closely related, followed by E13/5, SK4 and lastly Isolates 31-5, SK5 and E13/5 most likely share a common ancestor. Chk isolate was the most distant related of all the isolates as is indicated by the few conserved areas within the nucleotide compared to the other nucleotides. Segments between nucleotides 1- 185 and between positions 416 -606 are highly conserved in the sequence. The similarities, albeit, a few in some instances which are observed when all the nucleotide sequences are compared to each other suggest that gene transfer may have occurred at the ancestral level. The relationships between the isolates can be clearly seen in the phylogenetic tree which was constructed (figure 3.6). The greatest distance between the isolates is observed with the Chk sample while 31-5 and SK5 cluster together, representing high degree of relatedness. The cluster formed by 31-5, SK5 and E13/5 indicates that there is a greater level of relatedness between these phage isolates than between either of them to SK4 or Chk. Moreover, the phylogenetic tree further illustrates that E13/5, SK5 and 31-5 share a recent common ancestor with one another than with SK4 and are descended from an even more distant ancestor with Chk.

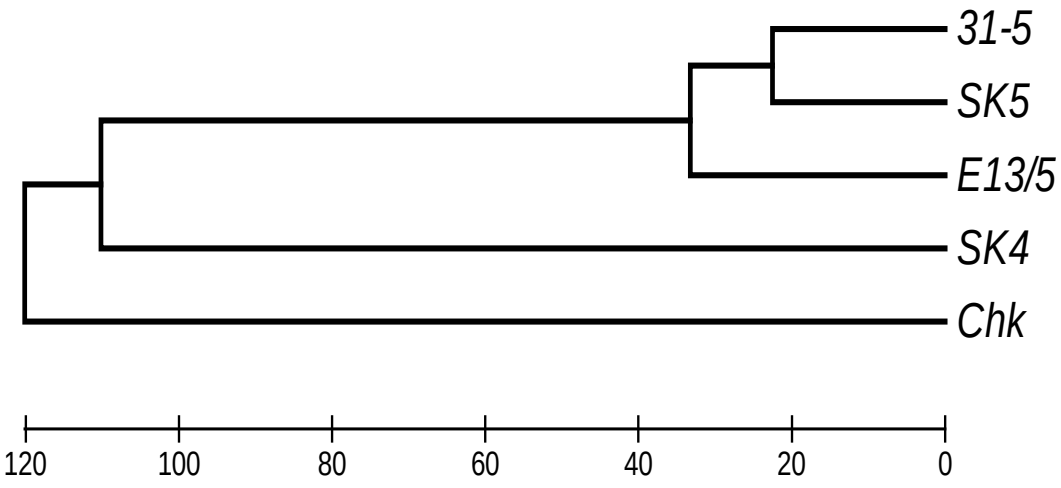


Figure 3.6: Neighbour-joining phylogenetic tree showing the relationship between the phage isolates.

All the phage isolates with the exception of Chk belong to the family *Myoviridae,* mostly identified as T4-like or T-even related phages*.* It is speculated that the distant relationship between Chk and the other isolates may be due to the fact that they were isolated from different sources. The likelihood of phages isolated from the same environment being similar is comparatively higher because there is a greater possibility of genes being transferred.

The high levels of homology may also be a result of gene transfer among the phages and between the phages and their hosts. Horizontal gene transfer continues because the phages tend to co-evolve with their hosts and this in turn contributes to diversity observed in genetic pool. Lawrence & Ochman (1998) estimate that approximately 16 kbp of *E. coli* genome is replaced through horizontal transfer of genes with other organisms. This apparently occurs every million years.

Classification of dsDNA has been a complex process partly because of extensive genetic variation. For example, phages may share similarities in morphology, modes of replication and general genomic structures and yet may be entirely dissimilar at the nucleotide level (Hendrix *et al*., 1999). Evidence from comparative analyses of newly characterised phages and cryptic prophages suggests that the majority of dsDNA phages have common ancestry and that exchange of genetic elements from a large shared pool does occur. Hendrix et al. (1999) believe that horizontal transfer is not restricted to phages whose hosts are closely related but should also occur between phages with overlapping host ranges irrespective of similarities or not in their host ranges. Bacterial DNA may be transferred by transformation, conjugation or transduction and phages serve as essential vectors for the horizontal transfer of bacterial DNA or mobile DNA elements (Canchaya *et al*., 2003). Among the phage groups, tailed phages are known to be the best vehicles for gene transfer and this is supported by their morphology which includes a phage tail and adsorption structures such as fibers and spikes (Canchaya *et al*., 2003; Guttman *et al*., 2005).

The morphology of some of the isolates can be seen in chapter 4 and they also confirm the identity of the isolates as T-even bacteriophages. Desplats & Krisch (2003) investigated the evolution of T4-type phages and they found that the genomes of these viruses are similar to the T4 genes that establish phage

morphology and many of the genes responsible for the T4 lytic cycle. It is generally accepted that the bacterial hosts of phages which are genetically related are closely related.

3.4. Conclusions

The use of molecular techniques for the identification of unknown phage isolates was investigated. Genomic fingerprinting by RAPD PCR provided useful information regarding the relatedness of isolates. It was inferred that phage SK5 and phage SK4 were closely related based on the banding patterns observed (see figure 3.4). In addition, RAPD PCR is also valuable as a starting point for further manipulation of amplicons in cloning and transformation reactions. Positive clones obtained from transformation procedures can then be used for sequencing purposes which subsequently provides identity of the isolates. The phages were insensitive to endonuclease activity by several enzymes, most likely due to modification of the viral DNA by methylation or glucosylation. This is a property that requires further investigation**.**

The identities of phage isolate SK5 and some of the viral community in chicken faeces was determined to be T4-like and T7-like podoviruses respectively by using specific primers which amplify the g23 capsid protein of T4 phages and DNA polymerase of T7-like podophages**.** A close relationship between E13/5, 31-5 and SK5 was also determined based on the presence of highly conserved motifs in their nucleotide sequences as well as the phylogenetic tree.

The results from this chapter suggest that successful identification of all the phage isolates by molecular procedures particularly conventional PCRs may require the use of information obtained from transmission electron microscopy. Electron micrographs provide an initial classification which can then be used to select the appropriate primers for amplification and subsequently, lead to identification of the isolates.

CHAPTER 4

MORPHOLOGICAL CHARACTERISATION OF ISOLATED BACTERIOPHAGES BY TRANSMISSION ELECTRON MICROSCOPY

4.1. INTRODUCTION

Morphological characterisation of any microorganism is one of the essential elements in establishing its identity. Microscopes are typically used for this purpose. However, owing to the ultramicroscopic size of viruses, transmission electron microscopy (TEM) has proven useful. TEM is a powerful tool which has played a significant role in the classification of isolated phages through the visualisation of morphological features. Electron microscopy and interpretation of genome sequences are the only methods which can be employed to place an unknown phage into a family. Furthermore, identification can go as far as species or genus level with some phages for example, phages of bacilli, vibrios, and enterobacteria (Ackermann, 2005).

From the time negative staining was first used on bacteriophages in the 1950s by Brenner and Horne (1959), there has been a vast improvement in the visualisation of macromolecules and biological particles using the transmission electron microscope. This technique owes its success to the fact that it enables one to better differentiate between molecules due to high contrast created by the stain (Frank, 2006) and thus allow the observer to make certain conclusions about the morphology of the sample in question.

Currently, about 5568 bacterial viruses have been described (Ackermann, 2007). Bacteriophage classification initially involved the identification of six basic types: tailed, filamentous and cubic phages which possess either DNA or RNA. The International Committee for Taxonomy of Viruses (ICTV) currently classifies phages into the order *Caudovirales* comprised of tailed phages, 13 families and 'floating genus' *Salterprovirus.* Approximately 96% of described bacterial viruses belong to the families *Myoviridae, Siphoviridae* and *Podoviridae*. Their virions consist of a head

(capsid) with a cubic symmetry and helical tails. The remaining 4% is made up of the polyhedral, filamentous and pleomorphic phages. The capsids of these phages may be enveloped or unenveloped (Ackermann, 2003, 2007).

According to Maniloff and Ackermann (1998) the ICTV defines a virus family as “a group of genera with definite common features.” The differences in tail morphology that exist between the 3 families in the order *Caudovirales* are an indicator of differences in the way they infect their hosts, how their virions are assembled and mature and lastly, the organisation of their nucleic acid (Maniloff & Ackermann, 1998).

A majority of phages are double-stranded viruses and they all possess a capsid surrounding their nucleic acid. Nevertheless, the shape and size of the capsid may differ significantly. The nucleic acid may be single- or double stranded DNA or RNA (Bradley, 1967). Various morphology types have been determined for coliphages and other enterobacteria.

The aim of this section was to determine the morphology of the phages which were isolated and reported on in chapter 2, and thus place them in their respective families.

4.2. MATERIALS AND METHODS

Information regarding preparation of media used is described in appendix A.

