

**Study on Isolation, Characterization and Antibacterial Potential of
Bacteriophage against Bovine Mastitis**



**THESIS IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE AWARD OF THE DEGREE**

OF

DOCTOR OF PHILOSOPHY

IN

VETERINARY MICROBIOLOGY

BY

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Enrollment No.: V-1628/16

COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY

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(2020)

CERTIFICATE

This is to certify that the thesis entitled “**Study on Isolation, Characterization and Antibacterial Potential of Bacteriophage against Bovine Mastitis**” submitted by **Dr. Ruchi Tiwari**, Enrollment No. **V-1628/16** in partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy in Veterinary Microbiology** of the **U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura (UP)**, India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated: 30/09/2020



(Prof. Sharad Kumar Yadav)

Major Advisor and Chairman

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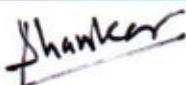

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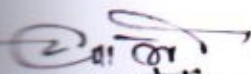
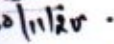
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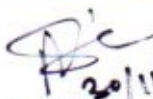
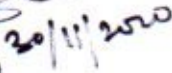
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LIST OF ABBREVIATIONS

| Abbreviation | : | Full Form |
|------------------------|----------|--------------------------------------|
| % | : | per cent |
| µg | : | microgram |
| µl | : | microlitre |
| ABST | : | Antibiotic Susceptibility testing |
| ARCAT | : | A rapid coliphage analysis technique |
| <i>B. subtilis</i> | : | <i>Bacillus subtilis</i> |
| cfu | : | colony forming units |
| cm | : | centimeter |
| CM | : | Clinical mastitis |
| CMT | : | California mastitis test |
| CNS | : | Coagulase negative Staphylococci |
| DAL | : | Double Agar Layer |
| <i>E. coli</i> | : | <i>Escherichia coli</i> |
| EcD | : | <i>E. coli</i> DUVASU Phage |
| EM | : | electron microscope |
| gm | : | gram |
| h | : | hour |
| I | : | Intermediate |
| i/m | : | Intramuscular |
| i/v | : | Intravenous |
| lb | : | pound |
| <i>Klebsiella</i> spp. | : | <i>Klebsiella</i> species |
| LA | : | Lactose agar |
| LB | : | Luria Bertani broth |
| LBA | : | Luria Bertani agar |
| mg | : | milligram |
| min | : | minute |
| ml | : | millilitre |
| NB | : | Nutrient broth |
| NSS | : | normal saline solution |
| °C | : | degree Celsius |
| <i>P. aeruginosa</i> | : | <i>Pseudomonas aeruginosa</i> |
| PFU | : | plaque forming units |

| | | |
|-----------------------|---|-----------------------------------|
| Phage | : | Bacteriophage |
| R | : | Resistant |
| RAPD | : | Random Amplified Polymorphic DNA |
| rpm | : | revolutions per minute |
| S | : | Susceptible |
| <i>S. aureus</i> | : | <i>Staphylococcus aureus</i> |
| <i>S. epidermidis</i> | : | <i>Staphylococcus epidermidis</i> |
| <i>S. typhimurium</i> | : | <i>Salmonella typhimurium</i> |
| SaD | : | <i>S. aureus</i> DUVASU Phage |
| SCC | : | Somatic cell count |
| SCM | : | Sub-clinical mastitis |
| SP | : | Streak plate/Spot inoculation |

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ABSTRACT

Current study was planned with the objective of isolation of major bacteria from cases of mastitis from lactating cows and buffaloes, thereafter isolation of bacteriophages against these bacterial isolates from samples of animal wastewater to assess the *in vitro* antibacterial potential of phage isolates. For this, collection, isolation and preliminary characterization of major bacteria (*Staphylococcus aureus* and *E. coli*) from milk samples of clinical mastitis (CM) and sub-clinical mastitis (SCM) cases from various farms and goshalas in and around Mathura, U.P. India was conducted and antibiotic sensitivity testing was performed. After this, isolation and characterization of specific phages from various locations including goshala/farms was attempted as part of a search for phages which might prove useful as antibacterial agents against mastitis pathogens. To achieve this, a total of seven hundred seventy composite milk samples from lactating cows and buffaloes were screened for SCM and CM and tested for bacterial isolation. The prevalence of SCM in lactating cows was found to be 32.94% by CMT and 50.9% by SCC, while in buffaloes 23.43% by CMT and 29.68% by SCC. The prevalence of CM was 87.5% in lactating cows and 73.47% in lactating buffaloes. Among 770 lactating cows and buffaloes, 61.94% animals were found positive for bacterial growth and no bacteria could be isolated from 38.06% animals. In the present study, a total of 85 *S. aureus*, 271 Coagulase negative Staphylococci (CNS) and 121 *E. coli* isolates were recovered. When antibiotic susceptibility testing was done, antibiotic resistant strains of *S. aureus* (resistant to kanamycin, methicillin, vancomycin and cotrimoxazole) and *E. coli* (resistant to ampicillin, amoxycillin, penicillin, methicillin and co-trimoxazole) were recovered. Among coagulase negative staphylococci, 100 % resistance was recorded against methicillin and co-trimoxazole, while more than 50% resistance was observed against ampicillin, amoxycillin, ampicillin/sulbactam, amikacin, erythromycin, gentamicin, kanamycin, penicillin-G, chloramphenicol, streptomycin, tetracycline, vancomycin, ceftriaxone, cefotaxime including methicillin and co-trimoxazole.

For rapid and preliminary detection of Staphylococcal phage and coliphage, a total of 55 representative samples of animal wastewater (urine/dung) constituting various body excretions of animals (cattle, buffalo, goats, sheep and poultry) were collected from various places including ILFC farm DUVASU Mathura, TVCC, DUVASU Mathura, different Goshalas of Mathura, Barsana, Goverdhan and Vrindavan, Goat farm, Sheep unit and Poultry farm. For rapid detection of phages, among 55 samples of wastewater, 45 (81.8%) samples were found positive by turbidity reduction method, while 30 (54.5%) were positive by spot inoculation method. A total of 51 phage isolates were obtained including 21 phage strains isolated against *S. aureus* while 30 against *E. coli*. Phage isolates (SaD₁, SaD₂, SaD₃, EcD₁, EcD₂, and EcD₃) were tested against bacterial isolates of *S. aureus* (85) and *E. coli* (121) to assess the *in vitro* lytic activity as a means of phage sensitivity testing and maximum lytic efficacy was shown by EcD₂ (100%), followed by EcD₁ (94.21%), EcD₃ (90.08%), SaD₁ (81.17%), SaD₃ (78.64%) and SaD₂ (75.29%). Electron microscopic findings indicated that coliphages belonged to family *Siphoviridae*. Coliphages were found more efficacious and can be explored for cocktail preparation in treating cases of SCM and CM.

This study provided first ever effort in Braj region (U.P.) focusing on investigation of the *in vitro* lytic strength of bacteriophages as natural antibacterial agents against *S. aureus* and *E. coli* involved in bovine mastitis.



Introduction

CHAPTER-1

INTRODUCTION

Mastitis is considered as one of the expensive diseases of milch animals in dairy sector which affect the profitability of reared animals, in terms of production losses (Seegers et al., 2003). Bovine mastitis is the inflammation of one or more quarters of the bovine mammary gland/udder caused by bacteria and/or yeast or molds and aggravated by physical/environmental factors like trauma, extreme weather, soil and contaminated water. The term 'mastitis' is derived from two latin words; Mammae (Mammae=mammary gland) and itis (Itis=inflammation). Mastitis is characterized by warm reddish painful swelling of teat/udder/quarter and deterioration of milk quality as well as reduced milk yield in the affected animals (Khan and Khan, 2006). Inflamed udder reduces the cost of animal in the animal market and exerts economic burden over the owners along with expenses of treatment costs (Bansal and Gupta, 2009). Mastitis, not only causes lowered milk production but if not treated timely may lead to loss of functional quarter and/or death of cow (Abebe et al., 2016; Dufour et al., 2019).

Mastitis is categorized into clinical mastitis (CM), sub clinical (contagious) mastitis (SCM) and environmental mastitis (Sudhan and Sharma, 2010). Clinical and subclinical mastitis are with known aetiology of host factors while environmental mastitis is related to the ambient environment. The environmental mastitis is caused by bacteria which spread primarily outside of the milking parlor *i.e.* the causative bacteria spreads from the cow's environment such as bedding, soil, manure, etc. (Garcia, 2004; Al-Haddadi et al., 2020). CM can be identified easily based upon clinically visible symptoms in terms of udder inflammation showing redness in affected part or complete udder, warmth, swelling, pain upon touch, presence of clots in milk, discoloration of milk and change in consistency of milk. Usually 5-10% mastitis cases are of CM type. The general symptoms are increased body temperature (> 39.5°C) and loss of appetite. As per the severity and intensity of symptoms, clinical form can be sub-categorized into mild, moderate and severe. In mild form of CM, colour, volume and consistency of milk gets abnormal; in moderate form, swelling of the teat or udder, warmth, pain and redness may be evident, while the severe form of

CM is characterized by acute toxic mastitis depicting symptoms of fever, anorexia and shock. Acute form of mastitis needs immediate corrective measures as compared to chronic form (Sol et al., 2000).

As compared to clinical form, in SCM no clinically visible symptoms appear, however, change in milk composition can be an indicator sign with increased somatic cell count (SCC). Somatic cells are largely leukocytes including neutrophils, macrophages, lymphocytes and sometimes alveolar epithelial cells as a part of defence mechanism to fight local infection and support fast healing of damaged teat or udder tissue (Bradley and Green, 2005; Akers and Nickerson, 2011). The SCC in the milk should be less than 2 lakh per ml in the udder of healthy cow. Studies suggested that milk samples with SCC between 2 to 20 lakh per ml have been found to be infected with coagulase-negative staphylococci (CNS), *Staphylococcus aureus* (*S. aureus*) along with *Streptococcus* spp (Plastridge, 1958; Malinowski et al., 2006). As the animal remain apparently healthy and milk appears normal, SCM is recognized and confirmed by laboratory examination of milk or by animal-side tests such as California mastitis test (CMT) followed by laboratory isolation of bacterial agent. SCM causes three times more production loss than CM, hence is responsible for significant economic loss (60-70%) of total economic losses associated with all mastitis infections (Samanta and Prasad, 1998; De Vliegher et al., 2012; Sinha et al., 2014; Mpatswenumugabo et al., 2017). Major financial burden witnessed in cases of SCM is due to reduced milk production (78%), cost of treatments (8%) and culling of affected animals (14%) (Abrahmsen et al., 2012; Romero et al., 2018). During the last century, significant advances to control mastitis have been made but changing population dynamics and variable herd structure make it a complicated disease and continues to remain a foremost problem of small and large dairy industry (Bergonier et al., 2003). Thus, further extensive research in the area is demanded (Ruegg, 2017).

Mastitis is one of the largest production concerns in the dairy sectors worldwide (Aghamohammadi et al. 2018; Dalanezi et al., 2020). Mastitis has a long-lasting effect on the milk yield because the cows that encountered mastitis in past generally do not recuperate pre-mastitis milk yields in their rest part of the lactation after onset of the disease (Rajala-Schultz et al., 1999). In spite of following advanced management practices in the cattle and buffalo rearing in dairy sector, mastitis is still a daunting disease and among the major economic issues of farmers and dairy owners.

