EVALUATION OF SAFETY AND THERAPEUTIC POTENTIAL OF ENVIRONMENTAL WASTE WATER LYTIC BACTERIOPHAGE AGAINST MULTIDRUG RESISTANT *Staphylococcus aureus* (MDRSA) IN BALB/c MICE

ODUOR JOSEPH MICHAEL OCHIENG’

REG. NO.: P150/20415/2012

A thesis submitted in partial fulfillment for the award of the Degree of Master of Science in Infectious Diseases (Virology) of Kenyatta University

April, 2016
DECLARATION

Candidate's declaration

This thesis is my original work and has never been presented for a degree award in any other University.

Oduor Joseph Michael Ochieng

Dept. of Medical Laboratory Science,

School of Medicine-Kenyatta University,

Signature: ........................ Date: 27/04/2016

This thesis has been submitted for examination with our approval as supervisors

1. Dr. Washington Ouma Arodi, PhD.

Dept. of Medical Laboratory Science,

School of Medicine-Kenyatta University,

Signature: ........................ Date: 27/04/2016

2. Dr. Atunga Nyachieo, PhD.

Institute of Primate Research (IPR),

WHO Collaborating Centre.

Signature: ........................ Date: 27/04/2016
DEDICATION

In honour of my parents (Mr. Oduor and Mrs. Akoth) and siblings: Paul Santana and Roseanne Vincesa for their support.

And for the greater glory of God!!!
ACKNOWLEDGEMENT

I wish to pass my heartfelt gratitude to my supervisors; Dr. Atunga Nyachieo (IPR) and Dr. Washington O. Arodi (KU). They gave me invaluable intellectual advice. Also, wish to pass my gratitude to the staff of IPR, UoN-Medical School, Aga Khan University Hospital and Nairobi Water & Sewage Treatment Company for the support accorded to me during this work.

I wish to acknowledge Mrs. Rose Kavurani, Mr. Kennedy Waititu, Ms Caroline Jerono, Mr. Eric, Mr. Deya Mr. Tom Adino and Mr. Jefwa provided lab technical assistant. Dr. Michael Ndungu (IPR-ASD) and Dr. Feredrick Maloba (Zoo-Dept., KU) assisted with the analysis of histological tissues. Dr. Pius Adoyo (IPR-RHB) donated lab materials for the experiments. Dr. Lucy Ochola (IPR-T.I.D) facilitated drafting of this work initial concept paper. Prof. Walter Jaoko, Prof. Oumu Anzala, Mr. Jonathan Oloo (UoN-Medical School), and Dr. George Omuse and Dr. Allan Njoroge (Aga Khan University Hospital) provided bacteria ATCC controls for the experiments.
# TABLE OF CONTENTS

DECLARATION ............................................................................................................................ ii  
DEDICATION ............................................................................................................................... iii  
ACKNOWLEDGEMENT ............................................................................................................... iv  
TABLE OF CONTENTS ................................................................................................................. v  
LIST OF TABLES ........................................................................................................................... viii  
LIST OF FIGURES ....................................................................................................................... ix  
ABBREVIATIONS AND ACRONYMS ........................................................................................... xii  
ABSTRACT ................................................................................................................................... xiii  

## CHAPTER ONE: INTRODUCTION ......................................................................................... 1  
1.1 Background of the study ........................................................................................................ 1  
1.2 Problem statement .................................................................................................................. 3  
1.3 Rationale .............................................................................................................................. 3  
1.4 Research questions ............................................................................................................... 4  
1.5 Hypothesis \(H_0\) ..................................................................................................................... 4  
1.6 Objectives ............................................................................................................................ 5  
1.6.1 General objective ............................................................................................................. 5  
1.7 Study output ......................................................................................................................... 6  

## CHAPTER TWO: LITERATURE REVIEW ........................................................................... 7  
2.1 Description of bacteriophage ............................................................................................... 7  
2.2 Life cycle of phages ................................................................................................................ 8  
2.3 Phage abundance and diversity .......................................................................................... 9
2.4 Application of phages .......................................................... 11
2.5 Phage therapy ........................................................................ 12
2.6 Emergence of MDRSA ............................................................ 14

CHAPTER THREE: MATERIALS AND METHODS ............................. 17

3.1 Study area .............................................................................. 17
3.2 Sampling ................................................................................ 17
3.2.1 Sampling design ................................................................. 17
3.2.2 Sample size determination .................................................. 18
3.2.3 Sample collection ............................................................... 18
3.3 Isolation of S.aureus bacteria, S.aureus lytic phage and in vitro
  study .......................................................................................... 19
3.3.1 Isolation of S.aureus bacteria ................................................. 19
3.3.2 Identification of S.aureus bacteria ......................................... 19
3.3.3 Antibiogram test .................................................................. 20
3.3.4 Preservation of isolated multidrug resistant S.aureus (MDRSA) .. 20
3.3.5 Isolation of S.aureus lytic phages .......................................... 20
3.3.6 In vitro screening for phage against MDRSA ......................... 21
3.4 In vivo Study ........................................................................... 22
3.4.1 Study animals ....................................................................... 22
3.4.2 Safety and efficacy study of phage therapy ............................. 22
3.5 Statistical analysis .................................................................... 26
CHAPTER FOUR: RESULTS ........................................................................................................27

4.1 Bacterial and phage isolation ................................................................................. 27

4.1.1 Identification of *S. aureus* bacterial isolated from waste and sewage water ............................................................................................................................... 27

4.1.2 Isolation and screening for phage isolates activity against MDRSA *in vitro* ......................................................................................................................... 32

4.2 *In vivo* Study ............................................................................................................. 33

4.2.1 Safety of phage therapy ......................................................................................... 33

4.2.2 Efficacy of phage therapy ..................................................................................... 36

CHAPTER FIVE: DISCUSSION, CONCLUSIONS, RECOMMENDATIONS AND SUGGESTION FOR FURTHER RESEARCH ............................................................. 62

5.1 Discussion .................................................................................................................. 62

5.2. Conclusions ............................................................................................................. 68

5.3 Recommendations .................................................................................................... 68

5.4 Suggestion for further research ............................................................................... 69

REFERENCES .................................................................................................................. 70

APPENDICES ................................................................................................................. 80

Appendix I ....................................................................................................................... 80

Appendix II ...................................................................................................................... 81

Appendix III .................................................................................................................... 82

Appendix IV .................................................................................................................... 83
LIST OF TABLES

Table 1: Physical appearance score .................................................................24

Table 2: Interpretation of zones of inhibition (clear patches) around the antibiotics
used in the antibiogram test .............................................................................31

Table 3: Cardiac blood bacteria count (mean log CFU/ml + SE) at day 10 post
infection ..............................................................................................................39

Table 4: End point (day 10) viable bacteria count (mean log10 CFU/g + SE) a
from organs. .......................................................................................................45

Table 5: End point phage count (mean log10 PFU/ml + SE) a from the liver .......47
LIST OF FIGURES

Figure 1: Life cycle of phages. ................................................................. 8

Figure 2: Experimental design for in vivo work. ........................................ 25

Figure 3: *Staphylococcus* species bacteria isolate colonies in (A) mannitol salt agar and (B) modified plate agar medium ......................................................... 27

Figure 4: Gram positive cocci (A), catalase positive (B) and coagulase positive (C) tests ................................................................................................................... 28

Figure 5: Positive API STAPH test for *Staphylococcus aureus* bacteria .......... 29

Figure 6: Negative API STAPH test for *Staphylococcus aureus* bacteria. ....... 29

Figure 7: Antibiogram test of *S.aureus* isolates was resistant to commonly used local antibiotics. ................................................................. 32

Figure 8: Spot assay showing the most virulent phage isolate patch .............. 33

Figure 9: Physical appearance graph of mice treated at 24 hrs post infections ...... 34

Figure 10: Physical appearance graph of mice treated at 72 hrs post infection. .... 35

Figure 11: Survival rates of mice treated at 24 hrs post infection (A) and 72 hrs post infection (B). ........................................................................................................... 36

Figure 12: A graph of 24 hrs p.i treatment bacteremia and viremia levels in treated mice. ................................................................................................................. 37

Figure 13: A bacteremia and viremia level graph of mice treated 72 hrs p.i. ...... 38

Figure 14: Brain homogenate pour culture plates. ........................................ 41

Figure 15: Lungs homogenate culture plates ............................................... 42

Figure 16: Liver homogenate pour plate cultures ........................................... 43
Figure 17: Kidney homogenate pour plate cultures.........................................................44
Figure 18: Liver homogenate plaque assay cultures. .......................................................46
Figure 19: Brain tissues from; (i) Non-infected group, (ii) Phage infected, non-treated group..................................................................................................................48
Figure 20: Brain tissues from MDRSA infected non-infected group.................................49
Figure 21: Brain tissues; i) MDRSA infected non-treated group. ii) MDRSA infected Antibiotic treated group. ........................................................................................................50
Figure 22: Brain tissues; i) MDRSA infected phage treated mouse. ii) MDRSA infected AntiPhag treated mouse........................................................................................................51
Figure 23: Lung tissues i) Non-infected mouse ii). Phage infected non-treated groups .................................................................................................................................52
Figure 24: Lung tissues; i) MDRSA infected non-treated mouse. ii) MDRSA infected antibiotic treated mouse ........................................................................................................53
Figure 25: Lung tissues; i) MDRSA infected mouse treated with phage. ii) MDRSA infected mouse treated with combination therapeutic agent.............54
Figure 26: Liver tissues from; i) Non-infected mouse. ii) Phage infected non-treated group.................................................................................................................................55
Figure 27: Liver tissues; i) MDRSA infected non-treated mouse. ii) MDRSA infected mouse treated with antibiotic.................................................................56
Figure 28: Liver tissues from; i) MDRSA infected mouse treated with phage. ii) MDRSA infected mouse treated with combination therapeutic agent.............57
Figure 29: Kidney tissues from; i) Non-infected mouse. ii) Phage infected non-treated mouse.................................................................58

Figure 30: Kidney tissues from; i) MDRSA infected, non-treated group and ii) MDRSA infected, antibiotic treated group.................................................59

Figure 31: Kidney tissues from; i) Kidney tissue from MDRSA infected mouse treated with phage. ii) Kidney tissue from MDRSA infected mouse treated with combination therapeutic agent. .................................................................60
ABBREVIATIONS AND ACRONYMS

