Isolation, characterization and evaluation of *Staphylococcus aureus* bacteriophage(s) as surface sanitizer

THESIS

SUBMITTED TO THE

BIHAR AGRICULTURAL UNIVERSITY

(FACULTY OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY)

Sabour, (Bhagalpur), BIHAR

In partial fulfilment of the requirements

FOR THE DEGREE OF

Master of Veterinary Science

IN

(VETERINARY PUBLIC HEALTH)

BY

Archana

Registration No – M/VPH/326/BVC/2015-16

(Veterinary Public Health & Epidemiology)

BIHAR VETERINARY COLLEGE

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DEPARTMENT OF VETERINARY PUBLIC HEALTH & EPIDEMIOLOGY
Bihar Veterinary College, Patna-800014.
(Bihar Agricultural University, Sabour, Bhagalpur, BIHAR)

CERTIFICATE-I

This is to certify that the thesis entitled **"Isolation, characterization and evaluation of *Staphylococcus aureus* bacteriophage(s) as surface sanitizer"** submitted in partial fulfilment of requirement for the degree of **Master of Veterinary Science (Veterinary Public Health)** of the faculty of post-Graduate Studies, Bihar Agricultural University, Sabour, Bhagalpur, Bihar is the bonafide research carried out by **Dr. Archana**, **Registration No- M/VPH/326/BVC/2015-16,** under my supervision and guidance. No part of the thesis has been submitted for any other Degree or Diploma.

It is further certified that the assistance and help received during the course of this investigation and preparation of the thesis have been fully acknowledged.

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CERTIFICATE- II

We the undersigned members of the Advisory Committee of **Dr. Archana**, **Registration No- M/VPH/326/BVC/2015-16**, a Candidate for the award of degree of Master of Veterinary Science with major in **Veterinary Public Health**, have gone through the manuscript of the thesis and agree that the thesis entitled "**Isolation, characterization and evaluation of *Staphylococcus aureus* bacteriophage(s) as surface sanitizer**" may be submitted by **Dr. Archana**, in partial fulfillment of the requirements for the degree.

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Microbiology, **Dr. Pankaj Kumar**, Asstt. Prof.-cum-Jr. Scientist, Department of Veterinary Microbiology, **Dr. Pallav Shekhar**, Asstt. Prof.-cum-Jr. Scientist, Department of Veterinary Medicine, **Dr. Anil Kumar**, Asstt. Prof.-cum-Jr. Scientist, Department of Veterinary Medicine and **Dr. G. D. Singh**, Asstt. Prof.-cum-Jr. Scientist, Department of Veterinary Surgery, during the period of research work and for his moral support.

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Archana
(ARCHANA)

Date 24/06/2017

Place BVC, Patna

DEDICATED

TO

MY PARENTS

&

HUSBAND

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mM	:	Millimole
MOI	:	Multiplicity of infection
MSA	:	Mannitol Salt Agar
Mw	:	Molecular weight
NA	:	Nutrient Agar
NaCl	:	Sodium chloride
°C	:	Degree centigrade
NSS	:	Normal Saline Solution
p mol	:	Pico mole
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
Pfu	:	Plaque forming units
pH	:	- Log hydrogen ion concentration
rpm	:	Revolutions per minute
TAE	:	Tris Acetate EDTA
Taq	:	*Thermus aquaticus*
Tris	:	Tris-hydroxy methyl aminoethane
UV	:	Ultraviolet
V	:	Volts
Viz.	:	Namely
w/v	:	Weight by volume

Introduction

1 Introduction

Staphylococcus aureus is a gram-positive coccal bacterium frequently present as commensal in the nostrils, respiratory tract, and on the skin. *S. aureus* is reported as opportunistic pathogen commonly associated with humans and animals, and is capable of causing serious diseases and death including sepsis, pneumonia, meningitis in humans and mastitis, keratitis, osteomyelitis in animals (Jensen *et al.*, 2015).

S. aureus is primarily transmitted through direct contact with a colonized or infected individual or through a fomite intermediate (Kazakova *et al.*, 2005; Miller & Diep, 2008; Chambers and DeLeo, 2009). Hands are the critical disseminator, particularly of healthcare workers (Bhalla *et al.*, 2004; Gordon & Lowy, 2008). Fomites become contaminated through hand-to-fomite contact, bioaerosol settling, or splashing/contamination with infectious fluids. Once established on a surface, *S. aureus* can survive for weeks to months (Dietze *et al.*, 2001; Sexton *et al.*, 2006; Williams & Davis, 2009).

S. aureus possesses a variety of adhesion mechanisms that facilitates their adhesion to different materials including the biomaterial surface (Herrmann *et al.*, 1993). Once the bacterium attaches to a surface, it is difficult to dislodge them (Gristina, 1994) as teichoic acids present on the cell wall of *S. aureus* carry a negative charge, and have a key role in the first step of biofilm formation (Gross *et al.*, 2001). Biofilm formation is a two-step process that requires the adhesion of bacteria to a surface followed by cell-cell adhesion, forming multiple layers of the bacteria (Cramton *et al.*, 1999). Once a biofilm has formed, it can be very difficult to treat clinically and to removal from the other surfaces because the bacteria in the interior of the biofilm are protected from recommended dose of antibiotics and disinfectants (Hoyle and Costerton, 1991).

Bacteriophages (or phages), small viruses of about 20–200 nm in size, are probably the most ancient and ubiquitous existing organisms on Earth. The word "bacteriophage" means to eat bacteria, and is so called because virulent bacteriophages can cause the compete lysis of a susceptible bacterial culture. Bacteriophages, like bacteria, are very common in all natural environments and they represent the most

abundant 'life' forms on Earth, with an estimated 10^{32} bacteriophages on the planet (Wommack & Colwell, 2000).

Bacteriophages were discovered more than a century ago. In 1896, Ernest Hanbury Hankin, a British bacteriologist, reported that something in the waters of rivers in India had unexpected antibacterial properties against cholera and this water could pass through a very fine porcelain filter and keep this distinctive feature (Hankin, 1896). In 1915, the British bacteriologist Frederick Twort, Superintendent of the Brown Institution of London, discovered a small agent that killed colonies of bacteria in growing cultures but the subsequent work was interrupted by the beginning of World War I and shortage of funding. Further, in 1917, Felix d'Herelle at the Pasteur Institute in France discovered the agent killing bacteria and observed that cultures of the dysentery bacteria disappear with the addition of a bacteria-free filtrate obtained from sewage. Since, then many scientists have been involved in developing techniques to study phages and using them for various purposes.

As, phage is the most abundant organisms found in the biosphere it can be classified on the basis of morphology, nucleic acid characteristics, and properties, although other factors such as host spectrum, virus-host interaction, and immunological features should be considered (Henry *et al.*, 2015). The key classification factors of phage are morphology and nucleic acid properties. Concerning morphology, there are phages with icosahedral protein capsid and no tail, phages with contractile tails, phages without contractile tails, and filamentous phages. The phages with or without tail belonged to the order *Caudovirales* which constitute 96% of phages prevalent in the biosphere. the order caudovirales have three family viz: *Siphoviridae*, *Myoviridae* and *Podoviridae* which can be distinguished by their tail morphologies. In all characterized phage 60% of the characterized phages belong to the family *Siphoviridae*, with long, flexible tails; 25% to *Myoviridae*, with double-layered contractile tails; and 15% to *Podoviridae* with short and stubby tails (Ackermann, 2007). The phage genome can be represented by either DNA or RNA and majority of phages contain double strand DNA (dsDNA), while few phage have ssRNA, dsRNA, or ssDNA.

All known phages can be divided into two groups depending on the type of infection, i.e., lytic and lysogenic or temperate infection. Lytic phages causes lysis of the bacterial cell at the end of their life cycle, disrupting its metabolism and releasing

newly formed phage particles. During the lytic cycle, an ecliptic phase occurs where the bacterial cell does not contain any complete virion but the virus replicates and both the host and early viral components are involved. Later on the viral proteins, necessary for new particle assembly formation and lysis of the cell, are formed during maturation (Danner and Belasco, 2001). During the lysogenic cycle, the viral genome does not take control of host machinery but remains inside the cell and replicates together with the host genome in order to generate clones of infected cells, which are then able to grow and divide for long periods of time. Latent forms of the viral genome are named "prophage" (Danner and Belasco, 2001).

Phages offer a number of advantages viz., high specificity to target their host determined by bacterial cell wall receptors, leaving untouched the remaining microbiota, a property that favours phages over other antimicrobials that can cause microbiota collateral damage; self-replication and self-limiting, meaning that low or single dosages will multiply as long as there is still a host threshold present, multiplying their overall antimicrobial impact; as bacteria develop phage defense mechanisms for their survival, phages continuously adapt to these altered host systems; low inherent toxicity, since they consist mostly of nucleic acids and proteins; phages are relatively cheap and easy to isolate and propagate but may become time consuming when considering the development of a highly virulent, broad-spectrum, and nontransducing phage and have prolonged shelf life. These special characteristics of lytic bacteriophages, make them excellent tools for various applications (Rohwer and Edwards, 2002; Hagens and Loessner, 2010; Mahony *et al.*, 2011).

Phages represent great diversity in their host range (Wommack and Colwell, 2000; Srinivasiah *et al.*, 2008). They exhibit a narrow host range, which is usually restricted to a single genus of bacteria (Ammann *et al.*, 2008), more frequently restricted to either a limited number of species within a genus or to a limited number of bacterial strains within a species (Jarvis *et al.*, 1991). However, the best lytic phages for biocontrol applications are those that have broadest host range known as polyvalent or wide host range bacteriophages (Bielke *et al.*, 2007a).

Phages were discovered well before the development of antibiotics. The idea of using phage as a potential therapeutic tool has been around for as long as phage have been known to exist as some eastern European countries continued using phage as medical treatments and in some countries physicians still regularly practice phage

therapy (Summers, 2001; Sulakvelidze and Kutter, 2005). Bruynoghe and Maisin (1921), first described the efficacy of bacteriophages in the treatment of a staphylococcal skin infection by injecting the phage preparation around surgically opened lesions with regression of infection within 24–48 hours.

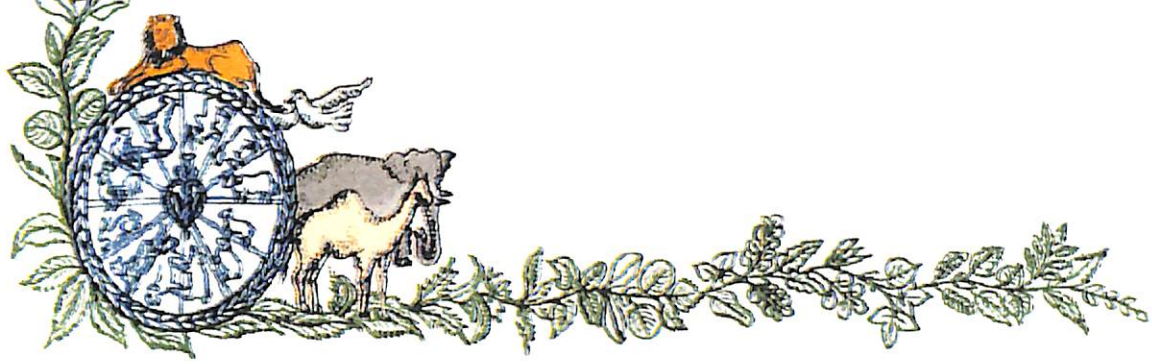
Recently, phages are reported for different applications that includes a reduction of pathogens colonization in animals during primary production (phage therapy), reduction of colonization on foods (biocontrol) during industrial food processing, lyse hosts at low temperatures (biopreservation) and viable cell reductions of biofilm formed by spoilage and pathogenic bacteria (biosanitation).

In recent years, there is an increasing consensus about improvement in the disinfection and sanitization of environmental surfaces in healthcare and food processing industries (Rutala and Weber, 2013; Donskey, 2013; Dancer, 2014, Han *et al.*, 2015). However, there are a number of areas of disagreement and controversy regarding best practices for disinfection and sanitization of environmental surfaces that can be resolved by the use of biological agents. A novel approach to reduce the need for harsh chemical sanitizers is the use of lytic bacteriophage(s) as biocontrol agents, which relies on the lytic activity of environmentally isolated lytic bacteriophages that are capable of killing specific bacterial strains or a subgroup of strains, usually within the same genus. The use of phage for biosanitation is promising although very challenging due to the diversity of bacteria found in different settings. There are limited research trials conducted at few places of the world regarding application of lytic bacteriophages as biosanitizer against different hosts (Abuladze *et al.*, 2008; Rashid *et al.*, 2012; Chen *et al.*, 2013; Woolston *et al.*, 2013; Jensen *et al.*, 2015). Thus, keeping in the view of this introduction, the present work was designed with the following objectives:

Objective:

1. To isolate and characterize *S. aureus* lytic bacteriophages.
2. To determine the potential of lytic phage(s) as surface sanitizer.

Review of Literature



2 Review of Literature

Staphylococcus aureus is a Gram-positive, spherical coccus having a diameter of 1- 1.3µm. On microscopic examination, the organisms appear in clusters, like bunches of grapes. *S. aureus* is responsible for food-borne infections (Garcia-Alvarez *et al.*, 2011) and various other disease conditions like, wound infections, septicaemia and toxic shock syndrome. Besides, skin pustules, impetigo, osteomyelitis, renal abscess, pneumonia, endocarditis, meningitis, gastroenteritis and sometimes serious conditions in patients undergoing hemodialysis, diabetis mellitus etc. may also be caused by *S. aureus* (Lewis and Jorgensen, 2005). About 60% of human population is estimated to be colonized by *S. aureus* and 20% of humans are persistent carriers. The nose is most favourable site (Zorgani *et al.*, 2009) but the organism can also survive on the skin and in the environment for a long time. Colonization of methicillin resistant *S. aureus* (MRSA) also occurs at sites other than nose e.g. pharynx, axilla, rectum, perineum (Eveillard *et al.*, 2006) which might play an important role in the transmission of infection. Until recently, MRSA has been primarily considered as nosocomial infection, acquired in hospital settings mainly affecting healthcare workers (Zorgani *et al.*, 2009).

S. aureus are also involved in the formation of biofilm on contact surfaces. Formation of biofilm is a two-step process that requires the adhesion of bacteria to a surface followed by cell-cell adhesion, forming multiple layers of the bacteria (Cramton *et al.*, 1999). Biofilms are aggregates of microbial cells surrounded by a matrix of exopolymers, which confers resistance to the microorganisms (Costerton *et al.*, 1999). Once a biofilm has formed, it is difficult to clinically treat because the bacteria in the interior of the biofilm are protected from antibiotics and phagocytosis (Hoyle and Costerton, 1991). Bacteria that aggregate to form biofilms are known to possess greater resistances to stress conditions than their planktonic counterparts, which are dispersed in the environment, including the susceptibility to sanitizers and other antimicrobials (Fux *et al.*, 2004). To remove biofilm organisms, the solution of sanitization must penetrate the matrix of exopolymers and gain access to the microbial cells causing biofilm inactivation and removal.

2.1. History of bacteriophages

In 1896, E. H. Hankin, a British bacteriologist demonstrated that the waters from the Indian rivers Ganga and Yamuna contained a biological principle that destroyed cultures of cholera-inducing bacteria. He proposed that an unidentified chemical substance was responsible for the decline in the spread of cholera (Hankin, 1896). A few years later, other researchers made similar observations although they did not investigate their findings further (Sulakvelidze *et al.*, 2001). Nearly 20 years after Hankin's report, Frederick W. Twort reported on a phenomenon referred to as the 'glassy transformation' while working with Vaccinia virus which had been contaminated by micrococci. He concluded that the glassy transformation was caused by an infectious agent that killed bacteria and multiplied itself in the process (Duckworth, 1976). Later on, in 1917, Felix d'Herelle independently discovered ultra viruses that resulted in the death of bacteria while studying patients suffering or recovering from bacillary dysentery (Summers, 2001). He proposed the name 'bacteriophage' from 'bacteria and phagein' (Phagein - Greek word for 'to eat or devour') therefore, implying that bacteriophages eat or devour bacteria. D'Herelle believed that a phage was an obligate parasite which is particulate, invisible, filterable, and self-reproducing in nature (Stent, 1963). He also introduced the term plaque to describe the circular area of clearing caused by infection of a single phage on double-layered agar plates. The first report of the therapeutic use of phage was a note by Bruynoghe and Maisin (1921) from Louvain, who noticed reduction in swelling and pain after injection of staphylococcal phage in the local region of cutaneous boils. In 1925, Bordet and Bail discovered the phenomenon of lysogeny or bacteriophages may infect bacteria without the induction of lysis. They confirmed the idea that the capacity of reproducing phages within bacteria necessitated the insertion of phage-encoded material into the hereditary units of the host microbe. Frank Macfarlane, an Australian scientist awarded the Nobel Prize in 1960 for his work on immunity, also worked on lysogeny and confirmed the viral nature of phages as well as the nature of its interactions with bacterial hosts (Sankaran, 2010).

