ISOLATION, CHARACTERIZATION AND DETERMINATION OF THE LYTIC PROPERTY OF BACTERIOPHAGES ON SALMONELLA SPECIES

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BY

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DEPARTMENT OF MICROBIOLOGY,

FACULTY OF LIFE SCIENCES,

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

NOVEMBER, 2016

DECLARATION

I hereby declare that the work in this dissertation entitled, "**Isolation, Characterization andDetermination of the Lytic Property of Bacteriophages on *Salmonella* Species**" was carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria, Kaduna State, Nigeria under the supervision of Prof. S.E. Yakubu and Prof. I.O. Abdullahi. The information derived from this literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Donna Tige BALA

_____	_____	_____
Name of Student	**Signature**	**Date**

CERTIFICATION

This dissertation entitled: "ISOLATION, CHARACTERIZATION AND DETERMINATION OF THE LYTIC PROPERTY OF BACTERIOPHAGES ON *SALMONELLA* SPECIES" by Donna Tige BALA meets the regulations governing the award of Masters of Science degree in Microbiology of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to the Glory of God, the service of humanity and my late father, Mr Bala P. Duna.

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ABSTRACT

The control of pathogens such as *Salmonella* is vital to control economic loss, animal and human morbidity and mortality. The use of antibiotics in the control of these pathogens is facing challenges such as development of resistance and adverse side effects. There is need for other antibacterial agents to be used together with or as alternative to antibiotics. Bacteriophages which infect specific bacteria show specialty in this aspect and is known to be a potential bio-control agent against pathogenic bacteria like *Salmonella*. This study was aimed at isolating, characterizing and determining the lytic property of bacteriophages on *Salmonella* species obtained in the course of the study from an egg and clinical isolate from the University's Sickbay. Two *Salmonella* species were used for the study -one was a clinical isolate obtained from the University's health services which was isolated from human source and the other was isolated from inside an egg. Seven Bacteriophages {three for each and the reference phage (Salmonelex which was purchased from Microes Food Safety, Netherlands) for both} that are lytic against the two *Salmonella* species were isolated (from the central collection point of the university's drainage system, the dam at Kwanan kurmi and soil from the farmlands and open fields where animals usually graze around the dam), cultivated and concentrated. Their antibacterial effects on the two *Salmonella* isolates were compared *in vitro* with that of two antibiotics (Ciprofloxacin and Chloramphenicol) and that of their combination with each antibiotic (1DWC, 1DWCip, 1KSC, 1KSCip, 1KWC, 1KWCip, 1RC, 1RCip, 2DWC, 2DWCip, 2KSC, 2KSCip, 2KWC, 2KWCip, 2RC and 2RCip). The phages were found to be as effective as the antibiotics (like in the comparison between 1R and Chloramphenicol, 2DW and Ciprofloxacin, 2KS and Ciprofloxacin and between 2R and Chloramphenicol); in some cases they were more effective than the antibiotics (such as the comparison between

1DW and Chloramphenicol or Ciprofloxacin, 1R and Chloramphenicol or Ciprofloxacin and between 2R and Ciprofloxacin) and in some cases (such as comparison between Chloramphenicol and 1KS, 1KW, 2DW, 2KS or 2KW and between Ciprofloxacin and 1KW or 1KS) they were less effective. In some cases the combination of the phages and the antibiotics (such as the combination of Chloramphenicol with 1KS, 1KW, 1R or 2KW and Ciprofloxacin with 1KS, 1KW, 2KS or 2KW) gave synergistic effect. It was evident these phages and their combinations with antibiotics can be potential control agents of *Salmonella* species. Bacteriophages can be used as bio-control agents against *Salmonella* and they can equally be used as complement or alternative to antibiotics such as Chloramphenicol and Ciprofloxacin in the control of *Salmonella* infection.

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LIST OF ABBREVIATIONS

ACMSF	-	Advisory Committee on the Microbiological Safety of Food
CFSPH	-	Center for Food Security and Public Health
CDC	-	Centre for Disease Control and Prevention
CLSI	-	Clinical and Laboratory Standards Institute
CiWF and WSPA	-	Compassion in World Farming and World Society for the Protection ofAnimals
EFSA	-	European Food Safety Authority
FDA	-	Food and Drug Administration
FAO	-	Food and Agriculture Organisation
FSIS	-	Food Safety and Inspection Service
USDA	-	United States Department of Agriculture
WHO	-	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Bacteriophages (phages) are viruses that infect bacteria. Their studies as the simplest biological systems set the foundations for molecular biology and microbial genetics (Cairns *et al.,* 1992; Kropinski, 2006). This provided information on central processes of bacterial cell (DNA replication, transcription and translation) and transduction (Cairns *et al.,* 1992; Kropinski, 2006). Phages are ubiquitous and regarded as the most abundant biological entities with estimates of $\geq 10^{31}$ phage particles on earth (Chibani–Chennoufi *et al.,* 2004a). Their genetic material encapsulated in a protein coat is either DNA or RNA which can be single or double stranded (Whitman *et al.,* 1998; Willey *et al.,* 2008).

Based on life cycle, they are classified into two: Temperate phages and virulent phages (Willey *et al.,* 2008; Ryan *et al.*, 2011). Temperate phages have lysogenic life cycle in which the nucleic acid is replicated together with the host gene for several generations without major metabolic consequences to the host cell. Instead, they establish mutualism with the lysogen which results to mutation and immunity to superinfection (Benett and Howe, 1998; Mathur *et al.*, 2003; Willey *et al.*, 2008). Under appropriate conditions, such as higher number of host cells to phages, the temperate phages can initiate lytic cycle to lyse the host cell and release phage particles (Benett and Howe, 1998; Willey *et al.*, 2008).

Virulent phages on the other hand possess the lytic life cycle in which there is penetration of phage nucleic acid into the host intracellular development and final

release of daughter phage particles after the lysis of the host cell (Hanlon, 2007; Willey *et al.,* 2008; Ryan *et al.,* 2011). Various phages specifically infect specific bacterial groups. This specificity can be exploited in the control of the bacteria they infect, for example, the *Enterobacteriaceae* which are infected by several coliphages.

Salmonella, a facultative intracellular pathogen and a member of the family *Enterobacteriaceae* is probably the most important pathogenic bacteria transmitted by oral route. Although it can be transmitted through contaminated food and direct contact from infected people, the most serious and common means of transmission is water borne (Madigan *et al.*, 1997). *Salmonella enterica*, one of the *Salmonella* spp whose medically important subspecies is *enterica* has serovars that include Enteritidis, Paratyphi, Typhi, and Typhimurium (Murray *et al.,* 2009). *Salmonella* species other than *Salmonella* Typhi are known to cause salmonellosis which is more frequent but less serious than typhoid fever (Madigan *et al.,* 1997). Sources of food borne *Salmonella* are humans and warm blooded animals. The organism reaches food by contamination from food handlers like in the case of Typhoid Mary (Madigan *et al.*, 1997; Willey *et al.*, 2008).

Another serovar of *Salmonella* that is of medical importance but to poultry is *Salmonella enterica* subspecies *enterica* serovar Pullorum. Pullorum disease is usually symptomatic only in young birds but in growing birds and adults, *Salmonella* Pullorum infections are likely to be unapparent. The mortality rate varies, but it can be as high as 100% (CFSPH, 2009; Hoelzer *et al*., 2011).The control of these disease is complicated by vertical transmission because hens can become subclinically infected carriers and pass the infections to their embryos in the egg. *Salmonella* Pullorum infections can be found in many avian species including chickens, turkeys, quail, guinea fowl, pheasants, ducks, pigeons, sparrows, canaries, bullfinches and parrots;

however, pullorum disease is uncommon except in chickens, turkeys and pheasants (CFSPH, 2009; Hoelzer *et al*., 2011).

Phage host specificity and lytic properties caught the interest of scientists like Frederick Twort, an English bacteriologist in 1915 who observed them (Twort, 1915; Topley and Wilson, 1929) and Felix d'Herelle, a French-Canadian microbiologist in 1917 who officially studied, recorded and named them (D'Herelle, 1917; Summers, 1999), giving birth to phage therapy (Topley and Wilson, 1929; Summers, 2001; Sulakvelidze *et al.*, 2001; Kropinski, 2006; Ryan *et al.*, 2011). Phage therapy is the use of bacteriophages to kill or control the bacteria in infected host (Bull *et al.*, 2002). It was abandoned for decades by the Western countries as a result of emergence and commercialization of antibiotics but survived in the former Soviet Union (Mathur *et al.*, 2003; Kutter and Sulakvelidze, 2005; Kropinski, 2006).

Antibiotic resistance due to abusive use and resultant decline in investment and development of novel antibiotics by pharmaceutical industries led to revival in the interest of phage therapy in human medicine and veterinary fields (Chopra, 1997; Mathur *et al.*, 2003; Kropinski, 2006; Veiga-crespo *et al.*, 2007; Morel and Mossialos, 2010). Subsequently, interest in its ability to control bacterial population extended from medical application into the fields of agriculture (Leverentz *et al.*, 2003; O'Flaherty *et al.*, 2009), aquaculture (Park *et al*., 2000; Nakai and Park, 2002), ecology (Chibani-Chennoufi *et al.*, 2004a; Withey *et al.*, 2005) and food industry (Montville and Chikindas, 2007).

1.2 Statement of research problem

Several outbreaks of food borne illnesses were traced to *Salmonella* whose prime vehicles were fruits, uncooked vegetables, contaminated fish, meat, dairy, eggs and their product from infected animals or cooked and canned food (such as chips, pastries, and chocolates) handled by pathogens carriers. Other vehicles include meat and field produce that were contaminated directly or indirectly with faeces of domestic or wild animals (CDC, 1997; Madigan *et al.*, 1997; D'Aoust and Maurer, 2007; Meng *et al.*, 2007).

Clinical conditions caused by Salmonella spp aside enteric fevers, enterocolitis and salmonellosis include complications such as systemic infections and Salmonella-induced chronic conditions such as aseptic reactive arthritis, Reiter's syndrome and ankylosing spondylitis (Cheesbrough, 2006; Willey *et al*., 2008). Though most patients recover from salmonellosis, it can cause problems in children, elderly and immunocompromised persons (Cheesbrough, 2006; Willey *et al*., 2008).

On the other hand, infection with *Salmonella* Pullorum has drastic economic impact as birds hatched from infected eggs hardly survive to productive adults since they die shortly after being hatched or become underweight and poorly feathered and eventually die before reaching full maturity. It also causes decreased egg production, fertility or hatchability in unapparent carriers as well as in birds with systemic signs (CFSPH, 2009; Hoelzer *et al*., 2011).

Several classes of antibiotics such as the aminoglycosides (gentamycin and streptomycin) (Bischoff *et al*., 1977; Paradelis *et al*., 1980), beta-lactams (penicillins and cephalosporin) (Grill and Maganti, 2008), quinolones (ciprofloxacin and levofloxacin) (Akahane *et al*., 1989; Isaacson *et al*., 1993; Kushner *et al*., 2001; Hakko

et al., 2005), chloramphenicol (Venegas-Francke *et al*., 2000) and tetracyclines (Kesler *et al*., 2004) have been used as remedy to prevent and treat infections caused by pathogenic bacteria such as *Salmonella*. Occurrences of positive outcomes are high but not without accompanying challenges. Antibiotics can have multiple side effects which can be serious (Smith and Huggins, 1982; Mathur *et al*., 2003) such as intestinal disorders, allergies, promotion of secondary infection (Prins *et al*., 1994; Yao and Moellering, 1995) and neurotoxicity (Snavely and Hodges, 1984; Chow *et al*., 2004; Grill and Maganti, 2011). Marked global increase in multiple antibiotic resistant microbes among which are multiple drug resistant *Salmonella*, have been reported at different times down history lane (CDC, 2004). While resistance is increasing in many pathogens, the number of new antimicrobial agents approved for use has slowed. Thus, the ability to control outbreaks of infectious diseases through antibiotic use alone has been diminishing (Fred and John, 1998).

1.3 Justification of the study

One of the agents of prevention and treatment of infections caused by pathogenic bacteria that has high efficacy and promises a future of better control of pathogenic bacteria is the bacteriophage or simply, phage. Phages have long been considered as bio-control agents for food production. They were used as antimicrobial systems for food at pre-harvest and post-harvest level (Barrow and Soothill, 1997; Leverentz *et al.*, 2001 and 2003b) to eliminate or reduce contaminating bacteria on various foods including fruits and vegetables (Sharma *et al.*, 2009). Commercial phage preparations have been approved as antimicrobial food additives by the U.S Food and Drug Administration in 2006 (FDA, 2006) and US Department of Agriculture (USDA) to prevent bacterial contamination of livestock, food crops, meat and other food products

by microbes such as *Salmonella* (O'Flaherty *et al*., 2009), *Listeria monocytogenes* (Fortuna *et al*., 2008) and *E.coli* (O'Flaherty *et al*., 2009).

A vital area of phage research that shows possibility of its deployment to control human pathogens such as *Salmonella* is its use to treat infections in animals as well as to prevent carriage of zoonotic pathogens that might subsequently get into the food chain system (Smith and Huggins, 1983; Nakai and Park, 2002; Sheng *et al.,* 2006; Tan *et al*., 2014). Clinical trials have been carried out with phage preparations to treat different infections in humans such as ear infection, burn wounds and leg ulcers which showed about 80% decline in mean bacterial cell count at infection site in phage-treated patients (Fortuna *et al.,* 2008). Other trials showed that phage can be used in combination with antibiotics to treat certain infections resistant to antibiotics alone (Alisky *et al.,* 1998). Phage preparations have been administered to patients whose infections were unresponsive to antibiotics therapy. The results showed a high percentage full recovery (about 93%) and improvements in condition (Slopek *et al.,* 1987; Weber-Dabrowska *et al*., 2000; Gorski *et al*., 2007).

Concerns about problems such as multi drug resistance by pathogenic bacteria, side effects and high cost of treatment have resulted into research on other agents of prevention and treatment of bacterial infection (Church *et al*., 2006; McVay *et al*., 2007). Bacteriophages can be the best answer to antibiotic resistance in the treatment of bacterial infections (Matsuzaki *etal*., 2005; Hanlon, 2007; Malik and Chhibber, 2009; Bedi *et al*., 2009; Kumari *et al*., 2011). These phages are considered to be economical, safe, self- replicating and effective bactericidal agents (Inal, 2003; Bradbury, 2004).

Reports of successful phage treatment are encouraging and indicate a high degree of efficacy of phage therapy to combat bacterial pathogens. In this light, the activities and

properties of bacteriophages need to be explored locally and exploited to improve clinical and public health which is threatened daily by pathogenic microorganisms.

1.4 Aim

The aim of this study was to isolate, characterize and determine the lytic property of bacteriophages on *Salmonella* species.

1.5 Specific objectives

The specific Objectives of the study were to:

1. obtain clinical isolates as well as isolate some *Salmonella* speciesand characterize them using conventional method and rapid identification kits (Microgen GnA-ID System and *Salmonella* Polyvalent O agglutinating serum for groups A-S).
2. isolate, concentrate and partially characterize bacteriophages from waste water, a dam and soil using filtration, differential centrifugation and plaque assay techniques.
3. determine the lytic activity of bacteriophages on *Salmonella* using lysis in tube assay by progeny phage release and Optical density measurement.
4. compare antibacterial activities as well as determine the combined activities of bacteriophage with standard antibiotics on the isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Bacteriophages

Bacteriophage literarily means "eater of bacteria" and refers to the remarkable ability of "bacteria-specific viruses" to bring about lysis of growing bacterial cultures. This is accompanied by production of more phages which are transmissible in series from culture to culture of susceptible bacteria (Adams, 1959; Griffiths *et al*., 2004; Orlova, 2012; Hyman and Abedon, 2015). The name was given by Felix d'Herelle and his wife in 1916 to bacteriolytic substance that he isolated from the feaces of a patient with dysentery (Adams, 1959; Summers, 1999; Stone, 2002; Orlova, 2012). Under certain conditions, some phages behave as antibiotics rather than viruses when they attack and kill susceptible bacteria with no evidence of bacterial lysis or of phage multiplication (Adams, 1959; Summers, 1999).

Bacteriophages, being ubiquitous like bacteria, are common in all natural environments – including our bodies- and are directly related to the numbers of bacteria present. Consequently, they represent the most abundant 'life' forms on earth with an estimated 10^{32} bacteriophages on the planet (Wommack and Colwell, 2000; Rohwer and Edwards, 2002; Hyman and Abedon, 2009; Hyman and Abedon, 2015). The number of phages so far isolated and characterized- a little above 5000 are only a tiny fraction of the total phage population and out of these, those whose complete genome sequence have been determined are just about 750 corresponding to only 12 different bacterial host (Ackermann, 2007; Hatfull and Hendrix, 2011; Orlova, 2012). They are readily isolated from different environments related to their host such as water, manure, animal and produce farms, different processing plant effluent, faeces and sewage, thus very

common in soil (Orlova, 2012; Akhtar *et al*., 2014). The sequencing of bacterial genomes reveals that phage genome elements are important source of sequence diversity and potentially influence pathogenicity and the evolution of the bacteria. There may be many phages that can't be recovered by standard laboratory techniques and are therefore missed. Nevertheless, those already with sequenced genomes can be used for future genetic, biochemical and structural studies (Hatfull and Hendrix, 2011; Orlova, 2012; Rohwer *et al*., 2014).

Bacteriophages form the roots of molecular biology and molecular genetics and are essential tools for genetic engineering (Rohwer *et al*., 2014; Hyman and Abedon, 2015). For example, they provide experimental proof that nucleic acid, not protein, was the genetic material, they assisted in the recognition of triplet code and later were used to uncover mechanisms of gene regulation, protein binding to DNA, protein folding, assembly of macromolecular structures and genetic recombination, they demonstrated evolution by flagrant horizontal gene transfer and provided proof that mutations arise independent of- not as a response to- the pressure of natural selection, their genomes were used for the first genomic and shotgun genomic/metagenomic sequencing, they were used for advances in cancer biology and they contain majority of all varied genes and global pool of genetic diversity encoded in phages and are also essential bionts within the human (Rohwer *et al*., 2014).

2.2 History of Bacteriophages

More than a century ago, bacteriophages were earlier noticed by different bacteriologist due to their action in bacterial cultures but they didn't pursue their findings to a conclusive end due to different restraining factors. They include Ernest Hanbury Hankin (a British bacteriologist) in 1896 who noticed strange antibacterial properties of water from river India that was filtered through porcelain filter against cholera (Hankin,

1896; Abedon *et al*., 2011b; Orlova, 2012; Wittebole *et al*., 2014), Gamaleya (a Russian bacteriologist) who noticed similar activity on *Bacillus subtilis* in 1898 (Samsygina and Boni, 1984; Sulakvelidze *et al*., 2001; O'Flaherty *et al*., 2009) and Frederick William Twort (a british microbiologist) in 1915 who observed similar activity of "filterable agent" which could be passed in series from colony to colony on cultures of *Staphylococcus aureus* and *Micrococcus*. He attributed it to a virus different from viruses of plants and animals but didn't go further because of the shortage of funds and the onset of world war I (Twort, 1915; Adams, 1959; Sulakvelidze *et al*., 2001; Clokie *etal*., 2011; Orlova, 2012; Wittebole *et al*., 2014).

The official breakthrough in the discovery of bacteriophages came when Felix d'Herelle (a French-Canadian medical bacteriologist) who was considered the founder and father of bacteriophages and phage therapy independently discovered, isolated, characterized the viral nature and named the bacteriophage. He also went ahead to use it as biological therapeutic agent in treating infections such as a boy with dysentery who recovered after being treated with phage and in outbreaks such as the severe haemorrhagic dysentery among French troops at Maisons-Laffitte in 1915 and the epidemic of fowl typhoid in 1919 in France (D'Herelle, 1917; Adams, 1959; Summers, 1999; Sulakvelidze *et al*., 2001; Dublanchet and Fruciano, 2008; O'Flaherty *et al*., 2009; Orlova, 2012; Wittebole *et al*., 2014) thereby attracting the attention of many medical scientists to bacteriophage as resistant/preventive and recovery tool from disease and as a tool for various researches and purposes.

Felix shares the credit of phage discovery with Twort since the discoveries which happened at the same time were independent of each other. He presented his findings in September 1917 at the meeting of the academy of sciences which were later published (D'Herelle, 1917). He also took part in establishing the International Bacteriophage

Institute in Tbilisi, Georgia in 1923 (now George Eliava Institute of Bacteriophage, Microbiology and Virology) where researches are still going on for application of phage therapy and phages are supplied for treatment of various bacterial infections (Summers, 1999; Sulakvelidze, 2001; Sulakvelidze *et al*., 2001; O'Flaherty *et al*., 2009). D'Herelle presented many subsequent findings which ignited the interest in the production and use of phage in treating common infectious disease. Since then, the use of bacteriophages became widespread in the control and elimination of infection until the 1940s when it became unpopular especially in western medicine. This was as a result of clinical failures in phage therapy which could be attributed to factors such as phage biology and its different mode of action not understood, poor techniques of experiments, poor quality of phage preparation, lack of understanding and difficulty in accurately identifying the etiological agent of ailment being treated, over-expectation from and therefore misuse of phages, absence of established protocol for *in vitro* testing of bacterial susceptibility to the phages, publications of discordant opinions about the efficacy of phage therapy as well as unreliable, inconsistence and shortage of data from clinical trials (Sulakvelidze *et al*., 2001; Summers, 2004; Skurnik *et al*., 2007; Kutateladze and Adamia, 2010; Wittebole *et al*., 2014).

The main factor that coincided with this factors and caused the abandonment of phages in western medicine -though it was still held in high esteem in the former Soviet Union and the Poland, was the discovery, emergence, implementation and widespread of broad spectrum antibiotics for treatment (Alisky *et al*., 1998; Clark and March, 2006; Dublanchet and Fruciano, 2008; Chan *et al*., 2013). Phage therapy remains mostly inactive until the 1980s when the emergence of antibiotic-resistant bacteria started posing threat to the medical world. The search for alternatives to conventional drugs coupled with the advent of the electron microscope –which enhance better

understanding of phage biology and application spurred the revival of researches into the world of bacteriophages (Ruska, 1940; Luria and Anderson, 1942; Luria *et al*., 1943; Smith and Huggins, 1982 and 1983; Kruger *et al*., 2000; Rice, 2008; Wittebole *et al*., 2014). The human experiments started in the 2000s (Bruttin and Brussow, 2005; Rhoads *et al*., 2009; Merabishvili *et al*., 2009).

Since the renewed interest and increased knowledge in phage therapy, the potentials and application of bacteriophage- though still undergoing intensive research, has gained recognition not just in phage therapy from the medical world alone. The need for its application has overflowed into the fields of Veterinary medicine and animal husbandry, agriculture, water, sewage and environmental treatment and assay, food industry, aquaculture, education and biotechnology as potential bioagents for detection, inactivation, prevention, control and cure of pathogenic bacteria as well as research tools and models in virology, genetic engineering and molecular biology (Watson and Eveland, 1965; Smith, 1985; Benhar, 2001; Sulakvelidze *et al*., 2001; Thomas *et al*., 2002; Nakai and Park, 2002; Willats, 2002; Inal, 2003; Petrenko and Vodyanoy, 2003; Walter, 2003; Clark and March, 2004; Jepson and March, 2004; Goodridge, 2004: Wang and Yu, 2004; Sharma *et al*., 2005; Bruttin and Brussow, 2005; Withey *et al*., 2005; Clark and March, 2006).

2.3 Classification of Bacteriophages

Like other viruses, taxonomic classification of bacteriophages has been a challenge especially to the International Committee on the Taxonomy of Viruses (ICTV) due to the lack of a common protein or genetic locus as that of 16s rRNA in bacteria on which to base a phylogenetic tree (Nelson, 2014). Another challenge is the continual discovery of new phages which are difficult to allocate into species of already established characteristics due to their distinct characteristics (Adams, 1959).

Nevertheless, phages can be classified base on several factors which includes host range, phage genome characteristics, phage morphology and auxiliary structures and infection type.

2.3.1 Host range

Classification of phages based on host range is valuable for individual phage strains but its limitation is that it can change by single step mutation (Adams, 1959). It is also less useful toward obtaining a facile understanding of common themes of phage biology. For example, Phages P1 and Mu can switch between different host ranges by utilising alternate phage-encoded adsorption protein. Also, it is possible to characterize phages without prior phage culturing by using nucleic acid sequencing or whole phage genome sequencing and so the use of phage host range is not appreciated (Hyman and Abedon, 2015).

2.3.2 Phage genome characteristics

Phages can be classified based on genome characteristics in that they can be single stranded (ss) DNA such as M13, double stranded (ds) DNA which is the most common in nature such as T4 and Mu, ssRNA such as MS2, and dsRNA such as $\phi 6$. The genomes could be segmented (multipartite) or non- segmented (monopartite) (Willey *et al*., 2008; Orlova, 2012; Hyman and Abedon, 2015).

The differentiation of phages based on their genome nucleotide sequences (phage genomics) increasingly aids access to a wealth of information useful in inferring their functionality and evolutionary history. It has aided the International Committee on Taxonomy (ICTV) in classifying phages at genus and sub-family levels leading to the development of new genera which so far, were up to 39 genera as at 2013 (Hyman and Abedon, 2015).

2.3.3 Phage morphology and auxiliary structures

Phages can be classified based on morphology and this makes them the largest viral group in nature. The can be filamentous phages, helical phages, or pleomorphic phage, Icosahedral phages without tail, those with tails and those that have lipid-containing envelope or have lipids in the particle shell (Ackermann, 2007). For those that have tail, the tail can be long or short, flexible or rigid and contractile or non-contractile (Hyman and Abedon, 2015). Majority of the phages isolated so far are tailed. Based on the knowledge so far, all tailed phages have dsDNA, monopartile genome and most are lytic phages though some are temperate (Hyman and Abedon, 2015).

The key factors for classifying phages into genera and family are the phage morphology and genomic (nucleic acid) characteristics (Ackermann, 2007; Orlova, 2012). For example, tailed phages which make up 96% of known phages are classified into the order *Caudavirales* and have dsDNA. The order is classified into three families *Myoviridiae, Siphoviridae*, and *Podoviridiae* which are shown in table 2.1 with other examples.

2.3.4 Infection type

Based on the type of infection, bacteriophages are of two types; Virulent (Lytic) bacteriophages and Temperate (Lysogenic) bacteriophages (Lenski, 1988; Orlova, 2012). The virulent phages are phages that undergo productive or lytic cycle in which many free virions are released by the phage-infected bacterial host after the phage genome replication resulting to the lysis and destruction/death of the bacterial host (Lenski, 1988; Willey *et al*., 2008; Hyman and Abedon, 2009). These bacteriophages are good for use in phage therapy and other bacterial control and typing/detection (Jones *et al*., 2007; Bahador *et al*., 2007; Smartt and Ripp, 2011; Lu and Koeris, 2011;

Hyman and Abedon, 2015) but not in fermentation industries and breweries where they cause slow or incomplete fermentation as well as product downgrading (Petty *et al*., 2006; Quiberoni *et al*., 2011; Marcó *et al*., 2012; Suárez *et al*., 2012; Hyman and Abedon, 2015).The temperate bacteriophages on the other hand are the phages that undergo a lysogenic cycle in which the viral DNA is integrated into the host cell DNA as it is replicated along with the host genome. In this case, viral DNA is segregated into and inherited by the daughter bacterial cells and so, no prophages are released. The prophages can maintain this lysogenic relationship with the host (lysogens) as they confer immunity to super infection to the lysogen or under appropriate conditions such as damage to host DNA and abundance of susceptible host, the prophages can initiate induction which leads to/ initiate lytic cycle (Willey *et al*., 2008; Yuole, 2014b; Hyman and Abedon, 2015).

Table 2.1: General characteristics of some phage types

Family	Genome	Morphology	Examples
Plasmaviridae	dsDNA	Pleomorphic	
Inoviridae	ssDNA, circular	Rod-shaped/helical/ Filamentous	M13
Leviviridae	ssRNA, linear	Icosahedral	MS2
Corticoviridae	dsRNA	Icosahedral	
Microviridae	ssDNA, circular	Icosahedral	ϕX174
Myoviridae	dsDNA, linear	Tailed, contractile	Mu, T4
Siphoviridae	dsDNA, linear	Tailed, non-contractile, long, flexible or rigid	T1, T5
Podoviridae	DsDNA, Linear	Tailed, non-contractile, short	P22, T3

Source: Ackermann (2006), Orlova (2012), Hyman and Abedon (2015).

These types of phages are not suitable for phage therapy as they can incorporate pathogenicity or increased virulence as well as serve as a vehicle for antibiotic resistance into the host through transduction (Brussow *et al*., 2004; Hanlon, 2007; Orlova, 2012; Hyman and Abedon, 2015). Instead, they are good tools in biotechnology, molecular biology and genetic engineering. They can be used for fine mapping of gene as good transducers, for developing series of gene expression and as cloning vectors (Zinder and Lederberg, 1952; Ikeda and Tomizawa, 1965; Bahador *et al*., 2007; Hyman and Abedon, 2015).

2.4 Life Cycle of Bacteriophages

Like Most viruses, the life cycle of bacteriophages is in five stages which includes attachment/ adsorption of phage to host, penetration of phage into the host, synthesis of viral genome, assembly of phage particles (for lytic) and release of phage particles.

2.4.1 Attachment/Adsorption of phage to host

This is the first step in the infectious cycle of the phage particle to the host cell. The phage comes across its host during random motion and collision and it attaches itself to specific receptor sites of the host which may be any of the cell surface components (such as protein, oligosaccharide, teichnoic acid, peptidoglycan and lipopolysaccharide) (Lenski, 1988), on the cell capsule, flagella or conjugative pili depending on the type of phage (Willey *et al*., 2008). Attachment is first reversible but later becomes irreversible (Hanlon, 2007; Youle, 2014a; Hyman and Abedon, 2015). After the receptor-mediated attachment to its host, the phage finds its way into the cell by using proteins on its surface to bind to and enter the cell (Lopez and Arias, 2010). It should be noted that absence of a specific receptor or alteration of the receptor (possibly by mutation) makes adsorption of the virus to the host impossible hence it cannot infect the host. Other

crucial factors that help adsorption are the ionic environment and the pH such that unfavourable salt concentration and pH can prevent adsorption. Binding may be as a result of electrostatic interactions which can be influenced by pH and presence of ions such as Mg^{2+} and Ca^{2+}. In the presence of salts, phages speedily attach to bacteria and can't be readily separated by centrifugation (Adams, 1959). It is after binding that penetration occurs (Adams, 1959; Willey *et al*., 2008).