ASD : Animal Science Department

CA-MRSA: Community acquired methicillin resistant *Staphylococcus aureus*

CFU : Colony forming unit

DNA : Deoxyribonucleic acid

DRSA : Daptomycin resistant *Staphylococcus aureus*

HA-MRSA: Hospital acquired methicillin resistant *Staphylococcus aureus*

ICTV : International Committee on Taxonomy of Viruses

LA-MRSA : Livestock acquired methicillin resistant *Staphylococcus aureus*

MDR : Multi-drug resistant

MDRSA : Multi-drug resistant *Staphylococcus aureus*

MRSA : Methicillin resistant *Staphylococcus aureus*

PFU : Plaque forming unit

RNA : Ribonucleic acid

RHB : Reproductive Health Biology

T.I.D : Tropical Infectious Disease

*S.aureus* : *Staphylococcus aureus*

UoN : University of Nairobi

U.S.A : United State of America

VRSA : Vancomycin resistant *Staphylococcus aureus*

WHO : World health organization

WPR : World Population Review
ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) pose a great threat to the global public health. Control of these bacteria has become difficult due to acquisition of resistance against even some of the best antibiotics. Thus, phage therapy could be the better alternative as they are easy to isolate and produce in mass within a short time. However, phage therapy has been a subject of debate over the years but recently there has been a renewed interest due to their proved therapeutic potential and have, therefore, found commercial application in some countries. Thus, this study was specifically designed to evaluate the efficacy of phages against MRSA both *in vitro* and *in vivo*. A litre of environmental waste water and sewage samples were collected around the county of Nairobi. The MRSA isolates were obtained from environmental waste water and sewage samples from Nairobi and its environs and evaluated for drug resistance using antibiogram test. In addition, lytic phages were isolated from these samples too. Thereafter, the *in vitro* efficacy of the phages against MRSA was done by spot assay and tube culture tests. Only the most virulent phage isolate was used for *in vivo* efficacy study which involved six groups of mice of n=5 per group (BALB/c mice; both sex). The first three groups acted as controls (group 1=only physiological saline, group 2=MRSA bacteria only and group 3=phage only) while the remaining groups were used for efficacy studies using a dosage of $10^8$ CFU/ml for MRSA bacteria, $10^8$ PFU/ml for phage and clindamycin at 8mg/kg. The efficacy study groups (groups 4-6) were first infected by MRSA and observed for 3 days before treatment with either antibiotic (group 4), or phage (group 5) or phage + antibiotic (cocktail therapy group 6). The mice were then observed for an additional 7 days. During the entire 10 days of observation blood samples were collected daily for bacteremia level determination before being euthanized. Different organs including the liver, brain, kidney and lungs were harvested for histopathological studies. All studies were done in accordance with the IPR Institutional Ethical Committee approved protocols. Antibiogram test indicated that MRSA isolated was a multidrug resistant strain (Figure 1). While the *in vitro* test showed the virulence of the phage isolates after 24 hrs culture at 37°C. Toxicity test showed that phages were safe. There was no significant difference in survival rates between phage infected group and non-infected control group ($p>0.05$). Bacteremia was significantly lower in phage treated group as compared to other treatment groups and bacteria non-treated group ($p<0.001$) (Figure 2). In addition, pathological results show that phage prevented organ damage by the bacteria (Figure 3). Thus, a single dose of phage was more effective than other therapeutic agents used in the study. Results of this study show that phage therapy is safe and its application should be considered for the treatment of multidrug resistant bacterial infections.
CHAPTER ONE: INTRODUCTION

1.1 Background of the study

Bacteriophages (phages) are obligate intracellular prokaryotic viruses that devour bacteria and are ubiquitous (Clokie et al., 2011). Lytic bacteriophages are species specific, as they only invade bacteria that contain compatible surface attachments (Deghorain et al., 2012).

In the quest of vaccine development and drug discovery, phage have been widely used in phage therapy, phage typing and bio-control (Chhibber et al., 2012; Haq et al., 2012). After the discovery of antibiotics, phage therapy which was widely practiced all over Europe and North America ceased (Summers, 2012). However, this was not the case in Georgia and Russia where phage therapy is still practiced (Reardon, 2014).

Several antibiotics have been produced for human medication and animal health care (Davies et al., 2010). However, this has not been easy due to a lot of challenges such as continuous bacterial antibiotic resistance and high cost of research and production. The first case of antibiotic resistance was recorded in 1955, when S. aureus was reported to be resistant to penicillin and later methicillin (a derivative of penicillin) (Chambers et al., 2009). Methicillin resistant S. aureus (MRSA) bacteria have also been documented to possess some resistance to other classes of antibiotics like quinolones and macrolides (Yıldız et al., 2014).
Surprisingly, this is the *S. aureus* strain that predominantly infects humans and livestock making it a great public health risk (Fitzgerald, 2012).

Due to high cost involved; only a few pharmaceutical companies are willing to invest in the research and development of new alternatives geared towards treatment of bacterial infections (Jagusztyn-Krynicka *et al.*, 2008). Therefore, the rate at which bacteria is developing resistance against the available antibiotics is higher than rate of production of new antibiotics (Castanon, 2007). This has made the need for alternative measure towards combating these bacterial stains necessary.

The present study sought to establish whether MDRSA isolate exist in the environment of the Nairobi County. The work further explored an alternative way of combating the ever emerging MDR bacterial isolates using phages as antibacterial agents. Phage therapy is not a new way of treatment (Keen, 2015), but in this study we chose to extract the lytic phages from sewage and waste water as an economical and cheap way of phage generation. The safety and *in vivo* antibacterial potential of environmentally derived phages against MDRSA has also been investigated. Bacteriophages were considered in this study because they can be obtained, manipulated, and produced easily in the laboratories found in developing countries.
1.2 Problem statement

Antibiotic resistance developing amongst bacterial isolates is an eminent public health risk toward human health and food security (Laxminarayan et al., 2006). The problem has resulted into hospital and community epidemics (Dukic et al., 2013; Stefani et al., 2012). These bacteria have caused economic losses that are as a result of purchasing antibiotic that are not efficient against most of the bacterial infections. In sub-Saharan Africa, antibiotic resistance is on the rise due to uncontrolled dispensation of antibiotics by public health service providers, veterinarians and drug stores. Humans and animals are largely infected with antibiotic resistant bacterial strains such as MDRSA due to their association and cross contamination as a result of trade, food production and animal rearing. Management of these bacterial infections is difficult since they are prevalent in the environment, food sources and water. This problem should be solved or else more MDRSA strains will be causing death in humans and livestock resulting into poor global economic status (Walsh, 2013). The W.H.O has called upon pharmaceutical companies to engage more on research, development and production of new antibiotics or alternative therapeutic agents like phages against the new and old emerging multidrug resistant bacteria.

1.3 Rationale

The present study sought to determine the safety, efficacy and therapeutic potential of phages against environmentally obtained MDRSA bacteria in mice. Lytic phages against \textit{Vibrio cholerae} have been isolated locally and their \textit{in vitro}
therapeutic potential against the *Vibrio cholerae* isolate studied (Maina et al., 2014). This findings show that lytic phages are locally available in our ecosystems. They can be used in research, development and production of new therapeutic agents. In addition, lytic phages that can be manipulated easily in laboratories with minimal equipment compared to manufacturing of new and broad acting antibiotics. Phages are effective in the control and treatment of bacterial infections caused by multidrug resistant bacteria. The study’s findings were to provide empirical protocols for isolating phages with therapeutic potential as the ones used in some parts of Eastern Europe.

**1.4 Research questions**

i. What is the antibiotic susceptibility of *S.aureus* bacteria in the environmental waste water and sewage of Nairobi County?

ii. Does the environmental waste water and sewage in Nairobi County harbor lytic phages against MDRSA bacteria?

iii. What is the *in vitro* susceptibility of *S.aureus* to lytic phages?

iv. What is the safety and efficacy level of lytic phages against MDRSA in mice?

**1.5 Hypothesis (H₀)**

- The environmentally obtained *S. aureus* bacteria are not multi-drug resistant
- Lytic phages are neither safe nor efficacious against MDRSA in mice.
1.6 Objectives

1.6.1 General objective

To evaluate the safety and efficacy of environmentally obtained lytic phages against MDRSA isolates in waste and sewage water within the Nairobi County.

1.6.2 Specific objectives

i. To determine the presence of MDRSA isolate from environmental waste water and sewage drainage systems of Nairobi County.

ii. To determine the existence of lytic phage against environmental MDRSA isolate from Nairobi County.

iii. To determine the *in vitro* antibacterial potential of lytic phages against environmental MDRSA isolate from Nairobi County.

iv. To evaluate the efficacy and safety of phage therapy against environmental MDRSA isolate from Nairobi County *in vivo* in BALB/c mice.
1.7 *Study output*

The study was geared towards generation of new information on the presence of MDRSA strains in the environment of Nairobi County. Thereafter, determine the safety and efficacy of the lytic phages against the MDRSA isolated within Nairobi County. This has been achieved by providing the baseline information on the antibacterial mechanism of lytic phage against MDRSA bacteria. The study has further shown that lytic phage is safe and has therapeutic potential against MDRSA *in vivo* using BALB/c mice.
CHAPTER TWO: LITERATURE REVIEW

2.1 Description of bacteriophage

Bacteriophages are also known as phages. The word “phage” comes from the Greek word “phagein” which means to eat or devour (Wilkinson, 2001). Thus, bacteriophages mean “bacteria eating viruses”. The microbes were christened by d’ Herelle as bacteriophage, after he observed the virus create clear patches on a bacterial lawn. In addition, phages are referred to as obligate intracellular parasites of prokaryotic microbes (Drulis-Kawa et al., 2012). Phages parasitize archeabacteria and eubacteria (Sime-Ngando, 2014).

Phages are classified based on their genomic make-up either as double or single stranded DNA or RNA (Ackermann, 2007; Rohwer et al., 2002). The international committee on taxonomy of viruses (ICTV) classifies phages depending on their genome, morphology, host specificity, habitat and life cycle. Thus all phages fall into order Caudoviridae which consists of Myoviridae, Podoviridae and Siphoviridae families (ICTV, 2014).

The most ubiquitous phages are the T₄ type which are polyhedral virus that are composed of icosahedral and helical structural components. Phages are host specific and they are named according to the bacterial species or genus they devour (Deghorain et al., 2012). Phages can also be isolated from harsh habitats like hot geysers, ocean beds, Polar Regions and volcanic vents. In addition,
animals’ guts, fecal matter and water samples from the rivers are other sources of phages (Dabrowska et al., 2005).

### 2.2 Life cycle of phages

Phages have different life cycles that are used to classify them. This includes; lytic, lysogenic, pseudo-lysogenic and chronic life cycle, as shown in the life cycle below (Drulis-Kawa et al., 2012; Weinbauer, 2004).