Schlesinger confirmed the biochemical nature of phages made of nucleoproteins allowed the existing theories to join together: phages are viral particles that are made of nucleoproteins (Pennazio, 2006). Finally, the invention of the electron

microscope (EM) allowed Helmut Ruska, a German doctor, to first describe round particles as well as "sperm-shaped" particles from a phage suspension adhering to a bacterial membrane (Ruska, 1940). One year later, Luria and Anderson visualized different types of phages and described their common structure: a non-homogeneous round head with a much thinner tail, giving the peculiar sperm-like appearance (Luria, 1943). They also described the various stages of bacterial lysis: adsorption which, increased with time, extensive bacterial damage and appearance of a large number of newly formed bacteriophages. The study of phages played a central role in some of the most significant discoveries in biological sciences, from identification of DNA as the genetic material, over the deciphering of the genetic code and the phenomenon of restriction and modification, to the development of molecular recombinant technology. Phage-derived proteins are currently being used as diagnostic agents (Smith *et al.*, 2001), therapeutic agents (Loeffler *et al.*, 2001; Schuch *et al.*, 2002) and for drug discovery (Liu *et al.*, 2004). Further, the bacteriophages have also been recognized for their potential use along the food chain towards an increased food safety as biotherapy, biosanitation, biopreservation and biocontrol agents (Coffey *et al.*, 2010; Mahony *et al.*, 2011; Sillankorva *et al.*, 2012).

2.2. Bacteriophage biology

Bacteriophages are the most abundant forms of life on the planet with an estimated 10^{31} phages in the biosphere (Sulakvelidze and Kutter, 2005). The phages measure between 20 and 200 nm (Ackermann and DuBow, 1987). Like other viruses, phages are infectious particles that have a genome comprising nucleic acid surrounded by protein subunits that form a protective capsid (Ackermann, 2003). It was suggested that the capsid plays important role in the phage life cycle as protecting the phage genome from DNA-degrading enzymes; effecting phage adsorption to a susceptible bacterium and uptake of the phage genome into the cytoplasm of the infected bacterium (Gill and Abedon, 2003). The capsid encloses a single copy of the genome, which is usually one molecule of double-stranded DNA, single-stranded DNA, double-stranded RNA, or single-stranded RNA (Kutter *et al.*, 2005).

In the tailed phages, the tail is a hollow tube, through which the phage nucleic acid passes during infection. The size of the tail can vary and some phages do not have

a tail structure. In some phages, the tail is surrounded by a contractile sheath, which contracts during infection of the bacterium. At the end of the tail, the more complex phages, like T4, have a baseplate and one or more tail fibers attached to it. Tail fibers contain proteins that recognize molecules on the surface of bacterial cell walls, which provide the ability to attach only to host cells (Guttman *et al.*, 2005; Ackermann, 2009).

Phages may be categorized by their morphology into tailed, polyhedral (icosahedral or quasi-icosahedral bodies), filamentous and pleomorphic phages (Ackermann, 2012). Tailed phages represent 96% of known phages and belong to the order *Caudovirales* and separated into three main phylogenetically related families. The three families of the tailed phages are *Myoviridae* with contractile tails consisting of a sheath and a central tube (25% of tailed phages); *Siphoviridae*, long, noncontractile tails (61%) and *Podoviridae*, very short tails (14%). The majority of bacteriophages are stable in a wide range of environmental conditions as the most tailed phages are stable in the pH range from 5 to 9 and are inactivated by heating at 60°C for 30 min (Ackermann, 2007).

2.3. Life cycle of bacteriophage

The phage life cycle can be one of two types, the productive or virulent cycle and the temperate or lysogenic cycle. Accordingly, phages are classified as lytic (virulent) or lysogenic (temperate).

Lytic/virulent cycle: Lytic phages infect bacterial cells causing inhibition of host metabolism and production of phage progeny. The lytic cycle results in the lysis of the bacterium accompanied by the release of multiple phage particles. The new progeny phages produced by the host bacterium spread to infect other cells. The time for the whole cycle is usually within 1–2 h and the number of phage produced depends upon the phage type (Guttman *et al.*, 2005). The typical lytic cycle of phages consists of the sequential steps of adsorption, penetration, latent period, maturation, and lysis.

Lysogenic cycle: Some phages infect cells and incorporate their nucleic acid into the genome of the host cell or exist as an episomal element, leading to a permanent association as a prophage with the cell and all its progeny. During

lysogeny, phages neither produce virions nor lyse bacteria. These phages are called temperate, and the cells that harbor a prophage are known as lysogenic. The lysogenic relationship between a temperate phage and its host bacterium provides a safe home to the temperate phage genome, blocks replication of nonvirulent homologous phages, and has the potential to alter the phenotype of the host cell (Gill & Abedon, 2003). The lysogenic host bacterium may carry prophage for many generations until it is reactivated and produce new copies of phages that lead to lysis and release of progeny phages. The mechanism of this reactivation varies between phages, but is usually triggered when the host cell is placed under adverse environmental conditions (Strauch *et al.*, 2007).

2.4. Isolation and characterization of *S. aureus* bacteriophages

Garcia *et al.* (2009) assessed the prevalence of bacteriophages infecting *S. aureus* in dairy samples by using fourteen *S. aureus* strains as enrichment cultures of 75 dairy samples and reported that all samples grew specific *S. aureus* bacteriophages. According to the host range, 8 different phages were isolated and 3 of them, phages PhiH5, PhiG7, and PhiA72, were found in 89% of the samples; all the isolated phages were temperate.

Yoon *et al.* (2013) isolated six *S. aureus* phages from soils of poultry/livestock farms and selected two phages, designated SP5 and SP6 based on the results of host range determination with 150 *S. aureus* strains and restriction enzyme treatment of phage DNA, for further characterization and genome sequencing. Both SP5 and SP6 were classified as members of the family *Siphoviridae*.

Li and Zhang, (2014) studied isolation and characterization of a highly virulent phage SPW from wastewater of dairy farm, which possessed a strong lytic capability against mastitis-associated *S. aureus*. The phage SPW produced large, round and clear plaques on bacterial culture plates and had an icosahedral head 62.5 nm in diameter and long tail of 106 nm, head and tail classified as a member of the *Myoviridae* family. Restriction analysis indicated that phage SPW was a dsDNA virus with an approximate genome size of 65–69 kb. The phage SPW was relatively stable in a wide range of temperature and pH values and resistant to chloroform and isopropanol. The optimal multiplicity of infection (MOI) was 0.01. When phage SPW was used to infect five other clinically isolated pathogenic isolates, it showed relatively wide spectrum

host range thus reported with potential for an effective approach to prophylaxis or treatment of *S. aureus*-associated mastitis in dairy cows.

Chang *et al.*, (2015) isolated and characterized a novel bacteriophage SA97 that infects *S. aureus*. The phage SA97 belongs to the *Siphoviridae* family, and the cell wall teichoic acid was found to be a host receptor of the phage SA97. They suggested that the phage SA97 may be a promising candidate for control of *S. aureus*.

Mohammed-Ali and Jamalludeen, (2015) isolated and characterized the phages effective against a wide range of methicillin resistant *S. aureus* (MRSA). They adopted enrichment method and used a mixture of ten MRSA isolates for isolation of phage from wastewater treatment plants. They characterized three phages and reported that all three phages belonged to the *Siphoviridae* family with long non-contractile flexible tails. They concluded that all three phages have a wide host range against *S. aureus* and are suitable candidates for future use in phage therapy against MRSA.

Rasool *et al.* (2016) examined a total of 15 sewage water samples for isolation of phage, collected from different areas located in and around Faisalabad. The isolated phage phage pq/48 exhibited a good lytic activity against MRSA when tested in-vitro, and the highest activity was attained within three to six hours of phage infection. The protein analysis using SDS-PAGE of this phage revealed 10 proteins of between 20 kDa and 155 kDa in size. They concluded that bacteriophages can be further characterized and appear to be a promising candidate for phage therapy against MRSA in the future.

Estrella *et al.* (2016) isolated and identified 7 novel phages with broad lytic activity for *S. aureus*. They screened the phages with a diverse collection of 170 clinical isolates by efficiency of plating (EOP) assays and reported that the phages effectively prevent growth of 70– 91% of MRSA and methicillin sensitive *S. aureus* (MSSA) isolates. The results electron microscopy indicated that the phage belonged to the *Myoviridae* family of viruses and were distinct from, but closely related to, phage K as characterized by restriction endonuclease analysis. Furthermore, they reported that the growth rate analysis via OmniLog microplate assay indicated that a combination of phage K, with phage SA0420□1, SA0456□1 or SA0482□1 have a synergistic phage-mediated lytic effect on MRSA and suppress formation of phage resistance. Finally, they concluded that a broad spectrum lytic phage mixture can

suppress the emergence of resistant bacterial populations and hence have great potential for combating *S. aureus* wound infections.

Rahimzadeh *et al.* (2017) isolated and characterizeed two MRSA phages from the sewage at a tertiary pediatric hospital. They performed the electron microscopy and reported that both MRSA phages' resembled to members of the family *Siphoviridae*, serogroups A and F. They exhibited a latent period of 70 minutes and with a burst size of 2,400 plaque-forming units (PFU)/infected cell.

Cui *et al.* (2017) isolated *Staphylococcus* phage JD007 and classified as belonging to the *Myoviridae* family based on its morphology, as observed by transmission electron microscopy. They reported that the lytic activity of phage was stable between pH 5–11 and below 42 °C and was able to lyse 95% of *S. aureus* isolates, including the prevalent ST239-MRSA and ST59-MRSA strains isolated from different hospitals in Shanghai, China.

2.5. Application of bacteriophages

Bacteriophages generally exhibit a narrow host range, which is usually restricted to one genus of bacteria (O'Flaherty *et al.*, 2005; Ammann *et al.*, 2008), but more frequently restricted to either a limited number of species within a genus or to a limited number of bacterial strains within a species (Jarvis *et al.*, 1991). The best virulent bacteriophages for biocontrol applications are those with the broadest possible host range. These are termed polyvalent bacteriophages (O'Flaherty *et al.*, 2005) or WHR (wide host range) bacteriophages (Bielke *et al.*, 2007a) as they are usually active against many species within a bacterial genus. Thus they can be applied to specifically target and eliminate that genus in foods or other environments.

To lyse the host, bacteriophages can be used by two different approaches, either as passive treatment or active treatment (Biological Hazards Panel, 2009). In passive treatment, bacteriophages are added in sufficient quantities to lyse all target organisms by primary infection "lysis from without" (lysis of the target cell without phage replication). This happens when sufficiently high numbers of phage particles adhere to the cell, and lyses it through alteration of the membrane electric potential, and/or the activity of cell wall degrading enzymes. Although, this treatment requires much higher number of bacteriophages, they should be able to eliminate even sparse

population of target bacteria. The other advantage of this treatment is that, the natural resistance due to restriction enzymes present in host bacteria will not be seen because the elimination is as a result of "lysis from without" phenomenon. By the application of this method, several bacterial taxa can be susceptible, which may not normally be susceptible to bacteriophages because the attachment antigen may be shared between them.

In active treatment a relatively small dose of bacteriophages is required for efficacious elimination of the target bacteria as a result of phage replication "lysis from within". In this treatment the timing of bacteriophage application and the number of host cells is important which, must be in excess of a predicted critical replication threshold to propagate enough bacteriophages to kill all target cells. If this threshold is not achieved the bacteriophages are unable to multiply and may disappear.

Phages offer several advantages as biocontrol agents for several reasons: (i) high specificity to target their host determined by bacterial cell wall receptors, leaving untouched the remaining microbiota, a property that favours phages over other antimicrobials that can cause microbiota collateral damage; (ii) self-replicating and self-limiting, meaning that low or single dosage will multiply as long as there is still a host threshold present, multiplying their overall antimicrobial impact; (iii) as bacteria develop phage defense mechanisms for their survival, phages continuously adapt to these altered host systems; (iv) low inherent toxicity, since they consist mostly of nucleic acids and proteins; (v) phages are relatively cheap and easy to isolate and propagate but may become time consuming when considering the development of a highly virulent, broad-spectrum, and non transducing phage; (vi) phages can generally withstand food processing environmental stresses including food physiochemical conditions; (vii) phages have been proved to have prolonged shelf life.

2.6. Phage as bio sanitizer national and International scenario

Bacteriophage effectively destroy the bacteria, while leaving the rest of the body unharmed, as opposed to current sanitizers that rely on high concentrations of alcohol that destroy all bacteria, including the good kind. A bateriophage sanitizer would thus be more healthful in the long run.

Abuladze *et al.* (2008) studied a bacteriophage cocktail of 3 *Myoviridae* phages (ECP-100) lytic for *Escherichia coli* O157:H7 to reduce experimental contamination of hard surfaces (glass coverslips and gypsum boards), tomato, spinach, broccoli, and ground beef by three virulent strains of the bacterium. They reported a significant reduction of 99.99%, 98%, and 94% after 5 min of treatment at concentration of 10^{10}, 10^{9}, and 10^{8} PFU/ml, respectively, from the glass coverslips and reductions of 100%, 95%, and 85% at similar concentration from the gypsum board surfaces.

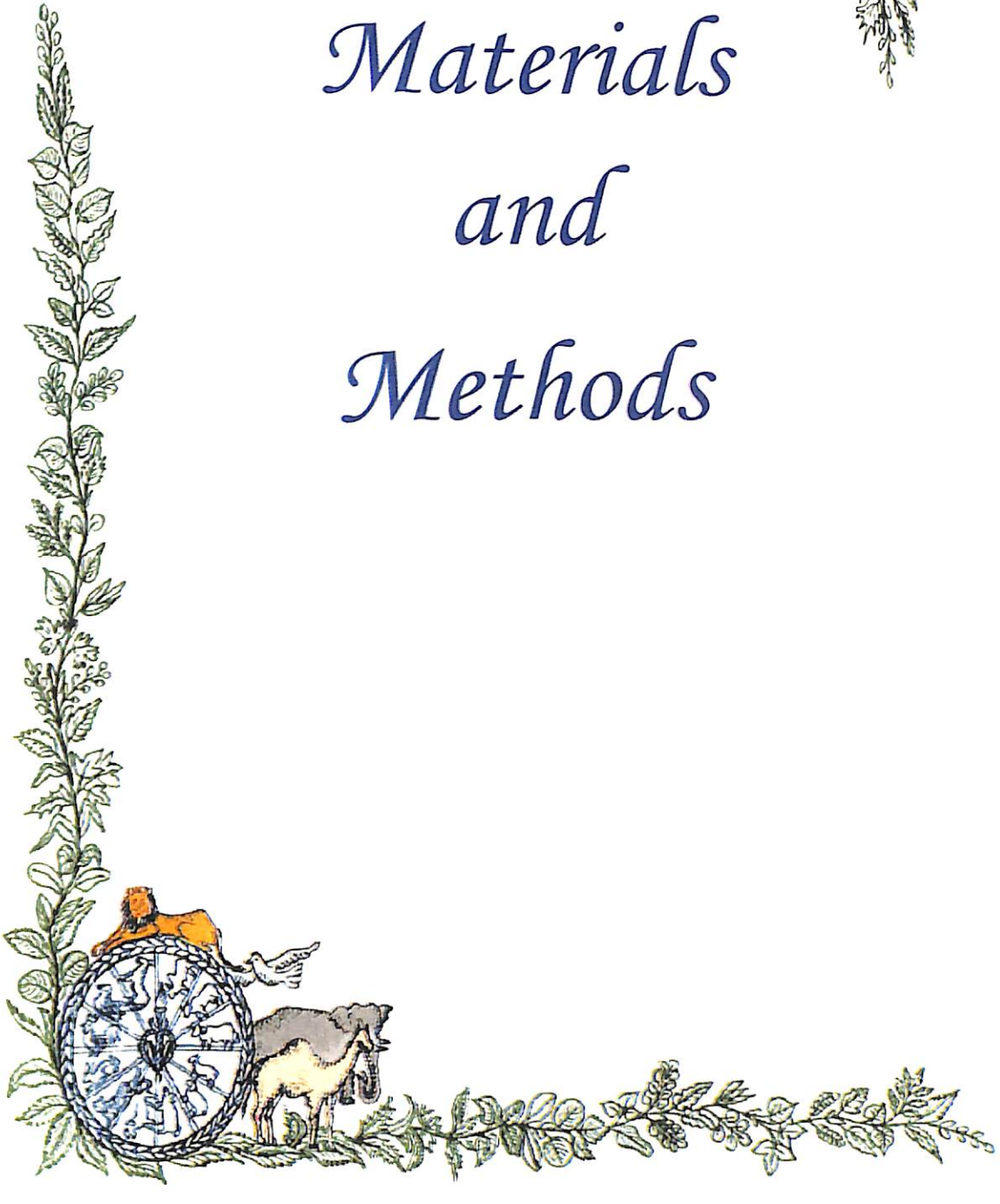
Rashid *et al.* (2012) studied by application of *Yersinea pestis* phage (YPP - 100) on three different hard surfaces (glass, gypsum board and stainless steel) experimentally contaminated with a mixture of three genetically diverse *Y. pestis* strains (CO92, KIM and 1670G). They reported that at concentration 10^{9}, 10^{8} and 10^{7} PFU/mL after 5 minutes exposure period completely eliminate all viable cells.

