Molecular characterisation of *Escherichia coli* O157:H7 specific bacteriophages from cattle faeces

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Thesis submitted in fulfilment of the requirements for the degree *Doctor of Philosophy in Biology* at the North-West University

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Graduation: April 2019

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ABSTRACT

Escherichia coli (*E. coli*) O157:H7 is the main cause of food-borne diseases including haemorrhagic colitis, which can progress to a complication referred to as haemolytic uremic syndrome (HUS) especially in children. The bacteria colonize the gastrointestinal tract of ruminants, particularly cattle, and contamination of carcasses and meat can arise at abattoirs if hygienic procedures are compromised and the bacteria in the intestine or cattle hide is transferred to sterile meat surfaces. The current methods of meat decontamination have various problems and limitations, especially regarding the organoleptic impact and the environmental effect of these methods. A novel, safe and environmentally friendly approach to mitigate *E. coli* O157:H7 involves the use of bacteriophages. The success of such strategy requires an in-depth understanding of the bacteriophages that infect *E. coli* O157:H7. This thesis therefore describes the isolation and detailed characterisation of *E. coli* O157:H7 bacteriophages as a basis to investigate their biocontrol potential.

Prior to the isolation and characterisation of virulent phages, the host bacteria – *E. coli* O157:H7 was isolated from cattle faecal samples obtained from some commercial farms in the North-West province. The isolates were characterised by determinining their virulence profiles and genetic relationships using pulsed-field gel electrophoresis (PFGE). The characterised bacteria were used as host strains for the isolation of phages and phages were further characterised by lytic profile determination, TEM, PFGE analysis, growth characteristics, stability under different physico-chemical conditions and finally by genome sequencing and analysis.

A total of 69 virulent *E. coli* O157:H7 isolates that was obtained in the study were genetically similar by PFGE grouping even though they originated from different farms. Using the characterised *E. coli* O157:H7 as host strains, 15 virulent phages were isolated from cattle faeces that were obtained from different farms. The spot test assay facilitated the identification and

selection of three lytic bacteriophages designated V3, V7 and V8 as potential candidates for the control of *E. coli* O157:H7. Further characterisation indicated that the three phages displayed a high degree of similar characteristics despite isolation from different farms. Transmission Electron microscopy (TEM) of the phages revealed that they all had isometric heads of about 73 – 77 nm in diameter and short tails of about 20-25 nm in diameter. Accordingly, phages V3, V7 & V8 were assigned to the family *Podoviridae* based on their morphology. Pulsed-field gel electrophoresis (PFGE) genome estimation of the 3 phages demonstrated identical genome sizes of ~ 69 nm. The latent periods and the burst sizes of these phages were 20 min, 15 min, and 20 min for V3, V7 and V8 respectively while the burst sizes were 374, 349 and 419 PFU/ infected cell respectively. While all the phages were relatively stable over a wide range of salinity, temperatures and pH values, their range of infectivity was rather narrow on environmental *E. coli* O157:H7 strains isolated from cattle feaces.

However, a novel *Escherichia coli* phage G17 (originally designated V3) that showed promising features was submitted for genomic sequencing. The obtained DNA sequence of phage G17 has allowed for genomic characterisation with bio-informatics analysis identifying the functions of a number of coding sequences. These molecular tools revealed high homology to the newly assigned *G7cvirus* genus making the novel phage G17 a possible member of the genus. Moreover, the genome did not show any homology to genes encoding virulence such as Shiga toxin genes, antibiotic resistance genes and other genes that are involved in lysogeny. Thus, phage phi-G17 possessed favourable characteristics and could be utilized as an alternative in the control of *E. coli* O157:H7.

DECLARATION

I, the undersigned, declare that the thesis hereby submitted to the North-West University-Mafikeng Campus for the degree of PhD in Biology and the work contained therein is my own original work. The thesis has not previously, in its entirety or in part, been submitted to any other institution for an academic qualification. All materials used have been duly acknowledged.

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DEDICATION

This work is dedicated to the memories of my late father and mother, who despite all odds gave me the head start I needed in life by providing and teaching me the importance of education. How gratifying would it have been for you to have witnessed this achievement? Nevertheless, your memories remain in my heart forever – “Sun re oh iba-omo ati Iye-omo rere.”

ACKNOWLEDGEMENTS

Habakkuk 3:19 – “the sovereign Lord is my strength; He makes my feet like the feet of a deer, He enables me to tread on the heights.” Thank you Lord for you provided the strength that sustained me throughout this turbulent period of my life.

I would also like to express my eternal gratitude to my supervisor, Prof CN Ateba for his leadership, continued support, patience and encouragement during my PhD program.

I also wish to thank and acknowledge all the members of staff in the School of Biological Sciences at the North-West University, Mafikeng campus. Both the academics and support staff are appreciated for their contribution towards my research program. I am deeply indebted to Prof HP Drummond for her willingness to edit this thesis despite her busy schedules, her thoroughness and the valuable insights that were provided to make this work of good quality would not be forgotten.

The assistance offered by Mrs T Ateba and Dr L Ngoma from the Agricultural Department of the North-West University especially during the collection of samples is fully appreciated. I would also like to acknowledge Dr S Aremu, Dr B Ojuederie, Dr B Isong, Dr A Kumar and Mr D. Kaptchouang for their kind help and support during this program. In addition, I am eternally grateful to Dr B Aremu and Dr L Chidamba at the University of Pretoria for the guidance and support provided especially in the analysis of the bacteriophage genomes.

My deep appreciation goes to Dr A Jordan from the Laboratory for Electron Microscopy Chemical Resource Beneficiation at the North-West University Potchefstroom Campus for her assistance in providing the transmission electron micrographs of the phages.

My deep gratitude also goes to everyone in all the different research labs in the School of Biological Sciences and most importantly to the members of the molecular microbiology laboratory for their continued support that made my research years memorable. They have all been instrumental in the accomplishment of this degree.

The financial support provided by the North-West University institutional aid and National Research Foundation (NRF UID- 89228) is mostly acknowledged.

Special thanks are extended to all the members of Rhema New Life Church particularly the Motshewane Home-cell members who have been my spiritual cornerstone. Thanks for your prayers, love and unending support throughout this time.

Finally to my family and all my friends who have been there for me all this years. To my late father and mother for the seed you planted in me; the seed of hard work, dedication and commitment. And also to Ma Vero, my husband and children; thanks guys for everything, without which this would have been impossible.

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CHAPTER ONE

INTRODUCTION AND PROBLEM STATEMENT

1.1 Introduction

Although the actual burden of food-borne illnesses is generally difficult to estimate, the frequency of suspected hospitalizations and deaths that result from exposure to food-borne pathogens all over the world is overwhelming (Newell *et al.*, 2010). In the USA for instance, an estimate of over 48 million cases of food-borne illnesses resulting in 128 000 hospitalizations and 3000 deaths are recorded annually (Control & Prevention, 2010; Scallan *et al.*, 2011). Likewise, a high incidence of food-borne diseases has been reported annually in most developing countries where about 2.0 million children die annually due to diarrhoea related illnesses most of which are associated with contaminated food and water (Boschi-Pinto *et al.*, 2008). Moreover, African countries in particular are experiencing a surge of outbreaks of bacterial-linked food-borne diseases including cholera, salmonellosis, campylobacteriosis, EHEC-related food-borne infections and the most recent outbreak of listeriosis in South Africa that resulted in 674 cases of food-borne illness and claimed the lives of 183 individuals was a case in point (Effler *et al.*, 2001; Niehaus *et al.*, 2011; Mengel *et al.*, 2014; WHO, 2018).

In addition to the impact on public health, food-borne infections have profound global economic implications to patients, industries and governments resulting from the considerable resources they expend in the prevention and control of the diseases and associated repercussions (Scharff, 2012; Hessain *et al.*, 2015). While the cost for individual loss of well being to the patient is difficult to estimate, medical costs in the form of hospital bills and medication costs can be catastrophic on patients and their household particularly in developing countries where patients and relatives are responsible for the cost of health care (McIntyre *et al.*, 2006). Similar economic

loss incurred by the food industry is in form of product recall, litigation costs resulting from outbreaks, and hampered consumer confidence resulting in the loss of demand for the products, and this may finally lead to loss of business (Hussain & Dawson, 2013). Further devastating effects on the economy of many countries include for example the cholera outbreak in Tanzania that resulted in 36 million USD in lost revenue and the costs of food-borne illnesses linked to bacteria commonly associated with food animals in the United States are estimated to be over 40 billion USD. Comparable economic losses are reported across many other countries (Scharff, 2010; Raz & Haasnoot, 2011; Scharff, 2012). Thus, food-borne diseases represent a significant public health and economic problem all over the world.

Generally, the majority of food-borne diseases are attributed to pathogenic bacteria with strains belonging to *E. coli* O157:H7, *Salmonella*, *Listeria monocytogenes* and *Shigella* known to be the most relevant groups that have received considerable attention worldwide (Newell *et al.*, 2010; Mahony *et al.*, 2011; Vongkamjan & Wiedmann, 2015). Among these food-borne pathogens, *E. coli* O157:H7 is of particular significance with regard to meat safety and has been kept under close surveillance by the Centre for Disease Control and Prevention due to its low infectious dose and the severity of its associated diseases (Rivas *et al.*, 2010).

Infections caused by *E. coli* O157:H7 range from watery and/or bloody diarrhoea to the more serious haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Rivas *et al.*, 2010; Lee & Park, 2015). These clinical manifestations can result in severe morbidity and mortality in some high-risk individuals, mostly in children less than five years of age, pregnant women, immune-compromised individuals and the elderly (Griffin *et al.*, 1988; Tserenpuntsag *et al.*, 2005). Due to the increasing number of these high-risk populations, notably HIV/AIDS patients in many African countries including

South Africa, *E. coli* O157:H7 remains a relevant food-borne pathogen that presents potential threat mostly to this group of people and to the entire populace (Kharsany & Karim, 2016).

Ruminant animals including cattle have been recognised as important reservoirs of *E. coli* O157:H7 that present an important source of contamination (Rivas *et al.*, 2010; Ferens & Hovde, 2011; Sasaki *et al.*, 2011). The bacterium colonizes the lower intestinal tract of cattle to high levels resulting in its spread among cattle, contamination of hides and the surrounding environment like the troughs, water and animal feed (Katani *et al.*, 2015). Therefore, the contamination of hides and the faecal shedding of *E. coli* O157:H7 by cattle has been linked with the adulteration of carcasses (Soon *et al.*, 2011). The pathogen finds its way to carcasses and derived meat products when standard hygienic procedures and meat manufacturing practices are compromised during the production process (Rivas *et al.*, 2010; Ateba & Mbewe, 2011; Soon *et al.*, 2011). This problem of contamination is aggravated by certain processing procedures such as grinding, needle tenderization, maceration and brine injection that allow the internalization of potential pathogens normally located on the surface of meat. Also contributing is the increasing consumer demand for meat cooked to a medium or very rare degree of doneness as they are rated higher for flavour and tenderness (Cox *et al.*, 1997; Goodson *et al.*, 2002). However, with the internalization of the contaminating pathogen, meat cooked to such aforementioned degree may result in undercooked meat, which can pose a serious health risk to the consumers (Adler *et al.*, 2012; Geornaras *et al.*, 2012). Consumers should therefore be educated about the risk posed by undercooked meat and should ensure that beef and its related products be cooked to well-degree of end point internal temperatures to prevent diseases associated with this formidable pathogen.

Given the zoonotic nature of *E. coli* O157:H7, an approach to control it on the farm prior to slaughter is attractive but previously described pre-harvest control measures are unable to completely eliminate the pathogen from cattle before harvest (Sofos, 2008; Soon *et al.*, 2011).

This can be attributed to the inability of the pathogen to initiate a disease state in its primary host and such asymptomatic carriers can serve as a continuous source of contamination in the farm (Pruimboom-Brees *et al.*, 2000). Moreover, colonised cattle shedding the pathogen do it intermittently with periods of high shedding interspaced with those of low shedding which then exacerbate control as cattle are constantly exposed to the pathogen (Hancock *et al.*, 1997). Even if pathogen-free cattle were raised on farms, the possibilities of cattle and hide contamination during transportation to and lairage at slaughterhouses highlights the continuous need for post-harvest pathogen reduction strategies to combat *E. coli* O157:H7 contamination and prevent human exposure to the pathogen (Arthur *et al.*, 2007; Soon *et al.*, 2011).

One such suggested pathogen-reduction strategy in a variety of food-related environments involves the use of bacteriophages which are viruses that infect specific bacterial cells as a biocontrol agent (Hudson *et al.*, 2005; Hagens & Loessner, 2010; Kazi & Annapure, 2016). Due to their strong antibacterial property, phages offer a promising alternative in the control of bacterial pathogens; hence, they have found various potential applications in biotechnology including the biocontrol of spoilage and pathogenic bacteria on food surfaces (García *et al.*, 2008; Mahony *et al.*, 2011; Sillankorva *et al.*, 2012). Additionally, they can be used as bio-sanitizers where they are applied against bio-films on equipment surfaces to destroy bacteria or as bio-preservatives, and as natural alternatives to antibiotics in animal health (Sillankorva *et al.*, 2012; Kazi & Annapure, 2016). Phages are described as a better substitute for antibiotics particularly with the increasing problem of antibiotic resistance in food animals, and studies evaluating their efficacy in food animals have reported a significant prophylaxis and therapeutic effects (Sheng *et al.*, 2006a). Moreover the application of phages as bio-sanitisers against bacterial pathogens and its associated biofilms on the contact surfaces and equipment used in the food industry has produced comparable encouraging results (Viazis *et al.*, 2011b).

The current interest in phage as a biocontrol tool is because of many appealing properties possessed by these natural antibacterial agents (Sillankorva *et al.*, 2012). Some of these attributes are: i) phages demonstrate selective toxicity by exhibiting specific activity against their host including food-borne pathogens *E. coli* O157:H7 while not disturbing the natural microfloral of the food substance; ii) since they are mostly comprised of nucleic acid and proteins they have low toxicity to humans; iii) Because of their natural occurrence in the environment they are relatively easy to produce and offer a cost effective and sustainable solution to the control of pathogens; iv) they are self replicating and self limiting and therefore do not require repeated dosing since a single dosage will continue to multiply provided the host is available; v) they are stable under various physiochemical conditions and hence can survive harsh conditions associated with food processing; vi) they occur naturally along the food chain and have been isolated from a wide variety of food products including beef (Hsu *et al*., 2002), chicken meat (Shousha *et al.*, 2015), seafood (DePaola *et al.*, 1998) and fermented products (Shin *et al.*, 2011), suggesting that they are regularly consumed by humans. Not only are they consumed, they have been isolated from different parts of the human body, again indicating their close association with humans (Hyman *et al.*, 2012). Thus, the introduction of phages onto food surfaces can be likened to naturally occurring phages on food substances that are unknowingly consumed with food with no adverse effect; consequently, such biocontrol application should not constitute a health hazard towards humans.

Despite these aforementioned desirable characteristics of phages, they possess undesirable features, which can impede their biocontrol applications (Brovko *et al.*, 2012). These attributes include their limited host range, the possibility for the transduction of virulence determinants among different bacterial strains and the risk of the development of phage resistant strains (Bandara *et al.*, 2012; Brovko *et al.*, 2012). While these concerns should not be disregarded, they can be circumvented in various ways, for instance, the problem of limited host range can be

overcome by screening for broad host range or using a mixture of different phage types in phage cocktails so as to increase their spectrum of activity (Hagens & Loessner, 2010). The resistant mutants are not a major problem that can influence treatment efficacy because the resistance mechanism commonly found in bacteria can be controlled by the use of broad host range phages or phage cocktails (Hagens & Loessner, 2010). Moreover the development of resistance ability in some pathogenic bacteria has been associated with loss of virulence (Santander & Robeson, 2007). Lastly, transduction of virulence determinants can be avoided by screening for phages with minimal transduction ability especially at the genomic level. Therefore since the desirable features of phages outweigh the disadvantages, which with meticulous preparation can be avoided, these limitations are not real threats to the biocontrol applications of phages in the food system.

In view of the great biocontrol potential that is inherent in phages, the FDA has approved the application of bacteriophages as natural intervention in foods (Akhtar *et al.*, 2014; Knoll & Mylonakis, 2014). Thus bacteriophage-based biocontrol strategies have a great potential to improve microbiological safety of food due to their long history of safe use, relatively easy handling and their high and specific antimicrobial activity.

1.2 Problem statement

Food producers and food processors are striving to ensure that they provide consumers with high quality and safe food products. In this regard, they have put in place various control measures at different levels along the food chain to establish hygienic and sanitation standards and therefore curtail the problems associated with contaminated food (Newell *et al.*, 2010; Spricigo *et al.*, 2013). In meat production for example, various physical and chemical decontamination systems are utilized throughout the beef production chain to reduce pathogen contamination on cattle

hides, carcasses, meat cuts, beef trimmings and ground beef (Dubal *et al.*, 2004; Koohmaraie *et al.*, 2005; Spricigo *et al.*, 2013).

Even with the current decontamination strategies employed to inactivate bacterial food-borne pathogens, cases of illnesses and even deaths associated with the consumption of *E. coli* O157:H7-contaminated foods are regular occurrences throughout the world (Browning *et al.*, 1990; Effler *et al.*, 2001; Abong'o & Momba, 2009; Viazis & Diez-Gonzalez, 2011). This is owing to the fact that these conventional procedures have shown a limited effect at reducing the presence of *E. coli* O157:H7 on meat and therefore cannot be relied on for the complete removal of the pathogen. Moreover, these decontamination methods have a negative effect on the organoleptic properties of food and hence are not well accepted by consumers (Spricigo *et al.*, 2013).

Therefore, it is suggested that developing other safe strategies that are efficient at eliminating the bacterial pathogens including *E. coli* O157:H7 on carcasses, and which at the same time, do not have a negative effect on the organoleptic properties of meat, are required to reduce the risk of meat contamination. Recently, interest has increased in using lytic bacterial viruses (bacteriophages) as biocontrol agents in foods because they hold enormous opportunities as a safe weapon for fighting infectious agents (Bueno *et al.*, 2012; Spricigo *et al.*, 2013; Zinno *et al.*, 2014). This thesis therefore describes the isolation and detailed characterisation of bacteriophages that are associated with an important food-borne pathogen *E. coli* O157:H7 phages as a basis to investigate their biocontrol potential. The findings of this research will add to knowledge and understanding of bacteriophages and their application in food safety.

1.3 Aims and Objectives

1.3.1 Aim

The aims of this study were to isolate and provide detailed characterisation of bacteriophages that are associated with an important food-borne pathogen *E. coli* O157:H7 phages. This will afford us a basis for future application of the phage isolates in the control of *E. coli* O157:H7 in South Africa and Africa.

1.3.2 Objectives

The specific objectives of the study were to;

- isolate and identify environmental/non-clinical *E. coli* O157:H7 strains
- determine the virulence gene determinants of the isolates
- characterise the isolates at the molecular level using pulsed field gel electrophoresis (PFGE)
- isolate *E. coli* O157:H7 specific bacteriophages from cattle faecal samples
- characterize isolated phages by their morphology, virulence potentials, host range and stability under different physico-chemical conditions
- determine the genome size of the isolated phage and perform PFGE
- determine the sequence and analysis of the genome

CHAPTER TWO

LITERATURE REVIEW

2.1 General characteristics of *Escherichia coli*

Escherichia coli, which was originally called *Bacterium coli commune*" is a member of the Enterobacteriaceae family, together with other enteric Gram-negative bacteria, such as *Shigella* and *Salmonella* (Welch, 2006). Later, the genus *Escherichia* was named after Theodor Escherich - a German paediatrician, who discovered *E. coli* in 1885 (Escherich, 1885; Welch, 2006). Currently, the genus *Escherichia* consists of five members: *E. coli, E. fergusonii, E. vulneris, E hermannii, E. albertii* some of which are known pathogens of humans that are associated with various gastrointestinal and extra-intestinal diseases (Fanning *et al.*, 2015). Among these species, *Escherichia coli* (*E. coli*) is regarded as the most extensively studied and well characterised living organisms that has been used as a model strain in scientific research (Fanning *et al.*, 2015). Research conducted on this strain and its derivatives have been vital in the advancement of the fields of genetics, molecular biology and physiology (Taj *et al.*, 2014).

E. coli is a predominantly Gram-negative, non-spore forming, facultative anaerobic bacterium. *E. coli* cells are typically rod-shaped and their dimensions are in the range of 0.25-1.0 μm wide and 2.0 μm long (Kubitschek, 1990). Strains of the bacterium that possess flagella are motile with peritrichously arranged flagella.

The growth and survival of *E. coli* depends on many environmental factors including pH, water activity and temperature. The organism can survive and grow at pH values of 4 to 10 while optimum growth occurs at near pH 7 and in the presence of 8% sodium chloride concentration

(Desmarchelier & Fegan, 2003). However, studies have indicated that some pathogenic strains are endowed with an intricate acid resistance system that enables them to survive exposure to low pH (Richard & Foster, 2003; De Biase & Lund, 2015). Such ability to withstand acid challenge is relevant in the survival of the organism (De Biase & Lund, 2015). A minimum water activity of 0.95 is required for growth (ICMSF, 1996). Despite their ability to grow over a wide range of temperature, they grow best under mesophilic temperatures with an optimum of 37 to 42 ºC. However, *E. coli* O157:H7 has been shown to survive in ground beef stored at -20 ºC for over 9 months (Doyle & Schoeni, 1984; Fanning *et al.*, 2015).

They are non-fastidious organisms that can live on a wide variety of substrates including complex carbohydrates with the use of the β-glucoronidase enzyme (Fanning *et al.*, 2015). The enzyme facilitates the breakdown of a flourogenic substrate - 4- methyl-umbeliferone glucoronide (MUG) and it forms the basis for detecting B-glucoronidase producing *E. coli* in a fluorescent assay when their MUG activity is determined. However, some pathogenic strains of *E. coli* such as *E. coli* O157:H7 lack B-glucoronidase. Moreover, in contrast to most other *E. coli* strains that can ferment Sorbitol, *E. coli* O157:H7 is unable to ferment this substrate (Riley *et al.*, 1983). These two phenotypic characteristics of *E. coli* O157:H7 i.e. MUG negative and sorbitol-negative are useful in its identification in the laboratory (March & Ratnam, 1986; Thompson *et al.*, 1990).

In addition to carbohydrate metabolism, serotypes of *E. coli* are widely differentiated based on specific markers found on the bacterial cell surface with the use of the modified Kauffman serotyping scheme (Kauffmann, 1947). According to this typing method three main surface antigens comprising of O (somatic or cell surface lipopolysaccharide layer), H (flagellar) and K (capsular) surface antigens are identified as biomarkers in the classification of *E. coli* (Schmidt, 2010). However most laboratories are not capable of typing the K antigen and as such, typing

based on O- and H- antigens are considered the gold standard for *E. coli* typing. With the use of this typing method 182 O-antigens and 53 H-antigens have currently been described among the members of genus *E. coli* and for *E. coli* serotype O157:H7 for instance, the O antigen was the 157th somatic lipopolysaccharide antigen to be discovered while the H antigen was the seventh flagellar antigen identified (Iguchi *et al.*, 2014; Joensen *et al.*, 2015). In most developing countries where access to DNA-based typing tools of bacteria pathogen is limited, serotyping remains the gold standard for the detection of *E. coli* and for characterizing outbreak-related strains because serotypic markers have in most cases been associated with pathogenicity (Dembele *et al.*, 2015).

Although most strains of *E. coli* are not human pathogens, certain serotypes particularly the serotype O157 have acquired virulence genes that enhance their disease causing ability and are therefore responsible for infections in humans (Armstrong *et al.*, 1996; Effler *et al.*, 2001; Kaper *et al.*, 2004). The genomes of the pathogenic *E. coli* have revealed evidence of horizontal gene transfer and plasticity resulting in the exchange of new genetic materials (Lawrence & Ochman, 1998; Perna *et al.*, 2001; Iguchi *et al.*, 2009). One possible means of gene transfer is conjugation because most pathogenic factors contained in different *E. coli* strains are on plasmids which are extra-chromosomal mobile genetic elements (Lawrence & Ochman, 1998). Additionally, bacteriophage-encoded virulence genes and pathogenicity islands encoding virulence factors have been associated with pathogenic strains of *E. coli* and demonstrated to play a key role in their pathogenesis (Novick, 2003; Fortier & Sekulovic, 2013). For instance the genomes of *E. coli* O157:H7 and some other pathogenic bacteria have many integrated bacteriophage genomes encoding virulence determinants notably the Shiga toxins (Perna *et al.*, 2001; Iguchi *et al.*, 2009). Therefore, the combination of different virulence genes within its genome defines the pathogenic potential of a particular *E. coli* strain (Manning *et al.*, 2008).

While previous studies carried out by Escherich associated the pathogenic strains with infant diarrhea and gastroenteritis, subsequent research indentified six main pathotypes of intestinal pathogenic *E. coli* groups that are commonly implicated in various types of gastrointestinal tract infections (Percival & Williams, 2014). These six main pathotypes areclassified as enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), diffusely-adhering *E. coli* (DAEC), and enterohemorrhagic *E. coli* (EHEC) (Nataro & Kaper, 1998; Bacon & Sofos, 2003; Schmidt, 2010). Even though all these classes of pathotypes, with the use of multiple systems of pathogenesis, can cause diarrheal disease in humans, the strains producing Shiga toxins commonly called Shiga-toxin producing *E. coli* (STEC) and specifically the EHEC group, cause the most severe types of infection in humans (Nataro & Kaper, 1998).

EHEC is defined as a subgroup of Shiga-toxin producing *E. coli* (STEC) otherwise known as verotoxin producing *E. coli* (VTEC) since verotoxins and Shiga toxins are different names for identical virulence determinants that cause illness in humans (Gyles, 2007; Karmali *et al.*, 2010). These toxins are named Shiga toxins because of their similarities to Shiga toxins produced by Shigella type 1, and are alternatively named verotoxin due to their cytotoxic activity on cultured Africans green monkey kidney cells (also known as vero cells) (Konowalchuk *et al.*, 1977). The ability to produce these toxins is not only a main determinant of the pathogenicity of EHEC but also an important attribute of this serotype in comparison to other pathogenic *E. coli*. (Gyles, 2007). In addition to shiga toxin production, the EHEC subgroup possesses the ability to induce the attaching and effacing effect in host cells (Tzipori *et al.*, 1986). This characteristic of attaching and effacing is also shared with the EPEC group, but unlike the EPEC, EHEC lack the genes and therefore the ability to produce bundle-forming pilus (Bardiau *et al.*, 2009). The detailed descriptions of these virulence determinants and their mechanism of action will be provided later in the review.

The largest outbreaks of EHEC are caused by a single serotype, *E. coli* O157:H7; although in recent years, other newer serotypes have also been implicated in infections and considered to possess outbreak potential (Johnson & Russo, 2002; Luna-Gierke *et al.*, 2014; Lee *et al.*, 2018). *E. coli* O157:H7 remains the best recognised and the most notorious human pathogen in many parts of the world (Williams *et al.*, 2000; Tarr *et al.*, 2005; Pennington, 2010; Meng *et al.*, 2013; Smith *et al.*, 2014).

2.2 *Escherichia coli* (*E. coli*) O157:H7

In 1982, a novel serotype of *E. coli*, classified as *E. coli* O157:H7, was first identified as a human pathogen during an outbreak enquiry of suspected food-borne illness in Oregon and Michigan, USA (Riley *et al.*, 1983; Wells *et al.*, 1983). Although this pathogen had rarely been documented at that time, it was detected in the faecal samples from the patients (Riley *et al.*, 1983). The patients also developed similar symptoms, including severe abdominal cramps and initial watery diarrhoea followed by bloody diarrhoea. Subsequent investigation into the source of the infection revealed an association between outbreak and eating of undercooked hamburgers at a particular fast-food restaurant. A decade after, the connection of *E. coli* O157:H7 to a large multi state outbreak linked to undercooked ground beef patties sold from a fast-food restaurant chain (Bell *et al.*, 1994b), resulted in *E. coli* O157:H7 being broadly recognised as an important and threatening emerging foodborne pathogen (Besser *et al.*, 1999; Viazis & Diez-Gonzalez, 2011). Since that time, *E. coli* O157:H7 has established itself on farms and food processing facilities worldwide as a serious public health concern (Viazis & Diez-Gonzalez, 2011).

The recent emergence of *E. coli* O157:H7 as a major human pathogen has raised the important question of whether it was a new organism or one that was previously present but unrecognised

or undetected (Armstrong *et al.*, 1996). Although the reports of *E. coli* O157:H7 in major outbreaks in the USA in 1982 has brought the pathogen into the attention of researchers and the public, documentation of its role in human infection was dated back to many years before 1982 (Johnson *et al*., 1996; Chart *et al*., 1991; Riley *et al*., 1987. Moreover, HUS, the major clinical outcome of *E. coli* O157:H7 infection was described in medical literature many years before the emergence of *E. coli* O157:H7, making it a possible cause of the syndrome. However the possibility of other serotype of *E. coli* cannot be ruled out (Gasser *et al.*, 1955).

In an effort to elucidate the origin of *E. coli* O157:H7, Armstrong *et al*.(1996) then suggested three hypotheses for its emergence in humans: i) emergence of the bacteria in animal populations, ii) previous existence of the bacteria in animal populations but changes in slaughtering and meat handling procedures resulted in increased contamination of food products, and iii) previous existence of the *E. coli* O157:H7 in meat supply but changes in the eating habits of consumer leading to increased infection rates. Even with the above explanations, it was still difficult to know when the first human case was reported or to decipher issues relating to the evolution of the pathogen. However, with the advent of genetic tools and an improved understanding of some of the important pathogenic mechanisms, the questions and mysteries around the origin and evolution of this important pathogen are now beginning to be unravelled.

With the use of molecular techniques such as the analysis of DNA sequence or multi-locus enzyme electrophoresis, researchers determined that *E. coli* O157:H7 evolved from enteropathogenic *E. coli* (EPEC) strain of serotype O55:H7, a non-Stx-producing strain but a cause of non-bloody diarrhea (Whittam *et al.*, 1993; Mead & Griffin, 1998; Zhou *et al.*, 2010). This evolution occurred relatively recently through a series of genetic recombination events that resulted in the acquisition of Shiga toxin encoding prophages, a large virulence plasmid (pO157), and additional chromosomal mutation (Reid *et al.*, 2000; Lathem *et al.*, 2003; Zhou *et*

al., 2010). The rate of genetic mutation shows that the current *E. coli* O157:H7 clades recently diverged from its ancestor some 400 years ago, since *E. coli* strains have a lower frequence of recombination when compared to *Vibrio cholera* lineage (Zhou *et al.*, 2002). Therefore, *E. coli* O15:H7 is a constantly evolving organism, continually mutating and acquiring new features, such as virulence factors that make the emergence of more dangerous serotypes or strains a potential threat.

2.2.1 Sources of *E. coli* O157:H7 infection in Humans

In the spread of *E. coli* O157:H7, various routes for human infection have been identified and they include food (Pennington, 2010), recreational and unchlorinated drinking water (Swerdlow *et al.*, 1992; Friedman *et al.*, 1999; Licence *et al.*, 2001; Verma *et al.*, 2007) and secondary person-to-person transmission among infected individuals (Belongia *et al.*, 1993).

2.2.1.1 Contaminated Foods

Due to the zoonotic nature of *E. coli* O157:H7, foods of animal origin are prone to contamination and could therefore serve as major agents in the spread of this pathogen to human consumers (Armstrong *et al.*, 1996; Arthur *et al.*, 2010). Therefore, food products including beef, lamb, chicken and pork are regarded as the principal sources for the transmission of *E. coli* O157:H7, however ground beef remains the predominantly implicated source of the pathogen (Gyles, 2007; Smith *et al.*, 2013). Other food products derived from cattle, including unpasteurised milk and dairy products have also been associated with *E. coli* O157:H7 infections (Goh *et al.*, 2002; McIntyre *et al.*, 2002; Liptakova *et al.*, 2004; Espie *et al.*, 2006).

Dairy products and raw beef may become contaminated during milking or slaughtering processes, when the pathogen from the skin, intestinal tracts and faeces of infected animals contaminates raw milk and animal carcasses (Elder *et al.*, 2000; McEvoy *et al.*, 2003) especially

during hide removal and evisceration (McEvoy *et al.*, 2003). It has been documented that contamination of hides and the faecal shedding of *E. coli* O157:H7 by cattle correlates with the adulteration of carcasses (Soon *et al.*, 2011). Once present in carcass, *E. coli* O157:H7 can become internalized and dispersed throughout the meat when certain processing methods such as needle tenderization, brine injection and grinding are applied. Moreover ground beef often is comprised of meat sourced from many carcasses, thus a few infected animals could contaminate a large quantity of ground beef. Consequently, ground or mechanically tenderised meats are efficient transmission vehicles due to the ease of cross contamination and are considered more dangerous than intact cuts of meat. This explains why most of the earlier reported cases have been linked to minced or ground meat (Belongia *et al.*, 1993; O'Brien *et al.*, 1993; Bell *et al.*, 1994a; Stevenson & Hanson, 1996; Rangel *et al.*, 2005). Therefore, ground or minced meat should be considered a potential risk and cooked to a high enough temperature (70 °C) to inactivate the pathogen prior to consumption.