Bacteriophage isolation is described in detail in section 2.4 of chapter 2. Briefly, raw sewage sample was centrifuged at 4 000 *g* and filtered with a 0.20 µm membrane filters (Sartorius ®). One millilitre of pure sewage sample was mixed with 1 ml of overnight bacterial cultures in 5 ml of 0.8% TSA and poured onto TSA plates and incubated overnight at 37°C. After the incubation period, well isolated plaques were picked from the agar plates using sterile toothpicks, inoculated into 5 ml TSB with 50 µl *E. coli* and incubated at 37°C for 6 hours, a period during which the bacteria cells lysed releasing phage particles. The lysates were treated with 200 µl of chloroform to

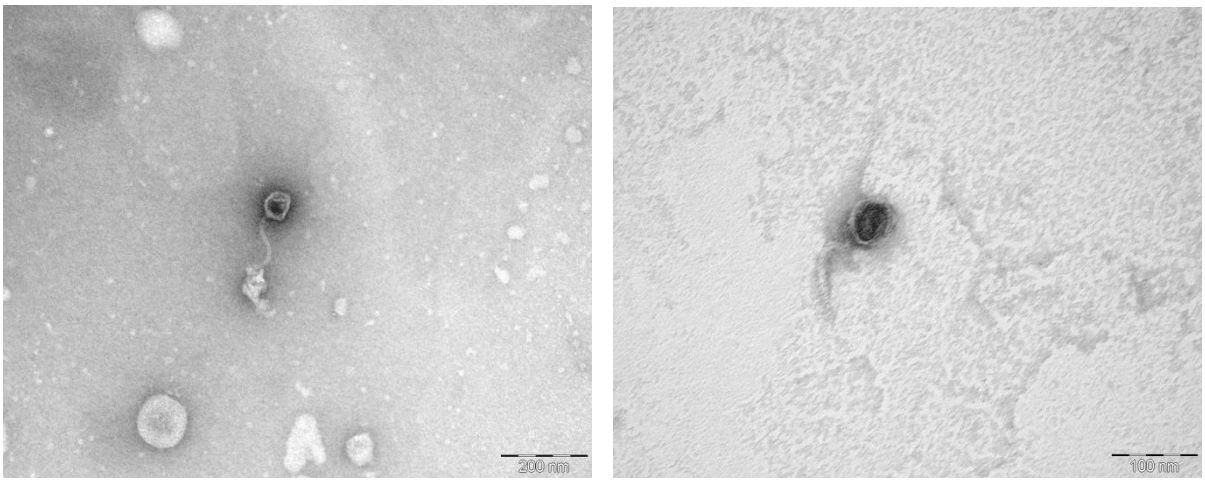
lyse bacterial cells. Centrifugation step was performed at 14 500 *g* for 10 min and samples were filtered with 0.20 µm membrane filters. A drop of the phage sample was added to a formvar-coated carbon grids and left to stand for 3 min. The grid was dried with a filter paper and a drop of 3% uranyl acetate was placed on the grid for 30 seconds, after which the grid was dried once again. The samples were examined in a Philips CM100 transmission electron microscope at 60 kv (Centre for Microscopy, UFS).

Alternatively, plaques were picked and placed in 4ml TSB (media described in appendix A). A volume of 200 µl of chloroform were added and incubated at 37 °C for 10 min. The phage suspension was then centrifuged at 14 500 *g* for 10 min and filtered using 0.45 µm and 0.20 µm membrane filters. Negative staining was then applied as described above. In some instances (chicken faecal matter samples) the original samples were centrifuged at 4000 *g* for 20 min and filtered with a 0.20 µm filters. Samples were then stained and examined under the transmission electron microscope, Philips CM100 transmission electron microscope at 60kv (Centre for Microscopy, UFS). Samples were used instead of lysates because the plaques initially obtained were too tiny to be picked off the agar plates.

4.3. RESULTS AND DISCUSSION

4.3.1. PHAGES ISOLATED FROM SEWAGE SAMPLE

Phage particles which were tailed and non-tailed in structure were successfully observed using TEM (refer to figures 4.1 to 4.7). Sewage sample was found to be rich in diversity and abundance of coliphages as demonstrated by the plaque assay results reported on in chapter two of this study which deals with the isolation and host range determination of *E. coli* bacteriophages. This observation was not particularly surprising because *E. coli,* the bacterial host of coliphages is found in the intestine of many animals. It is released into the environment in high concentrations through faecal material (Barnes *et al.,* 2003).



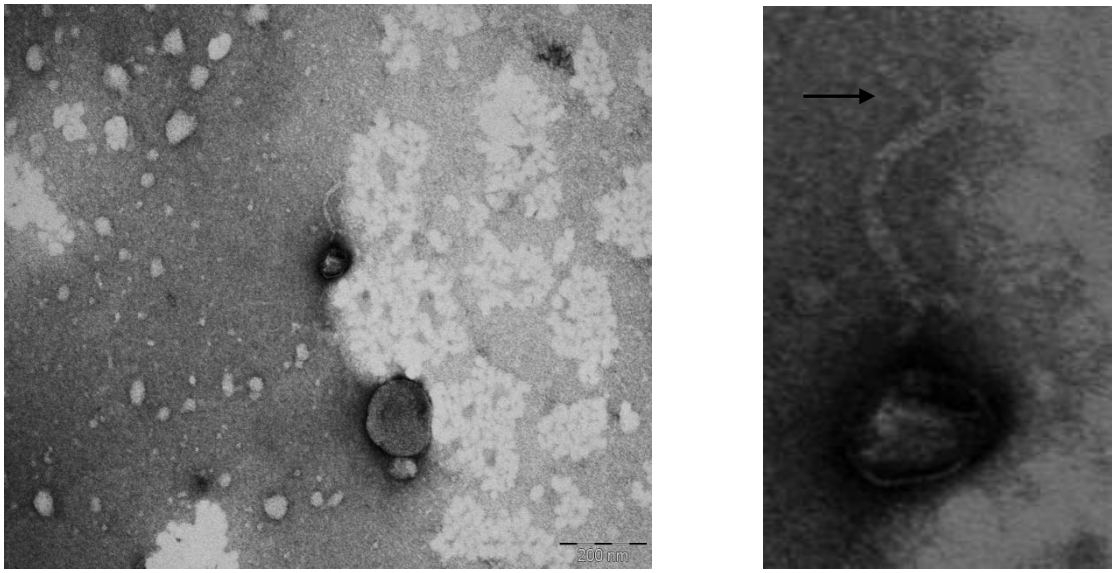
(a) (b)

Figure 4.1: Electron micrograph of phages with flexible tails. Scale bar corresponds to 200 nm in (a) and 100 nm in (b).

Viruses in the electron micrographs in figure 4.1 above have icosahedral capsids and flexible tails which appear similar to representatives of the morphotype β4 according to descriptions by Ackermann and Nguyen (1983). Dimensions for (a) are capsid diameter approximately 56 nm by 72 nm and tail about 120 nm. The capsid diameter and tail in (b) was found to be approximately 41 nm and 95 nm respectively. Based on tail morphology, these viruses can be placed into the *Siphoviridae* family. Phage in (b) may tentatively be placed into the T1-like genera although this still requires confirmation. T1-like phages strictly infect enterobacteria. The differences in capsid size suggest that the phage isolates belong to different morphoypes within the same family. Long, non-contractile tails are typical features of members in the *Siphoviridae* family. The nucleic acid can be clearly seen as the darkened region within the capsid. Uranyl acetate, one of the common reagents used to stain phage samples was used. Uranyl cations tend to bind to dsDNA and this leads to capsids appearing black due to their positive staining (Ackermann, 2005). It was not possible to conclude whether the tails had any attachment structures such as fibers or spikes.

Price and van Rooyen (2001) noted that the presence of debris in the sample can easily mask certain structures on the virion and as a result useful information regarding the isolated phages may be lost. The magnification at which the electron

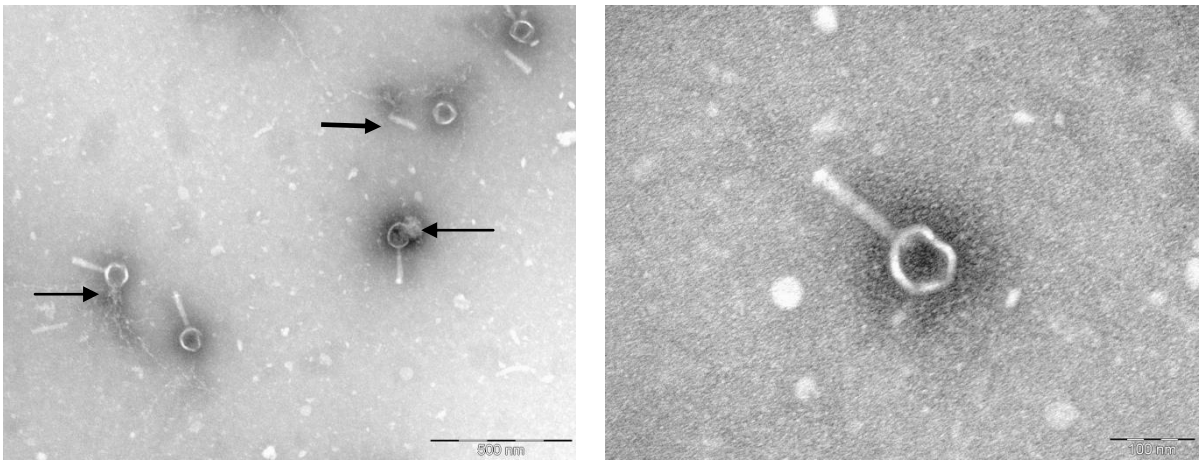
micrograph was taken can also affect the interpretation of results. Similar challenges were experienced in this study for some of the images.