Although, the cost of mastitis involving various factors may vary from farm to farm and from country to country, but the financial burden causing monetary and animal losses are more or less similar in all countries (Halasa et al., 2007; Azooz et al., 2020). In cattle and buffalo, mastitis is an important economic problem of most of the developing as well as developed countries including India (Tiwari et al., 2013; Das et al., 2018), Canada (Aghamohammadi et al., 2018), Germany (Hamann, 2001), Bangladesh (Kader et al., 2002), United Kingdom (Bennett et al., 1999), Netherlands (Hogeveen et al., 2011), United States (Hadrich et al., 2018) and Kenya (Anakalo et al., 2004; Mbindyo et al., 2020). Also, it has been reported that bovine mastitis is associated with a daily loss of 1.0 to 2.5 kg of milk during the first 2 weeks following the onset, and a total loss of 110 to 552 kg milk during the entire period of lactation depending upon the parity and time of occurrence (Rajala-Schultz et al., 1999). In India, the economic losses are due to mastitis is about Rs. 57.5 crore per annum which can be ascertained from the estimation that mastitis reduces milk production by 21% (<https://shodhganga.inflibnet.ac.in/>). India ranked top among milk producing countries (including cattle and buffalo milk production) and hence mastitis is very important disease to be considered in India having largest population of bovines (Bradley, 2002). In addition to the economic losses, the consumption of mastitis affected milk is harmful and is a major public health concern/hazard (Auldist and Hubble, 1998). Also, the antibiotic residue in milk due to injudicious use of antibiotics to treat and prevent mastitis is an important concern for human health. Another aspect of worry is the possibility of transmission of zoonotic threats due to consumption of mastitis affected animal's milk. Thus, the milk of affected animal can neither be consumed nor the animal is preferred for sale and therefore no profit is generated, moreover such situation add on burden to the farmers and dairy owners.

Mastitis is a multi-etiological disease and sometimes flawed milking habits of milkers and defective machine milking techniques also favour the pathogens to gain entry inside the udder to propagate and produce mastitis. As vacuum pressure is applied, this may broaden the teat orifice and hence make animal more prone to contagious and environmental mastitis (Mein et al., 2001, 2004; Tiwari et al., 2013). The involvement of bacterial agents in mastitis aetiology was first discovered in 1887 but major pathogenic bacteria involved were confirmed nearly around 1940s (Watts, 1988). According to the available literature, more than 137 species and subspecies of

microbes including bacteria, fungi and viruses can be associated with udder infection (Blajan, 1985; Kirk and Bartlett, 1986; Singh and Singh, 1994; Rajala-Schultz et al., 1999; Ndlela et al., 2016; Mbindyo et al., 2020). In clinical form of mastitis, mostly *Staphylococcus* or *Escherichia coli* (*E. coli*) bacteria overcome the normal microbiota of udder (Bradley and Green, 2001; Demme and Abegaz, 2015). The most common bacteria involved are *S. aureus*, *Streptococcus agalactiae* (*Str. agalactiae*), *Streptococcus pyogenes* (*Str. pyogenes*), *Corynebacterium pyogenes* (*C. pyogenes*), *E. coli*, *Klebsiella pneumoniae* (*K. pneumoniae*), *Klebsiella oxytoca* (*K. oxytoca*), *Enterobacter aerogenes*, *Pasteurella* spp etc (Green and Bradley, 2004; Bradley et al., 2007). Among the contagious pathogens, the most common are *S. aureus*, *Str. dysgalactia* and *Str. agalactiae*, and *S. aureus* is the predominant organism, while major environmental pathogens are members of *Enterobacteriaceae* particularly *E. coli*, and *Str. uberis*. Usually, subclinical mastitis and intra-mammary infections in heifers during calving are predominantly caused by CNS leading to heifer mastitis (Pyorala and Taponen, 2009). In primiparous cows, the highest incidence of mastitis is caused by *S. aureus*, *Klebsiella* spp. and *E. coli*. In older cows, significant losses are caused due to infections by *Streptococcus* spp., *Arcanobacterium pyogenes*, *S. aureus*, *Klebsiella* spp., and *E. coli* (Dufour et al., 2012). Overall, *S. aureus*, *Str. agalactiae*, and *Str. uberis* are the most common pathogens causing mastitis, while coliform mastitis accounts for 16.4% of the total mastitis cases (Grohn et al., 2004; Petersson-Wolfe et al., 2010; Vakkamaki et al., 2017; Nuesch-Inderbinen et al., 2019; Yu et al., 2019). Also, it has been proposed that either because of establishment of udder infection normal microbiome get upset due to which opportunistic pathogens prevail and mastitis is developed, or due to prolonged antimicrobial therapy normal inhabitant of udder get disturbed, hence mastitis begins and establishes (Ruegg, 2012; Oikonomou et al., 2014; Falentin et al., 2016; Hoque et al., 2019). Once established, if mastitis persists due to consistently disturbed normal microbiota and presence of pathogenic bacteria for long, it may convert into recurrent clinical mastitis (RCM) (Derakhshani et al., 2018; Hoque et al., 2020).

As time progressed, anti microbial therapy was made available by 1945, but it was not effective against all mastitis pathogens. The recognition of the multi-factorial etiology of bovine mastitis in the 1960s paved way for further research in mastitis and since then research is going on in identifying the causal agents and finding

appropriate therapy to cure and control mastitis. To prevent the mastitis, along with management practices antibiotic therapy is the most widely used procedure to control infection. Bovine mastitis is treated through medication via intramammary route along with intramuscular (I/M) administration of antibiotics. Both routes are preferred over single route to achieve the effective concentration of antibiotics in the udder. Moreover, high doses of antibiotics negatively influence milk quality and antibiotic residues in the milk are another problem. Researchers have documented that development of multidrug resistance bacterial strains obtained from cases of SCM and CM are growing and spreading world-wide. As host, pathogen and environment all three components are responsible for the outcome of udder infection. Bacteria undergo many drastic changes to survive the periods of starvation which increases their resistance to a variety of environmental insults (Kolter, 1992). Now-a-days, antibiotics have been so overused in medicine and as growth promoters in farm animals that many potentially pathogenic bacteria species are becoming resistant to them (WHO, 2014). In current situation, occurrence of multi drug resistant (MDR) strains, Methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA), vancomycin-resistant *S. aureus* (VRSA), livestock acquired (LA), community acquired (CA) and human acquired (HA) resistant strains of bacteria are grave concern emphasizing the necessity of adopting appropriate effective control strategies (Walther et al., 2006; van Loo et al., 2007; Fessler et al., 2012). As resistance to antibiotic therapy and number of resistant strains of bacterial pathogens are consistently increasing with the time, therefore alternative means of disease control have become essential (Shi et al., 2010; Gomes and Henriques, 2016; Francoz et al., 2017). One of the effective, nature friendly alternatives way is to explore natural enemies of bacteria, such as bacteriophages or phages for biological control of various bacterial infections like wounds, septicemia, meningitis, diarrhea, metritis and mastitis (Slopek et al., 1987; Smith and Huggins, 1987; Barrow et al., 1998; Hanlon, 2007; Tiwari and Hirpurkar, 2011; Tiwari et al., 2014; Titze et al., 2020). The alarming increase in antibiotic resistant bacteria coupled with problems of antibiotic residues has proved renewed interest in phage therapy (Dixon, 2004). It is, thus, foresighted that phage ‘the real conqueror of bacteria’ will provide potential tool to face the crisis of antibiotic overuse and will surely be the best choice to replace antibiotics for sustainable livestock production and human welfare (Douglas, 1975; Lorch, 1999; Weinbauer, 2004; Bolte et al., 2020; Geng et al., 2020).

In 1896, Hankins studied the effect of water of river Ganga and Yamuna of India on *Vibrio cholerae* and revealed that the holy water of these rivers possessed marked antibacterial activity (Hankin, 1896). Hankins (1896) also documented that, what so ever the antibacterial agent was; it kept the cholera epidemic away from being spread through ingestion of water from these rivers. This opinion invited a great many scientists to come forward and ponder over the situation. A British bacteriologist, Twort (1915) appeared as first person to recognize these agents as viruses which infect bacteria, followed by D'herelle in 1917, who virtually gave them the name Bacteriophage (D'Herelle et al., 1922). Phages are cultured in host bacteria by conventional microbiological methods. They are identified and classified primarily by their morphology in electron microscopy (EM), plaque (hole or clear areas of bacterial lysis developed over bacterial lawn as a result of phage replication) morphology and bacterial host range (Ackermann and Nguyen, 1983). Most of phages belong to *Siphoviridae* group (61.7%), followed by *Myoviridae* (24.5%), *Podoviridae* (13.9%), *Leviviridae* and *Microviridae*. On account of their cosmopolitan distribution, the phages have had a profound positive impact on the reduction of microbial population in the poultry and animal houses. They do not let bacteria to grow and so they became part and parcel of natural check and balance (Ngassam-Tchamba et al., 2020). Bacteriophages are specific for their target bacterium and hence create no negative impact on the surrounding mammary tissues or the environment and hence can also be used as udder wash before milking as they are safe for animals, handlers and ecosystem. Narrow spectrum host specific phages kill only target bacterium in exponential mode until all the similar strains of bacteria get destroyed; this property makes them a potential candidate against the pathogenic bacteria (Carson et al., 2010; Kasman and Porter, 2020). Such remarkable benefits distinguish bacteriophage therapy from antibiotic therapy and make the phage an ideal candidate for the biocontrol of bovine mastitis (Gill et al., 2006a; Tiwari et al. 2014; Ngassam-Tchamba et al., 2020).

A number of bacteriophages have been isolated and investigated for their potential in eliminating pathogenic bacteria such as *S. aureus* and *E. coli* associated with mastitis (Barrow, 2001; Kwiatek et al. 2012; Dias et al. 2013; Porter et al. 2016; Varela-Ortiz et al. 2018; Ganaie et al. 2018). Since all of the evaluations were based on *in-vitro* studies, further *in-vivo* studies have to be conducted to prove their efficacy in clinical cases. Literature reveals that bacteriophages can be used as a promising

candidate in preventing infections due to MRSA (Mann, 2008). Likewise, researchers have isolated and characterized novel coli phages to control antibiotic-resistant pathogenic strains of *E. coli* such as enterohemorrhagic *E. coli* and enteropathogenic *E. coli* (EHEC and EPEC) (Viscardi et al., 2008). Dias et al. (2013) reported that the isolated bacteriophages had features like thermostability and high lytic potential which make them suitable candidates against the *S. aureus* antibiotic resistant strains. As mastitis is mostly poly-microbial disease, hence cocktail of lytic phages can be used more efficiently. When phage cocktails were used for the treatment of *S. aureus*-induced mastitis in mouse model, it was found superior to either of the individual phages used alone (Geng et al., 2020). However, currently literature regarding the composition of therapeutic phage cocktail preparation against bovine mastitis is scanty and no reports of phage therapy against bovine mastitis are available from Braj region, Uttar Pradesh (U.P.), India. In view of the above facts, it is imperative that there is a clear need of a sustainable control option to manage the mastitis, but at present no single phage-based commercial product is available to be used against bovine mastitis. Hence, appropriate phage/phages need to be identified and tested for their antibacterial potential under laboratory conditions at initial level before their application in the biological system. Therefore, the present study was planned and conducted with the following objectives:

Objectives:

1. Isolation and characterization of the major bacteria (*Staphylococcus aureus*/*E. coli*) from milk samples of mastitis cases of cattle and buffalos.
2. Detection, isolation and characterization of the lytic bacteriophages against these bacterial isolates, from samples of animal wastes.
3. Assessment of *in vitro* antibiotic sensitivity pattern and *in vitro* Phage sensitivity testing against bacterial isolates.