**Figure 1:** Life cycle of phages (Weinbauer, 2004).
Lytic phages are the most destructive types, as they burst the bacterial cell to complete their life cycle. The lysogenic phages establish mutual associations with their host bacteria to form prophages. These kind of phages integrates their genomes into host bacteria chromosomes or exist as plasmids in the bacteria (Canchaya et al., 2003). They in turn establish stable and long lasting mutual associations with their host bacteria. In this state the phage is known as a prophage. This association of the lysogenic phage with the host bacterium results in the alteration of bacterial phenotype and genetic expression (Das et al., 2011). The pseudo-lysogenic phages form unstable association with their host bacteria which ends when the habitat environment changes like exposure to ultra-violet rays or environmental temperature fluctuations (Chibani-Chennoufi et al., 2004; Lunde et al., 2005). The chronic infection life cycle is found in archaeal viruses where the filamentous phages and plasmaviruses like those that infect *Mycoplasma* spp (Clokie et al., 2011). The life cycle Mycoplasma bacteriophages involves slow continuous shedding of phage copies by budding from the host bacteria (*Mycoplasma* spp) for a long time without lysing the host (Weinbauer, 2004).

### 2.3 Phage abundance and diversity

Phage abundance within the biosphere varies biogeographically. There is more phage in the world ocean than on land. The ocean has about $3.5 \times 10^{29}$ phage copies per milliliter while a gram of soil sample on land has $10^8$ phage copies (Wilhelm et al., 1999). However, phage copies on land vary from place to place.
Where a gram of marine sediments has about $10^{10}$ phage copies as compared to one gram fresh water lake sediment which an estimate of $10^9$ phages (Danovaro et al., 2002). In addition, fresh water lakes have about $10^9$ phage particles per milliliter than sea water of the same volume which has $10^7$ phage particles (Breitbart, 2012). Phage are also present in marine snow (algal flocs), in which there about $10^{10}$ phage copies (Peduzzi et al., 1993). Furthermore, phages are abundant in sea ice; which has an estimate of $10^{6-8}$ phage particles (Weinbauer, 2004) and air about $10^5$ phage copies (Chibani-Chennoufi et al., 2004).

In animals and plants phage are found where they help to maintain bacteria floral balance. Amongst animals ruminant bovine have phage count of about $10^7$ per gram of their feces while human’s feces has $10^9$ phage copies per gram (Niu et al., 2009; Rohwer, 2003). Phages ensure that the floral bacterial balance is stable within the animal host (Reyes et al., 2013). A stable bacterial flora in an organism prevents the upsurge of pathogenic bacteria such as *Clostridium difficile* which might result to opportunistic infections (Focà et al., 2015). Opportunistic infections are often as a result of broad spectrum antibiotics that eradicate useful bacteria that colonize the gut (Buffie et al., 2012). Phages are also abundant in certain food eaten by humans and especially dairy products such as yogurt, cheese and raw milk. Cheese has been found to higher phage content of about $10^9$ per gram more than yogurt which has $10^3$ phage particles per milliliter. While raw milk has $10^4$ phage particles per ml (Madera et al., 2004).
2.4 Application of phages.
Phages are versatile micro-organisms that have been utilized in biomedical research, and food bio-processing, medical and veterinary medicine (Haq et al., 2012). In biomedical research, phage display is a technology based on direct linkage between phage phenotype and its encapsulated phenotype that results to presentation of molecules libraries on the phage surface. The technique is vital in processing polypeptides which are useful in drug design (Sidhu, 2000). Phages such as lambda phages, T7 and Escherichia coli (E.coli) filamentous phage (M13) (Mead et al., 1988) are used as vectors in conjunction with Escherichia coli plasmid vectors to create a cosmid vector (Lodish et al., 2000). The cosmid vectors carry large DNA fragments and have the ability to replicate into several copies. This property makes it useful in the discovery and development of vaccines (Benhar, 2001). In addition phages are used in environmental cleaning to remove deadly pathogenic bacteria such as Bacillus anthracis from contaminated places (Jepson & March, 2004; Jończyk-Matysiak et al., 2014).

In the medical and veterinary field, phages have been extensively used as diagnostic tool and therapeutic agents. In diagnosis application of bacterial infections, phage typing is as an efficient way of detecting Mycobacterium tuberculosis, E.coli, Pseudomonas, Salmonella, Listeria, and Campylobacter species microbial strains (Barry et al., 1996).
2.5 Phage therapy

Obligate lytic phages are efficient antibacterial agents due to their bactericidal ability to burst open their host bacterium resulting into cell death (Pouillot et al., 2010). In the available literature, phages have been used to treat skin, subcutaneous and systemic bacterial infections caused by *E.coli, Staphylococcus aureus, Shigella dysenteriae* and *Klebsiella pneumoniae* (Kutter et al., 2010). These has been achieved due to phages’ specificity, bactericidal activity, auto-dosing capability, ability to evolve against phage resistant bacterial strains, and environmental availability (Koskella et al., 2013). Phages specificity allows them to specifically destroy target bacterium unlike antibiotics that clear even non-target bacteria. This makes phages impetus agents to counter balance the microbiome in the host lumen (Abedon et al., 2010). Most antibiotic have bacteriostatic ability that makes them inefficient in achieving full therapeutic potential which is not the case with lytic phages used for treatment purpose (Vinodkumar et al., 2008). Therefore, phages can be used to eliminate multidrug resistant strains of bacteria which include; multidrug resistant *Staphylococcus aureus* strains such as methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Staphylococcus aureus* (VRSA), carbapenem resistant *E.coli, Pseudomonas aeruginosa, K. pneumoniae* and *Shig. dysenteriae* (Marza et al., 2006).

In phage therapy, a small titer of phage is enough to cure a bacterial infection because the phages are capable of auto-dosing. Phages replicate rapidly and get
disseminated throughout the entire body system (Łusiak-Szelachowska et al., 2014). They have the ability to evolve to overcome the emergence of new phage resistant bacterial strains (Marraffini, 2013). This makes it easy to handle bacterial strains that are phage resistant. In addition, phage therapy is considerably safe as phages forms part of the human microbiota and ecosystem (Gupta et al., 2011). Antibiotics have been associated with serious adverse side effects which includes; kidney failure, allergies, nausea and liver damage (Alqahtani et al., 2015; Kishor et al., 2015; McMullan & Mostaghim, 2015). None of these has been documented from patients on phage therapy. Lytic phages do not carry toxin genes and have minimal tendency to induce DNA transduction between bacterial strains (Guenther et al., 2012). These phages are highly specific and will only destroy target pathogenic bacteria but, spare useful normal bacterial flora (Ahmed, 2012). The specificity nature of phages inhibits the occurrence of secondary infections such candidiasis and Clostridium difficile colitis which are often associate with broad spectrum antibiotics (Sulakvelidze et al., 2001).

Phage therapy also, has got its own challenges which include need for thorough identification of the pathogenic bacteria before treatment. This is due to their specific nature, since a phage strain for another bacterial genus will not lyse bacteria from another genus. The property therefore makes phage therapy cumbersome to some extent.(Loc-Carrillo & Abedon, 2011). This issue however, has been solved through the development of cocktail phage preparation which
consists of more than one phage strain or genus. In some case there are also, phage – antibiotic cocktails which have proven to be effective against bacterial infections (Jikia et al., 2005). Not all phages are suitable for therapeutic purpose as some of them carry toxic genes that make the bacteria more pathogenic (Brabban et al., 2005).

Phage therapy has been in use in Eastern Europe even after the introduction of broad spectrum antibiotics. The emergence of multidrug resistant bacteria isolates within the hospital and community setups has further cemented the practice of phage therapy in the region (Keen, 2015). Initially multidrug resistant bacteria isolates such as MDRSA strains were thought to be acquired only from hospitals (González-Castillo et al., 2013). However, recently more cases on MDRSA have been reported from the community setups than hospitals (David & Daum, 2010). This is an indication that there is a community strain of MDRSA circulating in livestock as well as fomites, soil and water (Pantosti, 2012).

2.6 Emergence of MDRSA

Staphylococcal infections were preferentially treated with β-lactam antibiotics which were considered to be effective against most of the gram positive bacteria (Lowy, 2003). Before emergence of resistant bacterial strains, penicillin was once the drug of choice for treatment of gram positive bacterial infections. Thereafter, *S.aureus* evolved and begun to synthesise β-lactamase enzyme which hydrolyses penicillin making them resistant against penicillin (Buzaid et al., 2011). This
resistance has been acquired due to over prescription of penicillin by public health service providers. In combating the emerging resistance, β-lactamase inhibiting drugs such as methicillin were developed. However, some *S. aureus* isolates have been reported to possess some resistance to methicillin. The methicillin-resistant *S. aureus* (MRSA) had acquired methicillin resistance gene (*mecA*) (Parker & Jevons, 1964).

The *mecA* gene is thought to have been acquired from distantly related species of *Staphylococcus*, which is *Staphylococcus fleuretti*. *Staphylococcus fleuretti* is common in animals (Tsubakishita et al., 2010). The resultant MRSA strains forms part of the normal human and animal bacterial flora (David & Daum, 2010; Mole, 2013). Despite the *S. aureus* being resistant to methicillin, cases of resistance with other drugs has been documented creating isolates that are multidrug resistant (MDR) (McDougal et al., 2010). These multi-drug *S. aureus* (MDRSA) strains includes; methicillin resistant *S. aureus* (MRSA), vancomycin resistant *S. aureus* (VRSA) and daptomycin resistant *S. aureus* (Stefani et al., 2015).

The MDRSA strains have become a global problem in hospitals and communities (Chiarello *et al.*, 2012). Stefani *et al.*, (2012) have reported that the global prevalence of MDRSA varies from continent to content and country to country. MDRSA prevalence in the USA, Japan and Taiwan was above 50%, while in Scandinavia and southern Europe it was between 1% and 50%. In Africa MDRSA
prevalence ranged from 10% to 50%, while in East Africa and especially Kenya the MRSA prevalence had raised from 10% from 40% in 2009 (Kariuki et al., 2011). However, another epidemiological study done in Kenya a year later after Stefania and colleagues studies showed that MDRSA prevalence in country was 81% in public funded hospitals (Maina et al., 2013). Mean while in private hospitals, it is relatively low. (Omuse et al., 2014).

The increase in MDRSA prevalence may be attributed to other sources of contamination apart from the hospital and communities (Ombui et al., 2009; Shitandi et al., 2004). New sources of MDRSA infections are livestock and pets which have resulted to novel strain known as livestock acquired MRSA (LA-MRSA). Thus, zoonotic sources of contamination cannot be ruled out (Broens et al., 2012; de Boer et al., 2009). This creates a major threat to the global public health and economy (WHO, 2013). In order to avert this antibacterial crisis the WHO has urged pharmaceutical companies to increase their efforts towards research, development and production of new antibiotics (Spellberg, 2010).