2.4.2 Penetration of phage into the host

After the irreversible interaction, the tail sheath contracts, phage enzymes degrade the cell wall forming a pore. The tail tube penetrates the cell envelope to cell membrane and the phage genome (sometimes accompanied by additional proteins) is injected into the bacterial cytoplasm where replication is optimal (Adams, 1959; Orlova, 2012; Hyman and Abedon, 2015). This happens leaving the capsid outside the host (Willey *et al*., 2009; Yuole, 2014a). Most of the bases on the viral genome are chemically modified to confer protection against attack by cellular restriction and nuclease enzymes (Hanlon, 2007).

2.4.3 Synthesis of viral genome

As a phage infects a host, it expresses certain genes which can be metabolically classified into six major classes. They include the genes involved in taking over the metabolic activity of the host, those that are involved in establishing and maintaining latent state (for lysogenic phages), those involved in preparing the infected cell for phage-progeny maturation, those that express phage-virion structural protein and virion-assembly protein, those that process and package newly replicated phage genomes into virions and those that are involved in either release of assembled virions

from host by lysing the host cell or exporting newly assembled virions through the cell membrane (Yuole, 2014a; Hyman and Abedon, 2015).

Once it enters the cell, the phage starts expressing the early genes which may shut down host gene expression and degrade host genome (for obligate virulent phages). Temperate phages do not completely disrupt the host cell functions since they rely on them (Willey *et al*., 2008; Yuole, 2014b; Hyman and Abedon, 2015). The host cell metabolism is redirected to the viral gene expression and phage production (Yuole, 2014a). The host RNA polymerase is used to synthesize the viral early mRNA which in turn synthesizes proteins and enzymes that take over the host metabolism. Some phages that encode their own DNA polymerase synthesize the enzyme along with the proteins involved in nucleic acid metabolism (Hyman and Abedon, 2015). The next action is the expression of the middle genes which initiates phage genome replication and other functions (Hyman and Abedon, 2015).

In the case of temperate phages, proteins are produced that bring about the integration of the phage genome into the host genome which instead stabilizes the phage genome in an episomal (plasmid) form. Proteins are also produced which prevent the expression of the remaining phage genes by inhibition or termination till it's ready to initiate lytic cycle when it will then express lytic genes and block expression of genes for lysogeny (Hyman and Abedon, 2015).

During the replication, viral DNA is synthesized and its replication is initiated from several origins of replication and it proceeds bidirectionally from each (Willey *et al*., 2008). In temperate phages, the integrated genome is being replicated as the bacterial genome is replicated and it is inherited along by the new daughter bacteria for generations till at certain stage when condition warrant, the phage genes will revert to

lytic cycle (Willey *et al*., 2008; Orlova, 2012). The transcription, translation and replication process yield copies of the viral genome in copies of 25, 50, or hundreds which exchange genome segments with one another or with host chromosome through recombination (Yuole, 2014a).

2.4.4 Assembly of phage particles

Immediately after DNA replication, the late genes are expressed which are dependent on phage-encoded functions for expression than the early genes. They are responsible for making the phage virions, maturing the virion, placing newly replicated phage genomes into the phage particles, cell lysis and release (Willey *et al*., 2009; Hyman and Abedon, 2015). The proteins are assembled into procapsids and the genome is incorporated into each. This process requires much energy in the form of ATP which is supplied by the metabolic activity of the host. The tail is also assembled independently. The tail and the tail fibres are spontaneously added after the head is completed (Willey *et al*., 2009; Yuole, 2014a).

2.4.5 Release of the phage particles

Mature virions accumulate until infection is terminated by lysis of host cell which is deliberately timed and executed by the phage (Yuole, 2014a). With the aid of enzymes earlier produced to attack the peptidoglycan of the host cell wall and create holes in the host membrane, the phages lyse the host cell at the end of the intracellular phase and over 100 exits the lysed cell which is now completely destroyed. The escaping virions set out to search for new host to infect thereby repeating the cycle all over again (Willey *et al*., 2008; Willey *et al*., 2009; Yuole, 2014a). Lysis of host cell can be induced from without before the end of the latent period of the phages by addition of chemicals like Cyanide (Cohen, 1949) or Proflavine (Foster, 1948), massive doses of

phages (Delbruck, 1940), sonic vibration (Anderson and Doermann, 1952) and addition of enzymes like lysozymes (Lwoff *et al*., 1950).

2.5 Potential Application of Bacteriophages

Since its discovery and later renewed interest in it, bacteriophage has been undergoing research in different fields for its use to control bacteria and other uses. Some of the fields in which bacteriophages are already in use or have potentials include phage therapy (human and veterinary medicine), agriculture and aquaculture, water and sewage assay and treatment, biofilm removal, molecular biology and genetic engineering and food safety.

2.5.1 Phage therapy in human and veterinary medicine

Phage therapy is the use of lytic or virulent bacterial viruses (bacteriophages) for treatment of bacterial infections especially those caused by antibiotic resistant bacteria (Sandeep, 2006; Borysowski and Gorski, 2008; Chhibber and Kumari, 2012). It has been used in the treatment of human and animal infections as a complement or alternative to antibiotic therapy (Alisky *et al*., 1998; Matsuzaki *et al*., 2005; Kysela and Turner, 2007; Curtin and Donlan, 2006). It has also been used since phage study started and remained in use in Eastern Europe and the former Soviet Union but abandoned by the western medicine due to some reasons especially the discovery of antibiotics. The emergence of resistance to antibiotics by bacteria especially the major pathogenic ones such as *Vibrio cholera*, *E. coli* 0157:H7 and *Salmonella enterica* led to the search for alternative or complementary antibacterial agents one of which is the use of bacteriophages as a biocontrol agent (Willey *et al*., 2008; Jassim and Limoges, 2014; Hyman and Abedon, 2015). Since then, revival in the interest in phages, researches and clinical trials have been going on in the use and efficacy of bacteriophages as natural

and reliable antibacterial weapon. The successes recorded promise a great future in the control of pathogenic bacteria. Therapeutic application of bacteriophages in the treatment of human eyes, ears, and nose infections, wound infection, gastroenteritis has been on-going for decades in Eliava Institute in Tbilisi, Georgia (Kutter *et al*., 2010; Abedon *et al*., 2011a). Other clinical trials include the use of phage preparations to treat chronic Otitis infection caused by antibiotic resistant *Pseudomonas aeruginosa* in humans (Wright *et al*., 2009) and in dogs (Hawkins *et al*., 2010), treat colibacillosis in poultry (Oliveira *et al*., 2010), decrease concentrations of Campylobacter and Salmonella on Poultry (Fiorentin *et al*., 2005; Atterbury *et al*., 2003; Kittler *et al*., 2013), treat urinary infection in monotherapy and /or in combination with antibiotics (Boratynska *et al*., 1994; Perepanova *et al*., 1995), to treat enteritis in calves, piglets and lambs (Smith and Huggins, 1983), treat suppurative and ulcers bacterial infections by *E.coli*, *Proteus*, *Pseudomonas*, *Staphylococcus* and *Streptococcus* (Lazareva *et al*., 2001; Markoishvili *et al*., 2002) and also treat Potato soft rot (Adriaenssens *et al*., 2012) and tomato bacterial wilt in Plants (Iriarte *et al*., 2012; Bae *et al*., 2012). The veterinary application of phage treats infections in animals and also prevents the carriage/transmission of zoonotic pathogens (O'Flaherty *et al*., 2009; Garcia *et al*., 2008).

2.5.2 Agriculture and aquaculture

Various phages have been isolated and used to detect/diagnose pathogens in cropping ecosystems and in growing crops. They have been also used to eradicate pathogen from contaminated soil or prevent infection in crops especially the economically important crops. As biological control agents, it can also prevent problems associated with use of chemical agents against pathogens such as environmental pollution, ecosystem disruption and residual chemicals on crops (Yamada, 2012). Phages have been used as

biocontrol against etiological agents of plant disease such as bacterial wilt by *Ralstonia solanacearum* in tobacco (Tanaka *et al*., 1990; Toyoda *et al*., 1991) and tomato (Iriarte *et al*., 2012; Bae *et al*., 2012), Potato soft rot by *Dickeya dianthicola* (Adriaenssens *et al*., 2012), Fire blight of pear and apple by *Erwinia amylovora* (Schnabel *et al*., 1998; Schnabel and Jones, 2001; Gill *et al*., 2003), leaf blight in onions and black spots in peppers and citrus fruits (Frampton *et al*., 2012).

The challenges of using phage in this setting is the rapid inactivation of the phage particles by UV-A and UV-B radiation at 280-400nm, high temperature, high and low pH, oxidation conditions, washing down by water, location and accessibility of bacteria in the plant, moisture level on the leaf and the environment and presence of other chemicals that may compromise the phage viability (Ignoffo and Garcia, 1992; Gill and Abedon, 2003; Iriarte *et al*., 2007). In order to curb these challenges, the time of application is to be put into consideration such that it is done in the evening when it will be more effective (Keary *et al*., 2013), the properties and behaviour of each phage-bacterium system should be understood to optimise the phage biocontrol (Nagai, 2012), application of phages should be made directly to the aerial tissues of the plant (phylosphere) or the rhizosphere to prevent sunlight UV from destructive and oxidative inactivation (Jones *et al*., 2007). Phages can also be used to control the level of competitors of beneficial bacteria like nitrogen fixing strains in horticulture (Novikova and Simarov, 1990). Even though phage has the advantages of relatively easy application, low cost, has no negative effect on the ecological systems, safety for humans, animals and plants, it's purpose as a biocontrol agent is not a panacea against plant pathogens (Nagai, 2012). Phages have been studied in aquaculture and their potentials cannot be underestimated. They have been used in different researches to control or prevent infections caused by *Lactococcus garviae* and *Pseudomonas*

plcoglossicida by administering them i.p or orally as phage-impregnated feed (Park and Nakai, 2003; Park *et al*., 2000), some *Vibrio* Species strains especially *Vibrio anguillarum* which cause vibriosis in many fish and invertebrate species (Tan *et al*., 2014), *Aeromonas hydrophila* and *Edwardsiella tarda* in ell (Hsu *et al*., 2000) and *Vibrio vulnificus* which infect and contaminates fish and shellfish (Cerveny *et al*., 2002). The use of phage in plants, animal feed and fish feed instead of antibiotic whose use as a prophylasis and growth enhancer has been banned by US FDA (Inal, 2003) can prevent or treat infection or disease in the plants, fish and higher animals as well as prevent transmission of zoonotic diseases and antibiotic resistant bacteria to humans that consume them (Cerveny *et al*., 2002; Inal, 2003; Kropinski, 2006; O' Flaherty *et al*., 2009; Tan *et al*., 2014). It can also reduce drastically economic loss associated with pre and post-harvest loss of crops to bacterial infection and loss of animals and fishes to bacterial disease due to its ability to prevent/ reduce morbidity and mortality rate (Pyle, 1926; Barrow *et al*., 1998; Park and Nakai, 2003; Vinod *et al*., 2006; Imbeault *et al*., 2006; Castillo *et al*., 2012; Tan *et al*., 2014).

2.5.3 Water and waste water assay and treatment

Water that is considered bacteriologically safe might not be fully safe owing to the fact that the detection of coliforms does not confirm the presence of water contaminating viruses such as enterovirus (Geldenhuys and Pretorius, 1989; Merrett *et al*., 1989; Havelaar *et al*., 1993). The ability of a class of bacteriophages known as coliphages (phages of coliforms) unlike bacterial indicators to resist environmental stress and chlorination like enterovirus, to be removed at comparable rates with enteroviruses during treatment process and exhibit seasonal variation similar to of enteroviruses makes them good candidates to serve as indicators of enteroviruses (Kott *et al*., 1974; Berg, 1974; Simkova and Cervenka, 1981; Settler, 1984; Duran *et al*., 2003; Lucena *et*

al., 2003; Langlet *et al*., 2009). Therefore, they can be good indicators of microbiological quality of water and water pollution (Guelin, 1948; Hilton and Stotzky, 1973; Borrego *et al*., 1987; Hyman and Abedon, 2015) as well as possible models for enteroviruses during drinking water and waste water treatment (Hilton and stotky, 1973; Kott *et al*., 1974; Scarpino, 1978; IAWPRC, 1991; Shirasaki *et al*., 2009a and b). They can also serve as viral indicators of faecal contamination which may provide source-specific information for impacted environmental water (Cole *et al*., 2003; Lucena and Jofre, 2010) and of virus reduction in waste treatment (Sharon *et al*., 2006). Because of their host specificity, phages can be used in microbial source tracking or identification of the source of faecal contamination of water especially environmental water by genotypic, phenotypic and chemical methods (Hsu *et al*., 1996; Simpson *et al*., 2002; Jofre *et al*., 2011). Identifying the source determines the solutions to be applied. This microbial source tracking is applied in recreation water, public water supplies, aquifer protection and propagation of fish, shellfish, and wildlife (Simpson *et al*., 2002). Phage treatment is not only seen as an indicator but also have potential to control problems of wastewater process like sludge dewaterability and digestibility, pathogenic bacteria, foaming in activated sludge plants and to reduce competition between nuisance bacteria and microbial population of interest (Withey *et al*., 2005).

2.5.4 Biofilm removal

Biofilms are the accumulation of microbial cells and their excreted products such as exopolysaccharide attached to living or inert surfaces. They can coat different surfaces like food, pharmaceutical and environmental processing equipment, medical catheters, implants shunts and prostheses (O' Flaherty *et al*., 2009). The presence of these biofilm contaminates these surfaces resulting in subsequent health-care associated infection and mortality (Donlan and Costerton, 2002). Its removal is a major challenge as the bacteria

embedded in its matrix are less accessible to antimicrobial agents (O' Flaherty *et al*., 2009). Different researches and reviews such as Bayer *etal*. (1979), Doolittle *et al.* (1995 and 1996), Lu and Collins (2007), Cerca *et al*. (2007), Donlan (2009) and Brussow (2012) showed evidence of the potential of phage to be used to control biofilms especially if they are engineered to express certain genes which penetrates biofilms and lyse bacteria or weakens adhesion factors in bacteria and aids biofilm formation. They have been used alone (Doolittle, 1995; Sillankorva *et al*., 2004; Cerca *et al*., 2007) or in combination with other anti-biofilm sanitizers/disinfectants such as quaternary ammonium compound (Roy *et al*., 1993), alkaline cleanser and hypochlorite (Sharma *et al*., 2005) which produced synergistic effect in reducing drastically the biofilm bacterial cell count.

2.5.5 Molecular biology and genetic engineering

Bacteriophages especially the tailed ones are not only used as model systems for understanding the general features of virus replication and vital cellular processes, they are also used as convenient tools in microbial genetics and genetic engineering (Cairns *et al.,* 1966). They also make easier the understanding of animal viruses. Their packaging signals, variety of enzymes and promoters and terminators are in use in laboratories of molecular biology (Roberts *et al*., 2003). Some have been used to integrate foreign DNA in mammalian cells (Thyagarajan *et al*., 2001; Hollis *et al*., 2003; Chalberg *et al*., 2005) and *Drosophila* (Groth *et al*., 2004) in order to produce transgenic animals or cure biochemical defects (Groth and Calos, 2004; Ginsburg and Calos, 2005; Held *et al*., 2005). They have been used as tranducers to carry cloned genes and for genetic recombination (Hyman and Abedon, 2010). Phages have been used in phage display to express peptides and proteins just like in standard protein chemistry and it was noticed that the determination of the sequence of the peptide insert

by DNA sequencing is more rapid and less expensive (Winter, 1994; O' Neil *et al*., 1994; Makowski, 1994; Sternberg and Hoess, 1995; Bradbury and Cattaneo, 1995; Ren *et al*., 1996; Sidhu *et al.*, 2003; Takakusagi *et al*., 2005; Kropinski, 2006). They have been used in phage typing through the use of phage amplication system by intracellular phage replication and resulting lysis of host bacterium reading to increase free phage which is easily measured (Badrinath *et al*., 2004; Isaacs *et al*., 2005) and phage-luciferase fusions where phages with wide host range are tagged with luciferase cassettes which when expressed, emits visible light are measured (Oda *et al*., 2004; Sasahara *et al*., 2004) to identify presence of specific pathogens in food and food products.

2.5.6 Food safety

In the food sector, phages have the potentials of preserving, controlling and decontaminating food against food borne pathogens on fresh fruits, vegetables, ready-to-eat food like milk, fish, meat and their product which do not undergo any further processing or cooking before consumption (Goodridge, 2004; Sulakvelidze and Barrow, 2005; Garcia *et al*., 2008; O'Flaherty *et al*., 2009). There are many studies where the use of phage as biocontrol against pathogens have been used to extend the shelf-life and/or storage quality of food (Bahador *et al*., 2007; O'Flaherty *et al*., 2009). These studies among many include Greer (1986) who illustrated that phage multiplied on steak surface and increased significantly the steak case-life suggesting that it is a good potential for the biological control of beef spoilage, he and his colleague studied at low temperature (2^0C) the biocontrol of phages against *Brochotrix thermospactos* on pork and phage as good approach for extending storage quality of chilled meat (Greer and Dilts, 2002), Wall *et al*. (2010) who tested the efficacy of a cocktail of phages to treat *S. Typhimurium* experimentally infected pigs before processing so that the carcass

contamination can be reduced and they found that the *Salmonella* colonization reduction was up to 2-3 Log10, Whichard *et al*. (2003) recorded a reduction of up to 2.1 Log10 in *Salmonella* levels on chicken sausages, Leverentz *et al.* (2001, 2003, 2004) showed that phages controlled a variety of bacterial pathogens on freshly cut honey dew melons and apples. It is as a result of these ground breaking researches and others that there was breakthrough in phage related biocontrol. International agencies such as FDA in 2006 approved two phage cocktails-Listshield (produced by intralytix) and Listex (produced by MICROES formerly EBI Food Safety) for prevention of *Listeria* contamination on ready-to-eat food (Fortuna *et al*., 2008; O'Flaherty *et al*., 2009; Keary *et al.,* 2013), European Food and Safety Authority (EFSA) in 2009 approved phage related preparations as additives to organic food (Keary *et al*., 2013) and Food Standards Australia and New Zealand (FSANZ) in 2012 approved phages as food processing aid (Keary *et al*., 2013).

2.6 Bacteriophage Based Companies

Though the use of bacteriophages as a biological antibacterial agent first caught the world's attention for and got its primary motivation from the treatment or prevention of human infections, its application in other field like veterinary, agriculture and food microbiology gained earlier approval for use globally (Jassim and Limoges, 2014). Its regulatory requirements are still challenging (Potera, 2013). Therefore its use in treatment of human infection might not extend to life threatening infections (Jassim and Limoges, 2014). Clinical trials on the application in human medicine are still going on but the use and commercialization of phages in other fields have been approved and are in use to control bacterial pathogens. Table 2.2 shows the examples of some approved companies and institutes that are into the production and commercialization of phage and phage related preparation for biocontrol of some pathogenic bacteria.

2.7 *Salmonella*Species

Salmonella, named after the veterinary bacteriologist Daniel. E. Salmon, is a genus of microorganism that belongs to the family *Enterobacteriaceae* (Willey *et al*., 2008; Gast, 2008). It consists of two species: *Salmonella enterica* and *Salmonella bongori* (formerly called *S. enterica* subsp. *bongori)*. S. enterica is divided into six subspecies: *S.enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*,*S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *Indica* (Popoff and Minor,

Table 2.2: Commercial phage preparations approved for use

Company	Phage Preparation	Targeted bacteria	Range of products	Year	Approved by	Reference
Intralytix Inc, Baltimore, MD, USA	LMP-102™	*L. monocytogenes*	Meat, poultry	2007	FDA	McVay *et al*., 2007
MICROES, Netherlands	LISTEX™ P100	*L. monocytogenes*	All food products	2007	FDA; USDA	Gorski *et al*., 2007
OmniLytics, Salt LakeCity, UT, USAss	BacWash	*Salmonella*	Hides of livestock	2007	USDA FSIS	Verbeken *et al*., 2007
OmniLytics, Salt LakeCity, UT, USA	AgriPhage™	*Xanthomonas capestris*, *Pseudomonas syringae*	Tomato, Pepper	2006	FDA; USDA FSIS	Fortuna *etal*., 2008; Golkar, *etal*., 2014.

Key:

FSIS; Food Safety and Inspection Service

FDA; Food and Drug Administration

USDA; United States Department of Agriculture

2001; Hendriksen, 2003; Grimont and Weill, 2007). Salmonella strains are classified into serovars base on extensive diversity of lipopolysaccharide (LPS) antigens (O) and flagellar protein antigens (H) in accordance with the Kauffmann–White scheme; currently over 2,500 serovars are recognised (Kayser *et al*., 2005; Grimont and Weill, 2007; Freitas *et al*., 2010). The serovars that are usually associated with causing disease in humans and food animals are in the subspecies *enterica* (Irving, 2005; CiWF and WSPA, 2013) and the most important foodborne strains in terms of number of cases and the severity of infection are *Salmonella* Typhimurium and *Salmonella* Enteritidis (Braden, 2006; Fernandes *et al*., 2006; CiWF and WSPA, 2013).

Salmonellae are facultative anaerobes, non-lactose fermenting, Gram negative, non-spore forming rods, ranging from 0.4-1.5 to 2-5 μm in size. Except for S. pullorum and S. Gallinarum, they are motile with peritrichous flagellae. They ferment D-glucose with the production of acid and usually gas. They also ferment L-arabinose, maltose, Dmannitol, D-mannose, L-rhamnose, D-sorbitol (except ssp VI), trehalose, D-xylose and dulcitol. They are oxidase negative, catalase positive, indole and Voges Proskauer (VP) negative, methyl red and Simmons citrate positive, H_2S producing and urea negative (Hendriksen, 2003; Yousef and Carlstrom, 2003; Montville and Matthews, 2008; Willey *et al*., 2008). Salmonellae are non-fastidious as they can multiply under various environmental conditions outside the living hosts. They do not require NaCl for growth, but can grow in the presence of 0.4% - 4% NaCl. The temperature range that most *Salmonella* serotypes grow is between 5°C and 47°C with optimum at 35°C - 37°C though some can grow at temperature as low as 2°C to 4°C or as high as 54°C (Gray and Fedorka-Cray, 2002). They are sensitive to heat and often killed at temperature of 70°C or above. Salmonellae grow in a pH range of 4 to 9 with the optimum between 6.5 and 7.5. They require high water activity (aw) between 0.99 and

0.94 (pure water aw =1.0) yet can survive at aw less than 0.2 such as in dried foods. Complete inhibition of growth occurs at temperatures less than 7°C, pH less than 3.8 or water activity less than 0.94 (Hanes, 2003; Bhunia, 2008).

Salmonellae are usually found in the intestinal tracts of birds and other animals. They can also be found in farm effluents, human sewage and in/on any material such as fruit and vegetables subject to faecal contamination. As zoonotic organisms, they are acquired by humans through contaminated foods and food products such milk, milk products, pork, pork products, beef, beef products, poultry, eggs, egg products, or water (Willey *et al*., 2008; Freitas *et al*., 2010; EFSA, 2010; CiWF and WSPA, 2013). They can cause infection in both humans and animals- young and pregnant being more susceptible. Some animals especially pigs and poultry may be infected but show no clinical illness and so become important factors in spreading infection between flocks and herds as well as cause human food poisoning when they enter the food chain (Wray and Wray, 2000; Freitas *et al*., 2010). Some diseases caused by *Salmonella* include:

2.7.1 Enteric fever

Enteric fevers are fevers caused by *S.* Typhi and *S.* Paratyphi A, B, or C with bacteraemia namely Typhoid and Paratyphoid respectively. They are usually found only in humans (Cheesbrough, 2006; Willey *et al*., 2008). They are acquired by ingestion of food or water contaminated by faeces of infected humans or person-to-person contact especially where sanitary conditions are inadequate and access to clean water is limited. S. Typhi is mainly water-borne and S. Paratyphi is mainly food-borne (Parry, 2006; Cheesbrough, 2006; Willey *et al*., 2008). The incubation period of Typhi in the small intestine is about 10 to 14 days. The bacteria colonize the small intestine,

penetrate the epithelium, and spread to the lymphoid tissue, blood, liver, and gallbladder. Symptoms include persistent high fever with low pulse rate, severe headache, abdominal pain, anorexia, and malaise, which last several weeks. Bacteria then re-infect the gastrointestinal tract, producing abdominal pain, diarrhoea, inflammation and ulceration, epistaxis, intestinal haemorrhage and perforation, toxaemia and renal failure which mayoccur in untreated late typhoid (and it is often fatal). After approximately 3 months, most individuals stop shedding bacteria in their faeces. However, a few individuals continue to shed S. Typhi for extended periods but show no symptoms. In these carriers, the bacteria continue to grow in the gallbladder and reach the intestine through the bile duct (Cheesbrough, 2006; Willey *et al*., 2008). Paratyphoid fever is a milder form of the fever with lower mortality rate (Cheesbrough, 2006; Willey *et al*., 2008; Pui *et al*., 2011a).

2.7.2 *Salmonella* enterocolitis or gastroenteritis

This is caused mainly by *S.* Typhimurium and *S.* Enteritidis especially in developing countries. Common sources of infection are poultry, meat and meat products, eggs and egg products. Symptoms like diarrhoea, vomiting, fever, and abdominal pain occur 12–36 h after eating contaminated food. In acute infections, blood and mucus can be present in faeces. Infants, the elderly, persons already in poor health, those with ulcerative colitis, malignancy, and immunosuppressed persons are at greater risk of developing serious disease (Cheesbrough, 2006).

2.7.3 Other non-typhoidal salmonellosis in humans

This is caused by other serovars of *Salmonella* that are non-typhoidal which are important food pathogens. It can be gastroenteritis, bacteremia and subsequent focal infection. Salmonellosis is especially problematic in a wide variety of

immunocompromised individuals, including (but not limited to) patients with malignancy, human immunodeficiency virus, or diabetes, and those receiving corticosteroid therapy or treatment with other immunotherapy agents. Endovascular infection and deep bone or visceral abscesses are important complications that may be difficult to treat (Hohmann, 2001).

2.7.4 Pullorum disease and fowl typhoid in birds

These two diseases have many similarities. Pullorum disease is caused by *Salmonella* Pullorum while Fowl typhoid is caused by *Salmonella* Gallinarum (Shivaprasad and Barrow, 2008). Both are septicemic diseases affecting primarily chickens and turkeys, but other birds such as quail, pheasants, ducks, peacocks, and guinea fowl are also susceptible (Shivaprasad and Barrow, 2008; CFSPH, 2009). They can be transmitted horizontally or vertically. Horizontal transmission happens when birds ingest them via the respiratory and oral routes, from contaminated environment or during cannibalism, through wound infections and from fomites including contaminated feed, water and litter since the bacteria may survive in a favourable environment for many months and up to several years. They are transmitted vertically when birds that are chronic carriers pass the bacteria to their offspring in eggs by transovarian infection (Beaudette, 1925; Beach and Davis, 1927; Hinshaw, 1930; Shivaprasad and Barrow, 2008; CFSPH, 2009). The disease or infection is characterized by moribund or dead birds immediately or within short time after hatching, somnolescence, weakness, depressed appetite, poor or retarded growth, gasping or laboured breathing, blindness and lameness in chicks (Beaudette, 1930; Evans *et al*., 1955; Johnson *et al*., 1992; Mayahi, 1995; Shivaprasad and Barrow, 2008) or sudden drop in feed consumption, ruffled feathers and pale combs, drop in egg production, decreased fertility, diminished hatchability, anorexia, diarrhoea, increased temperature, dehydration and sometimes death in growing and

mature birds. Mortality is highest at first outbreak followed by less severe losses in intermittent recurrence (Hinshaw *et al*., 1926; Erbeck *et al*., 1993; Shivaprasad and Barrow, 2008).

2.7.5 Salmonellosis in other animals

The nature and severity of Salmonella infections in different animal species varies enormously and is influenced by many factors such as the infecting Salmonella serovar, strain virulence, infecting dose, host animal species, age and immune status of the host, and the geographical region (Wallis, 2006). For example, the serovars associated with salmonellosis in cattle are usually serovars Dublin and Typhimurium, route of infection is usually oral, respiratory and conjunctival route and in terms of immune status, calves deprived of colostrum are more susceptible to infection. Likewise in pigs, the typical salmonelloses are septicaemia caused by serovars like Choleraesuis which cause disease in both adults and young and enterocolitis by serovars like Typhimurium which cause disease in pigs aged between 6-12 weeks but rarely in adults (Wallis, 2006). Salmonellosis in these animals is usually characterized by pyrexia, dullness, anorexia, diarrhoea with blood, mucus, or necrotic intestinal mucosa, septicaemia, pneumonia, abortion in pregnant animals, hepatomegaly, high temperature and high mortality (Wallis, 2006).

2.8 *Salmonella*Outbreaks and Economic impacts

Salmonella as a food borne pathogen is of global significance. There are 16 million annual cases of typhoid fever (Pui *et al*., 2011a). Salmonellosis is the most economically important foodborne disease of man and acute gastroenteritis caused by *Salmonella* spp remains a major global public health problem with an annual incidence of 1.3 billion cases with 3 million deaths (Pang *et al*., 1995; Thong *et al*., 1998; Pui *et*

al., 2011a). Nontyphoidal salmonellae in Africa are the most common cause of bloodstream infections in children younger than five years (Graham *et al*., 2000). Meningitis caused by nontyphoidal salmonellae is uncommon in economically developed countries (Saphra and Winter, 1957) but more frequent in tropical countries especially in children younger than six months, and associated with higher case-fatality rates than meningitis caused by other bacteria (Barclay, 1971; Graham *et al*., 2000; Molyneux *et al*., 2000; Sirinavin *et al*., 2001). *Salmonella* is among the most commonly isolated foodborne pathogens associated with fresh fruits and vegetables. Recently, the incidence of foodborne outbreaks caused by the contamination of fresh fruits and vegetables has increased and become a great concern in industrialized countries. Outbreaks of salmonellosis have been linked to a wide variety of fresh fruits and vegetables including apple, cantaloupe, alfalfa sprout, mango, lettuce, cilantro, unpasteurized orange juice, tomato, melon, celery and parsley (Pui *et al*., 2011b). Other vehicles of *Salmonella* that resulted in outbreaks include shell eggs, peanut butter and mixed salad (D'Aoust and Maurer, 2007; Montville and Matthews, 2008).