Review

of

Literature

2.1 Mastitis: Causative agents and Economic concerns

Mastitis is one among the most economically devastating diseases of dairy cows and buffaloes mainly for the small scale animal owners in developing countries, with various levels of economic losses reported by different countries. As it is a production disease the loss associated with bovine mastitis can be reduction in milk production, poor altered quality of milk and milk-products, expenses over preventive measures of mastitis, treatment cost including cost of medication, veterinary services, culling of infected cows, cost of labour etc. All over the world researchers have documented various aspects of mastitis.

Dohoo and Meek (1982) illustrated the role of somatic cell counts in assessing the quality of bovine milk as number of SCC usually remain below 2 lacs but due to udder infection, a significant rise in SCC is recorded.

Harmon (1994) mentioned that there are multiple factors including physiological and environmental conditions responsible for change in somatic cell counts, hence while predicting SCM or CM in correlation to SCC, all should be taken into account.

Honkanen-Buzalski et al. (1994) isolated a total of 168 coagulase-negative staphylococci (CNS) strains from milk samples of cows suffering with clinical mastitis from Ambulatory Clinic, College of Veterinary Medicine, Finland and suggested that *Staphylococcus hyicus*, *S. simulans* and *S. epidermidis* were the most commonly isolated CNS. CNS induced mastitis mostly seen during early lactation in young cows.

Saini et al. (1994) documented the prevalence and etiology of subclinical mastitis among crossbred cows and buffaloes in Panjab state.

Samanta and Prasad (1998) stated that subclinical mastitis causes three times more production loss than clinical mastitis, leads to significant economic loss (60-70%) of total economic losses associated with all mastitis infections.

Giannechini et al. (2002) determined the incidence of clinical mastitis, prevalence of sub-clinical mastitis and identified bacterial causative agents in the West Littoral Region of Uruguay. The prevalence of SCM was 52.4% and among bacterial isolates 62.8% were *S. aureus* isolates, 11.3% *Streptococcus agalactiae*, 8% *Enterococcus* sp., 7.4% coagulase-negative staphylococci, 6.4% *Streptococcus uberis*, 1.8% *Streptococcus dysgalactiae* and 1.5% were *E. coli*.

Boerlin et al. (2003) performed study on isolation of Staphylococcus spp. from cases of bovine mastitis and isolated 272 staphylococcal isolates from cases of bovine mastitis, tested for catalase, DNase, and coagulase activity by using two rapid identification kits for slide coagulase test (Slidex Staph Plus kit and RAPIDEC Staph from Bio-Merieux) and confirmed 159 strains of *Staphylococcus aureus*.

Burvenich et al. (2003) reported that *S. aureus* and *E. coli* cause mastitis in dairy cows around parturition and during early lactation as bacteria, cow and environmental factors are interdependent and influence animal susceptibility to mastitis.

Pitkala et al. (2004) selected 3282 cows from 216 farms in Finland to conduct a national survey for estimating prevalence of bovine mastitis, to find out distribution of mastitis pathogens and performed in vitro antimicrobial susceptibility of different mastitis pathogens. Somatic cell count, bacterial isolation and antimicrobial sensitivity testing was done and obtained results were compared with the previous results documented in 1995. Findings concluded that prevalence of mastitis reduced from 38% in 1995 to 31% in 2001, presence of bacterial agents increased notably from 21.0 to 33.5% and coagulase-negative staphylococci were the most common bacteria, followed by isolates of *Staphylococcus aureus*. Result of *in vitro* antimicrobial sensitivity testing revealed that 52.1% *S. aureus* strains showed penicillin resistance while it was reduced to 32.0% by coagulase-negative staphylococci.

Adwan et al. (2005) documented that Staphylococci (68.3%) were the predominant cause of subclinical mastitis, followed by coagulase-negative staphylococci accounted for 35.6%, *Micrococcus* spp (18.3%), *Proteous mirabilis* (9.4%) and *Bacillus* spp (4.0%).

Bradley and Green (2005) suggested that somatic cell count of higher than 2 lac cells/ml is an indication of either existing bacterial infection or is linked with increased susceptibility of animal towards clinical mastitis.

Dhakal (2006) have reported that there is difference in somatic cell count of the milk in normal and subclinical mastitis cases in Murrah buffaloes.

Haltia et al. (2006) collected quarter milk samples from 3,166 cows from 25 dairy herds for SCC and bacterial isolation studies. Results proposed high prevalence of mastitis as 52.7% milk samples showed SCC more than 200,000/ml and upon bacterial analysis *Staphylococcus aureus*, *Corynebacterium bovis* and coagulase negative staphylococci were the most common isolates of bacteria.

Joshi and Gokhale (2006) documented that subclinical mastitis was more important (10–50% in cows and 5–20% in buffaloes) than clinical mastitis (1–10%) in India.

Moroni et al. (2006) studied relationship between somatic cell count and intramammary infection in buffaloes and suggested that deviation in values of SCC can be accounted as important indications of teat/udder infection.

Batavani et al. (2007) described effect of subclinical mastitis on the quality and composition of milk in dairy cows.

Bradley et al. (2007) screened 97 dairy farms in England and Wales for clinical and subclinical mastitis by collecting milk samples aseptically and performing somatic cell counts. Result concluded that mean incidence of clinical mastitis as per farm records was 47 cases per 100 cows per year while from sample testing it was higher with 71 cases per 100 cows per year. Among bacterial isolates, 23.5 % were *Streptococcus uberis* and *Escherichia coli* were 19.8 %, however most common isolates from the samples with high cell counts were coagulase-negative staphylococci.

Kivaria and Noordhuizen (2007) performed a study to determine the etiology and distribution of bovine clinical mastitis in smallholder dairy herds in Tanzania. They reported that contagious mastitis pathogens were isolated from 45.6% of the culture-positive samples, while environmental pathogens were isolated from 48.2%.

Oviedo-Boyso et al. (2007) evaluated factors involved in the immunological mechanisms against the main pathogenic bacteria causing mastitis and highlighted the innate immune response of the mammary gland. They suggested that inflammation is a consequence of the activity of a number of cell and soluble factors that function altogether to eliminate invading microorganisms and factors involved in such inflammatory response vary depending upon the infectious agents.

Sharif et al. (2007) investigated the effect of severity of sub-clinical mastitis on somatic cell count and lactose contents in the buffalo milk.

Taponen et al. (2007) based on amplified fragment length polymorphism-analysis reported that in ovine species intra-mammary infections caused by coagulase-negative staphylococci may remain present throughout the lactation period.

Miranda-Morales et al. (2008) suggested that *Staphylococcus aureus*, coagulase-negative Staphylococci bacteria, *Streptococcus agalactiae*, and *Mycoplasma* spp. are primarily responsible for clinical and subclinical mastitis.

Abdel-Rady and Sayed (2009) performed epidemiological studies on subclinical mastitis over 350 cows of different breeds and ages in Assiut governorate, Assiut, Egypt during the period from June 2006 till July 2007 by using California mastitis test (CMT) and bacteriological examination was conducted. Results revealed that major causative agents were *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* with prevalence of 52.5, 31.25 and 16.25%, respectively.

Mdegela et al. (2009) reported prevalence of subclinical mastitis (51.6%) and effect of mastitis on milk quality on smallholder dairy farms in Tanzania.

Thorberg et al. (2009) described that subclinical mastitis caused by coagulase-negative staphylococci (CNS) is common in dairy cows and due to enormous number of species of CNS it is imperative to have knowledge of epidemiology of different CNS species in dairy herds. Persistent form of subclinical mastitis is oftenly caused by *Staphylococcus chromogenes*, *Staphylococcus epidermidis* and *Staphylococcus simulans*.

Kumar et al. (2010) studied incidence and economical aspects of clinical mastitis.

Nam et al. (2010) determined the distribution of different mastitis pathogens in 1255 milk samples from 368 lactating cows on 24 dairy farms in Korea during 2008. Out of 368, 200 (54.3%) cows showed SCC of 2,00,000 cells/ml and out of 1255 milk samples, 356 (28.3%) samples were positive for bacterial isolation and total 415 bacteria were isolated. The most commonly isolated bacteria was coagulase-negative staphylococci (40.7%), followed by Gram-negative bacteria (19.5%) other than *E. coli*, *S. aureus* (12.2%), *Streptococcus uberis* (5.3%), *Enterococcus* spp. (4.8%), *E. coli* (4.5%), and environmental streptococci (3.1%) other than *S. uberis*.

Petersson-Wolfe et al. (2010) elaborated that due to mastitis milk yield may get reduced and quality of milk is also deteriorated as bacterial enterotoxins can contaminate the milk.

Turkyilmaz et al. (2010) performed molecular identification of bacterial isolates obtained from dairy herds with mastitis and suggested that sub-clinical mastitis was more common than clinical mastitis.

Piessens et al. (2011) described that there is variation in the occurrence of kind of coagulase-negative Staphylococci species in the milk and environment of dairy cows between the herds. They reported that *Staphylococcus chromogenes* and *S. epidermidis* were seldom found in the environment while *S. haemolyticus* and *S. simulans* were mostly present in the environment

Supre et al. (2011) performed study over three dairy herds of twenty five animals in each, reported that among various species of coagulase-negative Staphylococci; *Staphylococcus chromogenes*, *Staphylococcus xylosum*, *Staphylococcus cohnii* and *Staphylococcus simulans* were the most prevalent and among all CNS few species causes persistent infection by affecting udder more than other CNS species.

Abera et al. (2012) enlisted major causes of mastitis and associated risk factors in smallholder dairy farms in and around Hawassa, Southern Ethiopia and mentioned that incidence of subclinical mastitis was more frequent than the clinical mastitis.

Abrahmsen et al. (2012) reported prevalence of subclinical mastitis as 87.9% in dairy farms in urban and peri-urban areas of Kampala, Uganda.

Carrillo-Casas and Miranda-Morales (2012) studied the prevalence of major mastitis pathogens responsible for bovine mastitis and described their effects on somatic cell count and on milk production.

Hussain et al. (2012) investigated the biochemical and cellular changes occurring in milk due to mastitis by performing California Mastitis Test (CMT) and somatic cell count on 592 buffaloes and 453 cattle. Results revealed that SCC was higher with considerably higher number of neutrophils as compared to macrophage and lymphocytes in the milk samples obtained from mastitic buffaloes and cattle.