However, this has not been the case since the venture is expensive and demands a lot of time to carry out. While the bacteria strains acquire resistance against new antibiotics faster than the rate at which these antibiotics are developed and produced (Huttner et al., 2013).
CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

The study was conducted in Nairobi County, the most populous county in the Kenya with an estimated population of about 3.37 million (KNBS, 2015). The county has an area of 696.1 sq Km and it has nine sub-counties namely; Starehe, Kamukunji, Kasarani, Makadara, Embakasi, Njiru, Dagoretti, Langata and Westlands (ASDSP, 2016). Majority of its inhabitants dwell in slums such as Mathare, Kibera and Mukuru Kwa Jenga. Slum dwellers have poor access to basic human needs such as water, basic sanitation and health care services (WPR, 2016). This make them prone to numerous bacterial infections and in most cases these bacteria are drug resistant strains (Maina et al., 2013; Njuguna et al., 2013). The slums’ drainage systems and sewage treatment plants in the county were selected for the study as they provided rich sources of multidrug resistant bacteria.

3.2 Sampling

3.2.1 Sampling design

Nairobi County was selected as study site using convenient sampling technique. Cluster sampling technique was used to pick the sampling sites within the county. The names of particular areas’ of the sites to be studied were selected using simple random sampling methods.
3.2.2 Sample size determination
Sampling size was determined using the formula used by (Kothari, 2004).

\[ n = \left( \frac{Z^2 \cdot \rho \cdot q}{\ell^2} \right) \]

Where \( n \) = desired number of water samples to be collected.

(Population is infinite)

\( Z \) = standard deviation is usually 1.96 which corresponds to 95% confidence interval.

\( \rho \) = the proportion of Kenyan slum dwellers who cannot access basic sanitation such as piped water is estimated to be 93% (Undie et al., 2006).

\( q = 1 - \rho \rightarrow (1 - 0.93) = 0.07 \)
\( \ell = 0.05 \) (5% absolute precision)

\[ n = \left( \frac{1.96^2 \cdot 0.93 \times 0.07}{0.05^2} \right) \]
\[ n = 100.035264 \geq 100 \text{ samples} \]

3.2.3 Sample collection
Environmental waste and sewage water were collected in sterile dark containers from the Nairobi City Sewage and Treatment plant at Dandora and drainage systems of Kibera, Mathare, Korogocho, Burma market and Dagoreti abattoir. These water samples were chosen for the study as they often host numerous strains of multidrug resistant bacteria (Blaak et al., 2015; Okemo et al., 2013).
3.3 Isolation of *S.aureus* bacteria, *S.aureus* lytic phage and *in vitro* study

3.3.1 Isolation of *S.aureus* bacteria (Chessbrough, 2000)

The water sample was sieved to remove debris and then centrifuged at 2500 g for 10 minutes. The supernatant was collected and preserved at +4°C. The sediment was slightly mixed with sterile 0.85% saline water to form a paste. The sediment paste was inoculated on to mannitol salt agar (Liofilchem®, Italy) and incubated at 37°C 12 – 24 hours (hrs). Single bacteria colonies from the overnight plate culture were further sub-cultured on mannitol salt agar plates to obtain pure bacteria isolates.

3.3.2. Identification of *S.aureus* bacteria (Chessbrough, 2000)

Isolates from different plates were gram stained. The gram positive isolates were cultured overnight on modified plate agar. The plate agar was modified adding 7.5% sodium chloride per kilogram of the medium, to make it more selective for *S.aureus*. These bacteria isolates were then tested for catalase enzyme. Coagulase test was done for all isolates that were catalase positive.

Analytical profiling index (API) test was done for all the isolates that were coagulase positive using API STAPH kit (BIOMERIEUX-France). This was to ascertain whether they were *S.aureus* isolates.
3.3.3 **Antibiogram test** (Wikler *et al.*, 2007)

Kirby- Bauer Disk diffusion test (Bauer *et al.*, 1966) was done as stipulated by Clinical standard laboratory institute (CLSI). The antibiotics used in the test were Ceftazidime (CAZ) 30µg, Oxacillin (OX) 1 µg, Vancomycin (VAN) 30 µg, Netilmicin (NET) 30 µg, Gentamicin (CN) 10 µg, Erythromycin (E) 15 µg, Trimethoprim-Sulfamethoxazole (SXT) 25 µg and Cefuroxime (CXM) 30 µg (Liofilchem®, Italy). The antibiotics disks had international standardized drug concentration as recommended by CLSI (Wikler *et al.*, 2007).

3.3.4 **Preservation of isolated multidrug resistant *S.aureus* (MDRSA)**

The isolates that were multidrug resistant were stored at +4°C for two weeks if they were to be used within ten days. Other bacteria samples were preserved in 15% glycerol – nutrient broth mixture at -20°C for long term storage (6 months to 3 years).

3.3.5 **Isolation of *S.aureus* lytic phages** (Adams & Wade, 1955)

Forty milliliters (40 ml) of the previous collected supernatant (**3.3.1 procedure** was centrifuged at 12000 g for 10 minutes (Centrific™ - Centrifuge Fisher scientific, USA). The second supernatant was collected, mixed with equivalent volume of nutrient broth (NB) (HiMedia-India) and one milliliter of the previously
overnight nutrient broth cultured MDRSA isolate. This mixture was incubated overnight at $37^\circ$C and 120 revolutions per minute (rpm) in a shaker incubator (LAB-LINE® incubator-shaker, USA). The overnight culture was centrifuged (10000 X g for 10 minutes), filtered (0.2 µm filter) and serial diluted tenfold. 250 µl of the tenfold serial diluent was combined with a fresh MDRSA isolate (300µl) overnight culture mixed with nutrient agar (NA) (0.7%) overlay and dispensed on dry nutrient agar (1.5%) base (HiMedia-India) medium. Thereafter, incubated at $37^\circ$C overnight after which huge plaques were aseptically picked and purified through the previous double layer method. Purified plaques were then cultured with an overnight MDRSA isolate culture for 18 hrs at $37^\circ$C. Afterwards this culture was ultra filtered with 0.2 µm to get pure lysate (MDRSA lytic phage isolate).

3.3.6 In vitro screening for phage against MDRSA (Golkar et al., 2013)

A lawn of MDRSA isolate was prepared using McFarland standard 2 ($6.0 \times 10^8$ CFU/ml) inoculum. The inoculum was made by serial diluting an overnight broth culture of the MDRSA isolate in a normal saline solution to much the McFarland standards (BIOMERIEUX-France). The inoculum was inoculated on to the nutrient agar plates by spread method and allowed to air dry. 5 µl of the pure lysate was spotted on MDRSA isolate plates. The media plates were air dried again then incubated at $37^\circ$C overnight. Clear patches (plaques) observed on the MDRSA lawn after 18 hours of incubation were measured to determine the most
virulent phage isolate. The most virulent lytic phage was stored at +4°C while waiting to used during in vivo studies.

3.4 In vivo Study

3.4.1 Study animals
Thirty BALB/c mice aged 6 to 8 weeks were sourced and maintained in the animal facility of the Institute of Primate Research (IPR) Karen-Nairobi. The mice were fed on antibiotic free food ration (Unga Feeds – Kenya Limited) and water was provided ad libitum. All study protocols and procedure were reviewed and approved by the Institutional Review Committee of the IPR (IRC/02/14) and Kenyatta University.

3.4.2 Safety and efficacy study of phage therapy

3.4.2.1 Inocula preparation of MDRSA isolate for infection and phage lysate for both infection and treatment.
The dosing inocula were prepared from an overnight MDRSA isolate culture. This culture was centrifuged at 2000 X g for 5 minutes, washed three times, and suspended in sterile physiological saline to the desired concentration (10^8 CFU/ml) (Yao et al., 1997)

The phages (10^8 PFU/ml) to be used in the study was prepared as described by McVay et al., (2007). 10^8 CFU/ml of the isolate S.aureus in 20 ml NB was added
20 ml of sterile NB and to this mixture 1 ml of lytic phage at $10^8$ PFU/ml was included. This mixture of *S.aureus*, lytic phage and sterile NB was incubated at 37°C, 120 r.p.m. for 3.5 hrs. The culture was then centrifuged at 10 000 X g for 10 minutes and the supernatant ultra-filtered using 0.2 µm filters. The filtrate was maintained at +4°C before use.

### 3.4.2.2 Experimental design

Mice were randomly assigned into 6 groups of five animals per group (Figure 1). The number of mice used in the study was determined by GPower 3.1 software (*Faul et al.*, 2009). 30 mice were used during this experiment, but it was done in triplicate to eliminate biasness. Previously 20 mice had been used to determine lethal dosage for phage therapy. In total the study made use of 200 mice; that is 180 for experimental work and 20 for phage lethal dosage determination.

Lethal dose determination involved intravenous infection of the 16 mice (male and female) via caudal tail vein with 0.20 – 0.25 ml varied lytic phage PFU/ml ($10^4$, $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$ and $10^{11}$). In this experiment 4 mice were used as controls.

In the main experiment mice were infected with 0.25 ml of either MDRSA ($10^8$ CFU/ml) or lytic phage ($10^8$ PFU/ml) intravenously via tail vein (*Sheen et al.*, 2010). The mice were treated at 24 hrs or 72 hrs post infection (p.i) with a single
dose of either clindamycin (8 mg/kg bwt), phage (10^8 PFU/ml) at 0.25 ml or a combination of both phage (10^8 PFU/ml) and clindamycin (8 mg/kg/bwt). This was done intravenously (Figure 1).

The mice physical appearance was observed daily and the appearance scored as indicated below (Table 1).

**Table 1: Physical appearance score**

<table>
<thead>
<tr>
<th>Physical appearance</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Normal</td>
<td>5</td>
</tr>
<tr>
<td>b) Slight illness; defined by lethargy and ruffled fur.</td>
<td>4</td>
</tr>
<tr>
<td>c) Moderate illness; defined by severe lethargy, ruffled fur and hunched back.</td>
<td>3</td>
</tr>
<tr>
<td>d) Severe illness; defined by all the above signs and exudative accumulation around partially closed eyes.</td>
<td>2</td>
</tr>
<tr>
<td>e) Moribund; defined as near death status or extreme end of all the above signs.</td>
<td>1</td>
</tr>
<tr>
<td>f) Death</td>
<td>0</td>
</tr>
</tbody>
</table>

Mice at moderate illness, severe illness and moribund conditions were euthanized on ethical grounds. This was done to minimize the pain the mice were undergoing (Kilkenny et al., 2010). 0.5 µl of blood was got from the mice from day 1 to 10. These blood samples were used to determine the viremia levels in phage infected mice, and bacteremia level MDRSA control and treatment mice groups. On the
tenth day all the surviving mice were euthanized. The cardiac blood and organs (brain, liver, kidneys and lungs) were then harvested.