2.9 Antibiotics

Antibiotics are low molecular weight natural or synthesized chemotherapeutic agents which are microbial metabolites or their derivative that can either kill ("cidal" antibiotics) susceptible microorganisms or inhibit ("static" antibiotics) their growth (Willey *et al*., 2008; Masurekar, 2009; Safe food, 2010). They are used to treat or prevent infectious diseases in animals, humans and plants. Ideally, chemotherapeutic agents used to treat infectious disease destroy pathogenic microorganisms or inhibit their growth at concentrations low enough to avoid undesirable damage to the host (Willey *et al*., 2008; Safe food, 2010). The discovery of antibiotics started with the discovery of Penicillin which was originally discovered in 1896 by a 21-year-old

French medical student named Ernest Duchesne. His work was forgotten until Alexander Fleming accidentallly rediscover the antibiotic in September 1928 when he noticed the activity of substance produced by a mould-*Penicillium notatum* whose spore accidently found its way into a petri plate of *Staphylococcus* he left before going for a weekend vacation. He named the active diffusible substance that can kill several pathogenic bacteria Pennicillin (Willey *et al*., 2008; Masurekar, 2009). Since then, the search for other antibiotics was stimulated and the development of newer, more powerful drugs revolutionized the world of medicine, alleviated human suffering, saved countless lives and aid useful microbiological research (Willey *et al*., 2008; Masurekar, 2009; Davies and Davies, 2010).

Available antibiotics can be classified as natural products, chemically modified natural products (also called semisynthetic antibiotics) and those made by total chemical synthesis. The majority are either natural products or semisynthetic compounds derived from them. Therefore, microorganisms remain today a major focus of the search for new antibiotics (Masurekar, 2009; Safe food, 2010).

2.10 Classes of antibiotics and their mode of action

2.10.1 Inhibitors of cell wall synthesis

Antibiotics that interfere with bacterial cell wall synthesis are the most selective antibiotics and have high therapeutic index because they target only prokaryotic cell structures. Classes of antibiotics that do this include the penicillins, cephalosporins, vancomycin, and bacitracin (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.1.1 Penicillins

These are derivatives of 6-aminopenicillanic acid and differ from one another with respect to the side chain attached to its amino group. Although their complete mechanism of action is still not completely known, it has been proposed that this structural similarity blocks the enzyme catalyzing the transpeptidation reaction that forms the peptidoglycan cross-links thereby blocking the formation of a complete cell wall causing osmotic lysis. They also bind to several periplasmic proteins and destroy bacteria by activating their own autolytic enzymes (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.1.2 Cephalosporins

This family of antibiotics contain a β-lactam structure that is very similar to that of the penicillins. Like penicillins, they also inhibit the transpeptidation reaction during peptidoglycan synthesis. They are broad-spectrum drugs frequently given to patients with penicillin allergies. Cephalosporins are broadly categorized into four generations (groups of drugs that are sequentially developed) based on their spectrum of activity. First generation cephalosporins are more effective against gram-positive pathogens than gram-negatives. Second-generation drugs, developed after the first generation, have improved effects on gram-negative bacteria with some anaerobe coverage. Third-generation drugs are particularly effective against gram-negative pathogens, and some reach the central nervous system. Though, antimicrobial agents do not cross the blood-brain barrier. Finally, fourth-generation cephalosporins are broad spectrum with excellent gram-positive and gram-negative coverage and, like their third-generation predecessors, inhibit the growth of the difficult opportunistic pathogen *Pseudomonas*

aeruginosa (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.1.3 Vancomycin

Vancomycin's peptide portion blocks the transpeptidation reaction by binding specifically to the D-alanine-D-alanine terminal sequence on the pentapeptide portion of peptidoglycan. The antibiotic is bactericidal for *Staphylococcus* and some members of the genera *Clostridium*, *Bacillus*, *Streptococcus*, and *Enterococcus*. It is given both orally and intravenously and has been particularly important in the treatment of antibiotic-resistant staphylococcal and enterococcal infections. It has been considered the "drug of last resort" in cases of antibiotic resistant *S. aureus* (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.2 Protein synthesis inhibitors

These antibiotics inhibit protein synthesis by binding with the prokaryotic ribosome. Their therapeutic index is fairly high, but not as that of the cell wall inhibitors because they can discriminate between prokaryotic and eukaryotic ribosomes. Some drugs bind to the 30S ribosomal subunit while others bind to the 50S subunit. They also affect different steps in protein synthesis such as aminoacyl-tRNA binding, peptide bond formation, mRNA reading, and translocation. Families of antibiotic with this mode of action include the Aminoglycosides, Tetracyclines and Chloramphenicol (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.2.1 Aminoglycosides

They contain a cyclohexane ring and amino sugars. They bind to the 30S ribosomal subunit and interfere with protein synthesis by directly inhibiting the synthesis process

and also by causing misreading of the mRNA. These antibiotics are bactericidal and tend to be most effective against gram-negative pathogen (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.2.2 Tetracyclines

These antibiotics are similar to the aminoglycosides and combine with the 30S subunit of the ribosome. This inhibits the binding of aminoacyl-tRNA molecules to the A site of the ribosome. Because their action is only bacteriostatic, the effectiveness of treatment depends on active host resistance to the pathogen. Tetracyclines are broad-spectrum antibiotics that are active against gram-negative, as well as gram-positive, bacteria, rickettsias, chlamydiae, and mycoplasmas (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.2.3 Chloramphenicol

It was first produced from cultures of *Streptomyces venezuelae* but it is now synthesized chemically. It binds to 23S rRNA on the 50S ribosomal subunit to inhibit the peptidyl transferase reaction. Due to its high toxicity, it is used only in life-threatening situations when no other drug is adequate (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.3 Metabolic antagonists

Some antibiotics they antagonize or block the functioning of metabolic pathways by competitively inhibiting the use of metabolites by key enzymes. They act as structural analogs (molecules that are structurally similar to naturally occurring metabolic intermediates). These analogs compete with intermediates in metabolic processes

because of their similarity, but are just different enough so that they prevent normal cellular metabolism. As such they are bacteriostatic but broad spectrum. They include classes of antibiotics like Sulfonamides or Sulfa Drugs and Trimethoprim (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.3.1 Sulfonamides or Sulfa Drugs

These drugs are structurally related to sulfanilamide, an analog of ρ-aminobenzoic acid, or PABA. PABA is used in the synthesis of the cofactor folic acid (folate). When sulfanilamide or another sulfonamide enters a bacterial cell, it competes with PABA for the active site of an enzyme involved in folic acid synthesis, causing a decline in folate concentration. This decline is detrimental to the bacterium because folic acid is a precursor of purines and pyrimidines, the bases used in the construction of DNA, RNA, and other important cell constituents. The resulting inhibition of purine and pyrimidine synthesis leads to cessation of protein synthesis and DNA replication, thus the pathogen dies. Sulfa drugs have a high therapeutic index because they are selectively toxic for many pathogens since these bacteria manufacture their own folate and cannot effectively take up this cofactor, whereas humans do not synthesize folate (we must obtain it in our diet) (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.3.2 Trimethoprim

It interferes with the production of folic acid by binding to dihydrofolate reductase (DHFR), the enzyme responsible for converting dihydrofolic acid to tetrahydrofolic acid, competing against the dihydrofolic acid substrate. It is a broad-spectrum antibiotic often used to treat respiratory and middle ear infections, urinary tract infections and traveller's diarrhoea. It can be combined with sulfa drugs to increase efficacy of

treatment by blocking two key steps in the folic acid pathway (Lambert, 1998; Madigan *et al.*, 2000;Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.4 Nucleic acid synthesis inhibitors

Antibacterial drugs that inhibit nucleic acid synthesis function by inhibiting DNA polymerase and DNA helicase or RNA polymerase and thus block the process of replication or transcription. These drugs are not as selectively toxic as other antibiotics because procaryotes and eucaryotes do not differ greatly with respect to nucleic acid synthesis. Drugs with this mode of action include Quinolones which are synthetic drugs that contain the 4-quinolone ring. Generations of fluoroquinolones have been produced and more are still being synthesized and tested. One of them, Ciprofloxacin was used during the 2001 bioterror attacks in the United States as one treatment for anthrax (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008). Quinolones act by inhibiting the bacterial DNA gyrase and topoisomerase II. DNA gyrase introduces negative twist in DNA and helps separate its strands. Inhibition of DNA gyrase disrupts DNA replication and repair, bacterial chromosome separation during division, and other cell processes involving DNA. Fluoroquinolones also inhibit topoisomerase II, another enzyme that untangles DNA during replication. It is not surprising that quinolones are bactericidal (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

The quinolones are broad-spectrum antibiotics. They are highly effective against enteric bacteria such as *Salmonella* species, *E. coli* and *Klebsiellapneumoniae*. They can be used with *Haemophilus, Neiserria, P. aeruginosa,* and other gram-negative pathogens. The quinolones also are active against gram-positive bacteria such as *S. aureus, Streptococcuspyogenes,* and *Mycobacterium tuberculosis*. Currently, they are used in

treating urinary tract infections, sexually transmitted diseases caused by *Neisseria* and *Chlamydia*, gastrointestinal infections, respiratory infections, skin infections and osteomyelitis (bone infection) (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002; Willey *et al*., 2008).

2.10.5 Disruptors of the cytoplasmic membrane

The balance of noncovalent interactions between the constituents involving ionic, hydrophobic and hydrogen bonding which maintains the stability of all membranes can be disturbed by the intrusion of molecules (membrane-active agents) which destroy the integrity of the membrane, thereby causing leakage of cytoplasmic contents or impairment of metabolic functions associated with the membrane. Most membrane-active agents have poor selectivity and so, can't be used systemically because of their damaging effects upon mammalian cells; instead they are used as skin antiseptics, disinfectants and preservatives. A few agents can be used therapeutically: the polymyxins, which act principally upon the outer membrane of Gram-negative bacteria (Lambert, 1998; Madigan *et al*., 2000; Talaro and Talaro, 2002).

2.11 Antibiotic resistance

There is a growing need to develop or discover new antibiotics. Microorganisms are evolving rapidly to attain resistance to the prevailing antibiotics. Antibiotic resistance poses serious threats to human and animal health. The widespread of drug resistant pathogens is one of the most serious threats to public health in this century (Willey *et al*., 2008; Vaishnav and Demain, 2009; WHO, 2014). The indiscriminate use and prescription of antibiotics in human and veterinary medicine, in animal feeds as growth promoters, in soaps and deodorants and disposition of unused or unmetabolised therapeutic drugs which has not been degraded in sewage systems and hospital

effluents that subsequently reach surface water then underground water and potentially drinking water has caused increase in number and spread of disease resisting treatment due to spread of drug resistance in microorganisms (Kummerer, 2003; Willey *et al*., 2008; Vaishnav and Demain, 2009).

Antibiotic resistance are classified into two types which can result in treatment failure; Intrinsic (Inherent) and acquired resistance (Power, 1998; Talaro and Talaro, 2002; Romero *et al*., 2012). Intrinsic resistance is the use of the inherent properties of the bacterium to prevent antibiotic action (Godfrey and Bryan, 1984) making them normally not susceptible to the antibiotic. This is possible due to inability of the antibacterial agent to enter the bacteria cell and reach its target site, or a lack of affinity between the antibacterial and its target (site of action), or the absence of the target in the cell (Romero *et al*., 2012). It is chromosomally mediated (Russell and Chopra, 1996). Acquired resistance occurs when bacteria that is usually susceptible to an antibiotic becomes resistant after exposure to the antibiotic. It occurs by mutations in the chromosome or by the acquisition of genes coding for resistance from an external source normally via a plasmid or transposon (Russell and Chopra, 1996; Power, 1998). Acquired resistance is more of a threat than the intrinsic in the spread of antibiotic resistance due to its transmissible nature of the resistance mechanism (Russell and Chopra, 1996; Power, 1998; Romero *et al*., 2012). The genetic elements responsible for acquired resistance are chromosomes, plasmids and transposons (Lewis 1989; Talaro and Talaro, 2002).

2.11.1 Chromosomal mutations

Mutations to chromosomal genes due to changes in the DNA sequence can result in resistance to some antibiotics. The mutation could be by transition which is the

substitution of one purine (A or G) for another and therefore one pyrimidine (C or T) for another, transversion which involves a change from a pyrimidine to a purine and vice versa and occurs when a segment of the DNA is reversed and duplication occur when a segment of the DNA is repeated, frameshift mutations which happens when one or two bases are inserted into the DNA sequence, resulting in an altered reading frame and therefore an altered gene product, macrolesions which are more extensive changes in the DNA sequence, deletions which result to loss of part of the DNA or insertions in which extra base pairs are added to a gene (Power, 1998).

2.11.2 Plasmids

These are additional circular elements of the DNA aside the normal genes of the chromosome necessary for the growth and replication of cells which are capable of replicating and transferring independent of the chromosome. They can code for properties that include antibiotic resistance. They have the ability to transfer within and between species and can therefore be acquired from other bacteria or by cell division. This property makes plasmid-acquired resistance much more threatening in terms of the spread of antibiotic resistance than resistance acquired due to chromosomal mutation and the ability to transfer antibiotic resistant genes is enhanced by the presence of transposons. Plasmids transfer can be done by conjugation (the transfer of DNA from a donor cell to a recipient cell by cell to cell contact), transduction (the transfer of DNA by bacteriophages and less significant in transfer of antibiotic resistance than conjugation since it is limited to organisms of the same species) and transformation (the ability of certain microorganisms to acquire naked DNA from the environment and also limited to organisms of same or related species, thus less significant than conjugation) (Power, 1998; Romero *et al*., 2012).

2.11.3 Transposons

These are mobile genetic elements capable of transferring or transposing independently from one DNA molecule (chromosome or plasmids) to another. They are normally flanked by short regions of almost identical DNA sequence known as repeats which function as recognition sequences for enzymes involved in transposition (the ability of transposons to transfer and integrate into the recipient DNA molecule). The central region of the transposon often codes for antibiotic resistance genes. Transposons do not require homologous regions of DNA in order to integrate into a DNA molecule unlike the normal recombination process in bacterial cells and are therefore a major cause of the transfer and spread of antibiotic resistance genes among different bacterial species. it is possible for bacteria to acquire a series of transposons coding for different antibiotic resistances by insertion in existing plasmids or the chromosome. Conjugative transposons which can mediate their own transfer have been described in the last 10 years. These make the transfer and spread of resistance genes more likely since they do not depend on insertion into conjugative plasmids for their mobilization (Power, 1998).

The mechanisms of drug resistance to antibiotic action by microorganisms can be achieved in different ways. Gene can be expressed to synthesize enzymes that inactivate the drug, decrease cell permeability and drug uptake, change the number or affinity of the drug receptor sites or modify an essential metabolic pathway (Power, 1998; Talaro and Talaro, 2002). Other mechanisms include lapsing into dormancy by bacteria to indirectly become resistant, converting cell wall orientation to the form that the antibiotic cannot affect (Talaro and Talaro, 2002).

Aside resistance to antibiotics by microorganisms, antibiotics have other limitations that gave rise to the need for alternative or complement to antibiotics. Allergies that are

sometimes fatal have been reported after treatment with antibiotics like pennicillins, aminoglycosides (Willey *et al*., 2008). Toxicity such as neurotoxicity and bone marrow toxicity due to consumption of antibiotics like the aminoglycosides, quinolones and cephalosporins has been reported (Isaacson *et al*., 1993; Grill and Maganti, 2008; Willey *et al*., 2008; Grill and Maganti, 2011; EFSA, 2014). Adverse effects like deafness, diarrhoea, after taking tetracycline, fever, hives rashes after taking sulfa drugs and quinolones had happened (Talaro and Talaro, 2002; Willey *et al*., 2008). Other complications such as renal damage, kidney and liver damage after medication with tetracycline, cardiovascular collapse, haematologic and aplastic anaemia and decreased white blood cells after taking chloramphenicol have been reported (Balbi, 2004; Willey *et al*., 2008; EFSA, 2014). Also, problems such as high cost of treatment and inability to restore initial appearance of skin have resulted in research on newer agents for the treatment (Church *et al*., 2006; McVay *et al*., 2007).

2.12 Advantages of bacteriophages

It is the search for solutions to these problems that motivated the researches into other fields including the use of bacteriophages as a biological control for bacteria. Though it can't completely replace antibiotics, Bacteriophage has some advantageous features that make it suitable to be used as an alternative or complement antibiotics. They include: bacteria infected by obligately lytic phages are unable to gain their viability (Carlton, 1999; Stratton, 2003), no side effect of phage application has been reported since its long use for human therapy (Chanishvili, 2009; Kutateladze and Adamia, 2010), they exhibit auto dosing since they are self-replicating to a high concentration in the presence of their host and might not need multiple applications. They are also self-limiting as soon as bacterial cells are eliminated (Abedon and Thomas-abedon, 2010; Kutateladze and Adamia, 2010), since they consist of mostly nucleic acid and proteins,

they are inherently non-toxic. They are only harmful to the bacterial host but not to the animal or plant. Though they may interact with the immune system resulting to harmful immune responses, there is little evidence of it being of concern during phage treatment. Nevertheless, highly purified phage preparations are used in certain phage therapy protocols to prevent such immune responses (Skurnik and Strauch, 2006; Skurnik *et al*., 2007; Gorski *etal*., 2007; Kutter *et al*., 2010), due to their host specificity, phages only minimally affect health-protecting normal flora bacteria and so, don't induce super infection to the animal or plant (Hyman and Abedon, 2010; Gupta and Prasad, 2011), they have narrower potential for inducing phage-resistance due to their relatively narrow host range. Therefore, the number of bacterial types with which selection for specific phage-resistance mechanisms can occur is limited. Like bacteria, phages mutate and therefore can evolve to counter phage-resistant bacteria. Furthermore, some mutations to resistance negatively impact bacterial fitness or virulence due to loss of pathogenicity-related phage receptors (Ho, 2001; Matsuzaki *et al*., 2005; Skurnik and Strauch, 2006; Hyman and Abedon, 2010; Cappareli *et al*., 2010), Because phages infect and kill using mechanisms that differ from those of antibiotics, specific antibiotic resistance mechanisms do not translate into mechanisms of phage resistance. Phages consequently can be readily employed to treat antibiotic-resistant infections (Carlton, 1999; Skurnik *et al*., 2007; Gorski *et al*., 2007; Kutter *et al.*, 2010), being part of the natural ecosystem, phages against many pathogenic bacteria are easily discovered often from sewage or other waste materials that contain high bacterial concentrations (Loc-Carrillo and Abedon, 2011), like antibiotics, they can be versatile in terms of formulation development, such as being combined with certain antibiotics. They are also versatile in application form, as liquids, creams, impregnated into solids, etc., in addition to being suitable for most routes of

administration. Different phages furthermore can be mixed as cocktails to broaden their properties, typically resulting in a collectively greater antibacterial spectrum of activity (Alisky *et al*, 1998; Carlton, 1999; Krylov, 2001; Gorski *et al*., 2007; Kutateladze and Adamia, 2010; Kutter *et al*., 2010; Goodridge, 2010), the ability of phages to increase in density in situ, given sufficient bacterial densities, could potentially reduce treatment costs by reducing phage doses required to achieve effiacy. Application of phages in low doses may also improve product safety, since phages will only increase in density if they are actively killing bacteria and do not otherwise linger long within the body (Abedon and Thomas-abedon, 2010; Kutter *etal*., 2010), because they are composed predominantly of nucleic acids and proteins and possess relatively narrow host ranges, discarded therapeutic phages- unlike broad-spectrum chemical antibiotics, will at worst have an impact on only a small subset of the environment and environmental bacteria. Phages not adapted to degradative environmental factors, such as sunlight, desiccation, or temperature extremes, also can be rapidly inactivated (Ding and He, 2010; Hyman and Abedon, 2010; Abedon and Thomas-abedon, 2010; Loc-Carrillo and Abedon, 2011) the use of phages is effective and safe in the treatment of especially antibiotic resistant infections (Perepanova *et al*., 1995; Bahador *et al*., 2007; Borysowski and Gorski, 2008) and it has been shown that successful phage therapy of staphylococcal infections, including those caused by MRSA, is much less expensive than antibiotic treatment impliying that wider phage application could also be beneficial for the economy of healthcare, a factor that must be considered in every country (Miedzybrodzki *et al*., 2007; Borysowski and Gorski, 2008).

2.13 Limitations of Bacteriophages

Bacteriophages also have some limitations on which researches are still on-going to curb in order to use them effectively to combat pathogenic bacteria. Some of them

include: bacteriophages can only be used effectively to control bacterial population if they are obligately lytic, stable under typical storage conditions and temperatures, subject to appropriate effiacy and safety studies, and, ideally, fully sequenced to confirm the absence of undesirable genes such as toxins (Krylov, 2001; Skurnik *et al*., 2007). Therefore, temperate phages cannot be used due to a combination of display of superinfection immunity (Hyman and Abedon, 2010), which converts phage-sensitive bacteria into insensitive ones, and the encoding of bacterial virulence factors, including bacterial toxins (Krylov, 2001; Skurnik and Strauch, 2006; Skurnik *et al*., 2007; Gill and Hyman, 2010). In addition to characterizing phages prior to use by virion morphology (via electron microscopy), protein profies, or genotype characterization other than via full-genome sequencing (e.g., PFGE profies of restriction digested genomes) (Krylov, 2001) , to curb this problem, it is necessary to identify those phages that display good primary pharmacodynamics (that is, antibacterial virulence), minimal secondary pharmacodynamics (low potential to do harm to patients), and good pharmacokinetics (an ability to reach target bacteria in situ) (Abedon and Thomas-Abedon, 2010). Phages that do not adequately meet these criteria should in most circumstances not be employed as therapeutics. Minimally this should entail avoiding temperate phages and, ideally, full genome sequencing should be used to rule out virulence factor carriage (Loc-Carrillo and Abedon, 2011) and the narrowness of phage host ranges place limitations on presumptive treatment with phages. However, as phages can often be employed in combination with other antibacterial agents, including other phages (known as phage cocktails), the lytic spectrum of phage products can be much broader than the spectrum of activity of individual phage types (Kutaladze and Adamia, 2010; Kutter *et al*., 2010; Goodridge, 2010) and even broader than antibiotics (Loc-Carrillo and Abedon, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Collection of Samples

The samples assumed to contained the bacteria and bacteriophages of interest were obtained from various sources as follows:

3.1.1 Bacterial collection

A total of four clinical isolates (samples 2, 4, 8 and 14) suspected to be *Salmonella* Typhi and *Salmonella* Paratyphi from stool samples were collected from the Laboratory of the University health services (Sick Bay) Ahmadu Bello University, Zaria after obtaining permit from the authority of the University Health Services. Thirty six samples consisting of fishes, cabbages, eggs, onion leaves, beef entrails and lettuces were obtained on different occasions from Samaru market. The clinical isolates and food samples were respectively transported immediately on collection to the laboratory, Department of Microbiology, Faculty of Life Science, Ahmadu Bello University, Zaria for confirmation and isolation respectively of *Salmonella* species.

3.1.2 Phage source collection

Duplicate waste water samples of 100ml eachwere collected into clean plastic containers from the central collection point of the university's drainage system. Duplicate waste water samples of 100ml each were also collected from the dam (used for fishing and farming activities) at Kwanan kurmi, Zaria. Wet soil samples were also collected from the farmlands and open fields where animals usually graze around the dam. All water and soil samples were transported in iced water pack to the laboratory, Department of Microbiology, ABU, Zaria for further use. 'Salmonelex' -A broad

spectrum *Salmonella* bacteriophage preparation of 2 x 10^{11} pfu/ml, obtained from Microes Food Safety, Netherlands was used as reference phage stock.

3.2 Sample Processing

After collection, the samples were processed as follows:

3.2.1 Bacterial isolation, identification and characterization

The samples were passed through a series of test to obtain isolates typical of Salmonella species and further characterized as follows:

3.2.1.1 Isolation of pure culture

Three whole eggs were each placed in 20ml sterile distilled water, allowed to stand for 10 minutes after which they were removed from the water. From each mixture, 1ml was taken and added to nine millilitres of nutrient broth each which is the pre-enrichment broth. Three other whole eggs were washed, disinfected with hypochlorite solution and allowed to dry. A sterile syringe was used to puncture each egg, make a pool of or mix the egg content (yolk and white), and 1ml of each egg was drawn out and aseptically injected into 9ml each of the pre-enrichment broth. One gram each of the other food samples (fishes, cabbages, lettuces, beef entrails and onion leaves) were each placed into 9ml of pre-enrichment broth. All the broths were incubated at 37^0C for 24 hours (Andrews and Hammacks, 2001; Loongyai *et al*., 2010; Al-ledani *et al*., 2014).

After 24 hours, 1 ml of each pre-enriched culture was inoculated into 9ml of Selenite F broth and incubated for 15 hours. All broths that were turbid and were reddish in colour were streaked on XLD, SSA, and BSA. Isolates obtained from Sick bay were also streaked directly on XLD, SSA and BSA. All inoculated plates were incubated at 37^0C for 24 hours and colonies formed were examined for characteristics such as

morphology and colour formation typical of *Salmonella* species. Discrete colourless colonies or colonies with black centre on SSA and XLD and brown colonies with metallic sheen on BSA were taken for Gram staining and were also inoculated in sterile nutrient broth and incubated for 24 hours at 35^0C for further analysis (Andrews and Hammacks, 2001; Willey *et al*., 2008; Loongyai *et al*., 2010; Al-ledani *et al*., 2014).

3.2.1.2 Gram stain of bacterial isolates

Smear of pure culture of the test organisms were prepared, air-dried and heat fixed on clean grease-free slides. The smears were covered with crystal violet stain for 60 seconds and washed off with clean water. They were covered with Lugol's Iodine for 60 seconds and washed off. Acetone was added drop wise and washed off immediately with water. They were covered with Safranin stain for 60 seconds and washed off. The slides were air dried and examined with microscope using the 40x objective and then 100x objective with oil immersion (Woodland, 2004; Cheesebrough, 2006).

3.2.1.3 Biochemical characterization of bacterial isolates

The Biochemical tests carried out for the identification of the isolates include the following:

3.2.1.3.1 Indole test

One and the half grams of Tryptone water media was measured and added to 100ml of distilled water. Three millilitres of the mixture were each dispensed into several bijou bottles and tightly covered. All bottles were autoclaved at 121^0C for 15 minutes after which they were allowed to cool and the test organisms were each inoculated and incubated at 37^0C for 48 hours. The control was left uninoculated. To each bottle, 0.5 ml Kovac's reagent was added and shaken gently. They were examined for red

coluration at the surface layer which indicates positive reaction (Woodland, 2004; Cheesebrough, 2006; Hemraj *et al*., 2013).

3.2.1.3.2 Citrate utilization test

Seven grams of Simmon's citrate agar powder was added to 300ml of distilled water and boiled to dissolve. Ten millilitres of the mixture were each dispensed into several test-tubes and covered with cotton wool. They were all autoclaved at 121^0C for 15 minutes, slanted and cooled to solidify. Each slant was inoculated with an isolate and incubated at 37^oC for 24 hours. They were thereafter examined for colour change from green to blue (Woodland, 2004; Tadesse and Alem, 2006; Hemraj *et al*., 2013).

3.2.1.3.3 Methyl Red and Voges-Proskauer tests (MR-VP)

Three and the half gram of MR-VP medium was added to 200ml of distilled water and allowed to dissolve. Five millilitres of the broth was each dispensed into several bijou bottles. The bottles were tightened and autoclaved at 121^0C for 15 minutes. After cooling the broths, the isolates were each inoculated in the broths and incubated for 48 hours at 35^oC. To a small tube, 1ml of the broth was transferred into which 3 drops of methyl red was added and observed for colour change. To the remaining broth, 15 drops of 5% **α-** Naphtol was added and shaken, 5 drops of 40% potassium hydroxide was added and shaken and the cap of the tube was loosen then the tube was placed in a sloping position as red colour formation is observed at the air-liquid interface for one hour (Tadesse and Alem, 2006; Hemraj *et al*., 2013).

3.2.1.3.4 Motility test

Four and the half gram of nutrient agar was added to 300 ml of distilled water to make it half strength. It was boiled to dissolve and dispensed -10ml in each test tube. The

media were autoclaved at 121^0C for 15 minutes and allowed to cool. Each isolate was inoculated in the motility medium by making a fine stab with a sterile straight wire to a depth of 2cm short of the bottom of the tube. It was incubated at 35^oC for 48 hours and examined for radiating turbidity away from the line of inoculum (Woodland, 2004).

3.2.1.3.5 Triple Sugar Iron (TSI) test

Nineteen and a half grams of TSI medium was added to 300ml of distilled water and boiled to dissolve. Ten millilitres each of the mixture was dispensed into several test tube, covered with cotton wool and autoclaved at 121^0C for 15 minutes. The media were slanted and cooled to solidify. Each isolate was aseptically stabbed in TSI agar to the depth of 2cm short of the bottom and also streaked on the slant surface. It was incubated for 24 hours at 37^0C and examined for colour change from red to yellow at slant and butt, gas formation and H_2S formation (Woodland, 2004).

3.2.1.3.6 Urease test

To 95ml of distilled water, 2.4g of Urea agar base was added. The mixture was boiled to dissolve, autoclaved at 121^0C for 15 minutes and allowed to cool to 50^0C when it was warm to touch but not solidified. Meanwhile, 20g of Urea salt was added to 50ml sterile distilled water to make 40% urea solution. The 40% urea solution was filtered through a 0.45µm pore sized membrane filter to sterilize. Five millilitres of the urea solution was aseptically added to 95ml molten urea agar base, swirled to mix, dispensed into sterile bijou bottles, slanted and allowed to solidify. Each isolate was inoculated on a urea agar slant and incubated for 24 hours. It was examined for colour change from peach to pink. Those that show no colour change were subjected to further test (Tadesse and Alem, 2006; Hemraj *et al*., 2013).