Woolston *et al.* (2013) studied by the use of a cocktail of six lytic bacteriophages, (SalmoFresh), for reduction of *Salmonella* Kentucky and *S.* Brandenburg from stainless steel and glass surfaces and reported > 99% reduction in the number of both organisms (2.1- 4.3 log) from both surfaces.

Chen *et al.* (2013) studied for control of Multidrug-resistant *Acinetobacter baumannii* (MDRAB) by addition of ϕAB2 phage at a concentration of at least 10^{5} PFU/ml. They reported that at 10^{5} PFU/ml >99.9% of *A. baumannii* M3237 was lysed after 5 min, regardless of concentration of bacteria. They further reported that the addition of ϕAB2 phage at a concentration of 10^{8} PFU/slide (>10^{7} PFU/cm^{2}) to glass slides containing *A. baumannii* M3237 at 10^{4}, 10^{5}, or 10^{6} CFU/slide, significantly reduced bacterial numbers by 93%, 97%, and 99%, respectively and they recommended the use of >10^{7} PFU/cm^{2} for decontamination of glass surfaces.

Jensen *et al.* (2015) isolated 12 phages lytic against *S. aureus* and MRSA and performed experiments to assess the utility of some phage to decontaminate fomites (glass and cloth) and reported a significant reduction in colony forming units of MRSA following phage treatment, including tests of a phage cocktail against a cocktail of MRSA isolates. They finally concluded that phage treatment can be used as an effective tool to decontaminate human MRSA from both hard surfaces and fabrics.

Materials and Methods



3 Materials and Methods

3.1.1 Aim of study

The aim of study was to isolate and characterize bacteriophage (s) against *Staphylococcus aureus* and evaluation of bacteriophage (s) as surface sanitizer.

3.1.2 Bacterial strains

All *S. aureus* isolates included in the study were available in the Department of Veterinary Public Health and Epidemiology, Bihar Veterinary College, Patna. A total of forty two (42) *S. aureus* and sixty seven (67) Methicillin resistant *S. aureus* (MRSA) isolates from different sources were used for evaluation of lysis efficiency or host range determination of bacteriophage (s) (Table 1). All *S. aureus* isolates were tested for their purity, morphology, biochemical characteristics and confirmation by molecular method.

3.1.3 Media, buffers and reagents

The media and chemicals used in this study were procured from reputed firms. Some of them included mannitol salt agar (Himedia, India), nutrient agar (Himedia, India), nutrient broth (Himedia, India), agar powder purified (Himedia, India) and trypticase soya broth (Himedia, India). Saline magnesium buffer (Himedia, India), TAE buffer (Thermoscientific, EU), PBS buffer, peptone water (Himedia, India).The detail of the preparation of media, buffers and reagents used in this study were listed in Appendix 1.

3.1.4 Chemicals used for molecular studies

All the chemicals used in this study were of molecular biology grade. Chemicals were procured from Amresco (USA), Qiagen (Germany), ThermoScientific (USA), Fermentas (India), SRL (India), Xcelris (India) and other reputed firms.

The chemicals used in bacteriophage study included magnesium sulphate (S. D. fine chem. Pvt ltd), calcium chloride (Merck), gelatin (Milk Elisa kit PD-ADMAS,

Table 1. List of Methicillin sensitive and Methicillin resistant *S. aureus* isolates used

SL. No.	Source of isolates	Methicillin resistant *S. aureus* isolates	Methicillin sensitive *S. aureus* isolates
1.	**Buffalo nasal carriage**	17B(N), 189B(N), 190B(N), 207B(N), 218B(N), 226B(N), 243B(N), 253 B(N)	115B(N),187B(N),197B(N),200B(N),203B(N), 211B(N),213B(N),222B(N),255B(N)
2.	**Buffalo skin carriage**	19B(S), 116B(S), 198B(S), 199B(S), 210B(S), 214B(S), 244B(S), 250B(S)	196B(S),221B(S),256B(S)
3.	**Cattle nasal carriage**	10C(N), 174C(N), 176C(N), 178C(N), 180C(N)	1C(N),135C(N),148C(N),156C(N)
4.	**Cattle skin carriage**	46 C(S), 165C(S), 171C(S)	139C(S),143C(S),147C(S),149C(N),155C(S), 163C(S),175C(S),181C(S)
5.	**Dog nasal carriage**	6 D(N), 8D(N), 11D(N), 20D(N), 21D(N), 28D(N), 33D(N), 35D(N), 38D(N), 43D(N), 51D(N)	48D(N),69D(N),103D(N),106D(N)
6.	**Dog skin carriage**	3D(S),7D(S),13D(S),16D(S),29D(S),32D(S),39D(S),44D(S),49D(S),52D(S),58D(S),95D(S),101D(S)	83D(S),89D(S),104D(S),107D(S)
7.	**Dog Wound**	4D(W),42D(W)	5D(W)
8.	**Livestock handler**	18(H),47(H),159H,183H,231H	167(H),169(H),230(H),236(H),242(H),258(H), 261(H)
9.	**Dog handler hand swab**	15D(H),27D(H),30D(H),37D(H),40D(H),41D(H),53D(H), 59D(H),71D(H),80D(H),99D(H),108D(H)	90D(H),105D(H)

Bangarori), PEG 8000 (Amresco, USA), chloroform (Qualigens, India) and sodium chloride (Himedia, India).

The chemicals used in PCR study included Dream *Taq* DNA polymerase (Thermoscientific), 10 x PCR buffer (Fermentas), dNTP mixture (Fermentas). 6X gel loading dye (Thermoscientific), Gene ruler 100 bp plus DNA ladder (Thermoscientific), Gene ruler 1.0 kb ladder plus ruler (Thrmoscientific), λ DNA/HindIII (Thermoscientific), nuclease free water (Thermo Scientific, USA), and ethidium bromide (Himedia, India).

The chemicals used in SDS PAGE study included Tris base (Himedia, India), bromophenol blue (Himedia, India), β-mercaptoethanol, ammonium persulfate (Himedia, India), coomassie brilliant blue G-250 (Himedia, India) , methanol (Qualigens), glacial acetic acid (SRL, India), acrylamide (Himedia, India), bis-acrylamide(Himedia, India), TEMED (Himedia, India), sodium dodecyl sulphate (Qualigens, India) and 4 colour prestained ladder (Puregene, USA).

The firms associated with other chemicals include sodium hydroxide pellets (Himedia, India), hydrochloric acid (NICE), tris base (Himedia, India), sodium dihydrogen phosphate (MERCK,India), disodium hydrogen phosphate(MERCK, India), potassium dihydrogen phosphate (MERCK, India), potassium chloride (Himedia, India), and ethylene diamine tetra acetic acid (Himedia, India) .

3.1.5 Kits and enzymes

The molecular biology kit DNeasy blood and tissue kit Qiagen (Germany) and XcelGen, Bacterial gDNA mini kit (Xcelris) were used in the study for isolation of genomic DNA of bacteria.

3.1.6 Plasticwares and glasswares

Plasticwares used in this study were procured from Himedia (India), Tarson (India), and glasswares were obtained from Borosil (India), plasticwares and glassware's were thoroughly washed and sterilized wherever necessary as per the standard procedures.

3.1.7 Equipments

Some important equipment used in the study were electronic balance (Denver, USA), centrifuge (REMI, India), deep freeze (-20°C) (Blue Star, India), gel documentation system (Biorad, Japan), Incubator (Remi, India), pH meter (Labman), variable pipette set (Thermoscientific), horizontal gel electrophoresis apparatus (Thermoscientific, China), vertical SDS-PAGE apparatus (Tarson, Imdia), refrigerated centrifuge (Remi, India), shaker incubator (Julabo-Shake Temp, SW22, Switzerland), water bath (YSI, India), vortexing (Tarson, India), autoclave (Instrumentation India, India), and water distillation apparatus (Millipore, India), laminar air flow bench (Ikon instruments, India), microscope (Olympus, India) and microwave oven (LG, India).

3.1.8 Oligonucleotide Primers

Primers used in the study were custom synthesized from Xcelris and Eurofins Genomics Pvt. Ltd. (India).

Table 2: List of primers used in the study

SL No.	Primers	Neucleotide base 5'-3'	Product size	Reference
1	16SrRNA F	GTAGGTGGCAAGCGTTATCC	228 bp	Karmakar *et al.*, 2016
	16SrRNA R	CGCACATCAGCGTCAG		
2	RAPD5	AACGCGCAAC		Gutierrez *et al.*, 2011

3.2 METHODS

3.2.1 Revival of the isolates

A total of forty two (42) *S. aureus* and sixty seven (67) Methicillin resistant *S. aureus* (MRSA) isolates present in department were revived by inoculation of a loopful of preserved culture into Nutrient broth with overnight incubation at 37°C. All isolates from nutrient broth were further streaked on mannitol salt agar with overnight incubation at 37°C and observed for development of yellow colour mannitol fermenter

colonies. A single colony from mannitol salt agar was used for biochemical and molecular confirmation.

3.2.2 Characterization of *Staphylococcus* isolates

3.2.2.1 Biochemical characterization

All *S. aureus* isolates were processed for microscopic examination after Gram staining and further confirmed by catalase and tube coagulase test using human plasma as per methods described by Agarwal *et al.* (2003).

The catalase test was performed by taking a fresh colony on a clean and dry glass slide and placing a drop of 3% H_2O_2 on the colony. The evolution of oxygen bubbles after addition of 3% H_2O_2 was considered as positive reaction for catalase test.

Tube coagulase test was performed on overnight grown culture in 0.8 ml of TSB-S followed by addition of 0.2 ml of human plasma of EDTA-blood. The mixture was kept in water bath preheated at 37^0C and observation was made after 4 h. If the test remains negative until four hours at 37^0C, the mixture was kept at room temperature and observed after 24 h.

3.2.2.2 Molecular characterization

3.2.2.2.1 Isolation of genomic DNA:

The isolation of bacterial nucleic acid was performed with XcelGen, Bacterial gDNA mini kit (Xcelris) as per the instruction provided by the manufacturer:

1. 1-3 ml fresh culture of *S. aureus* was pelleted by centrifugation at 12,000 rpm for 2 min at room temperature and supernatant was completely discarded.
2. The pellet was resuspended in 180 µl TE Buffer or Elution Buffer.
3. 18 µl of 50 mg / ml lysozyme solution and 5 µl of RNase A were added followed by incubation at 30^0 C for 15-30 min.
4. The cell lysate were centrifuged at 5,000 x g for 5 min at room temperature and the supernatant was discarded with leaving a residue of 10 µl liquid in the tube. The pellet was resuspended by vortexing.

5. 25 mg glass beads and 200 µl of Buffer TL were added and vortexd at maxi speed for 5 min. The beads were allowed to settle down to the bottom of the tube and supernatant was transferred to a new 1.5 ml centrifuge tube.
6. 25 µl of Proteinase K was added and vortexed for 10 seconds. It was spin down briefly and incubated at 55^0 C in a shaking water bath for 30 min.
7. 220 µl Buffer BL was added and briefly vortexed to mix with incubation at 65^0 C for 10 min.
8. 220 µl absolute ethanol was added and mixed thoroughly by vortexing for 20 sec.
9. The entire sample from Step 8 was transferred into a DNA mini column, including any precipitate that may have formed and centrifuged at 10,000 rpm for 1 min to bind DNA. The collection tube with flow-through was discarded.
10. The column was transferred into a new 2 ml tube and 500 µl Buffer KB was added and, centrifuged at 10,000 x g for 1 min. The flow-through was discarded and the collection tube was reused.
11. The column was placed into the same collection tube and washed by adding 650 µl DNA Wash Buffer diluted with ethanol and centrifuged at 10,000 rpm for 1 min.
12. Step 11 was repeated again to remove contaminants.
13. The flow-through was discarded and the column was kept with the lid open, into a new collection tube and centrifuged at maxi speed (10,000 rpm) for 2 min to dry the column.
14. The column was placed into a nuclease-free 1.5 ml microfuge tube and 30 µl of pre- warmed (65^0 C) elution Buffer was added to DNA Mini column. The columns were allowed to incubate at 65^0 C for 2 min and centrifuged at 10,000 rpm for 1 min to elute the DNA. The harvested DNA was stored at -20°C.

The Bacterial DNA were run as per standard protocol (Sambrook and Russell, 2001) on 0.8% w/v agarose gel prepared in 0.5X TBE for submarine agarose gel electrophoresis (at potential difference of 5 volt/cm) to check the integrity and to determine approximate genomic size of nucleic acid. Ethidium bromide was added @ 0.5 µg/ml in melted agarose.

3.2.2.2.2 PCR for confirmation of *Staphylococcus aureus*

A PCR assay was standardized for amplification of 16SrRNA gene fragment of *S. aureus* isolates as per the method described by Karmakar *et al.*, (2016) with some modification. The PCR reaction mixture was prepared in 25 µl reaction volume each containing 2.5 µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM MgCl2), 0.5 µl of dNTP mixture (10 mM each), 2 µl (10 pmol/µl) of forward and reverse primers, 1µl (1 unit) Taq DNA polymerase, 2 µl of bacterial lysate and 15 µl nuclease free water.

The PCR amplification reaction was standardized with an initial denaturation at 94^0C for 5 min, followed by 35 cycles of denaturation at 94^0C for 1 min, annealing at 55^0C for 1 min and elongation at 72^0C for 1 min with a final elongation phase at 72^0C for 5 min.

The amplified product were analysed by agarose gel (1.5%) electrophoresis stained with 0.5µg/ml ethidium bromide. The gel image was visualized and documented in gel documentation system (Biorad).

3.2.3 Isolation, purification, propagation and titration of bacteriophages

3.2.3.1 Collection of samples

A total of 32 samples including livestock farm (ILFC, BVC) sewage, mastitic milk, laboratory drainage of BVC, dog pus and skin swab (TVCC, BVC), and Ganga water (Kali Ghat, Patna) were collected and processed for isolation of bacteriophage by enrichment and non enrichment method.

Table 3: List of samples used for isolation of bacteriophage

Sl. No	Sample source	No of samples
1.	Dog pus	06
2.	Dog skin	04
3.	Ganga water	04
4.	Mastitic milk	04
5.	Livestock farm sewage	10
6.	Emu farm sewage	02
7.	Laboratory sewage	02
Total		**32**

3.2.3.2 Isolation of bacteriophage and host

Approximately 25 ml of sewage water, Ganga River water and mastitic milk samples were inoculated with 2.5 ml of 10X TSB with 10% NaCl into two separate 50 ml pre-sterilized centrifuge tube. However, pus and skin swab samples were inoculated into 25 ml TSB with 10% NaCl.

In enrichment method of phage isolation 0.5 ml of overnight grown culture of *S. aureus* isolate was added before incubation of tube at 37^{0}C for 24 h. After incubation, 1 ml of enriched samples were transferred into 1.5 ml presterilized centrifuge tube and centrifuged at 5000 rpm for 10 min. The supernatant was filtered with 0.22µm syringe filter and phage filtrate was preserved at 4°c.