In addition to foods of animal origin, fresh vegetables such as alfalfa and radish sprouts (Michino *et al.*, 1999), lettuce (Ackers *et al.*, 1998; Slayton *et al.*, 2013) and spinach have not been spared in *E. coli* O157:H7 contamination and have been linked to sporadic and outbreaks of illness in humans (Grant *et al.*, 2008). The bacteria have also survived in unpasteurized fruit juices and apple cider has been implicated as a transmission vehicle (Buchanan *et al.*, 1999; Hilborn *et al.*, 2000). These products may become contaminated when grown in soils that have been enriched by manure from infected animals or contaminated irrigation water (Besser *et al.*, 1993; Cieslak *et al.*, 1993) .

2.2.1.2 Contaminated water

Epidemiological investigations of sources of infection have concentrated on foods and especially those from bovine products, but waterborne transmission of *E. coli* O157:H7 has been reported, both from contaminated drinking-water (Swerdlow *et al.*, 1992) and from recreational waters (Keene *et al.*, 1994; Hilborn *et al.*, 2000). In addition, the contribution of irrigation water in the spread of *E. coli* O157:H7 was also recently reported in Nigeria (Chigor *et al.*, 2010).

2.2.1.3 Direct transmission

E. coli O157:H7 infection can result from contact with infected persons by faecal-oral routes, for example, transmission within families, day-care centres, and health care institutions is well recognised (Spika *et al.*, 1986; Carter *et al.*, 1987; Rangel *et al.*, 2005). In many outbreaks, the infected individual could be traced to consumption of contaminated food or water indicating the importance of preventing or reducing the exposure of food and water to the pathogen. In humans, children are considered as the high-risk group because they have weak immune systems, and are not yet skilled in thorough hygiene practices; and therefore are most likely to both transmit and be infected by person- to-person contact and by animal to human contact. The association between children and infection is reflected in other studies where outbreaks are linked to day-care centers (Belongia *et al.*, 1993), petting zoos and swimming areas (Brewster *et al.*, 1994; Paunio *et al.*, 1999). This association is particularly important because of age-related morbidity and mortality that have been reported with *E. coli* O157:H7 infections in children (Rangel *et al.*, 2005).

Overall, animal reservoirs accounts for the highest level of spread of *E. coli* O157:H7 to humans and food related transmission is widespread, necessitating the need to control the pathogens in food of animal origin, particularly beef. Consequently, *E. coli* O157:H7 poses a serious public health concern that threatens food safety and human health.

2.2.2 *E. coli* O157: H7 occurrence in cattle

The gastrointestinal tracts of ruminants, particularly cattle, have been established around the world as the major reservoirs of *E. coli* O157:H7, which is shed in faeces of healthy animals (Grauke *et al.*, 2002; Blanco *et al.*, 2004; Low *et al.*, 2005). Consequently, the majority of studies investigating the epidemiology of this important pathogen have focussed on the animal and farm-level prevalence. *E. coli* O157:H7 prevalence studies in United State of America beef cattle range from 9% to 28%, while farm prevalence as high as 70% or more has been reported; in fact most, if not all, farms have positive animals at some point in time (Sargeant *et al.*, 2003; Alam & Zurek, 2006; Callaway *et al.*, 2006; Oot *et al.*, 2007). Related studies in Europe have reported similar animal prevalence estimates of 7.5% to 20% (Bonardi *et al.*, 1999; Čížek *et al.*, 1999; Gunn *et al.*, 2007). Other countries including China (Zhou *et al.*, 2002), India (Chattopadhyay *et al.*, 2001) and Japan (Sasaki *et al.*, 2011) have also reported varying prevalence estimates of this pathogen in their animals.

While there is a wealth of information available on the incidence of cattle-borne *E. coli* O157:H7 in developed nations, little is known about their occurrence in Sub- Saharan Africa, particularly in their primary host – cattle. Most studies on the incidence of *E. coli* O157:H7 in sub-Saharan Africa have focussed on food and water-borne *E. coli* O157:H7 while research into animal carriage is scanty (Müller *et al.*, 2001; Abong'o & Momba, 2009; Lupindu, 2018; Osuolale & Okoh, 2018). A few available studies have also highlighted the presence of the pathogen on food animals with results from these studies being comparable to other countries where the rate of incidence varies from place to place. In South Africa, a study conducted by Ateba *et al.* (2008) reported a prevalence range of 5.4% to 20 % on cattle faecal samples while another study in Nigeria indicated animal prevalence of 15.2% (Ojo *et al.*, 2010). A more recent study in

Tanzania on the other hand, reported an extremely low prevalence of 0.9% (Lupindu *et al.*, 2014).

The discrepancies in prevalence estimates in these studies could have arisen from the difference in methods of detection, and also that the bacteria may be harboured extra-intestinally with little correlation to feacal shedding bacteria (Islam *et al.*, 2014). Moreover, the seasonal shedding is another contributor to this variation since it is well documented that the faecal prevalence of *E. coli* O157:H7 in summer months is usually greater than in winter months (Ogden *et al.*, 2004). Given these reasons, comparing the prevalence of this pathogen from one place to another may be difficult and unrealistic, but knowledge of the prevalence in a particular area may assist in the development of control strategies and may also give an indication of the risk to public health.

In cattle, *E. coli* O157:H7 colonise the follicle-associated epithelium (FAE) in the terminal rectum of cattle without clinical manifestations (Naylor *et al.*, 2003; Lim *et al.*, 2007). At this site it produces a potent cytotoxin (Shiga toxin) that has no serious effect on its preferred host (cattle) since they lack toxin receptors (Pruimboom-Brees *et al.*, 2000), however the same toxins are responsible for serious illness in humans infected by *E. coli* O157:H7 (Brien *et al.*, 1993; Karmali *et al.*, 2010). On the other hand, previous research with this pathogen indicates that calves may develop diarrhea that can be associated with AE lesions (Mainil *et al.*, 1987; Dean-Nystrom *et al.*, 1997). Nonetheless, unlike in humans, such infections in calves do not extend beyond the intestinal region, and there is no development of systemic manifestation of the disease (Cray & Moon, 1995; Kolenda *et al.*, 2015). Other studies have shown that weaned calves can be clinically healthy but colonised with *E. coli* O157:H7 (Cray & Moon, 1995). Thus *E. coli* O157:H7 has coevolved with its host, and it is well suited to survive in the intestinal tract as a commensal organism (Whittam *et al.*, 1993; Law, 2000).

The absence of clinical signs and symptoms in animal reservoirs is a crucial factor enhancing the risk of meat contamination. Since these animals have no visible signs of infection, they are regularly slaughtered; and their meat may become contaminated at the slaughterhouse due to spillage from the intestine or because of contact between carcass surface and hide. Such animals may thus become the gateway through which the microorganism enters the food chain. Therefore, until proven otherwise, every animal that is offered to be slaughtered for human consumption presents a potential risk of transmitting food borne pathogens notably *E. coli* O157:H7 if efficient control procedures are not applied.

2.2.3 Pathogenicity/Virulence factors of *E. coli* O157:H7

E. coli O157:H7 possesses diverse mechanisms involved in pathogenicity and interaction with its host on the cellular and genetic levels, which as previously indicated are encoded by chromosomal pathogenicity islands, phage chromosomes integrated in the bacterial and in plasmids (Law, 2000). However, these processes and their mechanism remain unclear probably due to the lack of animal models that produce similar symptoms and disease outcome as in humans (Melton-Celsa & O'Brien, 2003). The major virulence determinants that areconsidered necessary for the pathogenicity of *E. coli* O157:H7 include toxin production, plasmid O157 and Locus of enterocyte effacement (LEE) (Schmidt, 2010; Kawano *et al.*, 2012; Smith *et al.*, 2014).

2.2.3.1 Shiga toxins

The unique and distinguishing feature of *E. coli* O157: H7 in comparison to other pathogenic *E. coli* is its ability to produce one or more Shiga toxins, which is one of the most potent toxins known to man (Law, 2000; Pennington, 2010). Although there are two major types of the toxin designated Stx1 and Stx2 that have received considerable attention, other variants have been described and implicated in disease conditions (Persson *et al.*, 2007). Stx1 was first identified from *Shigella dysentery* serotype 1 and afterwards found in a group of *E. coli* strains (O'Brien *et*

al., 1983; Trofa *et al.*, 1999). Although virulent strains of *E. coli* O157:H7 may express Stx1, Stx2 or both toxins, epidemiological evidence suggests that EHEC isolates producing Stx2 are more commonly associated with serious disease compared to isolates producing only Stx1 (Ostroff *et al.*, 1989; Law, 2000; Persson *et al.*, 2007; Manning *et al.*, 2008). The greater toxicity of Stx2 was also supported by animal studies that described an association between the administration of Stx2 alone and the development of the symptoms of HUS (Tesh *et al.*, 1993; Siegler *et al.*, 2003). This lead to the conclusion that Stx2 is more toxic and more often associated with the principal manifestation of HC and HUS in human infections than Stx1 (Ostroff *et al.*, 1989; Boerlin *et al.*, 1999). Although the two toxins have been described to be immunologically distinct, they have similar modes of action. Moreover, they are about 70% similar at the amino acid level and belong to the same toxin family (Jackson *et al.*, 1987).

Structurally, the Shiga toxins (Stx) are A-B toxins that inhibit protein synthesis (O'brien *et al.*, 1992). The A subunit acts as a ribosomal RNA N-glycosidase, whereby it destroys the ribosomal RNA (28s rRNA) and inhibits protein synthesis, and further induces apoptosis in the host cell, whereas the pentamer of B subunit binds to a glycolipid receptor in endothelial cellular membranes called globotriaosylceramide (Gb3) (Keusch *et al.*, 1986; O'brien *et al.*, 1992). The binding of the B subunit to the Gb3 receptors plays a crucial role in the entry of the A subunit into specific cells. Since the Gb3 receptors are mainly located in a wide range of endothelial cells, especially the kidney cells, they become the primary target of Shiga toxins with a resultant damage to the renal glomerular endothelial cells (Ray & Liu, 2001). This damage manifests as hemorrhagic colits in the human colon and as HUS in kidney cells (Kaper, 2005).

2.2.3.2 Attaching and Effacing

The locus of enterocyte effacement (LEE) Pathogenicity Island is another major factor implicated in the pathogenesis of *E. coli* O157:H7. The LEE is a chromosomal region that is present in many EHEC strains including *E. coli* O157:H7 and is responsible for attaching and effacing lesions. This region encodes a whole cluster of virulence factors including a type III secretion system, the adhesin called intimin (*eaeA*), the intimin receptor TIr and other effectors which are responsible for attaching and effacing (A/E) lesions that are characteristic of *E. coli* O157:H7 infections (Nataro & Kaper, 1998; Deng *et al.*, 2004; Tree *et al.*, 2009). The attachment and interaction of *E. coli* O157:H7 to the gut mucosa produces some histopathological changes in the epithelium, otherwise referred to as A/E lesions. These lesions are characterised by effacement of the epithelial brush border microvilli and the formation of actin-rich pedestals within the host cell underneath the attached bacterial cells. The suggested functions of this pedestal are prevention of dislodgement of the bacterium during the host diarrheal response and inhibition of bacterial phagocytosis (Perna *et al.*, 1998).

2.2.3.3 Plasmid pO157

Unlike the Stx and LEE that are chromosomally located plasmid pO157 of *E. coli* O157:H7 is not chromosomally located and range in size from 90 – 104 kb (Burland *et al.*, 1998). Proteins presumed to be involved in the pathogenesis of *E. coli* O157:H7 contained in the plasmid include hemolysin (ehxA) (Schmidt *et al.*, 1994), a catalase-peroxidase (katP) (Brunder *et al.*, 1996), a type ii secretion system T2SS (etp) (Schmidt *et al.*, 1997), a zinc metalloprotease (stcE) (Lathem *et al.*, 2002), a serine protease (espP) (Brunder *et al.*, 1997) and an eae conserved fragment (ecf) (Yoon *et al.*, 2005). While the role of pO157 in pathogenesis is not fully understood, some studies have suggested adherence to epithelial cells as a possible function (Sheng *et al.*, 2006b; Dziva *et al.*, 2007). However, evidence suggests that *E. coli* O157:H7 strains that possess an the plasmid or express high levels of plasmid-encoded products are more

associated with outbreaks and HUS than strains without it (Abu-Ali *et al.*, 2010; Karmali *et al.*, 2010).

In addition to the above pathogenic factors, *E. coli* produces a myriad of other putative virulence mechanisms including numerous adhesions, toxins beside Stx1 & Stx2, proteins that aid in the attachment and colonization of the bacteria in the intestinal wall as well as those that can lyse red blood cells and liberate iron to help support *E. coli* metabolism (Law, 2000). All these other factors may play a role in colonization or other steps in pathogenic process because the pathogenesis of the pathogen is not dependant on a sinlge gene but rather a multifactorial process requiring a wide repertoire of virulence mechanisms (Law, 2000).

2.2.4 Diseases associated with *E. coli* O157:H7 infection

The clinical manifestations associated with of *E. coli* O157:H7 infection are broad and vary from asymptomatic cases to non-bloody/watery diarrhea, hemorrhagic colitis (HC), haemolytic uremic syndrome (HUS), thrombocytopenia purpura (inadequate platelet count) and death (Griffin *et al.*, 1988; Armstrong *et al.*, 1996).

Hemorrhagic colitis is the principal disease associated with *E. coli* O157:H7 and is characterised by bloody diarrhea and abdominal cramps (Griffin *et al.*, 1988). This infection can progress systemically and cause complications such as Hemolytic Uremic syndrome (HUS) and Thrombotic Thrombocytopenic Purpura (TTP) (Riley *et al.*, 1983). Hemolytic uremic syndrome is characterised by capillary haemolysis, acute renal failure, and thrombocytopenia (Moake, 1994). Although HUS was first described by Konowalchuk *et al.* (1977), its association with human disease was only reported in 1982 and 1983 (Karmali *et al.*, 1983; Riley *et al.*, 1983). In HUS as previously indicated, shiga-like toxins can enter the blood stream where they are transported to target organs, most often kidneys, and can destroy these organs (Ray & Liu,

2001). The cells from these target organs are rich in Glycolipid Gb3 (globotriaosylceramide), the predominant membrane receptor for the Shiga toxins, which is likely to account for the kidney damage in HUS (Ray & Liu, 2001). About 6% of individuals infected with O157 develop HUS, and O157 is described as the aetiological agent of more than 70% of the HUS cases in North America (Siegler, 1995). Children are considered to have a higher risk of developing HUS since they express higher levels of Gb3 and *E. coli* O157:H7 have been suggested to be the most common causes of renal damage among this group (Lingwood, 1994).

Among the adult population however, *E. coli* O157:H7 infection more frequently progresses to Thrombotic Thrombocytopenic Purpura (TTP) which is characterised by capillary haemolytic anaemia, fever, renal failure, thrombocytopenia, and neurological abnormalities (Griffin *et al.*, 1988; Mead & Griffin, 1998). In comparison to HUS, TTP has been associated with more prominent neurological disorders, less marked renal damage and fewer cases of diarrhea episodes (Griffin *et al.*, 1988).

E. coli O157:H7 sporadic infection and outbreaks have been documented around the globe; however, it has been reported with a higher frequency in some parts of the world, and described as an emerging pathogen of the developed countries (Armstrong *et al.*, 1996; Besser *et al.*, 1999). In fact, as previously indicated, the first reported case of the disease was in the United State of America during an outbreak of severe diarrhea that was linked to the consumption of contaminated hamburgers in a fast food chain. Since then many other infections and outbreaks due to contaminated fresh pressed apple cider (Besser *et al.*, 1993), hamburgers (Bell *et al.*, 1994b), dry cured salami (Control & Prevention, 1995), leaf lettuce (Ackers *et al.*, 1998) and spinach (Grant *et al.*, 2008) have been reported in the USA. Other parts of the world affected by the pathogen include Canada (Cody *et al.*, 1999; McIntyre *et al.*, 2002; Gaulin *et al.*, 2015), the United Kingdom(Stevenson & Hanson, 1996; Hilborn *et al.*, 2000; Licence *et al.*, 2001; Goh *et*

al., 2002); France (King *et al.*, 2009); Slovakia (Liptakova *et al.*, 2004) and Japan (Michino *et al.*, 1999). Generally, food-borne diseases are commonly associated with developing nations due to low socio-economic status, however *E. coli* O157:H7 has defied this norm by being reported in developed countries with good standard of living and efficient standard measures on food safety making the pathogen a global threat to the production of safe food.

Similar to the developed countries, cases of *E. coli* O157:H7 outbreaks have been reported in sub-Sahara Africa with wide geographic distribution but at surprisingly low prevalence (Cunin *et al.*, 1999; Effler *et al.*, 2001). This can result from underestimation of disease outbreak that commonly plague African countries due to lack of efficient surveillance systems and also in some instances underreporting of disease when there is a mild case of infection, or small outbreaks which are not reported to public health officials (Abong'o & Momba, 2009). In 1992, the first documented case of *E. coli* O157:H7 outbreaks occurred in Swaziland where surface water contaminated with animal faeces was implicated (Effler *et al.*, 2001). Following this another outbreak associated with locally made meat pies was identified in Central Africa Republic (Germani *et al.*, 1997). In Cameroon, over 275 cases and 45 deaths were caused by the O157 serogroup (Cunin *et al.*, 1999) infection and, while the source of the outbreak was unknown, *E. coli* O157:H7 contaminated water and food were suggested as the possible cause of infection (Cunin *et al.*, 1999). In South Africa, *E. coli* O157:H7 made up 7.7% of the isolated strains from the stool of young children presented with diarrheal infection in the period of 1996-1997 (Galane & Le Roux, 2001). The pathogen has also been recovered from animal products, water and the environment in this area (Ateba *et al.*, 2008).

Other parts of Africa have not been excluded from the menace of *E. coli* O157 infections and outbreaks. Thus *E. coli* O157-related diarrhea has been reported in Democratic Republic of Congo (DRC) (Koyange *et al.*, 2004); Kenya (Sang *et al.*, 1996); Cote d'Ivoire (Dadié *et al.*, 2000) and Nigeria (Olorunshola *et al.*, 2000).

2.2.5 Approaches to reduce *E. coli* O157:H7 on Carcasses

In slaughterhouses, it is impossible to prevent the contamination of carcasses with intestinal bacteria and pathogens. This can serve as an important avenue for the introduction of important pathogenic bacteria into the food chain, therefore decontamination steps such as washing and sanitizing during the process of slaughtering and carcass handling is vital to minimise the exposure of humans to food-borne pathogens (Dorsa *et al.*, 1996; Sofos & Smith, 1998; Huffman, 2002; Loretz *et al.*, 2011). Aside from the control of pathogenic bacteria, an additional benefit in the form of inhibition of contaminating food spoilage organisms, with a resultant increased product shelf life, emphasises the need to make these decontamination steps an essential part of the slaughtering procedure (Dickson & Anderson, 1992; Huffman, 2002). This however should not replace good manufacturing, sanitation and hygienic practices which are endorsed by regulatory agencies and the beef processing industry, as they lay the foundation for preventing contamination of beef products throughout the harvest process (Huffman, 2002).

While any selected procedure should reduce or eliminate pathogenic organisms, it should not compromise the quality of the food product. Thus, the choice ofa suitable control method should be informed by certain factors such as the effect of the method on the organoleptic or nutritional value of the food. Moreover, the control method of choice should be safe, affordable and acceptable to the consumers (Corry *et al.*, 1995). Although, many decontamination strategies have been described but the widely researched ones could be generally divided into three groups: physical (hot water, steam pasteurisation, steam vacuuming), chemical (organic acids, chlorine,

and acidified sodium treatment) and biological treatment (Smulders & Greer, 1998; Sofos & Smith, 1998; Huffman, 2002; Loretz *et al.*, 2011).

2.2.5.1 Physical treatment

2.2.5.1.1 Hot water wash

Hot water treatment is one of the most common interventions applied to foods to inactivate food-borne pathogens including *E. coli* O157:H7 (Sofos *et al.*, 1999). This may be attributed to the antimicrobial action of heat in as much as irreversible inactivation or destruction is a possible outcome following bacteria exposure. To be effective water temperatures should exceed 74 °C, and effectiveness increases as temperatures reach 80-85 °C (Sofos & Smith, 1998; Smith, 2009). Using a range of temperatures and pressures, many studies have documented the beneficial effect of this technology in reducing bacterial populations on beef carcass tissues at both laboratory scale as well as under commercial conditions (Davey & Smith, 1989; Gorman *et al.*, 1995; Dorsa *et al.*, 1997). While the treatment of carcasses with hot water spray has offered a means to reduce bacterial contamination, they do not completely eliminate the pathogen. In fact, there is the possibility of recontamination and spread of bacterial contaminants to adjacent tissue surfaces and equipments (Sofos & Smith, 1998). Furthermore, the treatment of carcasses with moist heat offers no long term advantages because re-growth of *E. coli* O157:H7 can occur during storage if meat surfaces become decontaminated (Dorsa *et al.*, 1998). Heat damage on carcass surfaces due to this treatment has also been reported (Pipek *et al.*, 2005).

2.2.5.1.2 Steam pasteurization

Steam pasteurisation is an alternative to hot water treatment when carcasses are exposed to pressurised steam for a specified period with concomitant reduction in bacterial count (Belk, 2001). In contrast to hot water washing where the temperature is below 80 °C, pressurised steam at close to 100 °C is applied to the carcass surface for 10-15s until it is raised to about 90 °C

(Golan *et al*., 2004). Steam at 100 °C has been shown to have a greater heat capacity than water at the same temperature and therefore more effectively penetrates crevices on carcasses (Dorsa *et al*., 1996). However the effectiveness of this technology can be impaired at certain sites of the carcass such as the neck where there is a possibility of uneven heat distribution as compared to other parts (Nutsch *et al.*, 1998). Other concerns that have been raised are tendency for workers to reduce application time or temperature so as to minimise carcass discolouration and such reduction can consequently compromise the effectiveness of the method (Gill *et al.*, 1999).Moreover, the adverse effect on organoleptic properties such as the colour and texture of steam-pasteurised carcasess is another limitation of this method (Bolton *et al.*, 2001; Pipek *et al.*, 2005). To overcome these some authors have suggested that steam pasteurised carcasses should be rapidly cooled to prevent muscle heating and protein denaturation (Phebus *et al.*, 1996).

2.2.5.1.3 Irradiation

This is a non-thermal physical method of decontamination involving the exposure of food substances to ionizing radiations such as gamma rays, X-rays, or high-energy electrons. At doses between 1 – 10 kGy, radiation has been shown to significantly reduce spoilage and foodborne pathogens with no adverse effect on meat (Arthur *et al.*, 2005). Despite the effectiveness of this technology, problems such as consumer rejection and the cost of infrastructure remain as impediments to its widespread applications in the food industry (Wheeler *et al.*, 2014).

Other techniques including steam vacuuming and knife trimming are usually used on meat carcasses as a spot decontamination process and are applied to remove visible contamination from carcasses especially in areas of heavy contamination (Bolton *et al.*, 2001; Huffman, 2002). The two methods have produced comparable positive results in reducing visible contamination as well as bacterial loads on localized areas of beef carcasses (Dorsa *et al.*, 1996; Kochevar *et al.*, 1997). However, concerns have been raised about theantibacterial effect on non-visible

contaminations, and contaminants including bacteria that are not confined to areas of visible contamination may be missed during treatment. Furthermore, these techniques are not designed for decontaminating the entire carcass (Bolton *et al.*, 2001).

2.2.5.2 Chemical decontamination treatments

A wide variety of chemical compounds is generally used for the decontamination of carcasses. For decontamination of beef and poultry carcasses, the range of chemical compounds commonly used include organic acids, chlorine-based sanitizers, phosphate-based sanitizers and various other substances (Sofos *et al.*, 1999; Loretz *et al.*, 2011).

2.2.5.2.1 Organic acids

Organic acids such as acetic, citric and lactic acid at 1 – 3% are the most commonly used chemical agents for meat carcass decontamination due to their availability, cost effectiveness and high efficacy (Belk, 2001; Mataragas *et al.*, 2008; Loretz *et al.*, 2011). Lactic acid has been granted a generally recognised as safe (GRAS) status and widely applied in rinses or sprays in commercial practices to decontaminate carcasses due to its efficacy (Sofos *et al.*, 1999). Reports obtained from a number of studies evaluating the efficacy of a variety of acids as decontaminating agents for carcasses are surprisingly divergent from one another (Dickson & Anderson, 1992; Siragusa, 1995; Castillo *et al.*, 2001). While some researchers have documented a reduction in bacterial contaminants following treatment and demonstrated that the method is more effective than other methods such as trimming or washing alone; others have refuted the claim and indicate the lack of significant evidence for its antimicrobial activity (Cutter & Siragusa, 1994; Bosilevac *et al.*, 2006). Of particular importance is the inherent ability of some bacteria contaminants notably *E. coli* O157:H7 to withstand acid stress, rendering the treatment inefficient (De Biase & Lund, 2015). Other factors affecting the efficacy of the method include the type of acid used, pH of the acid, duration of treatment and the type of meat treated, since the

bactericidal effect is more pronounced on fat surfaces, and reduced efficacy has been reported in contact with organic matter (Greer & Dilts, 1995; Bolder, 1997)

2.2.5.2.2 Chlorine

This is one of the most traditional chemical decontaminants that have been used in the beef industry, and the evaluation of its antimicrobial activity at concentrations ranging from 200-800 part per million (ppm) shows substantial reduction of microbial pathogens (Sofos & Smith, 1998). However, application of such high amount of chlorine is prohibited in the food industry, and application concentration is currently within the range of 20-50 ppm in countries such as Canada and the USA where the use is approved (Hugas & Tsigarida, 2008). Despite the reported effectiveness when high concentration is used, reducing the concentrations of chlorine to these levels results in a concomitant decrease in its efficacy (Bolder, 1997).

Similar to organic acids, chlorine is generally counteracted by organic matter and since the surfaces of hide and carcasses are mostly associated with organic material, this hampers its effectiveness (Hugas & Tsigarida, 2008). Moreover, chlorine gas and chlorine by-products – trihalomethanes (THM) are considered toxic and carcinogenic, and are therefore unsafe for human exposure (Sofos & Smith, 1998; Mishra *et al.*, 2014).

Other chemicals used for meat decontamination include tri-sodium phosphate (TSP) (Dickson *et al.*, 1994; Sofos & Smith, 1998), peroxyacetic acid and acidified sodium chlorite (Ransom *et al.*, 2003), and acidic calcium sulphate (Zhao *et al.*, 2004).

2.2.5.3 Biological treatments or Biocontrol

Biological treatments are currently being proposed and used as a novel technique for food preservation and decontamination (Hagens & Loessner, 2010). Examples of these treatments include the use of bacteriophages, which will be detailed in the next section, and other biological agents such as probiotics, bacteriocins and plant-derived essential oils. The application of bacteriocin producing bacteria, *Lactobacillus* spp, to ground beef has been shown to be effective against *E. coli* O157:H7 and *Salmonella* spp and was considered an important intervention for controlling food-borne pathogens (Smith *et al.*, 2005). In addition, a preliminary study evaluating the antimicrobial activity of a plant derived extract, Carvacrol against *E. coli* O157:H7 has reported a biocontrol potential on bovine hide and carcass cut (McDonnell *et al.*, 2012). A number of other plant essential oils (EOs) have been shown to possess antimicrobial activity against many food-borne pathogens and have the potential as natural substitutes for the currently used chemical treatments (Friedman *et al.*, 2002). Moreover, since these biocontrol agents are considered non-toxic and environmentally friendly, and in some cases exhibit highly specific activity against the bacteria of interest; they offer a preferred choice for pathogen control and food preservation.

2.3 Bacteriophages

2.3.1 Discovery of Bacteriophages/History of Bacteriophages

Bacteriophages, otherwise known as phages, are viral parasites of bacteria which are ubiquitous like the bacteria they infect, and were first described in 1896 by Ernest Hankin, a British bacteriologist (Hankin, 1896). In his research, He observed the antibacterial effect of the Ganges and Jumna rivers against some bacteria, particularly *Vibrio cholerae*, in spite of filtering the water through porcelain filters that bacteria could not pass through. He concluded that the filterable and heat labile substance might be responsible for the reduced cases of gastro intestinal

infections especially cholera in the villages near the river (Hankin, 1896; Sulakvelidze *et al.*, 2001).

Following the work of Hankin's report, a British bacteriologist, Frederick William Twort and a Canadian medical bacteriologist, Felix d'Herelle independently discovered that phages are viruses (Twort, 1915; d'Herelle, 1917). Consistent with Hankin's report, Twort discovered while working with some bacterial colonies, an agent that was filterable with antimicrobial activity against other bacteria strains. However he was uncertain of the identity of the agent of bacterial death and speculated that it was either a living protoplasm or an enzyme with the ability to infect micrococcus cells (Twort, 1915).

Shortly after Twort's discovery in 1917, d'Herelle independently made similar observation; however, he proposed that a virus that could pass through bacterial filters, with bactericidal effect, was responsible for the occurrence (d'Herelle, 1917). Afterwards, he described them as "ultraviruses" that are obligate parasites of living bacteria and named them "bacteriophages" or "phages" for short (Sulakvelidze *et al.*, 2001). He devised several techniques that are still used in the study of bacteriophages and recognised their potential in the treatment of infectious diseases. d'Herelle's suggestion of bacterial virus was much debated and not until the advent of the electron microscope in 1940 was the viral nature of bacteriophages recognised (d'Herelle, 1949). The findings of these great scientists have laid the foundation for subsequent bacteriophage research (Duckwoth, 1976).

2.3.2 General characteristics of Bacteriophages

Much like their bacterial hosts, bacteriophages have been shown to occur universally in the environment. However, with an estimated range of 10^{30} to 10^{32} in existence, they surpass the global bacterial population and have been described as the most numerous biological entity on earth (Hendrix *et al.*, 1999; Kutter & Sulakvelidze, 2004). They are usually in close association with their host bacteria and have been isolated from a variety of habitats including aquatic environment, soil, oceans, sewage and even in the gastrointestinal tracts of animals (Danovaro & Serresi, 2000; Kutter & Sulakvelidze, 2004; Hudson *et al.*, 2005; Niu *et al.*, 2009; Owens *et al.*, 2013).

Phage sizes range between 20 and 200 nm. Generally, each phage particle is made up of its nucleic acid genome enclosed in a protein coat called a capsid (Guttman *et al.*, 2005). Although the genomes of the majority of phages are mostly double-stranded DNA (dsDNA), a few other groups possessed single-stranded DNA, single-stranded RNA or double-stranded RNA genomes (Ackermann, 2003).

Phages, similar to other viruses, are absolute parasites with the ability to replicate only in bacterial cells (Monk *et al.*, 2010). Despite the fact that they contain the entire information needed for their reproduction, phages depend on the host cellular machinery for generating energy and producing proteins (Monk *et al.*, 2010). They display a high level of host specificity since they infect specific groups of bacteria in a particular species or strain, however some can infect related bacterial species (Monk *et al.*, 2010). This specificity has been attributed to the specific interaction between the tails of phages and specific receptors such as teichoic acids and lipopolysaccharides displayed on the surface of the target bacteria (Guttman *et al.*, 2005).

Phages are highly heterogeneous in structural, physiological and biochemical properties (Kutter & Sulakvelidze, 2004). Based on their morphology, phages can be categorised into tailed, polyhedral, filamentous and pleomorphic phages. Phages vary widely in their response/sensitivity to various environmental conditions. While phages are generally considered to be stable at pH range of 5 to 8, some can survive exposure to lower pH of up to 3 (Litt & Jaroni, 2017). Thus, every newly isolated phage needs to be specifically characterised for its acid sensitivity. Similarly, temperature stability of phages varies although some phages have been isolated in extreme environments such as hot springs (Liu *et al.*, 2009).

Due to their close interaction with their host, bacteriophages play a vital role in regulation of the microbial balance in the ecosystems where they have a significant impact on bacterial communities and on the level of nutrient flow within the ecosystem (Weinbauer & Rassoulzadegan, 2004; Abedon, 2008). This occurs when they parasitize on competing bacteria allowing the lysogens to dominate and hence regulate microbial balance; they also exert a profund influence on the biogeochemical cycling of nutrients when the host cell is lysed and incorporated organic particles in them are released as dissolved organic matter especially in the oceans (Fuhrman, 1999; Suttle, 2007).

They are also a dominant player in regulating the diversity and evolution of bacterial communities, especially when the bacterial hosts are infected by temperate phage that can integrate and interact with their host genome (Hendrix, 2005; Pride *et al.*, 2006). These phages are able to facilitate horizontal gene transfer and enable bacterial genetic variability through the process of transduction when phages move genetic materials from one bacterium to another (Ochman *et al.*, 2000; Wick *et al.*, 2005). The process has been described in many different bacterial species and involves the exchange of virulence factors including antibiotic resistance genes (Colomer-Lluch *et al.*, 2011). In addition to transduction, new genetic materials can be

provided from the infecting temperate phage genomes which then confer a selective advantage to their host when expressed and result in the emergence of pathogenic bacteria in a process called lysogenic conversion (Novick, 2003). This is when mobile phage encoded genetic materials including toxin genes, adhesion genes and genes involved in metabolic process are transferred to the host bacteria (Novick, 2003). Many important bacterial pathogens have been shown to be lysogenic, possessing phage-encoded virulence factors that are implicated in their pathogenesis (Penadés *et al.*, 2015). For instance the toxins of *Staphylococcus aureus*, *Clostridium botulinum, Vibrio cholera* and *E. coli* are all phage encoded (Novick, 2003). Overall, phages greatly influence the physiology, ecology and diversity of microbial communities (Penadés *et al.*, 2015).