3.2.1.4 Confirmation of bacterial isolates

Confirmation of the bacterial isolates was done by subjecting the isolates to the Microgen GnA-ID biochemical identification system and slide agglutination test of the selected isolates

3.2.1.4.1 Microgen GnA-ID system

Identification using the Microgen Identification system was carried out according to the manufacturer's instruction. Two colonies of a 24 hour culture of each presumptive *Salmonella* species were emulsified in 3ml sterile saline solution and mixed thoroughly. Three drops of the suspension was added to each well of the 12-well strips. Wells 1, 2, 3 and 9 which are for lysine, ornithine, H_2S and Urease respectively were overlaid with mineral oil. The wells were sealed back and incubated at 37^0C for 24 hours. After incubation, two drops of Kovac's reagent was added to well 8-the well for indole, and left for 60 seconds before reading. To well 10, one drop of VPI reagent was added and then one drop of VPII reagent. It was left for 15-30 minutes before reading. To well 12, one drop of TDA reagent was added and the reading was taken. For the rest of the wells, the adhesive tape was removed and all the positive results were recorded in the report form after they were compared with the colour chart.

The octal codes were generated from each form base on the recorded result and entered into the Microgen identification system software as it provides the identity of the organism base on probability. Two isolates that had the highest assurance for identification and representation as members of *Salmonella* species were selected for further analysis. They were identified as *Salmonella* species and *Salmonella pullorum* designated as S1 and S2 respectively for the remaining part of the study.

3.2.1.4.2 Slide agglutination test of the selected isolates

On a grease-free glass slide -one for each, a drop of distilled water was placed on one side as control and a drop of the Polyvalent Agglutination serum for *Salmonella* groups A-S was placed on the other side. Each was emulsified with a 24 hour old colony of the isolate and rocked for a minute. Each mixture was examined for agglutination compared to the negative control on a white surface.

3.2.1.5 Antibiotic sensitivity test of the selected isolates

The antibiotic susceptibility test was performed using the Kirby-Bauer method as described by Cheesebrough (2006). The antibiotic discs used for this research include Gentamicin (10µg), Ampicillin (10 µg), Ofloxacin (5µg), Nitrofurantoin (300µg), Ceftazidime (30µg), Chloramphenicol (30µg), Ciprofloxacin (5µg) and Amoxicillin-clavulanate (30µg). These are the drug of choice commonly used for treatment of diseases caused by *Salmonella* species and this informed the choice of their selection.

Mueller Hinton agar plates were each inoculated with 0.1ml suspension of the test organisms earlier matched against Macfarland No.1 turbidity standard. The suspension was spread evenly with a sterile bent glass rod and allowed to stand for 10 minutes for the surface to dry. Flamed forcep was used to aseptically place the antibiotic discs on the surface of the plate and incubated for 24 hours at 37^0C. After 24 hours, the plates were examined and interpreted according to the Clinical Laboratory Standards Institute guideline (CLSI, 2014).

3.2.2 Phage isolation and analysis

The water and soil samples were processed and the phages were isolated base on the bacterial isolate (host) and the analysis was carried out as follows:

3.2.2.1 Phage isolation

Four hundred millilitres of distilled water was added to 200g of the wet soil, shaken and left for 30 minutes and shaken again (Payment, 2002). It was filtered with a clean fabric.

The waste water samples obtained from the University's drainage system and the dam at Kwanan Kurmi and soil eluent were centrifuged at 4000 rpm for 20 minutes to remove coarse particles and the supernatants were each filtered through a 0.45µm pore size membrane filter to remove debris and bacterial cells that might be present. The filtrates were considered as clean source of phage (Chibani-Chennoufi *et al*., 2004b; Willey *et al*., 2008; Akhtar*et al*., 2014).

The phages were concentrated by adding 100ml of each filtrate to 8 hour old exponential phase bacterial culture grown in 100ml double strength tryptone soya broth. For the reference phage stock, 50ml of the 8 hour old culture of the test organisms were each inoculated with 10ml of the phage preparation "Salmonelex". All the mixtures were kept rotating in a shaker at a speed of 120 rpm at room temperature for 48 hours. The mixtures were sonicated, centrifuged at 4000 rpm for 20 minutes, decanted and the supernatant filtered through a 0.45µm pore sized membrane filter. Chloroform was added to the filtrate at $20\mu lml^{-1}$ to kill any surviving bacterial cell in the filtrate. The filtrate was further centrifuged at 10, 000 rpm and 4^0C for 10 minutes. The supernatants were decanted to further concentrate or enrich the phage suspension (Chibani-Chennoufi *et al*., 2004b; Sharma *et al*., 2005; Willey *etal*., 2008; Middelboe *et al*., 2010; Tan *et al*., 2014; Akhtar*et al*., 2014).

3.2.2.2 Phage identification

Phage identification and specificity was determined using the spot and plaque assay techniques as follows

3.2.2.2.1 Spot assay

The presence of *Salmonella*-specific lytic phages for the two isolates in Salmonelex, the water and soil were revealed by spot assay using the double-layer agar method. The bacterial lawn was prepared by adding 1ml of the four hour old bacterial culture equivalent to Optical Density (OD_{600}) of 0.3 to 4ml half strength molten tryptone top agar (TTA). The bacterial-soft agar mixture was mixed and immediately poured onto an already prepared and solidified tryptone soya agar (TSA) plate. The plates were allowed to solidify. Few drops of each phage stock were placed on different spots on the plate. There was also a positive control on which sterile distilled water was dropped and negative control which had no bacterial lawn, just the sterile TTA agar. All plates were left to absorb liquid on them, incubated at 37^0C for 24 hours and examined for clear spot (plaque) formation (Adams, 1959; Middelboe *et al*., 2010; Viazis *et al*., 2011; Tan *et al*., 2014; Akhtar*et al*., 2014).

3.2.2.2.2 Plaque assay

A series of 10-fold serial dilutions of the phage stocks in sterile distilled water were set-up to the sixth time. The fifth and sixth time dilutions of each stock were used for the plaque assay. For each dilution, 1ml of a four hour old bacterial culture was mixed with 0.5ml of the diluted phage stock and 0.2ml of Calcium chloride solution (to enhance adhesion). The mixture was incubated for 15 minutes, added to and briefly vortexed with 4ml molten TTA and immediately poured aseptically on an already solidified TSA

plate. The plate was allowed to solidify, incubated at 37^0C for 24 hours and examined for plaque formation (Akhtar*et al*., 2014). For those that formed too many or a too large plaque to be quantified, the process was repeated with higher dilution factor.

The plaque spots were cut out with a spatula, meshed and placed in 50ml of buffer, centrifuged, filtered again and concentrated for further use. The phages were labelled 1KS (Phage isolated from soil at Kwanan kurmi and lytic against *Salmonella* species), 1KW (Phage isolated from dam water at Kwanan kurmi and lytic against *Salmonella* species), IDW (Phage isolated from waste water at the central drainage point, ABU, Zaria and lytic against *Salmonella* species), 1R {Reference phage (Salmonelex) concentrated using *Salmonella* species}, 2DW (Phage isolated from waste water at the central drainage point, ABU, Zaria and lytic against *Salmonella* Pullorum), 2R (Reference phage 'Salmonelex' concentrated using *Salmonella* Pullorum), 2KW(Phage isolated from dam water at Kwanan kurmi and lytic against *Salmonella* Pullorum) and 1KS (Phage isolated from soil at Kwanan kurmi and lytic against *Salmonella* Pullorum).

3.3 Determination of the Lytic Property of the Phages

The lyticproperty of the phages was determined by carrying out Progeny phage release using plague assay and lysis in tube as shown below:

3.3.1 Progeny phage release using plague assay

Tryptone Soya Broth (TSB) was prepared and 100ml of the broth was inoculated with a 24 hour colony of the test bacterium and incubated for four hours. One millilitre of 10% phage suspension was added to the four hour old bacterial culture making it 1% phage. Half millilitres of sterile $CaCl_2$ solution was also added. The phage-host mixture was

incubated at 37^0C in a shaker rotating 100 rpm at room temperature. Every 20 minutes, 1ml of the mixture is added to and mixed with 4ml molten TTA and immediately poured on TSA plate. The plate was allowed to solidify and then incubated at 37^0C for 24 hours. All the plates were examined and the readings taken every 20 minutes were recorded till the point at which the plaques were too numerous to count (Chibani-chennoufi *et al*., 2004b).

3.3.2 Lysis in tube

The ability of the bacteriophages to lyse the respective bacterial isolates was assessed in liquid media as follows:

3.3.2.1 Phage activity on isolates in liquid medium

One millilitre of 10% of each phage (1DW, 2DW, 1KS, 2KS, 1KW, 2KW, 1R and 2R) was added to 150ml of a 4 hour culture of its corresponding bacterial host which is at an Optical Density (OD) of between 0.2 and 0.5. Half millilitre of $CaCl_2$ solution was added and the mixture was incubated in a shaker rotating at 100 rpm for 24 hours at room temperature. Every hour, during the 24 hours incubation, the absorbance of the culture is measured at 600nm using a colorimeter in the absence of a spectrophotometer. The negative control (sterile TSB) was used as the blank to calibrate the colorimeter before the each series of assay. The positive controls that contain only the bacterium and no phages were subjected to the same condition as the infected cultures (Chibani-.chennoufi *et al*., 2004b; Atterbury *et al*., 2007; Synott *et al*., 2009).

3.3.2.2 Antibiotic activity on isolates in liquid medium

To 150 ml of each of the 4 hour old bacterial culture- S1 and S2, 0.4 ml of Ciprofloxacin (200mg/100ml) was added – and labelled 1Cip and 2Cip respectively,

making it equivalent to 5µg/ml. One millilitre of Chloramphenicol (0.048g injectable dissolved in 10ml sterile distilled water) was added to each 150ml of the 4 hour old culture of the two isolates making it equivalent to 32µg/ml –and labelled 1C and 2C. All the mixtures and their positive controls were incubated at room temperature for 24 hours in a shaker rotating at 100 rpm during which the absorbance were measured hourly.

3.3.2.3 Combined activity of phages and antibiotics on isolates in liquid medium

To each 150 ml of the 4 hour culture of either isolates in TSB, 1ml of 10% phage suspension, 0.5ml of $CaCl_2$ solution and either 1ml of chloramphenicol (32µg/ml) or 0.4ml Ciprofloxacin (5µg/ml) were added. They were labelled 1DWC (Combination of phage 1DW and Chloramphenicol), 1DWCip (Combination of phage 1DW and Ciprofloxacin), 2DWC (Combination of phage 2DW and Chloramphenicol), 2DWCip (Combination of phage 2DW and Ciprofloxacin), 1KSC (Combination of phage 1KS and Chloramphenicol), 1KSCip (Combination of phage 1KS and Ciprofloxacin), 2KSC (Combination of phage 2KS and Chloramphenicol), 2KSCip (Combination of phage 2KS and Ciprofloxacin), 1KWC (Combination of phage 1KW and Chloramphenicol), 1KWCip (Combination of phage 1KW and Ciprofloxacin), 2KWC (Combination of phage 2KW and Chloramphenicol), 2KWCip (Combination of phage 2KW and Ciprofloxacin), 1RC (Combination of phage 1R and Chloramphenicol), 1RCip (Combination of phage 1R and Ciprofloxacin), 2RC (Combination of phage 2R and Chloramphenicol), 2RCip (Combination of phage 2R and Ciprofloxacin), The mixtures were incubated at room temperature in a shaker rotating at 100 rpm for a 24 hour period during which the absorbances were measured hourly at OD of 600nm. The positive controls contain neither phage nor antibiotics.

3.4 Data Analysis

The activities of the bacteriophages and antibiotics as well as the combination of the phages and respective antibiotics where compared at $p \leq 0.05$ using one way Analysis of Variances (ANOVA) and Tukey Post Hoc test by International Business Machines SPSS Statistics version 21 and GraphPad Prism version 5.

CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of Bacterial Isolates

Seven isolates which were coded 2 (Clinical isolate), 4 (Clinical isolate), 7 (from egg content), 8 (Clinical isolate), 23 (from onion leaf), 28 (from lettuce) and 33 (from cabbage) exhibited characteristics typical of *Salmonella* species. Further test using a standard kit (Microgen GnA-ID System) yielded one *Salmonella* species, three *Salmonella* Pullorum, one *Enterobacter aerogenes*, and two *Citrobacter freundii* as shown in Table 4.1. The serology test for the confirmation of the two selected isolates (one *Salmonella* species and one *Salmonella* Pullorum, designated S1 and S2 respectively) showed that both agglutinated with the agglutinating serum for *Salmonella* groups A-S (Plate 4.1).

4.2 Plaques formed by Isolated and Cultivated Phages

The spot and plague assay as shown in Plates 4.2 and 4.3 respectively showed clearances (plaques) formed in the bacterial lawn of the isolates when compared to the positive and negative controls (Plates 4.4 and 4.5) indicating the lytic activity of the phages on the isolates. The plaques formed from plague assay varied after tube dilutions as shown in Table 4.2. Six virulent bacteriophages were obtained from three sources -three for each isolate, and the reference phage (Salmonelex) was lytic on both isolates totaling eight bacteriophages in this research. 2DW produce more plagues followed by 2KW then 2KS, 1KS, 2R, 1KW, 1DW and 1R in descending order. The phages were each coded according to the number of its bacterial isolation source (S1 or S2), the location of the primary source (University's drainage system, or Kwanan Kurmi) and the source (water or soil).

Table 4.1. Isolates Identification Using Microgen GnA-ID System

S/N	Isolate No (New No)	Octal code	Identity	Percent probability (%)
1	2(1)	7702	*Salmonella* species	80.89
2	7(2)	7700	*Salmonella* Pullorum	66.15
3	4(3)	7725	*Salmonella* Pullorum	33.03
4	8(4)	7725	*Salmonella* Pullorum	33.03
5	23(6)	7746	*Enterobacter aerogenes*	33.57
6	28(7)	1742	*Citrobacter freundii*	99.84
7	33(8)	1742	*Citrobacter freundii*	99.84

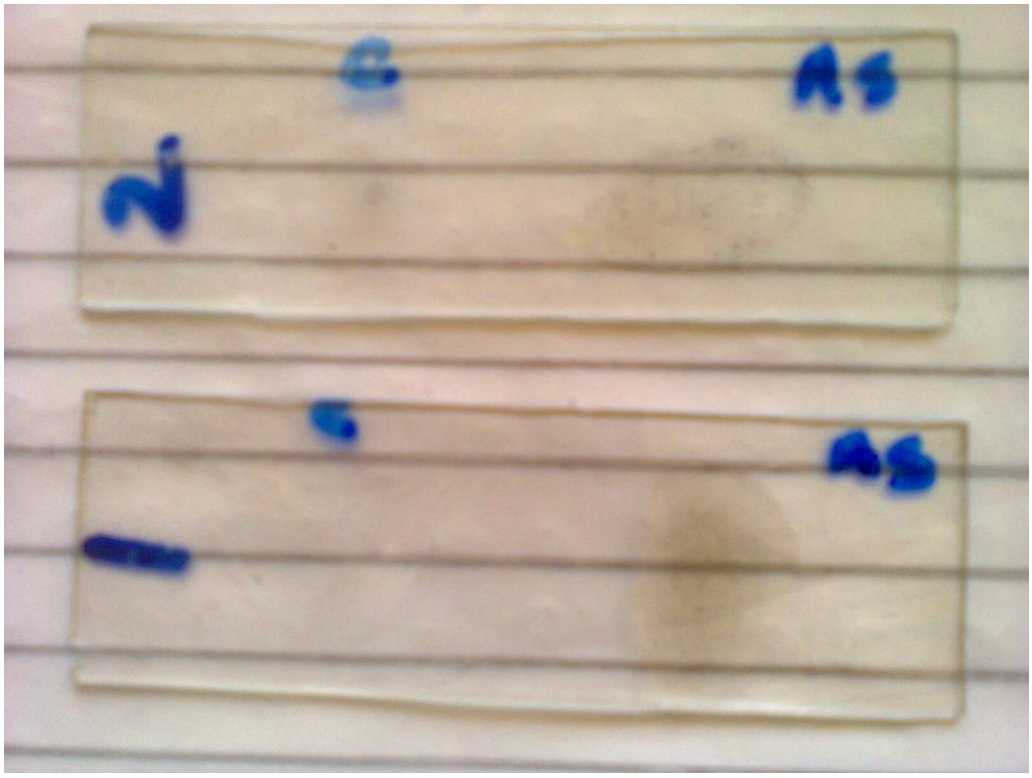


Plate 4.1: Confirmation of selected isolates (S1 and S2) using Polyvalent Agglutination serum for *Salmonella* groups A-S.

Key:

C- Control

AS- Agglutinating Serum

S1= *Salmonella* species

S2= *Salmonella* Pullorum



Plate 4.2: Plagues formed by phage on bacterial lawn after spot assay.



Plate 4.3: Plagues formed by phage on bacterial lawn after plaque assay.



Plate 4.4: Positive control showing bacterial lawn without phage.

Key:

+ve - Positive

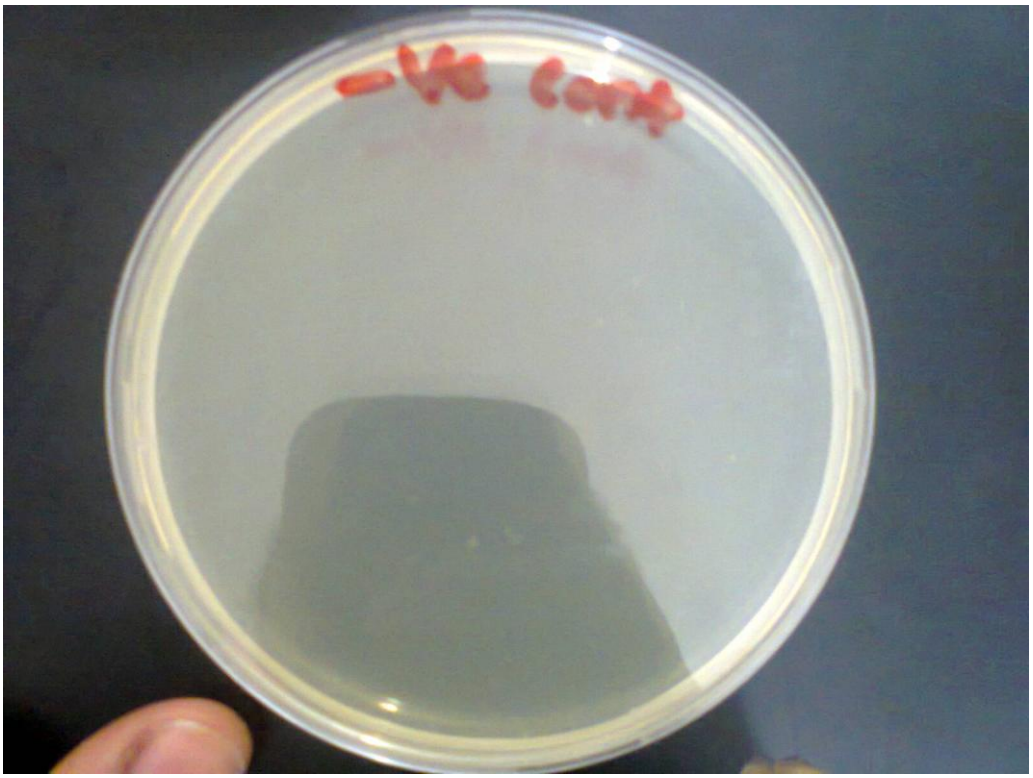


Plate 4.5: Negative control with neither bacterium nor phage.

Key:

-ve – Negative

Cont – Control

.

Table 4.2. Plagues formed after tube dilution

Bacteriophage	Plague (pfu/ml)
1KS	3.1×10^8
1KW	1.1×10^7
1DW	3.8×10^6
1R	7.3×10^3
2DW	1.1×10^{10}
2R	1.9×10^8
2KW	7.7×10^8
2KS	5.9×10^8

Keys

1KS – Phage isolated from soil at Kwanan Kurmi and lytic against *Salmonella* species

1KW - Phage isolated from dam water at Kwanan Kurmi and lytic against *Salmonella* species

IDW - Phage isolated from waste water at the central drainage point, ABU, Zaria and lytic against *Salmonella* species

1R – Reference phage (Salmonelex) concentrated using *Salmonella* species

2DW - Phage isolated from waste water at the central drainage point, ABU, Zaria and lytic against *Salmonella* Pullorum

2R – Reference phage (Salmonelex) concentrated using *Salmonella* Pullorum

2KW - Phage isolated from dam water at Kwanan Kurmi and lytic against *Salmonella* Pullorum

1KS – Phage isolated from soil at Kwanan Kurmi and lytic against *Salmonella* Pullorum

4.3 Antibiotic Sensitivity of *Salmonella* species (S1) and *Salmonella* Pullorum (S2)

The Antibiotic sensitivity test of the two selected isolates *Salmonella* species (S1) and *Salmonella* Pullorum (S2) (Table 4.3) showed that both isolates were susceptible to Gentamicin (100%), Chloramphenicol (100%), Ofloxacin (100%), Nitrofurantoin (100%), Ciprofloxacin (100%) and Amoxicillin- clavulanate (100) and were resistant to Ceftazidime (100%) and Ampicillin (100%) base on the standard laid out by CLSI (2014). Susceptibility by both isolates was highest to Ciprofloxacin followed by Ofloxacin then Chloramphenicol and Resistance was highest to Ceftazidime then Ampicillin.

4.4. Determination of the Lytic Property of the Phages by Progeny Phage Release

The progeny phage release from phage infected cultures showed infectivity from 20 minutes after infection and continuous increase in phage titer with time (Figure 4.1). The phage counts started at very high numbers ranging between $Log_{10}6$ and $Log_{10}10$.A peculiar pattern was displayed by 2KW. Its curve went down drastically at the 60^{th} minute and went up again before becoming too numerous to count. It should be noted that twenty minutes after the point at which each curve stopped, the plaque count became too numerous to count but could not be represented on the graph. Therefore, there was no definite peak for the phage infectivity as all became too numerous to count at one point or the other.

4.5 Determination of the Lytic Property of the Phages and/or antibiotics in Tube

All the assays carried out in tubes for 24 hours using either the phages alone, the antibiotic alone or the combination of the phages and either of the antibiotic displayed patterns of interest as explained below

Table 4.3: Antibiotic sensitivity of *Salmonella* species (S1) and *Salmonella* Pullorum (S2)

	Diameter of inhibition zone (mm) on	
Antibiotics	**S1**	**S2**
Gentamicin (10µg)	23 (S)	28 (S)
Ceftazidime (30µg)	R	R
Chloramphenicol (30µg)	27 (S)	22 (S)
Ofloxacin (5µg)	31 (S)	32 (S)
Nitrofurantoin (300µg)	22 (S)	13 (S)
Ampicillin (10 µg)	R	10 (R)
Ciprofloxacin (5µg)	35 (S)	40 (S)
Amoxicillin- clavulanate (30µg)	15 (I)	18 (S)

Key:

R- Resistant I- Intermediate S - Susceptible

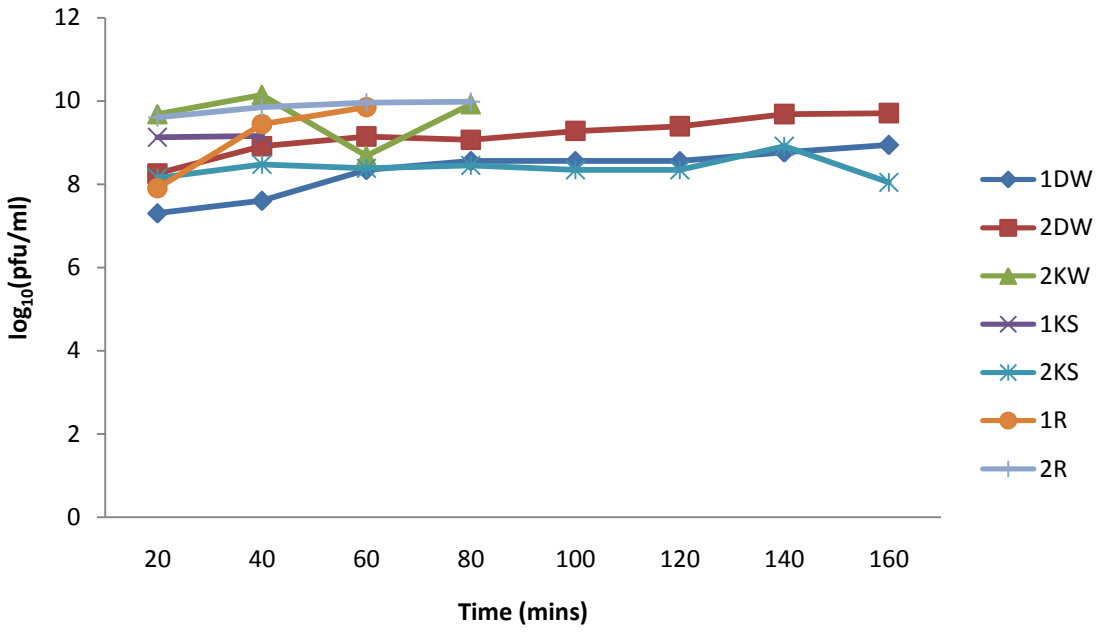


Figure 4.1: Progeny phage release from infected culture measured by plague assay and presented in logarithm.

Keys:

1KS – Phage isolated from soil at Kwanan Kurmi and lytic against *Salmonella* species

IDW - Phage isolated from waste water at the central drainage point, ABU, Zaria and lytic against *Salmonella* species

1R – Reference phage (Salmonelex) concentrated using *Salmonella* species

2DW - Phage isolated from waste water at the central drainage point, ABU, Zaria and lytic against *Salmonella* Pullorum

2R – Reference phage (Salmonelex) concentrated using *Salmonella* Pullorum

2KW - Phage isolated from dam water at Kwanan Kurmi and lytic against *Salmonella* Pullorum

1KS – Phage isolated from soil at Kwanan Kurmi and lytic against *Salmonella* Pullorum

4.5.1 Lytic Property of the Phages Alone by Lysis in Tube

All the phages involved in lysis in the tube assay significantly reduce the Optical Densities (OD) of both *Salmonella* species (S1) and *Salmonella* Pollurum (S2) growth after infection by phages while the uninfected controls maintain high OD_{600}. Figure 4.2 shows a clear difference between the OD_{600} of the positive control (S1) and that of the organism infected with phages 1DW, 1KS, 1KW and 1R. The decrease in OD_{600} of S1 infected with 1DW, 1R and 1KW were not significantly different from each other at $p \leq 0.05$ but were significantly different from 1KS except IKW which is not. The decrease in OD_{600} of S2 infected by the phages 2DW, 2KS, 2KW and 2R were significantly different from each other at $p \leq 0.05$ except 2DW and 2KW which are not significantly different from each other. The peak for 2DW, 2KS, 2KW and 2R were at the 2nd, 14th, 3rd and 1st hours respectively (Figure 4.3).

4.5.2 Activity of Antibiotics on Bacterial Isolates in Liquid Medium

The Antibiotics reduce the Optical Densities of *Salmonella* species (S1) and *Salmonella* Pullorum (S2) growth significantly compared to the controls when both isolates were subjected to tube assay, each treated separately with the two antibiotics. The decreases in OD600 readings by Ciprofloxacin and Chloramphenicol on S1 at $p \leq 0.05$ are not significantly different from each other. The OD_{600} of Chloramphenicol on S1 was high till the 9th hour when it drastically reduces with time up to the last hour and that of Ciprofloxacin reached its peak at the 6th hour and reduces gradually with time as shown in Figure. 4.4. The effects of the two antibiotics on S2 are significantly different from each

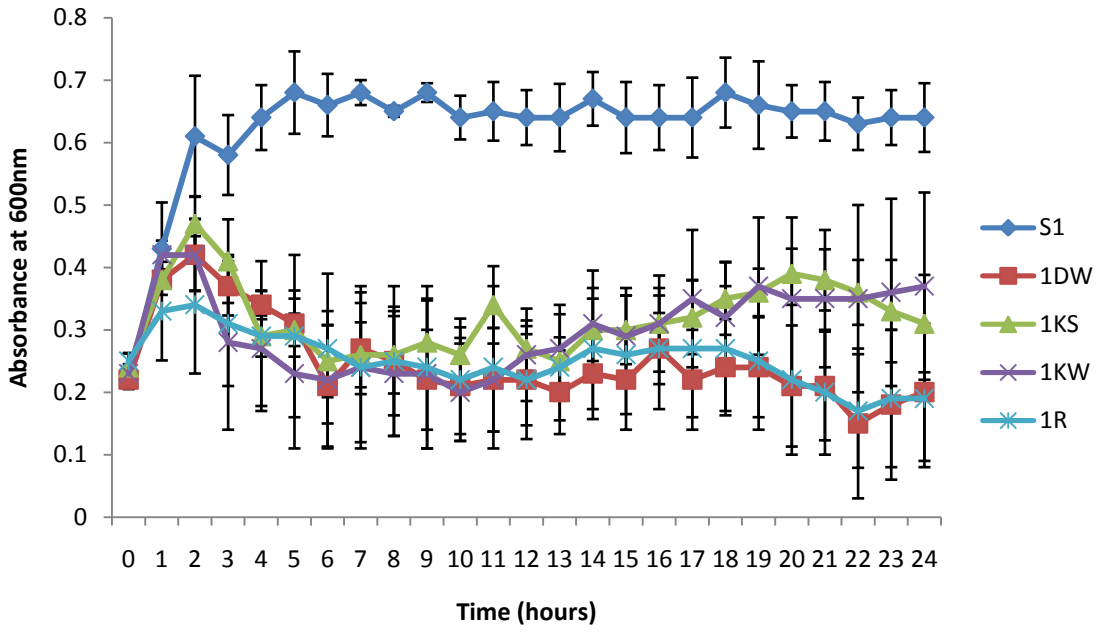


Figure 4.2. Optical Density (OD) development of uninfected control culture (S1) and parallel cultures infected with phages 1DW, 1KS, 1KW and 1R. The data represent the mean ± standard error.