However, in non enrichment method all tubes were incubated at 37^{0}C for 24 h without inoculation of *S. aureus* culture and enriched samples were used for isolation of *S. aureus* and their phage(s). A loopful of samples was streaked on mannitol salt agar and characteristic colonies from this were confirmed by Gram staining, biochemical tests and 16SrRNA PCR specific for detection of *S. aureus*. Further 1 ml of incubated samples were transferred into 1.5 ml pre-sterilized centrifuge tube and centrifuged at 5000 rpm for 10 min. The supernatant was filtered with 0.22µm syringe filter and phage filtrate was preserved at 4°c.

3.2.3.3 Detection of lytic phages by spot test

To detect phages 100 µl of overnight grown culture of *S. aureus* (For enriched method same isolate that was used for enrichment and for non enrichment method both the isolate i.e., isolate used of enrichment as well as *S. aureus* isolate isolated from the same samples), was mixed with 3 ml of top layer agar and poured onto pre-dried 3% nutrient agar plates and left to dry. Further 10 µl of phage filtrate was spotted over plates and allowed to be absorbed onto the plate. Once dried, plates were incubated at 37°C for 12-24 h and examined for presence of complete lysis/plaque formation/turbid lysis produced by phage. A sample was considered positive for lytic phage on observation of complete lysis/clear plaque/confluent or opalescent lysis, while appearance of turbid zone in the spot was considered negative.

3.2.3.4 Purification of bacteriophage plaque

The clear plaques produced by the sample was purified by serial dilution and plating on soft agar overlays as per the method described by Adams (1959) with slight modifications. The complete lysis zone/plaques formed was extracted using a sterile 1 ml pipette-tip, re-suspended in 1 ml salt magnesium (SM) buffer and incubated overnight at 4°C for eluting the phage in buffer. The phage suspension was filtered through 0.22 µm syringe filter. In order to obtain a suspension of 'pure' bacteriophage, which could be used for propagation, filtered phage suspension was 10 fold serially diluted and 100 µl of diluted phage and 100 µl host bacterium were mixed with 3.0 ml molten soft agar (0.7% agar, w/v) and poured quickly on top of solidified nutrient agar plate (3% agar, w/v). The plates were gently rotated and left to dry at room temperature for 20 min and incubated overnight at 37°C. The single plaque was passaged three times to obtain a single clone of each bacteriophage. The plaque morphology and initial concentration were observed and recorded.

3.2.4 Preparation of high titre phages stocks

3.2.4.1 Broth method

Large volume of high titre bacteriophage was propagated in nutrient broth. Plate harvested phage and overnight grown cultures were added in 1:10 ratio to nutrient broth and incubated at 37°C for 24 h and then left at room temperature for 2-3 days. This phage-bacteria suspension was then centrifuged at 7000 rpm for 10 min and then filtered through 0.45 µm syringe filter followed by 0.22 µm syringe filter into a 50 ml centrifuge tube and stored at 4°C for further use.

3.2.4.2 Titration of bacteriophage

To 900 µl of SM buffer in microfuge tube, 100 µl of bacteriophage suspension was added and mixed well to give a dilution factor of 10^{-1}. The suspension was further diluted by transferring 100 µl contents from tube 1 to 900 µl of SM buffer in tube 2 and so on. This ten-fold serial dilution was diluted down to 10^{-8} dilution factor and processed by the method as described above (section 3.2.3.4). The numbers of plaques formed by each bacteriophage was recorded. The numbers of plaque forming units (pfu) per ml in the bacteriophage suspension was calculated from the total dilution.

3.2.5 Nomenclature of bacteriophages

Nomenclature of bacteriophage was done as recommended by Adriaenssens and Brister, (2017).

3.2.6 Characterization of bacteriophages

3.2.6.1 Host range and lysis profiles determination

Host range of phage isolate was determined by using *S. aureus* (n=42) and methicillin resistant *S. aureus* (n=67) isolates of livestock and their handlers. The host range of bacteriophage was classified on the basis of number of isolates lysed by phage.

The host strain was inoculated in nutrient broth for overnight at 37°C and streaked on nutrient agar plates with the help of swab. Lysis effect was determined by spotting 10 µl of approximately 10^9 pfu/ml phage stocks within marked areas of streaked culture. Plates were examined for host cell lysis after incubation at 37°C for 16-18 h. The presence of clear zone on spotted area or small plaques was indicative of lysis and a score of plus (+) was assigned; phage isolates that produce turbid zone or did not yield plaques on a given host were assigned a score of minus (-).

3.2.6.2 Morphological characterization by transmission electron microscopy

The bacteriophage suspensions were concentrated using the protocol by Davis *et al.* (1986) with some modifications. In a 50 ml Oakridge tube, 10 ml of high titre bacteriophage filtrate was mixed gently with 10 ml of Tris Magnesium (TM) buffer and incubated for 15 minutes at room temperature. After incubation at room temperature, 2 ml of 5 M NaCl and 2.2 g of solid PEG-6000 or 8000 was added to the mixture and dissolved completely. The tube was then kept at 4°C for 2 h and centrifuged at 12000g at 4°C for 45 min. The supernatant was poured off and pellets were dissolved in 300 µl of TM buffer. About 200 µl of resuspended phage was then treated with 200 µl of chloroform and after proper mixing, centrifuged at 12000 rpm for 5 min. The supernatant was aliquoted into separate microfuge tube and stored at 4°C for transmission electron microscopy (TEM) analysis.

Isolate of bacteriophage was analyzed by TEM at Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi. The carbon-coated grid was held by forceps and 8 µl of phage particle suspension was absorbed onto the grid and left for 20 s. The excess liquid was removed by soaking with filter paper. The sample was negatively stained using a drop of 2.0% (w/v) uranyl acetate for 20 s and then the grid was rinsed with a drop of double distilled water. Excess liquid was removed by touching the grid with filter paper at right angles to the grid leaving a thin aqueous film. The grid was examined with JEM 1011 (JEOL, Japan) Transmission Electron Microscope operating at an acceleration voltage of 80 kV at different magnification.

Sample was visualized at different field and multi-images were recorded at different magnification along with the measurement of head diameter and tail size. The bacteriophage was classified into family as per the reference of Virus Taxonomy, classification and nomenclature of viruses, eighth report of the international committee on the taxonomy of viruses (Fauquet *et al.*, 2005).

3.2.6.3 Physiochemical characterization of phage

Bacteriophage was selected for physiochemical characterization by determination of their stability at temperature 4, 25, 37, 45 and 65°C.

One hundred microlitres of the bacteriophages suspension @ $6.2x10^8$ PFU/ml was added to a microfuge tube containing 900 µl SM buffer and placed in water bath preset to the required temperature (25, 37, 45 and 65°C) and at 4°C in refrigeration. Experiments were carried out in triplicate. The tubes were incubated at the test temperature for 60 min and then placed on ice for further 10 min before proceeding for titration of the suspension. To 900 µl of SM buffer in a microfuge tube, 100 µl of bacteriophage suspension was added and mixed well to give a dilution factor of 10^{-1}. A ten-fold serial dilution was made upto 10^{-4} dilution factor. 100 µl from each dilution (starting with the highest dilution) and 100 µl host bacterium were mixed with 3.0 ml molten soft agar (0.7% agar, w/v) and poured quickly on top of solidified nutrient agar plate (3% agar, w/v). The plates were gently rotated and left to dry at room temperature for 20 min and incubated overnight at 37°C. The number of plaques formed was recorded and the number of plaque forming units (pfu) per ml in the bacteriophage suspension was calculated from the total dilution.

3.2.6.4 Molecular characterization of phages

3.2.6.4.1 SDS-PAGE

SDS-PAGE of the phage samples was carried out according to the method of Laemmli (1970) with slight modification. A vertical slab gel with 12% acrylamide in separating gel and 5% in stacking gel was prepared loaded with phage sample. The phage sample was prepared by addition of 7 ml of purified phage lysate and 7 ml of ice cold acetone, mixed and kept at -20°C overnight for precipitation of protein. The pellet was centrifuged at 6000 rpm for 10 min and supernatant was discarded and the pellet was air dried. It was dissolved in 100 µl distilled water and mixed with 100 µl of the 2X Laemmli's sample buffer (with β-mercaptoethanol and SDS) and heated for 5 min at 100°C. The gel was run at 20 mA after loading 50 µl of prepared samples and 10 µl of 4 colour prestained protein ladder in separate well. After electrophoresis, the gel was carefully removed from the glass-plate sandwich and transferred to staining solution with 0.25% Coomassie Brilliant Blue G-250 stain for 4 h. The gel was then suitably destained for best visibility of the protein bands with several changes of destaining solution and photographed using a scanner.

3.2.6.4.2 Bacteriophage nucleic acid isolation and genomic determination size

The isolation of phage nucleic acid was performed using DNeasy blood and tissue kit (Qiagen).

1. In 50 ml Oakridge tube, 10 ml of high titer bacteriophage filtrate was mixed with 10 ml TM buffer and mixed gently by inversion.
2. After 15 min incubation period at room temperature, 2 ml 5M NaCl and 2.2 g of solid PEG- 8000 was added and dissolved completely. This was then kept at 4°C for 2 h and centrifuged at 12000 g at 4°C for 30 min.
3. The supernatant was poured off and pellets were re dissolved in 300 µl TM buffer
4. 200 µl of above suspension of bacteriophage was taken into a 1.5 ml micro-centrifuge tube and 200 µl ATL buffer was added.
5. Twenty µl proteinase K was added and mixed thoroughly by vortexing and incubated at 56°C for 2 h.

6. After incubation, the mixture was vortexed for 15 s and 200 µl AL buffer was added to the sample and mixed thoroughly.

7. Then 200 µl ethanol was added and mixed again thoroughly by vortexing. The mixture was then transferred into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm for 1 min.

8. The flow-through and collection tube was discarded and the DNeasy Mini spin column was transferred into a new 2 ml collection tube. 500 µl AW1 buffer was added and centrifuged for 1 min at 8000 rpm.

9. The flow-through and collection tube was discarded and the DNeasy Mini spin column was transferred into a new 2 ml collection tube. 500 µl AW2 buffer was added and centrifuged for 3 min at 14,000 rpm to dry the DNeasy membrane.

10. The flow-through and collection tube was discarded and the DNeasy Mini spin column was placed in a clean 1.5 ml centrifuge tube and 30 µl AE buffer was added directly onto the DNeasy membrane and incubated at room temperature for 30 min followed by centrifugation for 1 min at 8000 rpm to elute DNA.

11. The elution process was repeated with fresh addition of 30 µl AE buffer, incubation and centrifugation in the same tube. The harvested DNA was stored at -20°C.

The phage nucleic acid were run as per standard protocol (Sambrook and Russell, 2001) on 0.8% w/v agarose gel prepared in 0.5X TBE for submarine agarose gel electrophoresis (at potential difference of 5 volt/cm) to check the integrity and to determine approximate genomic size of nucleic acid. Ethidium bromide was added @ 0.5 µg/ml in melted agarose.

3.2.6.4.3 Random amplification of polymorphic DNA (RAPD)

RAPD-PCR was carried out with RAPD5 and OPL5 primers. The PCR reactions were performed using 25 µl reaction mixture containing 15.75 µl of NFW, 2.5 µl of 10× PCR buffer, 3 µl of dNTP mixture, 2 µl of 100 pmol of primer, 1.5 µl of DNA template and 0.25 µl (1 U) of *Taq* DNA polymerase. DNA amplification was performed in a BioRad Thermocycler, programmed as: 3 min at 94°C; four cycles of 45 s at 94°C, 2 min at 30°C, and 1 min at 72°C; 26 cycles of 15 s at 94°C, 30 s at 36°C, and 30 s at 72°C; and 10 min at 75°C. The DNA banding patterns were

examined by 1% agarose gel electrophoresis stained with 0.5 µg/ml ethidium bromide and a 100 bp ladder was used as size marker. The gel image was documented in gel documentation system (Biorad).

3.2.7 Effect of *S.aureus* phage on experimentally contaminated hard surface of cover slip

The study of evaluation of S. *aureus* bacteriophage as surface sanitizer was conducted as per the method described previously for *E. coli* O157:H7-specific phages by Abuladze *et al.* (2008) with certain modification.

3.2.7.1 Generation of MOI

A pfu against cfu was calculated to create a range of multiplicity of infection (MOIs) ranging from 0.01 to 1000 and was used to study the effect of *S. aureus* bacteriophage on the host inoculated on glass coverslip. Different MOI (PFU/CFU) used for inoculation study were constituted as follows:

1) **MOI 0.1:** 10 µl of 10^8 CFU/ml *S. aureus* culture and 100 µl of 10^6 PFU/ ml *S. aureus* phage.
2) **MOI 1:** 10 µl of 10^8 CFU/ml *S. aureus* culture and 100 µl of 10^7 PFU/ ml *S. aureus* phage.
3) **MOI 10:** 10 µl of 10^8 CFU/ml *S. aureus* culture and 100 µl of 10^8 PFU/ ml *S. aureus* phage.
4) **MOI 100:** 10 µl of 10^6 CFU/ml *S. aureus* culture and 100 µl of 10^7 PFU/ ml *S. aureus* phage.
5) **MOI 1000:** 10 µl of 10^6 CFU/ml *S. aureus* culture and 100 µl of 10^8 PFU/ ml *S. aureus* phage.

3.2.7.2 Preparation of inoculums

3.2.7.2.1 Bacterial inoculums

A single colony of *S. aureus* was inoculated into 5 ml of NB and incubated at 37^0C for 6hrs (log phase of growth). The desired concentration of bacterial inoculums was prepared using Mc Farland standard tubes. 1ml of grown culture was taken into a microfuge tube and centrifuged at 7000 rpm for 5 min. After that the supernatant was

discarded and bacterial pellet was dissolved in sterile Normal saline solution. The density of the suspension was matched with Mc Farland standard No. 1 by adding Normal saline solution. The stock concentration was calculated as nx3x10^8cfu/ml and the required concentration and volume of inoculums were prepared by ten-fold serial dilution in Normal saline solution.

3.2.7.2.2 Bacteriophage inoculums

Large volume of high titre bacteriophages filtrates were prepared in nutrient broth as per method described in section 3.2.4.1. The concentration of bacteriophage suspension (pfu/ml) was determined by titration of phage filtrates as described in section 3.2.4.2. The required concentration and volume of phage inoculums were prepared by ten-fold serial dilution in SM buffer.

3.2.7.3 Test matrices

The ability of *S. aureus* phage to reduce or eliminate *S. aureus* contamination of hard surface was examined using hard, inanimate surface of glass coverslips (22 × 22 mm in size). The glass coverslips was cleaned with 70% ethanol and rinsed with deionized water. They were further autoclaved and left with a drying cycle of 10 min. The matrices were cooled to room temperature and used immediately, or they were stored at room temperature until use.

3.2.7.4 General design of the studies utilizing *S. aureus* contaminated hard surfaces.

1. All cover slips were placed individually into properly labeled pre-sterilized petridishes in the laminar air flow.
2. All of the glass coverslips were pretreated with 5% (wt/vol) skim milk to "dirty" their surfaces, in order to mimic real-life settings where surfaces are often covered with dried organic matter.
3. Three experimental groups in triplicates at MOI of 0.1, 1, 10,100 and 1000 were examined during the study.
4. The glass coverslips in all three groups were contaminated with *S. aureus* by pipetting 10 µL of bacterial culture onto their surfaces and spreading them using

pipette tip, before allowing the inoculums to dry at room temperature for 15 to 25 min, or until visibly dry.

5. The glass coverslips in Group 1 were not treated with anything and served as the "dry control".
6. The glass coverslips in Group 2 were treated with PBS (0.1 mL/matrix) and were designated the "nonactive control".
7. The glass coverslips in Group 3 were treated with 0.1 mL of *S. aureus* phage of desired concentration.
8. After storage (5 min at room temperature), the excess PBS and phage solution was removed from the glass coverslips in Groups 2 and 3, respectively, by holding them vertically for 20–30 sec and allowing the excess liquid to drain into a disinfectant solution.
9. Subsequently, the test and control group glass coverslips were mixed gently (30 sec) in separate 50 ml capacity conical tubes containing 10 mL peptone water.
10. Three 10-fold serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) were prepared with peptone water.
11. To count the survival colonies spot of 10 µl of the undiluted and diluted mixtures were made in triplicate on mannitol salt agar and left for drying (Miles and Mishra, 1938).
12. All plates were incubated at 37^{0}C for 24 h and the plates with different dilution showing 3-30 colonies were counted.