2.3.3. Bacteriophage life cycles

Bacteriophages are classified into two distinct categories based on their life cycles – virulent phages and temperate phages (Guttman *et al.*, 2005; Ackermann, 2012). The virulent phages can only multiply by using the lytic life cycle, the outcome of which is always the lysis and death of the host cell. The cycle begins with adsorption of the phage tail fibres to the receptors on the bacterial cell surface, then phage DNA is injected into the host cell after degradation of the cell wall by phage encoded proteins known as lysozymes (Hagens & Loessner, 2007). The internalization of phage DNA results in the hijacking of the host biosynthesis mechanism and production of phage nucleic acid and structural components, which are then assembled before their release from the host. Using the same phage encoded lysozyme, the host cell wall is ruptured in order for the matured phage progeny to be released as exemplified in the life cycle of bacteriophages T4 (Fig 1.1).

Temperate phages however, have a different and more complex life cycle which can follow either of two pathways (Guttman *et al.*, 2005). Sometimes the phage may follow a lytic pathway resulting in lysis of the host cell and phage progeny release as described above. On the other hand, the infecting phage may instead initiate a lysogenic life cycle whereby the phage does not replicate, but the phage genome becomes integrated into the bacterial host genome as a prophage and remain dormant until stimulated by environmental factors to enter the lytic cycle and produce progeny (Guttman *et al.*, 2005; Monk *et al.*, 2010). As mentioned earlier, the integration of prophages into their host is implicated in the evolution of bacteria, since they enhance the horizontal transfer of genes including genes associated with virulence factors or antimicrobial resistance determinants (Boyd & Brüssow, 2002). As a result, temperate phages are not good candidates for biocontrol applications due to the possible undesirable outcome of horizontal gene transfer.

On the contrary, temperate phages have been shown to influence their host in several ways (Novick, 2003). They can help protect their host from infection by other phages and can also lead to changes in their properties that enhance the survival of the host. Properties such as restriction systems, antibiotic resistance and virulence factors of the bacterial host have been linked to the prophage they carry (Novick, 2003). A good example of a phage with temperate life cycle is lambda (Fig 1.1).

Contrary to the lytic and temperate life cycles, some bacteriophages replicate without the lysis of their host cell, which is done through continuous excretion of the progeny phage without lysis as demonstrated in phage M13 (Guttman *et al.*, 2005).

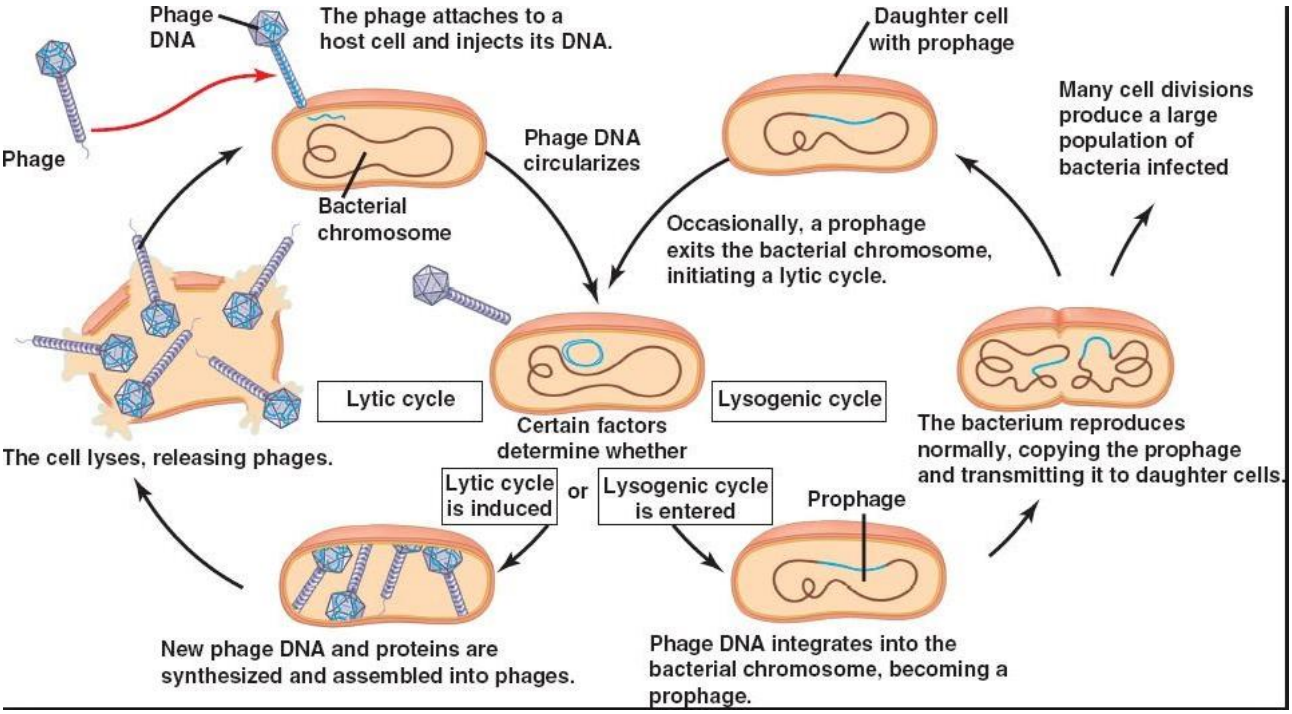

Figure 2.1: Phage life cycle depicting the lytic and lysogenic pathways (Reece *et al.*, 2011).

2.3.4 Structure and taxonomy of bacteriophages

The classification of phages has evolved since their first description by d'Herelle as one phage with many races (d'Herelle, 1917). With the discovery of the electron microscope, Ernest Ruska in 1942 proposed the first phage classification and demonstrated the morphological diversity of phages (Ruska, 1942). Since that time, various schemes of classification have been proposed by researchers (Burnett, 1933); however most of them were rejected by the scientific body until 1962,when Lwoff, Horne and Tournier suggested classification based on nucleic acid type (DNA or RNA) and virion properties including presence or absence of an envelope, capsid shape, and number of capsomeres (Lwoff *et al.*, 1962; Ackermann, 2003). This scheme was named the Lwoff, Home, and Tournier (LHT) system and became the basis for the present phage classification since the then virus classification committee (Provisional Committee on Nomenclature of Viruses (PCNV) adopted it. The PCNV was later developed into the International Committee of Taxonomy of Viruses (ICTV), which is the current organization in

charge of standardizing the classification of all viruses, including bacteriophages (Kutter & Sulakvelidze, 2004).

Currenlty, over 70 phage properties that are employed by the ICTV in characterisation of viruses have been described but emphasis is placed on criteria relating to their physical properties (Ackermann, 2009b; Ackermann, 2009a). Some physical features including the type of nucleic acid and virion morphology define the family of a phage while other criteria such as host range, sensitivity tests, and restriction endonuclease digestion patterns are used in differentiation into genera and species (Ackermann, 2009a). Although the ICTV adopted the polythetic species approach whereby a virus species is defined by a number of properties that may or may not be shared by all the members, there are still no universal criteria for phage classification hence phage taxonomic structure still remains open (Ackermann, 2009a; Van Regenmortel, 1990).

Based on the current classification system of the ICTV, viruses are placed into three orders comprising 61 families and 241 genera. Among these groups, bacteriophages account for one order (*Caudovirales*), with 14 families and 37 genera (Ackermann, 2003; Ackermann & DuBow, 2011). The *Caudovirales* are further grouped into three main families based on their tail structure: the *Myoviridae,* (24.5% of tailed phages) are made up of phages with unique contractile tails as seen in T4 phages; the *Siphoviridae* (61% of tailed phages) comprise of phages with long but non-contractile tails such as λ phage; and the *Podoviridae* (14% of tailed phages) which are made up of phages with short tails such as T7 phage (Ackermann, 2003; Monk *et al.*, 2010).

Morphological classification is the first step in the classification of a new phage. More than 6300 phages have been examined under the electron microscope, the majority of which are tailed (96%) and only 4% had polyhedral, filamentous or pleomorphic morphologies (Ackermann,

2003). Accordingly, tailed phages of the order *Caudovirales* represent the most abundant and widely distributed group of bacterial viruses, having a number of common features as well as differences. While these phages have been found to lack any type of envelope, they contain a linear double stranded DNA genome similar to their bacterial host, of different sizes ranging from 18 to 500 kb. These virions are also composed of a head that is icosahedral in shape, containing DNA and a long contractile or short tail usually with terminal adsorption structures including spikes or tail fibres. These structures are responsible for binding to the host cells (Casjens, 2005).

Although the *Caudovirales* comprise the majority of bacterial viruses, tailess phages are also in existence (Fig 2.2). They include about 190 known viruses, corresponding to less than 4% of the currently recognised bacterial viruses (Ackermann, 2005). They are classified into 10 small families, occur enveloped or non-enveloped, and are of three types: polyhedral phages that are icosahedral with cubic symmetry and either DNA or RNA constituted, filamentous phages with helical symmetry that are DNA constituted, and a few pleomorphic types without obvious symmetry axes that are also DNA constituted (Ackermann, 2005).

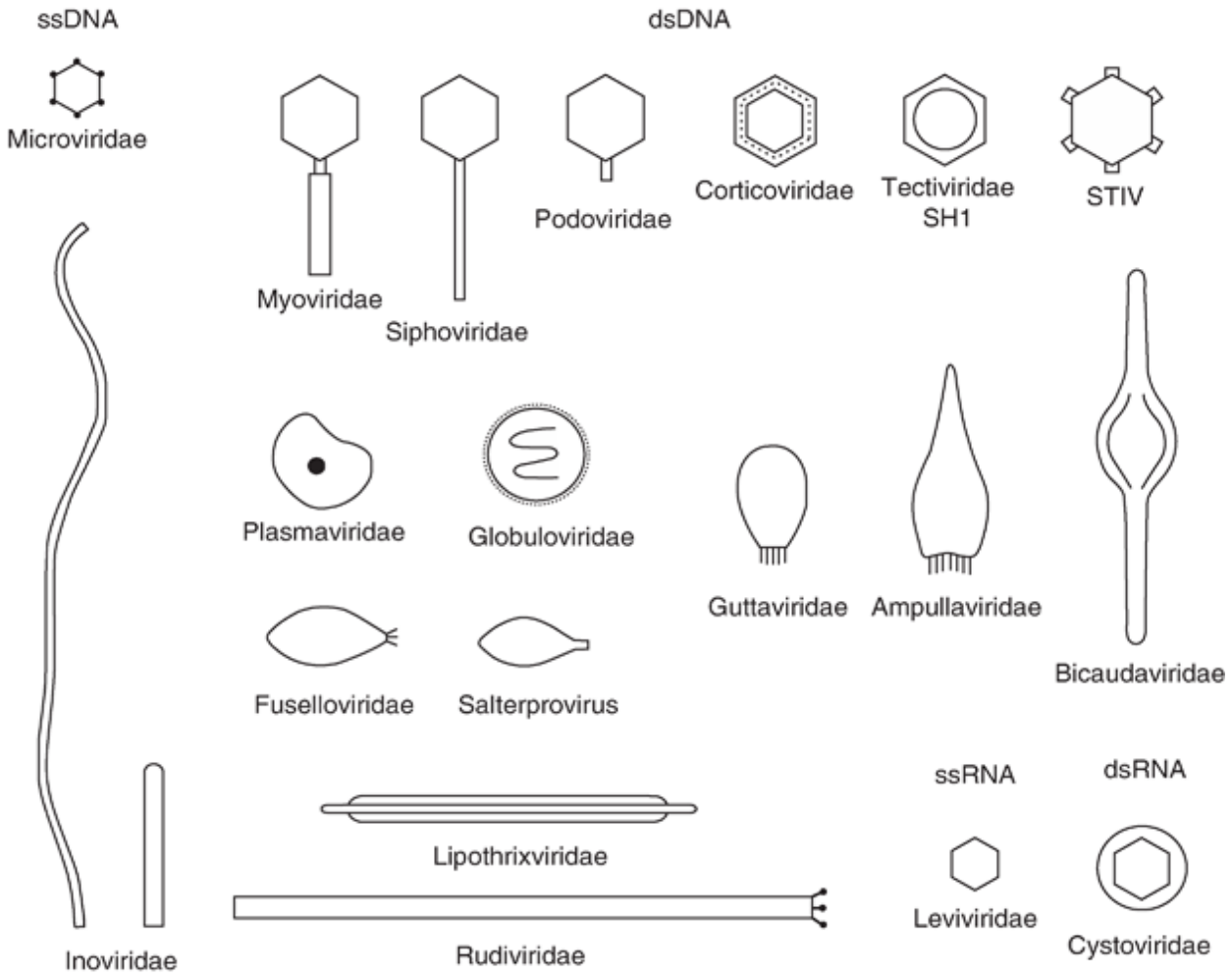

Figure 2. 2: Major Morphotypes of phages (Ackermann, 2007).

Other recently proposed alternative systems which are sequence-based classification schemes for phages include the construction of proteomic tree based on protein sequences and characterisation based on the genome content (Rohwer & Edwards, 2002). Unlike in bacteria where the analysis of 16S rRNA sequences aid in their taxonomic characterization and phylogeny determination, phages lack ribosomal sequence and therefore classification system based on a single gene is quite impossible (Rohwer & Edwards, 2002; Clokie *et al.*, 2011). To overcome this, a new sequence-based classification system involving the construction of phage proteomic tree that grouped related phage and identified signature genes in phage groups was proposed (Rohwer & Edwards, 2002). Although this proposed system was comparable with the ICTV system in its grouping and identification of phage families or genera, some potentially

inconsistent results were identified. Thus the proteomic tree approach was considered an independent confirmation of ICTV grouping but not a classification scheme (Ackermann, 2009a; Ackermann & DuBow, 2011).

A different modular approach was developed to classify phages using the modules of genes within the genome content (Lawrence *et al.*, 2002). This modular approach described the major role played by horizontal gene transfer in phage evolution and highlighted that phages are composed of modules, which are genes or groups of genes that are exchangeable. Moreover, it argues that phages have access to a common gene pool by which they acquire genes through horizontal transfer (Hendrix, 2002) challenging classification based on phylogeny and suggesting its replacement with the modular classification system (Lawrence *et al.*, 2002). Even though this proposal appeared attractive, it generated an enormous number of module combinations and was considered a non-feasible method for classification, and was finally abandoned by the ICTV(Ackermann, 2005; Ackermann, 2009a).

2.4 *E. coli* bacteriophages

In 1990, a few years after *E. coli* O157:H7 was reported in humans, bacteriophages specific to the pathogen were isolated from manure samples obtained from dairy farms (Ronner & Cliver, 1990). The isolated phage designated AR1 was reported as lytic against most strains of *E. coli* O157:H7 and *S. dysenteriae* but was unable to form plaques on other enterobacteria tested. The susceptible strain of *Shigella* produces an enteric toxin similar to that produced by *E. coli* O157:H7. Therefore, the authors suggested that a relationship exists between production of toxin and susceptibility to coliphage AR1. Further characterisation revealed a plaque size of 0.5 mm, a latent period of 20-25 min and a plaque size of 34 pfu (Ronner & Cliver, 1990).

In recent years, they have also been successfully isolated in many subsequent studies (Ronner & Cliver, 1990; Morita *et al.*, 2002; O'Flynn *et al.*, 2004; Raya *et al.*, 2006; Oot *et al.*, 2007; Niu *et al.*, 2009; Liao *et al.*, 2011; Niu *et al.*, 2012b; Park *et al.*, 2012; Dömötör *et al.*, 2016). Water sources, farm slurries, bovine and ovine faeces, as well as rumen samples and sewage samples have been popular sources for the isolation of *E. coli* O157:H7 phages since their hosts typically colonise the same environment. However, the pattern of prevalence indicated the abundance of phages in faeces compared to other sources samples. The ovine rumen alone has been estimated to contain 3×10^9 and 1×10^4 phage particles/ml of rumen fluid (Klieve & Swain, 1993). Thus cattle and/or ovine faecal samples might represent an ideal source for *E. coli* O157:H7 bacteriophages isolation (Morita *et al.*, 2002).

With the use of the TEM, researchers have examined the morphology of *E. coli* O157:H7 bacteriophages so as to characterise and also attribute them to a family (Ackermann, 2005). Unlike the phages of other genera, the coliphages have demonstrated relatively diverse distinct morphological types from tailed to filamentous phages; however, some morphological forms are more dominant and frequently encountered in the environment. In the same way, recent studies investigating the therapeutic potential of isolated phages have reported that the two forms most frequently encountered are the families of *Myoviridae* and the *Siphoviridae* (Kulikov *et al.*, 2012; Niu *et al.*, 2012b; Lee & Park, 2015). The *Podoviridae* are particularly uncommon since few studies have investigated *E. coli* O15:H7 infecting *Poviridae*, in fact, to the best of our knowledge there has been no report of them in Africa. The reason for this dominance is unclear but might be due to the method of isolation that targets long-tailed virulent phages with therapeutic potential. Even though the use of morphological characteristics in the classification of phages is widely accepted, it provides little or no information on their population genetics and evolutionary development, all of which can be revealed with genomic study.

Phage genome analysis has been very informative with respect to the biology of the individual phages (Casjens *et al*., 1992). However, it was the relatively recent results of high throughput sequencing technologies coupled to ever-expanding bioinformatics tools, which for the first time allowed scientists to study and compare multiple phage sequences at the whole genome resolution, thereby addressing fundamental biological questions relating to phage population genetics and evolution. In addition to comparative studies, information from individual phage genome sequence allows for genetic, biochemical and structural dissection of the phage; for instance sequencing of a biocontrol candidate genome makes it possible to assess for virulence related genes and genes involved in transduction before their application.

To date, the whole genome sequences of more than 147 *E. coli* phages are currently available in public databases (NCBI). Among them, only a few are specific to *E. coli* O157:H7 (Kropinski *et al.*, 2013). The majority of them are strictly lytic belonging to the *Myoviridae* family and having genome sizes ranging from 86.23 bp to 168.8 bp (Tiwari & Kim, 2013; Costa *et al.*, 2018). The genomes of other families such as the *Siphoviridae* and *Podoviridae* have also been reported, though with less frequency. While a few of these groups are lytic similar to the *Myoviridae*, others are temperate with smaller genome sizes. Temperate phages may play an important role in the pathogenicity of *E. coli* O157:H7 by carrying virulence factors, mediating lateral gene transfer and possibly facilitating adaptation of the pathogen during infection (Ochman *et al.*, 2000). In fact, the shiga toxin gene, which is the principal virulence gene in *E. coli* O157, has been shown to be transferred to the bacterial host when infected by a temperate phage. Other studies conducted by various researchers also on sequence analysis of *E. coli* phages have provided a solid background for future comparative studies (Kutter *et al.*, 2011; Kulikov *et al.*, 2012; Niu *et al.*, 2009; Tiwari & Kim, 2013; Niu *et al.*, 2014; Yesil *et al.*, 2017).

Presently, studies focussing on the isolation and characterisation of phages in Africa are limited (Ackermann & DuBow, 2011). To date there have been no investigations of *E. coli* O157:H7 specific bacteriophages in South Africa/Africa. Even the majority of lytic phages of *E. coli* O157:H7 that have been isolated elsewhere are not well studied since information relating to their biology, physicochemical as well as genomic characterisation is lacking (Kropinski *et al*., 2013). Only a few of them have been functionally characterised/comprehensively studied at the molecular level. The few numbers of *E. coli* O157:H7 genomes in the database indicate the need to sequence and analyse more genomes of this important food-borne pathogen using modern genome analysis techniques. Such investigations would provide a better understanding of phage diversity, phylogenetic and evolutionary relationship, with the potential to increase industrial, therapeutical and biotechnological applications of these phages.

2.5 Application of phages to control contaminants on meat surfaces

Although bacteriophages could be applied anywhere along the farm to fork chain, their use as a meat surface and carcasses decontaminant has been the focus of many studies, particularly in raw foods or ready to eat foods (Atterbury, 2009; Anany *et al.*, 2011; Guenther *et al.*, 2012; Tomat *et al.*, 2013). In *Listeria monocytogens* for instance, a combination of listeriophage LH7 and nisin was able to antagonise *Listeria monocytogens* strains and reduce their level without re-growth in broth medium (Dykes & Moorhead, 2002). However, the observed effectiveness could not be transferred to food systems as neither phage alone nor phage-nisin mixture had any effect on the pathogen. The researcher highlighted the biocontrol potential of listeriophage and nisin and concluded that there is need to have a better understanding of the interactions in complex food systems before their practical applications (Dykes & Moorhead, 2002).

Most studies investigating the efficacy of phage treatment on meat surfaces, particularly chicken meat, have focussed on the most frequently targeted zoonotic pathogens on chicken meat – *Campylobacter* and *Salmonella*. However, in an experiment to study the effect of phages on two different types of beef – cooked and raw, *Campylobacter spp* and *Salmonella* spp were the tested pathogens that were artificially inoculated on the beef. While phage treatment resulted in significant inactivation of the pathogens in comparison to the control, a greater inactivation was noted in raw meat than cooked meat (Bigwood *et al*., 2007).

A cocktail of three lytic bacteriophages, e11/2, e4/1c and pp01 was investigated for their ability to eliminate *E. coli* O157:H7. Addition of cocktails to experimentally contaminated beef surface resulted in significant control since the bacteria was inactivated in seven out of nine cases when the cocktail was applied. Although similar inhibitory effect of the treatment was reported in the in-vitro study, bacteria insensitive mutants (BIM) emerged following the challenge, albeit at low frequency. It was suggested that the formation of BIM should not hinder their application, as low levels of pathogens are usually present in the environment (Ofylyn *et al*., 2004). Similarly studies investigating the ability of phage cocktail ECP-100 to lyse *E. coli* O157:H7 on fresh produce and ground beef samples showed comparable effectiveness of phage because there was a significant reduction in pathogen load post-treatment (Abuladze *et al*., 2008).

The successful use of coliphages has led to the approval of a phage product, EcoShield (Intralytix Inc, Baltimore, MD), by the food and Drug Administration (FDA) in 2011. The product can be directly used on various foods, including ground beef, leading to a significant reduction of *E. coli* O157:H7 of 95% to 100%.

In addition to EcoShield, listShield is another commercial phage product for listeria from intralytix that can be sprayed on RTE foods and targets *Listeria monocytogenes* contamination in foods and food processing facilities. In a similar fashion, Litsex P100, a phage-based product from (EBI food safety) Micreos Food Safety designed to prevent listeria contamination on food products and food processing facilities.

Although the majority of investigations into the possible application of phages in the control of food-borne pathogens appear promising, the regulatory restrictions of phage application in bio-sanitation of food products are vast and may hamper their introduction into certain markets. However, the fact that there are some approved products commercially available for the biocontrol of pathogenic bacteria in food is an indication that we are heading in the right direction (Hungaro *et al*., 2014).

CHAPTER THREE

USE OF PULSED FIELD GEL ELECTROPHORESIS GENETIC TYPING FOR TRACING CONTAMINATION WITH VIRULENT *ESCHERICHIA COLI* O157:H7 IN BEEF-CATTLE PRODUCING FARMS

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3.1 Abstract: *E. coli* O157:H7 is an important causative agent of a variety of foodborne infections including hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) that may lead to renal failure in humans. The main source of infection is contaminated food of animal origin and a number of animal species especially cattle have been associated with this pathogen. Contamination usually occurs when faeces of animals comes into contact with carcass during slaughtering. The aim of the present study was to determine the virulence profiles and genetic relationships of *E. coli* O157:H7 strains isolated from cattle on some commercial farms in the North West Province, South Africa. A total of 260 faecal samples were collected from eight farms over a six-month period and assessed for the presence of *E. coli* O157:H7 through PCR analysis. Bacteria 16S rRNA gene fragments were amplified as an internal control while the rfb_{O157} and $flic_{H7}$ gene PCR were used to confirm identities of isolates. STEC virulence genes *stx1*, *stx2*, *eaeA* and the *hlyA* were

detected using PCR and the genetic relatedness of the isolates was determined using Pulse Field Gel electrophoresis (PFGE). A total of 69 (26.5%) *E. coli* O157:H7 isolates were detected in samples obtained from all the eight farms. A large proportion of the *E. coli* isolates possessed the stx_1 (66.7%) and stx_2 (97.1%) genes respectively. In addition, other accessory virulence genes *eae*A and *hly*A were detected in 30 (43.5%) and 43 (62.3%) of the *E. coli* isolates respectively. Pulsed-field gel electrophoresis (PFGE) analysis grouped these isolates into 6 clusters and the largest cluster contained 17 isolates originating from different farms. These results provide valid evidence that cattle harbor virulent and genetically similar *E. coli* strains particularly those belonging to the serotype O157 and therefore these animals may serve as a possible source for zoonotic transfer of these pathogens to humans. The implication is that these isolates may have severe public health consequences on consumers.

Keywords: *E. coli* O157:H7, Random amplified polymorphic DNA (RAPD), Pulse Field Gel electrophoresis (PFGE), cattle, zoonosis.

3.2 Introduction

Among the various pathogenic groups of *Escherichia coli* (*E. coli*), *E. coli* O157:H7 appear to be of greater virulence and therefore continue to be the dominant cause of illnesses in humans. Diseases caused by *E. coli* O157:H7 range from mild diarrhea to haemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) that presents more severe complications in children, elderly and immune-compromised individuals (Noris & Remuzzi, 2005; Tarr *et al.*, 2005; Gyles, 2007; Gould *et al.*, 2009; Pennington, 2010). HUS is characterized by impairment of the glomerulus resulting in renal failure and often leads to death (Gianantonio *et al.*, 1964).

The ability of this pathogen to cause the aforementioned diseases in particular HUS, has been attributed to the potential of individual cells to harbour a number of pathogenic determinants (Gyles, 2007). Although shiga-toxins have been identified as the principal virulence factors for

this pathogen, a number of accessory putative pathogenic determinants that include intimin encoded by *eaeA,* a plasmid-encoded enterohemolysin (*Ehly*) and pore-forming cytolysin encoded by the *ehx* gene have also been reported to contribute significantly to the pathogenicity of *E. coli* O157:H7 (Cookson *et al.*, 2007; Kawano *et al.*, 2012). Despite the fact that some studies have indicated that these virulence determinants seldom occur together in a given isolate the genetic combinations must be assessed to provide a clear indication of the impact of these determinants in *E. coli* O157:H7 isolates within a specific geographical area (Paton & Paton, 1998).

Healthy ruminants, in particular cattle, may harbour *E. coli* O157:H7 strains without showing any signs of disease (Cray & Moon, 1995; Brown *et al.*, 1997)**.** However, they have been identified as the principal source of human infections, and transmission may occur through a variety of routes (Karmali *et al.*, 2010). Although various routes of transmission of *E. coli* O157 serotypes have been reported in water, direct contact with shedding animals as well as person-to-person transmission have also been identified as potential modes of spreading these pathogens. Given that the proportion of food-borne infections, hospitalizations, and deaths caused by different pathogens have been reported to vary significantly and depend largely on the type of food product targeted, point of food chain analyzed and the degree of processing, some epidemiological studies have also revealed that there is a direct association between occurrences of *E. coli* O157:H7 food-borne outbreaks and the consumption of undercooked contaminated beef products (Riley *et al.*, 1983; Rangel *et al.*, 2005; Gyles, 2007). Despite the fact that to date no *E. coli* O157 food-borne outbreak has been reported in South Africa, previous surveillance studies revealed a very close genetic relationship between *E. coli* O157 strains isolated from water, animals, their corresponding meat products and diarrhoeal patients (Ateba & Mbewe, 2011; 2013; 2014). Moreover, the recent confirmation that the consumption of contaminated polony was responsible for the world's largest and most severe listeriosis outbreak that occurred

in South Africa highlights the need for constant surveillance studies. This implies that to prevent the transmission of *E. coli* O157:H7 along food production systems, a concerted effort consisting of effective control measures designed to implement strict control as well as recommended standard operating procedures either at the farm or slaughter houses should be enforced. Generally, these strategies are also centered on the studies that aim to provide indications of the distribution and population dynamics of the pathogen in animal species, since this is very critical in surveillance and control.

Bacterial genotyping methods are very important in epidemiological investigations of infectious agents because they reveal sources and routes of bacterial spread, especially in outbreaks, and thus assist in source tracking (Maslow et al., 1993; Olsen *et al.*, 1993). In addition, they assist in the assessment of genetic relationships among bacteria strains from different sources or geographic locations (Maslow *et al.*, 1993). A number of studies have been conducted in the study area in which the genetic relationship of *E. coli* O157 strains were assessed using the antibiotic resistant profiles (Ateba *et al.*, 2008); Enterobacterial Consensus Repetitive Sequences (ERIC) PCR analysis (Ateba & Mbewe, 2014) as well as ISR, BOX and REP-PCR analysis (Ateba & Mbewe, 2013). Results obtained from these studies were able to cluster isolates based on the sample stations and/or species from which they were isolated, and thus provide opportunities for source tracking.

Given that genetic variations among bacterial genomes is a valuable criterion for evaluating the genetic relationships, and Pulsed Field Gel Electrophoresis (PFGE) is considered a gold standard technique for the generation of more reliable isolate-specific genetic fingerprintings due to its very powerful resolution power (Prevost *et al.*, 1992; Gori *et al.*, 1996), the present study was designed to expand on our previous investigations. To the best of our knowledge this is the first study in South Africa to provide data on the genetic variations of pathogenic *E. coli* O157:H7

strains using PFGE. Data generated may provide an understanding of the epidemiology of this pathogen in the area.

3.3 Materials and Methods

3.3.1 Sample collection

A total of 260 faecal samples were collected from 260 healthy cattle in six farms (coded A-F) around Mafikeng in the North-West province, South Africa. These farms were selected based on the willingness of the farmers to participate in the study. These were commercial farms and animals received feed and dietary supplements regularly. Faecal samples were obtained from each animal through rectal palpitation and the samples were collected directly from the rectum of individual animals using sterile arm-length gloves. In order to avoid duplication, the animals were locked in their respective handling pens after sampling. The samples were labelled properly and were immediately placed on ice. The samples were transported to the microbiology laboratory of the North-West University where they were analyzed. Sampling for this study was done between November 2015 and April 2016.

3.3.2 Isolation and identification of *E. coli* O157:H7 strains

For isolation of *E. coli* O157:H7 strains, 2 g portions of the faecal samples were dissolved into 90 mL 2% (w/v) buffered peptone water (Oxoid, Basingstoke, Hampshire UK), and incubated aerobically at 37 ºC while shaking at 150 rpm for 24 hours (Zhao *et al.*, 1995). Tenfold serial dilutions were prepared from pre-enriched samples using 0.1% (w/v) Bacto Peptone water (Difco, UK) and aliquots of 100 µL from tubes with the dilution factor 10^4 were spread-plated onto sorbitol MacConkey agar (Merck, South Africa) supplemented with cefixime (50 µg/L) and potassium tellurite (25mg/L) (CT-SMAC). The inoculated CT-SMAC plates were incubated aerobically at 37 ºC for 24 hours. After incubation, plates were examined for the presence of non-sorbitol fermenting colourless colonies. The presumptive *E. coli* O157:H7 colourless

colonies were sub-cultured on CT-SMAC and plates were incubated aerobically at 37 ºC for 24 hours. Pure sorbitol-negative colonies exhibiting typical EHEC morphologies were stored at -80 ºC in 15% (v/v) glycerol and used for further bacteria identification tests.

3.3.3 Bacteria strains and serotyping of potential *E. coli* O157:H7 isolates

A total of 177 environmental presumptive *E. coli* O157:H7 isolates were subjected to serotype specific rapid slide latex agglutination serological assays using O157 (Oxoid, DR0620M) and H7 (Remel Europe Ltd., Kent, UK) monovalent antisera according to the manufacturer's instructions (Mast Diagnostics, UK).

3.3.4 Molecular characterization of *E. coli* O157:H7

Genomic DNA extraction from presumptive isolates

Bacterial chromosomal DNA was isolated from presumptive *E. coli* isolates using the ZR Genomic DNA™ Tissue MiniPrep kit (Zymo Research Corp, Irvine, CA, USA) according to the manufacturer's instruction. The eluted DNA was stored at −20 °C and used for molecular characterization of the isolates.

Determination of the concentration of DNA extracted

The quality and quantity of the chromosomal DNA extracted from the isolates was determined using a Nanodrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA) and values were recorded.

PCR for identification of *E. coli* O157:H7 isolates

Due to restriction on the importation of the *E. coli* O157:H7 ATCC positive control strains an environmental *E. coli* O157:H7 strain previously isolated within our research group and supplied by Prof CN Ateba was used as a positive control strain in all experiments while a no-template

PCR reaction tube was included in each run as negative control. In addition, as an internal control all DNA samples were screened for bacterial 16S rRNA gene fragments using the GM5F and 907R universal oligonucleotide primer sequences (Muyzer *et al.*, 1995) that are shown in Table 3.1. The identities of bacteria isolates were confirmed as *E. coli* O157:H7 through specific PCR amplification of the rfb_{O157} and $fliC_{H7}$ gene fragments (Morin *et al.*, 2004). The rfb_{O157} and $fliC_{H7}$ oligonucleotide sequences are shown in Table 3.1.