Keys:

S1 – Control uninfected culture of *Salmonella* species

1DW – Culture of *Salmonella* species infected with Phage 2DW

1R – Culture of *Salmonella* species infected with Reference phage (Salmonelex)

1KW - Culture of *Salmonella* species infected with Phage 2KW

1KS – Culture of *Salmonella* species infected with Phage 1KS

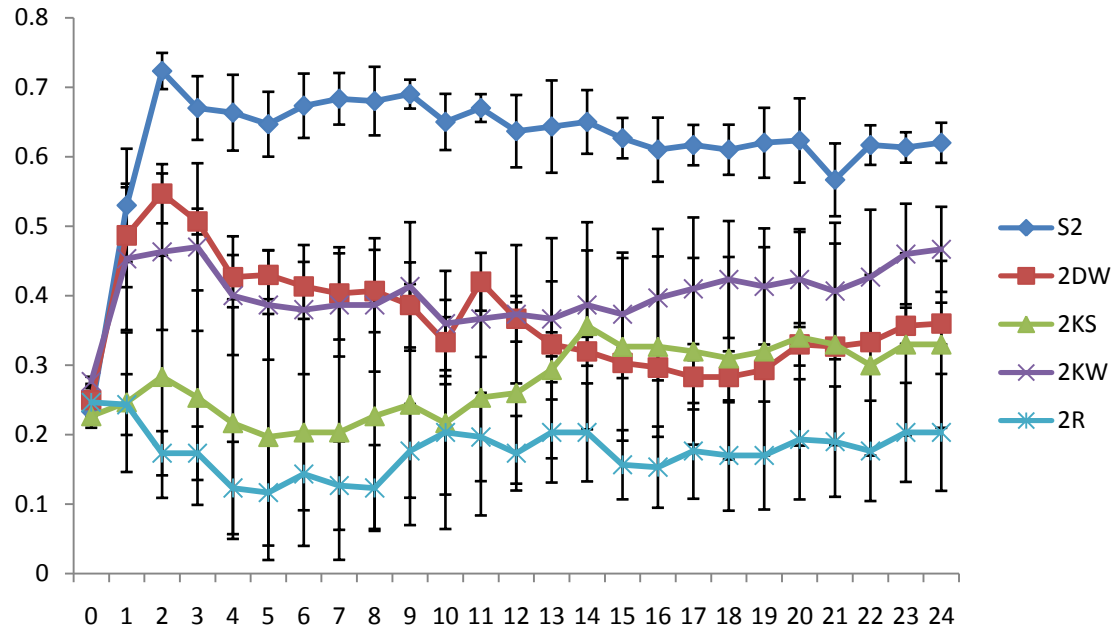


Figure 4.3. Optical Density (OD) development of uninfected control culture (S2) and parallel cultures infected with phages 2DW, 2KS, 2KW and 2R. The data represent the mean ± standard error.

Keys:

S2 – Control uninfected culture of *Salmonella* Pullorum

2DW – Culture of *Salmonella* Pullorum infected with Phage 2DW

2R – Culture of *Salmonella* Pullorum infected with Reference phage (Salmonelex)

2KW - Culture of *Salmonella* Pullorum infected with Phage 2KW

2KS – Culture of *Salmonella* Pullorum infected with Phage 2KS

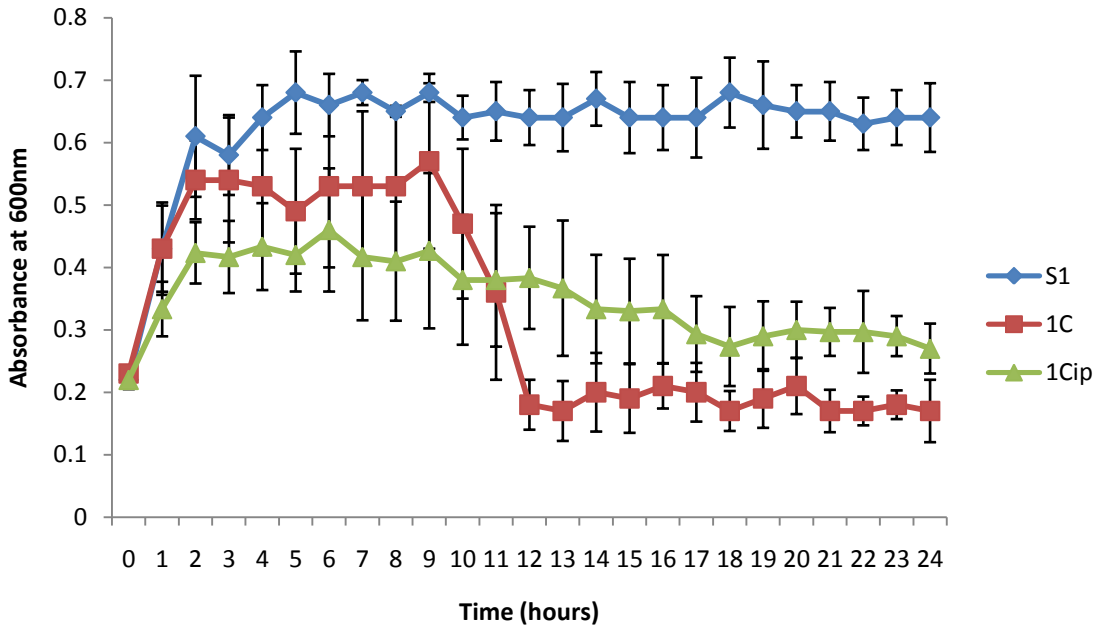


Figure 4.4. Optical Density (OD) development of untreated control culture (S1) and parallel cultures treated with antibiotics Chloramphenicol and Ciprofloxacin. The data represent the mean ± standard error.

Key:

S1 – Control untreated culture of *Salmonella* species

1C – Culture of *Salmonella* species treated with Chloramphenicol

1Cip - Culture of *Salmonella* species treated with Ciprofloxacin

other at $p \leq 0.05$. Figure 4.5 shows that the OD_{600} of Chloramphenicol treated culture dropped after its peak at the first hour and maintained low fluctuating OD_{600} while the OD_{600} of Ciprofloxacin treated culture increased steadily to the 6th hour and started reducing steadily till the last hour.

4.5.3 Activity of the Phages and Antibiotics on the Isolates Individually and in Combination

The absorbance of the bacteriophages alone, antibiotics alone and the combination of the phages and antibiotics were observe to decrease significantly compared to that of the untreated and uninfected cultures of the bacterial isolates. The reductions were also compared to each other.

4.5.3.1 Activity of IDW, Chloramphenicol, Ciprofloxacin, 1DWC and 1DWCip on S1

The absorbance of phage 1DW, Chloramphenicol (C) and 1DWC (combination of 1DW and Chloramphenicol) when the optical densities were measured, reduced significantly ($p \leq 0.05$) compared to the uninfected/untreated control (S1) as shown in Figure 4.6. The activity of Chloramphenicol (1C) was significantly different from that of Phage (1DW) and the combination of both (1DWC) at $p \leq 0.05$, but that of 1DW and 1DWC were not significantly different from each other. Similarly, the OD_{600} of S1 treated with Ciprofloxacin (Cip), 1DWCip (combination of 1DW and Ciprofloxacin) and the phage 1DW lagged below that of the control positive S1 (Figure 4.7). At $p \leq 0.05$, the activity of 1DW and 1DWCip on S1 were significantly different from that of Ciprofloxacin but both were not significantly different from each other.

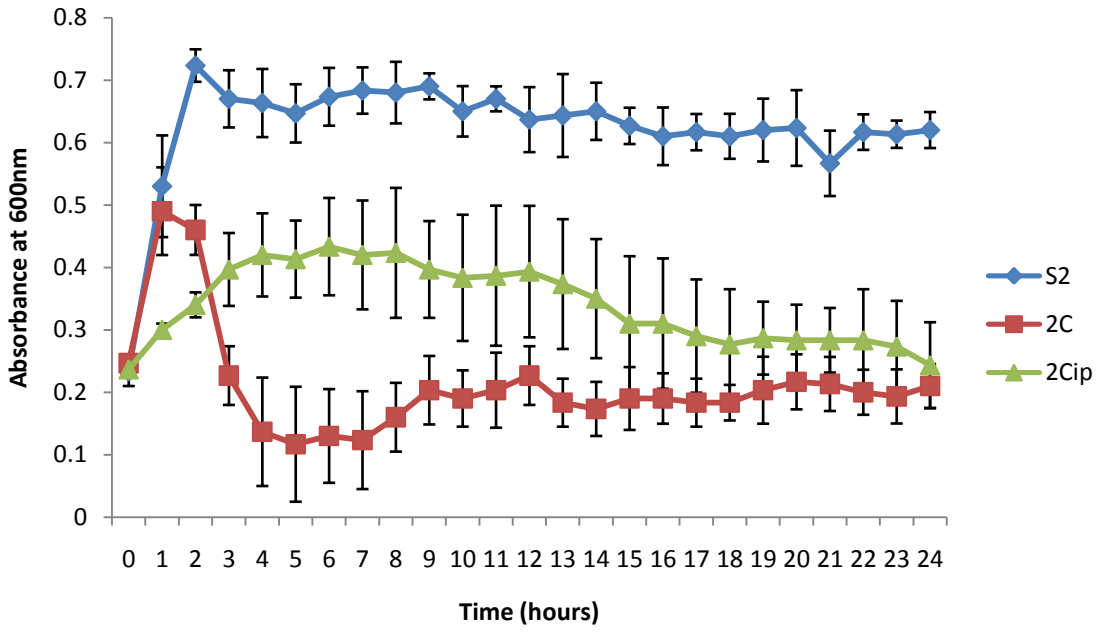


Figure 4.5. Optical Density (OD) development of untreated control culture (S2) and parallel cultures treated with antibiotics Chloramphenicol and Ciprofloxacin. The data represent the mean ± standard error.

Key:

S2 – Control untreated culture of *Salmonella* Pullorum

2C – Culture of *Salmonella* Pullorum treated with Chloramphenicol

2Cip - Culture of *Salmonella* Pullorum treated with Ciprofloxacin

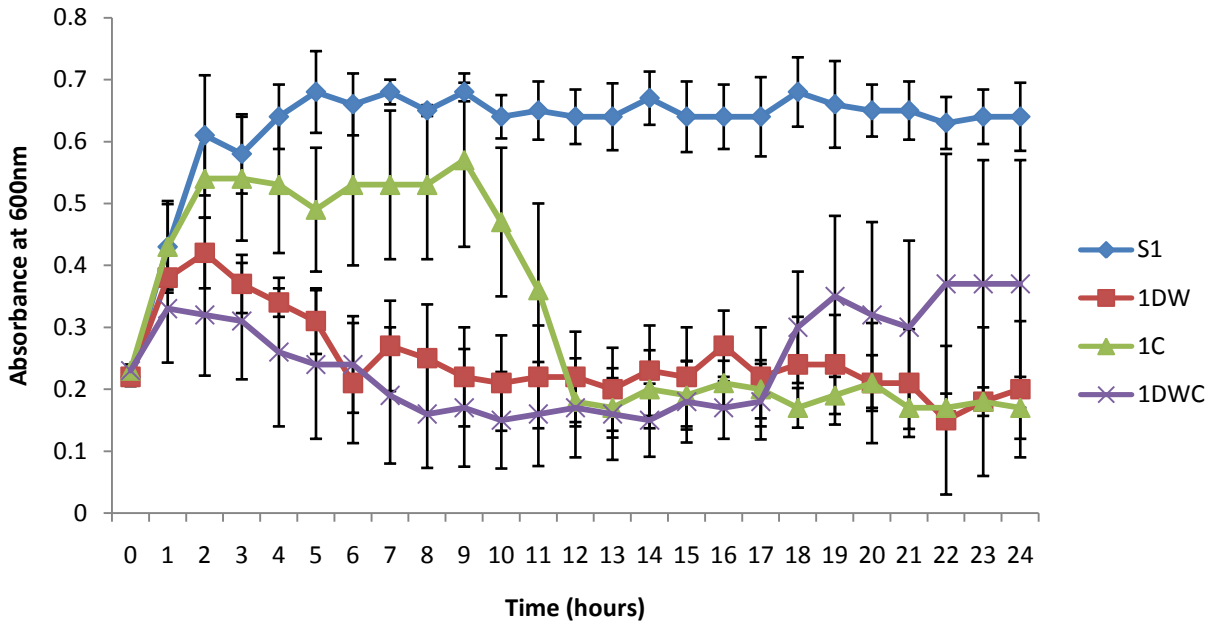


Figure 4.6: Optical Density (OD) development of uninfected control culture (S1) and parallel cultures infected/treated with 1DW, Chloramphenicol and both.The data represent the mean ± standard error.

Key:

S1 – Control uninfected/untreated culture of *Salmonella* species

1DW – Culture of *Salmonella* species infected with phage 1DW

1C – Culture of *Salmonella* species treated with Chloramphenicol

1DWC - Culture of *Salmonella* species infected and treated with both phage 1DW and Chloramphenicol

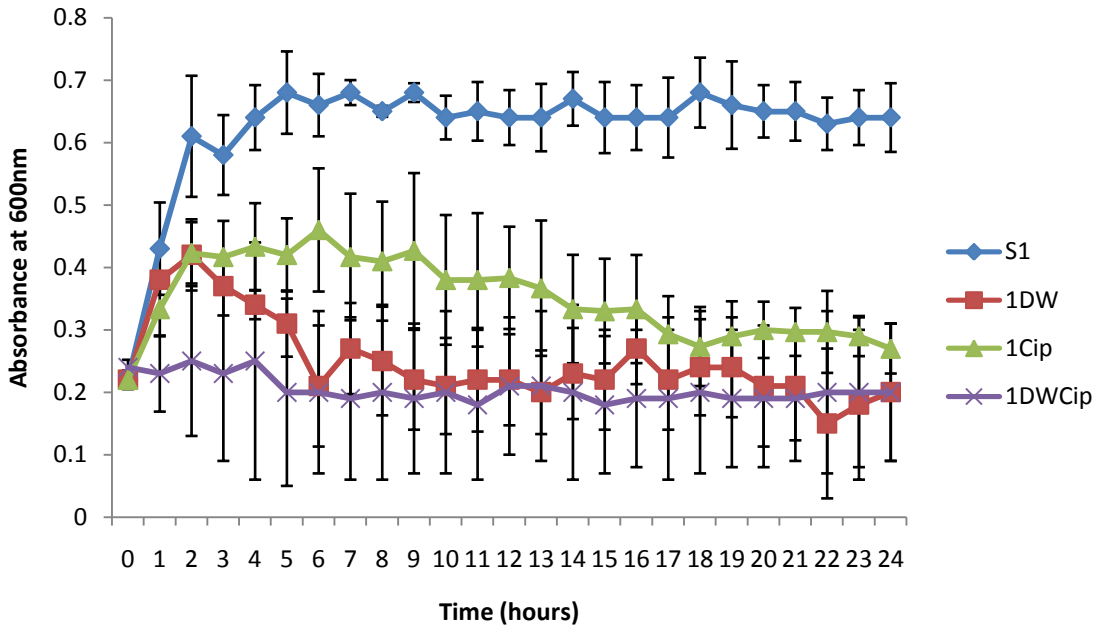


Figure 4.7: Optical Density (OD) development of uninfected control culture (S1) and parallel cultures infected/treated with 1DW, Ciprofloxacin and both.The data represent the mean ± standard error.

Key:

S1 – Control uninfected/untreated culture of *Salmonella* species

1DW – Culture of *Salmonella* species infected with phage 1DW

1Cip – Culture of *Salmonella* species treated with Ciprofloxacin

1DWCip - Culture of *Salmonella* species infected and treated with both phage 1DW and Ciprofloxacin

4.5.3.2 Activity of IKS, Chloramphenicol, Ciprofloxacin, 1KSC and 1KSCip on S1

There was significant decrease ($p \leq 0.05$) in OD_{600} of phage 1KS, Chloramphenicol and their combination (1KSC) compared with that of the control S1 which is uninfected/untreated (Figure 4.8). The effects of 1C and 1KS were not significantly different from each other at $p \leq 0.05$ but the effects of both are significantly different from that of 1KSC. The OD_{600} of the phage 1KS, Ciprofloxacin and the combination of the phage and the antibiotic -1KSCip, compared to the control S1 was significantly different (Figure 4.9). The activity of the phage 1KS and Ciprofloxacin were significantly different from that of their combination 1KSCip but not significantly different from one another at $p \leq 0.05$.

4.5.3.3 Activity of IKW, Chloramphenicol, Ciprofloxacin, 1KWC and 1KWCip on S1

There was reduction ($p \leq 0.05$) in the optical densities of cultures treated or/and infected with 1KW, C and 1KWC -Figure 4.10, compared to the positive control S1. There were significant differences at $p \leq 0.05$ between the reductions caused by Chloramphenicol and phage 1KW compared to 1KWC. Between 1KW and 1C, the actions are not significantly different. Figure 4.11 shows the difference between the absorbance of the control S1 and the cultures treated or/and infected with 1KW, Ciprofloxacin and their combinations 1KWCip. Statistically at $p \leq 0.05$, the actions by the 1KW and 1KWCip were significantly different from the antibiotic alone but not significantly different from each other.

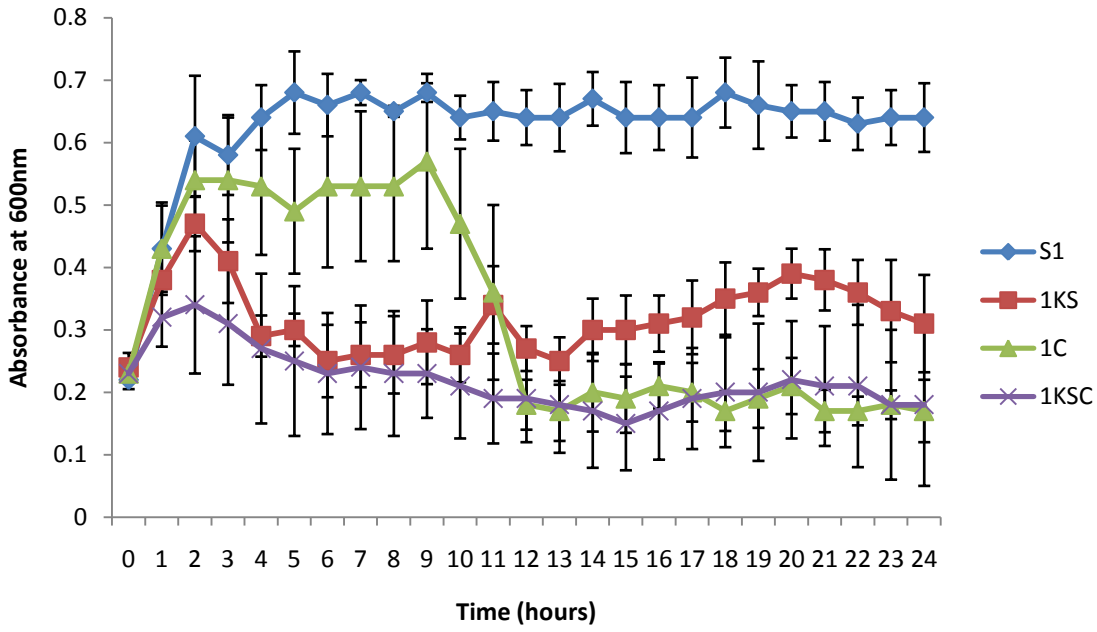


Figure 4.8: Optical Density (OD) development of uninfected control culture (S1) and parallel cultures infected/treated with 1KS, Chloramphenicol and both.The data represent the mean ± standard error.

Key:

S1 – Control uninfected/untreated culture of *Salmonella* species

1KS – Culture of *Salmonella* species infected with phage 1KS

1C – Culture of *Salmonella* species treated with Chloramphenicol

1KSC - Culture of *Salmonella* species infected and treated with both phage 1KS and Chloramphenicol

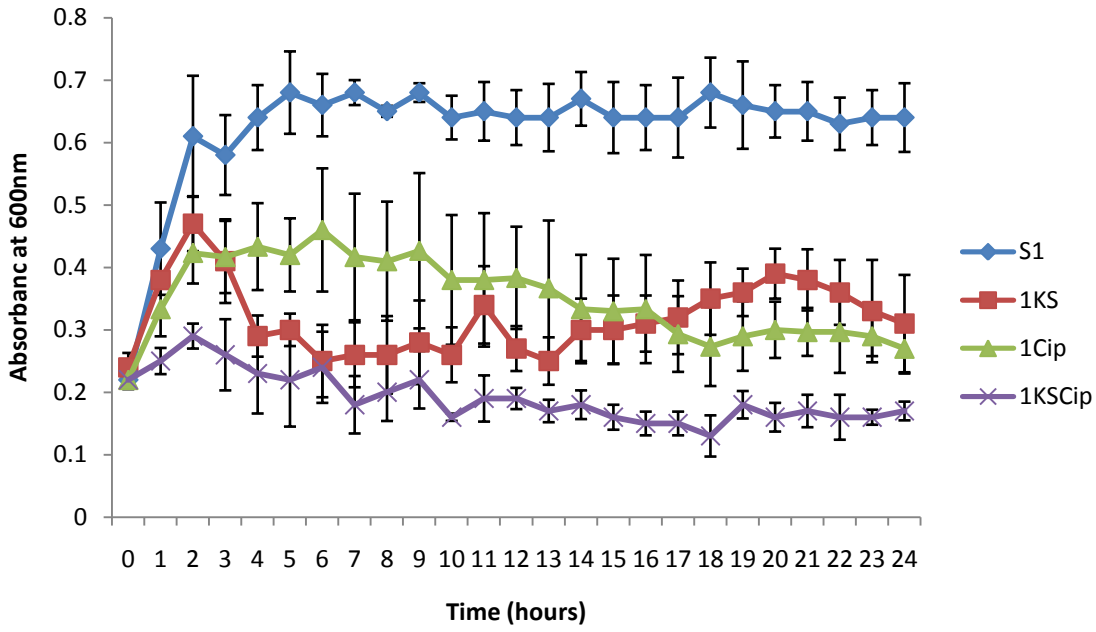


Figure 4.9: Optical Density (OD) development of uninfected control culture (S1) and parallel cultures infected/treated with 1KS, Ciprofloxacin and both.The data represent the mean ± standard error.

Key:

S1 – Control uninfected/untreated culture of *Salmonella* species

1KS – Culture of *Salmonella* species infected with phage 1KS

1Cip – Culture of *Salmonella* species treated with Ciprofloxacin

1KSCip - Culture of *Salmonella* species infected and treated with both phage 1DW and Ciprofloxacin

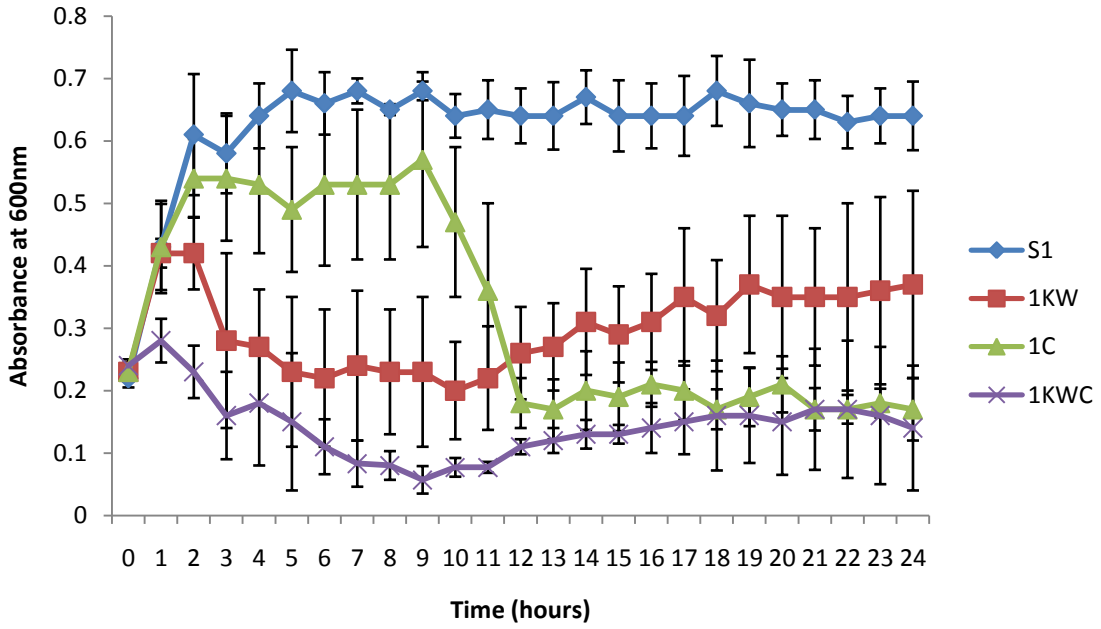


Figure 4.10: Optical Density (OD) development of uninfected control culture (S1) and parallel cultures infected/treated with 1KW, Chloramphenicol and both.The data represent the mean ± standard error.

Key:

S1 – Control uninfected/untreated culture of *Salmonella* species

1KW – Culture of *Salmonella* species infected with phage 1KW

1C – Culture of *Salmonella* species treated with Chloramphenicol

1KWC - Culture of *Salmonella* species infected and treated with both phage 1KW and Chloramphenicol

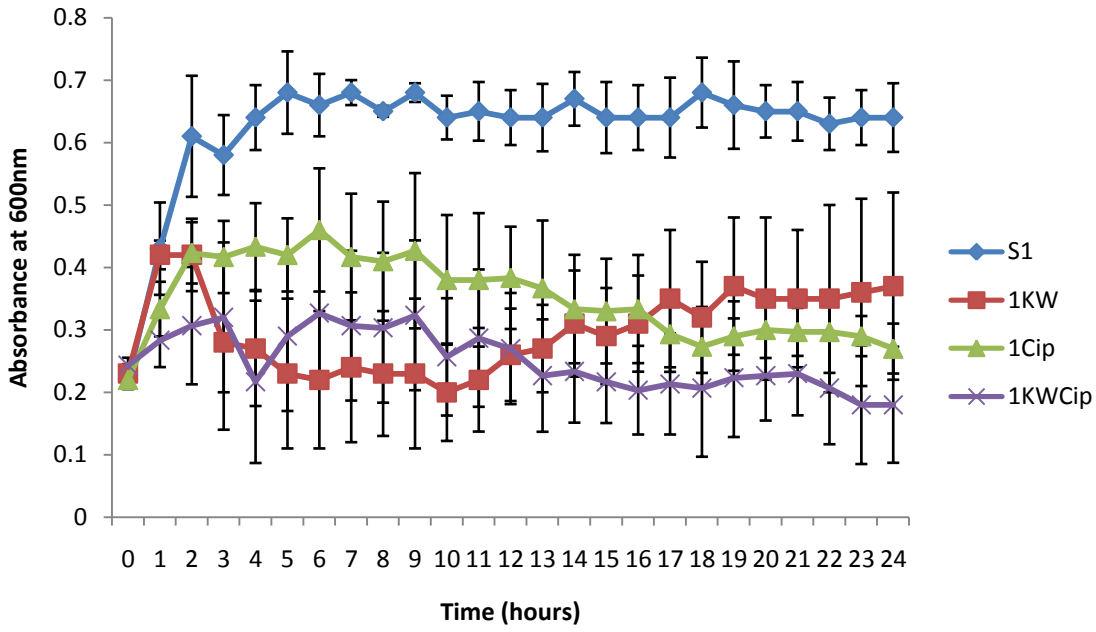


Figure 4.11: Optical Density (OD) development of uninfected control culture (S1) and parallel cultures infected/treated with 1KW, Ciprofloxacin and both.The data represent the mean ± standard error.

Key:

S1 – Control uninfected/untreated culture of *Salmonella* species

1KW – Culture of *Salmonella* species infected with phage 1KW

1Cip – Culture of *Salmonella* species treated with Ciprofloxacin

1KWCip - Culture of *Salmonella* species infected and treated with both phage 1KW and Ciprofloxacin

4.5.3.4 Activity of IR, Chloramphenicol, Ciprofloxacin, 1RC and 1RCip on S1

There was significant difference ($p \leq 0.05$) between the optical density of the control S1 and those of phage 1R, Chloramphenicol and 1RC as shown in Figure 4.12. There is no significant difference between the change in optical densities of 1R and 1C and of 1R and 1RC but there was significant difference between 1C and 1RC. Similarly, there was significant difference ($p \leq 0.05$) between the optical density of the control S1 and those of phage 1R, Ciprofloxacin and 1RCip as shown in Figure 4.13. The difference between the effect of 1R and 1RCip is not significant but that which is between 1R and 1Cip and 1Cip and 1RCip are significantly different from one another.

4.5.3.5 Activity of 2DW, Chloramphenicol, Ciprofloxacin, 2DWC and 2DWCip on S2

At $p \leq 0.05$, the optical densities of the cultures treated or/and infected with phage 2DW, the antibiotic -Chloramphenicol (2C) and the combination of the phage with the antibiotic (2DWC) were all significantly different from the optical density of the untreated and uninfected control culture (S2) as shown in Figure 4.14. The optical densities of 2C and 2DWC were not significantly different from each other but both are significantly different from that of 2DW at $p \leq 0.05$. Likewise, at $p \leq 0.05$, the optical densities of the cultures treated or/and infected with phage 2DW, the antibiotic -Ciprofloxacin (2Cip) and their combination (2DWCip) were all significantly different from the optical density of the untreated and uninfected control culture (S2) as shown in Figure 4.15. The optical densities of 2DW, 2Cip and 2DWCip are not significantly different from each other.

4.5.3.6 Activity of 2KS, Chloramphenicol, Ciprofloxacin, 2KSC and 2KSCip on S2

The optical densities of cultures treated or/and infected with the phage 2KS, 2C and the combination of both (2KSC) were all significantly different ($p \leq 0.05$) from the optical density of S2 (Figure 4.16). The optical densities of 2KS and 2C were significantly different from each other but both were not significantly different from 2KSC. The optical densities of cultures treated or/and infected with the phage 2KS, 2Cip and the combination of both (2KSCip) were all significantly different ($p \leq 0.05$) from the optical density of S2 as seen in Figure 4.17. The optical densities of 2KS, 2Cip and 2KSCip were all significantly different from one another.

4.5.3.7 Activity of 2KW, Chloramphenicol, Ciprofloxacin, 2KWC and 2KWCip on S2

The optical densities of the cultures containing either phage 2KW, Chloramphenicol or the combination of the two 2KWC were all significantly different ($p \leq 0.05$) from the control S2 as seen in Figure 4.18. The optical densities of 2KW, 2C and 2KWC were all significantly different from each other. The optical densities of the cultures containing 2KW, Ciprofloxacin and the combination of both 2KWCip were all significantly different ($p \leq 0.05$) from the control S2 (Figure 4.19). The OD_{600} of cultures containing 2KW, 2Cip and 2KWCip were significantly different from each other at $p \leq 0.05$.