3.2.7.5 Statistical analysis

All data were expressed as the mean±standard error (SE) unless otherwise specified. The statistical analyses were performed using online statistical tools GraphPad prism version 7. One-way analysis of variance (ANOVA) and Tukey multiple comparison tests were used to determine whether the observed differences in *S. aureus* recoveries from the *S. aureus* phage treated and PBS-treated hard surfaces were statistically significant. Differences were considered to be statistically significant when the P-values were < 0.0001.

Results

The present study was carried out to isolate and characterize *S. aureus* phage and to evaluate the potential of *S. aureus* phage as surface sanitizer.

4.1 Revival and characterization of isolates

4.1.1 Biochemical characterization

A total of 42 isolates of Methicillin sensitive *S. aureus* and 67 Methicillin resistant *S. aureus* isolates included in the study were streaked on Mannitol salt agar (MSA). All the isolates produced typical mannitol fermented golden, yellow or pale colour round colony (Fig.1) on MSA. A single characteristic colony of each isolate was selected for Gram staining, catalase (Fig.2) and tube coagulase test using human plasma (Fig.3). All the isolates were found positive for catalase and coagulase and also appeared in characteristic bunches of grapes under microscopic examination.

4.1.2 Molecular characterization

All *S. aureus* isolates included in the study were further subjected to PCR analysis for confirmation of *S. aureus* by targeting 16S rRNA gene. All isolates were found to produce 228 bp amplicon sizes corresponding to 16S rRNA gene specific for *S. aureus* (Fig.4 and 5).

4.2 Isolation, purification, propagation and titration of lytic bacteriophage(s)

4.2.1 Isolation of lytic bacteriophage(s)

A total of 32 samples were processed for isolation of *S. aureus* and their bacteriophages by enrichment and non enrichment methods (Table 3). Out of 32 samples processed *S. aureus* was isolated from 15 samples only, whereas only one sample processed, by non enrichment method was found positive for both *S. aureus* and its bacteriophage. The sample found positive for bacteriophage belonged to the dog pus sample collected from TVCC, Bihar Veterinary College, Patna which showed lytic activity in the form of clear zone of lysis against *S. aureus* isolated from the same

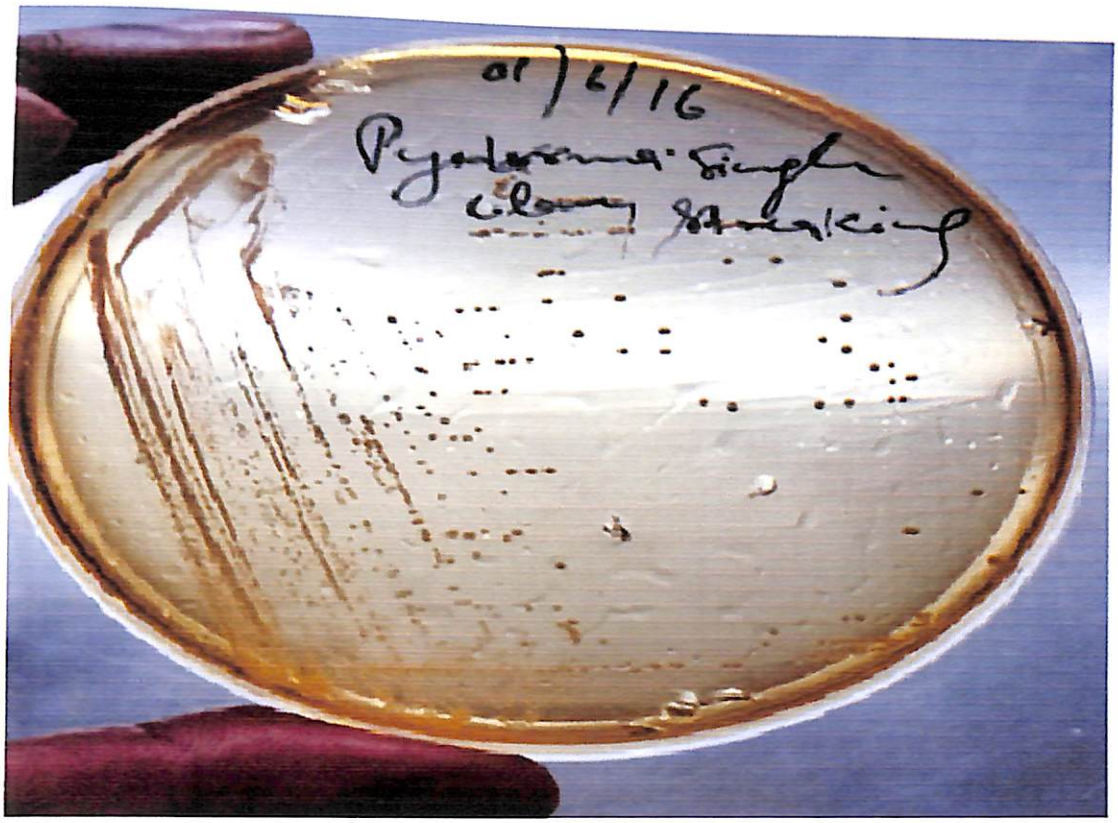


Fig. 1. *Staphylococcus aureus* colonies on Mannitol Salt Agar

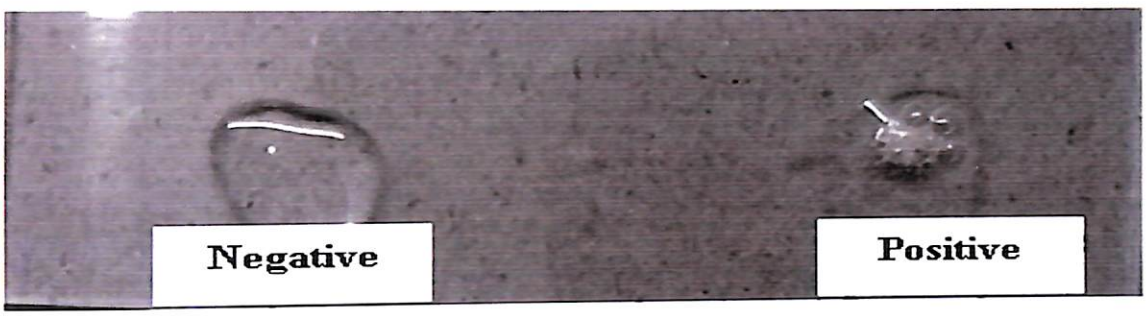


Fig. 2. Catalase test of *S. aureus* isolates

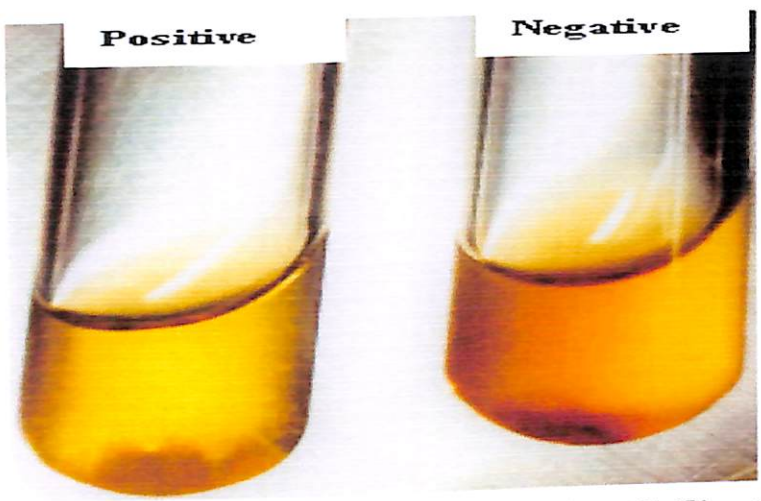


Fig. 3. Coagulase test of *S. aureus* isolates

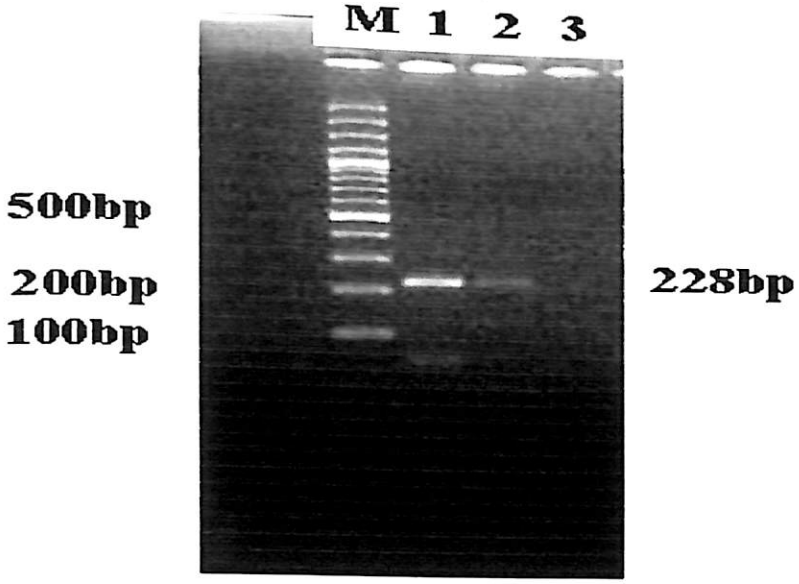


Fig. 4. Standardization of PCR for identification of *S. aureus* by 16srRNA amplification

M : 100 bp plus DNA ladder

L1-L2: Positive amplicon of 228 bp

L3 : Negative control

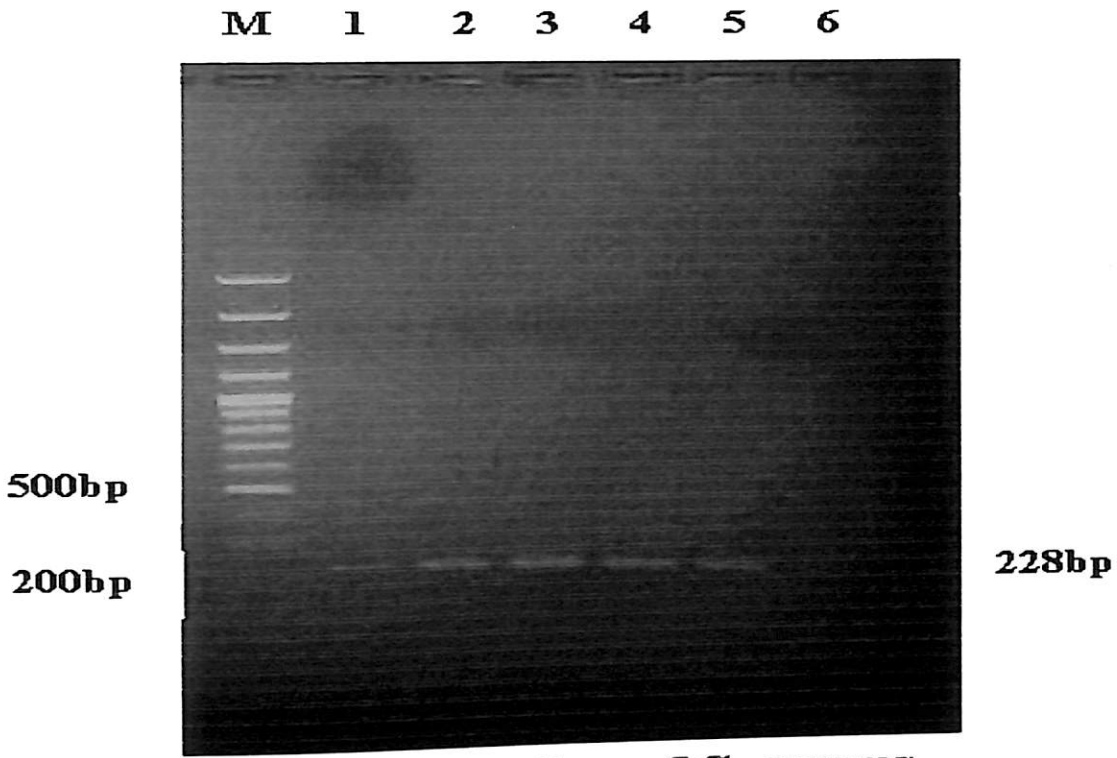


Fig. 5. PCR confirmation of *S. aureus* by amplification 16srRNA

M : 100 bp plus DNA ladder

L1-L5: Positive amplicon of 228 bp

L6 : Negative control

sample (Fig. 6). However, other 31 samples produced turbid zone either on *S. aureus* isolated from same sample or isolate used for enrichment (Fig. 6).

4.2.2 Purification, propagation and titration of phages

The only sample showing lytic activity against *S. aureus* isolate of the same sample (dog pus) was purified and propagated for phage through soft agar overlay method (Fig 7). The plaques produced by the phages were approximately 0.5 mm in size (Fig 7). Titration of phages showed that phage propagated through agar overlay method yielded a concentration of $1.6x10^6$ pfu/ml.

4.2.3 Nomenclature of phage

Nomenclature of phage was done as per the method recommended by Adriaenssens and Brister, (2017). The lytic phage isolated against *S. aureus* was named as *Staphylococcus* phage vB_Staph M-BVC1.

4.3 Characterization of bacteriophage

4.3.1 Host range and lysis profiles determination

To determine the host range, isolated *Staphylococcus* phage vB_Staph M-BVC1 was tested on a range of *S. aureus* (n=42) and methicillin resistant *S. aureus* (n=67) isolates. None of the *S. aureus* isolates were found susceptible to lysis by this phage, however, a lytic activity was found against 38.80% methicillin resistant *S. aureus* isolates (26/67). The phage *Staphylococcus* phage vB_Staph M-BVC1 showed lytic activity against 37.5% (3/8), 40.0% (2/5), and 45.45% (5/12) methicillin resistant *S. aureus* isolate from nasal carriage of buffalo, cow and dog, respectively (Fig. 8a and Table 4a-d). It also showed lytic activity against 37.5% (3/8), 66.67% (2/3), and 38.46% (5/13) methicillin resistant *S. aureus* from skin carriage of buffalo, cow and dog, respectively (Fig. 8a and Table 4a-d). However, no lytic activity was found against the isolates from dog's wound. When this phage was tested against methicillin resistant *S. aureus* isolates from human handler of cattle/buffalo and dog, it showed a lytic activity against 40% (2/5) and 25% (3/12) isolates, respectively (Fig. 8 and 8a).