Figure 2: Experimental design for in vivo work.
Samples of cardiac blood and organs were evaluated for bacteria CFU/ml, phage PFU/ml and histopathological adverse effects of the bacteria and phages. Organ samples (brain, liver, kidney and lungs) for histopathological analysis were collected in formalin. The tissues were stained with haematoxyline and counter stained with eosin as per IPR histopathological staining protocol.

3.5 Statistical analysis
The mice’ daily physical health status scores, blood bacteria (CFU/ml) and phage (PFU/ml) levels were collected, and keyed into Microsoft Office Excel 2007 software (Microsoft Word- Microsoft Corporation, USA). These data were analyzed using GraphPad Prism 5.0.1 (Graph pad software, San Diego. CA). A two- way analysis of variance (ANOVA) was used to determine the differences amongst groups and within the group. The level of significant difference was determined using Bonferroni post test and p-value (p<0.5) was considered significant.
CHAPTER FOUR: RESULTS

4.1 Bacterial and phage isolation

4.1.1 Identification of *S. aureus* bacterial isolated from waste and sewage water

Yellow colonies obtained on Mannitol salt agar indicated the presence of *Staphylococcus* species isolates (Figure 3 A) which were sub-cultured on modified plate agar and white colonies were formed (Figure 3 B).

![Image](image1.png)  
**Figure 3**: *Staphylococcus* species bacteria isolate colonies in (A) mannitol salt agar and (B) modified plate agar medium

Gram stain appearance of the isolated bacteria were grape-like bunches of purple cocci. The biochemical tests showed that the bacterium was catalase and coagulase positive (Figure 4 A, B and C).
Figure 4: Gram positive cocci (A), catalase positive (B) and coagulase positive (C) tests.

These isolates were further analyzed using API kit for *Staphylococcus* species. The test revealed that not all the isolates were *Staphylococcus aureus*, as others were identified as *Staphylococcus sciuri*, *Staphylococcus lentus*, *Staphylococcus xylosus*.
and *Staph.intermedius*. The confirmed positive *Staphylococcus aureus* isolate had distinct colour changes (Figure 5) unlike the negative test which indicated other *Staphylococcus* isolates such as *Staphylococcus lentus*, *Staphylococcus xylosus* and *Staph.intermedius* (Figure 6).

**Figure 5**: Positive API STAPH test for *Staphylococcus aureus* bacteria.

**Figure 6**: Negative API STAPH test for *Staphylococcus aureus* bacteria.
The antibiogram test showed that the isolated \textit{S.aureus} was as resistant as ATCC 43300 (MRSA control) when tested against the selected antibiotics. This indicated that the isolate was a multidrug resistant \textit{S.aureus} bacterium. The bacterium was resistant to; 1) Cephalosporin antibiotic (Ceftazidime) 2) Penicillin derived antibiotic (Oxacillin), 8) Macrolides (Erythromycin). 4) Glycopeptide (Vancomycin). 7) Gentamicin and 6) Netilmicin (aminoglycopeptides antibiotics). The isolate was sensitive to 3) trimethoprim-sulfamethoxazole (combination of two antibiotics), and 5) second generation cephalosporin antibiotic (Cefuroxime) (Figure 7). The drugs with smaller zones of inhibitions around the m than the resistant range were considered non-effective against the isolated \textit{S.aureus} and the well characterized MRSA (ATCC 43300). Sensitivity was considered when the zone of inhibition around the antibiotic was equal or larger to the standard sensitivity range (Table 2).
Table 2: Interpretation of zones of inhibition (clear patches) around the antibiotics used in the antibiogram test

<table>
<thead>
<tr>
<th>No.</th>
<th>Antibiotics</th>
<th>Isolated \textit{S.aureus}</th>
<th>ATCC 43300</th>
<th>Resistance range</th>
<th>Sensitivity range</th>
<th>Verdict</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ceftazidime</td>
<td>8 mm</td>
<td>7 mm</td>
<td>R \leq 14 mm</td>
<td>S \geq 18</td>
<td>R</td>
</tr>
<tr>
<td>2</td>
<td>Oxacillin</td>
<td>9 mm</td>
<td>0 mm</td>
<td>R \leq 10 mm</td>
<td>S \geq 13</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td>Trimethoprim-Sulfamethoxazole</td>
<td>33 mm</td>
<td>32 mm</td>
<td>R \leq 14 mm</td>
<td>S \geq 32</td>
<td>S</td>
</tr>
<tr>
<td>4</td>
<td>Vancomycin</td>
<td>16 mm</td>
<td>13 mm</td>
<td>R \leq 21 mm</td>
<td>S \geq 23</td>
<td>R</td>
</tr>
<tr>
<td>5</td>
<td>Cefuroxime</td>
<td>39 mm</td>
<td>30 mm</td>
<td>R \leq 14 mm</td>
<td>S \geq 28</td>
<td>S</td>
</tr>
<tr>
<td>6</td>
<td>Netilmicin</td>
<td>11 mm</td>
<td>10 mm</td>
<td>R \leq 12 mm</td>
<td>S \geq R</td>
<td>R</td>
</tr>
<tr>
<td>7</td>
<td>Gentamicin</td>
<td>9 mm</td>
<td>1 mm</td>
<td>R \leq 12 mm</td>
<td>S \geq 27 mm</td>
<td>R</td>
</tr>
<tr>
<td>8</td>
<td>Erythromycin</td>
<td>0 mm</td>
<td>0 mm</td>
<td>R \leq 13 mm</td>
<td>S \geq 22 mm</td>
<td>R</td>
</tr>
</tbody>
</table>
Figure 7: Antibiogram test of *S.aureus* isolates was resistant to commonly used local antibiotics.

4.1.2 Isolation and screening for phage isolates activity against MDRSA *in vitro*

Eight phages were isolated against the isolated MDRSA bacterium. The virulence of these phages against the MDRSA was determined by spot assay. These spots (clear patches on MDRSA lawn) are created when the phage parasitize a bacterium and replicate inside it. Many copies of this phage are produced which then produce lytic enzyme to digest the bacterium wall lyse. The phages escape into the surrounding to parasitize more bacteria and continue lysing them. This is what formed the patches or spots seen on the bacteria lawn (Figure 8).
Figure 8: Spot assay showing the most virulent phage isolate patch

The isolated virulent phage was active against MDRSA in vitro; it cleared the MDRSA bacteria in a broth media within 24 hrs.

4.2 In vivo Study

4.2.1 Safety of phage therapy
Results of trial study showed that phage was not toxic or pathogenic in mice, as none of the mice died. They showed no symptom of distress such as lack of appetite, ruffled fur and lethargy. The observation was made on all the involved in the study.

The mice hurdled in one of the cages after infection with a single of phage ($10^8$ PFU/ml) or MDRSA bacteria ($10^8$ CFU/ml). The mice infected with only phage
showed as active as non-infected mice with no major difference in physical appearance ($p > 0.5$). These mice (non-infected and phage infected) groups were significantly different from MDRSA infected mice groups ($p < 0.001$). The mice on treatment groups showed improvement on physical appearance. There was no significant appearance in all the treatment groups when mice were treated at either 24 hrs p.i (Figure 9) or 72 hrs p.i (Figure 10) ($p > 0.5$).

![Graph showing physical appearance scores over days for different groups of mice](image)

**Figure 9:** Physical appearance graph of mice treated at 24 hrs post infections
Survival rate was high in MDRSA infected mice groups treated 24 hrs p.i infection as compared to the once treated 72 hrs p.i. 100% survival rate was recorded in the non-infected and phage infected groups when the treatment was initiated at either 24 hrs p.i (Figure 11 A) or 72 hr p.i. (Figure 11 B). When treated at 24 hrs p.i 80% of the mice survived in the treatment groups (Figure 11 A), but not at 72 hrs p.i where only 60% of the mice survived (Figure 11 B).
Figure 11: Survival rates of mice treated at 24 hrs post infection (A) and 72 hrs post infection (B).

4.2.2 Efficacy of phage therapy

Results of blood culture for isolation of viable MDRSA bacteria showed that the MDRSA infected non-treated group had the highest bacteremia level at day 10.
This was significantly different when compared with treatment groups \((p > 0.001)\). The difference was observed when the mice were treated at either 24 hrs p.i or 72 hrs p.i. (Figure 12 and 13). The phage control group had no phage (0 PFU/ml) in their blood from day 4 to day 10 (Figure 12 and 13). There were no bacteria in phage treated mice at day 9 when the mice were treated either at 24 hrs p.i or 72 hrs p.i. (Figure 12 and 13).

![Graph](image)

**Figure 12:** A graph of 24 hrs p.i. treatment bacteremia and viremia levels in treated mice.
**Figure 13:** A bacteremia and viremia level graph of mice treated 72 hrs p.i.

In both treatments (24 hrs p.i. and 72 hrs p.i.) the MDRSA infected phage treated groups had no viable bacteria in their blood samples from day 9 to 10. This was very significant ($p < 0.001$) when compared with those mice that were treated with a single dose of either clindamycin or combination of clindamycin and phage (8 mg/kg/bwt clindamycin + $10^8$ PFU/ml of phage). The results further shows that there was no significant difference ($p > 0.5$) in bacteremia levels for mice treated with either clindamycin or the combination dosage.
The result of cardiac blood samples collected from the mice at study end point (day 10) from 24 hrs p.i. and 72 hrs p.i groups showed that phage treated mice had no viable bacteria count (0 CFU/ml). This was non-significantly different ($p > 0.5$) when compared with the samples from non-infected and phage infected control groups which also had no bacteria. While the mice on either clindamycin or combination treatment had viable bacteria count when treated 24 hrs p.i. This was different when the mice were treated at 72 hrs p.i., as there was trace of viable bacteria count in the blood of clindamycin treated mice. However, the mice that were on combination dosage treatment had no viable bacteria count in their blood samples (Table 3) at study end point.