4.5.3.8 Activity of 2R, Chloramphenicol, Ciprofloxacin, 2RC and 2RCip on S2

There was significant decrease ($p \leq 0.05$) in the optical densities of *Salmonella* cultures infected or/and treated with phage 2R, 2C and 2RC compared to the control S2 but there was a change at the seventh hour where the optical density of 2RC started increasing and

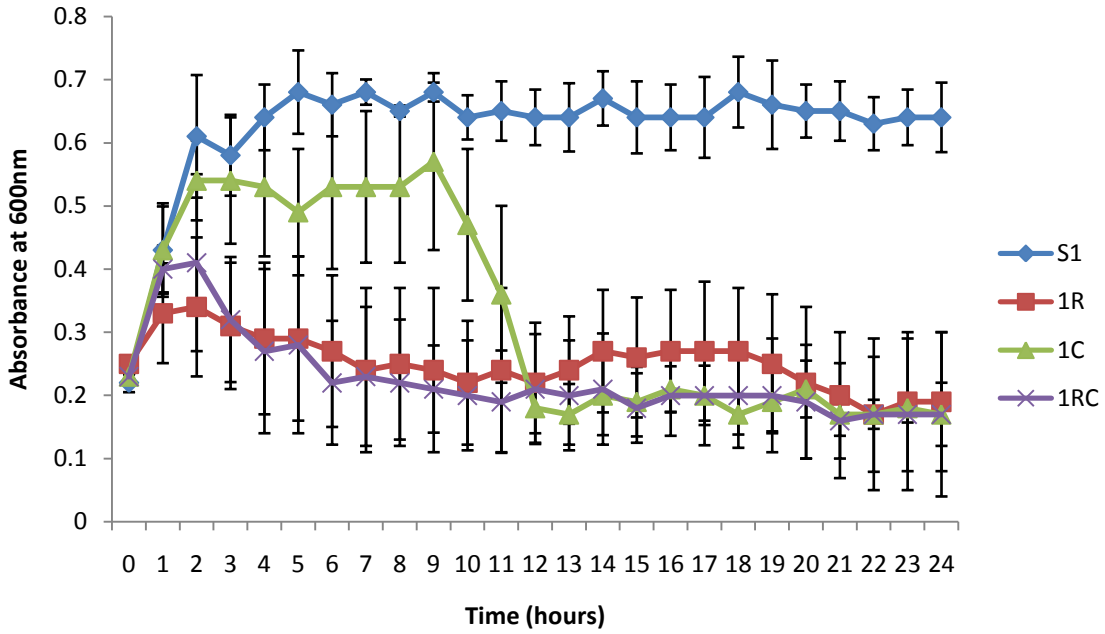


Figure 4.12: Optical Density (OD) development of uninfected control culture (S1) and parallel cultures infected/treated with 1R, Chloramphenicol and both. The data represent the mean ± standard error.

Key:

S1 – Control uninfected/untreated culture of *Salmonella* species

1R – Culture of *Salmonella* species infected with phage 1R

1C – Culture of *Salmonella* species treated with Chloramphenicol

1RC - Culture of *Salmonella* species infected and treated with both phage 1R and Chloramphenicol

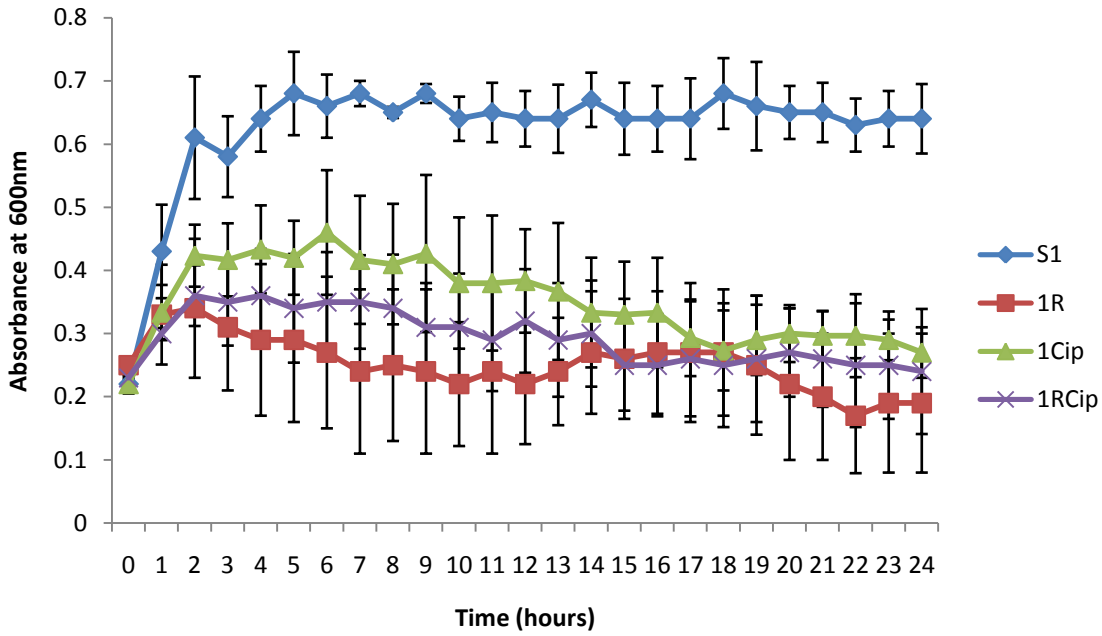


Figure 4.13: Optical Density (OD) development of uninfected control culture (S1) and parallel cultures infected/treated with 1R, Ciprofloxacin and both. The data represent the mean ± standard error.

Key:

S1 – Control uninfected/untreated culture of *Salmonella* species

1R – Culture of *Salmonella* species infected with phage 1R

1Cip – Culture of *Salmonella* species treated with Ciprofloxacin

1RCip - Culture of *Salmonella* species infected and treated with both phage 1R and Ciprofloxacin

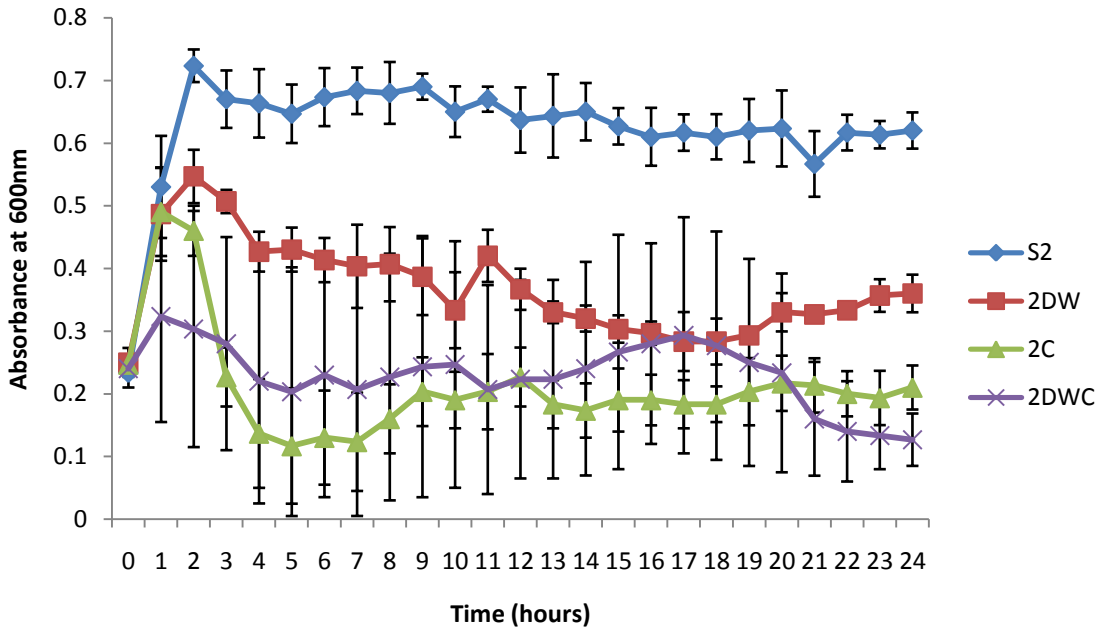


Figure 4.14: Optical Density (OD) development of uninfected control culture (S2) and parallel cultures infected/treated with 2DW, Chloramphenicol and both. The data represent the mean ± standard error.

Key:

S2 – Control uninfected/untreated culture of *Salmonella* Pullorum

2DW – Culture of *Salmonella* Pullorum infected with phage 2DW

2C – Culture of *Salmonella* Pullorum treated with Chloramphenicol

2DWC - Culture of *Salmonella* Pullorum infected and treated with both phage 2DW and Chloramphenicol

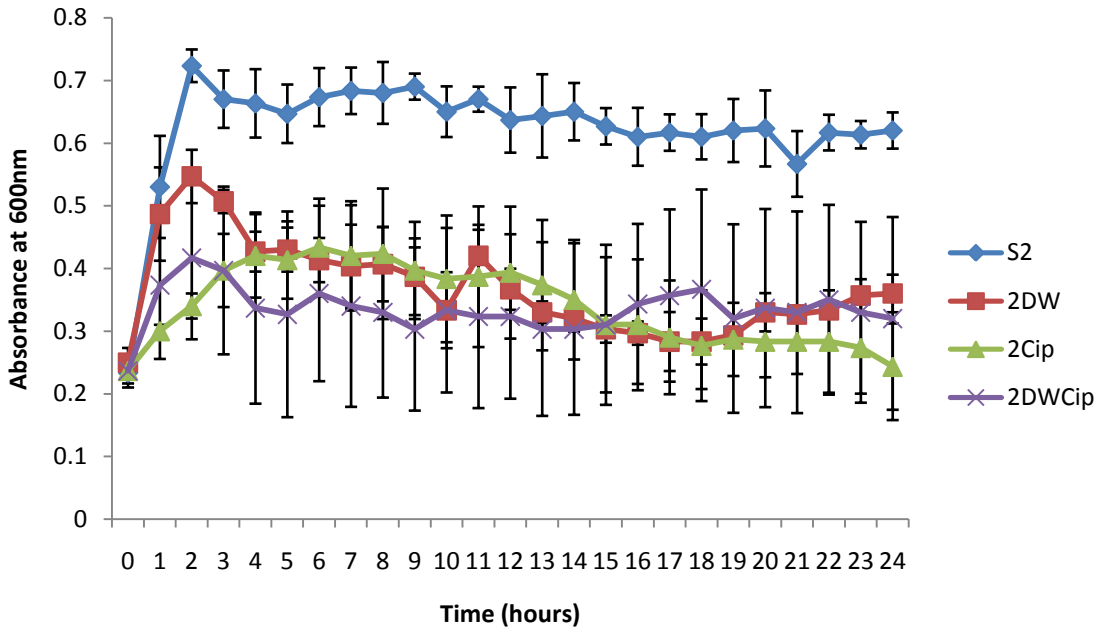


Figure 4.15: Optical Density (OD) development of uninfected control culture (S2) and parallel cultures infected/treated with 2DW, Ciprofloxacin and both. The data represent the mean ± standard error.

Key:

S2 – Control uninfected/untreated culture of *Salmonella* Pullorum

2DW – Culture of *Salmonella* Pullorum infected with phage 2DW

2Cip – Culture of *Salmonella* Pullorum treated with Ciprofloxacin

2DWCip - Culture of *Salmonella* Pullorum infected and treated with both phage 2DW and Ciprofloxacin

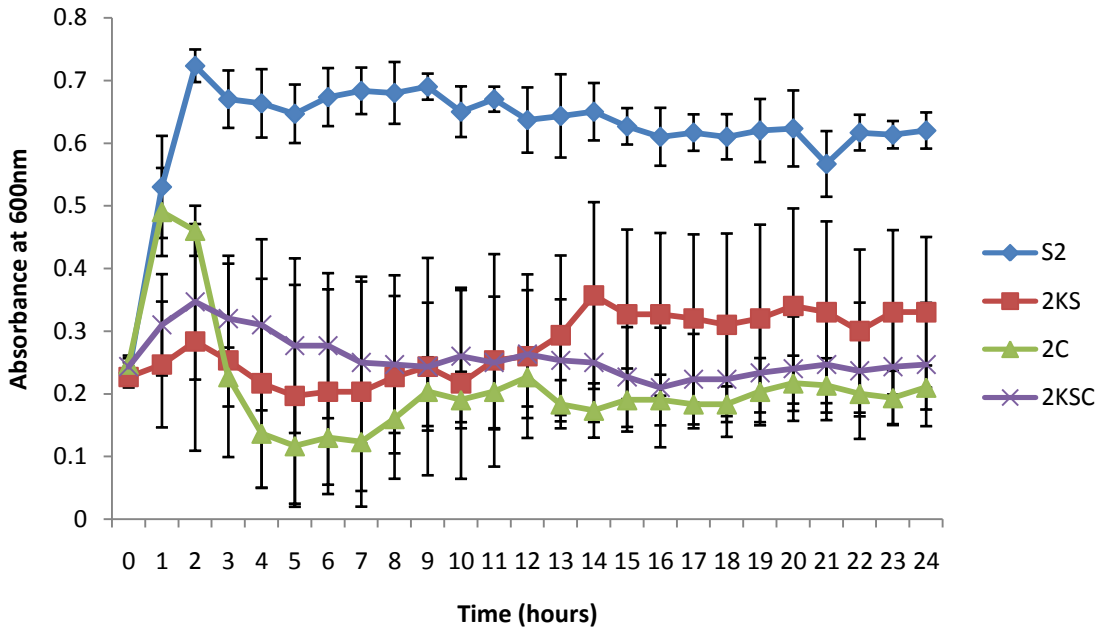


Figure 4.16: Optical Density (OD) development of uninfected control culture (S2) and parallel cultures infected/treated with 2KS, Chloramphenicol and both. The data represent the mean ± standard error.

Key:

S2 – Control uninfected/untreated culture of *Salmonella* Pullorum

2KS – Culture of *Salmonella* Pullorum infected with phage 2KS

2C – Culture of *Salmonella* Pullorum treated with Chloramphenicol

2KSC - Culture of *Salmonella* Pullorum infected and treated with both phage 2KS and Chloramphenicol

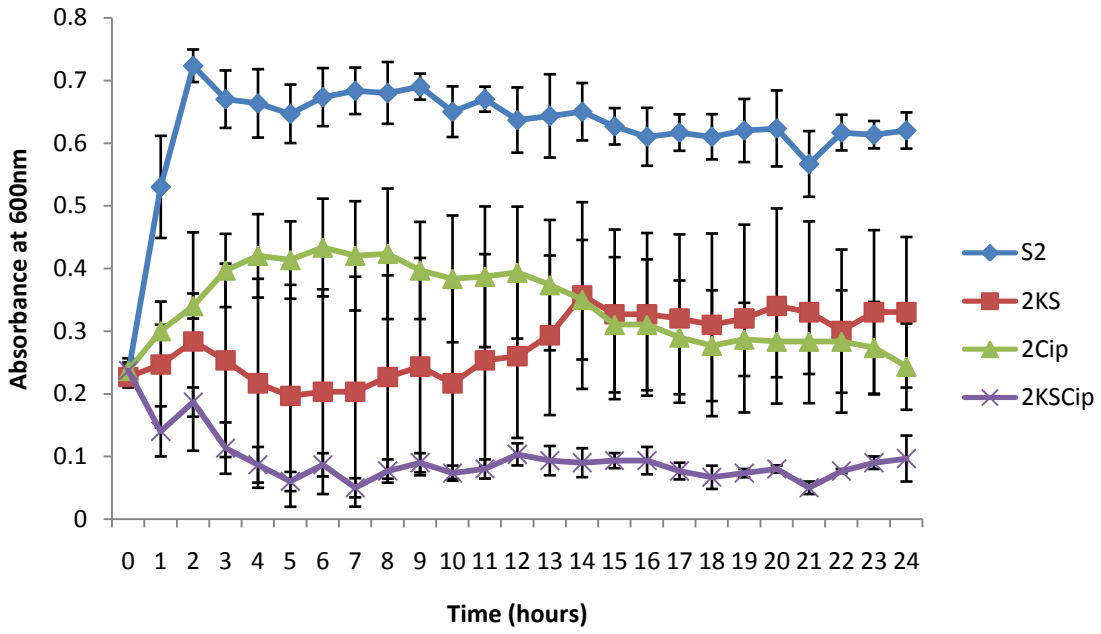


Figure 4.17: Optical Density (OD) development of uninfected control culture (S2) and parallel cultures infected/treated with 2KS, Ciprofloxacin and both. The data represent the mean ± standard error.

Key:

S2 – Control uninfected/untreated culture of *Salmonella* Pullorum

2KS – Culture of *Salmonella* Pullorum infected with phage 2KS

2Cip – Culture of *Salmonella* Pullorum treated with Ciprofloxacin

2KSCip - Culture of *Salmonella* Pullorum infected and treated with both phage 2KS and Ciprofloxacin

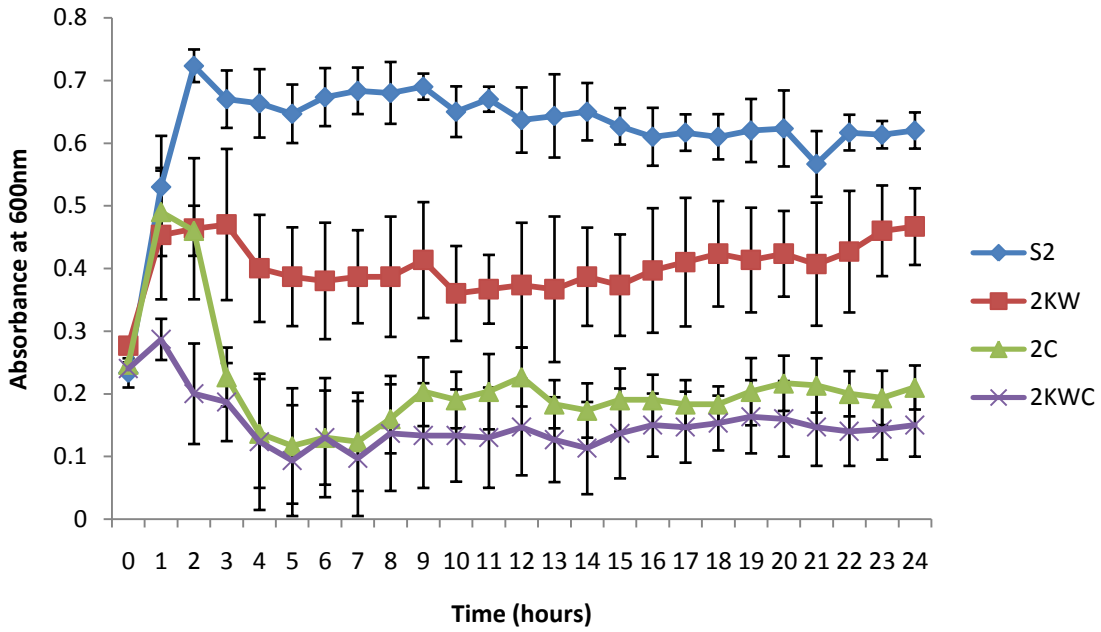


Figure 4.18: Optical Density (OD) development of uninfected control culture (S2) and parallel cultures infected/treated with 2KW, Chloramphenicol and both. The data represent the mean ± standard error.

Key:

S2 – Control uninfected/untreated culture of *Salmonella* Pullorum

2KW – Culture of *Salmonella* Pullorum infected with phage 2KW

2C – Culture of *Salmonella* Pullorum treated with Chloramphenicol

2KWC - Culture of *Salmonella* Pullorum infected and treated with both phage 2KW and Chloramphenicol

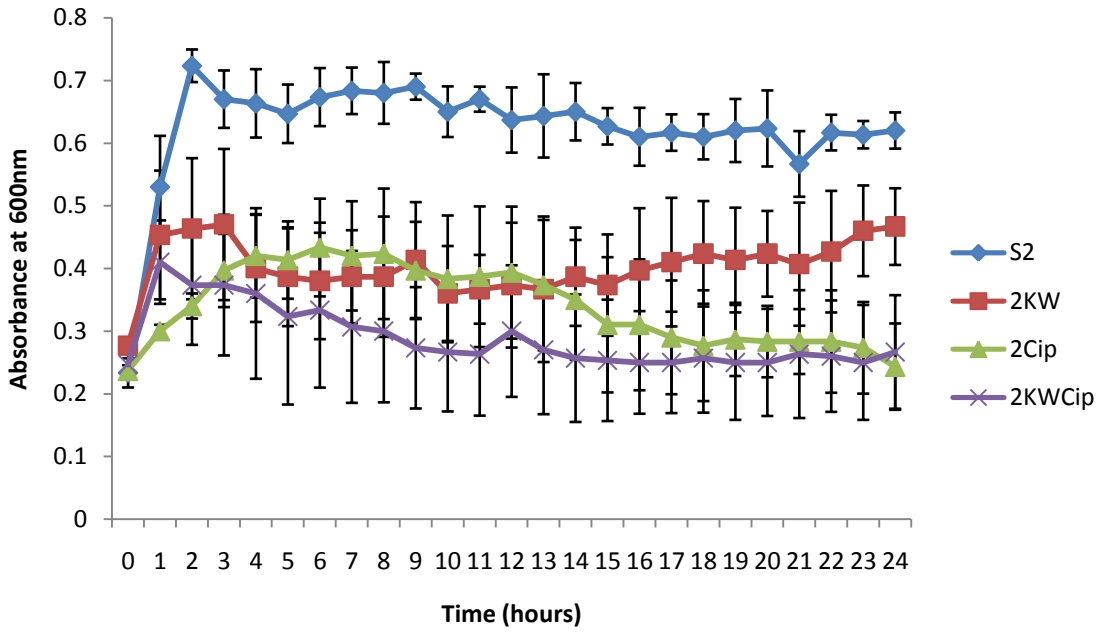


Figure 4.19: Optical Density (OD) development of uninfected control culture (S2) and parallel cultures infected/treated with 2KW, Ciprofloxacin and both. The data represent the mean ± standard error.

Key:

S2 – Control uninfected/untreated culture of *Salmonella* Pullorum

2KW – Culture of *Salmonella* Pullorum infected with phage 2KW

2Cip – Culture of *Salmonella* Pullorum treated with Ciprofloxacin

2KWCip - Culture of *Salmonella* Pullorum infected and treated with both phage 2KW and Ciprofloxacin

even superseded the control at the 17th hour and continued increasing steadily above the control as seen in Figure 4.20. The OD_{600} of 2R was not significantly different from that of 2C but both were significantly different from that of 2RC at $p \leq 0.05$. The optical densities of the cultures treated or infected with 2R, 2Cip and 2RCip were also significantly different ($p \leq 0.05$) from that of S2 as shown in Figure 4.21. At $p \leq 0.05$, the OD_{600} of 2Cip was significantly different from those of 2R and 2RCip which in turn were not significantly different from each other.

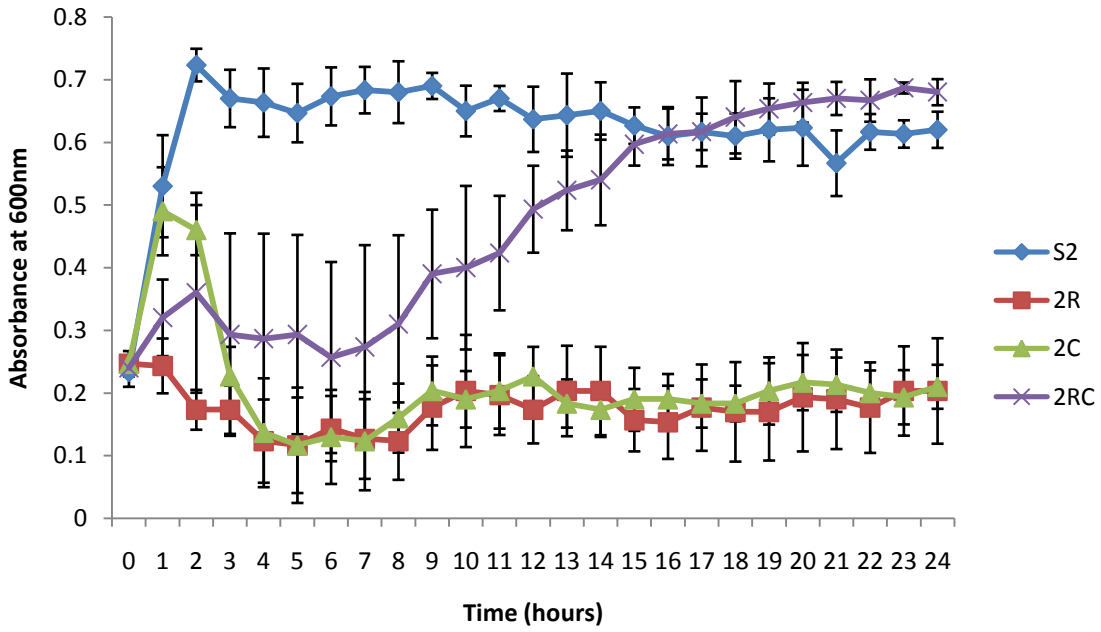


Figure 4.20: Optical Density (OD) development of uninfected control culture (S2) and parallel cultures infected/treated with 2R, Chloramphenicol and both. The data represent the mean ± standard error.

Key:

S2 – Control uninfected/untreated culture of *Salmonella* Pullorum

2R – Culture of *Salmonella* Pullorum infected with phage 2R

2C – Culture of *Salmonella* Pullorum treated with Chloramphenicol

2RC - Culture of *Salmonella* Pullorum infected and treated with both phage 2R and Chloramphenicol

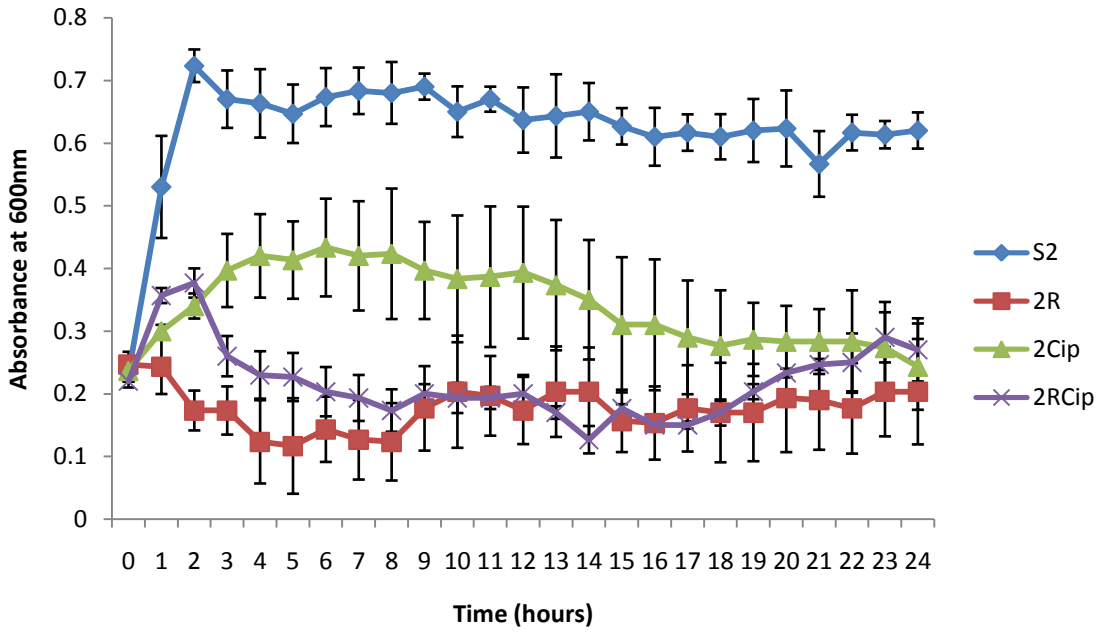


Fig. 4.21: Optical Density (OD) development of uninfected control culture (S2) and parallel cultures infected/treated with 2R, Ciprofloxacin and both. The data represent the mean ± standard error.

Key:

S2 – Control uninfected/untreated culture of *Salmonella* Pullorum

2R – Culture of *Salmonella* Pullorum infected with phage 2R

2Cip – Culture of *Salmonella* Pullorum treated with Ciprofloxacin

2RCip - Culture of *Salmonella* Pullorum infected and treated with both phage 2R and Ciprofloxacin

CHAPTER FIVE

5.0 DISCUSSION

The isolation of *Salmonella* Pullorum from egg (Sample 7) and human stool (Samples 4 and 8) shows the possibility of it being zoonotic. Since it is host restricted, it is considered to pose negligible risk to human health (McCullough and Eisele, 1951; Hoelzer *et al*., 2011). Its transmission to human may be due to consumption of contaminated food, raw/improperly cooked egg and egg products or contact/improper handling of contaminated vectors especially among poultry farmers. It probably got its way into the egg through transovarian (vertical) transmission (Shivaprasad, 2000; FAO/WHO, 2002; Anderson *et al*., 2006) or trans-shell (horizontal) transmission (ACMSF, 2001; FAO/WHO, 2002). The *Salmonella* species isolated from human stool (Sample 2) could also be of zoonotic origin since it is not *Salmonella* Typhi or *Salmonella* Paratyphi and therefore not of human origin.

The plaques formed after the spot and plaque assay indicates the presence of phage lytic on the host bacterial cells. It also indicates the presence of bacteriophages that are virulent against *Salmonella* species and *Salmonella* Pullorum in the university drainage system, the dam at Kwanan Kurmi and the soil of the farms around the dam. This is in line with many publications such as Filho *et al*. (2007), Hungaro *et al*. (2013), Akthar *et al*. (2014) and Piracha *et al*. (2014) where spot and plaque assay were the primary isolation, detection and lytic spectrum determination tools for virulent bacteriophages of some bacteria from sources such as chicken feaces, cattle and swine manure pit, sewage water, waste effluents and sewage sludge. The drastic reduction in the phage count of phage 2KW at the 60^{th} minute may be because at the specific time of assay before the media solidified, most of the phage particles were at the infective stage

inside the host cells. This pattern and the differences in the curves' patterns indicate that the burst time for bacteriophages varies.