Table4a. Host range of *S. aureus* bacteriophage

SL. No.	Source	Methicillin resistant *S. aureus* isolates	Phage lytic activity	Lytic efficiency (%)
1.	Buffalo nasal carriage	17B(N)	-	37.5
2.		189B(N)	-	
3.		190B(N)	-	
4.		207B(N)	+	
5.		218B(N)	-	
6.		226B(N)	+	
7.		243B(N)	-	
8.		253 B(N)	+	
9.	Buffalo skin carriage	19B(S)	-	37.5
10.		116B(S)	-	
11.		198B(S)	-	
12.		199B(S)	-	
13.		210B(S)	+	
14.		214B(S)	-	
15.		244B(S)	+	
16.		250B(S)	+	

Table4b. Host range of *S. aureus* bacteriophage

SL. No.	Source	Methicillin resistant *S. aureus* isolates	Phage lytic activity	Lytic efficiency (%)
1.	Cattle nasal carriage	10C(N)	-	
2.		174C(N)	-	
3.		176C(N)	+	
4.		178C(N)	+	
5.		180C(N)	-	
6.	Cattle skin carriage	46 C(S)	+	66.67
7.		165C(S)	-	
8.		171C(S)	+	

Table4c. Host range of *S. aureus* bacteriophage

SL. No.	Source	Methicillin resistant *S. aureus* isolates	Phage lytic activity	Lytic efficiency (%)
1.	**Dog nasal carriage**	6 D(N)	-	45.45
2.		8D(N)	-	
3.		11D(N)	+	
4.		20D(N)	-	
5.		21D(N)	+	
6.		28D(N)	+	
7.		33D(N)	+	
8.		35D(N)	-	
9.		38D(N)	-	
10.		43D(N)	+	
11.		51D(N)	-	
12.	**Dog skin carriage**	3D(S)	+	38.46
13.		7D(S)	-	
14.		13D(S)	+	
15.		16D(S)	-	
16.		29D(S)	+	
17.		32D(S)	+	
18.		39D(S)	-	
19.		44D(S)	-	
20.		49D(S)	+	
21.		52D(S)	-	
22.		58D(S)	-	
23.		95D(S)	-	
24.		101	-	
25.	**Dog Wound**	4D(W)	-	0
26.		42D(W)	-	

Table4d. Host range of *S. aureus* bacteriophage

SL. No.	Source	Methicillin resistant *S. aureus* isolates	Phage lytic activity	Lytic efficiency (%)
1.	**Livestock handler**	18(H)	-	40.00
2.		47(H)	+	
3.		159H	+	
4.		183H	-	
5.		231H	-	
6.	**Dog handler hand swab**	15D(H)	-	25.00

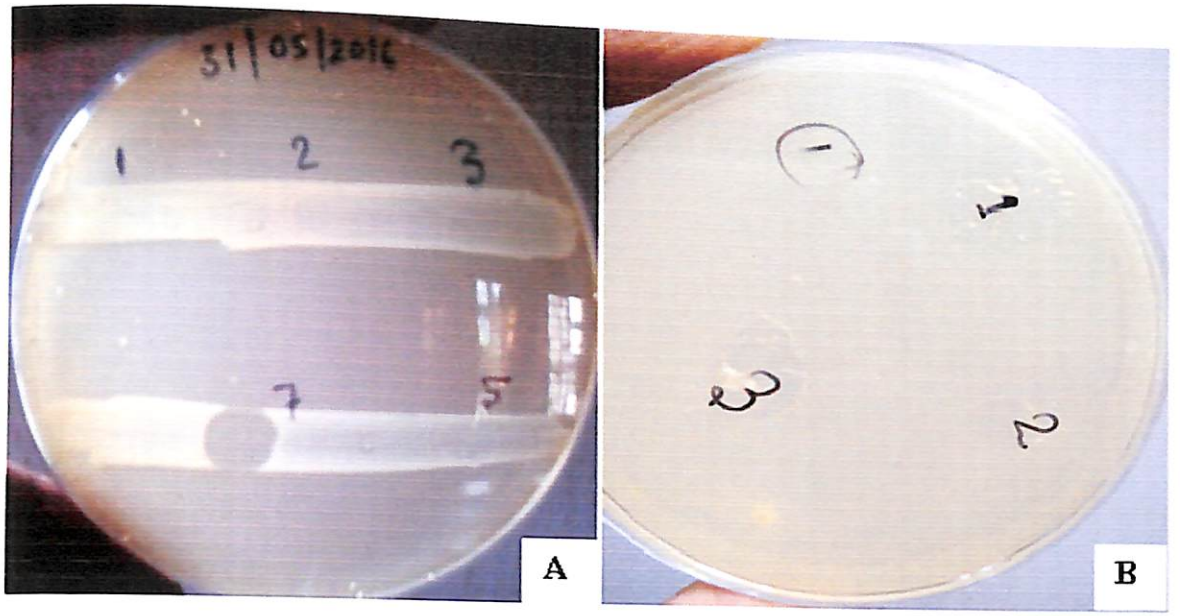


Fig. 6. Detection of phage in different samples

A. Spot test

B. Clear zone of lysis on top layer agar processed for purification

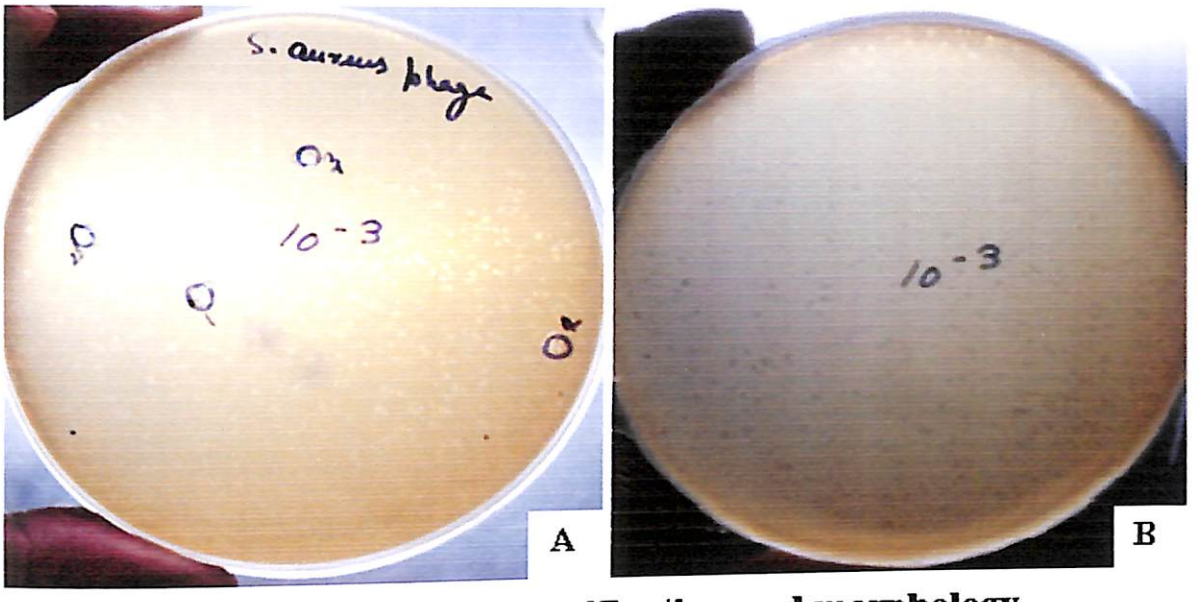


Fig. 7. Bacteriophage plaque purification and morphology

A. Single plaque of phage processed for purification

B. Uniform plaques of approximately 0.5 mm diameter produced after purification

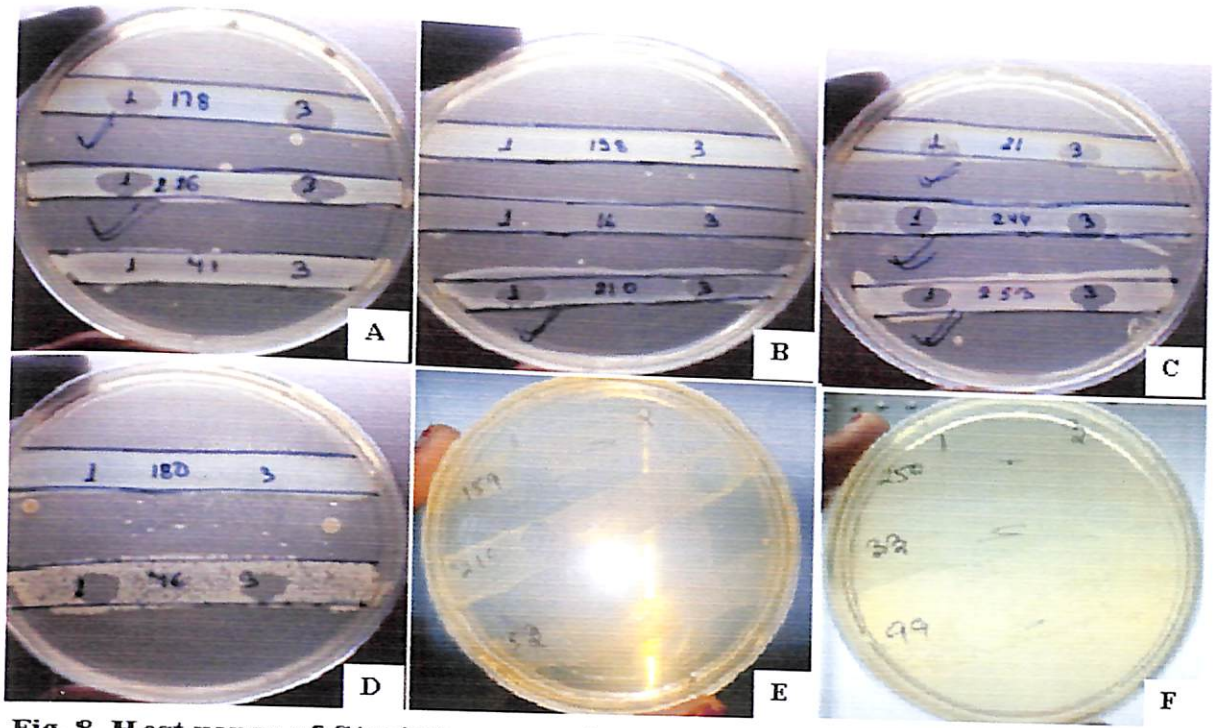


Fig. 8. Host range of *Staphylococcus* phage vB_Staph M-BVC1 by spot test

A. Phage showing lysis of cattle (178) and buffalo (226) nasal carriage *S. aureus*

B. Phage showing lysis of buffalo (226) skin carriage *S. aureus*

C. Phage showing lysis of dog nasal (21), buffalo skin (244) and buffalo nasal (253) carriage *S. aureus*

D. Phage showing lysis of cattle skin carriage (46) *S. aureus*

E. Phage showing lysis of cattle & buffalo handler hand isolate (159) and buffalo skin carriage (210) *S. aureus*

F. Phage showing lysis of cattle skin carriage (250), dog nasal carriage (33) and dog handler hand isolate (99) *S. aureus*

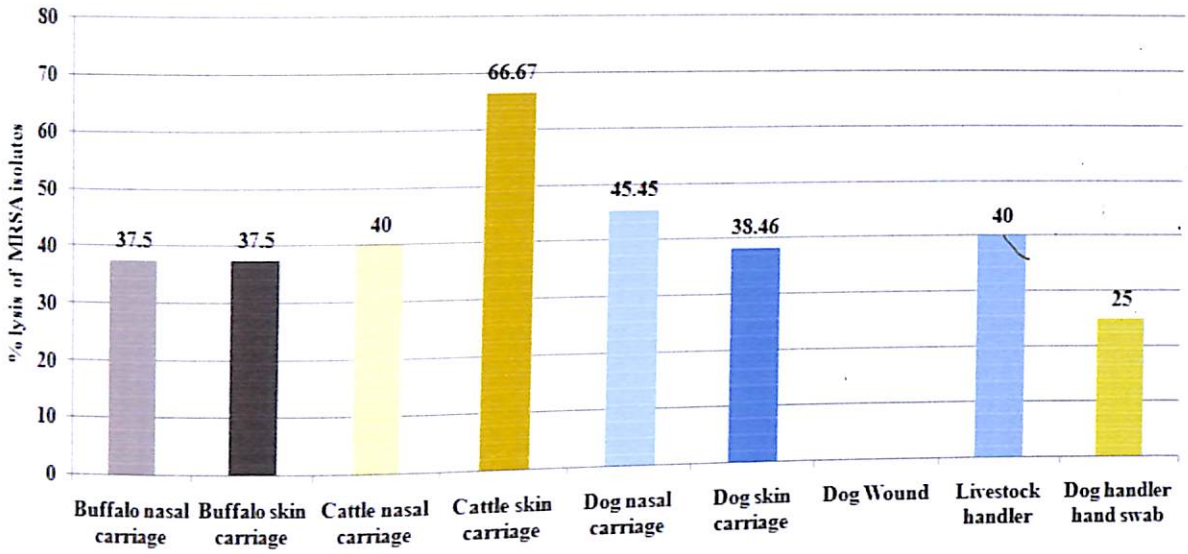


Fig. 8a. Lysis of MRSA isolates by bacteriophage

4.3.2 Morphological characterization of Phage by transmission electron microscopy

The morphology of *Staphylococcus* phage vB_Staph M- BVC1 under transmission electron microscopy showed that it belongs to family *Myoviridae* of order *Caudovirales* (Fig 9). The average diameter of head and length of tail was calculated on the basis of the means of head diameter and tail length of six representative phages. The head diameter was measured as 81.31 nm with a tail length of 92.08 nm (Fig.9). The structural analysis of phage revealed that it has a contractile tail consisting of a sheath and a central tube.

4.3.3 Physiochemical characterization of phage

The isolated phage, *Staphylococcus* phage vB_Staph M- BVC1, was characterized for its stability to different temperature range. The study revealed no or moderate inactivation of bacteriophages at 4°C, 25°C, 37°C and 45°C when incubated for 60 min. however the bacteriophage showed complete inactivation at 65°C temperature (Table 5, Fig.10).

4.3.4 Molecular characterization of phage

4.3.4.1 SDS-PAGE analysis

The structural proteins of host *S. aureus* was analyzed by SDS-PAGE, which allowed the detection of 8 major proteins of different size between 27 to 180 kDa with some minor proteins. The SDS-PAGE analysis of structural proteins of phage, *Staphylococcus* phage vB_Staph M- BVC1, allowed the detection of 13 protein bands comprising of seven major and 7 minor structural proteins. The major structural proteins included 01 (137-180kDa), 01 (91-137kDa) 03 (51-71kDa) 01 (27kDa), however, minor structural proteins were found between 35-51 kDa (Fig. 11). The study also revealed some common major structural protein between the host and phage (Fig. 11).

4.3.4.2 Bacteriophage nucleic acid isolation and genome size determination

The nucleic acid from *Staphylococcus* phage vB_Staph M- BVC1 phage was isolated using Qiagen blood DNA extraction kit. The genomic DNA was analyzed

Table 5. Temperature stability of bacteriophage

	Plaque count (log_{10} pfu/ml)				
Temperature	4^0C	25^0C	37^0C	45^0C	65^0C
Mean log_{10} pfu/ml	5.80 ± 0.01^a	5.77 ± 0.01^a	5.60 ± 0.01^a	5.71 ± 0.02^a	0.00 ± 00^b

* Mean (row wise) with different superscripts differ significantly (P<0.0001)

Table 6. Effect of phage as surface sanitizer (mean log_{10} cfu/ml)

Control	Treatment Group				
	T1(MOI 0.1)	T2(MOI 1)	T3(MOI 10)	T4(MOI 100)	T5(MOI 1000)
6.95 ± 0.03^a	5.95 ± 0.03^b	5.90 ± 0.03^{cb}	5.46 ± 0.09^d	----------	----------
4.69 ± 0.05^a	----------	----------	----------	00 ± 0.00^b	00 ± 0.00^b
Log reduction	1	1.1	1.5	No recovery	No recovery

* Log reduction was calculated by deducting log10 value of different treatment groups from control.

* Mean (row wise) with different superscripts differ significantly (P<0.0001)

Fig. 9. Transmission Electron microscopy of bacteriophage
A & B. *Staphylococcus* phage vB_StaphM-BVC1
C & D. Tail length and head diameter measurement of *Staphylococcus* phage vB_StaphM-BVC1

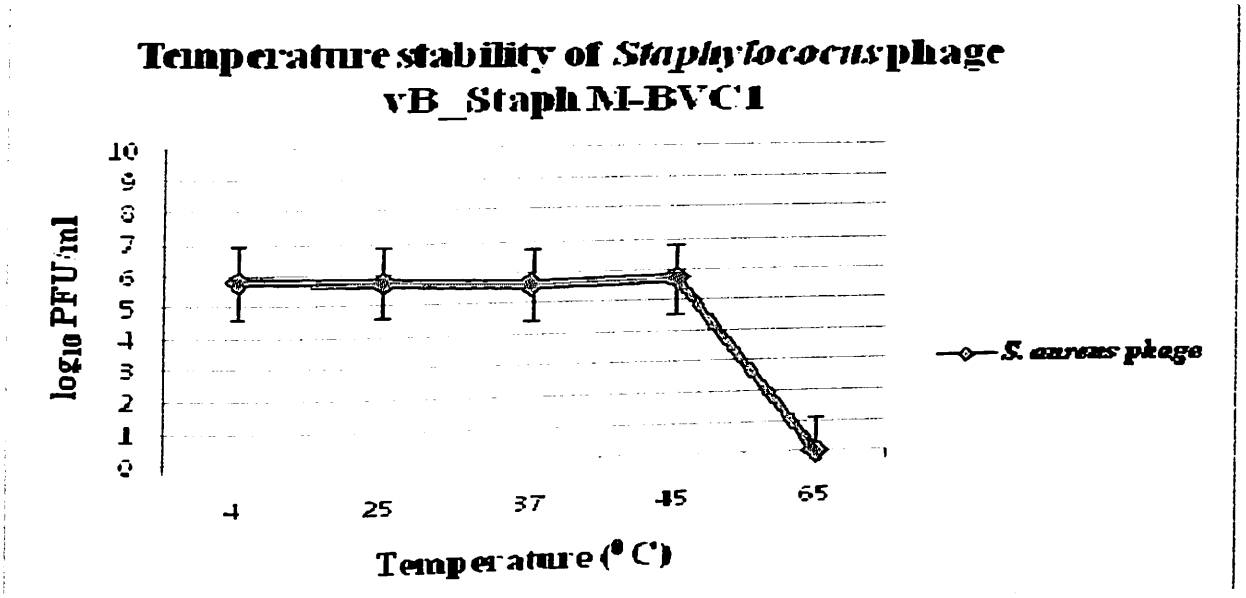


Fig. 10. Temperature stability of *Staphylococcus* phage vB_Staph M- BVC1

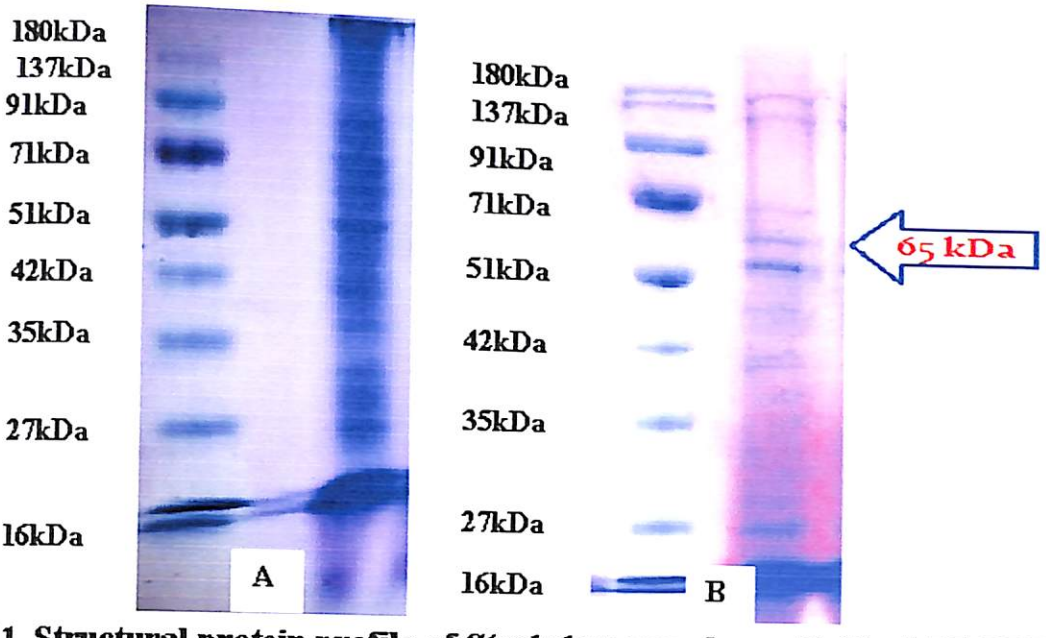


Fig. 11. Structural protein profile of *Staphylococcus* phage vB_Staph M- BVC1 and it's host