**Table 3: Cardiac blood bacteria count (mean log CFU/ml + SE) at day 10 post infection**

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>24 hrs post infection treatment</th>
<th>72 hrs post infection treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected (naïve) control group</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Phage control group</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Bacteria control group</td>
<td>8.0 ± 0.2</td>
<td>9.0 ± 0.2</td>
</tr>
<tr>
<td>Antibiotic therapy</td>
<td>3.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Phage therapy</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>AntiPhag therapy</td>
<td>2.0 ± 0.2</td>
<td>0.0</td>
</tr>
</tbody>
</table>
The MDRSA isolate was isolated from the tissues’ homogenate cultures of the MDRSA infected mice at day 10 post infection. Multi-drug resistant *S.aureus* infected phage treated mice in both treatments (24 hrs p.i. and 72 hrs p.i.) had 0 CFU/ml in the organs. While the organs from other MRDSA treated groups and non-treated group (control) had bacteria CFU/ml. The viable count of bacteria was higher in MDRSA infected mice treated with a dose of clindamycin than in the other treated groups. Combination treatment group had 0 CFU/ml of viable MDRSA isolate in the organs when they were given treatment 72 hrs post infection. However, this result could not be achieved if the mice were treated 24 hrs p.i. These results further shows that there were no viable bacteria (0 CFU/ml) in the brain tissues of phage treated and combination treated mice groups, as seen in the clindamycin treated mice group (Figure 14 and Table 4).
Figure 14: Brain homogenate pour culture plates; (i) Homogenate from non-infected group. (ii) Non-culture from Bactria control (R- bacteria colonies). (iii) Culture from antibiotic treated MDRSA infected group. (iv) Phage treated MDRSA infected group homogenate. (v) Homogenate of MDRSA infected groups treated with combination therapeutic agent. (vi) Homogenate of phage infected non-treated group.
Figure 15: Lungs homogenate culture plates: (i) Non-infected group. (ii) MDRSA infected non-treated group (K -bacteria colonies). (iii) MDRSA infected antibiotic treated group. (iv) MDRSA infected phage treated group. (v) MDRSA infected combination treated group. (vi) Phage infected non-treated group.
Figure 16: Liver homogenate pour plate cultures; (i) Non-infected group. (ii) MDRSA infected non-treated group (T-bacteria colonies). (iii) MDRSA infected antibiotic treated group. (iv) MDRSA infected phage treated group. (v) MDRSA infected combination treated group. (vi) Phage infected, non-treated group
Figure 17: Kidney homogenate pour plate cultures; (i) Non-infected group. (ii) MDRSA infected non-treated group (Q-bacteria colonies). (iii) MDRSA infected antibiotic treated group. (iv) MDRSA infected phage treated group. (v) MDRSA infected combination treated group. (vi) Phage infected, non-treated group.
Table 4: End point (day 10) viable bacteria count (mean log10 CFU/g + SE) * from organs’ homogenates.

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Brain</th>
<th>Lungs</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected control</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Phage control</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Bacteria control</td>
<td>7.2 ± 0.2</td>
<td>7.0 ± 0.2</td>
<td>9.0 ± 0.2</td>
<td>8.6 ± 0.2</td>
</tr>
<tr>
<td>Antibiotic therapy</td>
<td>3.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>Phage therapy</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Combination therapy</td>
<td>0</td>
<td>4.0 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>

* organs were homogenized in sterile physiological saline, diluted in the same saline and plated in a selective media.

Phage was isolated from the liver organs of the mice either infected or treated with phages. Multidrug resistant *S.aureus* infected mice that were treated with only phage had the highest plaque count (7 – 8 PFU/ml) compared to other groups that received phage. The mice on combination therapy had the least phage count (2 – 3 PFU/ml) (Table 5 and Figure 18).
Figure 18: Liver homogenate plaque assay cultures; (i) Non-infected groups had no plaques. (ii) Phage infected non-treated groups (S – plaques). (iii) MDRSA infected group treated with combination therapeutic agent. (iv) MDRSA infected group treated with phage.
Table 5: End point phage count (mean log10 PFU/ml + SE)\textsuperscript{a} from the liver.

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>24 hrs post infection</th>
<th>72 hrs post infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected control group</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Bacteria control group</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Phage control group</td>
<td>3.0 ± 0.2</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>Antibiotic therapy</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Phage therapy</td>
<td>7.0 ± 0.2</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td>Combination therapy</td>
<td>2.0 ± 0.2</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} organs were homogenized in sterile physiological saline, diluted in the same saline and plated on MRSA isolate lawn.

Haemotoxylin and eosin stained tissues (brain, lung, liver and kidney) depicted the pathology of the MDRSA bacteria on the infected mice. Histopathological findings showed the organs of non-treated MDRSA infected and clindamycin treated mice groups had severe inflammation (Figure 20, 24, 25-ii and 27). The organs of non-infected mice, phage infected mice and MDRSA infected – phage treated mice groups had no inflammation. While the lung and liver of MDRSA infected - combination treated mice groups had inflammation (Figure 25 – 28).
Figure 19: Brain tissues from; (i) Non-infected group, (ii) Phage infected, non-treated group (X- glial cell, H- Blood brain barrier). Magnification = X400.
Figure 20: Brain tissues from MDRSA infected non-infected group; B, E and G lymphocytic infiltrations (inflammation). Magnification = X400.
Figure 21: Brain tissues from: i) MDRSA infected non-treated group. ii) MDRSA infected antibiotic treated group. J and U lymphocytic infiltrations (inflammation), magnification = X 400.
Figure 22: Brain tissues; i) MDRSA infected phage treated mouse (R- glial cells). ii) MDRSA infected AntiPhag treated mouse (M- glial cells N- vacuolated blood brain barrier, and Y- dead glial cells), magnification = X 400.
Figure 23: Lung tissues i) Non-infected mouse ii). Phage infected non-treated mouse, magnification = X 400.
Figure 24: Lung tissues; i) MDRSA infected non-treated mouse (a) alveoli full of mucus, (b) deflated alveoli, (c) lymphocytic infiltrated septa, (d) pockets of serous fluid (pneumonia). ii) MDRSA infected antibiotic treated mouse (e) lymphocytic infiltrated alveoli, (f) pocket of serous fluid, (g) deflated alveoli and (h) perivascular fibrosis. Magnification = X400.
Figure 25: Lung tissues; i) MDRSA infected mouse treated with phage (K-minimal lymphocytic infiltrated septa and (L) ventilated alveoli. ii) MDRSA infected mouse treated with combination therapeutic agent (G) collapsed alveoli, (P) pockets serous fluid and (Y) perivascular fibrosis. Magnification = X 400.
Figure 26: Liver tissues from; i) Non-infected mouse. ii) Phage infected non-treated mouse, magnification = X 400.
Figure 27: Liver tissues; i) MDRSA infected non-treated mouse (W) severe perivascular lymphocytic infiltration (S) severe congestion. ii) MDRSA infected mouse treated with antibiotic (T) severe perivascular lymphocytic infiltration. Magnification = X 400.
Figure 28: Liver tissues from; i) MDRSA infected mouse treated with phage (W) non-inflammed hepatic blood vessel. ii) MDRSA infected mouse treated with combination therapeutic agent (Z) perivascular lymphocytic infiltration. Magnification = X 400
Figure 29: Kidney tissues from: i) Non-infected mouse. ii) Phage infected non-treated mouse. Magnification = X400.
Figure 30: Kidney tissues from: i) MDRSA infected, non-treated group and ii) MDRSA infected, antibiotic treated group. Magnification = X 400.
Figure 31: Kidney tissues from; i) Kidney tissue from MDRSA infected mouse treated with phage. ii) Kidney tissue from MDRSA infected mouse treated with AntiPhag. The tissues were normal. Magnification = X 400.
Kidneys histopathological findings showed no tissue damage (Figures 29 - 31). The homogenate culture of kidney tissues depicted that MDRSA infected non-treated, clindamycin and combination treated groups had viable MDRSA bacteria counts. While phage treated group did not have viable bacteria count (Table 4 and Figure 17).
CHAPTER FIVE: DISCUSSION, CONCLUSIONS, RECOMMENDATIONS AND SUGGESTION FOR FURTHER RESEARCH

5.1 Discussion
The present study has established that the *S.aureus* bacteria isolated from the waste water and sewage water samples from Nairobi County and its environs were resistant to Oxacillin, macrolides (Erythromycin), aminoglycopeptides (gentamycin and netilmicin) and glycopeptides (vancomycin) (Figure 7). An indication that the *S.aureus* bacterium isolate was a multidrug resistant strain. Thus, the study shows for the first time that the multidrug resistant (MDR) *S.aureus* bacteria isolate has been isolated (Oduor *et al.*, 2016) from environmental wastewater and sewage. Initially it was thought that multidrug resistant bacteria were nosocomial pathogens (Aiken *et al.*, 2014; Chambers & DeLeo, 2009; Omari *et al.*, 1997) on the contrary here the study shows that they are present in the waste and sewage water within the county of Nairobi.

The findings of this study corroborates those obtained in the USA and Sweden that reported presence of MDR *S.aureus* isolate in environmental waste water and sewage water at sewage treatment plants (Börjesson *et al.*, 2010; Naquin *et al.*, 2015). Thus, the antibiogram results of this study (Table 2 and Figure 7) indicate that environmental waste water and sewage water may be associated with MDR *S.aureus* acquisition in Kenya.
The prevalence of multidrug resistant *S.aureus* in Nairobi has been increasing from 39.8% (Omari *et al*., 1997b) to the current 81% (Maina *et al*., 2013) and probable cause might be the presence of the bacteria strains circulation in raw sewage or reclaimed waste water. This water is used in irrigating vegetable gardens in informal settlements of Nairobi County such as Mukuru Kayaba and Mathare slums. Multidrug resistant *S.aureus* strains have been isolated from environmental waste water in Mukuru Kayaba (Svabova *et al*., 2007) and this corroborates with the finding of this study.

Furthermore, the study successfully and confirmed the presence of *S.aureus* specific lytic phages (Figure 8) in the waste and sewage water collected within Nairobi County. These findings support Chhibber *et al.* (2013) who established that potent phages against ATCC 43300 MRSA strain can be obtained from sewage samples.

In mice, this study established that the lytic phages at a dose of $10^8$ PFU/ml were well tolerated, safe and eliminated from blood circulation in 3 days post infection (Figure 12 – 13). This was further shown by the mice physical appearance score (Figure 9 - 10) and survival rate (Figure 11), as these mice were as active as non-infected groups and all of them survived. The findings of this study also, suggest that phages were efficiently eliminated by the organs of the reticuloendothelial system such as the liver from the blood. This was shown by the presence of high
phage PFU/ml (2-3 PFU/ml) in the liver homogenate culture of phage infected non-treated group of mice 10 days p.i at the study’s end point (Table 5 and Figure 18). In this study phages were isolated from the liver tissue of phage infected non-treated mice, MDRSA infected phage treated mice and MDRSA infected combination treated mice (Figure 18). These finding corroborates with Westwater et al. (2003), who isolated phages from the liver tissues of bacteria infected mice treated with phages. The liver is one the reticuloendothelial organs in the mouse body; other organs includes the spleen and lymph nodes which traps the phages as they circulate in body through the blood (Biswas et al., 2002; Capparelli et al., 2007).