(a) (b)

Figure 4.2: Electron micrographs of one of the phage types isolated from sewage sample. (b) An enlarged micrograph of the same phage in (a) showing spikes at the tail end. Scale bar in (a) represents 200 nm.

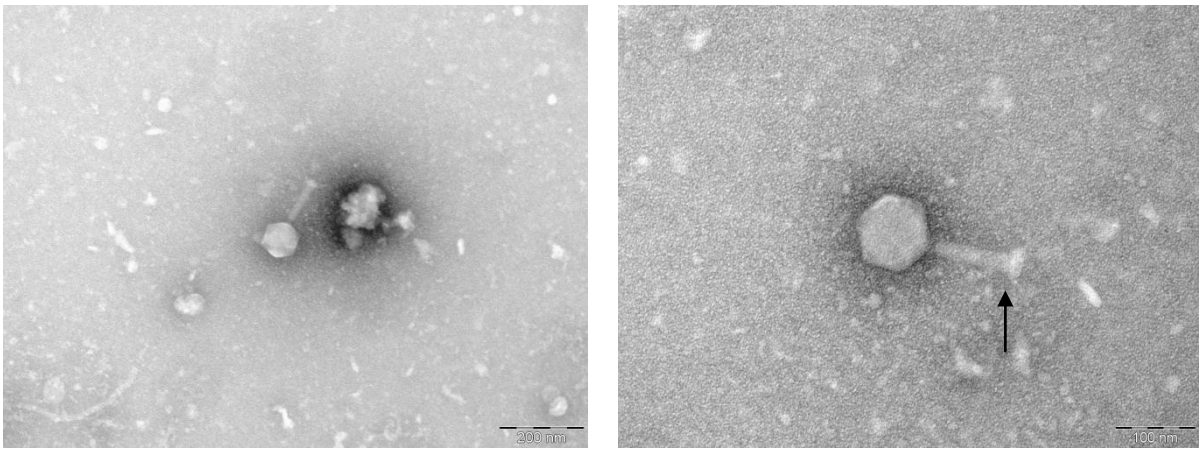
The phage in figure 4.2 also has a flexible tail. A closer inspection of the enlarged image in part b of figure 4.2 reveals what seem to be spikes at its end. These would be useful in adsorption to a suitable host. This phage has no contractile machinery and therefore does not belong to the *Myoviridae* family but rather the *Siphoviridae* family. The diameter of the capsid is about 72 nm and the tail is approximately 127 nm in length. Bacterial hosts include enterobacteria, Pseudomonas, Lactococcus, *Bacillus*, *Listeria* and *Streptomyces* (Ackermann, 2001). Dúran and colleagues (2002) also found *Siphoviridae* phages with flexible and curled tails among other isolates in their isolation of somatic coliphages from sewage samples. The presence of somatic coliphages serves as potential indicators for water pollution.



(a) (b)

Figure 4.3: Electron micrographs of phages propagated on *E. coli* K12. Plaque size was 5 mm. Note the presence of broken tails (see thick arrow on figure 4.3a) and some phages releasing DNA from broken capsids (see thin arrows in figure 4.3a).

During morphological examination, viral particles were found with contractile tails and capsids separated as indicated above. The phage head (figure 4.3b) has a diameter of about 72 nm and tail length of approximately 125 nm including the collar. This was probably due to changes in the ionic medium which subsequently resulted in osmotic shock of the phages. It has been reported that osmotic shock can destroy viral particles such that tail fibers and other structures may be lacking (Konopa & Taylor, 1973). The morphology observed in such cases offers incomplete information and can affect identification purposes somewhat. The nucleic acid is also seen exposed, outside the capsid in some of the particles. These samples were stained from lysates that had been added to Luria Bertani or Tryptone Soy broth. To minimise the chances of osmotic shock, lysates may be placed in buffered solutions such as Phosphate Buffered Solution. In an article on 'declining electron microscopy', Ackermann (2004) includes washing crude lysates with 0.1M ammonium acetate as the best option for removing proteins and sugars from the medium in which the phages are suspended. Intact virions may be obtained and better characterisation made possible such as with figure 4.3b. These phages resemble T4-like phages and this was confirmed by molecular analysis (refer to chapter 3). T4 phages belong to the *Myoviridae f*amily and infect enterobacteria and related bacteria such as *Acinetobacter, Pseudomonas,* and *Vibrio.*

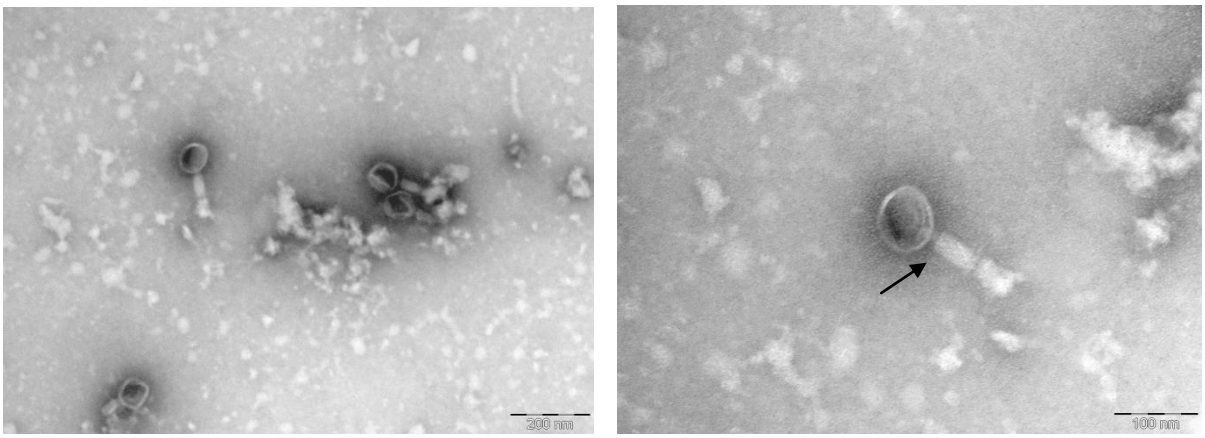


(a) (b)

Figure 4.4: Electron micrograph of phages propagated on *E.coli* K12 with the resulting plaque size of 2 mm (SK2 phage isolate). Arrow in figure 4.4b is pointing at the basal plate.

The viral particles in the figures above represent the morphology of isolate SK2, a phage which had been propagated on *E. coli* K12 and had a plaque size of 2 mm. The virion consists of a well defined icosahedral head and a contractile tail with a basal plate is clearly visible to which fibers are possibly attached. A range of appendages such as fibers or terminal structures may be found at the end of the tail (Bradley, 1967). The mentioned structures are important in allowing adsorption of phage to bacteria and genome transfer may then be possible. The base plate is usually complex and it may possess one to six fibers (Freifelder, 1983). The capsid diameter is about 88 nm, tail is about 97 nm long and the base plate is about 40 by 14 nm in its dimensions. These types of virions are characteristic of the *Myoviridae* members which were also isolated by Chibanni-Chennoufi *et al.* (2004b) from stool samples of patients suffering from *E. coli* diarrhea. The only difference is that the myophages from that study have elongated capsids compared to the ones depicted in figure 4.4a. These researchers noted that individual studies of phage isolation merely give an indication of the viruses present in an environment at the given moment when samples are collected and speculated on the possibility that phage titers reduce and move within an ecosystem. As a result, sewage samples or any other samples from the same location should not be expected to give the same results every time.

Goodridge *et al.* (2003) also found a coliphage, designated AR1 similar in morphology to figure 4.4a. However, capsid of bacteriophage AR1 is elongated in comparison. These phages both resemble T4 phages.