Genetic characterization of *E. coli* O157:H7 isolates for EHEC virulence gene determinants

The virulence profiles of the *E. coli* O157:H7 isolates was determined through specific uniplex PCR amplification of the stx_1 and stx_2 as well as duplex PCR assays of the *eaeA* gene and *hlyA* gene fragments (Pass *et al.*, 2000). PCR reaction mixtures were prepared as standard 25 µL volumes that constituted 12.5 µL of 2X Dream*Tag* Green Master Mix, 11 µL RNase free PCR water, 0.5 µL mixture of the forward and reverse primers (0.25 µL of each primer) and 1 µL of template DNA. All the PCR reagents were Biolab (New England, UK) products supplied by Inqaba Biotechnical Industry Ltd, Pretoria, South Africa. Amplifications were performed using a DNA thermal cycler (model- Bio-Rad C1000 Touch™ Thermal Cycler). Oligonucleotide primer sequences used in these PCR reactions were synthesized by Inqaba Biotec, South Africa and PCR amplification conditions used appear in Table 3.1. PCR amplicons were stored at 4°C until electrophoresis. PCR products were resolved by electrophoresis on a 2% (w/v) agarose (Seakme®, Rockland, USA) gel using 1X Tris-acetate-EDTA (40 mM Tris, 1 mM EDTA and 20 mM glacial acetic acid; pH 8.0) on a horizontal agarose gel equipment (BiocomDirect, Weir, UK). A 100 bp DNA molecular gene ruler (Fermentas, Glen Burnie, USA) was included in each gel and was used to confirm the sizes of the amplicons.

Pulsed-field gel electrophoresis (PFGE) analysis of *E. coli* O157: H7 isolate

In order to determine the genetic relatedness of *E. coli* O157:H7 strains isolated from cattle in the different farms, Pulsed-field gel electrophoresis was performed using the PulseNet Protocol (Hunter *et al.*, 2005). Log phase bacteria cultures were embedded in 1% (w/v) agarose plugs and cells in the plugs were lysed in 20% (w/v) sodium dodecyl sulfate (SDS) for 2 hours while shaking at 175 rpm. Bacteria chromosomal DNA was subjected to restriction digestion by embedding the plugs in *Xba*I (New England BioLabs, USA) following the manufacturer's instructions (MBI, Fermentas). Restricted genomic DNA was resolved by gel electrophoresis on a 1% (w/v) agarose gel using a CHEF DR II System (Bio-Rad) apparatus. The electrophoretic conditions were as follows 6 V/cm for 20 hours, pulse time ranging from 2.2 – 54.2 and 0.5 X Tris-borate EDTA (TBE) at 14 °C. A λ ladder DNA molecular weight maker (Boeehringer Mannheim, Germany) was included in all gels and used to determine the sizes of the fragments. After electrophoresis, the gels were stained with 50 ng/ml ethidium bromide, destained by washing with distilled water. Images were captured with a ChemiDoc imaging system (Bio-Rad ChemiDoc^{TM} MP imaging system, UK) using the GeneSnap (version 6.08) software. The PFGE patterns were analyzed using the GelCompar II program and the similarities between PFGE patterns was evaluated using the Dice coefficent similarity with a tolerance of 1%.

Table 3. 1: Oligonucleotide primer sequences used for amplification of 16S rRNA, *E. coli* rfb_{O157} and $fliC_{H7}$ genes as well as EHEC virulence genes in the study

Primers	**Sequence (5′-3′)**	**Targeted gene**	**Amplicon size (bp)**	**PCR conditions and cycles**
GM5F	TAC GGG AGG CAG CAG	*16S rRNA*	550	1 cycle of 3 minutes at 94°C, 25 cycles of 1 minute at 94°C, 1 minute at 55°C, 2 minutes at 72°C; 1 cycle of 10 minutes at 72°C(Muyzer *et al.*, 1995)
907R	CCG TCA ATT CCT TTG AGT TT			
RfbE F	CGG ACA TCC ATG TGA TAT GG	rfb_{O157}	259	95°C for 3 minutes, 10 cycles of 1 minute at 95°C for 1 minute, 65°C for 2 minutes, 72°C for 90 seconds; 95°C for 1 minute, 65°C for 2 minutes, 72°C for 90 seconds 1 cycle of 10 minutes at 72°C (Paton & Paton, 1998)
RfbE R	TTG CCT ATG TAC AGC TAA TCC			
FlicH7 F	GCG CTG TCG AGT TCT ATC GAG C	$fliC_{H7}$	625	94°C for 5 minutes, 35 cycles of 1 minute at 94°C, 30 seconds at 63°C, 1 minutes at 72°C; 1 cycle of 10 minutes at 72°C
FlicH7 R	CAA CGG TGA CTT TAT CGC CAT TCC			
Stx1 F	CCGATACGCTGCCAATCAGT	*stx1*	180	1 cycle of 5 minutes at 94°C, 40 cycles of 1 minute at 94°C, 1 minute at 63°C, 1 minutes at 72°C; 1 cycle of 10 minutes at 72°C (Paton & Paton, 1998)
Stx1 R	ACGCAGACCGTAGGCCAGAT			
Stx2 F	ATA AAT CGC CAT TCG TTG ACT AC	*stx* 2	255	95°C for 3 minutes, 10 cycles of 1 minute at 95°C for 1 minute, 65°C for 2 minutes, 72°C for 90 seconds; 95°C for 1 minute, 65°C for 2 minutes, 72°C for 90 seconds 1
Stx2 R	AGA ACG CCC ACT GAG ATC ATC			

				cycle of 10 minutes at 72°C (Paton & Paton, 1998)
Eae F	GAC CCG GCA CAA GCA TAA GC	*eaeA*	384	95°C for 3 minutes, 10 cycles of 1 minute at 95°C for 1 minute, 65°C for 2 minutes, 72°C for 90 seconds; 95°C for 1 minute, 65°C for 2 minutes, 72°C for 90 seconds 1 cycle of 10 minutes at 72°C (Paton & Paton, 1998)
Eae R	CCA CCT GCA GCA ACA AGA GG			
HlyA F	GCA TCA TCA AGC GTA CGT TCC	*hlyA*	534	95°C for 3 minutes, 10 cycles of 1 minute at 95°C for 1 minute, 65°C for 2 minutes, 72°C for 90 seconds; 95°C for 1 minute, 65°C for 2 minutes, 72°C for 90 seconds 1 cycle of 10 minutes at 72°C(Paton & Paton, 1998)
HlyA R	AAT GAG CCA AGC TGG TTA AGC T			

3.4 Results

3.4.1 Isolation and identification of *E. coli* O157: H7

A large proportion (177; 68%) of the 260 faecal samples analysed in this study, produced typical non-sorbitol fermenting colonies presumptive *E. coli* O157:H7 morphologies on CT-SMAC agar (Table 3.2). As shown in Table 3.2, 93 (52.5%) of the non-sorbitol fermenting isolates were positively identified as *E. coli* O157:H7 based on data generated using the O157 and H7 antisera. However, PCR assay with the use of specific primers for *rfb*O157 gene and *flic* H7 confirmed 26.5% (69/260) of the isolates as *E. coli* O157:H7. The prevalence of *E. coli O157:H7* was higher in farm F (100%) than E (85.6%), C (41%) and H (32.7%). In addition, Farms D (3%) and A (8%) had the lowest prevalence of *E. coli* O157:H7 contamination (Table 3.2).

Table 3. 2: The frequency of *E. coli* O157:H7 in faeces based on different detection methods

Farm code	Total no of Samples collected	Proportion of isolates positive for the different *E. coli* O157:H7 identification tests		
		No. of typical non-sorbitol fermenting *E. coli* O157:H7 colonies on CT-SMAC agar	No. of isolates positive for O157 and H7 antisera	No. of isolates positive for rfb_{O157} and $fliC_{H7}$ genes
A	50	15 (30%)	7 (14%)	4 (8%)
B	59	42 (71%)	20 (33.8%)	13 (22%)
C	29	24 (82.7%)	13 (44.8%)	12 (41%)
D	32	25 (78%)	5 (15.6%)	1 (3%)
E	14	13 (92.8%)	12 (85.7%)	12 (85.6%)
F	6	6 (100)	6 (100%)	6 (100%)
G	21	16 (76%)	10 (47.6%)	5 (23.8%)
H	49	36 (73%)	20 (40.8%)	16 (32.7%)
Total	**260**	**177 (68%)**	**93 (35.7%)**	**69 (26.5%)**

3.4.2 PCR detection of virulence genes in isolated strains of *E. coil* O157:H7

A total of 69 confirmed *E. coli* O157:H7 isolates were screened for the presence of STEC virulence genes stx_1, stx_2, *eaeA* and *hlyA* by PCR analysis. Overall, all (100%) the *E. coli* O157:H7 isolates analysed in this study harboured either of the shiga-toxins (Fig 3.1 – Fig 3.5). As indicated in Table 3, the proportion of the stx_2 gene detected was higher (97%) than the stx_1 (66.6%). Similarly, the *hlyA* was more prevalent (62.3%) among the isolates when compared to the *eaeA* gene (43.4%) (Table 3.3). In addition, a large proportion of these isolates harboured both stx_1 and stx_2 while (39.1%) possessed both *hylA* and *eaeA* genes. Despite this a cause for concern was the fact that 32 (46.4%) of the isolates harboured the stx_2, *eaeA* and *hlyA* genes while 14 (20.2%) possessed all the virulence genes analysed in the study. Figure 1 indicates an

agarose gel (1%) of bacterial 16S rRNA gene fragments (A), *rfb*$_{O157}$ (B), *stx*$_1$ (C), *stx*$_2$ (D) and the *eaeA* and *hlyA* gene fragments amplified from *E. coli* O157:H7 isolates in the study.

As shown in Table 3.4, eight major genotypes designated G1 to G8 were identified. A large proportion (20.2% - 26.1%) of the isolates belonged to the genotypes G2 (*stx*$_1^-$-*stx*$_2^+$-*eaeA*$^+$-*hlyA*$^+$), G3 (*stx*$_1^+$-*stx*$_2^+$-*eaeA*$^+$-*hlyA*$^+$), G5 (*stx*$_1^+$-*stx*$_2^+$-*eaeA*$^-$*hlyA*$^-$) and G7 (*stx*$_1^+$-*stx*$_2^+$-*eaeA*$^-$-*hlyA*$^+$).

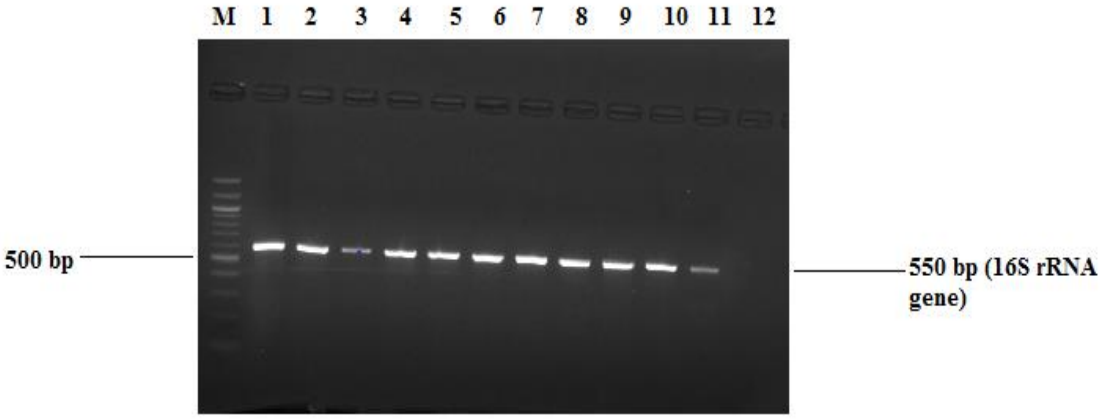

Figure 3. 1: Agarose gel (1%) of 16S rRNA gene fragments of *E. coli* O157:H7 isolates. Lane M: 100 bp marker; Lanes 1-11: 16S rRNA (550 bp) gene positive isolates; Lane 12: negative control

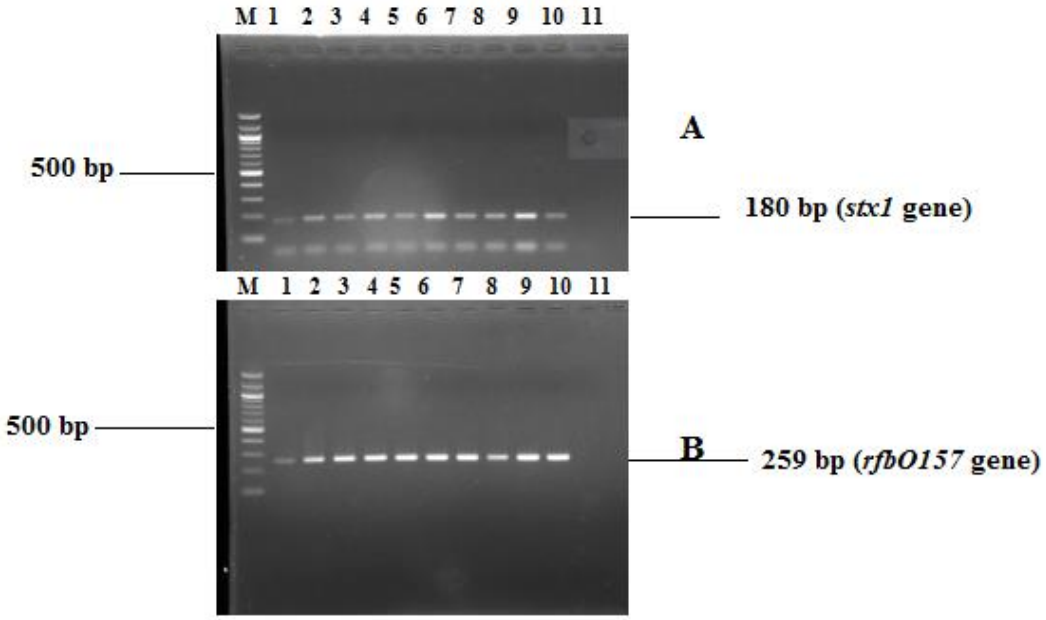

Figure 3. 2: Agarose gel (1%) of stx_1 (A) and rfb_{O157} (B) gene fragments of *E. coli* O157:H7 isolates. Lane M: 100 bp marker; lanes 1-10 A & B: stx_1 (180 bp) and rfb_{O157} (259 bp) genes positive isolates respectively. Lanes 11: negative control

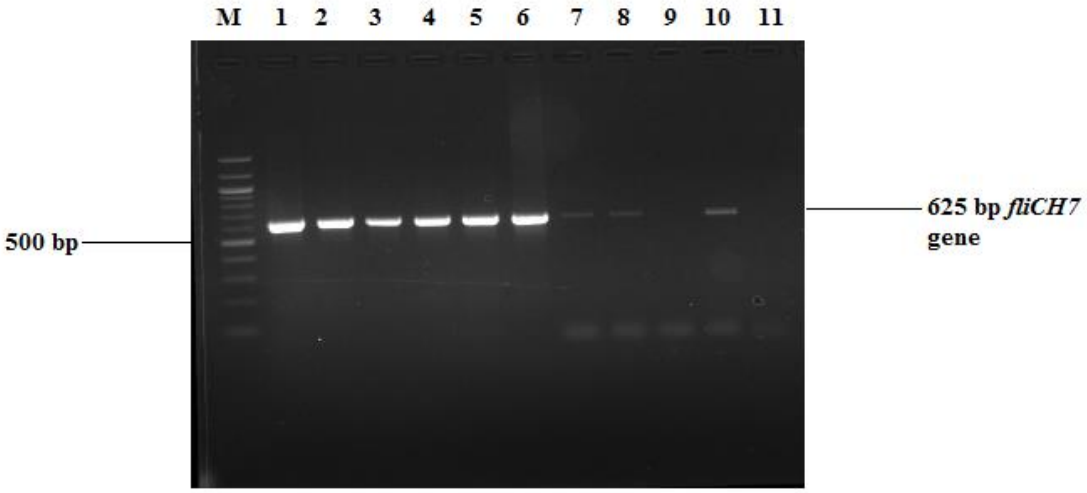

Figure 3. 3: Agarose gel (1%) of *fliC$_{H7}$* gene fragments of *E. coli* O157:H7 isolates. Lane M: 100bp marker; Lanes 1-8,10: fliCH7 (625 bp) gene positive isolates; Lane 11: negative control

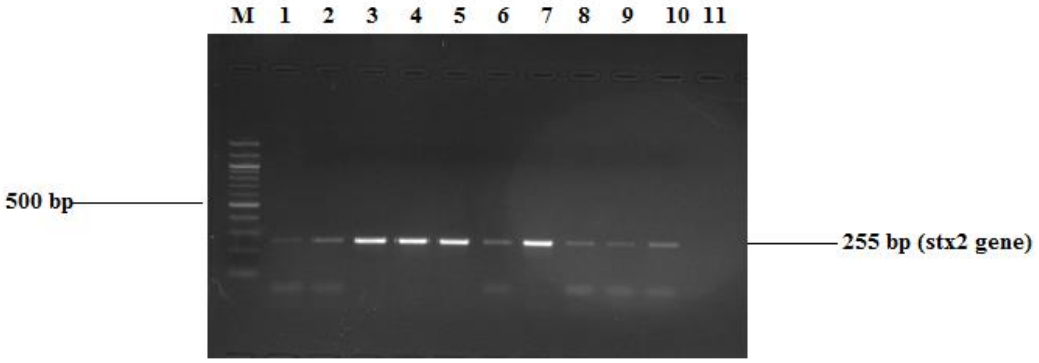

Figure 3. 4: Agarose gel (1%) of *stx$_2$* gene fragments of *E. coli* O157:H7 isolates. Lane M: 100bp marker; lanes 1-10: *stx$_2$* (255 bp) gene positive isolates; lane 11: negative control

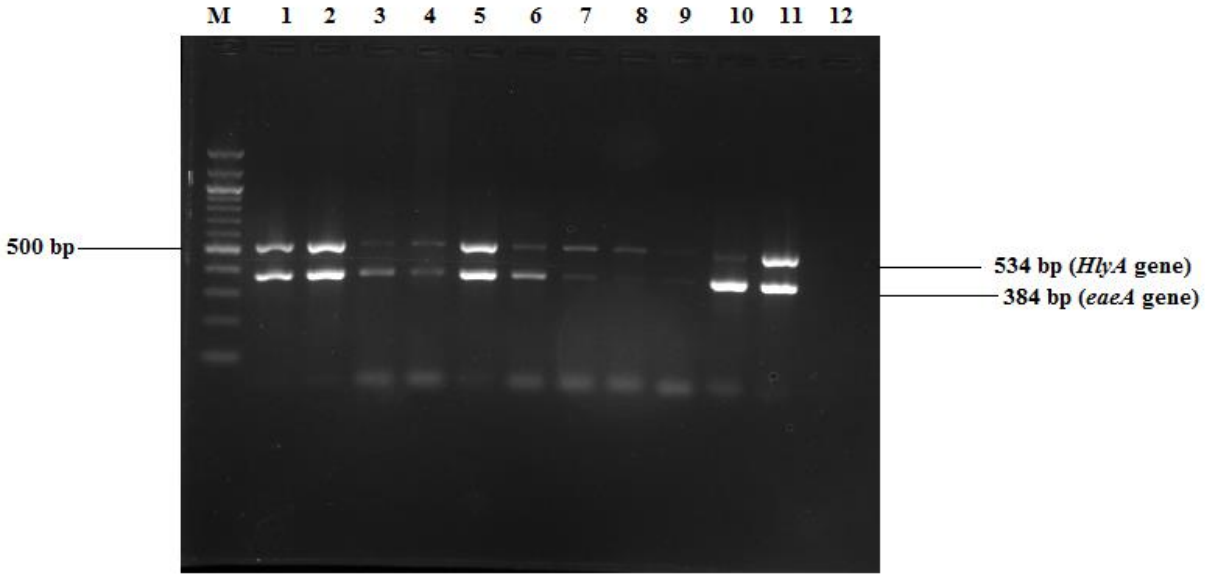

Figure 3. 5: Agarose gel (1%) of a 384-bp fragment of *eaeA* and 534-bp fragment of *HlyA* gene of *E. coli* O157:H7 isolates. Lane M: 100 bp marker; Lanes 1-11: *eaeA* and *HlyA* positive isolates: lane 12: negative control.

Table 3. 3: **Prevalence and distribution of virulence genes among *E. coli* O157:H7 isolates**

Farm Code	**No of isolates tested**	**No of isolates that were positive for the different virulence genes**			
		***sxt*$_1$**	***sxt*$_2$**	***eaeA***	***hlyA***
Farm A	4	1	4	3	3
Farm B	13	0	13	13	11
C	12	12	12	3	5
D	1	0	1	0	1
E	12	12	10	0	12
F	6	0	6	0	0
G	5	5	5	5	5
H	16	16	16	6	6
Total	**69**	**46**	**67**	**30**	**43**

3.4.3 Pulsed-field gel electrophoresis typing of *E. coli* O157:H7 isolates

PFGE molecular typing was carried out to investigate the genetic relationships of *E. coli* O157:H7 isolates from the different farms. The PFGE of *Xba*l-digested chromosomal DNA from the 69 isolates revealed that only 58 *E. coli* O157:H7 isolates produced stable and reproducible fingerprints. The PFGE patterns of these 58 isolates produced six clusters designated (I – VI) as shown in Figure 3.6. Clusters were further analysed for associations of isolates from the different farms and data was reported in Table 3.5. The largest cluster (cluster I) with 17 *E. coli* O157:H7 isolates possessed isolates from three (C, F and G) farms. Clusters V and VI possessed 12 and 16 isolates respectively while cluster III was the smallest cluster with 3 isolates. Generally, all the clusters except for clusters II, III and IV were mixed since they contained isolates from more than one farm (Figure 3.6). The findings of this study also indicate a high genetic similarity between isolates from the same farm. In addition, it was most often

observed that isolates from the same farm that cluster together possess similar virulence gene profiles

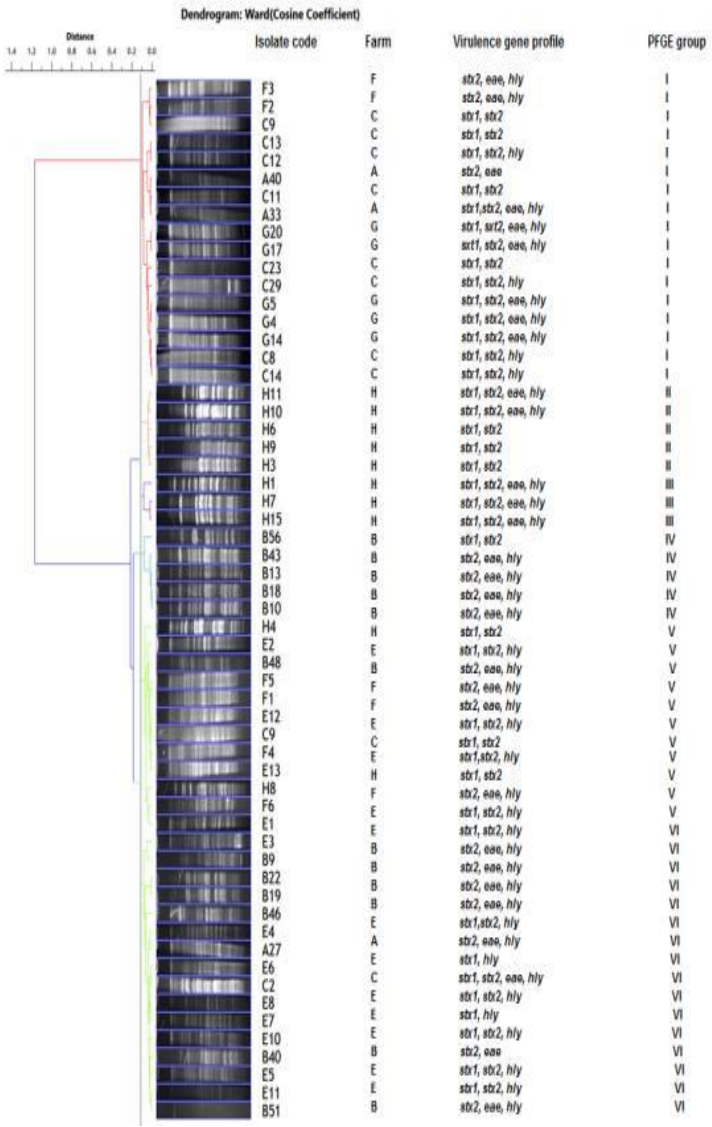

Figure 3. 6: Dendrogram of PFGE patterns of *E. coli* O157:H7 isolates. I, II, III, IV, V, VI are the main PFGE clusters.

Table 3. 4: **Proportion of eight different gene combinations (genotypes) identified in *E. coli* O157:H7 isolates**

Gene combinations for *E. coli* O157:H7 isolates							**Proportion of *E. coli* O157:H7 isolates with the different gene combinations per sample source**								
							Farm								
***rfb*$_{O157}$**	***flic*$_{H7}$**		***Stx*$_1$**	***Stx*$_2$**	***eaeA***	***hlyA***	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**Total**
+	+	G1	-	+	-	+	1	0	0	1	0	0	0	0	**2 (2.8%)**
+	+	G2	-	+	+	+	1	11	0	0	0	6	0	0	**18 (26.1%)**
+	+	G3	+	+	+	+	1	0	1	0	0	0	5	7	**14 (20.2%)**
+	+	G4	-	+	+	-	1	1	1	0	0	0	0	0	**3 (4.3%)**
+	+	G5	+	+	-	-	0	1	5	0	0	0	0	9	**15 (21.7%)**
+	+	G6	+	+	+	-	0	0	1	0	0	0	0	0	**1 (1.4%)**
+	+	G7	+	+	-	+	0	0	4	0	10	0	0	0	**14 (20.2%)**
+	+	G8	+	-	-	+	0	0	0	0	2	0	0	0	**2 (2.8%)**

+: Gene present, -: Gene absent, G1- G8 – eight different genotypes identified in the study

Table 3. 5: **PFGE clusters of *E. coli* O157 isolates from different farms**

PFGE clusters	No of isolate	Farms
I	17	A, C, F, G
II	5	H
III	3	H
IV	5	B
V	12	B, C, E, F, H
VI	16	A, B, C, E

3.5 Discussion

The main aim of this study was to isolate *E. coli* O157:H7 from cattle faeces and determine their virulence profiles. A total of 69 *E. coli* O157:H7 isolates were positively identified in the study. The occurrence of *E. coli* O157:H7 in cattle farms has been investigated in several studies (Ateba *et al.*, 2008; Arthur *et al.*, 2010; Ateba & Mbewe, 2011; Sasaki *et al.*, 2011; Soon *et al.*, 2011). Despite the fact that cattle are recognized as a primary natural reservoir of *E. coli* O157 strains (Gyles, 2007; Gunn *et al.*, 2007; Ferens & Hovde, 2011), previous findings in the present study area revealed that pigs rather than cattle are potential hosts for these pathogens (Ateba *et al.*, 2008; Ateba and Bezuidenhout, 2008; Ateba and Mbewe, 2011). This was also further confirmed by the higher prevalence of *E. coli* O157:H7 isolates in pork than beef samples (Ateba & Mbewe, 2011). Monitoring the occurrence and distribution of pathogenic organisms particularly, *E. coli* O157:H7 in cattle farms does not only assist in providing information on the prevalence in a given area but also assists in the development of on-farm control strategies against *E. coli* O157:H7. This is motivated by the fact that previous reports have revealed that there is a significant reduction in the transmission of *E. coli* O157:H7 to beef and its associated raw products when proper farm management techniques designed to reduce the prevalence and persistence of the pathogen in farm animals are implemented (Gunn *et al.*, 2007). Given that *E. coli* O157:H7 has a very low infectious dose (Tuttle *et al.*, 1999), the findings of this study indicate that these isolates may have severe public health implications on consumers if they are consumed in undercooked food products. Nevertheless, it has been reported that the actual incidence of *E. coli* O157:H7 in farms appeared to vary based on differences in geographical locations, variation in seasons during which sampling was performed, number of animals in the farm, the degree of hygienic practices and farm management practices in the farms as well as the sampling and isolation protocols used (Hancock *et al.*, 1998; Rugbjerg *et al.*, 2003; Gunn *et al.*, 2007). Notwithstanding this, the prevalence of *E. coli* O157:H7 in cattle farms from this study as well as previous reports (Ateba *et al.*, 2008; Ateba & Mbewe, 2011) provides microbiological

risk assessment data for the area.

Several studies have assessed the potential of *E. coli* O157:H7 isolates to harbour virulence gene determinants (Paton & Paton, 1998; Cobbold & Desmarchelier, 2001; Kobayashi *et al.*, 2001; Jo *et al.*, 2004; Çadırcı *et al.*, 2010). Despite the fact that the two shiga toxin genes (*stx*$_1$ and *stx*$_2$) as well as the intimin (*eaeA*) and enterohymolysin (*hlyA*) genes continue to receive a lot of attention, some epidemiological investigations of *E. coli* O157:H7 strains associated with disease outbreaks in humans have revealed that there are other genes as well as *stx*$_2$ variants that also play a significant role in the pathogenicity of this bacteria (Manning *et al.*, 2008; Sasaki *et al.*, 2011). In the present study, the *stx*$_2$ was more often detected (92%) among isolates than the *stx*$_1$.

This finding is similar to previous reports in which the *stx*$_2$ has been reported to be higly prevalent in bovine *E. coli* O157:H7 isolates (Cobbold and Desmarchelier, 2001). Despite this the detection of isolates with 8 different gene combinations indicates that the virulence gene profiles of pathogenic *E. coli* O157:H7 isolates are very diverse and thus the isolates in the present study may have severe public health complications in the area. In addition, it has been reported that there is a positive correlation between the production of shiga-toxin genes, particularly *stx*$_2$, by *E. coli* O157:H7 isolates and development of serious health complications such as HUS (Cimolai *et al.*, 1994; Boerlin *et al.*, 1998; Baker *et al.*, 2007). These pathogens may be transmitted to beef and its associated products if proper hygiene practices are not implemented in the abattoirs and this amplifies the need to control the level of contamination of animals with these pathogens.

Epidemiological investigations provide data that may assist in the detection and surveillance as well as control of bacteria strains that are responsible for both sporadic and outbreaks of disease in a given geographical location. Comparison of genetic fingerprints for isolates from difference

areas and/or species may provide an understanding of whether causative strains are resident or transient bacteria populations (Akinyemi *et al.*, 2010).

The findings of previous investigations carried out by Ateba and Mbewe (2014) provided baseline line data that is reliable for source tracking and that could be expanded on by using PFGE. PFGE is highly discriminatory and has been used in the development of guidelines that are very reliable in the interpretation of epidemiological data (Tenover *et al.*, 1995). In the present study, PFGE fingerprinting patterns grouped the pathogenic *E. coli* O157:H7 isolates into six clusters with three containing isolates from two or more farms. Given that analysis of PFGE patterns is based on similarity in the fingerprints generated (Lin *et al.*, 2008), the present findings indicate that a large proportion of the isolates were considered to be genetically related, regardless of the farms from which they were isolated. This study presents for the first time, the PFGE genetic profiles of South Africa *E. coli* O157:H7 isolates from cattle, and therefore provides opportunities for a large scale study involving isolates from different sources including humans in order to fully understand the epidemiology of the pathogen in the area. Data from such studies will provide an indication of the emergence of new clonal types and also enrich our understanding of the dominant genotypes in the area. Farms with identical or a closely related PFGE patterns indicate that the presence of these genetically similar strains in animal populations and farms may be as a result of the widespread spatial circulation of related or identical strains. These strains are therefore circulated between environmental matrices and animal populations through cross contamination which frequently occurs when standard operating procedues and proper hygiene are not implemented. Thus the presence of more then one type of PFGE on a farm is an indication of the propability of multiple sources of the pathogen to the farms.

In conclusion, this study has established that cattle reared in farms around the North-West province harbour genetically diverse and virulent *E. coli* O157:H7 which could serve as possible sources of zoonotic infections and environmental contamination. Although no cases of *E. coli* O157:H7 human disease has been reported in NW province, the need for suitable control measures to reduce the carriage in cattle cannot be underestimated, and moreso future research is needed to devise mitigation strategies at other stages in the transmission from animal to humans so as to limit or eradicate possible transmission to humans.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Funding: This research was funded by the North-West University Mafikeng Campus, South Africa and a scarce skills grant from the [National Research Fund (NRF)], South Africa under the grant number [NRF-UID 89228].

Acknowledgments: This study was supported by the North-West University, Mafikeng Campus, South Africa. We also acknowledge the financial support afforded by the National research fund, South Africa NRF – UID 89228. We also thank Dr L Ngoma and Mrs T Ateba for assistance with sample collection.