The study also established that hematogenous meningitis developed due to presence of inflammation and haemorrhage in the brain meninges (Figure 20 – 21), an indication that the MDR S.aureus was able to penetrate the blood-brain barrier (BBB). Sheen et al. (2010) also did get similar findings as the ones shown in this study when they intravenously challenged mice with S.aureus bacteria to show that the bacteria is capable of crossing the blood brain barrier (BBB). This finding has further been shown in this work by the isolation of viable bacteria count from the brain homogenate cultures of MDRSA infected mice; non-treated control and clindamycin treated groups (Table 4 and Figure 14). Hepatitis and pneumonia conditions were also evident due to severe lymphocytic infiltration and congestion of the organs. In the liver hepatitis was marked with severe perivascular
lymphocytic infiltration of the blood vessels that indicated zones of inflammation (Figure 27). Pneumonia was marked with severe congestion of the alveoli with serous fluid and mucus, inflammation of septea due to severe lymphocytic infiltration. In addition, the lung had haemorrhagic and deflated alveoli (Figure 24). These histopathological findings have been supported by the isolation of viable MDRSA bacteria from these organs’ homogenate cultures, which showed that they had high bacteria counts (Table 4 and Figures 15- 16).

No inflammation was observed in the organs of phage infected – none treated mice groups (Figures 19 ii, 22 i, 23 ii, 25 i, 26 ii and 28 ii). These mice also, had good physical appearance score and registered 100% survived rate during the experiment (Figures 9, 10 and 11.). These results findings shows that lytic phages are not pathogenic and do induce serious notable adverse side effects. Similar findings have been shown by Slopek et al.(1985) and Łusiak-Szelachowska et al. (2014) with human studies.

These results show that MDRSA infected mice treated with a single dose of S.aureus lytic phage did not have viable bacteria count in the blood. This was observed from day 7 for those mice treated 24 hrs p.i or from day 9 when treated at 72 hrs p.i treatment (Figure 12 – 13). These mice group’s organs had no inflammation or viable MDRSA count in their blood and organ homogenate cultures (Tables 3- 4, and Figures 14-17 and 19 - 28). This showed that a single
A single dose of phage at $10^8$ PFU/ml was a better remedy against the MDRSA than a single dose of clindamycin at 8mg/kg/bwt in clearing MDRSA. The results of this work negate the study’s hypothesis which states that phage therapy is neither safe nor effective against MDRSA infections. This result is a proof of concept that bacterial specific lytic phages may be used against multidrug resistant bacteria isolates. The findings agree with those obtained by Shivshetty et al. (2014) showing that bacteriophages conferred protection against lethal *Pseudomonas aeruginosa* infection in diabetic mice as opposed to use of antibiotics.

The mice that received phage treatment did not show any signs of meningitis or encephalitis implying that the phages cleared the *S.aureus* bacteria before it crossed the blood brain barrier. This was further confirmed by bacterial analysis that showed their brain homogenate culture had no viable bacteria count (0 CFU/ml) (Table 4). Pouillot et al., (2012) did get similar findings while studying efficacy of phage therapy against experimental *Escherichia coli* meningitis.

Findings of this study also showed that a single dose *S.aureus* lytic phage prevented the establishment of hematogenous MDRSA pneumonia and hepatitis in mice (Figure 25 i, and 28 i). Similar results have been observed in other studies done by obtained by Capparelli et al. (2007) and Matsuzaki et al. (2003) while studying therapeutic efficacy of lytic *S.aureus* phage against experimental systemic *S. aureus* infection in mice. A single dose of either clindamycin (8
mg/kg/bwt) or combination therapeutic agent ($10^8$ PFU/ml of phage + 8 mg/kg/bwt of clindamycin) was insufficient to cure the mice experimental bacterial infection (Figures 21 ii, 24 ii, 25 ii, 27 ii and 28 ii). The results were contrary to the expected findings as clindamycin is considered one of best drugs against community acquired MDRSA (Moellering, 2008). The failure of clindamycin to prevent MDRSA sepsis in these mice groups could have been due to resistance of the bacterium isolate against the drug. The inefficacy of combination therapy could be attributed to antagonism nature of clindamycin activities towards phages lytic mechanisms.

The efficacy level of lytic S.aureus phage against MDRSA infection was determined using the results of physical appearance score, daily blood bacteremia levels, counts of viable MDRSA bacteria colonies from the organs and histopathological findings. These findings showed that the infection had been cleared off. The results obtained herein support further research into the potential use of lytic phages in treatment of hematogenous S.aureus meningitis which is one of the major cause of death among HIV/AIDS patients in Kenya (Kwobah et al., 2015).
5.2. Conclusions

- The present study concludes that there exist multidrug resistant S. aureus (MDRSA) strains in the environmental waste water and sewage within the Nairobi County.

- A dosage of $10^8$ PFU/ml of S. aureus lytic phage was most effective than a single dose of Clindamycin (8 mg /kg/bwt) against experimental MDRSA infection in mice.

- Lytic S. aureus phage is safe as an antibacterial agent as it showed no toxic side effect on the mice tissues.

5.3 Recommendations

- There is need for constant surveillance of MDRSA bacteria in the environmental wastewater and sewage.

- There is need to create awareness on phage therapy to change perception of policy makers towards the use of phages as alternative therapeutic agent against multidrug resistant S. aureus infections.

- In order to practice phage therapy in future there is need to characterize and study the pharmacology of lytic phages with therapeutic potential against bacterial infections.
5.4 Suggestion for further research
Morphological and molecular characterization of lytic *S.aureus* phage of therapeutic potential isolated in Nairobi County and the entire country should be done in future.
REFERENCES


http://doi.org/10.1016/j.ijantimicag.2015.05.008


APPENDICES

Appendix I

Approval letter of research work by Kenyatta University graduate school:

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

FROM: Dean, Graduate School
TO: Mr. Oduor M. Ochieng
C/o Medical Lab Science,
Kenyatta University

DATE 25th November, 2013
REF: F150/20415/12

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board at its meeting of 14th November, 2013 approved your Research Proposal for the M.Sc. Degree, Entitled “Therapeutic Potential of Bacillus Strain in Treating Staphylococcus aureus Bacteremia in Mice”.

You may now proceed with your Data collection.

Thank you.

JULIA GITU
FOR: DEAN, GRADUATE SCHOOL

cc. Chairman, Medical Lab Science Dept.
Supervisors:

1. Dr. Washington Yumui Arodi, PhD
   C/o Department of Medical Lab Science
   KENYATTA UNIVERSITY

2. Dr. Nyakimo Atunga, PhD
   Senior Research Scientist
   Institute of Primate Research (IPR)
   WHO Collaborating Centre
Appendix II

Research permit from Institute of Primate Research

[Image of the research permit from Institute of Primate Research]
Appendix III
Sampling site permit from Nairobi Water and Sewage Company:

[Image of the permit]

NC/ASCH/RA/VOL 1405/RKJg

Kenyatta University
School Of Medicine
Department Of Medical Laboratory Science
P.O. Box 43844-00100
NAIROBI

9 January 2014

Dear Mr Odour Joseph

RE: PERMISSION TO CARRY OUT RESEARCH SAMPLES FROM DANDORA ESTATE WASTE WATER TREATMENT PLANT

Reference is made to your letter dated 7 January 2014 on the above mentioned subject.

Approval is hereby granted to collect research samples from Dandora Estate Waste Water Treatment Works. By a copy of this letter, Rural Coordinator is requested to give all the necessary support.

Kindly submit a research report upon completion to the office of the Training Coordinator Headquarters.

Rosemary Kijana
Director Human Resource and Administrative Services

Board of Directors:
P. Kuguru (Chairman), Dr. M. W. Kimani (Vice-Chair), L. Njairu, Prof. J. Kimani, Marla Nansio, Samuel Ogaya
Merry Motu, S. Motu, E. R. Onyido, J. Kiarie, Prof. M. Gachengeri, Reg. P. G. Gitahi (Managing Director)
Appendix IV
Conference attendance and work presented:

1) 9th Federation of African International Immunology Society Conference held 1st - 4th December 2014.

A STUDY OF THERAPEUTIC POTENTIAL OF Staphylococcus aureus BACTERIOPHAGE IN TREATING METHICILLIN RESISTANT Staphylococcus aureus BACTEREMIA IN BALB/C MICE

Oduor Joseph M. Ochieng¹,², Washington Arodi¹, Atunga Nyachieo²

1. Kenyatta University (Medical School), 2. Institute of Primate Research (IPR)

Background: Methicillin resistant Staphylococcus aureus (MRSA) is a global problem due to its high rate of resistance against present antibiotics. Although currently there are few antibiotics under development, they are expensive to produce and therefore alternative means of combating resistant bacterial infections such as those caused by MRSA are desirable. Phage therapy has been regarded as an alternative viable approach of treating multidrug resistant bacterial infections.

Objective: To isolate MRSA specific phages and establish their efficacy against MRSA both in vitro and in vivo.

Methods: A total of n=30 sewage samples were collected from different sites namely: Nairobi sewage and water treatment plant, Dagoretti slaughter house sewage, Nairobi River, Mathare slums, Burma market sewage and Institute of Primate Research sewage for isolation of both MRSA and phages. Whereas MRSA bacteria were isolated using mannitol salt agar selective media, the MRSA phages were isolated from the ultra-filtered sewage water and incubated with a 24hr old MRSA bacteria to identify to plaques. For in vivo study, five groups with n=6 mice each were used. Group 1 (no challenge); group 2 (MRSA infected); group 3 (phage challenge) group 4 (bacteria and phage treatment) and finally group 5 (bacteria and antibiotic treatment).

Results: Both MRSA bacteria and phage were successfully isolated and characterized in vitro. Currently in vivo study is ongoing.

Conclusion: Since the MRSA specific phage was very effective in vitro, this indicates the phage potentiality as a therapeutic agent. However, this needs to be studied in vivo to ascertain its efficacy.
Personal Details
Name: Oduor Joseph Michael Ochieng’
Title: M.Sc. student
Address: Institute of Primate Research (IPR),
        P.O. Box 24481 (00502), Karen- Nairobi.
Phone No.: (+254) 0726492239
E-mail: josemislredo@gmail.com

Certificate of Participation

This is to confirm that

Joseph Michael Ochieng’

Presented a Poster during the
9th FAIS CONFERENCE
held at Safari Park, Nairobi, Kenya

2nd to 4th December 2014

Certificate awarded for participation in the 9th FAIS conference.
Introduction: Methicillin resistant *Staphylococcus aureus* (MRSA) pose a great threat to the global public health\(^1\). Control of these bacteria has become difficult due to acquisition of resistance against even some of the best antibiotics\(^1\). Thus, phage therapy could be the better alternative as they are easy to isolate and produce in mass within a short time\(^2\). However, phage therapy has been a subject of debate over the years but recently there has been a renewed interest due to their proved therapeutic potential and have, therefore, found commercial application in some countries\(^3\). Thus, this study was specifically designed to evaluate the efficacy of phages against MRSA both *in vitro* and *in vivo*.