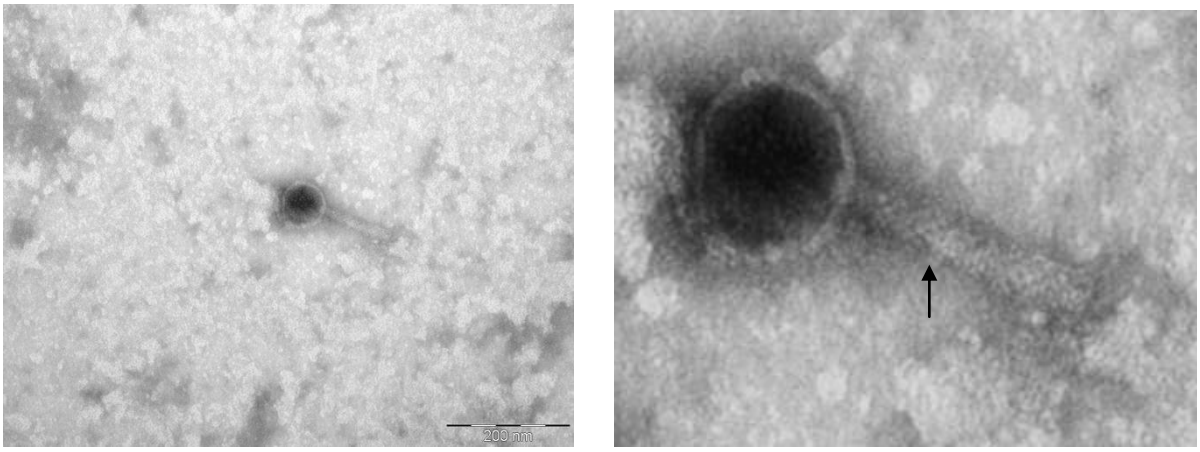


(a) (b)

Figure 4.5: Electron micrographs of phages isolated from sewage sample grown on *E. coli* K12. Phage with collar and contracted tail sheath seen in (b) (indicated by arrow). Plaque size was 4 mm. Scale bar in (a) is 200 nm; scale bar in (b) is 100 nm.

Contractile tails characteristic of *Myoviridae* are evident in phages seen in figure 4.5. These phages also possess a short collar between the capsid and tail. The tail sheath in the figures appears contracted. It is thought that the base plate of myophages is responsible for sheath contraction and binding of phages in this family to receptors on the bacterial cell. Sheath contraction results in the tail penetrating the cell membrane and injecting its DNA (Smith & Anderson, 1967). The base plate of T4, a typical example of myoviruses, is the part where the infection of bacteria is controlled. The first step of infection involves reversible binding of 6 long tail fibers to lipopolysaccharide receptors on the bacterial cell surface. Following this, short tail fibers located under the base plate are irreversibly attached to the cell and this leads to a structural change in the base plate which triggers sheath contraction and subsequently forces the tail tube through the cell membrane and phage DNA is transferred into the bacterial cytoplasm (Kostyuchenko *et al*., 2003). The phage type observed in figure 4.5 appears similar to some of the phages found in ruminal fluid from sheep and cattle (Klieve & Bauchop, 1988). Other phages observed from the rumen were also similar to those shown in figure 4.1 and figure 4.7 b below and also included other phages with unique features. One other important characteristic to

note in all the myoviruses is that they have larger capsids which contain more DNA than siphoviruses and podoviruses.

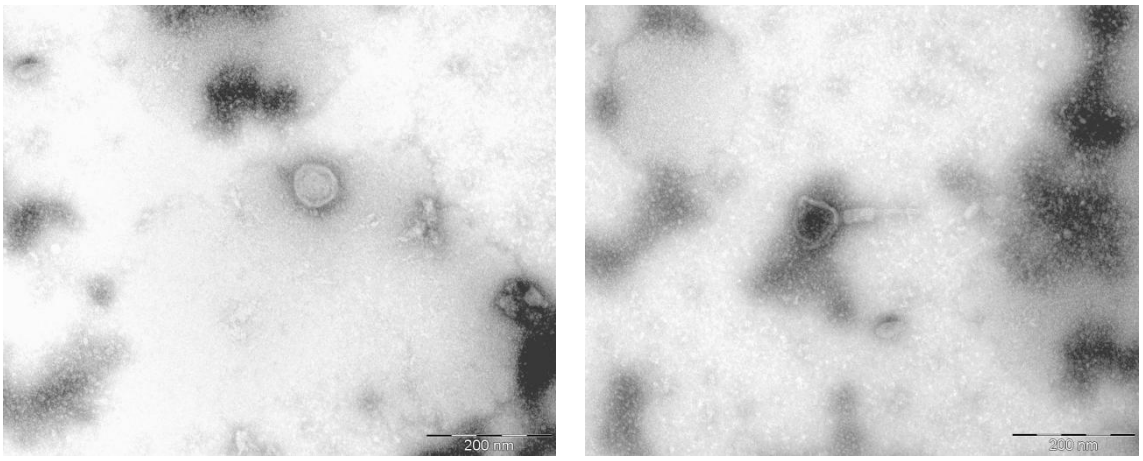


(a) (b)

Figure 4.6: Electron micrograph of phage grown on *E. coli* K12. Scale bar, 200 nm. Enlarged image of the same phage isolate is shown in (b) showing striation detail on the tail.

Virions in figure 4.6(a) appear to be members of the T-even group and belong to the *Myoviridae* family based on the contractile tail morphology. The tail dimensions are 120 nm while the capsid is 80 nm in diameter. The isolate bears a strong resemblance to the T2 type phages. Tailed phages were more abundant in the samples in comparison to their tailless counterparts. This is not a surprising observation since it has been widely reported that about 96% of all known phages belong to the tailed families, namely, *Siphoviridae, Myoviridae* and *Podoviridae.* A study by Muniesa and colleagues (1999) showed similar *Myoviridae* phage types with an isometric head and non-isometric heads. In addition, they also found phages similar in morphology to those shown in figure 4.1. Results from that study revealed that *Myoviridae* were the most abundant in human and animal sewage while *Siphoviridae* coliphages were the second most abundant. It was concluded from their investigations that contaminated water from human or animal sewage would exhibit similar morphological findings. This is an unexpected conclusion considering that siphoviruses are reportedly more widespread in nature and represent approximately 61% of the phage population, while myoviruses only constitute 24.5% of tailed phages. However, it is probably because the authors' (Muniesa *et al.,* 1999) work was only restricted to water polluted by human or animal sewage.

4.3.2. PHAGES ISOLATED FROM CHICKEN FAECAL MATTER SAMPLES



(a) (b)

Figure 4.7: Electron micrographs of tailless phage and tailed phage with a contracted tail which were obtained from the chicken faecal matter samples.

A tailless phage with an icosahedral head can be seen in figure 4.7a. In contrast, a phage with a rigid, contractile tail resembling a T-even phage which is part of the *Myoviridae* family was seen in figure 4.7b. The morphotype fits the A1 type description of T-even phages by Ackermann & Nguyen (1983). The tail sheath in the figure 4.7b appears contracted. This is believed to be so because the tail tube can be seen at the end of the sheath. During adsorption, the fibers on the end of the tail initiate attachment. Afterwards, the sheath contracts when the base-plate is in contact with the cell, its central part penetrates the bacterial cell and nucleic acid is transferred (Simon & Anderson, 1967). The phage isolate in figure 4.7 b is similar to those seen in figure 4.5 isolated from sewage. The capsids do however look slightly different in that the capsid appears deformed, probably due to reaction during the preparation steps involved in staining the samples. In addition, the sheath is contracted. Molecular studies showed that the phages isolated from chicken faecal matter had isolates that belong to the *Podoviridae* family. That could possibly be the phage in figure 4.7 a, probably with the short tail that is not visible. However, this is only speculative and cannot be concluded from the electron micrograph. Podoviruses are also known to infect enteric bacteria among other hosts (Ackermann, 2003).

Similarly, Oliveira and colleagues (2009) recently reported on the isolation of phages, phiF61E, phiF258E and phiF78E from chicken faeces. There are some similarities in some of the results obtained in this study and those reported by these authors with regards to morphology. Phage isolate phiF61E bears a striking resemblance to the phage in figure 4.7 b in that both possess contractile tails and short collars although the phage head sizes differ. Phage isolate phiF61E has a prolate head in comparison. phiF258E shares similarities with the phage in figure 4.7, specifically the long non-contractile tails. phiF78E and the viral particle shown in figure 4.6 also seem somewhat alike,however, the main difference is the elongated heads of phiF78E.

Campylobacter phages have also been isolated from chicken faecal excreta from free-range layers (Loc Carrillo *et al*., 2007). The morphological characteristics of bacteriophage CP8 appear somewhat similar to figure 4.7b although CP8 has a terminal bleb as an additional feature.

Horses' faeces have also been demonstrated as a source of coliphages by Golomidova *et al.* (2007). The large intestine of a horse is reported to consist of a variety of microorganisms such as bacteria, fungi, archaea and protozoa. An important difference with the microbes found in the rumen is that they remain unaffected by digestion and are found in the faecal matter. Phage isolates were all tailed and their morphological characteristics were in accordance with those of *Myoviridae* and *Siphoviridae* members. One of the phages (type IV) reported by Golomidova *et al.* (2007) is similar to a phage isolated in this study which can be seen in figure 4.6 a.