Hussein (2012) described prevalence (38.89%) and bacterial etiology of sub-clinical mastitis in dairy cows in district Al-Sulaimaniyah in Sudan.

McAdow et al. (2012) reported that coagulase test can differentiate highly pathogenic *S. aureus* from comparatively lesser pathogenic strains of staphylococci as due to deficiency of coagulase-encoding gene CNS strains fail to produce coagulase enzyme.

Shittu et al. (2012) calculated prevalence of sub-clinical mastitis as 85.3% in lactating cows in the Savannah Region of Nigeria and described risk factors associated with SCM.

Bardhan (2013) documented the estimates of economic losses due to clinical mastitis in organized dairy farms.

Hegde et al. (2013) analyzed the incidence of subclinical mastitis and distribution of major mastitis pathogens in organized farms and unorganized zones. Out of 246 milk samples, 323 bacterial isolates were obtained comprised of 95 *S. aureus*, 95 coagulase negative staphylococci (CNS), 48 *E. coli* and 85 strains of streptococci.

Jeykumar et al. (2013) emphasized on increased risk of transmission of communicable diseases such as tuberculosis, brucellosis, staphylococcal toxemia, septic sore throat etc through mastitis.

Katsande et al. (2013) calculated prevalence of mastitis (21.1%) in dairy cows from smallholder farms in Zimbabwe.

Kayesh et al. (2014) described that increase in somatic cell count (SCC) is helpful in prediction of subclinical mastitis, provided bacterial isolation can be made.

They have isolated *Staphylococcus* spp., *Streptococcus* spp. and *Escherichia coli* from cases of clinical and subclinical mastitis.

Sinha et al. (2014) performed study on subclinical mastitis in dairy animals to assess the incidence and economics of subclinical form of bovine mastitis in Central Region of India. They reported the losses due to mastitis in monetary terms were estimated to be Rs 1390/- per lactation, among which around 49% was due to loss of value from milk and 37% on veterinary expenses. Losses were comparatively higher in crossbred cattle due to their high production potential that was affected during mastitis period. The cost on treatment of an animal was approximately Rs 509 /- including expenditure on medicine (31.10%) and health services (5.47%). Antibiotics not only involve cost but they are not as effective as earlier, hence other therapeutic modalities need to be explored for the treatment of mastitis. He reported that losses were comparatively higher in crossbred cattle.

Iraguha et al. (2015) estimated the prevalence of bovine mastitis and discussed associated risk factors in dairy cows in Nyagatare District, Rwanda. They suggested that coliform mastitis occurs mainly due to contamination of teat/udder from soil and faecal material.

Patil et al. (2015) analyzed milk samples of forty apparently healthy lactating buffaloes for subclinical mastitis and clinical mastitis by performing CMT and SCC and reported that SCC was higher in buffaloes with subclinical mastitis than in buffaloes with normal milk and SCC was higher in buffaloes with clinical mastitis than those with subclinical mastitis.

Abebe et al. (2016) screened 529 lactating cows for SCM by CMT and estimated the prevalence and risk factors associated with bovine mastitis in dairy herds at South Ethiopia. Results revealed prevalence of 74.7% mastitis at herd-level and 62.6% at cow-level (59.2 % for sub-clinical and 3.4% for clinical mastitis). *Staphylococcus aureus* was isolated as an important causative agent of mastitis from 51.2% of the 172 CMT positive milk samples.

Argaw (2016) reviewed epidemiology of clinical and subclinical mastitis on dairy cows, mentioned the prevalence of mastitis cases and emphasized that antibiotic resistance is increasing due to inappropriate usage of antibiotic drugs.

Martins et al. (2016) evaluated the microbiological profile of milk samples collected before and after mastitis treatment with gentamicin and investigated antimicrobial susceptibility of *Staphylococcus* spp. They isolated 210 bacterial strains from milk samples of cows tested for clinical and subclinical mastitis and showed that among bacterial isolates, Coagulase-negative staphylococci (CNS) were the most frequent (47.61%) among the 63 (30.01%) *Staphylococcus* spp. recovered.

Sanotheran et al. (2016) investigated prevalence of subclinical mastitis (SCM) by CMT in 152 lactating cows of Batticaloa District, Sri Lanka. CMT positive milk samples were processed for microbiological and biochemical analysis. Result revealed 66 lactating cows (43%) were positive to CMT, however 93.9% samples showed bacterial growth upon culturing and *Staphylococcus* spp. (90.5%) was the most frequently isolated genera.

Gogoi et al. (2017) assessed the prevalence of subclinical mastitis in 92 (local and cross bred) cows belonging to unorganized farms of North Lakhimpur town in Assam. Milk samples were collected from 92 cows, 18 (19.56 %) cows were found CMT positive and out of 18, *E. coli* was isolated from 7 samples (38.88%) and coagulase negative *Staphylococcus* spp. from 5 samples (27.77%).

Saglam et al. (2017) conducted a study to determine the role of *Staphylococcus* in case of subclinical mastitis. Four hundred lactating cows were screened by California Mastitis Test (CMT) and bacterial isolation for confirming subclinical mastitis. Results showed out of 400, 235 milk samples were found positive for sub-clinical mastitis by CMT. A total of 117 (49.7%) *Staphylococcus* spp. were isolated, including 76 (64.95%) as *S. aureus*, 74 (63.24%) coagulase-positive staphylococci and 43 (36.75%) coagulase-negative staphylococci.

Mpatswenumugabo et al. (2017) conducted study in Western Rwanda and reported the prevalence of subclinical mastitis (SCM) was 50.4% and identified coagulase negative Staphylococci (51.5%), *Staphylococcus aureus* (20.6%), *Streptococcus* species (10.3%), *Bacillus* species (10.3%), *Streptococcus agalactiae* (5.8%), and *Escherichia coli* (1.5%) as the causative bacteria.

Zeryehun and Abera (2017) performed study in Eastern Ethiopia to measure the prevalence of CM and SCM in lactating cows. The prevalence of clinical and subclinical mastitis were 12.5% and 51.8%, respectively and microbiological

examination of milk sample revealed coagulase negative Staphylococci species (CNS) as the most predominant (34.2%) species while *Streptococcus faecalis* (2.1%) was identified as the least bacteria.

Jadhav et al. (2018) suggested that SCC is the most commonly used single consistent indicator of udder health after performing a study over 214 Holstein Friesian crossbred dairy animals and reported a threshold value of 3,10,000 somatic cells/ml of milk to differentiate case of sub-clinical mastitis from normal milk.

Romero et al. (2018) conveyed that subclinical mastitis (SCM) causes most of the financial losses due to reduced milk production (78%), cost of treatments (8%) and culling (14%).

Fahim et al. (2019) isolated *E. coli* strains from thirty lactating cows and fifteen lactating buffaloes from five dairy farms to estimate the prevalence of *E. coli* in case of sub-clinically mastitis (SCM) and also studied antibiogram of *E. coli* isolates. Before attempting bacterial isolation, all milk samples were screened by California Mastitis Test (CMT) and somatic cell count (SCC) techniques for SCM. They reported recovery of *E. coli* from 50% composite milk samples of cows and buffaloes both.

Nuesch-Inderbinen et al. (2019) isolated 82 strains of *E. coli* from milk samples of bovine mastitis cases of cows and assessed the antibiogram. Result of ABST showed resistance of 22 % to ampicillin and 14.6 % to tetracycline. They were susceptible to most of antibiotics and few strains showed mild resistance to gentamicin (3.7 %), amoxicillin/clavulanic acid (2.4 %) and ceftiofur (1.2 %).

Yu et al. (2019) documented high incidence of *E. coli* from 750 bovine milk samples from mastitis cases in China.

Mbindyo et al. (2020) investigated the prevalence and etiologies of clinical and subclinical mastitis in Kenya by screening 395 dairy cows for subclinical mastitis by using the California mastitis test (CMT) and bacterial isolation. The result revealed overall prevalence of mastitis based on CMT and clinical examination was 80% (316/395), out of which 6.8% (27/395) was clinical mastitis, while 73.1% (289/395) was subclinical mastitis. Based on culture, the predominant bacteria were coagulase-negative Staphylococci (CNS) with 42.8% (435/1016) followed by *Streptococcus species* with 22.2% (226/1016), *Staphylococcus aureus* with 15.7% (160/1016),

Pseudomonas aeruginosa with 5.1% (52/1016), and *Enterobacter* species 0.7% (7/1016), while 23.7% of the sample yielded no bacterial growth. The findings suggested a relatively high prevalence of subclinical mastitis with CNS as predominant bacteria.

2.2 Role of antibiotics in controlling SCM and CM

Chandler (1971a and b) performed experimental study on mouse model against bacterial mastitis to assess the relative efficacy of antibiotics.

Sandholm et al. (1990) described that many times due to diverse factors bovine mastitis does not respond to antibiotic therapy and hence alternative treatment options need to be thought of.

Owens (1998) evaluated various antibiotics for generation of L-forms from *S. aureus* isolated from cases of bovine mastitis.

Manie et al. (1999) determined the antibiotic-resistance pattern of the microbes recovered from freshly slaughtered beef and unpasteurized and pasteurized packaged milk. Obtained bacterial isolates of Staphylococci and Enterobacteriaceae were checked for resistance spectrum to gentamicin, vancomycin, streptomycin, penicillin, methicillin and tetracycline by using the disc diffusion susceptibility test. They suggested that resistant bacteria can be transferred through the consumption of raw beef and unpasteurized milk to human beings.

Sol et al. (2000) detailed numerous important factors responsible for early recovery of animal after therapy of clinical mastitis caused by *Staphylococcus aureus*.

Gentilini et al. (2002) performed ABST over 123 isolates of coagulase-negative staphylococci isolated from cases of clinical and subclinical bovine mastitis in Argentina from March 1998 to March 2000. Isolates of coagulase-negative staphylococci showed resistance to penicillin (27.6%), oxacillin (3.2%) and erythromycin (4.8%), while were sensitive to gentamicin, cephalothin and ampicillin-sulbactam.

Barkema et al. (2006) documented that success of treating *Staphylococcus aureus* associated bovine mastitis depends not only on the therapeutic regimen but interplay of cow, pathogenic bacteria and environment all plays active role in the recovery of animal.

Tenhagen et al. (2006) isolated mastitis pathogens from dairy cows and tested their antimicrobial resistance pattern against 6 selected antimicrobial agents in Brandenburg, Germany. Mastitis pathogens were isolated from 26.4% of the milk samples, where 9.1% were coagulase-negative staphylococci and percent of *S. aureus* was 5.7%. Cephalosporins were the preferably used drug and maximum resistance was shown to ampicillin by *S. aureus*.

John and Harvin (2007) markedly reported that CNS, which were normally considered as non-pathogenic, being part of normal microflora have shown more resistance to antimicrobials specially β -lactam antibiotics, as compared to *S. aureus*. Some studies have claimed 90% of oxacillin resistance in CNS and urge the need of newer antimicrobial agents with effective antistaphylococcal activity. As Staphylococcal biofilm formation is a common feature in case of CNS infections, hence therapeutic agent must have capacity to penetrate the biofilm in order to inhibit and eradicate adherent staphylococci.