M: Four colour prestained protein marker

L1(A) : Host of *Staphylococcus* phage vB_Staph M- BVC1

L1 (B) : *Staphylococcus* phage vB_Staph M- BVC1

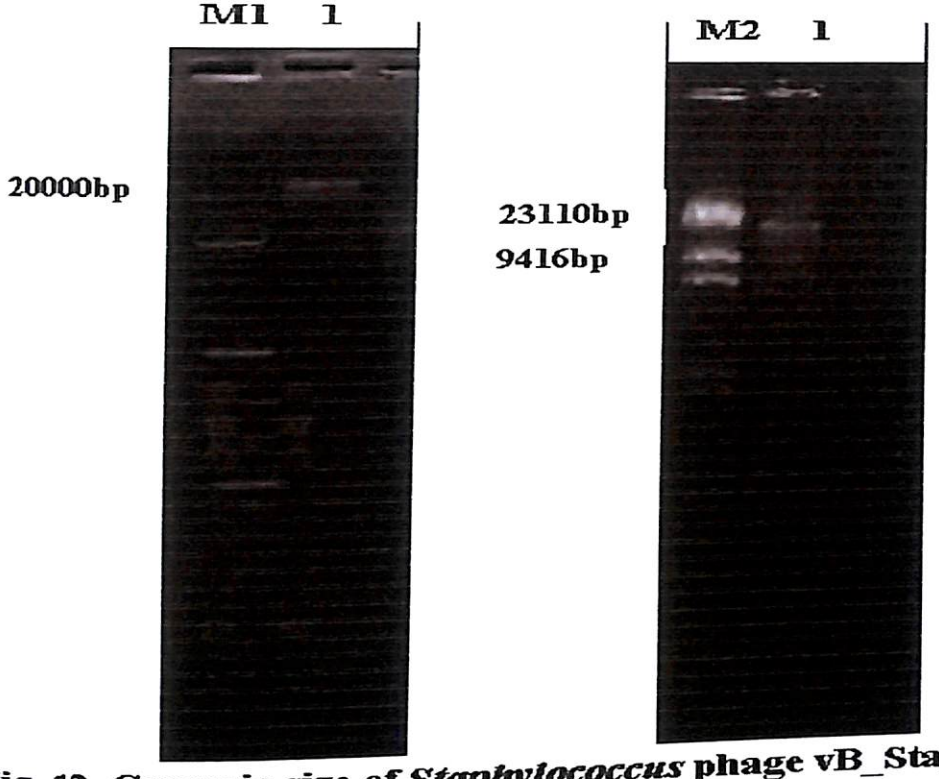


Fig. 12. Genomic size of *Staphylococcus* phage vB_Staph M- BVC1

M1: 1 Kb plus DNA ladder

M2: λDNA Hind III DNA marker

L1: 5µl DNA of *Staphylococcus* phage vB_Staph M- BVC1

with agarose gel electrophoresis using λDNA Hind III DNA marker and 1 Kb plus DNA marker. It revealed a genome size for the phage of more than 20 kb but less than 23 kb (Fig. 12).

4.4.4.4 Random amplification of polymorphic DNA (RAPD)

RAPD-PCR was performed with OPL5 and RAPD5 primers from the bacterial DNA-free bacteriophage genomic DNA extracted using blood and tissue kit (Qiagen).

RAPD-PCR analysis with OPL5 and RAPD5 primers of *Staphylococcus* phage vB_Staph M- BVC1 resulted in different banding patterns consisting of 2–10 bands. The RAPD 5 primer produced 10 bands between 200 to 2000 bp (Fig. 13) whereas, RAPD-PCR with OPL5 produced only two bands below 20 kb and 700bp (Fig. 13B).

4.3.4.3 Surface decontamination study by *Staphylococcus* phage vB_Staph M-BVC1phage.

To study the efficiency of phage as surface sanitizer we inoculated sterile glass coverslips with MRSA, as described in the section 3.2.7 and then treated with phage or mock treatments and measured the ability to decontaminate/ kill bacteria (section3.2.7.1). Glass slides inoculated with MRSA isolate and treated with phage showed significant reductions in CFUs ($p< 0.0001$) compared to control group (Table 6). The recovery of *S. aureus* from control group which has used a MOI of 0.1, 1, 10 was recorded as $\log_{10}$ 6.95±0.03 cfu/ml. It showed a significant reduction ($P <0.0001$) in *S. aureus* recovery from the glass surface (Table 6) treated with active phage at MOI 0.1, 1.0, 10, 100 and 1000. The log reduction recorded as 1.0 at MOI 0.1, 1.1 at MOI 1.0, 1.5 at MOI 10, where as *S. aureus* was found to be completely inhibited or no growth or recovery were recorded at MOI 100 and 1000 (Table 6, Fig. 14).

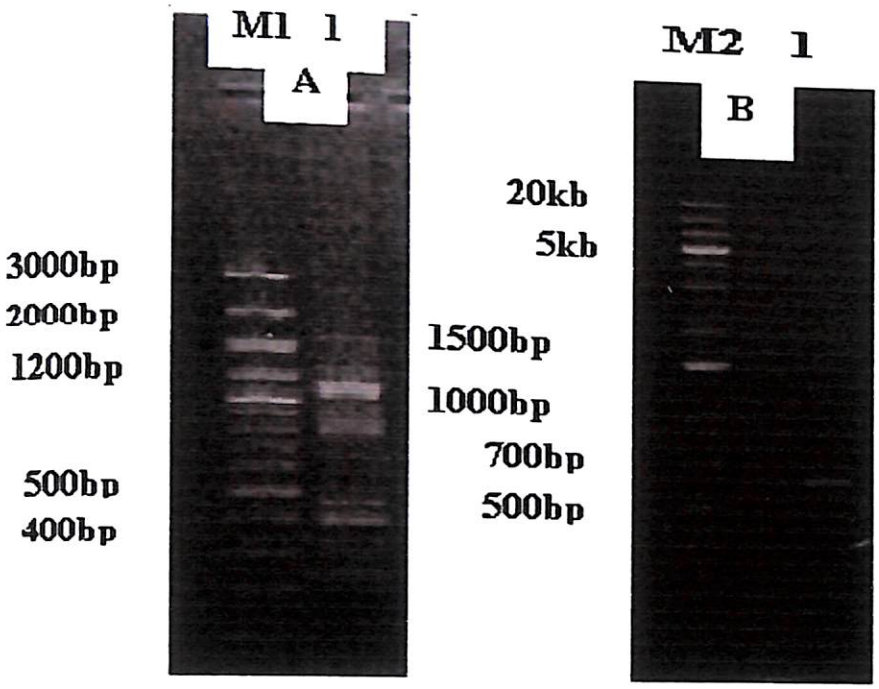


Fig. 13. RAPD analysis of genomic DNA of *Staphylococcus* phage vB_Staph M- BVC1

M1: 100bp plus DNA ladder

M2: 1kb plus DNA ladder

A1: Amplification with RAPD 5 primer

B1: Amplification with OPL 5 primer

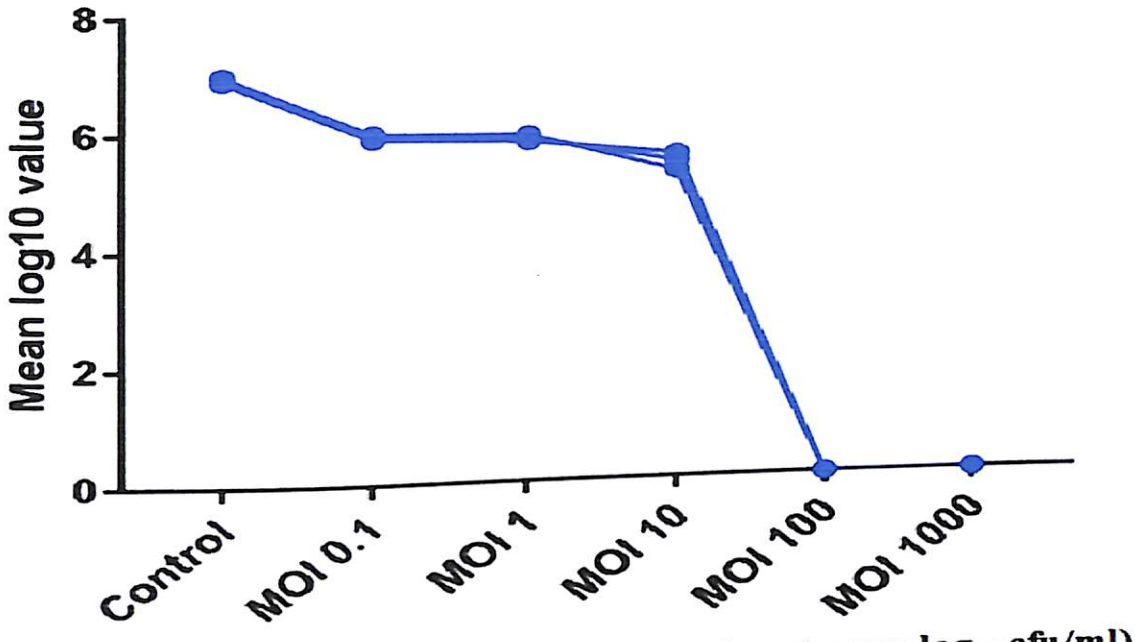
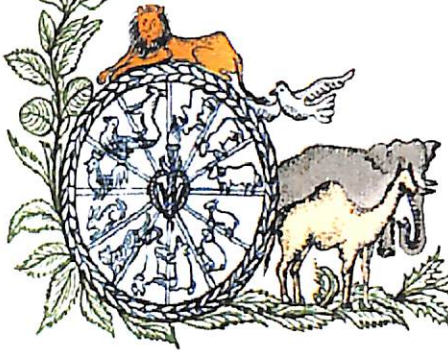


Fig. 14. Effect of phage as surface sanitizer (mean $\log_{10}$ cfu/ml)

Discussions



5 Discussion

S. aureus is a commensal bacterium capable of colonizing the nose and skin which is found transiently in ~50% of the human population whereas ~20% of human population are reported to colonize the organism permanently (Casewell *et al.*, 1986; Grice and Segre, 2011). *S. aureus* can cause life-threatening diseases in different tissues including bones, joints, blood, lungs, heart, and brain (Lowy, 1998). They are primarily transmitted through direct contact with a colonized or infected individual, or through a fomite intermediate (Chambers and DeLeo, 2009). The contaminated objects and surfaces also play an important role in *S. aureus* transmission and once it is established on a surface, can survive for weeks to months (Williams and Davis, 2009). It is also a principal biofilm former bacterium and once biofilm has formed, it can be very difficult to treat clinically and to remove from the other surfaces (Hoyle and Costerton, 1991).

Use of antibiotics has become a routine practice in human as well veterinary medicine resulting in development of antibiotic resistance among bacterium. Since their discovery in the 30's and 40's, antibiotics have made unprecedented improvements in humans and livestock health, worldwide. However in the past 20 years, the MDR 'super bugs' have evolved with resistance to even our most powerful miracle drugs. World is facing an alarming condition with a pandemic of antimicrobial resistant pathogens (Donovan, 2007; Ventola, 2015). The situation has further been complicated by the fact that since 1980 no new class of antibiotic has been discovered by any pharmaceutical industry or research organization throughout the world in spite of huge research efforts and inputs (Lorch, 1999 and Fernandes, 2006).

Although use of penicillin (1940's) initially led to a dramatic reduction in mortality from staphylococcal infections in human and veterinary medicine, later, this organism developed resistance to a number of antimicrobials including Penicillin (β-lactam antibiotic) and penicillin resistant strains were isolated (Rammelkamp *et al.*, 1942; Cookson *et al.*, 2003). Subsequent upon this penicillinase-resistant molecule, methicillin (the first semi-synthetic penicillin) was introduced in 1959 to combat penicillin resistance. Unfortunately methicillin-resistant *Staphylococcus aureus* (MRSA) strains were identified soon after its introduction on international scene and

the first MRSA was reported in 1961 in UK hospital, which was later on reported from other parts of the world (Barber, 1961; Jevons, 1961; Tiemersma *et al.*, 2004; Grundmann *et al.*, 2006; Boucher and Corey, 2008; Kim, 2009; Chandrasekaran *et al.*, 2014; Vishnupriya *et al.*, 2014).

Under such condition a multifaceted approach will be the most efficient strategy for the control of antimicrobial resistance. But no universally acceptable programme exists at present and it is very likely that specific policies/modalities will be required for such situation (Foucault and Brouqui, 2006). This has led scientists to re-consider the approaches and compounds, many of which were not thought to yield desirable results in the past and were eventually abandoned. It is now accepted that some of the previously discontinued approaches or compounds may prove invaluable tools for our on-going fight against multi-drug-resistant bacterial infections and lytic bacteriophages have therefore, become one of the most acceptable and potent option for research during the last 30 years. Bacteriophages/Phage – Therapy (PT) is rapidly re-emerging as a possible modality for treatment of bacterial infections (Lorch, 1999).

In recent years, there is an increasing consensus about improvement in the disinfection and sanitization of environmental surfaces in healthcare and food processing facilities (Donskey, 2013; Dancer, 2014; Rutala and Weber, 2013; Han *et al.*, 2015). In this regard a novel approach to reduce the need of harsh chemical sanitizers is the use of lytic bacteriophage(s) as biocontrol or biosanitizing agents. Bacteriophages (phages) are viruses capable of infecting and replicating in bacterial cells. Phages are the most common organism found on the planet and as such represent great diversity in their overall host range (Wommack and Colwell, 2000; Srinivasiah *et al.*, 2008). Since virus infectivity requires binding to a specific receptor, phage are specific for a small host range and are thus unable to infect human cells.

With the above considerations, the present study was designed with the objective to isolate and characterize wide host range lytic bacteriophages against *S. aureus* and to evaluate bacteriophage(s) as a surface sanitizing biological agent. The *S. aureus* were prioritized since they are among the most important target bacteria for biosanitizer study and have been involved as a major surface contaminants.

In the present study, *S. aureus* isolates of diverse origin maintained in the Department of Veterinary Public Health & Epidemiology were used. The isolates were

confirmed by morphological, biochemical and molecular method. All the 109 isolates of *S. aureus* used in the present study resulted in the amplification of desired amplicon size of 228bp of target gene (16s rRNA) of *S. aureus* (Fig. 5).