Visualising phages from the chicken faeces proved to be more challenging than the sewage samples. Several attempts were made for both sets of samples before virions that could be conclusively said to be phages were observed. However, once some of the problems had been identified and dealt with, greater success was obtained with sewage samples than with the chicken faeces samples. Hence, only two electron micrographs are presented for the chicken faeces samples unlike the

numerous ones seen in the previous section. When studies were initially conducted using the faecal samples, the plaques obtained were very small (see figure 2.5 from chapter 2) and the diameter could not even be determined. Therefore, the samples were simply filtered and stained or grown in liquid media, purified by centrifugation and filtration and then prepared for microscopy. It was difficult to visualise virions probably due to a low viral concentration. In contrast, sewage samples where lysates were used revealed better results with the images and in some cases several viral particles were seen. It is suggested that concentration methods should be used to increase the viral titer in the sample and more viral particles will be evident.

TEM results showed agreement with the fact that sewage samples are rich in bacteria and the viruses that infect them as reported in literature. A number of phage types were found, most belonging to the families *Siphoviridae* and *Myoviridae.* In the chicken samples, virions that could possibly belong to the *Podoviridae* were observed.

Bradley (1967) emphasised that no morphological type is unique to any particular bacterial genus or species and therefore one might find phages which are similar morphologically but infect different bacteria. However, some slight differences always exist even when phages do look alike. These may include the type of an appendage attached to the base plate of the tail.

Negative staining and TEM can be a challenging technique and the degree of success with results can be inconsistent depending on the phage titre, amount of stain used and timing involved in staining the sample during preparation. Despite all the challenges, it remains a useful technique for presumptive phage classification. It is important to include a wash step using either phosphate buffer solution or ammonium acetate to remove any proteinaceous substances and sugars which may be present in the samples. The possibility of including carbohydrates such as glucose or trehalose to negative stains has been suggested to improve the negative staining technique (Harris & Hornet, 1994). The combination of 1% trehalose and 4 or 5% ammonium molybdate gave better results than uranyl acetate combination which was less stable with the electron beam. It has also been suggested that the

addition of agents, polyethylene glycol (PEG) or octyglucoside (OG) may aid in controlling the amount of negative stain around the structure of interest.

Morphological characterisation is generally regarded as one of the important criteria used to place phages into specific families owing to the fact that clear differences in the size and structure of tailed phages which can be easily established and there is a great deal of information available for comparison purposes. Some of the challenges which are however faced with this criterion are: structures such as collars may be present in one instance and absent in another, related phages may have varying tail dimensions, the majority (about 61%) of tailed phages belong to the *Siphoviridae* family, poor electron microscope skills and lack of proper control in magnification. Internal standards or catalyse crystals may serve to deal with the problem of magnification control (Ackermann *et al.,* 1992).

Ackermann and Nguyen (1983) referred to the use of transmission electron microscope as the “method of choice for phage identification” for phages of enterobacteria which have varied morphological types.

4.4. CONCLUSIONS

TEM was found to be an extremely useful tool in the morphological characterisation of phages. This method may be used for identification purposes in the absence of nucleic acid information although the 2 features are best utilised together for a definite conclusion on identity to be made. Phages exist in abundance in ecological niches colonised by their bacterial hosts. Great diversity of phages is found in sewage samples. *E.* coli is part of the normal intestinal microbiota and therefore, coliphages in particular can usually be isolated from sewage samples, faecal samples of humans and animals or polluted waters.

Majority of the phages observed by transmission electron microscope belong to the order *Caudovirales*. Morphological differences were noted in the phages isolated including presence or absence of tails, tail lengths and other characteristics which allowed definite or tentative (in some cases) placement into families. About 5

different phage types were obtained from sewage while only 2 types were observed for the chicken faecal matter samples. Electron micrographs revealed the presence of members of the *Siphoviridae* and *Myoviridae* families of bacteriophages.

CHAPTER 5

GENERAL DISCUSSIONS AND CONCLUSIONS

Colibacillosis in poultry is a disease of significant economic importance to the poultry industry worldwide. The causative agent of the disease, avian pathogenic *Escherichia coli* (APEC), possesses several virulence determinants which act in combination to bring about varying degrees of pathogenesis in the affected hosts. The disease is usually opportunistic in nature and is thought to be the most widespread among broiler chickens of all ages although a marked increase in severity and susceptibility is common in young birds. Characteristic forms of the infection include air sacculitis, omphalitis, synovitis, pericarditis, peritonitis, salpingitis, and osteomyelitis. APEC is also responsible for cellulitis and colisepticaemia, the most severe form of colibacillosis (Barnes *et al.*, 2003). Economic losses are incurred once infection is established due to increased morbidity, mortality and an increase in condemnations of carcasses at slaughter.

The South African poultry industry alone produced an annual income of R13.5 million in 2007 and approximately 717 million broilers were produced in the same year (USDA GAIN report, 2007). There is a great demand for poultry products and broiler production rates increased from an average of 7.6 million in 1990 to 13.8 million broilers per week. These figures give an indication of the threat that bacterial infections pose to the industry in the event of an outbreak. In 2002, the US poultry industry which has an annual value of more than $50 billion, suffered economic losses amounting to more than $80 million due to *E. coli* infections caused by APEC in broilers (Kish, 2008). Prevention and treatment of colibacillosis has traditionally been in the form of antibiotics such as ampicillin, chloramphenicol, gentamicin and fluroquinolones to mention a few examples (Barnes *et al.,* 2003). The widespread use of antibiotics for the prevention and treatment of bacterial infections over long periods led to the development of antibiotic resistant bacterial strains. In the agricultural sector, antibiotics have commonly been used at sub-minimal levels for growth promotion purposes of animals. The problem of antibiotic resistance was exacerbated by factors such as abuse or excessive antibiotic use (Mellon *et al.,*

2001), incorrect dosage and duration for administration. The increasing crisis and prevalence of antibiotic resistance has prompted broad renewed interest into the possibilities of using bacteriophages for treatment and prophylaxis of bacterial infections. The use of antibiotics such as fluoroquinolones and antibiotic growth promoters (to increase growth and feed efficiency) in the agricultural industry has been banned in the European Union and the United States. In addition, further bans on antibiotic use are still pending in other countries and concerns over the possible transmission of resistant genes to consumers have contributed to the need for alternative forms of treatment. Moreover, there are concerns over the possible transmission of antibiotic resistance genes through the food supply which may render antibiotic therapy in humans ineffective. Apart from the concerns over antibiotic resistance, the broad spectrum activity of antibiotics whereby both the targeted bacteria and the normal microbiota are affected is also a major challenge. There are several reports on phage therapy success in animal studies by a number of researchers in the West. Considerable protection against *E. coli* infections and a decrease in mortality rates were found through the use of lytic phages (Smith & Huggins, 1982; 1983; Huff *et al.,* 2002; 2006). Additionally, data from studies into the control of *Salmonella* and *Campylobacter* infections by phage therapy have been encouraging (Barrow *et al.,* 1998; Goode *et al.,* 2003; Higgins *et al.,* 2005).

The use of lytic bacteriophages for the control of bacterial infections offers attractive advantages compared to the use of antibiotics. These include a high specificity of the phages for the bacterial host. Thus, the normal microbiota population is not destroyed together with the pathogens as is usually the case with broad-spectrum antibiotics. There are also no adverse reactions to treatment with phages. Phages also increase exponentially within the host thereby treatment is effective at the specific site of infection. Furthermore, phage resistance can be dealt with more rapidly because phages can co-evolve with their bacterial hosts and therefore efficacy may be better maintained compared to antimicrobial therapy (Carlton, 1999; Matsuzaki *et al.,* 2005). The high host specificity is also a limitation in cases where disease is caused by different strains of the same bacterial pathogen. Therefore, phage cocktails for treatment of bacterial infections are preferred (Brüssow, 2005).

The purpose of this study was to isolate and subsequently characterise different phages against *E. coli* isolates from diseased chickens. The results obtained would help serve as a precursor to the ultimate objective of using lytic phages for treatment and prophylaxis of colibacillosis in poultry.