Materials and Methodology: A litre of environmental waste water and sewage samples were collected around the county of Nairobi. The MRSA isolates were obtained from environmental waste water and sewage samples from Nairobi and its environs and evaluated for drug resistance using antibiogram test. In addition, lytic phages were isolated from these samples too. Thereafter, the *in vitro* efficacy of the phages against MRSA was done by spot assay and tube culture tests. Only the most virulent phage isolate was used for *in vivo* efficacy study which involved six groups of mice of n=5 per group (BALB/c mice; both sex). The first three groups acted as controls (group 1=only physiological saline, group 2=MRSA bacteria only and group 3=phage only) while the remaining groups were used for efficacy studies using a dosage of \(10^8\) CFU/ml for MRSA bacteria, \(10^8\) PFU/ml for phage and clindamycin at 8mg/kg. The efficacy study groups (groups 4-6) were first infected by MRSA and observed for 3 days before treatment with either antibiotic (group 4), or phage (group 5) or phage+antibiotic (cocktail therapy group 6). The mice were then observed for an additional 7 days. During the entire 10 days of observation blood samples were collected daily for bacteremia level determination before being euthanized. Different organs including the liver, brain, kidney and lungs were harvested for histopathological studies. All studies were done in accordance with the IPR Institutional Ethical Committee approved protocols.

Results and Discussion: Antibiogram test indicated that MRSA isolated was a multidrug resistant strain (Figure 1). While the *in vitro* test showed the virulence of the phage isolates after 24 hrs culture at 37°C. Toxicity test showed that phages were safe. There was no significant difference in survival rates between phage infected group and non-infected control group \((p>0.05)\). Bacteremia was significantly lower in phage treated group as compared to other treatment groups and bacteria non-treated group \((p<0.001)\) (Figure 2). In addition, pathological results show that phage prevented organ damage by
the bacteria (Figure 3). Thus, a single dose of phage was more effective than other therapeutic agents used in the study.

Conclusion: Results of this study shows that phage therapy is safe and its application should be considered for the treatment of multidrug resistant bacterial infections.

References


72 HOURS POST INFECTION TREATMENT COMPARATIVE GRAPH

Figure 2: A graph showing the sterility of the subjects’ blood samples on each day, for 10 days of the study. By day 10, all MRSA bacteria had been cleared in phage therapy group as compared to all other treatment groups.
Figure 3: Micrographs showing liver tissues from different groups of the study; W, T and Z represent inflammation of hepatic blood vessels. S represents edema. Phage treated group had normal liver tissues unlike other treatment groups.
Therapeutic efficacy of graphylophycin against dermatophagoides farinae

Joseph, W. O. Oduor

presented at the AIBBC 2015 held at the African Population and Health Research Center, Nairobi, Kenya from September 17th to 19th, 2015.

Co-organizer
University of Tokyo, Japan

Dr. Kibungo, OGW
Biotechnology Research Institute: KARI

Chair

Dr. Benson Njambi
Maeono University, Kenya

Co-organizer

Phage therapy against multidrug resistant *Staphylococcus aureus* (MDRSA) in BALB/c mice. A prospective study.

Joseph M. O. ODUOR (1),(2)*, Fredrick MALOBA (1), Washington O. ARODI (1), Atunga NYACHIEO (2)

(1) School of Medicine, Kenyatta University, Nairobi, Kenya
(2) Institute of Primate Research (IPR), Nairobi, Kenya

**Introduction:** Phage therapy has been a subject of debate over the years but recently there has been a renewed interest due to their proved therapeutic potential and have, therefore, found commercial application in some countries. Thus, this study was specifically designed to evaluate the efficacy of phages against MRSA both *in vitro* and *in vivo*.

**Materials and Methodology:** Phages and MDRSA were isolated from waste water around the county of Nairobi. The *in vitro* efficacy of the phages against MDRSA was evaluated and only the most virulent phage isolate was used for *in vivo* efficacy study which involved six groups of mice of n=5 per group (BALB/c mice; both sex). The first three groups acted as controls (group 1=only physiological saline, group 2=MDRSA bacteria only and group 3=phage only) while the remaining groups were used for efficacy studies using a dosage of 10^8 CFU/ml for MDRSA bacteria, 10^8 PFU/ml for phage and clindamycin at 8mg/kg. Bacteremia from these mice and histopathology after euthanasia was evaluated.

**Results and Discussion:** Antibiogram test indicated that MDRSA isolated was a multidrug resistant strain. While the *in vitro* test showed the virulence of the phage isolates after 24 hrs culture at 37°C. Toxicity test showed that phages were safe. There was no significant difference in survival rates between phage infected group and non-infected control group (p>0.05). Bacteremia was significantly lower in phage treated group as compared to other treatment groups and bacteria non-treated group (p<0.001). In addition, pathological results show that phage prevented organ damage by the bacteria. Thus, a single dose of phage was more effective than other therapeutic agents used in the study.

**Conclusion:** Results of this study shows that phage therapy is safe and its application should be considered for the treatment of multidrug resistant bacterial infections.

---

4) 100th anniversary of the bacteriophage discovery and 10th anniversary of the Phage Therapy Unit in Wroclaw (Poland). Clinical Phage Therapy 2015 conference (26th September 2015).
Evaluation of therapeutic efficacy of *Staphylococcus aureus* bacteriophage against methicillin resistant *Staphylococcus aureus* (MRSA) in BALB/c mice

Joseph M. O. ODUOR 1, 2*, Fredrick MALOBA1, Washington O. ARODI1, Atunga NYACHIEO2

(1) School of Medicine, Kenyatta University, Nairobi, Kenya
(2) Institute of Primate Research (IPR), Nairobi, Kenya

**Introduction:** Methicillin resistant *Staphylococcus aureus* (MRSA) pose a great threat to the global public health. Control of these bacteria has become difficult due to acquisition of resistance against even some of the best antibiotics. Thus, phage therapy could be the better alternative as they are easy to isolate and produce in mass within a short time. However, phage therapy has been a subject of debate over the years but recently there has been a renewed interest due to their proved therapeutic potential and have, therefore, found commercial application in some countries. Thus, this study was specifically designed to evaluate the efficacy of phages against MRSA both *in vitro* and *in vivo*.

**Materials and Methodology:** A litre of environmental waste water and sewage samples were collected around the county of Nairobi. The MRSA isolates were obtained from environmental waste water and sewage samples from Nairobi and its environs and evaluated for drug resistance using antibiogram test. In addition, lytic phages were isolated from these samples too. Thereafter, the *in vitro* efficacy of the phages against MRSA was done by spot assay and tube culture tests. Only the most virulent phage isolate was used for *in vivo* efficacy study which involved six groups of mice of n=5 per group (BALB/c mice; both sex). The first three groups acted as controls (group 1=only physiological saline, group 2=MRSA bacteria only and group 3=phage only) while the remaining groups were used for efficacy studies using a dosage of $10^8$ CFU/ml for MRSA bacteria, $10^8$ PFU/ml for phage and clindamycin at 8mg/kg. The efficacy study groups (groups 4-6) were first infected by MRSA and observed for 3 days before treatment with either antibiotic (group 4), or phage (group 5) or phage+antibiotic (cocktail therapy group 6). The mice were then observed for an additional 7 days. During the entire 10 days of observation blood samples were collected daily for bacteremia level determination before being euthanized. Different organs including the liver, brain, kidney and lungs were harvested for histopathological studies. All studies were done in accordance with the IPR Institutional Ethical Committee approved protocols.

**Results and Discussion:** Antibiogram test indicated that MRSA isolated was a multidrug resistant strain. While the *in vitro* test showed the virulence of the phage isolates after 24 hrs culture at 37°C. Toxicity test showed that phages were safe. There was no significant difference in survival rates between phage infected group and non-infected control group ($p>0.05$). Bacteremia was significantly lower in phage treated group as compared to other treatment groups and bacteria non-treated group ($p<0.001$). In addition, pathological results show that phage prevented organ damage by the bacteria. Thus, a single dose of phage was more effective than other therapeutic agents used in the study.

**Conclusion:** Results of this study shows that phage therapy is safe and its application should be considered for the treatment of multidrug resistant bacterial infections.
SAFETY AND THERAPEUTIC EFFICACY OF \textit{STAPHYLOCOCCUS AUREUS} SPECIFIC LYTIC PHAGE AGAINST MULTIDRUG-RESISTANT \textit{S.AUREUS} (MDRSA) IN BALB/C MICE: A PROSPECTIVE STUDY.

Oduor Joseph M. Ochieng\textsuperscript{a,b,*}, Onkoba Wycliffe Nyamongo\textsuperscript{a}, Maloba Fredrick\textsuperscript{c}, Arodi Washington Ouma\textsuperscript{b}, Atunga Nyachieo\textsuperscript{a}

\textsuperscript{a} Institute of Primate Research (IPR), Nairobi-Kenya, \textsuperscript{b} School of Medicine, Kenyatta University, Nairobi- Kenya, \textsuperscript{c} School of Applied Pure Science, Kenyatta University, Nairobi-Kenya.

Abstract

The use of phage therapy as an alternative method of treating infections caused by multidrug-resistant bacteria has been a controversial issue. The present study sought to determine safety and therapeutic efficacy of environmentally obtained \textit{Staphylococcus aureus} lytic phage against multidrug-resistant \textit{S.aureus} (MDRSA) in mice. Phages and MDRSA were isolated from sewage and waste water collected from within Nairobi County. The isolated \textit{S.aureus} bacterium was screened for resistance towards; Ceftazidime, Oxacillin, Vancomycin, Netilmicin, Gentamicin and Erythromycin, Trimethoproim-Sulfamethoxazole and Cefuroxime. Thirty BALB/c mice were randomly assigned into three groups; the MDRSA infection group (n=20), the phage-infection group (n=5) and non-infection group (n=5). After 24 or 72 hours post-infection (p.i.) with MDRSA, the infected mice were either treated with a single dose of clindamycin (8mg/kg/bwt) or $10^8$ PFU/ml of \textit{S.aureus} phage or a combination (clindamycin and \textit{S.aureus} phage). Safety was determined by monitoring animal physical health post-infection as well as gross pathology and histopathology. Bacteremia was determined daily for 10 days and used to establish therapeutic efficacy of the phage. Treatment with phage was efficacious (100\%) compared to clindamycin (62.25\%) at 24hrs p.i and 87.5\% at 72hrs p.i.) while combination therapy (75\% at 24hrs p.i. and 100\% at 72hrs p.i.). Efficacy of the treatment regimens was dependent on the time of treatment post infection. The mice infected with MDRSA and treated with phage had no bacteremia at day 7 post-treatment compared to those treated with clindamycin and combination therapy ($P < 0.001$). There were no tissue abscesses, inflammation in the brain, lungs and liver tissues of phage treated mice compared to those treated with clindamycin and combination therapy. The \textit{S.aureus} phage obtained from sewage and waste water from within Nairobi County was safe and possessed therapeutic efficacy against MDRSA bacterium.