Bacteriophages have been isolated from different sources including soil (Ashelford *et al.*, 2003), sewage, sewage sludge (Carey-Smith *et al.*, 2006; Oliveira *et al.*, 2009), and mammalian feces (O'Flynn *et al.*, 2004; 2006; Wongsuntornpoj *et al.*, 2014) demonstrating the natural occurrence of phages in environment and the mammalian intestinal tract.

A total of 32 samples from different sources were processed for isolation of *S. aureus* and their bacteriophages, but only one lytic phage of *S. aureus* was isolated from dog pus sample against the host which was isolated from the same sample. The finding of present study is in concordance with previous reports which states that phages are most widely distributed and diverse entities in the biosphere and ubiquitously present which can be found in all reservoirs populated by bacterial hosts (Mc Grath and Van Sinderen, 2007). Isolation of phage by enrichment method has been reported by various workers (Switt *et al.*, 2013; Wongsuntornpoj *et al.*, 2014), however, in the present study, phage was isolated against the host isolated from the same samples.

Host range and lysis profiles were determined on the basis of lysis of different Methicillin Sensitive *S. aureus* (n=42) and Methicillin Resistant *S. aureus* (n=67) isolated from different sources. The findings depicted that *Staphylococcus* phage vB_Staph M- BVC1 was highly specific to the MRSA with a lytic activity of 38.80%. The lysis profile study showed that the phage was not able to lyse any of the Methicillin Sensitive *S. aureus* isolates (n=42) of livestock and animal handlers origin. This indicates the high specificity of phage *Staphylococcus* phage vB_Staph M- BVC1 to MRSA which can act as a possible agent for the control of MRSA. The finding is in agreement with the report of Jensen *et al.* (2015) who isolated 12 phages from environmental samples and reported a lytic activity of phage namely CJ 17, M5, NS6, M1, M7 and DH1 against 28.5% to 42.8% methicillin resistant *S. aureus* isolates from human. However, in contrast to the findings of this study, Hsieh *et al.* (2011) isolated a phage from endo-tracheal tube washing namely Stau2, of *Myoviridae* family, with a lytic activity of 80% of *S. aureus* isolated from a hospital in Tiawan. Synnott *et al.*

(2009) isolated 52 phages from sewage effluent and reported that only two phages namely ΦSA039 and ΦSA012 belonged to myoviridae which had the widest host range with a lytic activity of 87% and 53%, respectively against *S. aureus* isolates from mastitic milk.

The host range study showed that the phage, *Staphylococcus* phage vB_Staph M- BVC1, tended to have greater lytic activity against cow skin carriage MRSA isolates followed by dog nasal carriage isolates. A slight low efficiency of lysis was observed by this phage against isolates of cow and buffalo nasal carriage and buffalo and dog skin carriage. The phage also showed a lytic efficiency of 25-40% against methicillin resistant *S. aureus* isolates from livestock handler. It was surprising to observe that the phage did not show any lytic activity against methicillin resistant *S. aureus* isolates belonging to dog wound. Therefore, it could be concluded that there might be involvement of variants of methicillin resistant *S. aureus* in the wounds of dog and similar strain of methicillin resistant *S. aureus* might be circulating among cattle, buffalo, dog and their handlers.

The morphology of the *Staphylococcus* phage vB_Staph M- BVC1 was determined by transmission electron microscopy. The Electron micrograph studies demonstrate the novel phage is members of tailed phage group of order *Caudovirales* and family Myoviridae, characterized by a polyhedral capsid, a long contractile tail. According to the International Committee on Taxonomy of Viruses, tailed phages are classified into three families including *Siphoviridae, Myoviridae* and *Podoviridae.* Over 95% of all phages described in the literature belonged to the order *Caudovirales* are tailed dsDNA phages. Further, the morphology of the phage isolated in this study was similar to *S. aureus* phages reported by many workers (Synnott *et al.*, 2009; Hsieh *et al.*, 2011). Thus, the findings of the present study suggested that *Myoviridae* lytic phages infecting *S. aureus* can be isolated from dog wound.

Thermal stability of phage revealed that the phage titre were stable or reduced non-significantly with 100% lytic activity at temperatures when incubated at 4-45°C, however the complete inactivation was observed at 65°C. The present finding was in accordance with reports of Mishra *et al.* (2014); Cui *et al.* (2017) who had reported that in thermal tolerance study, *S. aureus* phage and Salmonella phage were stable at temperature 30^0 and 40°C with 100% lytic efficacy and their activities reduced at 50°C

and declines sharply at 60°C with less than 5% efficacy. The temperature stability of phage P2 studied by Chandra *et al.* (2011) revealed that the phage survives below 50°C and as the temperature increases beyond 50°C the activity of phages decreases, thus exhibiting an inverse relationship between the temperature and survivability.

The SDS-PAGE analysis of structural proteins of *Staphylococcus* phage vB_Staph M- BVC1 allowed the identification of 13 major protein bands of 27-180 kDa as well as 35-51 kDa minor structural proteins. Rasool *et al.* (2016) performed proteome analysis of Phage pq/27 and pq/48 using SDS-PAGE and reported ten distinct proteins of size ranging between 20 kDa and 185 kDa. Kwiatek *et al.* (2012) and Rasool *et al.* (2016) reported a protein band of 65 kDa in phage MSA6 which was a major tail sheath protein on the basis of sequence homology with staphylococcal phage K, of the Myoviridae family. Another study reported similar finding of a tail protein which had 78 kDa size (Han *et al.*, 2013). On this basis, it can be suggested that *Staphylococcus* phage vB_Staph M- BVC1 resembles the phage of the *Myoviridae* family, which support the finding of transmission electron microscopy of this study.

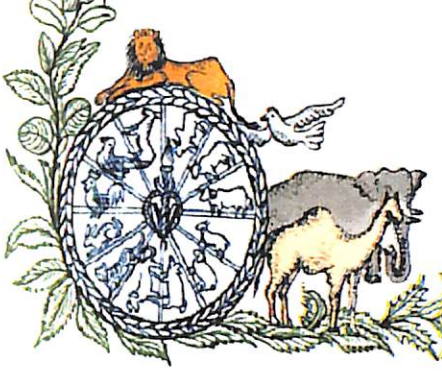
The agarose gel electrophoresis of the phage nucleic acid revealed the presence of genome size of more than 21 kb. This was in accordance with the finding of Ngangbam and Devi, (2012) however in contrast to the present finding an approximate genome size of 65–69 kb was reported by Li and Zhang, (2014).

In the present study, RAPD-PCR was performed with OPL5 and RAPD5 primers from the *Staphylococcus* phage vB_Staph M- BVC1 genomic DNA extracted by using blood and tissue kit. The RAPD-PCR of phage with OPL5 primer yielded different patterns of banding consisting of 2 bands between 200bp to 2kb. RAPD-PCR analysis with RAPD5 primer of phage yielded 10 bands ranging from 200bp to 2.0kb which is in agreement with previous finding (Gutierrez *et al.* 2011). RAPD-PCR using purified DNA has also been previously used to assess the genetic diversity of vibriophages (Comeau *et al.*, 2006; Shivu *et al.*, 2007), phages infecting *Escherichia coli* (Dini & de Urraza, 2010) and *Pseudomonas aeruginosa* (Li *et al.*, 2010). Gutierrez *et al.* (2011) evaluated RAPD-PCR technique using universal primers RAPD5, OPL5, P1 and P2 to produce unique and reproducible band patterns from 26 different bacteriophages infecting *Staphylococcus epidermidis*, *Staphylococcus aureus*,

Lactococcus lactis, *E. coli*, *Streptococcus thermophilus*, *Bacillus subtilis* and *Lactobacillus casei* bacterial strains. They finally reported that the use of RAPD-PCR for quick typing of phage isolates and preliminary assessment of their genetic diversity bypassing tedious DNA purification protocols and previous knowledge of their sequence. Winget and Wommack, (2008) reported that the RAPD-PCR technique appears to be a practical and efficient tool for routine use in high-resolution viral diversity studies by providing assemblage comparisons through fingerprinting, probing, or sequence information.

To study the efficacy of phage as surface sanitizer glass cover-slip was used to provide a model system for simulating hard surface contamination in hospital settings which has been used by other researchers, too, for bacterial decontamination using phage (Rashid *et al.*, 2012). The ratio of host cells and phages were denominated as "multiplicity of infection (MOI)" that has a great relevance to the application of phage based technology assesment (Kasman *et al.*, 2002; Whichard *et al.*, 2003). In the study, analysis of the effects of *Staphylococcus* phage vB_Staph M- BVC1 on glass matrix at room temperature revealed 1 log reduction of contamination at MOI 0.1. The treatment at MOI 1.0, the *S. aureus* count was reduced by $\log_{10}$ 1.1 while 1.5 log reduction was observed at MOI 10. At higher MOI of 100 and 1000, no count or complete reduction of *S. aureus* was observed for the entire period of study. The findings of the present study corroborates with previous study by Jensen *et al.* (2015) and Rashid *et al.* (2012) who had reported significant reduction in colony forming units of MRSA and *Y. pestis* following phage treatment respectively. Our findings suggest that phage treatment can be used as an effective tool to decontaminate MRSA from hard surfaces and the possibility can be explored for its therapeutic use to treat MRSA cases which needs further study.

Summary and Conclusions



6 Summary and Conclusions

S. aureus is a commensal bacterium capable of colonizing the nose and skin, and can cause life-threatening diseases in different tissues including bones, joints, blood, lungs, heart, and brain. The contaminated objects and surfaces play an important role in *S. aureus* transmission and once it is established on a surface, can survive for weeks to month. Use of antibiotics has become a routine practice in human as well as in veterinary medicine resulting in development of antibiotic resistance among bacterium. In recent years, there is an increasing consensus about improvement in the disinfection and sanitization of environmental surfaces in healthcare and food processing facilities. In this regard a novel approach, to reduce the need of harsh chemical sanitizers, is the use of lytic bacteriophage(s) as biocontrol or biosanitizing agents.

The present study was performed to isolate and characterize wide host range lytic bacteriophages against *S. aureus* and to evaluate bacteriophage(s) as a surface sanitizing biological agent. A total of 109 *S. aureus* isolates (comprising of methicillin sensitive and methicillin resistant *S. aureus*) were used in the study which were confirmed by biochemical and molecular test. The isolates resulted in the amplification of desired amplicon size of 228bp of target gene (16s rRNA). A total of 32 samples from different sources were processed for isolation of *S. aureus* and their bacteriophages, but only one lytic phage of *S. aureus* could be isolated from dog pus sample against the host which was isolated from the same sample. The phage were named as *Staphylococcus* phage vB_Staph M- BVC1. Host range analysis and lysis profiles of *Staphylococcus* phage vB_Staph M- BVC1 revealed the high specificity of the phage to the MRSA with a lytic activity of 38.80%. The lysis profile study showed that the phage was not able to lyse any of the Methicillin Sensitive *S. aureus* isolates of livestock and animal handler's origin. This indicates the high specificity of phage *Staphylococcus* phage vB_Staph M- BVC1 to MRSA which can act as a possible agent for the control of MRSA. The Electron micrograph studies demonstrated the novel phage as a member of tailed phage group of order *Caudovirales* and family Myoviridae. The thermal stability of phage revealed non-significant reduction in phage titre with 100% lytic activity at temperatures when incubated at 4-45°C, however the

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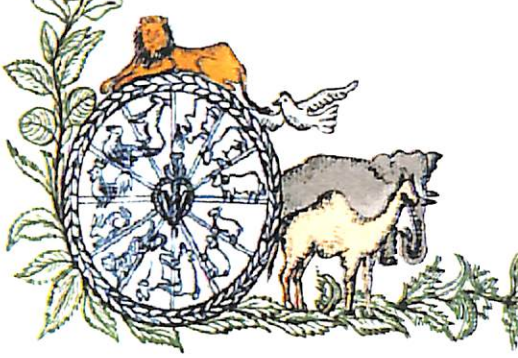
Appendix



Appendix

COMPOSITION OF MEDIAS AND GENERAL REAGENTS USED:

Mannitol salt agar

Proteose peptone	10.0 g
Meat extract	1.0 g
Sodium chloride	75.0 g
D-Mannitol	10.0 g
Phenol red	0.025 g
Agar	15.0 g
Distilled water	1000 ml

Sterilized by autoclaving at 15 psi for 15 minutes.

10X Tryptone soya broth

Pancreatic digest of casein	17.0 g
Papaic digest of soyabean meal	3.0 g
Sodium chloride 5.000 Dextrose	2.5 g
Dibasic potassium phosphate	2.5 g
NaCl	10 g
Distilled water	100 ml

Dispense 2.5 ml in test tubes and sterilized by autoclaving at 15 psi for 15 minutes.

Nutrient broth

Peptone	10.0 g
Beef extract	10.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml

Sterilized by autoclaving at 15 psi for 15 minutes.

Overlay/ top layer agar

Nutrient broth	1.3g
Agar purified	0.7g
Distilled water	100 ml

Dispense 3 ml in test tubes and sterilized by autoclaving at 15 psi for 15 minutes.

NA base plate (3%)

Nutrient broth	8 g
Agar powder purified	3g
Distilled water	100 ml

Sterilized by autoclaving at 15 psi for 15 minutes.

SM buffer

NaCl	5.8 g
MgSO4 .7H2O	2 g
Tris Cl 1 M (pH 7.5)	50ml
Gelatin 2%	5ml
Distilled water	upto 100ml

Sterilized by autoclaving at 15 psi for 15 minutes.

Normal Saline Solution (NSS)

Sodium chloride	8.5 g
Distilled water	1000 ml

Sterilized by autoclaving and stored at 4°C until used.

TM buffer

Tris HCl 1.0 M (pH 7.5)	5 ml
$MgSO_4$ 1M	1 ml
Distilled water	upto 100 ml

Sterilized by autoclaving at 15 psi for 15 min.

5M NaCl

Sodium Chloride	29.22 g
Distilled water	upto 100 ml

Sterilized by autoclaving at 15 psi for 15 min

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS:

TAE buffer (50 X)

Tris base	60.50 g
Glacial acetic acid	14.25 ml
0.5 M EDTA solution (pH 8.0)	25.0 ml

The final volume was made up to 250 ml with double distilled water.

Ethidium bromide stock (10 mg / ml)

Ethidium bromide	10 mg
Double distilled water	1 ml

The ethidium bromide was dissolved in water and stored at 4 °C in amber colour tubes.

REAGENTS AND SOLUTIONS FOR SDS- PAGE

30% Acrylamide- bisacrylamide mix

Acrylamide	29. 0 g
Bis- acrylamide	1.0 g

Heat the solution to 37°C to dissolve the chemicals. Adjust volume up to 100 ml using distilled water. Fliter the solution through nitrocellulose filter and stored the filtered solution in dark bottle at 4°C.

1.0 M Tris (pH 6.8)

Tris	6.05 g

Dissolve in 65 ml autoclave DW by gently heating. pH adjusted to 6.8 using 1N HCl and volume made up to 100 ml with autoclave DW and store at 4°C.

1.5 M Tris (pH 8.8)

Tris	18.15g

Dissolved in 65 ml autoclave distilled water by gently heating. pH adjusted to 8.8 using 1N HCl and volume made up to 100 ml with autoclaved distilled water and store at 4°C.

10% SDS

Sodium dodecyl sulphate	10 g
Distilled water	upto 100 ml

10% APS

Ammonium per sulphate	100 mg

Made volume up to 1.0 ml with distilled water. Mix and store at 4°C.

Tris Glycine Buffer (5X)

Tris base	15.1 g
Glycine	94.0 g
SDS 10% (w/v)	50 ml
Distilled water	upto 1000 ml.

Sample loading buffer (2X)

Tris HCl (1M, pH 6.8)	1.0 ml
Glycerol 20% (v/v)	2.0 ml
SDS 4% (w/v)	4.0 ml
2- Mercapto ethanol (14.3M)	0.14 ml
Bromophenol blue 0.2% (w/v)	20 mg
Distilled water	upto 10ml.

Mix and store at -20°C.

Staining solution

Comassie Brilliant Blue	1.25 g
Methanol	250 ml
Acetic acid	50 ml
Distilled water	upto 200 ml.

Mix and filter through Whatman filter paper no. 1 and store in amber colored bottle.

De-staining solution

Methanol	500 ml
Glacial acetic acid	100 ml
Distilled water	400 ml.