Bacteriophages are widely distributed in nature and are found in different types of environments where they can be isolated. Fourteen different bacteriophages were isolated during this project. Nine of these were isolated from sewage sample and five were isolated from chicken faecal matter by the double agar overlay procedure. The lytic spectra of each phage isolate were determined by infecting all of the different *E. coli* strains which were available and differences in host specificity were observed. Isolate E13/2 (see table 2.6) was the most highly virulent among the sewage isolates, lysing 8 of the 11 *E. coli* strains. E13/5, 31-5 and BS4 (see table 2.6) were lytic towards at least four of the bacterial strains. SK2 and SK5 (see table 2.6) exhibited similar lytic patterns and were therefore placed into one group. An interesting characteristic was observed with 31-5 and E13/5 (See table 2.6): both their original bacterial hosts (the strain that they were first isolated on) were resistant to lysis upon re-infection. The culturing of phages and their host bacteria over long periods, either in the laboratory or in nature can result in the selection of phage resistant bacteria (Mizoguchi *et al*., 2003). There are a number of possible explanations for this and these include the development of lysogeny, abortive infections or mutations on the phage or bacteria which prevents recognition of binding sites (Adams, 1959). This is not a unique situation and resistance has been noted in a number of studies such as Kim and co-workers (2008) who found resistance in some *Listeria monocytogenes.*

Phage isolates from chicken faecal matter were highly host specific and had a narrower host range compared to isolates from the sewage sample. Isolates βP2, βP5, βP6 and K3 (see table 2.9 for information on the phages) each lysed a maximum of 2 *E. coli* strains. Isolate CP4 only lysed one bacterial strain. Four of the five isolates did not destroy *E. coli* K12. This is of particular interest because K12 is a laboratory strain which may be used to represent non-pathogenic *E. coli.* In comparison, 5 of the phage isolates from the sewage sample were lytic to the laboratory strain. These results may tentatively indicate that the mentioned phages

would not harm the normal microbiota. Further investigations in this regard would be necessary for conclusive comparisons between known pathogenic and non-pathogenic *E .coli.* The high specificity of some of the phage isolates would necessitate the use of cocktails instead of single phage preparations to increase the target range for disease control. This has been well demonstrated by Tanji and colleagues (2005) when phage cocktails consisting of 3 phages were found to be effective in reducing *E.coli* 0157:H7 in the gastrointestinal tract of mice. The use of phage cocktails is not only for purposes of increasing efficacy of phage treatment but also aids in reducing the development of phage resistant bacteria. Oliveira *et al*. (2009), who also isolated phages against APEC from poultry faeces, determined the lytic spectra of the 5 phage isolates in different cocktail combinations of two, three, four and five phages. A combination of two phages yielded a lytic activity of 60.4%, followed by 70.5% from the cocktail of three phages. Little difference was observed between the combinations of four and five phages, 71.8% and 72.5% respectively. With regards to phage cocktails, Oliveira and fellow researchers (2009) concluded that a combination of three and not five phages was a more suitable option in view of the higher financial demands of characterising and producing five phages compared to only three. From our findings, the best isolates to include in a phage cocktail are E13/2, 31-5, and E13/5 or BS4 (these last two both lyse four *E. coli* strains). The determining factor as to which of the individual phages should be included in a cocktail preparation is the specific causative agent/s of colibacillosis in a given area. Therefore, knowledge of the common strains which cause disease in a particular region is important. Phages isolated in this study can then be tested against those strains. If results obtained are unsatisfactory, other phages would have to be isolated which specifically target those bacteria. The narrow host range and highly specific nature of phages means that phage preparations have to be customised for the different regions, except for cases where the disease is caused by the same strain. Future studies should include a larger collection of bacteria, for example, 100 strains isolated from diseased chickens from different flocks in a particular region/s. This would give an indication of how effective the phages from this study may be on a larger scale.

The high host strain specificity which was observed for some phages may also prove useful for phage typing in the long term. This is a natural method of identification unlike other procedures such as PCR and is based on the ability of the phage to

infect bacteria and cause lysis (Rees & Loessner, 2005). Phage typing has been commonly used to differentiate strains of bacteria such as *Listeria* and *Salmonella* (Loessner & Busse, 1991; Ward *et al.* 1987). Further investigations are necessary to exploit this potential.

The potential use of lytic phages to control *E. coli* infections in poultry and other animals has been well-documented. This includes research by Barrow *et al.* (1998), where a lytic phage was used to control experimental *E. coli* septicaemia and meningitis-like infection in chickens and delayed bacteremia in calves deprived of colostrum. Protection against morbidity and mortality was observed when chickens were experimentally infected with 10^6 cfu of *E. coli* and treated with 10^6 pfu of phage which was administered intramuscularly. Nearly 100% mortality was recorded when phages were not administered. Similarly, Huff and co-workers (2003) observed a decrease in mortality rate from 57% to 13% when 2 bacteriophage isolates were administered as a single intramuscular injection following a challenge with *E. coli* serotype 02. Multiple injections of the phages directly after the *E. coli* challenge, and at 8 and 9 days' old resulted in full recovery of the chickens. Other studies performed by researchers such as Goode *et al*. (2003) on reducing *Salmonella* and *Campylobacter* contamination and Bielke *et al.* (2007) on the reduction of *Salmonella* on broiler carcasses demonstrate that phage therapy is indeed a feasible substitute for antibiotics.

The ability of bacteria to cause disease is due to the presence of virulence genes. Facultative pathogens such as *E. coli, Vibrio cholera, Salmonella enterica* and *Staphylococcus aureus* carry some of these genes on mobile genetic elements which include plasmids, transposons and phages (Boyd, 2005). Once bacteria are infected with phage, the bacteriophage-encoded virulence genes are capable of altering the pathogenic potential of the host in a process called phage lysogenic conversion (Boyd & Brüssow, 2002). This is one of the safety challenges of phage therapy and for this reason, it is important to ensure that the selected phage is strictly lytic and non-transducing. Some lysogenic phages can transfer virulence genes between bacteria and their presence in a phage preparation to be used for treatment can increase the chances of the virulent genes being passed on to new bacterial strains and confer pathogenicity to previously non-pathogenic bacteria.

Another safety concern is the purity of phage preparations. Impure samples may contain endotoxins which can elicit adverse reactions or side-effects (Sulakvelidze & Kutter, 2005) in the chickens. Merabishivili *et al.* (2009) recently purified endotoxins from a bacteriophage cocktail using a commercially available kit called EndoTrap® Blue manufactured by Cambrex BioScience in Belgium. This would be a valuable means of ensuring purity of phage preparations in future studies and thus addressthe issue of adverse reactions which may be attributed to impure samples.

Morphological characterisation of phages was possible by transmission electron microscopy technique. A majority of phages which were observed were tailed and this is in agreement with literature where about 96% of all described phages have tails (Ackermann, 2007) Tail morphology may be long, and non-contractile as seen in Myoviruses or contractile as in Siphoviruses. Lastly, the tail may be short as is characteristic of Podoviruses. The phages observed during this study could be identified as belonging to the *Myoviridae* and *Siphoviridae* families of the order *Caudovirales.* Most of the myophages resembled T4-like phages. An interesting feature which was seen in some of the *Siphoviridae* members was the presence of flexible tails which were curled (see figures 4.1 and 4.2). The electron micrographs were useful in offering information regarding the possible identity of the isolates. For example, SK5 isolate is clearly seen as a T4-like virus from EM photographs (see figure 4.3 in chapter 4) and this was confirmed by molecular methods. Therefore, one can conclude that electron microscopy and molecular techniques work well together for the identification of unknown viruses.

Molecular techniques were established for identification of unknown isolated phages. The major challenge facing phage taxonomy by molecular characterisation is the lack of universal primers which would be useful in rapidly identifying unknown viruses. If one has prior knowledge about the viruses to be identified, specific primers for those phage groups can be selected. For example, MZIA1bis and MZIA6 primers which amplify the g23 of the major capsid protein of T4 and T4-like phages. From the TEM studies, phage SK5 was tentatively identified as a T4 or T4-like phage and this isolate was positively identified as a T4 phage using these primers. T7-like podophages were also identified using degenerate HECTORPol primers which are specific for the DNA polymerases of T7-like podoviruses (Breitbart *et al.,* 2004).

RAPD PCR was employed to infer relatedness to some of the isolates. It was found that SK5 and SK4 were closely related because they gave similar banding patterns. Cloning the different amplified products revealed that these isolates were indeed related in that they both belong to the family *Myoviridae* and they both showed high degrees of homology to T4-like phages, Felix 01 for SK4 and bacteriophage AR1 for SK5. The viral community in chicken faecal matter sample contained podoviruses among other yet to be identified viruses. It is highly likely that there are other phage groups in the faecal matter samples. This could be determined based on selected plaques. The main objective for the molecular section of the project was to establish techniques which could be used for the rapid identification of unknown phages. This was accomplished in that phage isolate 31-5 and E13/5 also gave high homology identities to T-even phages, bacteriophage KEP10 and CEV1. Multiple alignments of nucleotide sequences showed conserved regions for 31-5, SK5 and E13/5. This indicates that they are closely related. Chk showed the least degree of conserved regions to the other phage sequences and this indicated that it is a distant relative of the other isolates. The high level of homology may be as a result of horizontal gene transfer between the phages and their hosts. This may be possible because it has been reported that closely related phages usually infect closely related bacteria (Desplats & Krisch, 2003). The phylogenetic tree constructed also showed that E13/5, SK5 and 31-5 share a more recent ancestor with each other than with SK4 and Chk.