The continuous increase in phage titer with time indicates the increased production of progeny phages which in turn infect more bacterial cells as bacterial cell lysis continues. This self-replicating ability is one of the factors that give the bacteriophage an upper hand over antibiotic (Cerveny *et al*., 2002). The replication dynamics of each phage on its host indicates reasonable decrease of the host in the presence of its respective phage in 24 hours.

Decline in Optical Density ($OD_{600)}$ was considered to be due to the lytic activity phage on its host. Although statistically, the reference phage performed better than the other phages on S1 and S2 except 1DW which was similar in action to 1R; nevertheless, all were very active against the *Salmonella* isolates. This shows the effectiveness of the phages *in vitro* as biological antibacterial agents against *Salmonella* species and *Salmonella* Pullorum. The antibacterial patterns of the phages in this study are similar to earlier publications such as Chibani-Chennoufi *et al*. (2004b), Atterbury *et al.* (2007), Synnott *et al*. (2009), Hungaro *et al*. (2013) and Piracha *et al*. (2014) who all observed similar trends (increased phage titer by phage counts and significant decrease in OD) on several phage treated bacterial isolates compared to their respective untreated controls.

The susceptibilities of the two selected isolates were highest for Ciprofloxacin followed by Ofloxacin and then Chloramphenicol. This is similar to the trend in some publications such as Antunes *et al*. (2003), Smith *et al*. (2009) and Abdullahi *et al*. (2014) where Salmonellae were found to be totally or almost totally susceptible to these antibiotics. The two antibiotics with the highest activity were chosen for further studies

but since they were both Flouroquinolones, the next antibiotic with the highest activity –Chloramphenicol, was used to replace Ofloxacin for further studies. The significant reduction in OD of cultures of the isolates treated with Chloramphenicol and Ciprofloxacin compared to the controls also indicate the susceptibilities of the isolates to the antibiotics even in liquid medium.

Considering the significant reduction in OD_{600} of the infected/treated cultures in comparison to the uninfected/untreated cultures as the lytic activity of the phages on the host and likewise the detrimental effects of the antibiotics on the host, deductions were made that all the bacteriophages, the antibiotics and the combinations of the phages and antibiotics -except 2RC, have antibacterial effects on the two salmonellae used in this study.

The activities observed against *Salmonella* isolates in this study by the phages alone, antibiotics alone and their combinations were divided into five categories;

1. In some cases like comparison between 2R and Ciprofloxacin, the phage alone had a better effect on the isolate compared to the antibiotic alone and the combination of phage and antibiotic making it a better option as biocontrol agent against the isolate,

2. For some cases such as comparisons between 1R and Chloramphenicol and 2KS and Ciprofloxacin, the combination of phage and antibiotic gave synergistic effects making the combination therapy a better option than the phage or antibiotic alone,

3. In some cases like comparison between 1R and Chloramphenicol and the combination 1RC, the phage had similar or same effect with the antibiotic or the combination of both.

4. Meanwhile in cases such as comparison between 2KS and Chloramphenicol, the antibiotic proved to be a better antibacterial agent than the phage alone and the combination therapy,

5. A unique case -where phage alone and antibiotic alone were good antibacterial agent but the combination therapy (2RC) which started well but later exceeded the control in OD.

The phage 1DW, the antibiotic 1C and their combination 1DWC all had good observable effects on S1. Though the OD_{600} of 1DWC started increasing at the seventeenth hour (possibly due to contamination by another microbe in the process of assay) statistically the phage alone and the combination of both were better than the chloramphenicol alone. Also, they have similar degree of activity. For the same phage and ciprofloxacin on same isolate, the use of 1DW is the best option for control of S1 compared to ciprofloxacin alone and the combination of both.

The activity patterns of 1KS, Chloramphenicol and the combination of both indicate that all can serve as control agents of S1. The combination of phage and the antibiotic serves as a better option statistically for the control of S1. Similarly, the combination of the phage and Ciprofloxacin is a better option when compared to ciprofloxacin or the Phage alone. In the case of 1KW, the combinations of the phage and either antibiotic (1KWC and 1KWCip) have better effects compared to the phage or antibiotic alone. For 1R in relation to Chloramphenicol and Ciprofloxacin, the phage alone served as the best option compared to each antibiotic alone or the combination of the phage and the

respective antibiotic. The only exception is in the case of 1R and chloramphenicol where the combination therapy-1RC, serve a better option even though its effect is not statistically different from the phage alone which in turn is not significantly different from the antibiotic alone.

In the case of 2DW, the antibiotics and their respective combinations, all can serve as control agents for S2 except that 2C and 2DWC-which turn out not to be significantly different from each other, are better options than the phage alone. The phage, Ciprofloxacin and the combination of both can serve similar purpose without much difference. A different deduction was made for 2KS, Chloramphenicol and the combination where chloramphenicol is seen as a better option though it has close activity with its combination with the phage 2KS. As expected and unlike the previous results so far –except for the case of 1R, chloramphenicol and their combination, the combination of 2KS with ciprofloxacin and the combination of 2KW with each antibiotic produce synergistic effects which made them better potential control agents than the phages alone or antibiotics alone.

In the case of the reference phage on S2 -2R, there was a peculiarity observed where OD_{600} of culture infected and treated with combination of phage and chloramphenicol started increasing at a point and till it exceeded the control. The reason for this pattern is unknown since it didn't happen in the case of the phage alone or the antibiotic alone but it could be attributed to resistance to both phage and antibiotic which could result from mutation or adaptation of the host bacterium as replication continues due to processes such as transduction, development of resistant genes and loss of receptors and colonization factors for both phage and antibiotic (Mahony *et al*., 2011; Piracha *et al*., 2014). This can be a hindrance in using such approach for combination therapy and control measures. The other results for 2R alone and Chloramphenicol alone show that

either of them can be used since there is no significant difference between them. The use of 2R alone or in combination with ciprofloxacin gives a better option than the use of Ciprofloxacin alone.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Salmonella Pullorum and *Salmonella* Species isolated or obtained from egg and human sources suggest them to be zoonotic and may pose negative economic impact in the poultry industry as well as health hazard in humans respectively.

Bacteriophages isolated and characterized from waste water, dam and soil confirm their presence and abundance of virulent phages in such places.

The activity pattern of the phages on their respective *Salmonella* host indicates the ability of virulent phages to lyse *Salmonella* species and therefore their use in the control of *Salmonella* Species. They were seen as potential biocontrol agents that can be used against pathogens such as *Salmonella* for preventive, therapeutic and curative measures in the fields of food, industries, biosanitation, agriculture, veterinary and human medicine.

Based on the results and deductions from this research, phages were seen to perform well like antibiotics -and in some cases more, in the control of the *Salmonella* species. They were also seen to be efficient either alone or in combination with antibiotics. They can therefore be used as alternative to antibiotics or in combination with antibiotics in cases of adverse side effects to antibiotics, resistance to antibiotics, financial constrains to access antibiotics and so on.

The result of this study may not exactly reflect what will happen *in vivo* because the dynamics of phage-bacterium-environment interactions *in vivo* may be different from those *in vitro* due to the viscosity of the gut matrix (Weld *et a*l., 2004), complex

physicochemical environment, host defences (Connerton andConnerton, 2005), metabolic state of the bacterial host *in vivo* (Chibani – Chennoufi *et al*., 2004b) and so on. Therefore, caution must be taken when phages are to be used as this is not a conclusive study on the safe use of phages. Factors such as interaction of phage-host and food or environment, phage viability and security should be understood before reliable use.

6.2 Recommendations

In view of the results and discussions obtained from this study, the following measures are recommended:

1. Food should be properly processed/cooked before consumption to prevent infection by pathogenic organisms like *Salmonella* species.
2. Care should be taken in using and handling of objects associated with food and food products especially vegetables, meat, eggs, fish and their products.
3. Proper monitoring and care of poultry farms should be done.
4. Sensitization of the masses on prevention measures against *Salmonella* infection.
5. Personal (especially handwashing) and environmental hygiene are necessary in reducing contamination and transmission of pathogens.
6. Further short term and long term studies on phage activity *in vivo*, phage biology, phage lytic enzyme biology, phage engineering, phage genome analysis, emergence of bacterial host resistant mutants and consumer safety should be done on the bacteriophages to better understand and explore their potential as bio-control agents of *Salmonella* species.

7. Equipment necessary for research into the world of bacteriophages should be made readily available in institutions to enhance hitch-free researches.
8. Cocktail of different lytic phages should be used in the control of *Salmonella* using bacteriophages to curtail resistance of phages by *Salmonella*.
9. Virulent, non-transducing phage should be used for biocontrol of *Salmonella.*
10. Known bacterial virulent genes in phage genome should be checked before use.

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APPENDIX I: PRESUMPTIVE CHARACTERISTICS OF THE ISOLATES TYPICAL OF SALMONELLA

			Colony Morphology				Biochemical Reaction						Triple Sugar Iron (TSI) reaction			
S/N	Sample ID	Selenite F Broth (T/C)	SSA	XLD	BSA	Gram reaction	I	MR	VP	Cit	Mot	U	Slope	Butt	H_2S	Gas
1	2	NA	Colourless with black centre	Milky with black centre	White covered with metallic sheen	Short GNR	-	+	-	+	+	-	R	Y	+	+
2	4	NA	Colourless	Colourless	Brown	Short GNR	-	+	-	-	+	-	R	Y	-	+
3	6	+	Colourless	Red	Brown	Short GNR	-	+	-	-	-	-	R	Y	-	+
4	7	+	Colourless	No growth	White with brown centre	Short GNR	-	+	-	-	+	-	R	Y	-	+
5	8	NA	Colourless	Milky with black centre	White covered with metallic sheen	Short GNR	-	+	-	-	+	-	R	Y	+	+
6	14	NA	Colourless	Red	White covered with metallic sheen	Short GNR	-	-	+	+	+	-	R	Y	+	+
7	19	+	Colourless	Colourless	White	Short GNR	-	-	+	+	+	-	R	Y	-	+
8	21	+	Colourless	Red	White covered with metallic sheen	Short GNR	-	-	+	-	-	-	R	Y	+	-
9	23	+	Colourless with black colouration	Colourless	Brown	Short GNR	-	-	+	-	+	-	R	Y	+	-
10	27	+	Colourless with black colouration	Colourless	White covered with metallic sheen	Short GNR	-	-	+	-	-	+	Y	Y	+	-
11	28	+	Colourless with black colouration	Colourless	White	Short GNR	-	+	-	-	+	-	R	Y	+	-
12	30	+	Colourless	Colourless	White covered with metallic sheen	Short GNR	-	+	-	-	+	-	Y	Y	-	+
13	33	+	Colourless with black colouration	Colourless with black colouration	White covered with metallic sheen	Short GNR	-	+	-	-	+	-	R	Y	+	+
14	34	+	Colourless with black colouration	Colourless	White covered with metallic sheen	Short GNR	-	+	-	-	+	+	Y	Y	+	+
15	38	+	Colourless with black colouration	Colourless	White covered with metallic sheen	Short GNR	-	+	-	+	-	+	Y	Y	-	+

Key:

Y – Yellow R – Red T – Turbidity C - Colour change MR – Methyl red VP – Voges proskuaer I – Indole Cit – Citrate Mot - Motility SSA – *Salmonella-Shigella* Agar BSA – Bismuth Sulfite Agar XLD – Xylose Lysine Desoxycholate agar GNR - Gram negative rods U – Urease + - positive - - Negative NA – Not applicable

APPENDIX II: MICROGEN GN-A SUBSTRATE REFERENCE TABLE

Well	Reaction	Description	Positive	Negative
1	Lysine	Lysine decarboxylase-Bromothymol blue changes to green /blue indicating the production of the amine cadaverine	Green/Blue	Yellow
2	Ornithine	Ornithine decarboxylase-Bromothymol blue changes to blue indicating the production of the amine putrescin.	Blue	Yellow/Green
3	H_2S	H_2S production- Thiosulphate is reduced to H_2S that reacts with ferric salts producing a black precipitate		
4	Glucose	Fermentation- Bromothymol blue changes from blue to yellow as a result of acid produced from the carbonhydrate fermentation	Yellow	Blue/ Green
5	Mannitol			
6	Xylose			
7	ONPG	Hydrolysis- ONPG hydrolysis by B-galactosidase results in the production of yellow ortho-nitrophenol	Yellow	Colourless
8	Indole	Indole is produced from tryptophan and gives a pink/red complex when Kovac's reagent is added.	Pink/Red	Colourless
9	Urease	Hydrolysis of urea results in the formation of ammonia leading to an increase in pH which turns phenol red from yellow to pink/red	V.Deep Pink	Straw to pale salmon pink colour
10	VP	Acetoin production from glucose is detected by the formation of a pink /red complex after the addition of alpha naphthol and creatine in the presence of KOH.	Deep Pink/ Red	Colourless to pale pink
11	Citrate	Utilisation of citrate (only carbon source) leading to a pH increase giving a colour change in bromothymol blue from green to blue	Blue	Yellow/ Pale Green
12	TDA	Indolepyruvic acid is produced from tryptophan by tryptophan deaminase giving a cherry red colour when ferric ions are added. Indole positive isolates may give a brown colour- this is a negative result	Cherry red	Straw colour

APPENDIX III: ISOLATES IDENTIFICATION USING MICROGEN Gn-A ID SYSTEM

S/N	Isolate No (New No)	Lys	Orn	H_2S	Glu	Man	Xyl	O.N.P.G	Ind	Ure	V.P	Cit	T.D.A	Octal code	Identity	Percent probability (%)
1	2(1)	+	+	+	+	+	+	-	-	-	-	+	-	7702	*Salmonella species*	80.89
2	7(2)	+	+	+	+	+	+	-	-	-	-	-	-	7700	*Salmonella pullorum*	66.15
3	4(3)	+	+	+	+	+	+	-	+	-	+	-	+	7725	*Salmonella pullorum*	33.03
4	8(4)	+	+	+	+	+	+	-	+	-	+	-	+	7725	*Salmonella pullorum*	33.03
5	23(6)	+	+	+	+	+	+	+	-	-	+	+	-	7746	*Enterobacter aerogenes*	33.57
6	28(7)	-	-	+	+	+	+	+	-	-	-	+	-	1742	*Citrobacter freundii*	99.84
7	33(8)	-	-	+	+	+	+	+	-	-	-	+	-	1742	*Citrobacter freundii*	99.84

Key;

Lys – Lysine Orn – Ornithine Glu – Glucose Man – Mannitol

Xyl – Xylose Ind – Indole Ure – Urease O.N.P.G - 2-nitrophenyl-β-D-galactopyranoside

V.P – Voges Proskauer Cit – Citrate TDA – Tryptophan Deaminase Acid

APPENDIX IV : COLLECTION OF WASTE WATER AT THE UNIVERSITY'S DRAINAGE SYSTEM



APPENDIX V : DESIGNATION OF NAMES TO ISOLATED PHAGES IN THIS STUDY

Bacteriophage	Bacterium used for isolation (and code)	Location of Primary source	Source
1DW	*Salmonella* species (S1)	Drainage system	Water
2DW	*Salmonella* Pullorum (S2)	Drainage system	Water
1KW	*Salmonella* species (S1)	Kwanan Kurmi	Water
2KW	*Salmonella* Pullorum (S2)	Kwanan Kurmi	Water
1KS	*Salmonella* species (S1)	Kwanan Kurmi	Soil
2KS	*Salmonella* Pullorum (S2)	Kwanan Kurmi	Soil
1R	*Salmonella* species (S1)	Reference phage (Salmonelex)	Reference phage (Salmonelex)
2R	*Salmonella* Pullorum (S2)	Reference phage (Salmonelex)	Reference phage (Salmonelex)

APPENDIX VI: ACTIVITIES OF PHAGES AND ANTIBIOTICS ON SALMONELLA SPECIES AND SALMONELLA PULLORUM

APPENDIX VIa: Absorbance readings of Chloramphenicol and Ciprofloxacin on *Salmonella* species (S1) and the positive control at 600nm

	S1					1C					1Cip				
Time (hr)	**a**	**b**	**c**	**Mean**	**SE**	**a**	**b**	**c**	**Mean**	**SE**	**a**	**b**	**c**	**Mean**	**SE**
0	0.21	0.2	0.25	0.22	0.015	0.24	0.23	0.23	0.23	0.0033	0.23	0.2	0.23	0.22	0.01
1	0.34	0.38	0.58	0.43	0.074	0.29	0.48	0.51	0.43	0.069	0.3	0.28	0.42	0.3333	0.04372
2	0.42	0.69	0.73	0.61	0.097	0.41	0.6	0.6	0.54	0.063	0.34	0.42	0.51	0.4233	0.0491
3	0.48	0.57	0.7	0.58	0.064	0.34	0.61	0.67	0.54	0.1	0.32	0.41	0.52	0.4167	0.05783
4	0.6	0.57	0.74	0.64	0.052	0.31	0.62	0.67	0.53	0.11	0.32	0.42	0.56	0.4333	0.0696
5	0.65	0.59	0.81	0.68	0.066	0.29	0.56	0.63	0.49	0.1	0.31	0.44	0.51	0.42	0.05859
6	0.62	0.6	0.76	0.66	0.05	0.28	0.65	0.67	0.53	0.13	0.28	0.48	0.62	0.46	0.09866
7	0.64	0.68	0.71	0.68	0.02	0.29	0.63	0.68	0.53	0.12	0.25	0.4	0.6	0.4167	0.1014
8	0.65	0.67	0.64	0.65	0.0088	0.29	0.65	0.66	0.53	0.12	0.25	0.4	0.58	0.41	0.09539
9	0.68	0.71	0.66	0.68	0.015	0.3	0.67	0.75	0.57	0.14	0.22	0.41	0.65	0.4267	0.1244
10	0.7	0.58	0.63	0.64	0.035	0.28	0.7	0.42	0.47	0.12	0.2	0.38	0.56	0.38	0.1039
11	0.72	0.66	0.56	0.65	0.047	0.3	0.63	0.15	0.36	0.14	0.19	0.39	0.56	0.38	0.1069
12	0.72	0.57	0.62	0.64	0.044	0.26	0.13	0.15	0.18	0.04	0.23	0.41	0.51	0.3833	0.08192
13	0.74	0.56	0.61	0.64	0.054	0.26	0.1	0.14	0.17	0.048	0.2	0.33	0.57	0.3667	0.1084
14	0.75	0.61	0.64	0.67	0.043	0.32	0.11	0.16	0.2	0.063	0.19	0.32	0.49	0.3333	0.08686
15	0.75	0.61	0.56	0.64	0.057	0.29	0.1	0.18	0.19	0.055	0.19	0.32	0.48	0.33	0.08386
16	0.74	0.6	0.57	0.64	0.052	0.28	0.16	0.19	0.21	0.036	0.18	0.34	0.48	0.3333	0.08667
17	0.76	0.62	0.54	0.64	0.064	0.29	0.14	0.16	0.2	0.047	0.19	0.29	0.4	0.2933	0.06064
18	0.79	0.66	0.6	0.68	0.056	0.23	0.13	0.14	0.17	0.032	0.15	0.31	0.36	0.2733	0.06333
19	0.77	0.67	0.53	0.66	0.07	0.28	0.14	0.14	0.19	0.047	0.18	0.33	0.36	0.29	0.05568
20	0.7	0.69	0.57	0.65	0.042	0.3	0.16	0.17	0.21	0.045	0.21	0.34	0.35	0.3	0.04509
21	0.72	0.67	0.56	0.65	0.047	0.24	0.15	0.13	0.17	0.034	0.22	0.34	0.33	0.2967	0.03844
22	0.69	0.65	0.55	0.63	0.042	0.22	0.15	0.15	0.17	0.023	0.17	0.39	0.33	0.2967	0.06566
23	0.69	0.67	0.55	0.64	0.044	0.22	0.18	0.14	0.18	0.023	0.23	0.3	0.34	0.29	0.03215
24	0.7	0.69	0.53	0.64	0.055	0.27	0.12	0.12	0.17	0.05	0.19	0.31	0.31	0.27	0.04

Key:

a - First reading, b – Second reading, c – Third reading, SE - Standard Error, S1 - Salmonella species alone (control), 1C - Chloramphenicol treated culture of S1, 1Cip – Ciprofloxacin treated culture of S1

APPENDIX VIb: Absorbance readings of Phage 1DW and its combination with the two antibiotics (1DWC and 1DWCip) on *Salmonella* species (S1) at 600nm

Time	1DW					1DWC					1DWCip				
(hr)	a	b	c	Mean	SE	a	b	c	Mean	SE	a	b	c	Mean	SE
0	0.22	0.21	0.21	0.22	0.0033	0.24	0.21	0.24	0.23	0.01	0.22	0.26	0.24	0.24	0.012
1	0.38	0.37	0.39	0.38	0.033	0.35	0.47	0.17	0.33	0.087	0.12	0.33	0.24	0.23	0.061
2	0.36	0.53	0.55	0.42	0.057	0.39	0.45	0.13	0.32	0.098	0.1	0.49	0.17	0.25	0.12
3	0.32	0.46	0.49	0.37	0.047	0.34	0.45	0.13	0.31	0.094	0.05	0.51	0.12	0.23	0.14
4	0.32	0.39	0.43	0.34	0.023	0.37	0.39	0.03	0.26	0.12	0.06	0.62	0.07	0.25	0.19
5	0.26	0.42	0.46	0.31	0.053	0.34	0.37	0	0.24	0.12	0.05	0.5	0.05	0.2	0.15
6	0.11	0.40	0.43	0.21	0.097	0.26	0.37	0.1	0.24	0.078	0.05	0.47	0.09	0.2	0.13
7	0.2	0.42	0.45	0.27	0.073	0.18	0.38	0.01	0.19	0.11	0.05	0.45	0.06	0.19	0.13
8	0.16	0.42	0.43	0.25	0.087	0.11	0.33	0.04	0.16	0.087	0.05	0.47	0.08	0.2	0.14
9	0.14	0.38	0.43	0.22	0.080	0.11	0.36	0.05	0.17	0.095	0.07	0.43	0.08	0.19	0.12
10	0.13	0.36	0.41	0.21	0.077	0.09	0.3	0.05	0.15	0.078	0.06	0.45	0.08	0.2	0.13
11	0.14	0.39	0.48	0.22	0.083	0.11	0.32	0.04	0.16	0.084	0.06	0.42	0.07	0.18	0.12
12	0.15	0.37	0.40	0.22	0.073	0.1	0.33	0.08	0.17	0.08	0.1	0.44	0.1	0.21	0.11
13	0.13	0.33	0.38	0.20	0.067	0.11	0.31	0.07	0.16	0.074	0.09	0.46	0.09	0.21	0.12
14	0.16	0.38	0.44	0.23	0.073	0.11	0.27	0.08	0.15	0.059	0.06	0.47	0.07	0.2	0.14
15	0.14	0.38	0.54	0.22	0.08	0.13	0.31	0.1	0.18	0.066	0.05	0.39	0.09	0.18	0.11
16	0.21	0.38	0.51	0.27	0.057	0.11	0.27	0.13	0.17	0.05	0.05	0.42	0.11	0.19	0.11
17	0.14	0.38	0.49	0.22	0.080	0.14	0.3	0.1	0.18	0.061	0.04	0.45	0.08	0.19	0.13
18	0.16	0.39	0.53	0.24	0.077	0.13	0.35	0.43	0.30	0.09	0.05	0.45	0.09	0.2	0.13
19	0.16	0.40	0.51	0.24	0.080	0.13	0.34	0.57	0.35	0.13	0.07	0.42	0.09	0.19	0.11
20	0.11	0.40	0.51	0.21	0.097	0.06	0.34	0.57	0.32	0.15	0.06	0.41	0.09	0.19	0.11
21	0.12	0.38	0.47	0.21	0.087	0.08	0.26	0.57	0.30	0.14	0.11	0.4	0.07	0.19	0.1
22	0.03	0.40	0.45	0.15	0.12	0.01	0.38	0.73	0.37	0.21	0.06	0.46	0.09	0.2	0.13
23	0.06	0.41	0.45	0.18	0.12	0.02	0.37	0.73	0.37	0.20	0.07	0.43	0.1	0.2	0.12
24	0.09	0.42	0.43	0.20	0.11	0.01	0.4	0.69	0.37	0.20	0.09	0.42	0.09	0.2	0.11

Key:

a - First reading, b – Second reading, c – Third reading, SE - Standard Error, 1DW - Phage 1DW infected culture of S1, 1DWC – Phage and Chloramphenicol infected and treated culture of S1, 1DWCip – Phage and Ciprofloxacin infected and treated culture of S1.

APPENDIX VIc: Absorbance readings of Phage 1KS and its combination with the two antibiotics (1KSC and 1KSCip) on *Salmonella* species (S1) at 600nm

	1KS					1KSC					1KSCip				
Time (hr)	a	b	c	Mean	SE	a	b	c	Mean	SE	a	b	c	Mean	SE
0	0.24	0.28	0.20	0.24	0.023	0.22	0.21	0.25	0.23	0.012	0.22	0.2	0.23	0.22	0.0088
1	0.37	0.39	0.39	0.38	0.0067	0.36	0.38	0.23	0.32	0.047	0.22	0.24	0.29	0.25	0.021
2	0.42	0.44	0.56	0.47	0.044	0.38	0.52	0.13	0.34	0.11	0.31	0.31	0.25	0.29	0.02
3	0.43	0.28	0.51	0.41	0.067	0.38	0.44	0.12	0.31	0.098	0.2	0.37	0.2	0.26	0.057
4	0.31	0.23	0.34	0.29	0.033	0.39	0.39	0.04	0.27	0.12	0.19	0.36	0.15	0.23	0.064
5	0.3	0.26	0.35	0.30	0.026	0.36	0.38	0	0.25	0.12	0.18	0.37	0.12	0.22	0.075
6	0.14	0.26	0.34	0.25	0.058	0.31	0.35	0.04	0.23	0.097	0.18	0.35	0.18	0.24	0.057
7	0.16	0.30	0.33	0.26	0.052	0.31	0.36	0.04	0.24	0.099	0.15	0.27	0.12	0.18	0.046
8	0.14	0.29	0.35	0.26	0.062	0.34	0.33	0.03	0.23	0.1	0.14	0.29	0.17	0.2	0.046
9	0.16	0.30	0.39	0.28	0.067	0.28	0.32	0.09	0.23	0.071	0.16	0.31	0.19	0.22	0.046
10	0.17	0.29	0.31	0.26	0.044	0.26	0.33	0.05	0.21	0.084	0.15	0.16	0.17	0.16	0.0058
11	0.25	0.31	0.46	0.34	0.062	0.23	0.29	0.05	0.19	0.072	0.14	0.26	0.16	0.19	0.037
12	0.2	0.29	0.32	0.27	0.036	0.17	0.32	0.08	0.19	0.07	0.16	0.22	0.19	0.19	0.017
13	0.18	0.27	0.31	0.25	0.038	0.15	0.33	0.07	0.18	0.077	0.14	0.2	0.18	0.17	0.018
14	0.2	0.34	0.36	0.30	0.05	0.12	0.35	0.05	0.17	0.091	0.14	0.22	0.18	0.18	0.023
15	0.19	0.33	0.37	0.30	0.055	0.11	0.3	0.05	0.15	0.075	0.13	0.2	0.16	0.16	0.02
16	0.22	0.33	0.37	0.31	0.045	0.11	0.32	0.07	0.17	0.078	0.11	0.16	0.17	0.15	0.019
17	0.23	0.30	0.43	0.32	0.059	0.13	0.35	0.09	0.19	0.081	0.11	0.17	0.16	0.15	0.019
18	0.26	0.34	0.46	0.35	0.058	0.12	0.38	0.11	0.2	0.088	0.07	0.18	0.15	0.13	0.033
19	0.3	0.36	0.43	0.36	0.038	0.12	0.41	0.06	0.2	0.11	0.16	0.22	0.15	0.18	0.022
20	0.35	0.35	0.47	0.39	0.04	0.09	0.4	0.16	0.22	0.094	0.14	0.21	0.14	0.16	0.023
21	0.39	0.29	0.46	0.38	0.049	0.09	0.4	0.14	0.21	0.096	0.16	0.22	0.13	0.17	0.026
22	0.32	0.29	0.46	0.36	0.052	0.02	0.47	0.15	0.21	0.13	0.11	0.23	0.14	0.16	0.036
23	0.2	0.30	0.48	0.33	0.082	0.01	0.42	0.1	0.18	0.12	0.14	0.18	0.15	0.16	0.012
24	0.18	0.30	0.45	0.31	0.078	0	0.44	0.11	0.18	0.13	0.14	0.19	0.17	0.17	0.015

Key:

a - First reading, b – Second reading, c – Third reading, SE - Standard Error, 1KS - Phage 1KS infected culture of S1, 1KSC – Phage and Chloramphenicol infected and treated culture of S1, 1KSCip – Phage and Ciprofloxacin infected and treated culture of S1.