CHAPTER FOUR

ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES WITH LYTIC ACTIVITY AGAINST VIRULENT *ESCHERICHIA COLI* O157:H7

4.1 Abstract: Bacteriophages also known as phages can provide alternative measures for the control of *E. coli* O157:H7 that is currently an emerging foodborne pathogen of severe public health concern. This study was aimed at characterising *E. coli* O157:H7 specific phages as potential biocontrol agents for these pathogens. Fifteen phages were isolated and screened against 69 environmental *E. coli* O157:H7. Only three phages designated as V3, V7 and V8 displayed broad lytic spectra against environmental shiga toxin-producing *E. coli* O157:H7 strains. Transmission Electron microscopy (TEM) of the phages revealed that they all had isometric heads of about 73 – 77 nm in diameter and short tails ranging from 20 - 25 nm in diameter. Despite the fact that these phages were isolated from cattle in different farms, they displayed very high degree of similarities and were assigned to the family *Podoviridae* based on their morphology. Pulsed field gel electrophoresis (PFGE) genome estimation of the 3 phages demonstrated identical genome sizes of ~ 69 nm. The latent periods of these phages were 20 min, 15 min, and 20 min for V3, V7 and V8 respectively while the burst sizes were 374, 349 and 419 PFU/ infected cell respectively. Despite the fact that all the phages were relatively stable over a wide range of salinity, temperatures and pH values, their range of infectivity or lytic profile was rather narrow on environmental *E. coli* O157:H7 strains isolated from cattle faeces. This is the first study to provide information on the occurrence, morphologies and stability of *E. coli* O57:H7-specific phages in South Africa and demonstrated that *Podoviridae* bacteriophage bio-control agents are the dominant *E. coli* O57:H7-infecting phages in cattle. Phages in this

study possessed favourable characteristics and thus they can be exploited in the formulation of phage cocktails for the bio-control of *E. coli* O157:H7 in meat and other meat products.

4.2 Introduction

Despite the current decontamination strategies employed to inactivate bacterial pathogens especially *E. coli* O157:H7 in the food chain, cases of foodborne infections and even deaths associated with the consumption of food contaminated with *E. coli* O157:H7- occur regularly in many countries throughout the world (Browning *et al.*, 1990; Effler *et al.*, 2001; Reiss *et al.*, 2006). Most *E. coli* O157:H7 infections are associated with foods of animal origin and dairy products (Rangel *et al.*, 2005)**,** contaminated meat (Samadpour *et al.*, 1994), mince beef products (Rangel *et al.*, 2005) and fresh produce (Ackers *et al.*, 1998; Viazis & Diez-Gonzalez, 2011). However, ground beef is still the most frequently implicated source of *E. coli* O157:H7 infections and has been implicated in over 75% of outbreaks recorded worldwide (Rangel *et al.*, 2005; Viazis & Diez-Gonzalez, 2011). Although the majority of outbreaks occur in developed countries, some sporadic cases of infections and outbreaks have also been reported in developing countries (Browning *et al.*, 1990; Cunin *et al.*, 1999; Olorunshola *et al.*, 2000; Effler *et al.*, 2001; Gwavava *et al.*, 2001; Mandomando *et al.*, 2007; Ateba *et al.*, 2008; Chigor *et al.*, 2010; Lupindu, 2018). In contrast to developed nations, African countries are faced with the problem of poor health systems and inefficient surveillance or pathogen tracking systems exacerbating the burden of disease in these nations. In addition, the frequent occurrence of *E. coli* O157:H7 infections is a serious additional problem to disease-burdened nations that requires effective control measures.

In order to curb public health problems associated with *E. coli* O157:H7 contamination in meat, a number of preservation procedures and decontamination strategies have been utilized and continue to be developed and implemented (Sofos *et al.*, 1999; Hugas & Tsigarida, 2008; Newell

et al., 2010; Loretz *et al.*, 2011). Presently, conventional decontamination procedures, including but not limited to heat treatments, chemical wash, and irradiation, have been applied to meat carcasses to reduce bacterial contamination (Hugas & Tsigarida, 2008). Although some success has been reported with the application of these methods especially when combined in a multiple hurdle approach, limitations do exist for others. For instance, the application of some physical methods, such as steaming and hot water washing as bacteria control strategies has been observed not only to be costly but also to have a negative effect on appearance and quality of meat carcasses resulting in consumer rejection (Sofos & Smith, 1998). In the same way, the application of some chemical compounds as a decontamination procedure is also limited by several factors such as their non-selective mode of action against a wide range of microorganisms, negative effect on sensory properties of meat, the detrimental effects of chemical residues as well as their corrosiveness that could affect the food-processing environment (Sofos & Smith, 1998; Wheeler *et al.*, 2014). Moreover, the common use of chemical sanitizers has been shown to select for resistant strains rendering this procedure less effective (Berry & Cutter, 2000).

Against this background, there is the need to develop other environmentally safe strategies that are effective at reducing or eliminating bacteria pathogens particularly Shiga-toxin producing *E. coli* O157:H7 strains on beef carcasses and at the same time do not have a negative effects on the nutritional and sensory properties of the final raw food products unlike chemical and physical treatments. Recently, there has been growing interest in the use of lytic bacterial viruses, also known as bacteriophages, as biocontrol agents in humans, animals, plants and in food products because of their enormous antibacterial properties (Bueno *et al.*, 2012; Spricigo *et al.*, 2013; Zinno *et al.*, 2014). In addition to their bactericidal ability, they do not change the structure, odour and flavour of food products and are considered safe for humans to use since they only target susceptible bacterial cells. Moreover, bacteriophages can self-replicate, thus they do not

require repeated dosage, and they are present almost everywhere in the environment where they can easily be isolated (Bueno *et al.*, 2012; Spricigo *et al.*, 2013; Zinno *et al.*, 2014).

In the search for phages, it has been demonstrated that the chances of isolating them are higher where their hosts are highly prevalent, thus for phages infecting pathogens such as *E. coli* O157:H7 whose normal reservoir is the GIT of cattle, faeces of this ruminant represent an ideal source for isolation (Niu *et al.*, 2009). Numerous studies have reported the isolation of faecal-originating phages infecting *E. coli* O157 from countries all over the world including USA, UK, Canada and Spain (Kudva *et al.*, 1999; Niu *et al.*, 2009; Raya *et al.*, 2006; Viazis *et al.*, 2011a). The prevalence or occurrence of phages in animals that was reported in these studies varies from one region to another with a prevalence ranging from 10% - 28% but in some cases going up as high as 49% (Hallewell *et al.*, 2014; Anany *et al.*, 2015). The disparity in the abundance of phages in faecal sources could be as a result of monthly variations in the population of *E. coli* as well as *E. coli* O157:H7 in both the GIT of animals and the environment (Niu *et al.*, 2009). Nonetheless, these studies, have demonstrated that phages are most often present in the environment where their bacteria hosts are also present which then stimulates the need to continuously assess their bio-control potentials against antibiotic and virulent bacteria strains like *E. coli* O157:H7.

Given this renewed interest on phage biocontrol of pathogens in food products, several phage-based products that have shown considerable potential have been approved for use and are already commercially available in the market in continents like North America and Europe (Brovko *et al.*, 2012). LISTEX^{TM} P100 and Listshield^{TM} (LMP-103 TM) have been approved by the US Department of Agriculture (USDA) for the control of *Listeria monocytogenes*, and EcoShield^{TM} has also been permitted for the control *E. coli* O157:H7. Similarly, SalmoFresh, another phage-based preparation, received the highly desireable generally recognised as safe

(GRAS) recognition from the FDA in 2013 for direct applications onto meat products and fresh produce. In the West, all these products have been received and applied by the food producers as natural antimicrobials employed in reducing the risk of foodborne human diseases due to the consumption of contaminated foods (Hagens & Loessner, 2010; Monk *et al.*, 2010; Brovko *et al.*, 2012).

However, in Africa there is little or no information on the application of phages in the food sector, due to the fact that our understanding of bacteriophages and their possible biocontrol potentials is limited. In addition, the lack of reports on phage studies in most developing countries could be attributed to the ultramicroscopic nature of viruses making them difficult to handle in standard microbiology laboratories present in these countries. Therefore, phage research in Africa is relatively new and few studies have been reported in which these bio-control agents were isolated from the environment. Despite the fact that a few reports have described the isolation and characterisation of phages infecting *Bacillus*, *Pseudomonas*, *Enterobacteria* and *S. aureus* (Koko *et al.*, 2011; Basdew & Laing, 2015), to the best of our knowledge there have been no studies on the isolation and characteristics of phages infecting *E. coli* O157:H7. Therefore, this study is the first to present a detailed report on the isolation of strictly lytic (virulent) phages with antibacterial activity against environmental virulent *E. coli* O157:H7 isolates from cattle in some randomly selected farms in the North-West province (South Africa). In order for these phages to be considered as suitable candidates for biocontrol applications, they were further characterised based on their infectivity range, morphology and growth parameters, and the stability of the phage preparations *in situ* under different conditions was assessed as well as their genomic analysis. The main intention is to develop bacteriophage-based treatments that would be potentially useful in the biocontrol of *E. coli* O157:H7.

4.3 Materials and methods

4.3.1 Bacterial Strains

Phages were isolated from cattle faeces in farms using environmental *E. coli* O157:H7 strains that are listed in Table 4.1. These strains were also isolated from cattle faecal samples collected from eight (A, B, C, D, E, F, G, H) farms in the North-West province. These *E. coli* O157:H7 isolates were fully characterised and their virulent properties are outlined in a previous report (Akindolire & Ateba, 2018). A total of 14 *E. coli* O157:H7 strains were used as indicators to screen faecal samples for the presence of endemic phages. The selection was based on their genotypic variability as well as the different geographical locations from which the strains were isolated. *E. coli* O157:H7 strains were routinely grown in tryptic soy broth (TSB) at 37 °C and preserved at -80 °C in 20% (v/v) glycerol. During experimental procedures, bacterial stocks were held at room temperature for 1 hour and later sub-cultured on tryptic soy agar (TSA). Pure colonies were used as host strains for phage experiments.

Table 4. 1: List of *E. coli* O157:H7 strains and isolation sources used for this study

Farm/Source	Strain ID	PFGE group
A	A27	VI
	A40	I
B	B10	IV
	B56	IV
C	C9	V
	C13	I
E	E7	VI
	E13	V
F	F1	V
	F5	V
G	G17	I
	G5	I
H	H1	III
	H10	II

4.3.2 Enrichment and isolation of bacteriophages with *E. coli* O157:H7 as the host

The isolation and enrichment of phages was based on a previously described method (Twest & Kropinski, 2009) with modifications. Cattle faecal samples were collected between May 2017 to August 2017 from cattle in farms in North-West province, South Africa and used as bacteria host strains for phage isolation. Ten grams of faecal samples was dissolved in 100 ml of lambda diluent and mixed thoroughly. Faecal suspensions were centrifuged at 10 000 x g for 10 mins to remove particulate matter. The clarified sample (10 ml) was added to a 10 ml overnight culture of each of 14 *E. coli* O157:H7 host strains in double-strength TSB with 2 mM $CaCl_2$. The suspension was incubated for 48 h with gentle shaking at 50 rpm at 37 °C. After incubation, the suspensions were centrifuged at 5,400 x g for 15 min at 4 °C and supernatants were carefully filtered through 0.22 µm Millipore syringe filters (Fischer Scientific, Ottawa, ON) and the clarified crude lysates (filtrates) were stored at 4 °C.

The spot-test technique was used to detect for phage activity against *E. coli* O157:H7 (Sambrook *et al.*, 1989). Briefly, 100 µl of an overnight culture of the bacterial hosts were mixed with 3 ml of molten top agar (0.6% w/v agar) held at 50 ºC and then overlaid immediately onto Modified nutrient agar (MNA) plates so as to create a bacterial lawn and allowed to solidify for 15 mins. Phage activity was tested in the enriched samples by spotting with 10 µl from each lysate on the top of soft agar containing the bacterial lawn and allowed to adsorb for about 10 min. Plates were incubated at 37 ºC upside up for 24 h. After incubation, plates were observed for zones of cell killing or bacteria growth inhibition in the bacterial lawns at the sites where the lysate was dropped. If present, the cleared spots were collected from the overlays (soft-agar layers) by cutting using a one ml pipette tip and placing each plaque individually in a 2 ml Eppendorf tube containing 300 µl of phage buffer. The tubes were stored overnight at room temperature to allow

phage particles to diffuse out from the soft agar. The mixture was later filtered through 0.22 µm Millipore membrane syringe filters (Fischer Scientific, Ottawa, ON).

4.3.4 Purification of phages

Isolated phages were purified using the soft agar overlay method (Sambrook *et al.*, 1989). Ten-fold serial dilutions of each lysate that was positive for the spot test was prepared, and 100 µl of each dilution was mixed with 100 µl of an overnight culture of the host bacterium for 10 - 15 mins at 30 ºC. Three millilitres of molten top agar held at 50 ºC was added to the mixture and overlaid onto MNA agar plates and the plates were incubated at 37 ºC for 16 - 24 h in upright positions. Plaques from different bacteria hosts with varying sizes and morphologies were picked from the overlays and placed individually in 300 µl phage buffer [λ-buffer: 5.8g/L NaCl; 2g/L $MgSO_4$. $7H_2O$; 10ml/L 1M Tris-HCl (pH7.5)] and held overnight incubation at 4 ºC for phages to diffuse out of the agar. Bacteriophages in the lysates were purified three successive times in order to obtain single plaques with homogeneous plaque morphologies. Ten-fold serial dilutions of the purified phage lysates were prepared and phages titres were determined and tested using the soft agar overlay technique. The purified phage lysates were stored at 4 ºC.

4.3.5 Determination of the lysis profile of isolated phages

The isolated bacteriophages were screened for their ability to infect host bacterial cells in order to determine their infectivity range or lysis efficiency. This assay is based on the ability of the phage to either produce a clear plaque, turbid plaque or no lysis against a specific host cell. A total of 69 environmental *E. coli* O157:H7 strains previously isolated from cattle and characterised were used in this assay that employed the spot test (Carlson, 2005). Bacterial lawns of all the 69 *E. coli* O157:H7 strains were prepared on modified nutrient agar (MNA) and 10 µl droplets of phages lysates with titres of 1 x 10^7 PFU/ml were spotted on these lawns. The plates were incubated at 37 ºC for 24 h and checked for the presence of plaques. The most efficient

phages based on the lysis profiles, plaque clarity and size thus displaying zones of lysis against most of the isolates were selected for further studies.

4.3.6 Propagation of phage in liquid medium

Phage propagation was performed using the liquid propagation method (Sambrook *et al.*, 1989). Briefly, for each phage, 1 ml of the mid-exponential phase (OD_{600} = 0.45-0.55) culture of the host bacterium was inoculated to 100 ml of fresh modified tryptic soy broth (mTSB) containing 10 mM $CaCl_2$. To this 100 µl of phage lysate with at least 1 x 10^8 pfu/ml (MOI ~ 0.1) was added and incubated aerobically at 37 °C with shaking at 170 rpm for 6 - 8 h or until lysis occurred. The mixture was centrifuged at 6000 x g for 10 mins at 4 °C. The supernatant was neutralized with 0.1 N NaOH (pH = 7.0) and filtered with 0.2 µm syringe filter (Fischer Scientific, Ottawa, ON). The titres of phages present in the lysate was determined using the double layer agar method (34). Aliquots of 100 µl from each 10-fold serially diluted lysate was mixed with 100 µl of the bacterial host suspensions and 3 ml of 0.6% (w/v) top agar was added to the tubes. The contents of the tubes were immediately poured unto Modified nutrient agar (MNA) agar plates and plates were allowed to solidify for 15 mins. Plates were incubated aerobically at 37 °C for 24 h. Phage titration was done in triplicate. High titre phage lysates were stored at 4 °C until they were concentrated using polyethylene glycol (PEG).

4.3.7 Concentration and Purification with Polyethylene Glycol (PEG)

Phage concentration and purification was performed using previously described methods (Yamamoto *et al.*, 1970; Ackermann, 2009b). DNAse 1 and RNAse A to a final concentration of 1 µg/ml respectively were added to lysates to degrade residual bacterial DNA and RNA while 0.5 M NaCl was later added to the mixture and incubated at 40°C for 1 h. The suspension was centrifuged at 6000 x g for 10 min at 4 °C and the phage-containing supernatant was transferred into a clean flask. High titre propagated phage lysates were precipitated by adding PEG 8000

gradually with constant stirring, to a final concentration of 10% w/v. The mixture was incubated at 4 °C overnight to allow precipitation of the phage particles. A PEG pellet, containing phage particles, was obtained by centrifugation at 11,000 *x g* for 10 mins and at 4 °C. Supernatants were carefully removed using a pipette and care was taken to ensure that the pellet was undisturbed. The pellet was washed twice in 0.1 M ammonium acetate (pH=7.0) and re-suspended in 0.5 ml sterile distilled water. The re-suspended pellet was incubated overnight at 4 °C in order to soften it. The phage suspension was stored at 4 °C until transmission electron microscopy.

4.3.8 Transmission Electron Microscopy

The morphology of the isolated phages was investigated using the transmission electron microscope (Ackermann, 2009b). Sample preparation and electron microscopy was performed by Dr. Anine at the North-West University, Potchefstroom Campus in South Africa. Briefly, phage pellet obtained after PEG precipitation and ammonium acetate washing was re-suspended in 50 µl lamda diluent. A drop of the suspension was placed on carbon-coated formvar grids and allowed to stand for 2 min. The suspension was stained with 1% (w/v) ammonium molybdate (pH=7.0) for 5 min and excess fluid was drawn off with filter paper. Grids were allowed to air dry and were then examined with a FEI Tecnai G2 20 S-Twin transmission electron microscope operating at 120kV and a magnification range of 20000 – 100000. Micrographs were taken with a Gatan bottom mount camera using Digital Micrograph software at 80 kV and a magnification range of 20,000 to 250,000.

4.3.9 Phage stability under different chemical and physical conditions

Effect of different incubation temperatures on phage stability

To determine the effects of different incubation temperatures on phage stability, phage lysates of known titres were incubated at 45 °C. Titre of the phage was determined by the soft-agar overlay technique after 1 h incubation. The experiment was repeated at temperatures of 50 °C, 55 °C and 60 °C. Phages that were diluted in phage buffer was used as a control. Each treatment was performed in triplicate. The inoculated plates were incubated at 37 °C for 24 h and after 24 h the number of plaques was counted for each treatment and results were recorded.

Effect of different pH levels on phage stability

The pH stability of the phage was determined using a previously described method with some modifications (Harley & Prescott, 1993). Briefly, the pH of TSB was adjusted to the following ranges: 2, 4, 8, 10, and 12 by the addition of either 1M HCL or 1M NaOH. Aliquots of 900 µl of the pH adjusted TSB were transferred into Eppendorf tubes and 100 µl of phage lysates were added to the tubes. The tubes were gently mixed and left to stand at room temperature for 18 h. After 18 h the titre of the phages in each sample was determined using the soft agar overlay technique (Sambrook *et al.*, 1989). In order to achieve this, 100 µl of phage suspension was mixed with 100 µl of overnight culture, and the mixture plated using the double layer agar techniques. Phage diluted in TSB was used as a control. Each treatment was performed in triplicate. All plates were incubated aerobically at 37 °C for 24 h, the number of plaques was counted to determine phage titre for each pH treatment, and results were recorded.

Effect of salinity on phage stability

The effect of different saline concentrations on the stability of the phages was determined (Harley & Prescott, 1993). Aliquots of 100 µl of each phage lysate was added to 900 µl of TSB containing 0.5%, 10% and 15% NaCl respectively and incubated aerobically at 37°C for 18 h. Phage diluted in TSB was used as a negative control in the experiment. After incubation, both the treated and untreated phage lysates were plated using the soft-agar overlay technique. The number of plaques were counted and used to determine the phage titre for each of the different treatment and control samples.

Effect of chloroform on phage stability

The effect of chloroform on the stability of phages was determined using a previously described method but with minor modifications (Harley & Prescott, 1993). Optimal phage dilutions of 1 x 10^5 were prepared in lambda diluent. Aliquots of 1 ml of each dilution was treated with 10% (v/v) chloroform and stored at room temperature (25 °C) with gentle shaking for 1 hr. The suspension was centrifuged at 10000 x *g* for 10 mins at 4 °C. The phage titre of the supernatant was determined using an overnight culture of *E. coli* O157:H7 G17 as the host bacterium. A phage diluted in lambda diluent was used as a control sample. Treatments were performed in triplicates. Plates were incubated at 37 °C for 24 h and phage titres were calculated using average values for each treatment.

4.3.10 One-step growth curve

Burst sizes and latent periods of selected phages were determined by a one-step growth experiment according to a previous method (Ellis & Delbrück, 1939). Phages were diluted in 9.9 ml TSB in order to obtain 10^6 pfu/ml. The temperature of the diluted phage lysate and overnight culture was maintained at 37 °C in a water bath. Phages were added to host bacterium at MOI of 0.1 and the mixture was mixed well and incubated at 37 °C while noting the time 0. Two large tubes containing 9.9 ml of TSB were prepared for 10^2 and 10^4 dilutions and kept at 37 °C. After

10 mins of incubation, 100 µl of the adsorption mixture was transferred into the 10^2 tube and mixed thoroughly. Using a clear and sterile pipette, 0.1 ml of the mixture was transferred from the tube with dilution factor 10^2 to tube 10^4. The contents in the tube was mixed thoroughly and incubated at 37 °C for 90 mins. After 20 mins, samples were collected every 5 mins for 90 min and phages were titrated in each respective sample as previously described (Ellis & Delbrück, 1939). The relative burst size was determined by dividing the number of virus-like particles released from the cell with the number of virus particles initially added. The relative burst size obtained at different time intervals were plotted against time to determine the latent periods. The average counts obtained from triplicate treatments per sample, were plotted to obtain the one-step growth curve (Ellis & Delbrück, 1939).

4.3.11 Pulsed filed gel electrophoresis (PFGE) to determine bacteriophage genome size

Phage genome size was determined by pulsed field gel electrophoresis (PFGE) using a previous method (Sambrook *et al.*, 1989). Briefly, 200 µl of purified and concentrated phage suspension was mixed with 10 µl of 20 mg/ml proteinase K (Sigma-Aldrich, USA) solution and 200 µl of 1.2 % (w/v) molten plug agarose (Bio-Rad, Hemel Hempstead, UK) in TE buffer. These contents were mixed properly and dispensed into plug molds (BIO-RAD, UK) and allowed to solidify. Solidified plugs were removed and placed in Eppendorf tubes containing 5 ml of lysis solution [50 mM EDTA pH 8.0, 50 mM Tris-HCI (pH 8.0), 1 % (w/v) sarcosyl and 25 µl of 20mg/ml proteinase K solution] all obtained from Sigma-Aldrich, UK. Each tube was incubated at 55°C for 2 h with constant and vigorous agitation at 175 rpm. The lysis solution was discarded and 5 ml of warm sterile distilled water was added to each tube. The contents of the tubes were incubated at 55°C for 15 min, and then plugs were washed 3 times with TE buffer [20 mM Tris-HCI (pH 8.0), 50 mM EDTA (pH 8.0)] and incubated at 55°C for 15 mins between each wash. Each plug was cut into 3 mm slices, transferred to the wells of a 1 % (w/v) agarose (Bio-Rad,

UK) gel prepared in 0.5 X TBE (Sigma-Aldrich, UK). Chromosomes isolated from *Saccharomyces cerevisiae*, strain YPH80 and supplied by Sigma, UK was included in each gel and used as a PFGE marker. The wells were sealed with molten 1 % (w/v) agarose and allowed to set, after which they were transferred to a Bio-Rad CHEF-DR® II electrophoresis system (Bio-Rad Laboratories, UK). Electrophoresis was performed in 0.5 X TBE at 6 V/cm for 18 h with incremental pulses of 2.2 - 54.2 s and with the buffer circulating at 14°C. Gels were stained in 1 µg/ml of ethidium bromide for 30 min and images were captured with a ChemiDoc XRS Imager (Bio-Rad, UK) using the Quantity One program.

4.3.12 Restriction Enzyme analysis of bacteriophage DNA

The remaining bacteriophage genomes in agarose plugs previously prepared in the PFGE procedure were subjected to the Restriction Fragment Length Polymorphism (RFLP) protocol by digesting them with different restriction endonucleases obtained from Biolabs (New England Biolabs, UK). Restriction enzymes *Eco*RI, *Hind*III, *SSP*I and *NDE*I were used in the RFLP and the digestion was carried out according to the manufacturer's instructions. The reactions were incubated at 37°C for 16h. The plugs were then subjected to the PFGE for 16-18 h using a pulse time of 1s – 15s and 6V/s alongside with Lambda PFGE marker. Restriction digestion banding patterns were compared visually.

4.4 Results

4.4.1 Isolation of bacteriophages

During this study, 152 cattle faecal materials were screened separately for the presence of bacteriophages using environmental *E. coli* O157:H7 strains as hosts. The presence of different bacteriophages specific to *E. coli* O157:H7 from 152 faecal samples collected from cattle farms in the North-West Province, South Africa, was detected using the spot test and double layer agar techniques. Of the 152 samples analysed, only 15 possessed phages that were active against the

environmental *E. coli* O157:H7 strains. These 15 different phages were chosen for further purification, propagation and characterization. Details of phages isolated from the different farms are shown in Table 4.2.

Table 4. 2: Isolation of *E. coli* O157:H7 bacteriophages

Farm	Total Sample	No of Phage	Phage code
A	25	-	
B	47	4	V1, V2,V3, V4
C	50	3	V5, V6, V7
D	30	6	V8, V9, V10, V11 V12, V13,V14, V25
Total	152	15	

Table 4. 3: Morphological characteristics of the isolated phages

Phage	Plaque Morphology	size (mm)	Titre (PFU/ml)
V3	Clear, small	1.5	$2x\ 10^{10}$
V7	Clear, medium	2	2.5×10^{8}
V8	Clear, medium	2	1×10^{10}

4.4.2 Lytic profile

The lytic profile of the isolated phages was determined against a total of 69 different environmental *E. coli* O157:H7 strains that were previously obtained and characterised (Akindolire & Ateba, 2018). The results in Table 4.4 showed that a large proportion (86.9%) of the environmental stains were resistant to phage activity since no plaques were formed when phages were spotted on the bacterial lawn. However, some of the phages demonstrated narrow host range and were able to produce areas of clear zones on bacterial lawn. Phages V6, V12, V13, V14 and V15 were each able to lyse only two bacterial hosts while phages V1, V2, V9, and V11 were able to lyse 3 *E. coli* O157:H7 strains. Contrary to the low levels of virulence displayed by these phages, three phages (V3, V7 and V8) demonstrated the broadest spectrum of activity because they infected between 7 and 10 *E. coli* O157:H7 host strains. These three super-phages were selected for further characterisation.

Figure 4. 1: Plaque assay of phage lysates spotted on a bacterial lawn of *E. coli* O157:H7 G17 as indicator strain

Table 4. 4: Lytic effect of phages on *E. coli* O157:H7 strains. + = Lysis; - = No plaques

E. coli O157:H7 strains	Bacteriophages														
	V1	V2	V3	V4	V5	V6	V7	V8	V9	v10	V11	V12	V13	V14	V15
B17	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
B46	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
B48	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B52	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
C2	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
C9	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-
C10	-	-	-	-	-	-	+	-	-		-	-	-	-	-
C13	+	+	+	+	+	-	+	+	-	+	+	-	-	-	-
C29	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
E1	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
E2	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
G17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
G20	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H1	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
H2	-	-	+	-	-	-	+	+	-	+	-	-	-	-	-
H4	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
H5	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
H6	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-
No of sensitive isolates	3	3	7	4	4	2	9	7	3	5	3	2	2	2	2

All phages have unique lytic profile. Twelve bacterial strains: B 52, B46, B48, C2, C10, C29, E1, G20, H1, H4, H5 and H12 were lysed by only by one phage while B17, C9, H2, and H6 were each lysed by 2 or more different phages. *E. coli* O157 strains C13 and G17 exhibited the highest level of susceptibility to the phages and were lysed by 9 and 15 phages respectively.

4.4.3 Morphological characterisation of phages

Transmission electron microscopy (TEM) of the phages displayed morphologically similar structures. All the obtained phages possess icosahedral heads and short non-contractile tails with tail fibres (Fig 2). Based on these morphological characteristics, these phages were assigned to the family *Podoviridae* according to the classification system of Ackermann (Ackermann, 2003). The diameters of the capsids of V3, V7 and V8 were 75± 3.6 nm, 73±1.2 nm, and 77±3.1 nm respectively. Phages V3. V7 and V8 also had tail lengths of 23±3.1 nm, 25 ±3.2 nm and 20±1.0 nm respectively.

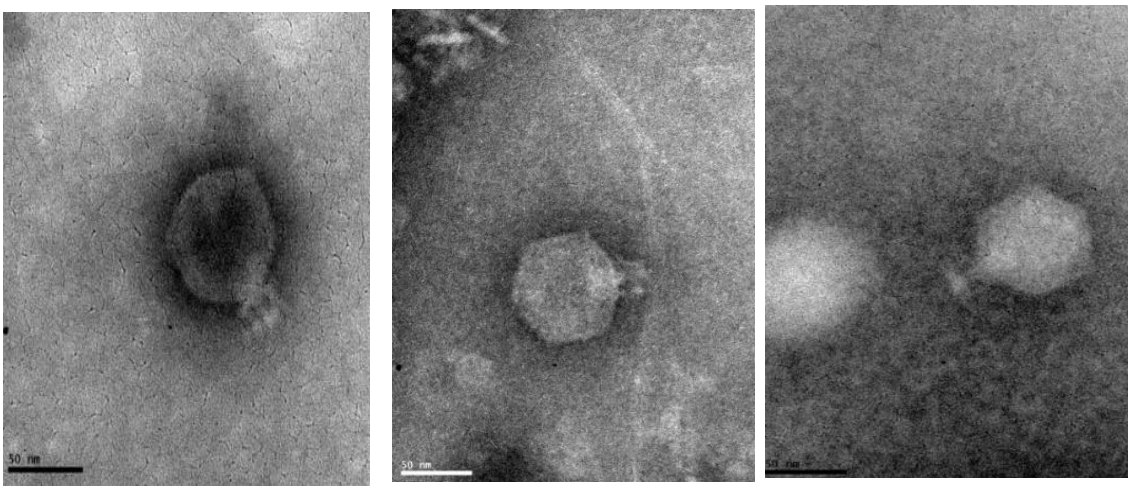

Figure 4. 2: Electron micrographs of *E. coli* O157:H7 phages. Bar = 50 nm. (A) V3, (B) V7, (C) V8

4.4.4 Phage growth characteristics

The single-step growth curve for the determination of the latent periods which is the time interval between the adsorption and the beginning of burst, and the burst sizes for the 3 phages is represented in figure 4.3. Phage V8 had the highest burst size of 419 phages per infected cell among the 3 phages with a latent period of 20 min, this was followed by V3 that had a burst size of 374 phages per infected cell with the same latent period as phage V8 (20min). Nevertheless, phage V7 had the lowest burst size of 349 phages per infected cell with a latent period of 15 min.

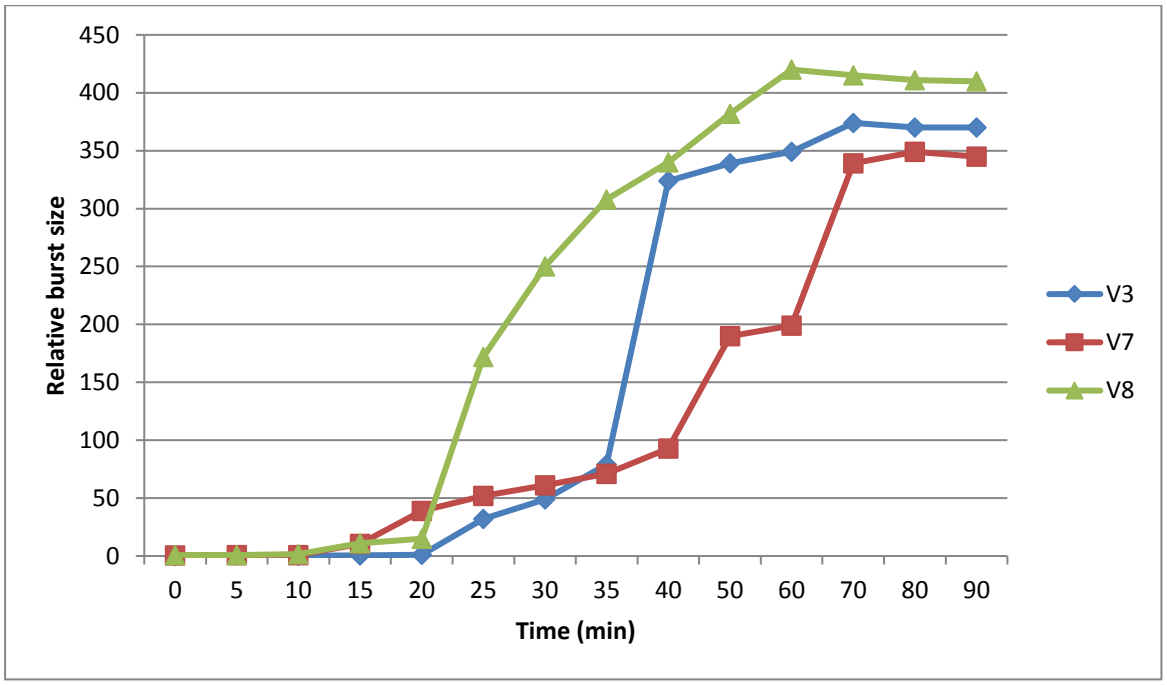

Figure 4. 3: One-step growth curve of phages V3, V7 & V8

4.4.5 Phage stability under different chemical and physical conditions

Temperature

Results showed that decreasing the incubation temperature resulted in an increase in phage titres particularly at temperatures below 60 °C (Fig 4.4). It was evident that 55 °C was the optimum temperature for V3 phage replication while phage V7 was most stable at 45 °C with just 20% reduction in titre, and that there was a drop in titres at temperatures above 60 °C. Exposure to

temperatures above 55 °C was the most damaging with a great reduction (100%) in phage titres for phage V7 and V8 while V3 was the most thermal stable among the phages with lowest reduction in titre even at 55/60 °C. Overall results showed that propagation of these phages is negatively affected by increased exposure to high temperatures.