Sharma et al. (2007) studied prevalence, etiology and antibiogram of microorganisms associated with cases of sub-clinical mastitis in buffaloes in Durg, Chhattisgarh, India.

Kahir et al. (2008) analysed prevalence and risk factors of subclinical bovine mastitis in some dairy farms of Sylhet district in Bangladesh.

Rodloff et al. (2008) described basis of tripartite classification scheme for categorization of bacterial strains into susceptible, intermediate and resistant group as per the *in vitro* efficacy to the bacterial strain.

Sumathi et al. (2008) investigated prevalence of clinical mastitis in bovines and studied the antibiogram of bacterial isolates recovered from mastitis cases.

Koksal et al. (2009) evaluated antibiotic resistance patterns of 200 strains of CNS in Turkey in between 1999 and 2006. Among 200 CNS isolates, most prevalent species was *Staphylococcus epidermidis* and 67.5 % CNS were methicillin resistant. Resistance of CNS to gentamicin was 90% while it was 80% to erythromycin. Likewise 72% to clindamycin, 68% to trimethoprim-sulfamethoxazole, 67% to ciprofloxacin, 60% to tetracycline, 56% to chloramphenicol and 25% to fusidic acid.

Nam et al. (2009) determined prevalence and antimicrobial susceptibility of gram-negative bacteria isolated from bovine mastitis between 2003 and 2008 in Korea.

Bal et al. (2010) reported prevalence and antimicrobial susceptibility pattern of Staphylococci and Streptococci from subclinical mastitis cases. A total of, 100 isolates of (CNS) were recovered, which showed resistance of 58% to penicillin-G, 48% to ampicillin and 20% to neomycin.

Virdis et al. (2010) studied antimicrobial resistance patterns in 25 *S. aureus* and 75 coagulase negative staphylococci (CNS) strains isolated from milk samples of goats suffering with subclinical mastitis. Result of ABST depicted that 56.0% of *S. aureus* and 41.3% of CNS isolates expressed multi-drug resistance. 28.0% of *S. aureus* were resistant to kanamycin, 16.0% to oxytetracycline, and 12.0% to ampicillin, while CNS showed maximum resistance to ampicillin (36.0%) and kanamycin (6.7%). Findings suggested that among CNS, one strain of *Staphylococcus epidermidis* was resistant to six antibiotics. In the study mostly strains were resistant to single drug and multidrug resistance was less frequently observed characteristic.

Bhatt et al. (2011) reported prevalence of clinical and subclinical mastitis in cattle to be 5.5% and 15.75%, respectively by using California Mastitis Test (CMT) kit in Anand and in nearby villages of Gujarat. Among bacterial isolates, *Staphylococcus aureus*, *B. pumilus*, *Staphylococcus chromogenes*, *Bacillus species*, and *Pseudomonas* species were recovered. Antibiogram showed highest bacterial resistance to penicillin G and oxacillin antibiotics.

Kaliwal et al. (2011) investigated the prevalence and antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in and around Dharwad region. Out of 310 samples screened, 180 isolates of coagulase negative staphylococci were acquired. The result of antimicrobial susceptibility of CNS illustrated maximum sensitivity of 83.88% to ceftriaxone, followed by 79.41% to cefotaxime, 76.47% to methicillin, 73.52% to ciprofloxacin, 70.05% to erythromycin, 66.11% to amikacin, 42.94% to gentamycin, 36.76% to amoxicillin, 29.41% to ampicillin and 23.23% to penicillin. The results indicated that CNS strains isolated from bovine mastitis showed highest susceptibility to ceftriaxone.

Kalmus et al. (2011) demonstrated that *Streptococcus uberis* and *E. coli* were the major pathogens associated with clinical mastitis, while *S. aureus* and CNS were mainly involved with subclinical mastitis. Most of strains of *S. aureus* and CNS were resistant to penicillin.

Mahami et al. (2011) evaluated the risks of antimicrobial resistant microbes associated with branded and unbranded milk sold in Accra, Ghana. Microbiological analysis showed occurrence of *S. aureus*, *S. epidermidis*, *Enterococcus faecalis*, *E. coli*, *Salmonella typhi*, *Klebsiella spp*, *Proteus vulgaris* and *Enterobacter spp*. Results of antibiotic susceptibility tests concluded that all the isolates were multi-resistant and showed no susceptibility to ampicillin, tetracycline, chloramphenicol, gentamycin, cotrimoxazole, ceturoxime and cefotaxime and found 100 % resistant to the above tested antibiotics.

Waller et al. (2011) compared prevalence and antimicrobial susceptibility of CNS species in clinical and subclinical mastitis and reported that *Staphylococcus chromogenes* and *Staphylococcus epidermidis* were the most frequently occurring CNS species followed by *Staphylococcus simulans* and *Staphylococcus haemolyticus*. *S. epidermidis* was notably more prevailing in subclinical mastitis than in clinical mastitis, whereas *Staphylococcus hyicus* was comparatively more common in clinical mastitis.

Haftu et al. (2012) determined the prevalence of bovine mastitis, isolated mastitis pathogens and studied the antibiotic resistance pattern of bacteria isolated from a total of 305 cows of dairy farms in Northern Ethiopia. The prevalence of clinical and subclinical mastitis was 3.6 and 33.8 %, respectively. Among bacterial isolates, 36 % were *S. aureus* and 27.3 % were *E. coli* isolates. Result of antibiotic susceptibility testing of *S. aureus* showed highest sensitivity to nalidixic acid (82.4 %) and chloramphenicol (58.8 %) and highest resistance to clindamycin and ampicillin. *E. coli* isolates were resistant to most of the antibiotic drugs tested.

Kenar et al. (2012) isolated 67 (11.7%) coagulase-negative staphylococci from 572 CMT positive milk samples of subclinical mastitis cases of bovine in Turkey. *S. epidermidis* (18 in number) was most prevalent among CNS. CNS isolates showed resistance to trimethoprim and sulphamethoxazole (76.2%), erythromycin (73.2%), oxacillin and ampicillin (70.2%), penicillin (58.3%), gentamicin (53.8%), tetracycline

(52.3%), vancomycin (51.8%), ciprofloxacin (26.9%), ceftiofur (23.9%), and cephalothin (13.5%). Results indicated that maximum resistance was shown to beta-lactam antibiotics due to their extensive use in prevention and treatment of mastitis.

Alekish et al. (2013) studied prevalence of bovine mastitis and antimicrobial resistance pattern of bacterial pathogens isolated from bovine mastitis cases in northern Jordan. They showed 92% resistance of CNS to macrolides in northern Jordan.

May et al. (2014) evaluated national resistance data obtained from the Surveillance Network (TSN) to know the national prevalence of CNS of 13 years (1999 to 2012) in USA. They studied the resistance pattern of CNS mainly *S. epidermidis* against ciprofloxacin, levofloxacin, and clindamycin and suggested continuous increase in the resistance with the passing years. They reported that *S. epidermidis* resistance to ciprofloxacin was increased from 58.3% to 68.4% and to clindamycin from 43.4% to 48.5% in the period of thirteen years.

Al Tayyar et al. (2015) studied the prevalence of coagulase-negative staphylococci (CNS) and their antimicrobial susceptibility pattern by using 18 antibiotics in Jordan. Results revealed *S. epidermidis* and *S. haemolyticus* as most common CoNS isolates. A total of two hundred twenty three (223) isolates of CoNS were found highly sensitive to vancomycin, rifampin, and linezolid while resistant to ampicillin, amoxicillin, penicillin, ceftiofur, cefazolin, amoxicillin-clavulanic acid and erythromycin.

Bansal et al. (2015) compared antibiogram of coagulase-negative Staphylococci (CNS) associated with subclinical and clinical mastitis in dairy cows. Findings suggested that CNS isolates were mostly susceptible to chloramphenicol, gentamicin and streptomycin, while they showed comparative resistance to penicillin group of drugs. Percentage sensitivity of CNS isolates was 98.3% to chloramphenicol, 93.1% to gentamicin, 91.4% to streptomycin, 91.4% to linezolid, 87.9% to ceftiofur, 86.2% to cloxacillin, 86.2% to clotrimazole, 86.2% to bacitracin, 84.5% to enrofloxacin and 70.7% to ceftiofur/tazobactam. However, 77.6% isolates shown resistance to amoxicillin, 75.9% to penicillin and 74.1% to ampicillin among Penicillin group and 51.7% to cefoperazone among cephalosporins. Workers also

emphasized that bacterial isolates recovered from clinical cases of mastitis showed higher resistance than the subclinical isolates.

Ventola (2015) described various causes of the antibiotic resistance and crisis originated due to AMR.

Attili et al. (2016) studied the role of Enrofloxacin in controlling the severity of the clinical mastitis caused by *Staphylococcus aureus* in a commercial dairy flock of 73 sheep and results showed that enrofloxacin help in reducing rectal temperature and somatic cell count and all isolated *S. aureus* strains were sensitive to enrofloxacin.

Martins et al. (2016) investigated the antimicrobial susceptibility profile of *Staphylococcus* spp. by the disc diffusion test on Mueller-Hinton agar by using ampicillin 10µg (AMP), gentamicin 10µg (GEN), kanamycin 30µg (KAN), oxacillin 1µg (OXA), penicillin G 10 IU (PEN), ceftiofur 30µg (CTF), and cefaclor 30µg (CEC). Findings revealed that The *S. aureus* strains were resistant to neomycin (37.5%), kanamycin (25.0%), and gentamicin (12.5%), *Staphylococcus* spp. showed high levels of resistance to neomycin (16.95%), penicillin G (10.17%), and ampicillin (10.17%), CPS showed resistant to neomycin (13.64%), penicillin G (13.64%), and ampicillin (13.64%), while CNS were resistant to all antimicrobial agents tested. Multidrug resistance to all antibiotics tested was observed in 1.69% of the *Staphylococcus* spp. isolates.

Beyene et al. (2017) described the prevalence and antimicrobial resistance profile of *Staphylococcus* spp including *S. aureus* and coagulase negative staphylococci in dairy farms.

Bhat et al. (2017) mentioned incidence of bovine clinical mastitis in Jammu region and discussed antibiogram of isolated pathogens.

Kalinska et al. (2017) revealed that approximately 90% of mastitis cases are caused by environmental bacteria, which showed lower susceptibility to antibiotics and this is alarming condition for the researchers to encourage them for finding new solutions towards mastitis prevention and treatment.

Amer et al. (2018) reported the prevalence of CM and SCM to be 12.0% and 60%, respectively in 250 cattle in Ecuador. Microbiological analysis of milk samples of CM revealed that 33.3% were coliforms, 25.8% coagulase-positive staphylococci,

20.4% coagulase-negative staphylococci (CNS), 9.7% streptococci, 7.5% *Bacillus* spp., and 3.2% *Klebsiella* spp. while samples of SCM showed that 55.4% were CNS higher than CM, 22.1% *Bacillus* spp., 9.3% streptococci, and 6.1% coagulase-positive staphylococci. In vitro antibiotic susceptibility testing of the bacterial isolates indicated that all strains were susceptible to amoxicillin, ampicillin, cefotaxime, enrofloxacin, sulfamethoxazole-trimethoprim, gentamicin, and neomycin. In the study, no multidrug-resistant strains were reported.