The BLAST results showed homologies to sequences of some phages which infect *E. coli* strains (e.g. bacteriophage CEV1), *Salmonella* (Felix 01) and *Erwinia* (*Erwinia* phage phiEa21-4). The phage isolates may be tested on these pathogens to increase the host range in future studies. *Salmonella* is considered to be one of the major food-borne diseases of humans and is transmitted through the consumption of poultry or eggs (Gast, 1997). Some members of the genus *Erwinia* which include *E. carotovora and E. amylovora* are opportunisitic pathogens which cause soft rot in crops, such as potatoes, and fire blight in apples and pears with significant economic implications (Slater *et al*., 2004). If lytic activity is observed on these bacteria, the phage isolates may have broader application in the food and agricultural industries.

To summarise the results obtained in this study, SK4 and SK5 are the only phage isolates which were characterised fully, that is, by their lytic activity, family based on electron microscopy and closest relative from molecular procedures. They both belong to the family *Myoviridae.* SK4 is a close relative of Felix 01 while SK5 is closely related to bacteriophage AR1. 31-5 and E13/5 are close relatives of bacteriophage KEP10 and bacteriophage CEV, respectively. Morphologically, SK2 was also found to be a member of the *Myoviridae* family while the sewage sample also revealed *Siphoviridae* members with flexible tails. No individual morphological determinations were made for isolates from chicken faecal matter. However, when the purified whole sample was examined by TEM, myoviruses were identified as one of the representatives of the phage community. Lytic activity was determined for all the isolates, with E13/2 (isolated from sewage sample and propagated on *E. coli* strain 1304/99) being the most lytic by destroying 8 out of 11 *E. coli* strains and would therefore be the best choice for therapy. CP4 (isolated from chicken faecal matter) has the least potential for phage therapy as it only lysed 1 *E. coli* strain. SK1, SK3, SK4, βP2, βP5, βP6, and K3 each lysed 2 strains, SK2 and SK5 lysed 3 strains while BS4 and E13/5 lysed 4 strains.

Table 5.1: Summary of phage lytic spectra, morphologies and closest relatives

Phage isolate	Lytic activity	Family (EM)	Closest relative
BS4	4/11	ND	ND
SK1	2/11	ND	ND
SK2	3/11	Myoviridae	ND
SK3	2/11	ND	ND
SK4	2/11	Myoviridae	Enterobacteria Felix 01
SK5	3/11	Myoviridae	Bacteriophage AR1
E13/5	4/11	ND	Bacteriophage CEV
E13/2	8/11	ND	ND
31-5	5/11	ND	Phage KEP10
βP2	2/11	ND	ND
βP5	2/11	ND	ND
βP6	2/11	ND	ND
K3	2/11	ND	ND
CP4	1/11	ND	ND

ND – Not determined; ratios for lytic activity represent the number of *E. coli* strains out of a total of 11 strains lysed by the phage isolates

Phage therapy has great potential not only in treating animal infections but can also be utilised in the human medicine, and plant agriculture sectors to combat infections caused by bacterial pathogens. Moreover, it can be employed for treatment or prophylaxis in the food and water industries to eliminate pathogens such as *Listeria* and *Vibrio cholerae* (Skurnik *et al*., 2007). It is of vital importance that the phages selected for therapeutic purposes be well-characterised before their application. This would include genomic sequencing, understanding the phage biology, and efficacy studies *in vivo* (Merril *et al.,* 2006). An example of a phage that has been characterised in detail is bacteriophage P100, which kills a significant number of *Listeria monocytogenes* strains. Sequencing, bioinformatic analyses, an oral toxicity study in rats, and proof of concept by application on cheese were performed in this investigation (Carlton *et al*., 2005).

In conclusion, the isolated phages have the potential to control *E. coli* infections in poultry as demonstrated from the different lytic spectra. Further characterisation studies are essential before this potential can be fully realised. Methods of the rapid identification of unknown phages are required before they can be used for the treatment of bacterial diseases in poultry. A number of molecular procedures were successfully used to identify some of the viruses. The use of the transmission electron microscope was shown to provide useful information on the morphology of the isolated viruses. It was shown in this work that a combination of TEM and molecular techniques can be used to identify the isolated phages.

Future Research

It is recommended that future research include shotgun cloning method, single step growth curves to study the phage-host interactions, and morphological characterisation of all the isolates. Further investigations should also involve testing the phage isolates on *Salmonella* strains, especially since this is one of the most important pathogens of poultry and results in high mortality rates. It has been noted that phage cocktails are more advantageous than single phage preparations in curbing the possible development of phage resistant bacteria. Therefore, research into the specific titers of each phage to effect optimal results (maximum bacterial death) will be an important factor to consider. Full genome sequencing of each isolate and investigations into whether any toxin-encoding genes or virulence factors are present must be also be perfomed. This is particularly important because these genes can be transferred to the bacterial host. Finally, the efficacy of phage therapy should be evaluated in controlled clinical trials using chickens experimentally infected with avian pathogenic *E. coli.*

SUMMARY

Avian pathogenic *Escherichia coli* (APEC), the causative agent of colibacillosis belong to the family enterobacteriaceae. The disease is manifested as localised or systemic infections which include peritonitis, airsacculitis, omphalitis, swollen head syndrome and colisepticaemia in poultry. Colibacillosis results in increased mortality, condemnations of carcasses at slaughter, reduced feed conversion and production due to morbidity and increased cost of treatment. The standard method for the control of colibacillosis has been the use of antibiotics either as a treatment or as prophylaxis through the addition of antibiotics into the animal feed at sub-minimal levels. The efficacy of antibiotics has drastically reduced over the years due to the emergence and increased prevalence of antibiotic resistance. The use of some antibiotics and antibiotic growth promoters such as fluoroquinolones has been banned and further bans are still impending. Therefore, colibacillosis can potentially cripple the poultry industry nationally and worldwide, if traditional treatment options are ineffective. There is a great demand for potential alternatives to the use of antibiotics and lytic bacteriophages offer attractive advantages over antibiotics in this regard.

This study focused on the isolation and characterisation of lytic bacteriophages with the aim of determining their potential to control *E. coli* infections, in poultry. Nine bacteriophages were successfully isolated from sewage samples and five from chicken faecal matter. The lytic patterns of the phages against *E. coli* K12 and *E. coli* strains isolated from diseased poultry (presumed to be pathogenic) were determined. Among the phages isolated from sewage samples, 31-5, E13/2, E13/5 and BS4 lysed four or more of the *E. coli* strains. The widest host strain range was exhibited by E13/2 which infected 8 of the 11 strains. Thus, this isolate may hold the greatest potential for therapy. In contrast, isolates from chicken faecal matter had a very narrow host strain range, lysing a maximum of two *E. coli* strains including the original host. An interesting observation was made in that the laboratory strain; *E. coli* K12 was resistant to lysis by four out of the five isolates (from chicken faecal matter). This is an important factor to consider in any potential form of treatment where only the pathogenic strains are targeted while the normal microbiota should remain unaffected. The varied lytic patterns observed for most of the isolates further

demonstrate the importance of employing phage cocktails rather than single preparations to treat or prevent colibacillosis. Additionally, some of the isolates may prove useful in phage typing due to their high discriminatory nature in lysis. Although much has been reported on APEC-specific coliphages isolated from sewage, this is one of a few studies where they have been successfully isolated from chicken faecal matter.

Examination of morphological characteristics by transmission electron microscopy revealed the presence of a majority of tailed phages which are members of the *Myoviridae* and *Siphoviridae* families. For some viruses definite conclusions could not be made concerning their identity and they were therefore only tentatively placed into certain families.

Molecular analyses included polymerase chain reaction (PCR) using primers specific for the *g*23 sequence of T4-type phages and the DNA polymerase of T7-like podophages. Although the identity of every phage isolate could not be inferred, the relatedness between isolates SK4 and SK5 was established using the PCR technique of random amplified polymorphic DNA (RAPD). Phylogenetic investigations revealed that 31-5 and SK5 were closely related followed by E13/5 and SK4.

In conclusion, this study demonstrated that some of the isolated phages have the potential to prevent, eliminate or reduce APEC in poultry. Further research is however, necessary to fully characterise the phage isolates and perform efficacy tests in *vivo.*

Keywords: Avian pathogenic *Escherichia coli* (APEC), poultry, colibacillosis, antibiotic resistance, bacteriophages, transmission electron microscopy, PCR, RAPD PCR

OPSOMMING

Voël potogeniese *Escherichia coli* (VPEC), wat die siekte colibacillosis veroorsaak, behoort in die familie Entrobacteriaceae. Die siekte, wat in pluimvee voorkom, manifesteer as ’n lokale of sistematiese infeksie wat peritonitis, airsacculitis, omphilais, geswelde kop sindroom en colisepicaemia insluit. Colibacillosis ly tot verhoogde mortaliteit, afkeuring van karkasse tydens slagting, verlaagde voer omskakeling en produksie as gevolg van morbiditeit en die verhoogde koste van behandeling. Die standaard metode vir die beheer van colibacillosis is die gebruik van antibiotika beide as behandeling of as profilakse deur die toevoeging van sub-minimal vlakke van antibiotika in die voer. Die effektiwiteit van antibiotika het in die afgelope jare drasties afgeneem as gevolg van teenwoordigheid van antibiotika weerstandige bakterieë. Die gebruik van sommige antibiotika en antibiotiese groei stimuleerders soos fluoroquinolones is al verban en nog verdere verbanning van ander antibiotika word verwag. Dus kan colibacillosis die pluimvee industrie kniehalter op buite nasionale as internasionale vlak as die tradisionele behandeling onefektief is. Daar is ’n groot aanvraag na alternatiewe behandelings in die plek van antibiotika en litiese bakteriofae bied ’n baie aantreklike voordeel bo antibiotika.