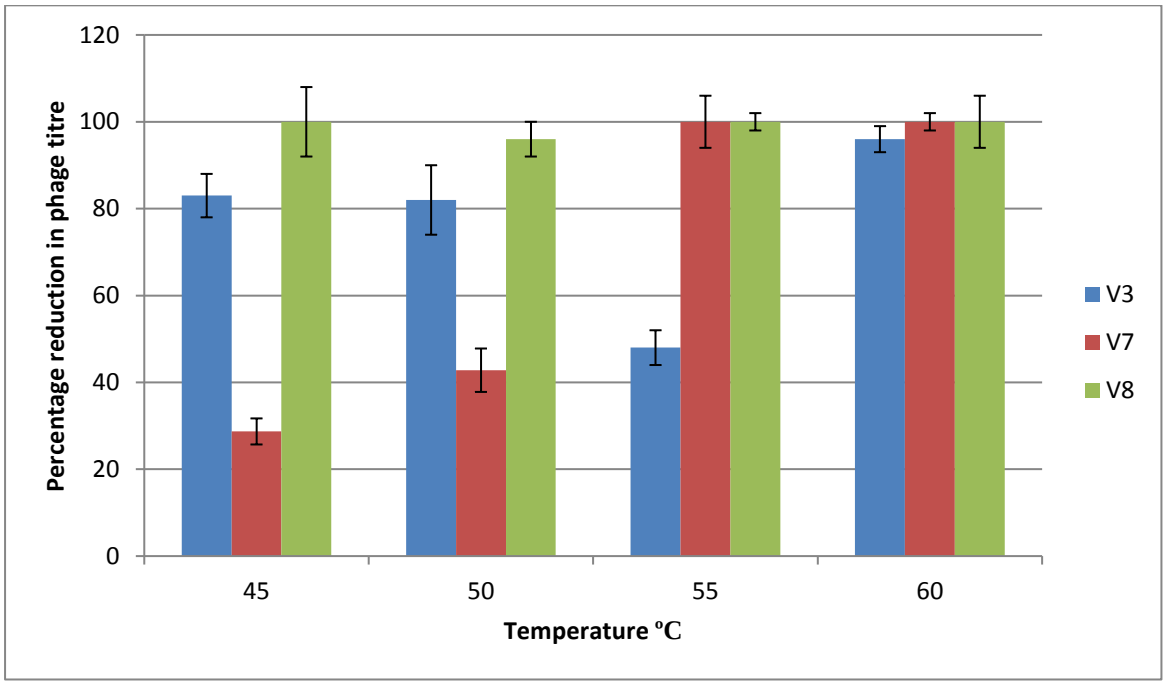

Figure 4. 4: Thermal stability of the isolated phages. Error bars indicate standard deviation among the triplicate samples

pH

The effect of the pH of the medium on the stability of phages was determined by suspending the phage in pH adjusted TSB medium and the percentage reduction in phage titre was determined. Overall, most of the phages were more sensitive to acidic than alkaline conditions. Phage particles showed great stability within the pH range of 8.0 to 10.0 while a considerable decrease in titres was observed at pH values below 6.0 and above 10.0. Phages V7 and V8 displayed similar pH stability but phage V3 was the least stable with a huge drop in the titre even at pH 6 and 8. At pH 2, 4 and 12, 100% inactivation of the 3 phages was observed (Fig 4.5).

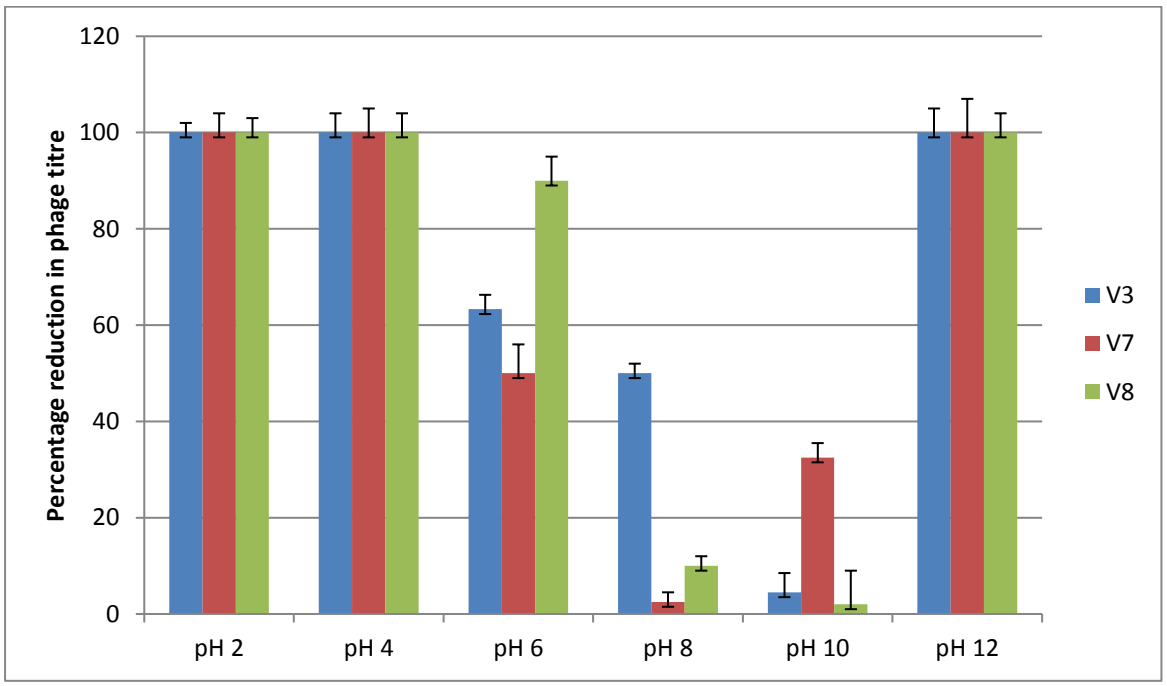

Figure 4. 5: pH stability of the isolated phages

Salinity

All the three phages showed similar activity to the different salt concentrations tested (Fig 4.6). Phages retained stability in the presence of 0.5% NaCl with less than 20% reduction in titres. Similar stability was displayed by phages V3 and V8 in the presence of 5% NaCl while phage V7 lost over 60% activity. In 10% NaCl, titres of the three phages were reduced by over 60%. Surprisingly, all the tested phages survived exposure to 15% NaCl albeit at a greater loss of activity.

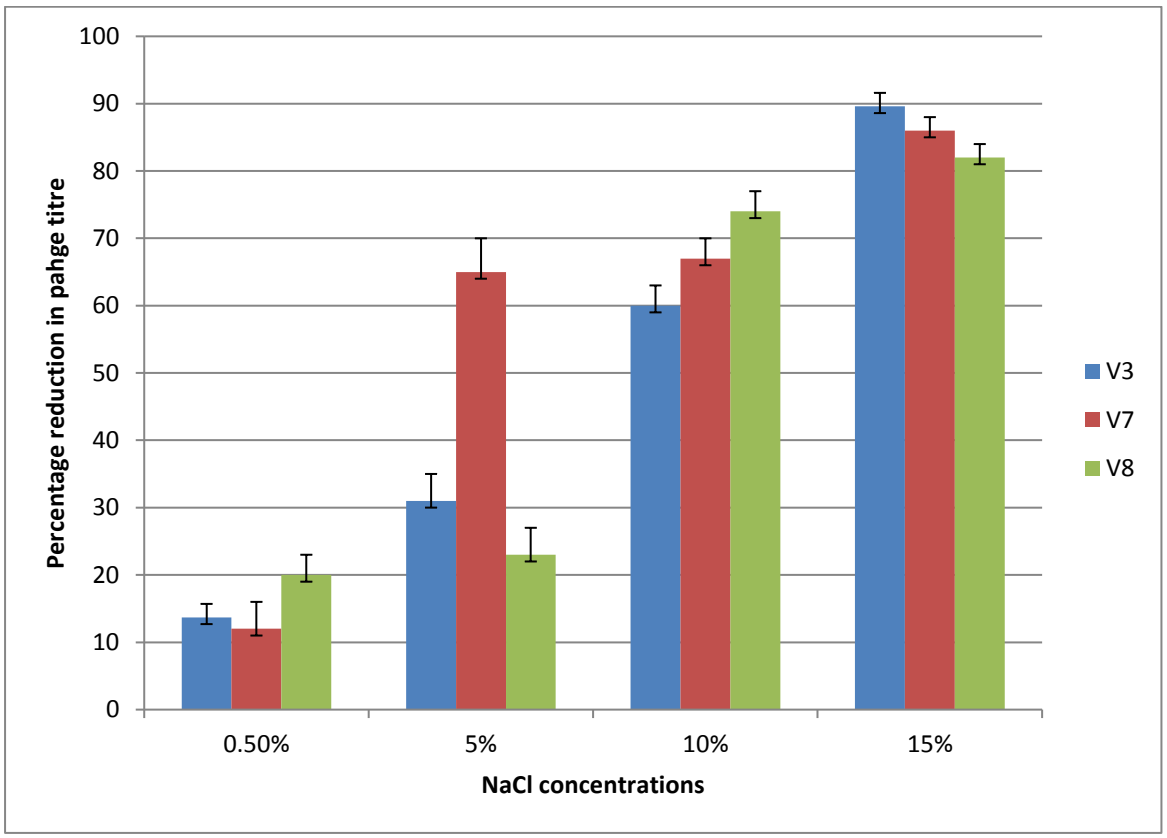

Figure 4. 6: Stability of the isolated phages under different NaCl concentrations

Chloroform

Chloroform exposure did not have a negative impact on phage titres of V3 and V7. They remained stable in 10% chloroform after incubation for 1 h. However, the addition of chloroform resulted in 40% reduction in phage titre of V8.

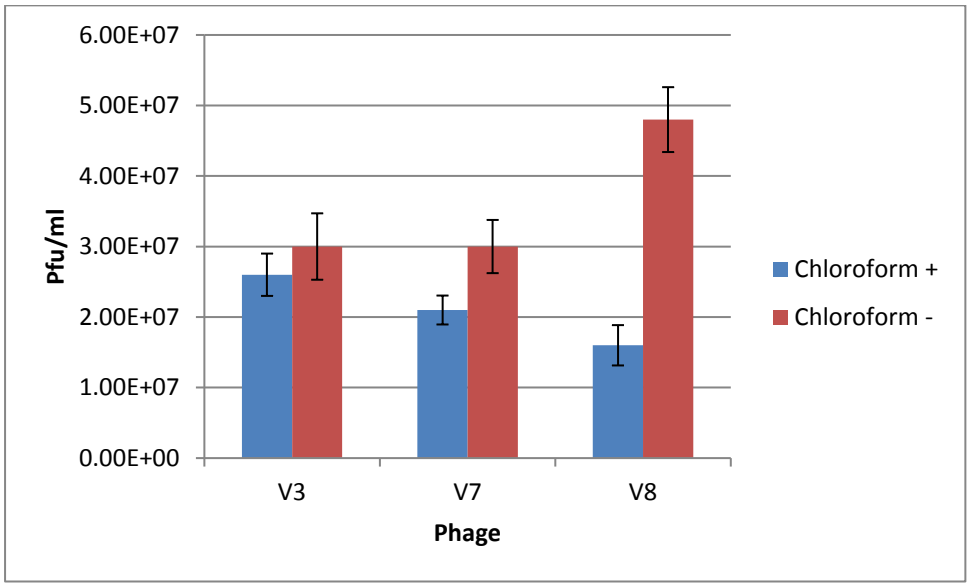

Figure 4. 7: Effect of chloroform on the stability of the isolated phages

4.4.6 Genomic characterisation of phages

PFGE determination of phage genome size

Gels showed that phages contained a discreet single DNA band indicating the purity of the phage stock and the band corresponding to the genomes of these phages. The genome sizes of the newly isolated phages were estimated to be ~ 69 Kb (Fig 4.8).

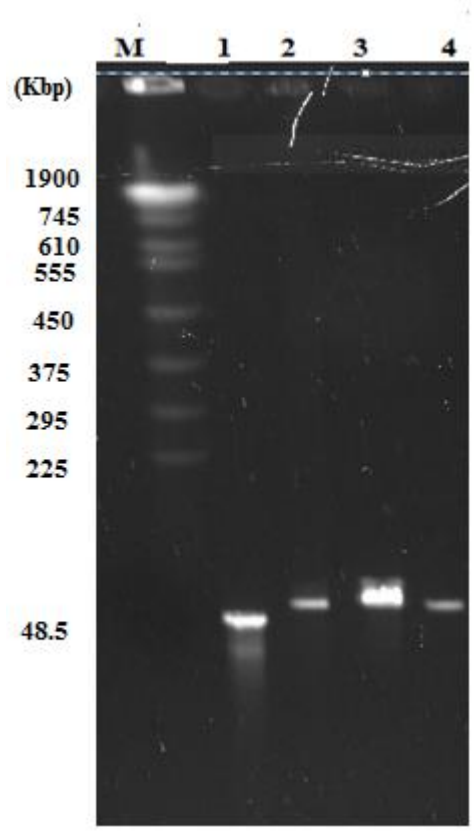

Figure 4. 8: PFGE of phages prepared in agarose plugs without DNA extraction. Lane M: Yeast DNA Marker, Lane 1: uncut lambda; lane 2: V3, Lane 3: V7, Lane 4: V8

4.4.7 Restriction endonuclease pattern

The phages in agarose plugs were digested in-situ with different restriction endonucleases. Agarose electrophoresis on 1% gels of the digested DNA indicated that the phages had double stranded DNA, and the enzymes *Ssp*I and *Nde*I produced the best digestion patterns (Fig 4.9). Enzymes *Hin*dIII, and *Eco*RI had no restriction sites on the phage DNA (data not shown). The restriction enzymes *Ssp*I and *Nde*I could not differentiate the phages based on banding patterns as similar patterns were observed in all of them, nevertheless the host range analysis and TEM analysis revealed subtle differences among them. Moreover the phages were obtained from different farms in the province.

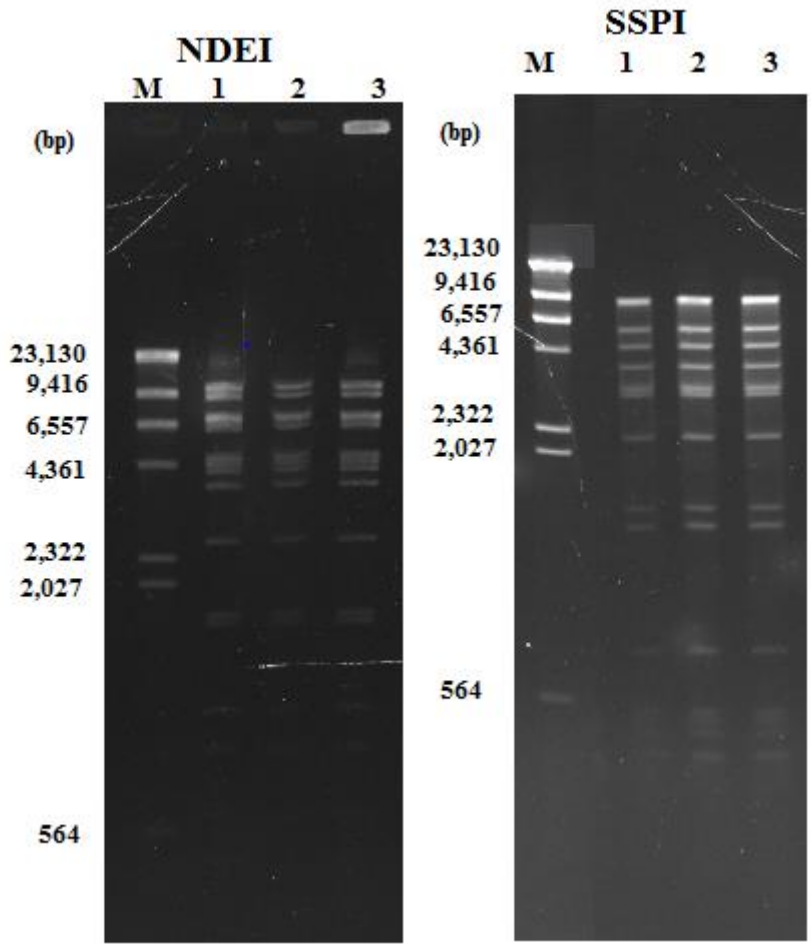

Figure 4. 9: Restriction digestion of phage using *Ssp*I and *Nde*I. M= marker; Lanes 1, 2, 3 = V3, V7, V8 respectively

4.5 Discussion

Bacteriophages have emerged as novel biotechnological tools that could be exploited in the control of foodborne pathogens such as *E. coli* O157:H7 (O'Flynn *et al.*, 2004; Hagens & Loessner, 2010; Mahony *et al.*, 2011). Key to the successful application of phages in food safety is ensuring that potential candidates are strong, stable and strictly lytic against the targeted pathogens. Therefore, newly isolated phages are screened and made to undergo a variety of phenotypic and genotypic characterisations so as to ensure that they meet these requirements. In this study, the main objective was to isolate and propagate virulent phages against *E. coli* O157:H7 so that they could be characterised in terms of biological and physical properties that

can be exploited for biocontrol purposes. For that purpose, cattle faecal material where the target bacteria are believed to be in abundance was used as a source for isolation of phages specific to *E. coli O157*:H7 (Niu *et al.*, 2009; Clokie *et al.*, 2011). Overall, 15 lytic *E. coli* O157:H7 phages designated V1 to V15 were successfully isolated from the 152 processed faecal samples using environmental host strains. These phages were selected based on their ability to form a clear zone of lysis (plaques) on bacterial lawn since this suggested that they were virulent, as these are preferred in biocontrol applications. The host strains were previously characterised in another study and selected after phenotypic and genetic characterisation to ascertain their diversity. The isolation rate of the phages in the study was relatively low in comparison to other studies that have reported high levels of *E. coli* phages in cattle faeces (Morita *et al.*, 2002; Viazis *et al.*, 2011a). One plausible explanation might be that there were low numbers/concentrations of the host strains in the faecal samples resulting in greatly reduced chances of phage detection, for the prevalence of phages is dependent on the host density threshold (Payne & Jansen, 2003; Niu *et al.*, 2009). This possibility is further affirmed by the fact that different samples were sourced for the isolation of the host strains and the phages because both isolations could not be done concurrently and therefore the occurrence of *E. coli* O157:H7 in the phage isolation samples was unknown in this study. Studies have previously described the isolation of lytic phages against pathogenic *E. coli* O157:H7 from different sources such as sewage, cattle faeces and environmental samples all over the world (Kudva *et al.*, 1999; Niu *et al.*, 2009; Viazis *et al.*, 2011b; Hallewell *et al.*, 2014; Litt & Jaroni, 2017) and hence demonstrated their abundance in nature. However, to the best of our knowledge, this is the first reported isolation and characterization of virulent phages against *E. coli* O157:H7 in South Africa/ Africa.

The lytic spectra of a phage, which can be described as one of the most significant biological characteristics of a phage, is defined by what bacteria genera, species and strains it can kill (Kutter, 2009). In the biocontrol applications of phages, it is important that phage candidates

which are virulent with broad lytic spectra are selected for, rather than temperate phages and those with narrow lytic activity (Carlson, 2005; Hagens & Loessner, 2010). This is due to the ability of temperate phages to transfer of virulence or antibiotic resistance genes (Fortier & Sekulovic, 2013; Haaber *et al.*, 2016) and narrowly lytic ones are unable to cover many bacterial strains. The lytic profiles of the phages in this study were to some extent distinguishable from each other when different host strains of environmental *E. coli* O157:H7 were used, indicating the diversity among the phages. The lytic spectra of the phages however, would be considered relatively narrow, since out of the 69 environmental host strains that were screened against, only 7 to 10 strains were lysed consistently by 3 of the 15 phages. Phages with a narrow host range have also been widely isolated against *E. coli* (Kulikov *et al.*, 2012) and other species of bacteria, including the lytic phage of *Achromobacter* - phiAxp-3 (Ma *et al.*, 2016), *Salmonella* infecting phage P7 (Bigwood *et al.*, 2008) FGSSA 2 (Carey-Smith *et al.*, 2006) and N4-like *Pseudomonas* infecting phages (Ceyssens *et al.*, 2010). Narrow spectrum of activity against bacteria hosts has been reported as a unique characteristic common to the N4-like phages (Kulikov *et al.*, 2012). The newly isolated phages are not only similar to the N4-like phages in terms of their host range; they also displayed morphological resemblance, suggesting possible relatedness to this group of phages.

Bacteria strains C13, E2 and G17 were the most susceptible, but a significant proportion of the bacterial strains were resistant. The differences in susceptibility of the bacterial strains could be due to various host factors/mechanisms that bacteria have employed to overcome the infection and killing action of phages. One such mechanism is losing phage receptor from bacterial surface. Phage attachment to a specific receptor on the bacterial surface is the first essential step in phage infection and any change in the surface profile either by regulated expression or mutation in the receptors will result in phage insensitivity (Shin *et al.*, 2012). Another host factor contributing to bacterial resistance is the presence of restriction endonucleases in some bacterial

cells (Seed, 2015). These restriction systems act by degrading foreign viral DNA with subsequent obstruction of the phage replication process. A further possibility, but in this case not related to host factor, is if the bacterial host strains are infected with temperate phages that render them lysogens and confer lysogenic immunity (Obeng *et al.*, 2016). Such lysogenized bacteria will be immune to super-infection by a related phage. In this study, it appeared that most of the bacteria strains employed in lytic profile assay could have possessed any of the resistance mechanisms and thus the wide resistance displayed by them. However, despite the observed bacterial resistance and the consequential narrow host range of the phages, they are not useless because they can still be used in developing phage cocktails to increase the breadth of bacteria strains that they can control. Three of the phages (V3, V7, and V8), although demonstrated moderate capability to lyse environmental strains of *E. coli* O157:H7, had the broadest lytic profile among the isolated phages and were selected for further characterisation.

The TEM analysis that is comparable to Gram-staining in bacteriology is the fastest and easiest characterisation method that aids in the attribution of phages to families and for the identification of novel phages (Ackermann, 2012; Aprea *et al.*, 2015). Observation under TEM revealed that all the three phages, despite being isolated from different farms, belonged to the family *Podoviridae* that is characterised by icosahedral head and short non-contractile tails. This family is one of the three main families of the *Caudovirales*, otherwise referred to as the tailed phages. Although earlier studies have demonstrated that the tailed phages (*Caudovirales*) represent the most diverse, numerous and wide spread of all bacterial viruses, the *Podoviridae* family are the least represented, accounting for about 14% of this group (Ackermann, 1998; Ackermann, 2001). Similarly, more recent studies investigating the morphology of environmental *E. coli* O157:H7 bacteriophages have supported the dominance of the other two families – *Myoviridae* and *Siphoviridae* (Raya *et al.*, 2006; Lu & Breidt, 2015; Niu *et al.*, 2012a). This evidence indicates that *E. coli* O157:H7 infecting *Podoviridae* are uncommon in the studied environment

and the isolation of this rare group in the present study could improve our knowledge and understanding of them prior to biocontrol application.

The phages were further characterised when the one-step growth experiment was employed in determining the infection characteristics such as the latent period and burst size that can be explored to check the efficiency of a phage's ability to infect and develop in a particular host. The burst sizes of the phages were 374, 349 and 419 particles per cell, with an average latent period of 15 min for V8 and 20 min for V3 and V7. Seven *E. coli* O157 – phage infecting also demonstrated short latent periods ranging from 12 to 30 min and large burst sizes between 89 - 631 particles per cell (Litt & Jaroni, 2017). Conversely, the burst sizes of the isolated phages in this study are considered large when compared to other *E. coli* phages where average burst size as small as 33 and 51 pfu/cell have been reported (Lee & Park, 2015). On the other hand, a burst size of 9000 pfu/cell has been reported for a Podovirus - phage phiAxp-3 (Ma *et al.,* 2016). Variations in latent period and burst size of different phages could be attributed to the differences in host cells, growth medium, pH and temperature of incubation (Guttman *et al.*, 2005). The relatively short latent period and large burst size of phage V3, V7 and V8 suggested they have a selective advantage over other competing phages whereby they could produce enough virions within a short time to lyse the host bacteria. Therefore, phages V3, V7, V8 obtained in this study possessed outstanding characteristics regarding their short latent periods (15-20min) and large burst sizes, which make them attractive candidates for a biocontrol treatment program.

In addition to characterisation based on biological properties, newly isolated phages should be assessed for their stability and persistence when exposed to different external conditions that they might encounter in the environment so as to confirm their biocontrol potential (Hagens & Loessner, 2010). Thus, a further objective of the study was to investigate the response of the

three phages to changes in physico-chemical stress factors that might be experienced during production of phage or during biocontrol application of formulated phages. The three phages showed almost similar behaviour and stability at different incubation temperatures and pH. They were stable between 45 °C and 55 °C for 60 mins, however temperatures above 55 °C resulted in the reduction of the titres, which might be due to the effect of these high temperatures on phage proteins (Ackermann *et al.*, 2004). Studies investigating the effect of temperature on phage stability have reported similar observation (Litt & Jaroni, 2017) of the ability of some phages to withstand high temperatures. The thermal stability of the phages to high temperature (45 °C – 55 °C) in this study suggested that they could be suitable for application on carcasses/meat of the phages particularly at the post-harvest stage, in combination with other food processing technologies involving thermal treatments to improve microbial safety of food.

Acidic environments affect the infectivity of *E. coli* phages and it was suggested that these conditions result in denaturation of phage proteins and consequently loss of phage viability (Hazem, 2002). Earlier research has reported that most tailed phages are stable at pH 5.0 to 9.0 (Fan *et al.*, 2017; Smolarska *et al.*, 2018) and this was consistent with the results obtained in this study. While all of the isolated phages were highly sensitive to overnight exposure to acidic conditions (pH 2.0 and pH 4.0), some phages especially phage V3 were found to be resistant to a higher alkaline environment (pH 10.0). This was similar to previous reports on the preference of Podoviruses for alkaline conditions and their sensitivity to acidic conditions (Jończyk *et al.*, 2011). This alkaline stability would consequently increase the area of application of these phages.

The isolated phages were also tested for their stability to varying salt (NaCl) concentrations, since some meat processing procedures require the incorporation of salt as either flavour enhancers or preservatives. The isolated phages showed high stability in low salt concentration

(0.5 – 5%), but increasing the concentration (10% -15%) led to a greater reduction in phage activity. The observed saline stability is in agreement with studies conducted by other researchers where high levels of salt, in some cases up to 5M did not affect the phage titre (Lu & Breidt, 2015; Smolarska *et al.*, 2018). The adverse effect of increasing salt concentration can be due to osmotic pressure exerted on phage capsids preventing the ejection of phage DNA (Evilevitch *et al.*, 2003), or the aggregating effect of high salt concentration on phage particles with concomitant reduction in bacterial binding sites. The ability of these phages to withstand high salt concentration is indicative of the possibility of applications in high-salt meat products that might be infected with *E. coli* O157:H7.

During the isolation, purification and preparation of phages, certain chemicals such as chloroform are incorporated into the growth medium to enhance lysis and are also included in the phage lysates to prevent bacterial contamination (Sambrook *et al.*, 1989). The isolated phages showed a high level of stability to chloroform treatment after 2 h. Thus, the incorporation of 10% chloroform into the phage medium during isolation and storage is recommended for these phages. Some authors have reported varying results on the susceptibility of phages to chloroform and suggested that the sensitivity of each phage is confirmed prior to chloroform treatment (Hazem, 2002; Guttman *et al.*, 2005).

In order to further characterise the phages, their genomic properties were studied. Firstly, the sizes of the genomes were determined using PFGE. PFGE can be used to separate large DNA fragments efficiently and it has been used for the genomic investigations of different strains of bacteria and phage (Carrillo *et al.*, 2007). In order to prevent DNA shearing that might result from handling large DNA molecules, the phage particle was embedded in PFGE plugs prior to lysis. Genomic DNA was estimated at 68,854 bp in size, which corresponds to the genomic sizes of the majority of the *Podoviridae* in the NCBI database. Contrary to the previous assumptions

that the *Podoviridae* are the least common phage among the *Caudovirales*, all of the isolated phages belong to this genus.

Further genomic characterisation was done in order to assess the diversity among the phages based on restriction fragment length polymorphism (RFLP) by the use of restriction enzymes to digest the phage DNA *in situ*. This was done in order to avoid shearing problems associated with restriction digestion of large DNA molecules. RFLP analysis has been employed in the assessment of genetic diversity among bacteriophages (Akhtar *et al.*, 2014). A previous study has reported the susceptibility of the phage genome to the enzymes (*Nde*I and *Ssp*I) employed in this study (Raya *et al.*, 2006). Successful restriction enzyme digestion observed in this study confirmed that indeed phage genome was a double stranded DNA, a defining characteristic of the tailed phages (*Caudovirales*). Despite this, the restriction enzymes employed for this analysis could not differentiate between the phages as similar patterns were observed in them. Similarly, morphological identification by TEM also revealed the similarity among these phages. Interestingly, phages with similar digestion profiles and comparable appearances differed in their lytic ability against environmental *E. coli* O157:H7 strains. This could be as a result of the high degree of homology that is shared among phages with similar genome sizes (Chopin *et al.*, 2001). Considering the similarities observed in these phages, it is suggested that they undergo genome sequencing and analysis to provide an insight into the relatedness observed in this study.

In conclusion, three strong, stable and virulent lytic bacteriophages against *E. coli* O157:H7 were isolated and characterised in this study. The phages belonged to the family *Podoviridae*, with rapid growth rates and similar restriction enzyme patterns. They demonstrated thermal stabilities and were able to survive in a wide range of pH levels. They can be considered as promising candidates in the biocontrol of an emerging foodborne pathogen *E. coli* O157:H7. Although the phages showed reduced lytic spectrum against environmental *E. coli* O157:H7,

they can be combined in phage cocktails to increase the area of application and control a wider range of *E. coli* O157:H7 strains especially the virulent strains. Future research should be directed to sequencing of the whole genomes to ascertain their safety in biocontrol, and to the optimization of cocktail concentrations that can be used to cause complete inhibition of *E. coli* O157:H7 in both artificial media and real food systems.

CHAPTER FIVE

GENOME SEQUENCING AND ANALYSIS OF *ESCHERICHIA* PHAGE ECOP VB G17, A NOVEL *E. COLI* O157:H7 PHAGE ISOLATED FROM CATTLE IN THE NORTH-WEST PROVINCE, SOUTH AFRICA

5.1 Abstract

Virulent phages possess great potentials as natural biocontrol agents against food-borne pathogens notably *E. coli* O157:H7. However, it is required that a selected biocontrol phage candidate is fully characterised, particularly at the genomic resolution to ensure their safe application in food production. *Escherichia* phage vB EcoP G17 (originally designated phage phi-G17) that shows activity against environmental but virulent *E. coli* O157:H7 was previously isolated from cattle faeces and characterised, and in this study was submitted for genomic analysis. The complete genome sequence of phage G17 acquired from Ilumina's MiSeq platform revealed a genome of 68,270 bp in size with a G+C content of 43.5%. A total of seventy-eight putative protein coding sequences (CDs) identified in the genome were further annotated. The majority (76) of these proteins showed homology to similar proteins from members of the *Podoviridae* in the NCBI non-redundant database while only two proteins were unique to phage G17. Although phage G17 genome possessed twelve putative rho-independent terminators and nine putative promoters, only one tRNA gene that encodes isoluecine was detected. Moreover, Phage G17 displayed a high level of sequence homology to *Escherichia* phages: EcoP vB PhAPEC7, EcoP vB PhAPEC5 and EcoP vB G7C, all belonging to the *G7Cvirus* genus, thus it was suggested that phage G17 be classified as a *G7Cvirus*. Analysis reveals the absence of toxin, virulence, antibiotic resistance, and transduction-related genes. The results suggest that *E. coli* O157:H7 phage G17 is a novel phage that might have potential use in biocontrol of *E. coli* O157:H7 in food production.