APPENDIX VId: Absorbance readings of Phage 1KW and its combination with the two antibiotics (1KWC and 1KWCip) on *Salmonella* species (S1) at 600nm

	1KW					1KWC					1KWCip				
Time (hr)	**a**	**b**	**c**	**Mean**	**SE**	**a**	**b**	**c**	**Mean**	**SE**	**a**	**b**	**c**	**Mean**	**SE**
0	0.25	0.20	0.24	0.23	0.012	0.25	0.22	0.25	0.24	0.01	0.22	0.26	0.25	0.24	0.012
1	0.38	0.43	0.46	0.42	0.11	0.35	0.24	0.25	0.28	0.035	0.2	0.34	0.31	0.28	0.043
2	0.31	0.50	0.46	0.42	0.077	0.31	0.17	0.21	0.23	0.042	0.12	0.38	0.42	0.31	0.094
3	0.31	0.51	0.02	0.28	0.083	0.3	0.08	0.1	0.16	0.07	0.09	0.45	0.42	0.32	0.12
4	0.28	0.43	0.11	0.27	0.084	0.38	0.12	0.03	0.18	0.1	0.07	0.48	0.1	0.22	0.13
5	0.23	0.44	0.03	0.23	0.067	0.37	0.07	0	0.15	0.11	0.07	0.47	0.33	0.29	0.12
6	0.14	0.44	0.08	0.22	0.03	0.19	0.1	0.04	0.11	0.044	0.09	0.48	0.41	0.33	0.12
7	0.13	0.48	0.10	0.24	0.037	0.13	0.11	0.01	0.083	0.037	0.08	0.48	0.36	0.31	0.12
8	0.16	0.44	0.10	0.23	0.04	0.12	0.08	0.04	0.08	0.023	0.07	0.44	0.4	0.3	0.12
9	0.12	0.47	0.11	0.23	0.012	0.1	0.04	0.03	0.057	0.022	0.09	0.38	0.5	0.32	0.12
10	0.12	0.36	0.13	0.20	0.064	0.08	0.1	0.05	0.077	0.015	0.07	0.37	0.33	0.26	0.094
11	0.14	0.39	0.14	0.22	0.058	0.08	0.09	0.06	0.077	0.0088	0.07	0.39	0.4	0.29	0.11
12	0.12	0.37	0.30	0.26	0.055	0.1	0.13	0.09	0.11	0.012	0.1	0.4	0.31	0.27	0.089
13	0.13	0.33	0.35	0.27	0.053	0.1	0.16	0.1	0.12	0.02	0.06	0.37	0.25	0.23	0.09
14	0.14	0.38	0.41	0.31	0.024	0.11	0.18	0.11	0.13	0.023	0.08	0.36	0.26	0.23	0.082
15	0.14	0.38	0.36	0.29	0.022	0.12	0.16	0.11	0.13	0.015	0.09	0.31	0.25	0.22	0.066
16	0.16	0.38	0.40	0.31	0.027	0.1	0.22	0.1	0.14	0.04	0.07	0.31	0.23	0.2	0.071
17	0.15	0.38	0.51	0.35	0.032	0.11	0.25	0.08	0.15	0.052	0.08	0.36	0.2	0.21	0.081
18	0.14	0.39	0.42	0.32	0.023	0.1	0.33	0.04	0.16	0.088	0.02	0.4	0.2	0.21	0.11
19	0.17	0.40	0.54	0.37	0.037	0.11	0.31	0.06	0.16	0.076	0.06	0.39	0.22	0.22	0.095
20	0.1	0.40	0.55	0.35	0.012	0.06	0.32	0.07	0.15	0.085	0.1	0.35	0.23	0.23	0.072
21	0.14	0.38	0.53	0.35	0.029	0.06	0.36	0.08	0.17	0.097	0.12	0.35	0.22	0.23	0.067
22	0.07	0.40	0.57	0.35	0.019	0.05	0.39	0.08	0.17	0.11	0.06	0.37	0.19	0.21	0.09
23	0.07	0.43	0.57	0.36	0.0058	0	0.38	0.09	0.16	0.11	0.07	0.37	0.1	0.18	0.095
24	0.09	0.43	0.59	0.37	0.012	0	0.34	0.08	0.14	0.1	0.05	0.36	0.13	0.18	0.093

Key:

a - First reading, b – Second reading, c – Third reading, SE - Standard Error, 1KS - Phage 1KW infected culture of S1, 1KWC – Phage and Chloramphenicol infected and treated culture of S1, 1KWCip – Phage and Ciprofloxacin infected and treated culture of S1

APPENDIX VIe: Absorbance readings of Phage 1R and its combination with the two antibiotics (1RC and 1RCip) on *Salmonella* species (S1) at 600nm

	1R					1RC					1RCip				
Time (hr)	a	b	c	Mean	SE	a	b	c	Mean	SE	a	b	c	Mean	SE
0	0.23	0.27	0.24	0.25	0.012	0.23	0.22	0.23	0.23	0.0033	0.23	0.22	0.23	0.23	0.0033
1	0.36	0.45	0.18	0.33	0.079	0.45	0.43	0.33	0.4	0.037	0.28	0.31	0.3	0.3	0.0088
2	0.35	0.52	0.14	0.34	0.11	0.49	0.61	0.14	0.41	0.14	0.26	0.41	0.4	0.36	0.048
3	0.35	0.47	0.12	0.31	0.1	0.38	0.46	0.13	0.32	0.099	0.22	0.45	0.39	0.35	0.069
4	0.39	0.42	0.05	0.29	0.12	0.31	0.47	0.02	0.27	0.13	0.25	0.5	0.33	0.36	0.074
5	0.4	0.43	0.03	0.29	0.13	0.38	0.46	0	0.28	0.14	0.23	0.51	0.28	0.34	0.086
6	0.33	0.43	0.04	0.27	0.12	0.26	0.36	0.03	0.22	0.098	0.23	0.5	0.32	0.35	0.079
7	0.28	0.43	0	0.24	0.13	0.29	0.39	0.01	0.23	0.11	0.24	0.49	0.31	0.35	0.074
8	0.26	0.46	0.04	0.25	0.12	0.25	0.38	0.03	0.22	0.1	0.23	0.51	0.29	0.34	0.085
9	0.21	0.47	0.04	0.24	0.13	0.2	0.33	0.09	0.21	0.069	0.2	0.44	0.3	0.31	0.07
10	0.17	0.41	0.08	0.22	0.098	0.21	0.34	0.04	0.2	0.087	0.18	0.47	0.29	0.31	0.085
11	0.16	0.5	0.06	0.24	0.13	0.18	0.34	0.06	0.19	0.081	0.16	0.44	0.28	0.29	0.081
12	0.16	0.41	0.1	0.22	0.095	0.2	0.36	0.06	0.21	0.087	0.19	0.47	0.29	0.32	0.082
13	0.17	0.41	0.14	0.24	0.085	0.21	0.35	0.05	0.2	0.087	0.15	0.46	0.27	0.29	0.09
14	0.15	0.46	0.19	0.27	0.097	0.18	0.37	0.07	0.21	0.088	0.16	0.45	0.28	0.3	0.084
15	0.14	0.45	0.2	0.26	0.095	0.17	0.28	0.09	0.18	0.055	0.13	0.38	0.25	0.25	0.072
16	0.17	0.46	0.17	0.27	0.097	0.19	0.32	0.1	0.2	0.064	0.11	0.39	0.24	0.25	0.081
17	0.16	0.48	0.17	0.27	0.11	0.18	0.35	0.08	0.2	0.079	0.12	0.43	0.22	0.26	0.091
18	0.2	0.47	0.14	0.27	0.1	0.17	0.36	0.08	0.2	0.083	0.11	0.44	0.2	0.25	0.098
19	0.18	0.47	0.1	0.25	0.11	0.18	0.37	0.06	0.2	0.09	0.12	0.46	0.21	0.26	0.1
20	0.12	0.45	0.08	0.22	0.12	0.14	0.36	0.06	0.19	0.09	0.18	0.41	0.23	0.27	0.07
21	0.15	0.39	0.05	0.2	0.1	0.13	0.33	0.02	0.16	0.091	0.16	0.41	0.21	0.26	0.076
22	0.12	0.35	0.05	0.17	0.091	0.05	0.4	0.05	0.17	0.12	0.11	0.44	0.21	0.25	0.098
23	0.11	0.41	0.06	0.19	0.11	0.04	0.42	0.06	0.17	0.12	0.12	0.41	0.22	0.25	0.085
24	0.1	0.41	0.07	0.19	0.11	0.04	0.43	0.04	0.17	0.13	0.1	0.43	0.18	0.24	0.099

Key:

a - First reading, b – Second reading, c – Third reading, SE - Standard Error, 1R - Phage 1R infected culture of S1, 1RC – Phage and Chloramphenicol infected and treated culture of S1, 1RCip – Phage and Ciprofloxacin infected and treated culture of S1.

APPENDIX VIf: Absorbance readings of Chloramphenicol and Ciprofloxacin on *Salmonella* Pullorum (S2) and the positive control at 600nm

	S2					2C					2Cip				
Time (hr)	**a**	**b**	**c**	**Mean**	**SE**	**a**	**b**	**c**	**Mean**	**SE**	**a**	**b**	**c**	**Mean**	**SE**
0	0.21	0.21	0.28	0.2333	0.02333	0.24	0.25	0.25	0.2467	0.003333	0.22	0.24	0.25	0.2367	0.008819
1	0.68	0.4	0.51	0.53	0.08145	0.35	0.55	0.57	0.49	0.07024	0.28	0.31	0.31	0.3	0.01
2	0.72	0.68	0.77	0.7233	0.02603	0.42	0.54	0.42	0.46	0.04	0.3	0.36	0.36	0.34	0.02
3	0.64	0.61	0.76	0.67	0.04583	0.32	0.17	0.19	0.2267	0.04702	0.28	0.45	0.46	0.3967	0.0584
4	0.63	0.59	0.77	0.6633	0.05457	0.31	0.04	0.06	0.1367	0.08686	0.29	0.51	0.46	0.42	0.06658
5	0.6	0.6	0.74	0.6467	0.04667	0.3	0.01	0.04	0.1167	0.09207	0.29	0.47	0.48	0.4133	0.06173
6	0.68	0.59	0.75	0.6733	0.04631	0.28	0.05	0.06	0.13	0.07506	0.3	0.43	0.57	0.4333	0.07796
7	0.61	0.71	0.73	0.6833	0.03712	0.28	0.05	0.04	0.1233	0.07839	0.26	0.44	0.56	0.42	0.08718
8	0.6	0.67	0.77	0.68	0.04933	0.27	0.1	0.11	0.16	0.05508	0.25	0.41	0.61	0.4233	0.1041
9	0.65	0.72	0.7	0.69	0.02082	0.3	0.11	0.2	0.2033	0.05487	0.3	0.34	0.55	0.3967	0.07753
10	0.65	0.58	0.72	0.65	0.04041	0.28	0.14	0.15	0.19	0.04509	0.21	0.38	0.56	0.3833	0.1011
11	0.65	0.65	0.71	0.67	0.02	0.32	0.17	0.12	0.2033	0.06009	0.22	0.34	0.6	0.3867	0.1122
12	0.63	0.55	0.73	0.6367	0.05207	0.32	0.17	0.19	0.2267	0.04702	0.23	0.36	0.59	0.3933	0.1053
13	0.64	0.53	0.76	0.6433	0.06642	0.26	0.14	0.15	0.1833	0.03844	0.19	0.38	0.55	0.3733	0.104
14	0.68	0.56	0.71	0.65	0.04583	0.26	0.13	0.13	0.1733	0.04333	0.19	0.34	0.52	0.35	0.09539
15	0.68	0.58	0.62	0.6267	0.02906	0.29	0.13	0.15	0.19	0.05033	0.14	0.28	0.51	0.31	0.1079
16	0.69	0.53	0.61	0.61	0.04619	0.27	0.16	0.14	0.19	0.04041	0.12	0.33	0.48	0.31	0.1044
17	0.67	0.57	0.61	0.6167	0.02906	0.26	0.14	0.15	0.1833	0.03844	0.11	0.36	0.4	0.29	0.09074
18	0.68	0.59	0.56	0.61	0.03606	0.24	0.16	0.15	0.1833	0.02848	0.1	0.36	0.37	0.2767	0.08838
19	0.72	0.58	0.56	0.62	0.05033	0.31	0.14	0.16	0.2033	0.05364	0.17	0.34	0.35	0.2867	0.0584
20	0.73	0.62	0.52	0.6233	0.06064	0.3	0.2	0.15	0.2167	0.0441	0.17	0.33	0.35	0.2833	0.05696
21	0.67	0.53	0.5	0.5667	0.05239	0.29	0.21	0.14	0.2133	0.04333	0.18	0.33	0.34	0.2833	0.05175
22	0.64	0.56	0.65	0.6167	0.02848	0.27	0.15	0.18	0.2	0.03606	0.12	0.37	0.36	0.2833	0.08172
23	0.64	0.57	0.63	0.6133	0.02186	0.28	0.15	0.15	0.1933	0.04333	0.13	0.37	0.32	0.2733	0.07311
24	0.62	0.57	0.67	0.62	0.02887	0.28	0.18	0.17	0.21	0.03512	0.11	0.34	0.28	0.2433	0.06888

Key:

a - First reading, b – Second reading, c – Third reading, SE - Standard Error, S2 - *Salmonella* Pullorum alone (control), 2C - Chloramphenicol treated culture of S2, 2Cip – Ciprofloxacin treated culture of S2.

APPENDIX VIg: Absorbance readings of Phage 2DW and its combination with the two antibiotics (2DWC and 2DWCip) on *Salmonella* Pullorum (S2) at 600nm

	2DW					2DWC					2DWCip				
Time (hr)	a	b	c	Mean	SE	a	b	c	Mean	SE	a	b	c	Mean	SE
0	0.25	0.29	0.21	0.25	0.02309	0.24	0.24	0.24	0.24	0	0.2	0.27	0.24	0.2367	0.02028
1	0.45	0.63	0.38	0.4867	0.07446	0.66	0.16	0.15	0.3233	0.1684	0.42	0.55	0.15	0.3733	0.1178
2	0.49	0.52	0.63	0.5467	0.04256	0.68	0.12	0.11	0.3033	0.1884	0.47	0.61	0.17	0.4167	0.1298
3	0.52	0.47	0.53	0.5067	0.01856	0.62	0.11	0.11	0.28	0.17	0.43	0.61	0.15	0.3967	0.1338
4	0.39	0.4	0.49	0.4267	0.0318	0.61	0.02	0.03	0.22	0.195	0.39	0.57	0.05	0.3367	0.1525
5	0.39	0.4	0.5	0.43	0.03512	0.6	0.01	0	0.2033	0.1984	0.41	0.56	0.01	0.3267	0.1641
6	0.4	0.36	0.48	0.4133	0.03528	0.62	0.03	0.04	0.23	0.195	0.4	0.58	0.1	0.36	0.14
7	0.29	0.4	0.52	0.4033	0.06642	0.61	0	0.01	0.2067	0.2017	0.42	0.57	0.03	0.34	0.1609
8	0.32	0.38	0.52	0.4067	0.05925	0.62	0.04	0.02	0.2267	0.1968	0.39	0.53	0.07	0.33	0.1361
9	0.29	0.37	0.5	0.3867	0.06119	0.66	0.03	0.04	0.2433	0.2084	0.32	0.52	0.07	0.3033	0.1302
10	0.23	0.33	0.44	0.3333	0.06064	0.64	0.05	0.05	0.2467	0.1967	0.4	0.52	0.08	0.3333	0.1313
11	0.36	0.4	0.5	0.42	0.04163	0.54	0.04	0.04	0.2067	0.1667	0.37	0.55	0.05	0.3233	0.1462
12	0.35	0.32	0.43	0.3667	0.03283	0.54	0.07	0.06	0.2233	0.1584	0.36	0.53	0.08	0.3233	0.1312
13	0.3	0.33	0.36	0.33	0.01732	0.54	0.06	0.07	0.2233	0.1584	0.36	0.51	0.04	0.3033	0.1386
14	0.28	0.33	0.35	0.32	0.02082	0.58	0.05	0.09	0.24	0.1704	0.34	0.52	0.05	0.3033	0.1369
15	0.33	0.32	0.26	0.3033	0.02186	0.64	0.06	0.1	0.2667	0.187	0.39	0.48	0.06	0.31	0.1277
16	0.31	0.32	0.26	0.2967	0.01856	0.6	0.11	0.13	0.28	0.1601	0.37	0.55	0.11	0.3433	0.1277
17	0.34	0.32	0.19	0.2833	0.04702	0.67	0.1	0.11	0.2933	0.1884	0.4	0.57	0.1	0.3567	0.1374
18	0.32	0.32	0.21	0.2833	0.03667	0.64	0.12	0.07	0.2767	0.1822	0.39	0.63	0.08	0.3667	0.1592
19	0.3	0.31	0.27	0.2933	0.01202	0.58	0.1	0.07	0.25	0.1652	0.34	0.57	0.05	0.32	0.1504
20	0.31	0.39	0.29	0.33	0.03055	0.55	0.09	0.06	0.2333	0.1586	0.39	0.58	0.04	0.3367	0.1581
21	0.34	0.33	0.31	0.3267	0.008819	0.34	0.05	0.09	0.16	0.09074	0.41	0.56	0.02	0.33	0.1609
22	0.33	0.33	0.34	0.3333	0.003333	0.3	0.06	0.06	0.14	0.08	0.39	0.59	0.07	0.35	0.1514
23	0.31	0.36	0.4	0.3567	0.02603	0.24	0.09	0.07	0.1333	0.05364	0.33	0.58	0.08	0.33	0.1443
24	0.3	0.39	0.39	0.36	0.03	0.21	0.08	0.09	0.1267	0.04177	0.34	0.59	0.03	0.32	0.162

Key:

a - First reading, b – Second reading, c – Third reading, SE - Standard Error, 2DW - Phage 2DW infected culture of S2, 2DWC – Phage and Chloramphenicol infected and treated culture of S2, 2DWCip – Phage and Ciprofloxacin infected and treated culture of S2.

APPENDIX VIh: Absorbance readings of Phage 2KS and its combination with the two antibiotics (2KSC and 2KSCip) on *Salmonella* Pullorum (S2) at 600nm

	2KS					2KSC					2KSCip				
Time (hr)	**a**	**b**	**c**	**Mean**	**SE**	**a**	**b**	**c**	**Mean**	**SE**	**a**	**b**	**c**	**Mean**	**SE**
0	0.22	0.21	0.25	0.2267	0.01202	0.27	0.21	0.25	0.2433	0.01764	0.21	0.25	0.25	0.2367	0.01333
1	0.05	0.38	0.31	0.2467	0.1004	0.31	0.45	0.17	0.31	0.08083	0.06	0.18	0.18	0.14	0.04
2	0.08	0.63	0.14	0.2833	0.1742	0.35	0.56	0.13	0.3467	0.1241	0.14	0.21	0.21	0.1867	0.02333
3	0.07	0.56	0.13	0.2533	0.1543	0.36	0.47	0.13	0.32	0.1002	0.05	0.19	0.1	0.1133	0.04096
4	0.05	0.55	0.05	0.2167	0.1667	0.41	0.48	0.04	0.31	0.1365	0.03	0.11	0.12	0.08667	0.02848
5	0.04	0.55	0	0.1967	0.177	0.34	0.48	0.01	0.2767	0.1393	0.03	0.08	0.07	0.06	0.01528
6	0.04	0.53	0.04	0.2033	0.1633	0.35	0.43	0.05	0.2767	0.1157	0.05	0.1	0.11	0.08667	0.01856
7	0.03	0.57	0.01	0.2033	0.1834	0.32	0.43	0	0.25	0.129	0.02	0.07	0.06	0.05	0.01528
8	0.09	0.55	0.04	0.2267	0.1623	0.33	0.38	0.03	0.2467	0.1093	0.04	0.1	0.09	0.07667	0.01856
9	0.08	0.59	0.06	0.2433	0.1734	0.36	0.33	0.04	0.2433	0.102	0.06	0.1	0.11	0.09	0.01528
10	0.09	0.52	0.04	0.2167	0.1524	0.38	0.35	0.05	0.26	0.1054	0.05	0.09	0.08	0.07333	0.01202
11	0.12	0.59	0.05	0.2533	0.1695	0.36	0.35	0.04	0.25	0.105	0.05	0.09	0.1	0.08	0.01528
12	0.2	0.51	0.07	0.26	0.1305	0.38	0.35	0.06	0.2633	0.102	0.07	0.11	0.13	0.1033	0.01764
13	0.35	0.48	0.05	0.2933	0.1273	0.33	0.37	0.06	0.2533	0.09735	0.05	0.1	0.13	0.09333	0.02333
14	0.48	0.53	0.06	0.3567	0.149	0.35	0.34	0.06	0.25	0.09504	0.05	0.09	0.13	0.09	0.02309
15	0.42	0.5	0.06	0.3267	0.1353	0.33	0.28	0.07	0.2267	0.07965	0.07	0.1	0.11	0.09333	0.01202
16	0.38	0.52	0.08	0.3267	0.1298	0.32	0.29	0.02	0.21	0.09539	0.05	0.11	0.12	0.09333	0.02186
17	0.36	0.53	0.07	0.32	0.1343	0.31	0.28	0.08	0.2233	0.07219	0.05	0.09	0.09	0.07667	0.01333
18	0.38	0.52	0.03	0.31	0.1457	0.3	0.33	0.04	0.2233	0.09207	0.03	0.09	0.08	0.06667	0.01856
19	0.4	0.53	0.03	0.32	0.1498	0.34	0.28	0.08	0.2333	0.0786	0.06	0.08	0.08	0.07333	0.006667
20	0.47	0.52	0.03	0.34	0.1557	0.36	0.28	0.08	0.24	0.08327	0.09	0.07	0.08	0.08	0.005774
21	0.48	0.47	0.04	0.33	0.145	0.35	0.32	0.07	0.2467	0.08876	0.07	0.04	0.04	0.05	0.01
22	0.44	0.42	0.04	0.3	0.1301	0.33	0.36	0.02	0.2367	0.1087	0.08	0.07	0.08	0.07667	0.003333
23	0.43	0.49	0.07	0.33	0.1311	0.33	0.34	0.06	0.2433	0.09171	0.07	0.1	0.1	0.09	0.01
24	0.46	0.44	0.09	0.33	0.1201	0.35	0.34	0.05	0.2467	0.09838	0.06	0.17	0.06	0.09667	0.03667

Key:

a - First reading, b – Second reading, c – Third reading, SE - Standard Error, 2KS - Phage 2KS infected culture of S2, 2KSC – Phage and Chloramphenicol infected and treated culture of S2, 2KSCip – Phage and Ciprofloxacin infected and treated culture of S2

APPENDIX VIi: Absorbance readings of Phage 2KW and its combination with the two antibiotics (2KWC and 2KWCip) on *Salmonella* Pullorum (S2) at 600nm

	2KW					2KWC					2KWCip				
Time (hr)	a	b	c	Mean	SE	a	b	c	Mean	SE	a	b	c	Mean	SE
0	0.29	0.27	0.27	0.2767	0.006667	0.24	0.24	0.24	0.24	0	0.22	0.28	0.24	0.2467	0.01764
1	0.53	0.25	0.58	0.4533	0.1027	0.35	0.27	0.24	0.2867	0.03283	0.53	0.4	0.3	0.41	0.06658
2	0.6	0.24	0.55	0.4633	0.1126	0.36	0.13	0.11	0.2	0.08021	0.51	0.42	0.19	0.3733	0.09528
3	0.57	0.23	0.61	0.47	0.1206	0.31	0.14	0.11	0.1867	0.06227	0.42	0.54	0.16	0.3733	0.1122
4	0.5	0.23	0.47	0.4	0.08544	0.34	0.03	0	0.1233	0.1087	0.42	0.56	0.1	0.36	0.1361
5	0.48	0.23	0.45	0.3867	0.07881	0.27	0	0.01	0.09333	0.08838	0.37	0.54	0.06	0.3233	0.1405
6	0.51	0.2	0.43	0.38	0.09292	0.32	0.04	0.03	0.13	0.09504	0.38	0.52	0.1	0.3333	0.1235
7	0.48	0.24	0.44	0.3867	0.07424	0.28	0.01	0	0.09667	0.09171	0.38	0.47	0.07	0.3067	0.1212
8	0.54	0.21	0.41	0.3867	0.09597	0.32	0.05	0.04	0.1367	0.09171	0.36	0.46	0.08	0.3	0.1137
9	0.57	0.25	0.42	0.4133	0.09244	0.3	0.06	0.04	0.1333	0.08353	0.38	0.36	0.08	0.2733	0.09684
10	0.48	0.22	0.38	0.36	0.07572	0.28	0.07	0.05	0.1333	0.07356	0.33	0.39	0.08	0.2667	0.09493
11	0.46	0.27	0.37	0.3667	0.05487	0.29	0.05	0.05	0.13	0.08	0.39	0.33	0.07	0.2633	0.09821
12	0.56	0.22	0.34	0.3733	0.09955	0.3	0.08	0.06	0.1467	0.07688	0.41	0.4	0.09	0.3	0.105
13	0.59	0.2	0.31	0.3667	0.1161	0.26	0.08	0.04	0.1267	0.06766	0.33	0.41	0.07	0.27	0.1026
14	0.53	0.26	0.37	0.3867	0.07839	0.26	0.05	0.03	0.1133	0.07356	0.31	0.4	0.06	0.2567	0.1017
15	0.53	0.26	0.33	0.3733	0.0809	0.28	0.07	0.06	0.1367	0.07172	0.36	0.34	0.06	0.2533	0.09684
16	0.59	0.26	0.34	0.3967	0.09939	0.25	0.09	0.11	0.15	0.05033	0.3	0.36	0.09	0.25	0.08185
17	0.61	0.27	0.35	0.41	0.1026	0.26	0.09	0.09	0.1467	0.05667	0.31	0.35	0.09	0.25	0.08083
18	0.59	0.32	0.36	0.4233	0.08413	0.24	0.1	0.12	0.1533	0.04372	0.27	0.4	0.1	0.2567	0.08686
19	0.58	0.34	0.32	0.4133	0.08353	0.28	0.1	0.11	0.1633	0.0584	0.31	0.37	0.07	0.25	0.09165
20	0.56	0.36	0.35	0.4233	0.06839	0.28	0.11	0.09	0.16	0.06028	0.32	0.35	0.08	0.25	0.08544
21	0.6	0.34	0.28	0.4067	0.09821	0.27	0.09	0.08	0.1467	0.06173	0.35	0.38	0.06	0.2633	0.102
22	0.61	0.39	0.28	0.4267	0.09701	0.25	0.08	0.09	0.14	0.05508	0.3	0.39	0.09	0.26	0.08888
23	0.59	0.45	0.34	0.46	0.07234	0.24	0.1	0.09	0.1433	0.04842	0.31	0.37	0.07	0.25	0.09165
24	0.58	0.45	0.37	0.4667	0.06119	0.25	0.09	0.11	0.15	0.05033	0.32	0.39	0.09	0.2667	0.09062

Key:

a - First reading, b – Second reading, c – Third reading, SE - Standard Error, 2KW - Phage 2KW infected culture of S2, 2KWC – Phage and Chloramphenicol infected and treated culture of S2, 2KWCip – Phage and Ciprofloxacin infected and treated culture of S2.

APPENDIX VIj: Absorbance readings of Phage 2R and its combination with the two antibiotics (2RC and 2RCip) on *Salmonella* Pullorum (S2) at 600nm

	2R					2RC					2RCip				
Time (hr)	a	b	c	Mean	SE	a	b	c	Mean	SE	a	b	c	Mean	SE
0	0.21	0.28	0.25	0.2467	0.02028	0.24	0.26	0.22	0.24	0.01155	0.22	0.22	0.22	0.22	0
1	0.19	0.21	0.33	0.2433	0.04372	0.2	0.4	0.36	0.32	0.0611	0.34	0.38	0.35	0.3567	0.01202
2	0.17	0.23	0.12	0.1733	0.0318	0.14	0.67	0.27	0.36	0.1595	0.33	0.4	0.4	0.3767	0.02333
3	0.14	0.25	0.13	0.1733	0.03844	0.19	0.61	0.08	0.2933	0.1615	0.32	0.25	0.21	0.26	0.03215
4	0.12	0.24	0.01	0.1233	0.06642	0.15	0.62	0.09	0.2867	0.1676	0.3	0.22	0.17	0.23	0.03786
5	0.09	0.26	0	0.1167	0.07623	0.16	0.61	0.11	0.2933	0.159	0.3	0.17	0.21	0.2267	0.03844
6	0.15	0.23	0.05	0.1433	0.05207	0.13	0.56	0.08	0.2567	0.1524	0.28	0.15	0.18	0.2033	0.0393
7	0.12	0.24	0.02	0.1267	0.0636	0.18	0.59	0.05	0.2733	0.1627	0.23	0.12	0.23	0.1933	0.03667
8	0.1	0.24	0.03	0.1233	0.06173	0.25	0.58	0.1	0.31	0.1418	0.24	0.15	0.13	0.1733	0.03383
9	0.2	0.28	0.05	0.1767	0.06741	0.33	0.59	0.25	0.39	0.1026	0.23	0.19	0.18	0.2	0.01528
10	0.36	0.2	0.05	0.2033	0.0895	0.46	0.59	0.15	0.4	0.1305	0.24	0.16	0.18	0.1933	0.02404
11	0.27	0.25	0.07	0.1967	0.0636	0.46	0.56	0.25	0.4233	0.09135	0.22	0.2	0.16	0.1933	0.01764
12	0.25	0.2	0.07	0.1733	0.05364	0.5	0.61	0.37	0.4933	0.06936	0.26	0.17	0.17	0.2	0.03
13	0.33	0.2	0.08	0.2033	0.07219	0.45	0.65	0.47	0.5233	0.0636	0.19	0.16	0.16	0.17	0.01
14	0.31	0.23	0.07	0.2033	0.07055	0.41	0.66	0.55	0.54	0.07234	0.17	0.1	0.11	0.1267	0.02186
15	0.14	0.25	0.08	0.1567	0.04978	0.53	0.64	0.62	0.5967	0.03383	0.19	0.17	0.17	0.1767	0.006667
16	0.1	0.27	0.09	0.1533	0.0584	0.54	0.68	0.62	0.6133	0.04055	0.17	0.14	0.14	0.15	0.01
17	0.14	0.31	0.08	0.1767	0.06888	0.52	0.71	0.62	0.6167	0.05487	0.16	0.15	0.14	0.15	0.005774
18	0.14	0.32	0.05	0.17	0.07937	0.54	0.74	0.64	0.64	0.05774	0.14	0.21	0.16	0.17	0.02082
19	0.13	0.32	0.06	0.17	0.07767	0.58	0.72	0.66	0.6533	0.04055	0.18	0.21	0.22	0.2033	0.01202
20	0.15	0.36	0.07	0.1933	0.08647	0.6	0.69	0.7	0.6633	0.0318	0.24	0.22	0.24	0.2333	0.006667
21	0.16	0.34	0.07	0.19	0.07937	0.62	0.71	0.68	0.67	0.02646	0.23	0.25	0.26	0.2467	0.008819
22	0.12	0.32	0.09	0.1767	0.07219	0.6	0.69	0.71	0.6667	0.03383	0.17	0.25	0.33	0.25	0.04619
23	0.17	0.34	0.1	0.2033	0.07126	0.67	0.69	0.7	0.6867	0.008819	0.21	0.33	0.33	0.29	0.04
24	0.14	0.37	0.1	0.2033	0.08413	0.71	0.64	0.69	0.68	0.02082	0.17	0.31	0.33	0.27	0.05033

Key:

a - First reading, b – Second reading, c – Third reading, SE - Standard Error, 2R - Phage 2R infected culture of S2, 2RC – Phage and Chloramphenicol infected and treated culture of S2, 2RCip – Phage and Ciprofloxacin infected and treated culture of S2