Hierdie studie fokus op die isolasie en karakterisering van litiese bakteriofae met die potensiaal om *E. coli* infeksies in pluimvee te beheer. Nege bakterifage was suksesvol geïsoleer uit riool en vyf vanaf hoender fekale materiaal. Die litiese patrone van die fage teenoor *E. coli* K12 en *E. coli* geïsoleer vanuit siek pluimvee (aangeneem as patogeniese *E. coli*) was bepaal. Die fage wat uit riool geïsoleer was, 31-5, E12/2, E12/5 en BS4 het vier en meer van die *E. coli* stamme liseer. Die E12/2 het die grootste gasheer reeks gehad wat 8 van die 11 stamme infekteer het. Hierdie isolaat toon die grootste potensiaal vir terapeutiese gebruik. In teenstelling hiermee het die isolate vanaf hoender fekale materiaal ‘n nouer gasheer reeks wat ‘n maksimum van twee *E. coli* stamme, insluitend die oorspronklike gasheer, liseer. ‘n Baie interessante observasie was geneem met die laboratorium stam, *E. coli* K12, wat bestand was teen lise van vier van die vyf isolate (hoender fekale materiaal). Dit is ‘n belangrike faktor wat in ag geneem moet word wanneer gekyk word na behandeling van slegs die patogeniese stamme waar die normale mikro-organisme

moet ongeaffekteer bly. Die verskeidenheid litiese patrone gesien met die meeste van die isolate demonstreer verder die noodsaaklikheid van die gebruik van verskillende fage as behandeling in stede van ‘n enkele faag om colibacillosis te voorkom. Sommige van die fage kan ook gebruik word tydens faag tipering as gevolg van hul wye verskeidenheid in litiese patrone. Alhoewel daar al baie inligting geraporteer was op VPEC-spesifieke colifage geïsoleer uit riool, is hierdie die eerste studie waar fage suksesvol geïsoleer was uit hoender fekale materiaal.

Tydens morfologiese karakterisering met behulp van transmissie elektron mikroskopie, is die teenwoordigheid van fage met sterte meestal gevind wat deel van die *Myoviridae* en die *Siphoviridae* families uitmaak. Vir sommige van die virusse kon daar nie ’n definitiewe gevolgtrek geneem word inverband met hul identiteit en was gevolglik net tentatief geplaas in sekere families.

Die molekulêre analise sluit in die polimirase ketting reaksie (PKR) waar primers spesifiek vir die *g*23 volgorde van die T4-tipe fage en die DNS polimerase van T7-tipe podofage gebruik was. Alhoewel die identiteit van al die fage wat geïsoleer was nie toegeken kon word nie, kon die verwantskap tussen die isolate SK4 en SK5 wel deur die gebruik van PKR tegniek, lukraak vermeerdering polimorfiese DNS (RAPD), bepaal word. Filogenetiese ondersoek het aangedui dat 31-5 en SK5 naby verwant is, gevolg deur E13/5 en SK4.

In opsomming, die studie demonstreer dat sommige van die geïsoleerde fage het die potensiaal om VPEC in pluimvee te voorkom, elimineer en te verminder. Verdere ondersoek is egter nodig om die fage verder te karakteriseer en om effektiwiteit toetse *in vivo* te doen.

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APPENDIX A

Brilliant Green Agar

Peptone	10 g
Yeast Extract	3 g
Lactose	10 g
Saccharose	10 g
Sodium Chloride	5 g
Agar	15 g
Brilliant Green	12.5 mg
Phenol Red	0.08g

The media components were suspended in 1000 ml distilled water, mixed thoroughly and autoclaved at 121°C for 15 minutes. The media was allowed to cool slightly and poured onto agar plates.

Cystine Lactose Electrolyte Deficient (CLED) Agar

Pancreatic Digest of Gelatin	4.0 g
Pancreatic Digest of Casein	4.0 g
Beef Extract	3.0 g
Lactose	10.0 g
L-Cystine	128.0 mg
Bromthymol Blue	0.02 g
Agar	15.0 g

The media components were suspended in 1000 ml distilled water, mixed thoroughly and autoclaved at 121°C for 15 minutes. The media was allowed to cool slightly and poured onto agar plates.

Eosin Methylene Blue (EMB) Agar

Peptone	10g
di-Potassium hydrogen phosphate	2g
Lactose	5g
Sucrose	5g
Methylene blue	0.07g
Eosin yellowish	0.4g
Bacteriological agar	15g

The media components were suspended in 1000 ml distilled water, mixed thoroughly and autoclaved at 121°C for 15 minutes. The media was allowed to cool slightly and poured onto agar plates.

Luria Bertani (LB) Broth

Sodium Chloride	5g
Tryptone	10g
Yeast extract	5
Bacteriological agar	15g

The media components were suspended in 1000 ml distilled water, mixed thoroughly and autoclaved at 121°C for 15 minutes.

MacConkey Agar

Peptone	20g
Lactose	10g
Bile salts	5g
Sodium chloride	5g
Neutral red	0.75g
Bacteriological agar	15g

Distilled water was added to a final volume of 1000 ml. Media was thoroughly mixed and sterilised by autoclaving at 121°C for 15 minutes. The media was allowed to cool slightly and poured onto agar plates.

50 X TAE BUFFER

Tris base	2.42 g
Glacial acetic acid	57.1 ml
EDTA	37.2 g

Components were made up to a volume of 1 000 ml with distilled water and pH was adjusted to 5.8. The buffer was diluted to a 1 X concentration for use as a running buffer for gels.

APPENDIX A contd.

Tryptone Soy Agar (TSA)

Sodium chloride	5g
Glucose	2.5g
Pancreatic digest of casein (tryptone powder)	17g
Pancreatic digest of soybean meal	5g
Bacteriological agar	15g

Media components were dissolved in a volume of 1000 ml distilled water, the pH was adjusted to 7.3 and the media was autoclaved.

Tryptone Soy Broth (TSB)

Sodium chloride	5g
Glucose	2.5g
Pancreatic digest of casein (tryptone powder)	17g
Pancreatic digest of soybean meal	3g
Di-Potassium hydrogen phosphate	2.5g

Media components were dissolved in 1000 ml distilled water, pH was adjusted to 7.3 and broth was autoclaved.

APPENDIX A contd.

Tryptone Soy Molten Agar (0.8%)

Sodium chloride	5g
Glucose	2.5g
Pancreatic digest of casein (tryptone powder)	17g
Pancreatic digest of soybean meal	5g
Bacteriological agar	8g

Distilled water was added to a volume of 1000 ml, pH was adjusted to 7.3 and the media was autoclaved.

Xylose Lactose Deoxycholate (XLD) Agar

Xylose	3.5 g
L-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium chloride	5 g
Yeast extract	3 g
Phenol red	0.08 g
Sodium Deoxycholate	2.5 g
Sodium thiosulfate	6.8 g
Ammonium ferric citrate	0.8g
Bacteriological Agar	15g

Distilled water was added to a volume of 1000 ml, pH was adjusted to 7.4 and the media was autoclaved

APPENDIX B

REAGENT	Supplier
Agarose	Laboratorius Conda
100 bp ladder	Fermentas
1 kb DNA Ladder Plus	Fermentas
6x Orange Loading Dye	Fermentas
Bacteriological agar	Merck
Centrifuge tubes	Sterillin
Chloroform	Merck
Crystal Violet solution	Merck
DNAse I	Roche
dNTPs	Roche
*Eco*RI	Fermentas
EDTA	SaarChem
Ethanol (99.5%)	Merck
GFX™ PCR DNA and Gel Band Purification Kit	Armensham Biosciences
Glucose	Merck
O'GeneRuler™ DNA Ladder Mix	Fermentas
O'GeneRuler™ 1 kb DNA Ladder Plus	Fermentas
PCR reaction buffer 10X concentration	Southern Cross Biotechnology
pGEM® T Easy Vector System I	Promega

QIAamp® DNA Mini Kit	Qiagen
RNAse A	Roche
Saffranin	Merck
Sodium chloride	Merck
SuperTherm DNA Polymerase	Southern Cross Biotechnology (PTY)
Syringes	Promex
Tryptone powder	Merck
Yeast extract powder	Merck
Zyppy ^{TM} Plasmid Miniprep Kit	Zymo Research