5.2 Introduction

Bacteriophages are viruses of bacteria that possess enormous potentials which could be fully exploited by humans in different areas of biotechnological processes. Phages are described as the most abundant biological entities on earth estimated to be ten times more than bacteria (Hendrix *et al.*, 1999; Kutter & Sulakvelidze, 2004). They are ubiquitous and consequently are isolated from various environments where their bacterial hosts are found including seas, soil and intestinal tracts of animals (Danovaro & Serresi, 2000; Kutter & Sulakvelidze, 2004; Hudson *et al.*, 2005; Owens *et al.*, 2013). In these environments, they play a significant role in the regulation of bacterial physiology, diversity, abundance and evolution or virulence which eventually affects such environment as a whole (Weinbauer & Rassoulzadegan, 2004; Hendrix, 2005; Pride *et al.*, 2006; Abedon, 2008). They demonstrate a high degree of host specificity resulting from the interactions between the tail fibres and specific receptor molecules on target bacteria surfaces, and this defines their host range, since different bacterial strains possess differing surface molecules (Monk *et al.*, 2010). It is this unique characteristic of host specificity that makes them an extremely useful discriminatory tool in the detection and typing of bacterial strains, and phage typing has been traditionally used in epidemiological studies to characterise outbreak-associated strains (Hagens & Loessner, 2007). Most recently, there has been a renewed interest in the use of phages as natural agents of control of undesirable bacteria.

Virulent bacteriophages have a great potential to improve food safety and they have been successfully applied as novel natural control agents against food-borne pathogens (Hagens & Loessner, 2007; Zinno *et al.*, 2014; Callaway *et al.*, 2008). However, before phages are approved for therapeutic use, it is essential that the genomes be screened for undesirable features (Hagens & Loessner, 2007; Klumpp *et al.*, 2013). Phage genomic features such as carriage of virulence genes, antibiotic resistance genes and genes that are implicated in transduction can all be revealed from genome sequencing data. Alternatively, genome sequencing can assist in

identification of genes encoding lytic enzymes in the genome which can be used to develop recombinant proteins exhibiting lytic activities that can be employed in the control of pathogenic bacteria (Drulis-Kawa *et al.,* 2015). From a taxonomic classification perspective, genome-sequencing information can also reveal the evolutionary history and lifestyle of phages, which will assist in more coherent classification (Liu *et al.,* 2009; Lorenz *et al.,* 2013). Thus whole genome sequencing and analysis of phage offers exciting new opportunities not only in the control of pathogenic bacteria but also in expanding of our understanding of phage biology.

To date, the National Center for Biotechnology Information (NCBI) have over 2000 phage sequences available in its database (NCBI, 2018). Of these, 500 *E. coli* phage sequences are available. The first complete genome sequence of an *E. coli* phage was obtained for the siphovirus Lambda (λ) (Sanger *et al.,* 1982) and many others have been reported, particularly the long-tail type (Sheng *et al.,* 2010; Niu *et al.,* 2012b; 2014; Park *et al.,* 2012; Costa *et al.,* 2018). However, there is underrepresentation of morphological and genomic data of the short-tailed podoviruses in databases, which could be due to their low occurrence as they represent only 14% of the order *Caudovirales* (Ackermann, 2003). Interestingly, this group of phages has been detected in many bacterial host including *Pseudomonas aureginosa*, *Listeria monocytogenes*, *Bacillus* spp, *Staphylococcus* spp and *Escherichia coli*, making them interesting potential biocontrol agents (Ceyssens *et al.,* 2010; Kulikov *et al.,* 2012; Lorenz *et al.,* 2013; Takemura-Uchiyama *et al.,* 2013; Akhtar *et al.,* 2014).

Among the phages infecting *E. coli* in the *Podoviridae* family is the previously assigned genus *N4-like* phages whose taxonomic status has recently been reassessed with the use of comparative genomics and proteomics analysis (Wittmann *et al.,* 2015). Earlier studies identified *Escherichia coli* K-12 strain-specific phage N4 that was referred to as a genetic orphan among tailed phages as the representative of the genus *N4-like* phages (Ceyssens *et al.,* 2010). Although GenBank

currently has over 56 phages classified as N4-like, with each phage encoding the unique feature of virion associated RNA polymerase, a recent comparative analysis of their genomes, proteome, and phylogenesis showed enormous diversity among them. Based on the observed variation, six new phage genera were defined among the N4-like viruses, and they including *G7cvirus*, *Ea92virus*, *Luz7virus*, *Lit1virus* and *N4virus* (Wittmann *et al.*, 2015). The *G7cvirus* currently comprises 8 officially assigned species namely: EcoP vB PhAPEC7, EcoP vB PhAPEC5, IME11, ECBP1, Bp4 EC1-UPM and vB EcoP G7C with vB EcoP G7C designated as the type species (Tsonos *et al.*, 2014; Wittmann *et al.*, 2015). Another unassigned member of the genus include bacteriophage St11Ph5 (Golomidova *et al.*, 2018).

The *G7Cvirus* has been reported in different species of *E. coli* including avian pathogenic *E. coli* (APEC), *E. coli* O78, and *E. coli* 4s, however, to the best of our knowledge there has not been any documentation of *G7cvirus* phage characterised for *E. coli* O157:H7 (Kulikov *et al.*, 2012; Gan *et al.*, 2013; Tsonos *et al.*, 2014). Moreover, research investigating the field of phage genomics is rare in Africa despite the aroused global interest in phage study (Ackermann & DuBow, 2011). Currently, no studies in South Africa are involved in genomic characterisation of *E. coli* phages or in the application of these agents against *E. coli* O157:H7. However, earlier in this study, three bacteriophages that are lytic against *E. coli* O157:H7 were isolated and characterised. Due to the desirable features possessed by these phages, one of them designated phi-G17 was selected for further genomic studies. Thus, this study aimed to determine the genomic sequence of a novel *E. coli* O157:H7 phage G17 and conduct bioinformatics analysis of the sequence data in order to assign functions to genes contained in the genome. This will assist in screening the phage at genomic resolution for any virulence and lysogenic attributes, which can help us to determine whether the phage is fit for use as a biocontrol component. Moreover, the genomic analysis could result in generating more sequence data so as to gain better knowledge on the molecular structure and taxonomy classification of *E. coli* O157:H7 phages.

5.3 Materials and methods

5.3.1Bacterial strains and bacteriophage

E. coli O157:H7 strain used in this study was obtained from the previous section of this study (Akindolire & Ateba, 2018). The host bacterial strain and phage G17 were grown and propagated in Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI); Tryptic Soy Agar (TSA); modified nutrient agar (MNA) and Top agar 0.6% agarose. Using earlier described techniques (chapter 4), phage G17 was isolated from cattle faecal sample and characterised by determining its lysis profile, stability to varying physiochemical conditions and morphology using TEM. Moreover, high titre lysate of phage G17 was prepared in liquid culture and filtered using 0.22 µm Millipore membrane syringe filters (Fisher Scientific, Ottawa, ON) prior to purification.

5.3.2 Phage purification and DNA extraction

Phage purification was done with the use of a previously described method (Yamamoto *et al.*, 1970). DNase 1 and RNase A each to a final concentration of l0 µg/ml was added to crude lysates, (Sigma-Aldrich, Oakville, ON) so as to digest residual bacterial DNA and 0.5 M NaCl was included; the mixture was then incubated at 40 °C for 1 h. The suspension was centrifuged at 6000 x g for 10 min at 4 °C and the phage containing supernatant collected. The obtained supernatant was precipitated by gradually adding 10%w/v (final concentration) PEG 8000 with constant stirring. The mixture was incubated at 4 °C overnight to allow precipitation of phage particles. Precipitated phage was centrifuged at 11000 x g for 10 min at 4 °C to sediment phage particles and the supernatant was gently removed. To remove residual PEG, the phage pellet was washed thrice using lambda diluent [5.8g/L NaCl; 2g/L $MgSO_4$.7H2O; 10ml/L Tris-HCl (pH 7.5)] and the pellet was re-suspended by adding 0.5 ml lamda diluents and incubating overnight at 4 °C to soften the pellet. Phage genomic DNA was extracted from purified concentrated phage lysate using a previously described method with some modifications (Sambrook *et al.*, 1989).

Then the phage genomic DNA was extracted and purified from the concentrated phage stock using Norgen phage DNA isolation kit (Norgen Bioteck Corp., Ontario Canada), following the manufacturer's instructions. Extracted DNA was quantified using NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific Limited). Once the quantity and purity of the extracted DNA was established, a 50 ng sample of the DNA was used to determine the DNA sequence.

5.3.3 Genome sequencing and annotation

Purified phage DNA was sequenced at Inqaba Biotechnical Industry (Pty) (Pretoria, South Africa) in South Africa. In brief, phage DNA was fragmented using ultrasonication using ultrasonication approach (Covaris) and resulting DNA fragments were size selected (300-800bp) using AMPure XP beads, end repaired and Ilumina specific adapter sequences ligated. Sample DNA was diluted to a standard concentration (4nM) before sequencing on Ilumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit, following the manufacturer's standard protocol which generated 50 Mb of data (2x300 bp long paired-end reads) per sample. The quality of the raw reads were assessed with FastQC (0.11.5) (Andrews, 2010) and thereafter reads were filtered and trimmed using Trimmomatic at default parameters (Bolger *et al.*, 2014). *De novo* assembly of the trimmed reads was carried out using SPAdes v.3.11.1 (Bankevich *et al.*, 2012) genome assembler software and the result was evaluated with QUAST.

5.3.4 Bioinformatics analysis and annotation of genome sequence data

Initial genome annotation was done using the Rapid Annotation Using Subsystem Technology (RAST) (version 2.0) server (Aziz *et al.*, 2008) and PHAge Search Tool (PHAST) (Zhou *et al.*, 2011) online analysis tools. Predicted proteins in the genome were further annotated by BLASTP against the NCBI non-redundant GenBank database (Altschul *et al.*, 1997). Genome sequence comparisons were carried out using the BLASTn (version 2.8.0) with the parameters

set at default values (Altschul *et al.*, 1997). A search for phage encoded tRNA genes was done with tRANscan-SE program (version 2.0) (Lowe & Chan, 2016) and ARAGORN (Laslett & Canback, 2004) using default parameters. A search for Rho-independent terminators was performed with Arnold (Naville *et al.*, 2011) while putative promoters were predicted using PHIRE (Lavigne *et al.*, 2004). Functional annotations and homology assignments between genes from other *G7cvirus* phages and predicted genes of phage G17 were based on amino acid sequence alignment searches (BlastP) using standard parameters.

Comparisons at the proteomic level were performed using CoreGenes (Zafar *et al.*, 2002). Mauve software was used for multiple genome alignments and defining sequence homology percentage with related phages (Darling *et al.*, 2010). DNA plotter software was used to generate a circular genomic map of the phage (Carver *et al.*, 2008).

The annotated genome sequence for the phage G17 was deposited in GenBank database under the accession number MH358458.

5.4 Results

5.4.1 Genomic characteristics of phage G17

The genome of phage G17 was revealed to be a double stranded DNA of 68 270 bp in length which is made up of 28.6% thymine, 27.8% adenine, 21.6% cytosine and 21.9% Guanine. The nucleotide distribution is depicted in Table 5.1. It has a G+C content of 43.5%, which is slightly lower than 50.5% G+C content reported for *E. coli* O157:H7 (Sakai strain) (Hayashi *et al.*, 2001). Previously determined GE analysis revealed that the genome is a linear double stranded DNA with an approximated size of ~ 69,000 (Fig 5.1) supporting the sequenced size obtained. The phage was from the family *Podoviridae*, proposed sub-family *Equartavirinae* and genus *G7cvirus*. The complete genome sequence of phage G17 was submitted to the NCBI Gene bank database where it appears under the accession number (MH358458).

Table 5. 1: Nucleotide distribution of phage G17

Nucleotide	Count	Frequency
Adenine (A)	19004	0.278
Cytosine (C)	14734	0.216
Guanine (G)	14983	0.219
Thymine (T)	19549	0.286
C+G	29717	0.435
A+T	38553	0.564

Table 5. 2: General features of phage G17 and members of the *G7Cvirus*

Feature	**Phage**								
	G17	**PhAPEC7**	**PhAPEC5**	**G7C**	**Bp4**	**ECBP1**	**EC1-UPM**	**pSb-1**	**IME11**
Genome size (Kbp)	68.27	71.78	71.25	72.92	72.61	69.86	70.91	71.63	72.57
G+C%	43.5	43.3	43.5	43.4	42.8	42.7	42.9	42.7	43.1
No ORFs	78	83	83	79	98	82	80	103	91
No tRNAs	1	1	1	0	2	2	0	0	0
GenBank accession no	MH358458	KF562340	KF192075	HQ259105	KJ135004.2	JX415535	KC206276	KF620435	JX880034

Table 5. 3: Genomic features of phage G17

Feature	
Genome size (bp)	**68270**
Total number of sequences	**2**
G+C content (%)	**43.5**
Total ORFs	**78**
Average ORF length (bp)	**860**
Protein coding region (% of genome size)	**98.2%**
No of gene products similar to Known proteins	**75**
No of gene products similar to known *G7cvirus*	**71**
No of conserved hypothetical protein with unknown function	**4**
tRNA	**1**
No of promoters	**9**
No of rho-independent terminators	**16**

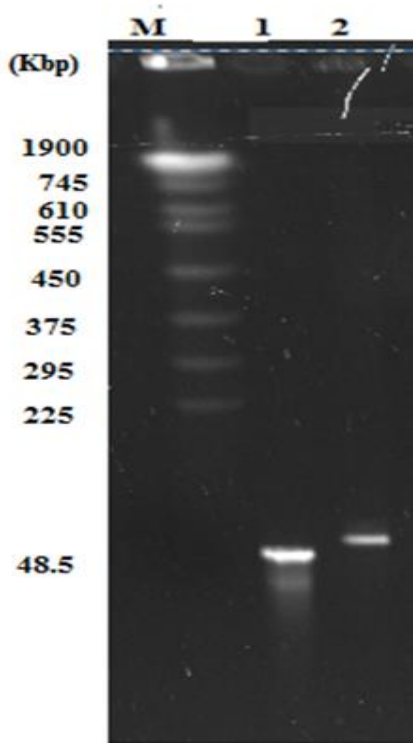

Figure 5. 1: PFGE pf phage G17 prepared in agarose plugs without DNA extraction; 1: PFGE marker; 2: uncut lambda, 3: phage G17

5.4.2 Identification and analysis of open reading frames (ORFs)

The genome was analysed using online analyses tools such as PHAST and RAST. Open reading frames (ORF) were determined on the basis of a start codon (ATG, GTG, and TTG). G17 genome contained a total of 78 putative ORFs ranging in length from 114 bp (ORF 37) to 10 353 bp (ORF 15, virion RNA polymerase) with the greater part of them (60/78) leftward oriented while the rest are rightward oriented (Table 5.4). The majority (75/80) of the identified ORFs utilized ATG as their start codon, while, only 3 and 2 ORFs started with a GTG and a TTG start codon respectively. The predicted ORFs represent 98.2% of the phage sequence, demonstrating high coding percentage and compactness of the genome organization as generally observed in other tailed dsDNA phages. Genes encoding toxins and those implicated in transduction were absent in the genome of phage G17.

Interestingly, most of the protein encoding sequences (72/78) were identified as phage proteins possessing homologous with proteins of the newly assigned *G7cvirus* genus in the non-redundant NCBI database (Wittmann *et al.*, 2015), particularly *Escherichia* phage vB EcoP PhAPEC7 and *Escherichia* phage vB EcoP PhAPEC5 (Tsonos *et al.*, 2014). Additionally, a handful of the proteins showed similarity to other G7cviruses, including *Shigella* phage pSb-1, *Escherichia* phage vB EcoP G7C, *Escherichia* phage Bp4 and an unassigned *G7Cvirus* phage *Escherichia* phage St11Ph5 (Tsonos *et al.*, 2014; Golomidova *et al.*, 2018; Kulikov *et al.*, 2012). On the contrary, only a small number of the proteins (4/78) showed homology to phages from other genera including *Escherichia* phage PMBT57 (*N4-like* genus), *Escherichia* phage PGN829.1 (unclassified *Podoviridae*) and *Escherichia* phage 121Q (unclassified *Myoviridae*) (Koberg & Brinks, 2018; Chaudhary & Taneja, 2018; Lapin *et al.*, 2014). Only two proteins (2/78) (Genes 1 & 52) were hypothetical proteins with no significant matches and were thus considered unique to phage G17.

Genes that constitute the core genomes of *G7cvirus* are determined by coreGenes comparison of some G7cviruses (*Escherichia* phage APEC7, *Escherichia* phage APEC5, *Escherichia* phage G7C, *Escherichia* phage Bp4) and 20 conserved proteins obtained (Table 5.5). Of these genes, 19 were identified in phage G17, while only one (supper-infection immunity) of the conserved genes was absent. CoreGenes analysis also revealed that phage G17 shares 73.1% homologs with *Escherichia* phage vB EcoP PhAPEC7 while 43.6% of its genome is similar to phage *Shigella* phage pSb-. Taken together, these data suggest that the newly isolated phage shares a great level of similarity with *G7cvirus*, only gene 27 (putative phosphatase kinase of *Escherichia* phage PMBT57), gene 33 (hypothetical protein of *Escherichia* phage PMBT57) and gene 69 (hypothetical protein of Erwinia phage EamMY3) could be associated with phages outside this genus.

In support of the virulent nature of phage G17, no lysogeny module was found in the genome. Common to the many *Podoviridae*, the genome structure of phage G17 displayed a modular organisation genome with organised discrete functional modules (Fig 5.2), containing genes involved in virion morphogenesis, DNA replication, and cell lysis module.

Table 5. 4: General features of putative ORFs of phage G17 and homology to proteins in the database

Gene (CDs)	Cordinates (bp)	Length (bp)	Strand	Predicted Function	Homology to genes of other phages
1	13..1143	1131	+	Hypothetical protein	-
2	1465..1698	234	+	Phage tail length tape measure	YP 009031976.1 putative tail length tape measure (*Escherichia* phage Bp4)
3	1683.. 1943	261	+	Putative holin protein	YP009031977.1 putative Holin protein (*Escherichia* phage Bp4)
4	1933..2568	636	+	Putative N-acetylmuramidase	YP009055575.1 putative N-acetylmuramidase (*Escherichia* phage vB EcoP PhAPEC5)
5	2549..3049	501	+	Putative RZ/RZ1 spanin protein	AGC31575.1 Putative RZ/RZ1 spanin protein (*Escherichia* phage EC1- UPM)
6	3058..5328	2271	+	Phage portal protein	YP009055573.1 putative portal protein (*Escherichia* phage vB EcoP PhAPEC5)
7	5342..5686	345	+	Conserved hypothetical protein	YP009055572.1 Hypothetical protein (*Escherichia* phage vB EcoP PhAPEC5)
8	5706..6926	1221	+	Phage tape measure protein	YP009031079.2 Tape measure protein (*Escherichia* phage Bp4)
9	6943..8145	1203	+	Putative major capsid protein	YP 009055570.1 Putative coat protein (*Escherichia* phage vB Ecop PhAPEC5)
10	8219..8854	636	+	Conserved hypothetical Phage protein	YP009055569.1 Hypothetical protein (*Escherichia* phage vB EcoP PhAPEC5)
11	8931..9767	837	+	Phage protein	YP009056190.1 putative structural protein (*Escherichia* phage EcoP PhAPEC7)
12	9769..12411	2643	+	Phage protein	YP009055567.1 Hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
13	12425..12868	444	+	Phage protein	YP009031992.1 putative structural protein (*Escherichia* phage Bp4)
14	12881..14836	1956	+	Phage protein	YP009055565.1 putative structural protein (*Escherichia* phage vB Ecop PhAPEC5)
15	14940..25292	10353	+	Translation intiation factor 2/ Virion RNA	YP 009055564.1 Virion RNA polymerase (*Escherichia* phage vB

				polymerase	Ecop PhAPEC5)
16	25546..25343	204	-	Hypothetical protein	YP 009008501.1 Hypothetical protein (*Shigella* phage pSb-1)
17	26051..25671	381	-	Phage protein	YP009055562.1 Hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
18	26193..26035	159	-	Hypothetical protein	YP009008499.1 Hypothetical protein (*Shigella* phage pSb-1)
19	27782..27531	252	-	Hypothetical protein	YP009056182.1 Hypothetical protein (*Escherichia* phage vB Ecop PhAPEC7)
20	28433..27933	501	-	Hypothetical protein	YP009056181.1 Hypothetical protein (*Escherichia* phage vB Ecop PhAPEC7)
21	28989..28435	555	-	Hypothetical protein	YP009055558.1 Hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
22	29786..28989	798	-	Putative single-stranded DNA binding protein	YP 009055557.1 s-s DNA binding protein (*Escherichia* phage vB Ecop PhAPEC5)
23	30580..29828	753	-	Phage protein	YP009056177.1 Hypothetical protein (*Escherichia* phage vB Ecop PhAPEC7)
24	32790..30637	2154	-	DNA pimase	YP009055555.1 Putative DNA primase (*Escherichia* phage vB Ecop PhAPEC5)
25	33767..32787	981	-	Phage protein	YP 009055553.1 Hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
26	34240..33767	474	-	3'-phosphatase, 5'-polynucleotide Kinase	AUV59045.1 Kinase (*Escherichia* phage PMBT57)
27	34545..34240	306	-	Phage protein	YP009055551.1 Hypothetical (*Escherichia* phage vB Ecop PhAPEC5)
28	35513..34542	972	-	DNA polymerase I	YP009055550.1 DNA polymerase 1 (*Escherichia* phage vB Ecop PhAPEC5
29	36150..35494	657	-	Phage protein	AXY82613.1 Hypothetical protein (*Escherichia* phage PGN829.1)
30	37964..36147	1818	-	DNA polymerase I	YP004782168.1 putative DNA polymerase 1 (*Escherichia* phage EcoP G7C)
31	38504..37974	531	-	Phage protein	YP009056170.1 Hypothetical protein (*Escherichia* phage vB

					Ecop PhAPEC7)
32	39825..38515	1311	-	DNA helicase	YP009056169.1 DNA helicase (*Escherichia* phage vB Ecop PhAPEC7)
33	40197..39859	339	-	Phage protein	AUV59112.1 Hypothetical protein (*Escherichia* phage PMBT 57)
34	40639..40244	396	-	Phage protein	YP009055544.1 Hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
35	42747..40702	2046	-	Phage rIIB lysis inhibitor	YP009056166.1 rIIB-like protein (*Escherichia* phage vB Ecop PhAPEC7)
36	45316..42752	2565	-	Phage rIIA lysis inhibitor	YP009056165.1 rIIA-like protein (*Escherichia* phage vB Ecop PhAPEC7)
37	45457..45344	114	-	Hypothetical protein	YP004782161.1 Hypothetical protein (*Escherichia* phage Ecop G7C)
38	45782..45450	333	-	Phage protein	Yp009055540.1 hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
39	45990..45775	216	-	Phage protein	YP009056162.1 Hypothetical protein (vB Ecop PhAPEC7)
40	46224..46000	225	-	Hypothetical protein	YP004782158.1 Hypothetical protein (EcoP G7C)
41	46523..46284	240	-	Hypothetical protein	YP009056161.1 Hypothetical protein (vB EcoP PhAPEC7)
42	47464..46520	945	-	Putative thymidylate synthase	YP009055536.1 putative thymidylate synthase vB Ecop PhAPEC5)
43	47907..47464	444	-	Phage protein	YP009056159.1 hypothetical protein (vB EcoP PhAPEC7)
44	48242..48036	207	-	Phage protein	YP009055532.1 hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
45	48758..48252	507	-	Deaminase	YP009032026.1 dCTP deaminase (*Escherichia* phage Bp4)
46	49933..48758	1176	-	Hypothetical protein	YP009055530.1 hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
47	50993..49941	1053	-	Phage protein	YP009055529.1 hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
48	51357..51028	330	-	Hypothetical protein	YP004782148.1 Hypothetical

					protein (EcoP G7C)
49	51543..51358	186	-	Hypothetical protein	YP004782147.1Hypothetical protein (EcoP G7C)
50	51728..51540	189	-	Hypothetical protein	YP009056150.1 Hypothetical protein (vB EcoP PhAPEC7)
51	52428..51832	597	-	Hypothetical protein	YP09056149.1 Hypothetical protein (vB EcoP PhAPEC7)
52	53671..52430	1242	-	Hypothetical protein	-
53	53942..53760	183	-	Hypothetical protein	YP009056148.1 Hypothetical protein (vB EcoP PhAPEC7)
54	54143..54003	141	-	Hypothetical protein	YP006990663.1 Hypothetical protein (*Escherichia* phage IME11)
55	55347..54130	1218	-	Phage protein (RNAP2)	YP009055523.1 RNAP2 (*Escherichia* phage vB Ecop PhAPEC5)
56	56221..55403	819	-	RNA polymerase	YP005055521.1 RNAP1 (*Escherichia* phage vB Ecop PhAPEC5)
57	56619..56272	348	-	Phage protein	ATS924831.1 Hypothetical protein (*Escherichia* phage St11Ph5)
58	56944..56624	321	-	Hypothetical protein	YP009055515.1 hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
59	57198..56941	258	-	Hypothetical protein	ATS92479.1 Hypothetical protein (*Escherichia* phage St11Ph5)
60	57416..57195	222	-	Phage protein	YP00482135.1 Hypothetical protein (EcoP G7C)
61	57721..57413	309	-	Phage protein	YP00482134.1 Hypothetical protein (EcoP G7C
62	58067..57855	213	-	Hypothetical protein	ATS92474.1 Hypothetical protein (*Escherichia* phage St11Ph5)
63	58201..58070	132	-	Conserved hypothetical protein	YP004782133.1 Hypothetical protein (EcoP G7C)
64	58364..58194	171	-	Conserved hypothetical protein	YP00482132.1 Hypothetical protein (EcoP G7C)
65	58606..58364	243	-	Hypothetical protein	YP009055509.1 hypothetical protein (*Escherichia* phage vB Ecop PhAPEC5)
66	59556..59263	294	-	Hypothetical protein	YP009056130.1 Hypothetical protein (vB EcoP PhAPEC7)
67	59970..59647	324	-	Conserved hypothetical Phage protein	YP004782125.1 Hypothetical protein (EcoP G7C)

68	60463..60176	288	-	Conserved hypothetical protein	YP009056212.1 Hypothetical protein (vB EcoP PhAPEC7)
69	60822..60460	363	-	Conserved hypothetical protein	YP 009101802.1 Hypothetical protein (*Escherichia* phage 121Q)
70	61037..60918	120	-	Conserved hypothetical protein	YP009055587.1 Hypothetical protein (vB EcoP PhAPEC5)
71	61341..61027	315	-	Conserved hypothetical protein	ATS92548.1 Hypothetical protein (*Escherichia* phage St11Ph5)
72	61661..61341	321	-	Conserved hypothetical protein	YP009055584.1 Hypothetical protein (vB EcoP PhAPEC5)
73	61979..61665	315	-	Conserved hypothetical protein	YP009056212.1 Hypothetical protein (vB EcoP PhAPEC7)
74	62343..61945	399	-	Conserved hypothetical protein	YP009055582.1 Hypothetical protein (vB EcoP PhAPEC5)
75	62836..62546	291	-	Putative Terminase subunitA	YP009055580.1 putative terminase (vB EcoP PhAPEC5)
76	63007..63696	690	+	Putative tail protein	YP 0090562021.1 putative structural protein (vB Ecop PhAPEC 7)
77	63689..65278	1590	+	Putative Tail protein	AGC31500.1 putative tail fibre protein (*Escherichia* phage EC1-UPM)
78	65285..65995	711	+	Putative tail fibre protein	YP 0090562021.1 putative structural protein (vB Ecop PhAPEC 7)

Table 5. 5: Comparison of some conserved orthologs in *G7cvirus* to phage G17.

Ortholog Group	**Homology in phage G17**
RNAP 1	√
RNAP 2	√
Capsid decorating protein	√
dCTP deaminase	√
Super-infection immunity protein	-
Thymidilate synthase	√
rIIA-like protein	√
rIIB-like protein	√
DNA helicase	√
DNA Primase	√
DNAP1	√
Single-stranded DNA binding protein	√
Virion RNA polymerase	√
Major capsid/coat protein	√
Portal protein	√
N-acetyl muramidase	√
Tail fibre protein	√
Tail protein	√
RZ/RZ1 spanin protein	√
Tail length tape measure protein	√

DNA Packaging and morphogenesis homologs

A number of genes encoding proteins directly involved in DNA packaging were identified in phage G17 genome. Terminase subunit-A was determined to be gene *78* while genes encoding head protein were determined to be *6* for portal protein and gene *9* for putative major capsid protein. Phage tail proteins were encoded by a set of genes including gene *2* for tail length tape measure protein, and gene *8* for tape measure protein. Four genes encoding putative structural

proteins were *11, 13, 14* and *78*; and they were all found to be similar to the protein encoded by APEC7.

Nucleotide metabolism and DNA replication genes

Sequence analysis of G17 genome has identified many genes that are involved in G17 DNA multiplication. In addition, the phage genome was found to consist of three major genes involved in nucleotide metabolism: 3'-phosphatase, 5'-polynucleotide kinase (gene *26*), Deoxycytidine triphosphate deaminase (gene *45*) and putative thymidylate synthase (gene *42*). Genes involved in DNA replication were identified as gene *24* for DNA primase; *28* for DNA polymerase 1; *30* for putative DNA polymerase; *32* for DNA helicase and gene *22* for single-stranded DNA binding (ssb) protein. Genes involved in transcription were *55* for RNA ploymerase 2 (RNAP2); *56* for RNA polymerase 1 (RNAP1) and Gene *15* was determined to code for translation initiation factor 2 otherwise referred to as virion RNA polymerase (vRNAP).

Lysis and lysis inhibition genes

The phage G17 genome contains sequences homologous to a putative N-acetylmuramidase (endolysin) in gene *4* and gene *3* is identified by BLASTP analysis as a putative holin protein. Moreover, gene *5* was determined to be a putative RZ/RZ1 spanin protein that is shown to be involved in lysis in phages that infect Gram-negative bacteria. Interestingly, genes *35* and *36* were determined to be lysis inhibitors rIIA & rIIB respectively.

Transfer RNA (tRNA) and Regulatory genes

Both Aragorn and tRNAscan-SE could only detect a 74 bp tRNA gene that was located at position 26406 – 26481 in the genome of phage G17. This tRNA gene encodes for amino acid Isoluecine with anticodon AAT and its GC content was 43.7%. Promoters' prediction by PHIRE

revealed nine putative promoters in the genome (Table 5.6) while 16 putative Rho-independent transcription terminators were detected using Arnold (Table 5.7).