Al-Haddadi et al. (2020) reported that bovine mastitis cases in Oman are mainly associated with environmental bacteria and they express high resistance to commonly used antibiotics.

Bolte et al. (2020) performed a study on 58 Northern German dairy farms to investigate the susceptibility of frequently occurring mastitis pathogens isolated from clinical mastitis cases against commonly used antimicrobials. They reported presence of 51 isolates of *Streptococcus agalactiae*, 54 isolates of *Streptococcus dysgalactiae*, 50 isolates of *Streptococcus uberis*, 85 isolates of *Staphylococcus aureus*, 88 isolates of non-aureus staphylococci, 54 isolates of *Escherichia coli* and 52 isolates of *Klebsiella* species and calculated minimal inhibitory concentration (MIC) against cefquinome, cefoperazone, cephapirin, penicillin, oxacillin, cloxacillin, amoxicillin/clavulanic acid and cefalexin/kanamycin. Results revealed that growth of most of Gram positive mastitis bacteria was hindered at higher cloxacillin concentration than oxacillin concentration and MIC values of Gram negative pathogens were higher as compared to earlier MIC values of the drugs.

2.3 Bacteriophage

Bacteriophages are viruses that naturally infect bacteria. Hankin (1896) documented that the waters of the Ganges and Yamuna rivers in India had a marked antibacterial action. He particularly studied the effect of holy water on *Vibrio cholerae* and reported that the substance was responsible to keep cholera epidemics from being spread by ingestion of the water of these rivers. D'Herelle et al. (1922) discussed about nature and properties of phages with regard to plaque formation and composition, infective centers, the lysis process, host specificity of adsorption and multiplication, the dependence of phage production on the precise state of the host, isolation of phages from sources of infectious bacteria and the factors controlling

stability of the free phage. For the first time D'Herelle coined the term bacteriophage, commonly called as phage (Borysowski et al., 2006).

2.3.1 Morphology and Classification of phage:

Literature reveals countless reports on morphological studies of different bacteriophages.

Adams (1953) first time formulated considerations about phages and accordingly he considered all available criteria for classification. On the basis of these criteria he gave biological classification of phages.

Ackermann and Nguyen (1983) performed a morphological study on sewage coliphages by electron microscopy. Based on different morphology, they classified them into ten varieties and also suggested that electron microscopy is the method of choice for investigation of phage geography and phage ecology and these phages were classified into four different families; namely, *Myoviridae*, *Styloviridae*, *Podviridae* and *Microviridae* based on different morphology.

Adeles et al. (1984) grouped bacteriophages into different clusters on various basis such as, morphology, host range, nucleic acid type, strategies of infection, physico-chemical characteristics and dependence on properties of hosts and the environment.

Phages replicate only inside the susceptible host bacterial cells. Goyal et al. (1987) described in detail the replication of lytic phages. They documented that phage use the ribosomes, protein synthesizing factors, amino acids and energy generating systems of host cell to replicate, and hence phages can multiply only in actively metabolizing host bacteria.

Pelczar et al. (1988) documented that phages which are of particular interest in water quality assessment are classified into following six families on the basis of phage morphology and their nucleic acid content-*Myoviridae*, *Siphoviridae*, *Podoviridae*, *Microviridae*, *Inoviridae* and *Leviviridae*.

Ackermann et al. (1994) described nine new species of tailed *Bacillus* phages. He reported phage P-10 as one of the largest virus known.

Pedroso and Martins (1995) performed an experiment for the detection of coliphages in water by employing the ARCAT technique. The study over 45 different

plaque morphology indicated that the phages involved belonged to 3 families *Myoviridae*, *Podoviridae* and *Siphoviridae*. He showed presence of broad spectrum coliphage in water and concluded that there were no apparent relationship between coliphage family and specific plaque morphology.

In a separate study, Ackermann and Krisch (1997) broadly defined the T-4 type of bacteriophage on the basis of phage morphology. He showed that 18 apparently unrelated phages with prolate heads and contractile tails were found in a wide range, a descriptive catalogue of these phages was presented in the study.

Various reports are available on the detection of most of the members of different phage families in a wide variety of water environments. Grabow et al. (1998) reviewed such condition and reported that counts of coliphages may increase in certain water environments and they can be detected by relatively simple, inexpensive and rapid plaque assays.

Maniloff and Ackermann (1998) in a separate study reported the classification of phages. They classified phages into following families on the basis of phage morphology and their nucleic acid content-*Myoviridae*, *Siphoviridae*, *Podoviridae*, *Microviridae*, *Inoviridae* and *Leviviridae*.

Grabow (2001) described the structure of bacteriophage. He detailed that the phages basically consist of a nucleic acid molecule (genome) surrounded by a protein structure (capsid). The capsid is made up of morphological subunits called capsomers.

Ackermann (2003) studied more than 5100 bacteriophages and based on electron microscopic study, grouped them into one order *Caudovirales* and 13 families. He also stated that *Siphoviridae* is by far the most frequent phage group (61.7%), followed by the *Myoviridae* (24.3%) and *Podoviridae* (13.9%). In addition, there are number of families such as *Cystoviridae*, *Lipothrixviridae*, *Carticoviridae*, *Tectivividae*, *Plasmaviridae*, *Rudiviridae* and *Fuselloviridae*. He also observed that phages with long, non contractile tails predominate polyhedral filamentous and pleomorphic phages. He reported that at least 4950 (96%) were tailed phages.

Han et al. (2013) performed isolation and characterization of virulent bacteriophage SAH-1 against *Staphylococcus aureus* from sewage near a dairy cow farm and classified it under *Myoviridae* family. They also suggested the therapeutic potential of phage SAH-1 in treating *Staphylococcus aureus* infections.

Litt and Jaroni (2017) studied morphological characteristics of bacteriophages isolated against *Escherichia coli* O157:H7 by using transmission electron microscope. Based on morphological features, phages were grouped into *Myoviridae* and *Siphoviridae* family.

2.3.2 Source of Phage: Animal waste and sewage as rich resource

Ewert and Paynter (1980) performed an experiment on enumeration of bacteriophages in sewage treatment plant and he reported that the maximum total phage concentration in the fluid phase of sewage was 2.2×10^7 /ml.

Goyal et al. (1987) documented that phages exist wherever bacteria occur and they share a common ecology with their respective bacterial hosts.

Duran et al. (2002) reported that bacteriophages were more resistant than their host bacteria to the environmental changes and persist even in the absence of host bacteria.

Brussow (2005) have recently reviewed importance of phage typing and suggested that phages or phage like genomic sequences accounted for the major genomic differences between members of bacterial species.

Kutter and Sulakvelidze (2005) studied about the titer of phage in biosphere and they consider these phages to be the most abundant entities in the biosphere with total number estimated from 10^{30} to 10^{32} .

McLaughlin et al. (2006) worked on isolation of *Salmonella* bacteriophages by an enrichment protocol as well as directly from effluents and observed that phage titres of 2.9×10^8 to 2.1×10^9 PFU/ml and 12 to 148 pfu/ml were obtained, respectively.

Voluminous literature is available which suggest that phages of bacterial pathogens of animals are shed in animal feces and can be isolated from soil, aqueous environment and sewage. The incidence and survival of phages in sewage is subjected to many variables such as temperature, pH, organic matter *etc.* (Aidan et al., 2009; Al-Khafaji, 2012). However, cent percent recovery of phages is difficult.

Ibrahim (1969) reported that lytic bacteriophages against *E. coli* and *Salmonella* species were isolated from fecal samples of turkey poult.

Dhillon et al. (1976) worked on distribution of bacteriophage and showed that phages for different bacteria have been isolated from domestic sewage containing

mammalian feces (cows, pigs and humans) and also performed enumeration of coliphages and *Salmonella* phages.

Goyal et al. (1980) described method for concentration of coliphages from large volumes of water and wastewater and reported that coliphages occurring naturally in raw and secondarily treated sewage were recovered with increase average efficiencies of 56.5 % and 55.0%, respectively.

Calvo et al. (1981) performed a study on waterborne bacteriophages active on *Yersinia enterocolitica* (YE), which were isolated from the samples of surface water. These new waterborne phages were used for phage typing of YE and results indicated that the isolation of waterborne bacteriophages might reflect the presence of lysogenic YE.

Ackermann and Nguyen (1983) made a study on coliphages, recovered from sewage. By enrichment of sewage culture they obtained 10 morphologically different varieties of coliphages and grouped them under 4 different families.

Havelaar et al. (1986) attempted an experiment to explain the presence of F-specific RNA phages in waste water and fecal material from human and animals. They suggested that phages were detected in appreciable numbers only in feces from pigs, broiler chickens, sheep and calves but not from dogs, cows, horses and humans.

Tartera and Jofre (1987) performed study on detection of bacteriophages active against *Bacteriodes fragilis* in sewage polluted waters and reported that phages were always recovered from sewage and sewage polluted samples of water and sediments but not from non-polluted samples of water.

The concentration of phage in natural unpolluted waters is in general, believed to be low. Bergh et al. (1989) developed a new method for quantitative enumeration and reported that in natural unpolluted waters bacteriophage concentration was found up to 2.5×10^8 phage/ml. These concentrations indicated that phage infection might be an important factor in the ecological control of planktonic micro-organisms.

Salama et al. (1989) documented that 49 *Campylobacter* phages were isolated from 272 effluent samples of which 42 produced lysis with *C. jejuni* strains and 7 with *C. coli* strains. Phages were recovered from pig manure, abattoir effluents, poultry manure, sewage and human feces or farm pasture soil.

Grabow (2001) documented a wealth of information on phages which were found in sewage. He reported that counts of somatic coliphages (in sewage) was in order of 10^6 - 10^8 per litre where as slaughterhouse waste water contained counts of somatic coliphages as high as 10^{10} per litre.

Huff et al. (2003a) isolated bacteriophages from municipal waste treatment and poultry processing plants in his study on bacteriophage used for treatment of a severe *E. coli* respiratory infection in broiler chickens.

Klaus et al. (2003) analyzed different sources of environmental water for isolation of bacteriophages of T-4 type morphology and they suggested that highest abundance of coliphages were present in wastewater and sewage.

Muniesa et al. (2004) conducted an experiment for the detection of phages by using a lab strain of *E. coli* as host and with the help of plaque-hybridization based method phages were isolated from a range of sewage samples.

Carey-Smith et al. (2006) found that phages infecting *Salmonella* species were isolated from sewage using soft agar overlays containing three *Salmonella* serovars.

McLaughlin et al. (2006) reported isolation of *Salmonella* bacteriophages from nine swine manure lagoons.

Zhilentov et al. (2006) identified a *C. jejuni* flagellum specific phage PV22 from *Proteus vulgaris* in sewage by sampling of the drainage sewage water.

Jamalludeen et al. (2007) performed isolation of nine bacteriophages from sewage of 38 different pig farms which lyse 0149 enterotoxigenic strain of *E. coli*.

Yoon et al. (2010) isolated two bacteriophages against *S. aureus* from sewage, soil and straw samples collected from cowsheds. Phages were confirmed by performing plaque assay over lawn of *S. aureus*.

2.3.3 Rapid detection, enrichment, Isolation, characterization and lysate preparation:

Adams (1959) designed the principles of plaque assay technique. Numbers of phage particles are generally determined by direct quantitative plaque assays. The quantitative detection of phages in numbers below the detection limit of direct plaque assay is therefore carried out by direct plaque assays using large Petri dishes.

Rima and Steensma (1971) performed a study on comparison of different isolation techniques for *B. subtilis* phages. They observed that when different techniques were used for the isolation of phages of *B. subtilis*, a number of different phages were also obtained. They reported presence of certain defective phages in the old cultures of all strains of *B. subtilis* tested.

Parisi and Talbot (1974) used an improved rapid plate method by which isolation of bacteriophages become easier even from lysogenic bacteria.

Berman et al. (1980) described molecular filtration technique for increasing the concentration of viruses in water, hence recovery was enhanced.

Goyal et al. (1980) developed membrane filter adsorption-elution technology for the concentration of coliphages from large volumes of wastewater and suggested that coliphages could be concentrated from 17 litre volumes of tap water with recoveries ranging from 34 to 100%.

Divizia et al. (1989) also documented that, among different methods used for recovery of phages, ultrafiltration technique has a high efficiency of recovery. Efficiency of recovery has been recorded as 100% for various commercial filter devices with different diameters of flat sheet ultrafiltration membranes.

Salama et al. (1989) have demonstrated an improved method for the isolation of *Campylobacter jejuni* and *E. coli* bacteriophages from different effluent samples and 49 *Campylobacter* phages were isolated.

Grabow et al. (1998) conducted an experiment and reported that small number of phages in large volumes of water may also be detected by qualitative enrichment procedures.

McLaughlin et al. (2006) used phage enrichment technique and reported that phage titer obtained was increased by the enrichment protocol.

Tiwari et al. (2010) detailed the protocol for isolation and characterization of lytic phages from natural waste material of livestock. They used turbidity reduction method for rapid detection of lytic activity of phage present in wastewater.

Kwiatek et al. (2012) isolated lytic bacteriophages against *S. aureus* from milk samples through enrichment protocol by using a mixed culture of three arbitrarily selected bacterial strains *S. aureus* American Type Culture Collection (ATCC) 43300,

ATCC 25923 and *S. aureus* MRSA 643. Presence of phage was detected by formation of lytic zones of plaques over the bacterial lawn.

Rehman et al. (2016a) isolated and purified bacteriophages against *S. aureus* from the sewage water by following enrichment protocol.

2.3.4 Factors affecting replication and characterization of phages:

Ellis and Delbruck (1939) described the growth pattern of phages. There are several factors which can promote the replication of phages in their host bacterial cells.

Havelaar and Hogeboom (1983) reported that cations such as calcium and magnesium ions, promote the phage adsorption over the host bacteria. These cations in appropriate concentration have an impact on infection of bacterial cell by phage.

Goyal et al. (1987) documented the effect of several variables on phage survival under laboratory condition. They studied about the factors which affect the number and behaviour of phages in water environment and reported that the densities of both bacteria and phages; the association of phages and bacteria with solids; the presence of organic matter, especially organic matter that influences the metabolic activity of the host bacteria; ultraviolet and visible light; temperature; pH; the concentration and type of ions; and the metabolic activities of micro-organisms other than the host bacteria are important factors which can affect the phage replication. They also evidenced that successful phage replication requires at least 10^4 host bacteria per ml.

Brenner et al. (1999) investigated the effect of season on replication and recovery of phages from rural watershed. They used plaque assay technique for detection of enteric phages against *Salmonella typhimurium* and *Bacteroides fragilis* and reported the presence of host specific phages, especially during the summer months.

Several important factors include pH, temperature, chloroform, organic matter, relative number of host bacteria and phage, ionic concentration, etc.

Effect of pH:

Hydrogen ion concentration (pH) is an important criteria which is used for characterization of phages as it affects the burst size of phage. Grilione and Carr

(1959) reported that phage appears to be stable to the basic range of pH but inactivation was evident at the lower pH levels. Rogers and Sarles (1963) also studied the effect of pH in order to characterize the *Enterococcus* bacteriophage.

Phelps (1967) used the criteria of pH inactivation for characterization of newly isolated phages for *Neisseria*.

Colasito and Rogoff (1969) reported that no significant difference was seen due to pH effect on the various phages. They suggested that stability was maximal between pH 6 and pH 8 and all the phages were completely inactivated at pH values of 4 and 10.

Goyal et al. (1987) have documented that pH of water may not have a major effect on phage adsorption and replication.

Kinoshita et al. (1993) investigated the effect of pH on attachment and detachment of 2 phages in sandy soil in a series of laboratory experiments and found that attachment of the lipid-containing phage was insensitive to pH (5.7-8.0) but an increase in pH had a moderate effect on enhancing detachment.

Basdew and Laing (2014) performed study to assess the effect of pH on six *S. aureus* phages sabp-p1, sabp-p2, sabp-p3, sabp-p4, sabp-p5 and sabp-p6 and findings concluded that all six phages showed optimal lytic ability at pH 6 to 7 and reduced activity above or below pH of 6 to 7.

Rehman et al. (2016b) reported the effect of pH on the stability of lytic bacteriophage isolated from the sewage water of dairy farm against *staphylococcus aureus* causing mastitis and findings of the study showed that the lytic activity of bacteriophage was highest at pH of 7.0.

Litt and Jaroni (2017) tested stability of isolated coliphages against a wide range of pH (1–11). By using 1 M NaOH and/or HCl (Fisher Scientific, NJ) pH range of 1–11 was maintained in PBS in which phages were suspended and incubated at 37°C to determine their viability at time period of 1, 2, 4, 6, 12, and 24 h.

Oduor et al. (2020) isolated *Staphylococcus* phages Stab20, Stab21, Stab22, and Stab23. Characterization studies based on pH-stability experiments were carried out by incubating phage suspensions at pH of 1.4, 3.4, 5.4, 7.4, 9.4, 11.4 and 12.9 at 37 °C for one h and results suggested that phages showed tolerance to pH range of 5.4

to 9.4. Findings suggested that an increase in acidity or alkalinity both had negative effect on the viability of phage as all four phages Stab20, Stab21, Stab22, and Stab23 were inactivated below the pH of 5.4 and above pH 9.4.

Effect of Temperature:

Several reports are available on the effect of temperature on physiology and ecology of phages, hence, it has been considered by various workers for characterization of phages. Bronfenbrenner (1932) in his study performed on phages detailed about the temperature inactivation of phages due to effect of heat.

Rogers and Sarles (1963) discussed about the inactivation of *Enterococcus* bacteriophages due to effect of temperature. They reported that more than 99% phages were inactivated at 60⁰C and completely inactivated at 70⁰C after 1 hour exposure.

Nishihara and Romig (1964) conducted an experiment on temperature sensitive mutants of *B. subtilis* phages to observe the effect of temperature and they concluded that the burst size get changed as a result of effect of higher temperature on the metabolism of the host because the bacterial growth rate differ at different temperature scales.

Phelps (1967) performed a study in which he discussed about characterization of *Neisseria* phages based on temperature studies.

Colasito and Rogoff (1969) in a separate study found that lytic phages of *Bacillus thuringiensis* get inactivated at 60⁰C when exposed for 5 min.

Charles et al. (1970) reported the effect of elevated temperature on synthesis of DNA in bacteriophage ϕ 29-infected *Bacillus amyloliquefaciens*, hence replication was affected.

Lamontagne and McDonald (1972) performed an experiment on *B. subtilis* phage which forms plaques only at temperature above 50⁰C. In the study, adsorption of *B. subtilis* phage TSP-1 to *B. subtilis* strain W168 was tested with cells grown at 37, 45 and 53⁰C and results indicated that irreversible adsorption occurs only with cells grown at 53⁰C. If TSP-1 was allowed to infect cells at 53⁰C subsequent shifts to 37⁰C inhibited phage replication and resulted in the inactivation of plaque forming units.

Seeley and Primrose (1980) studied the effect of temperature on the ecology of aquatic bacteriophages. They classified the coliphages according to effect of temperature on their efficiency of plating into: low temperature (LT) phages with optimum plating temperature at or below 30⁰C, Mid temperature (MT) phages in the range of 15 to 42⁰C and high-temperature (HT) phages in the range of 25 to 42⁰C or 30 to 45⁰C and concluded that the feces of warm blooded animals contained only MT and HT phages.

Mendez et al. (2002) found that titers of phages either in pure culture phage suspension or in naturally occurring phage suspensions were stable at (-70±10)⁰ C and at (-20±5)⁰C, when protected with glycerol.

Lu et al. (2003) performed an experiment and reported that phages against *Lactobacillus plantarum* get inactivated above 70⁰C temperature.

Jonczyk et al. (2011) summarized the influence of diverse external factors like temperature, acidity and ion concentration on survival of phages under unfavorable conditions and documented that morphology of phage has correlation with survival rate of phage. Knowledge of these properties of phages are important for their therapeutic and agricultural applications under variable environmental conditions.

Basdew and Laing (2014) studied the effect of temperature on six phages of *S. aureus* and showed that phages sabp-p1, sabp-p2 and sabp-p3 were most stable at increasing temperatures (45 °c to 70°C), in comparison to phages sabp-p4, sabp-p5 and sabp-p6. They also documented that 4°C was the minimum temperature at which phages could be stored without a significant reduction in their lytic and replication abilities.

Rehman et al. (2016b) reported the effect of temperature on the stability of lytic bacteriophage isolated from the sewage water of dairy farm against *Staphylococcus aureus* causing mastitis. Study showed that the lytic activity of bacteriophage was optimally highest at 37 °C temperature.

To assess the thermal stability, Litt and Jaroni (2017) analysed the effect of range of temperature on phage viability. Phages were suspended in PBS and incubated at 40, 60, 70, and 90°C for 60min to determine their viability. For cold storage stability, 1ml phage solution was stored at 4, -20, and -80°C and tested after 0, 1, 30, 60, and 90 days duration for activity and viability.

Oduor et al. (2020) conducted thermo-tolerant studies on *Staphylococcus* phages Stab20, Stab21, Stab22, and Stab23 by incubating phages at 35, 40, 45, 50, 55 and 60 °C in phosphate buffered solution (pH 7.4) for time period of one h and after that tubes were cooled on ice for 30 min. Results revealed that phages were active below temperature of 40°C.

Effect of chloroform:

Basdew and Laing (2014) demonstrated that all six *Staphylococcus aureus* phages sabp-p1, sabp-p2, sabp-p3, sabp-p4, sabp-p5 and sabp-p6 showed variable sensitivity to chloroform exposure and sabp-p5 exhibited the highest level of reduction in activity (74.23%) as compared to the other phages.

Oduor et al. (2020) showed that *Staphylococcus* phages Stab20, Stab21, Stab22, and Stab23 were 100 % resistant to chloroform treatment as no loss or reduction in viability was recorded.

Host range:

A comprehensive study has been made on isolation and characterization of different phages from various sources to determine wide host range in which the phage life cycle can be completed.