Peculiar genes

Unlike most reported phage genomes that contained a small number of known genes, a very high proportion (96%) of phage G17 genes had homologs either with phage proteins that have predicted functions or with conserved hypothetical phage proteins whose functions are yet to be determined. Accordingly, only 2 hypothetical genes without homologs to other known genes were found in the genome (Table 5.4). These genes are located in ORFs *01* and *052*. A search for homologs using BLASTP produced no significant hit to sequences in the database. However, most of the other identified genes were similar to structural and enzymatic genes from phages belonging to the genus *G7cvirus.*

Table 5. 6: Putative promoters in the genome of phiG17 phage

Name	Location	Sequence
P1	05252...05277	ACCCAGCTTATTCACTTGGTTCCCAA
P2	30862...30887	CTGTACTTCCTTCATCTGGTTCTGGA
P3	43329...43354	TCATACCATTTCCACATGGTTCCAAT
P4	44044...44069	ATGTACCCTCTTCACGCGTTTCCTTA
P5	58841...58866	TGATACCTTCTTCACCTGGTTCCAGG
P6	Complement (05934…05909)	CTGCATCTTCTTCATCTGGTTCGGTT
P7	Complement (16113...16088	GAATACCTTCTTCAACTCTTTCACGC
P8	Complement (16932...16907)	GTTTACCTGCTTCAGCCTGTTCCTGA
P9	Complement (66583...66558)	TTAGAACTTCTTCACCGGCTTCCAAT

Table 5. 7: Putative Rho-independent terminators in the genome of G17

Name		**Sequence**	**ΔG**
T1	1138	AGTTAACAAAAACCCCGCTTCGGCGGGGTTAGTTTTATCATT	17.00
T2	2578	TTATCTTCTGGTGGTGGTCCTTGGGGCCACCATTTACTTTTGGG	14.60
T3	8151	TTTTGTTAAGAGGGGGACATGAGTCCCCCTTGTTTTATTTCG	15.10
T4	8866	CAGTTCAACACGGCTCACTGGGGTAACTCCGTGGGCCgTTTCTTTTTGAAC	11.30
T5	25289	CTAAATAAAAAAGCCCCTCTAATGAGGGGCTTTGCTTTTTAAAA	16.20
T6	25559	CAGATATGAGAAAGCCCTCACTAGGAGGGCTTgTTTTGTTTTGGCT	13.80
T7	40515	AAGGTCACGGTCGTTGGGTGAACACCCAATGTTTTAGCAATGG	10.90
T8	42620	GTATATCCTCAGAGCGACCCACGGGTTGCTCgTATTGTATCTGA	15.30
T9	46247	AGTAAACCTAAGACCTCCTCCGGGAGGTCTTTTTTTGGTTTC	13.30
T10	51749	CCCTCAAGTTTGACCTCCTAGAAATAGGAGGTCTTTTTTTGACTT	14.10
T11	53717	AGCTGAAACTAGCCTCACACCTAAACAGTGTGGGGCTTTTTTATTTCCA	13.20
T12	53959	GTTAATAAACTGGCCTCATTCCTTCGGGAGTGGGGCCTTTTATTTTTTG	12.40
T13	58708	AATCAACAACTGGACTACCTTCGGGTAGTCCTTTTATTTTTAA	17.20
T14	59606	AATCAATATAGGAGGCTCTTCGGAGTCTCCTTTTATAAAGAT	16.90
T15	62356	GAAAACGGTAAGGGGTAGAGTCGAGAGGCTCTACCCCaTTTCTTTTTCTGG	14.50
T16	62359	GAAAAAGAAATGGGGTAGAGCCTCTCGACTCTACCCCTTACCGTTTTCAA	13.30

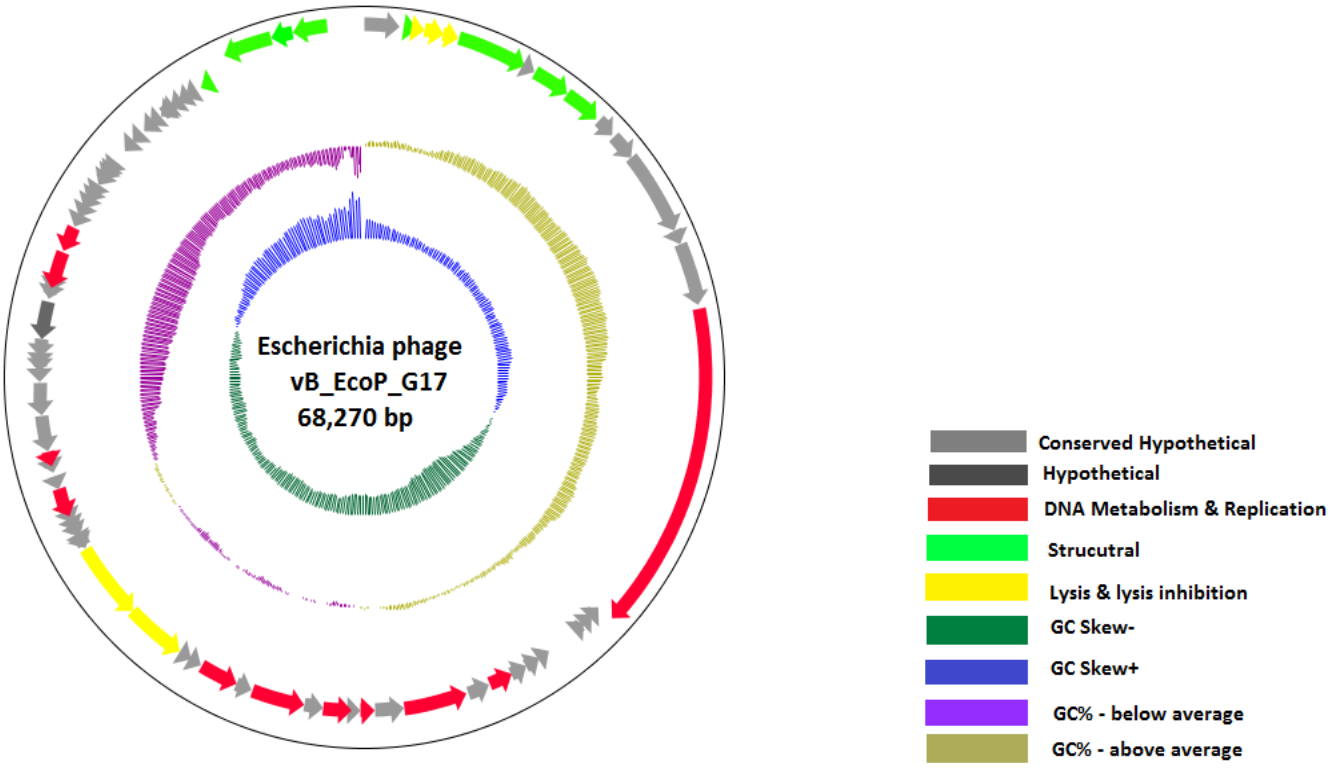

Figure 5. 2: The genome map of phage G17. The arrows represent predicted genes and the sense of coding, while the colours indicate the various functional groups as indicated on the legend of the gene products and the GC content and GC skew.

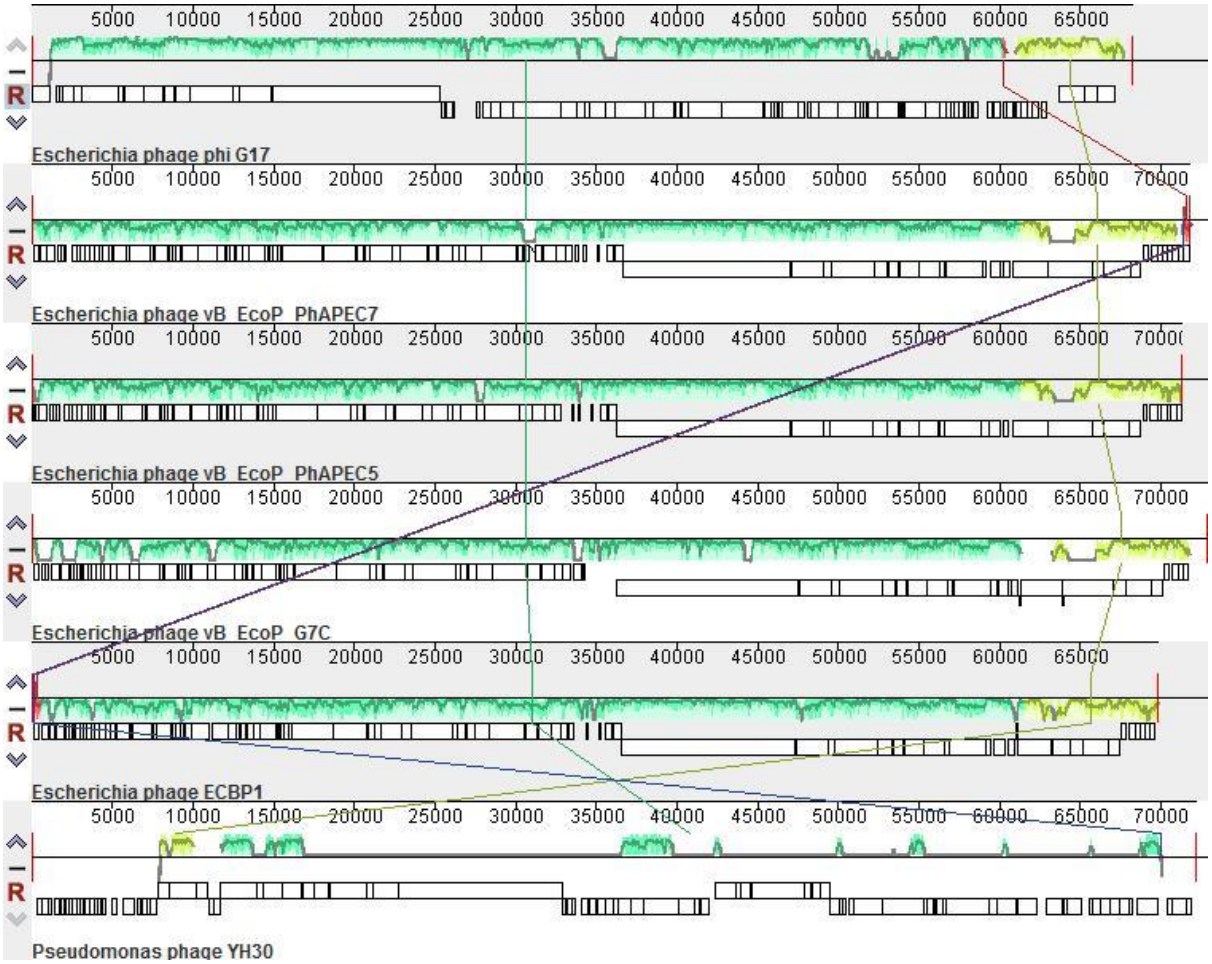

Figure 5. 3: Whole genome alignment of phage G17 and some podoviruses including the related *G7Cvirus* using the progressive mauve. Blocks with similar colours represent areas of high synteny between the different genomes

5.5 Discussion

Recent developments in sequencing technologies and protein similarity tools have offered enormous opportunities in the advancement of phage taxonomy resulting in more accurate classification system based on relatedness between phages, and also assisting in the selection of safe biocontrol candidates (Hatfull, 2008; Hatfull & Hendrix, 2011). In fact, genome analysis and bioinformatics studies are among the prescribed prerequisite prior to the application of phages as biocontrol agents. This will ascertain the absence of any undesirable traits such as antibiotic resistance genes or virulence genes in a potential biocontrol candidate.

This is the first investigation into the evaluation of the eligibility of phage G17 for biocontrol by analysing its genomic content. The main purpose of this study was to obtain, study and screen the sequence of phage G17 for any toxin, antibiotic resistance or transduction-associated genes that would be considered unsafe for its biocontrol application. We have determined the sequence and genome structure of the virulent *E. coli* O157:H7 phage G17. The non-redundant genome has a size of 68 270 bp with a G+C content of 43.5%. Phages in the *G7cvirus* genus have genomic size ranges between 72.92 to 69.86 kb and GC content of 43.5 to 41.3. In addition, the genome shared 94% and 95% nucleotide identity with APEC7 and APEC5 respectively, both of which are members of the *G7cvirus*. Although the genome of G17 is slightly smaller than that of the *G7cvirus*, analyses with a number of bio-informatic tools revealed considerable homologies with this genus in terms of gene content and functions.

Annotation of the genome using PHAST (Zhou *et al.*, 2011) and RAST (Aziz *et al.*, 2008) and BLASTP (Altschul *et al.*, 1997) identified 78 genes which is also similar to the previously sequenced *Escherichia* phage (Patel *et al*., 2015). The majority of predicted coding sequences (CDs) (76) code for products with high homologies to a number of phages, most notably members of the aforementioned *G7cvirus* phages, suggesting a high degree of significant

similarity with this group. Further computational analysis of core-Genes showed that G17 shared 57 (73%), 54 (69%), 53 (68%) and 52 (62%) homology with *Escherichia* phage vB EcoP PhAPEC7 (NC_024790), *Escherichia* phage vB EcoP PhAPEC5 (NC_024786), ECBP1 (NC_018854) and G7C (NC_015933) respectively while for comparison, it only had 4 (5%) sequence similarity with phage N4.

Escherichia phage N4 was the representative species for the previously assigned *N4-like* genus. As earlier indicated, the old *N4-like* genus comprised of many groups of distantly related phages before their recent re-classification. In 2015, some phage researchers re-examined the classification of these groups using BLASTN, CoreGenes and phylogenetic analysis. Their results suggest that although some similarities exist among these groups of phages, genomic analysis revealed that the *G7cvirus*, a newly proposed genus, is distinct from the other genera in the N4-group of viruses (Wittmann *et al.*, 2015).

Phages belonging to the newly assigned *G7cvirus* were isolated from locations that were geographically distant and they were also ecologically diverse, infecting varying bacterial hosts. *Escherichia* phages PhAPEC5 and PhAPEC7 were isolated from river water in Belgium (Tsonos *et al.*, 2014), while phages G7C and Bp4 were obtained from horse faeces in Russia (Kulikov *et al.*, 2012) and faecal samples in China respectively (Ma *et al.*, 2016). Phage G17, a new addition to the group, was isolated from cattle faecal samples in South Africa (Chapter 3). This supports the notion that similar phages are present in geographically distinct locations (Hatfull *et al.*, 2006).

Similar to the genomic organization of other phages, the genomes of the G7cviruses have been shown to contain genes encoding typical phage essential functions including DNA packaging and morphogenesis, DNA metabolism and replication and host cell lysis gene products.

According to their functions, these genes are usually in a modular arrangement of two large clusters which is transcribed in opposite directions (Wittmann *et al.*, 2015). Similarly, genomic structure of phage G17 conforms to a similar modular arrangement where the early genes (DNA metabolism, host interaction) and middle genes (replication genes) have one orientation while late genes (structural and cell lysis genes) are transcribed in the opposite direction (Fig 5.2). Additionally, phage G17 also displayed a high degree of homology to these essential genes, indicating the conserved nature of the genes and the cellular function they perform among the G7C viruses (Kulikov *et al.*, 2012).

Additionally, unlike other phages that rely on the host enzyme for the transcription of their early proteins, G7C viruses and other N4-like phages possess a very large virion encapsulated RNA polymerase (vRNAP) that is injected with the phage genome into the host (Garcia-Heredia *et al.*, 2013; Ma *et al.*, 2016). This remarkable feature is unique and conserved among these genera of podoviruses, which is suggestive of the vital role it plays in the propagation of the phages. In addition to vRNAP, the G7C viruses and N4-like viruses also encode two more polymerases RNAP1 and RNAP2, which are required for phage transcription (Ma *et al.*, 2016). Similarly, phage G17 harbours 2 RNA polymerases located at gene 56 and 57; it also contains a giant 3450 aa vRNAP positioned at gene 15 representing approximately 7% of the whole genome length. The high level of homology displayed by phage G17 in these polymerases suggests that just like the G7Cviruses, phage G17 does not rely on host RNA for the transcription of its early proteins and DNA replication genes and as such utilizes a similar transcription mechanism to the *G7Cvirus.*

Bioinformatics analyses of each of the genes contained in phage G17 against nucleotide collection of NCBI revealed no evidence of sequences that encode for antibiotic resistance or toxin-related genes. In the same way, lysogenic-related proteins were not found supporting the

virulent nature demonstrated by the formation of clear plaques in earlier studies (Chapter 3). Similar results were obtained when related G7cviruses such as APEC7, APEC5 and G7C were reported to be lytic and were further assessed for their biocontrol potential (Kulikov *et al.*, 2012; Tsonos *et al.*, 2014). As such, the new phage G17 does not contain any homology to harmful genes or proteins that are undesirable and this therefore increases the potential use of the phage in biocontrol application. Members of the genus *G7cvirus* have been genomically shown to be safe and effective in experimental phage therapy. Thus, phages belonging to the genus *G7cvirus* can be a useful therapeutic agent in phage therapy. Currently, there are two *E. coli* O157:H7 podoviruses described in the literature, however, this study is the first study to report these phages in Africa.

Despite the genomic similarities displayed by the *G7cvirus* there are some discrepancies between these phages. For instance, genomes of Podoviruses and particularly the G7cviruses have been shown to possess varying numbers and types of tRNA genes. The genomes of Bp4 and ECBP1 contain 2 tRNAs each (Nho *et al.*, 2012), whereas vB EcoP PhAPEC7 and PhAPEC5 have 1 tRNA (Tsonos *et al.*, 2014) coding for leucine and isoleucine respectively and others such as G7C and EC1-UPM even lack tRNA genes (Kulikov *et al.*, 2012; Gan *et al.*, 2013). The detection of one tRNA gene that encodes same amino acid– Isoleucine as vB EcoP PhAPEC5 and vB EcoPAEC7 in the genome of phage G17 provided further evidence in support of the homology between these phages (Tsonos *et al.*, 2014). While the exact role of tRNA genes in phages is not fully understood, some suggested functions are related to the efficient translation of phage proteins, which thereby provides a greater growth advantage to phage carrying its own tRNA genes (Weiss *et al.*, 1968; Delesalle *et al.*, 2016). However, the presence of just one tRNA in the genome of phage G17 is suggestive of their dependency on host tRNA genes for the synthesis of other phage proteins.

CoreGenes comparisons of the *G7cvirus* phage genome has revealed that 20 proteins with known functions are conserved in them. Based on their respective functions the conserved proteins are predominantly grouped into nucleotide metabolism proteins, DNA morphogenesis proteins, DNA packaging, as well as host lysis proteins. These conserved proteins were all identified in phage G17 genome with the exception of just one – the super-infection immunity gene. This further supports the assumption that phage G17 is closely related to the *G7cvirus*.

Superinfection immunity is a phage-encoded bacterial resistance mechanism which occurs when an infected bacterial cell is protected from further infection by other closely related phages (Seed, 2015). This has been described among other phages, particularly the T-even phages, where the immunity (*imm*) gene facilitates superinfection exclusion by preventing DNA injection of superinfecting phages into the cytoplasm and therefore making the incoming phage non-infectious (Lu & Henning, 1994; Seed, 2015). Although the function of this gene is unclear, it is suggested that they provide adaptive/selective advantage to both the host cell and the infecting phage by blocking DNA transfer across the cell membrane (Lu & Henning, 1994; Seed, 2015). Thus, the lack of this gene in phage G17 is indicative of their susceptibility to superinfection by other phages and reduced survivability, however, it is possibly a non-essential gene for their propagation.

Further analysis of the whole genome using the progressive mauve algorithm showed that phage G17 shared similar synteny charcateristcs with the related *G7Cvirus* phages as compared to an unrelated pseudomonas phage YH30, supporting the similarity to the same group of viruses.

In addition to the genomic similarity displayed by this phage, the previously observed morphological resemblance (chapter 4) of phage G17 to this genus corroborated their relatedness. Therefore it is suggested that phage G17 be classified as a member of the *G7Cvirus*.

Using the proposed ICTV nomenclature system the newly characterised phage G17 is suggested to be named *Escherichia* phage vB_EcoP_G17 (Kropinski *et al.*, 2009).

In conclusion, phages have been reported to have potential applications as biocontrol agents against pathogenic bacteria. This study focussed on the whole genome sequencing and analysis of *Escherichia* phage G17, a novel and lytic phage infecting an important foodborne pathogen- *E. coli* O157:H7. The genome analysis showed that phage G17 is genetically related to the order *Caudiovirales*, family *Podoviridae*, and genus *G7Cvirus*. Access to the genome sequence has provided us genome level information that has not only assisted in the classification of this novel phage but also revealed the absence of any virulent and transduction genes. This makes the phage a unique and safe candidate for biocontrol, where it might be used in a cocktail of several phages to increase the possibility of reducing a wide range of *E. coli* O157:H7 strains.

CHAPTER SIX

GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 General conclusions

Food safety in particular meat safety is a matter of growing public concen, considering the fact that meat is a nutrient-rich food substance that provides excellent environment for the growth of important spoilage and pathogenic microorganisms including *E. coli* O157:H7 (McDonald & Sun, 1999; Sofos, 2008). In an effort to counter the microbiological threat posed on meat safety, various regulatory standards and acts are in place to minimise contamination of carcasses and meat with pathogenic microorganisms and thereby ensure production of safe meat and meat products (Attenborough & Matthews, 2000; Ropkins & Beck, 2000; Sofos, 2008). Unfortunately, in most developing countries including South Africa, proper regulatory and hygienic standards are not strictly adhered to and can sometimes be compromised at farms and slaughter-houses, particularly in the informal sector, increasing the risk of carcass contamination with pathogens such as *E. coli* O157:H7 (Kang'ethe, 1993; Adzitey *et al.*, 2011; Rani *et al.*, 2017). The most recent outbreak of listeriosis in South Africa has revealed not only a relaxation of regulations and protocols involved in the production of safe meat-derived products but also the inadequacy of the current conventional intervention strategies (WHO, 2018).

E. coli O157:H7 is one of the important meat-borne pathogens that present a significant menace to meat safety, since the control of the pathogen in foods and even the infections arising from the pathogen have proven difficult to manage. Although researchers have investigated various methods of mitigating this pathogen at the abattoirs, especially after harvest, but currently no single method is without a limitation (Sofos & Smith, 1998; Berry & Cutter, 2000; Belk, 2001; Wheeler *et al.*, 2014). Of particular importance is the ability of the pathogen to initiate disease at

a low infectious dose, and treatment of such infections with antibiotic is contraindicated, making disease management a complex process (Tuttle *et al.*, 1999; Tarr *et al.*, 2005; Smith *et al.*, 2012; Wong *et al.*, 2012). Currently the treatment for *E. coli* O157:H7 infection in humans is mostly based on supportive therapy, notably rehydration; however, the outcome of infection could be fatal in some immuno-compromised individuals including young children, elderly, and people with HIV and AIDS (Kelleher *et al.*, 1996; Noris & Remuzzi, 2005; Tserenpuntsag *et al.*, 2005; Michael *et al.*, 2009). This therefore amplifies the need to implement strategies that are directed at controlling the occurrence of this pathogen in cattle or its by-products to minimise or prevent human exposure through the consumption of contaminated food.

Recently, enormous interest has been directed towards the use of biological agents or their by-products in the control of pathogenic bacteria (Dykes & Moorhead, 2002; Leverentz *et al.*, 2003; Viazis *et al.*, 2011b; Drulis-Kawa *et al.*, 2015). This is due to the numerous benefits associated with the biocontrol agents. The application of lytic phages in the control of important food-borne pathogens such as *E. coli* O157:H7 could offer a natural, sustainable, environmentally friendly alternative to traditional control strategies (Hagens & Loessner, 2010; Mahony *et al.*, 2011; Spricigo *et al.*, 2013; Tomat *et al.*, 2013; Kazi & Annapure, 2016; Plaza *et al.*, 2018). Given the considerable advantages associated with phages, this study wasinitiated in an attempt to later develop phage-based products that could be used in the control of *E. coli* O157:H7 on food surfaces particularly beef. The research reported in this thesis therefore forms the basis for the development of such products, since it describes the isolation and detailed characterisation of *E. coli* O157:H7 phages prior to their possible formulation into a commercial product. The importance of such a study cannot be over-emphasised because it improves our understanding of phage-*E. coli* O157:H7 dynamics and more importantly reveals phage attributes before application in the food system.

Bacteriophages are recognized as obligate parasites only able to replicate in the presence of its bacterial host (Monk *et al.*, 2010). As such, studies investigating them require a susceptible bacterial host either isolated from the environment of study or commercially procured strains (Carey-Smith *et al.*, 2006; Niu *et al.*, 2009; Viazis *et al.*, 2011a; Akhtar *et al.*, 2014; Fan *et al.*, 2017; Litt & Jaroni, 2017; Mahmoud *et al.*, 2018). In this particular study, a collection of cattle-borne *E. coli* O157:H7 was employed as the host organism for phage isolation. In order to do so, cattle faecal samples were screened for the presence *E. coli* O157:H7 and the pathogen was detected in 26.5% of the samples. The results of *E. coli* O157:H7 isolation and characterization indicated that the animals tested were colonised by a genetically diverse and virulent population of the pathogen. This has important public health implications when undercooked beef and other cattle-derived products such as raw or non-pasteurised milk from these animals are consumed. Of equal importance are the ecological and epidemiological impacts, since colonised cattle provide an environment that facilitates the exchange and transfer of genetic elements among the varied isolates; and this could result in emergence of new and more deadly strains. It is also of economical concern, especially for the food industry, considering the loss that can result from food recalls or from consumer rejection of the products associated with contamination. The isolation of the pathogen in the study supports the premise that cattle are the natural reservoir and their carriage can provide an avenue for pathogenic strain evolution (Blanco *et al.*, 2004; Low *et al.*, 2005; Gyles, 2007). This calls for improved food hygiene procedures to minimise food safety risks to consumers.

Many studies have described the isolation and characterisation of phages that could be employed for the biocontrol of bacterial pathogens (Ronner & Cliver, 1990; Raya *et al.*, 2006; Viazis *et al.*, 2011a; Kulikov *et al.*, 2012; Owens *et al.*, 2013; Krasowska *et al.*, 2015; Litt & Jaroni, 2017; Huang *et al.*, 2018; Mahmoud *et al.*, 2018; Smolarska *et al.*, 2018). This study likewise focussed on the isolation and characterisation of *E. coli* O157:H7 bacteriophages. A total of 15 virulent

phages were successfully isolated from cattle faeces obtained from different farms using the previously isolated *E. coli* O157:H7 as host strains. A spot test assay was used to determine the lytic profile of the isolated phages against environmental *E. coli* O157:H7. This enabled the identification and selection of three good phage candidates with broadest host range for the control of *E. coli* O157:H7. The phages were designated V3, V7 and V8. While the characterised phages displayed distinctive lytic profile, they had a narrow range of infectivity against environmental *E. coli* O157:H7 strains since none of them was able to infect all the host strains tested. One suggested solution is to increase the number of samples screened or the types of samples, for instance, environmental samples like sewage and farm slurry could be included to improve the chances of isolating a broad host range phage. Additionally, the limited host range phage obtained in the study can be mixed as a cocktail to increase the breadth of *E. coli* O157:H7 strains that can be controlled by the phage mixture.

Morphological analysis conducted by TEM revealed that the three phages displayed similar morphology of icosahedral heads of about 73-77 nm in diameter and short tails of about 20 - 25 nm in length and belonged to the order *Caudovirales* (Tailed phages), family *Podoviridae*. This family is the least studied so far among the *Caudovirales* and they comprise 14% of all tailed phages (Ackermann, 2007). The isolated phages displayed a narrow range of infectivity against environmental *E. coli* O157:H7 strains. Nevertheless, the one-step growth experiment revealed that all the phages exhibited relatively high burst sizes ranging from 349 to 419 pfu/cell and short latent periods of 15 to 20 minutes.

Stability studies also revealed similar behaviour under different physical and chemical conditions. They were stable between 45 °C and 55 °C; however, temperature above 55°C negatively influenced their stability. Similarly, they were surprisingly stable in high salt concentrations and maintained their activity in up to 5% NaCl. However, they had similar pH

stability range from pH 6 to pH 10. Interestingly, phage V8 was more stable at a higher alkaline pH. They also displayed a high level of stability to chloroform treatment.

To complete the characterisation of a newly isolated phage, it is important to analyse the sequence of its DNA. This will not only provide information that will aid in taxonomic classification but also reveal any virulence and lysogenic attributes genetically, which can help us to determine whether the phage is fit for use as a biocontrol component. In the last part of the study (Chapter 5), one of the phages, V3 now designated phi G17 was selected for whole genome sequencing and analysis. Genomic analysis confirmed that phage G17 is of the order *Caudovirales*, and family *Podoviridae.* Further analysis of the sequence and protein homology of phage G17 showed that the genome consisted of 68 270 bp with a GC content of 43.5% and over 98.2% of the phage genome are involved in coding for putative proteins.

Although 78 ORFs were identified and annotated, the majority (75) of the ORFs code for products with homology to similar proteins from members of the *Podoviridae* phage in the NCBI database. Among these 75 podoviridae-related genes, 72 belonged to the newly assigned genus -*G7Cvirus* while only two were unique to phage G17. Although phage G17 genome possessed sixteen putative rho-independent terminators, nine putative promoters and one tRNA gene was detected in the genome.

More detailed genome analysis indicated that the phage shared sequence homology to *Escherichia* phage EcoP vB PhAPEC 7, *Escherichia* phage EcoP vB PhAPEC 7 and *Escherichia* phage vB EcoP G7C with over 73% sequence homology. Thus the proposed classification for the new phage G17 was: genus - *G7cvirus*, family – *Podoviridae*, order - *Caudovirales* phage. Using the naming guideline that has received approval from the ICTV, this newly characterised phage was assigned *Escherichia* phage vB_EcoP_G17 (Adriaenssens & Brister, 2017).

In conclusion, this thesis describes the successful isolation and comprehensive characterisation of *E. coli* O157:H7 bacteriophages, which have enhanced our understanding of phage biology and the interactions between them and their bacterial host. Moreover, further genomic analysis of the novel phage G17 has revealed its safety as proteins and genes considered dangerous in biocontrol applications were absent in the genome.

6.2 Recommendations for Future Research

Future studies should be carried out to identify the routes of transmissions and determine other reservoirs for *E. coli* O157:H7 on farms aside from cattle so that efficient control measures can be implemented at these sites on farms and beyond, and thereby reduce the risk of animal carriage, carcass contamination as well as human infections. Moreover, in order to have a better understanding of the epidemiology an all-inclusive surveillance information that reflects the flow of the pathogen from farm to fork, investigations should be directed at isolates from other sources including clinical associated strains and environmental isolates.

It is recommended that other environmental samples such as sewage and farm slurry be screened for *E. coli* O157: H7 phage and results obtained with respect to host range, physiochemical and genomic properties compared to faecal-borne phages. Although the biocontrol potential of phages is widely acknowledged, a major limitation to their applications is the lack of assigned functions to most of their genes as observed in this study; therefore, further analyses are needed to determine the functions of these genes before application.

Even though the abundance and pervasiveness of phages in the universe has been documented and substantial research into the biotechnological capability of phage has been conducted in Europe and the western world, our knowledge of phages in Sub-Saharan Africa is still

rudimentary and their applications in the control of pathogens non-existing. Thus, this study has provided the foundation for further phage research. However, isolation and detailed characterisation of more phage isolates together with investigations into their application potentials could enhance our understanding of this novel biocontrol tool.

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APPENDICES

Appendix 1

Culture media and Buffer

Lambda diluent (Per litre)

NaCl	5.8g
$MgSO_4.7H_2O$	2g
1M Tris-HCL (pH 7.5)	10 ml
Distilled water	up to 1L

Aliquot diluent into 100 ml vials. The solution was sterilized by autoclaving and was stored at room temperature. Lamda diluent was used in phage experiment.

Modified Nutrient Agar (MNA) per liter

Nutrient broth	180g
NaCl	76.5g
Agar No1 (Oxoid)	90g
CaCl	74.7mg
FeCl	9.9mg
$MgSo_4$	4.5g
30% sterile glucose	10 ml – (filter sterilized and added after autoclaving agar)

Appendix 2

Table 1: Effect of chloroform on phage G17

Phage	Chloroform +	Chloroform -
V3	2.60E+07	3.00E+07
V7	2.10E+07	3.00E+07
V8	1.60E+07	4.80E+07

Table 2: Single-step growth curve data

	Phage count pfu/ml (Relative burst size)		
Time (min)	V3	V7	V8
0	0.1	0.4	0.8
5	0.2	0.6	0.86
10	0.36	0.98	1.8
15	0.51	0.72	11
20	24	0.98	99
25	19	8.5	200
30	49	39	399
35	79	30	419
40	324	61	299
50	259	29	249
60	349	93	210
70	374	180	199
80	299	189	178
90	250	180	170

Table 3: Effect of Temperature on phage G17

	Phage count pfu/ml (% reduction)			
Phage	45 ºC	50 ºC	55 ºC	60 ºC
V3	1.1 x 10^5 (45%)	10 x10^5 (82%)	30 x 10^5 (48%)	6 x10^5 (96%)
V7	7.7 x 10^5(28.7%)	100 x 10^5 (42.8%)	ND (100%)	ND (100%)
V8	ND (100%)	2 x10^5 (96%)	ND (100%)	ND (100%)

Table 4: Effect of pH on phage G17

Phage	Ph - Percentage reduction in phage count (%)					
	2	4	6	8	10	12
V3	100	100	63.3	50.0	40.0	100
V7	100	100	50.0	2.5	32.5	100
V8	100	100	90.0	10.0	2.0	100

Table 5: Effect of Different NaCl concentrations of phage G17

Phage	Percentage Reduction in phage count at Different NaCl concentration			
	0.5%	5.0%	10.0%	15.0%
V3	13.7	31	60	89.6
V7	12	65	67	86
V8	20	23	74	82

Appendix 3

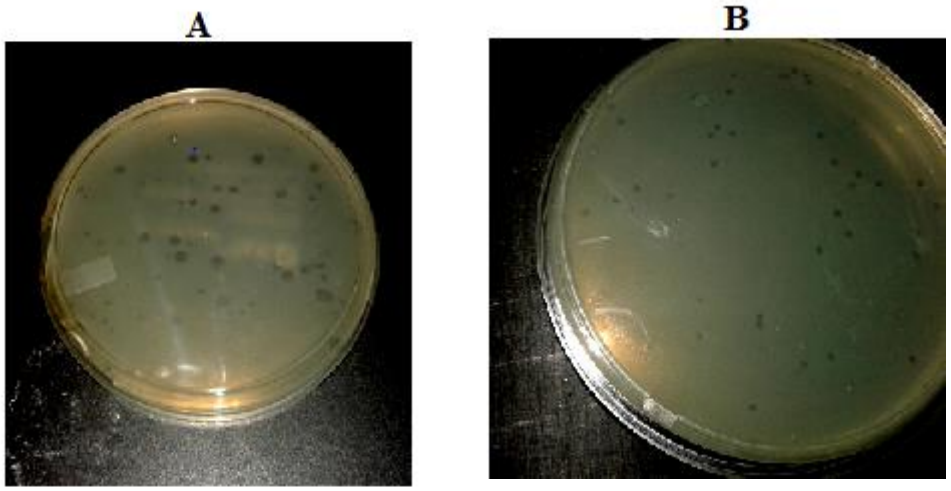

Fig 1: Morphologies of the isolated phages, A: Different-sized plaques comprising of different phages; B: A pure phage with similar plaques

Appendix 4

Table 6: Phage G17 sequencing statisitcs

Name	AA_phagemerge (30865/12/1)
Uploaded File	AA-phage_S14_L001_R1_001.fastq AA-phage_S14_L001_R2_001.fastq
Date Uploaded	Thu Oct 11 15:17:26 2018
Number of Reads	154,330
Type	Paired End
Platform	Illumina
Single Genome	Yes
Insert Size Mean	Not Specified
Insert Size Std Dev	Not Specified
Outward Read Orientation	No
Number of Reads	154,330
Total Number of Bases	42,433,303
Mean Read Length	274.9517
Read Length Std Dev	51.1159
Number of Duplicate Reads(%)	57696 (37.38%)
Phred Type	33
Quality Score Mean	36.18
Quality Score (Min/Max)	2.0/38.0
GC Percentage	43.27%
Base Percentages	A(28.405%) C(21.7292%) T(28.2955%) G(21.5454%) N(0.0249%)