Phages for *Staphylococcus* spp:

Wentworth (1963) conducted bacteriophage typing of the Staphylococci to characterize them.

Brown et al. (1972) studied morphology and physical properties of *Staphylococcus* bacteriophage.

Talbot and Parisi (1976) performed phage typing for confirmative identification of *Staphylococcus epidermidis* by using specific *Staphylococcus* phage.

Jefferson and Parisi (1979) conducted phage typing of ten species of coagulase-negative staphylococci by bacteriophages isolated against *Staphylococcus epidermidis*.

Barcs et al. (1992) emphasized that Coagulase-negative staphylococcal (CNS) strains are mainly part of normal microbial flora, however their involvement in nosocomial infections is reported. Scientists isolated a total of 152 coagulase-negative

staphylococcal strains mainly; *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* from clinical samples of 14 patients hospitalized after bone-marrow transplantation and characterized these CNS strains by bacteriophage typing, biotyping, and determining antibiogram, extracellular products like biofilm generation, plasmid or exoprotein pattern. Phage typing was done by using 16 polyvalent staphylococcal phages.

Boussard et al. (1992) reported phage typing as one of the technique for characterization of coagulase-negative staphylococci.

O'Flaherty et al. (2005) isolated and characterized two anti-staphylococcal phages namely DW2 and CS1 from farmyard slurry and effluents and assessed their lytic activity against pathogenic *Staphylococcus aureus* associated with bovine infections. Researchers recommended that because isolated phages were active against pathogenic *S. aureus*, hence they can be utilized in form of teat dips or as teat washing solution as means of non-antibiotic prophylactic against staphylococcal bovine mastitis.

Balasubramanian et al. (2007) discussed use of lytic phages as a specific and selective probe for detection of *Staphylococcus aureus*.

Garcia et al. (2009) investigated prevalence of bacteriophages infecting *Staphylococcus aureus* in dairy samples and described the applications of phages as biocontrol agents.

Synnott et al. (2009) isolated and characterized *Staphylococcus aureus* bacteriophages from sewage influent and illustrated their wide host ranges along with potent lytic activity.

Gutierrez et al. (2010) isolated and characterized bacteriophages infecting *Staphylococcus epidermidis*.

Mishra et al. (2011) performed a study to characterize *Staphylococcus aureus* phage SA4 and assessed *in vitro* lytic activity of phage SA4.

Deghorain and Melderren (2012) described that Staphylococci phages family has phages used for typing of *Staphylococcus aureus* and CNS both. Based on morphological studies, these phages belong to the order *Caudovirales* and family *Siphoviridae*.

Deghorain et al. (2012) interestingly revealed that genomic studies indicated close evolutionary relationship of *Staphylococcus aureus* phages with novel phages isolated for coagulase-negative staphylococci.

Wisniewska et al. (2012) described phage typing protocol for characterization of clinical isolates of methicillin-resistant *Staphylococcus aureus* by using a standardized panel of bacteriophages tested internationally. By means of infecting each bacterial strain systematically with a set of phages claimed for that species, all strains can be identified individually by observing the pattern of susceptibility and resistance to each phage type.

Melo et al. (2014) characterized *Staphylococcus epidermidis* phage named vB_SepS_SEP9 and grouped into *Siphoviridae* family.

Melo et al. (2018) isolated lytic bacteriophages named vB_SauM_LM12, against *S. aureus* from environmental sewage waters by using different clinical MRSA isolates.

Moller et al. (2019) described that all the known staphylococcal phages belong to order *Caudovirales* containing linear double-stranded DNA as genome and grouped into three families as per their distinguishing morphological features; members of *Siphoviridae* with long, noncontractile-tail, *Myoviridae* with the contractile-tail and *Podoviridae* with short but noncontractile-tail.

Phages for *E. coli*:

Stirm (1968) performed a study on *E. coli* K-bacteriophages regarding isolation and characterization of 5 *E. coli* K-phages.

Ellis and Winters (1969) reported isolation of potential MS2- bacteriophage strains of *E. coli*.

Kudva et al. (1999) reported the isolation of *E. coli* 0157 phages from bovine and ovine fecal samples and tested their lytic activity against cultures of *E. coli* 0157:H7 in animals. Based on antigen specificity and plaque morphology, three phage isolates KH1, KH4 and KH5 were isolated. When a mixture of these three 0157 specific phages (cocktail) were tested for lytic activity, this cocktail lysed all the *E. coli* 0157 cultures tested.

In a separate study, Jothikumar et al. (2000) discussed for the first time isolation of coliphages specific to enterotoxigenic strains of *E. coli* (ETEC). All fifteen isolated phages were grouped into 3 different phage strain based on RAPD pattern. These three phages yielded clear plaques when tested for morphology. They prepared lysate of high phage titer and plaque assay was performed.

Goodridge et al. (2003) conducted an experiment on isolation, propagation and characterization of two coliphages. Phages were characterized on the basis of morphological and host determination studies.

Huff et al. (2003a) also reported isolation of phage to a nonmotile *E. coli* serotype from municipal waste treatment and poultry processing plants and used these isolated phages for treating a severe *E. coli* respiratory infection.

Sajjad et al. (2004) made a study on isolation, purification and titration of coliphages T₁ and T₄. They also detailed about the different plaque morphology for characterization purpose.

Jamalludeen et al. (2007) investigated isolation and characterization of nine bacteriophages from pig farm sewage which can lyse 0149 enterotoxigenic *E. coli*. They reported that phages produced large, clear plaques and phage titer was maintained even after 16 h exposure of pH 5-9.

Begum et al. (2010) isolated 49 phages from 12 different surface water samples in Dhaka. Among forty nine, one phage named IMM-001 showed lytic activity to Enterotoxigenic *Escherichia coli* (ETEC) due to specificity towards CS7 fimbrial proteins which acted as colonization factor (CF) and potential receptor for IMM-001.

Manohar et al. (2018) performed a study on isolation, enrichment and characterization of *E. coli* phage myPSH1131 equipped with broad host range.

Phages for *Salmonella* spp:

Ibrahim (1969) made the study regarding the isolation and host range determination of *Salmonella* phages from fecal samples of turkey poults.

Alavidze et al. (2000) also reported isolation and characterization of lytic phages against selected *Salmonella* serotypes.

In a separate study, Carey-Smith et al. (2006) reported isolation of two types of phages infecting *Salmonella* from sewage. Characterization was done on the basis of plaque morphology and host range studies. Phage FGCSSa1 had the broadest host range infecting six of eight *Salmonella* isolates and neither of two *E. coli* isolates.

McLaughlin et al. (2006) discussed in detail about isolation of *Salmonella* phages from swine effluent lagoons.

Bielke et al. (2007) investigated host range of *Salmonella* phages and reported that phage host range is not always genera-restricted and phages could be of wide host range.

Phage for *Bacillus* species:

Grilione and Carr (1959) reported that phages for the genus *Bacillus* have rightfully gained popularity as virological research tools. They isolated a bacteriophage for *B. pumilus* from a manure sample and investigated the general properties of this phage.

Romig and Brodetsky (1961) devised a simple method for isolation of phages active against various strains of *Bacillus* and reported preliminary steps for their characterization. Based on plaque morphology they named isolated phages as SP 5, SP 6, SP 7, SP 8, SP9 and SP 13.

Nishihara and Romig (1964) performed a study on isolation of twelve temperature-sensitive mutants of *B. subtilis* phage SP 3. The classification of temperature sensitivity was based on the inability of isolates to form plaques at 43⁰C.

Anderson et al. (1966) studied the detail structure of *B. subtilis* phage. The criteria of plaque morphology, temperature and pH inactivation and host range were used in preliminary characterization of newly isolated *Bacillus* phages.

Colasito and Rogoff (1969) characterized six lytic phages of *B. thuringensis* on the basis of phage morphology, plaque morphology, host range, calcium requirements, thermal inactivation and stability.

Shimizu et al. (1970) worked on isolation, characterization and morphological studies of *B. subtilis* phage Nf. Based on electron microscopic observation, morphology of phage was described. Nf phage has an oblong head with a six-sided outline and a tubular tail. Twelve wedge shaped appendages were attached to the

lower part of two collars. They made physico-chemical characterization of Nf phage by performing sucrose density gradient centrifugation.

Rima and Steensma (1971) worked on isolation of *B. subtilis* phage by employing various techniques and reported that numbers of different phages could be obtained by using different techniques.

Yasbin and Ledbetter (1977) conducted a study on isolation of a derivative of *B. subtilis* strain 168 which is sensitive to defective phage PBSX and PBSZ. They reported that *B. subtilis* strain 168 and W 23 maintain the defective phages PBSX and PBSZ. Phage PBSX kills *B. subtilis* strain W23 and phage PBSZ is bactericidal to strain 168.

Ackermann et al. (1994) defined nine new species of tailed *Bacillus* phages, based on morphological and physico-chemical properties and reported that total number of tailed phage species for *Bacillus* species was 33.

Phages for *Pseudomonas*:

Postic and Finland (1961) isolated and characterized seven pseudomonas phages from clinical sources and used them for typing of strains of *P. aeruginosa*.

Alavidze et al. (1999) performed isolation of lytic phages against multidrug resistant *Pseudomonas aeruginosa*.

Park et al. (2000) isolated phage specific to a fish pathogen. *Pseudomonas plecoglossicidia*, as a candidate for disease control.

Harper and Enright (2011) advocated use of bacteriophages for the treatment of *Pseudomonas aeruginosa* infections.

Other phages:

Rogers and Sarles (1963) performed characterization of *Enterococcus* bacteriophage isolated from the small intestine of the rat.

Phelps (1967) reported the isolation and characterization of phages for *Neisseria*. Characterization was based on plaque-morphology, temperature and pH effect on phages and host range studies.

Stevenson and Airdrie (1984) worked on isolation and titration of *Yersinia ruckeri* phages.

Salama et al. (1989) made study on isolation of *Campylobacter jejuni* and *Campylobacter coli* phages from the samples of pig manure, abattoir effluents, sewage and poultry manure.

Bhimani and Freitas (1990) documented the isolation and characterization of phages of lactic *Streptococci*. They also determined host range of *Streptococci* phage.

Atterbury et al. (2003) detailed the isolation and characterization of *Campylobacter* phages from retail poultry.

Lu et al. (2003) studied the isolation and characterization of *Lactobacillus plantarum* phage Ø JL-1.

2.4 Clinical application: Phage therapy in Veterinary Field:

The careful study of the host range, lytic spectrum, cross resistance and other fundamental properties of the phage being used is a major factor in the reported successes of the phage therapy work.

The first known report of successful phage therapy came from Bruynoghe and Maisin (1921), who used phage to treat *Staphylococcal* skin infections.

Welkos et al. (1974) investigated the effect of phage lysate on *Salmonella* strains tested. They reported that phage lysate with a titer of 10^{12} pfu/ml was found to be optimal. This preparation lysed 98.2% of *Salmonella* strains tested, while maintaining high specificity of phage O-1 for *Salmonellae*, however, 5.9% of *E. coli* strain tested were found susceptible.

In France, Vieu (1975) led the therapeutic phage efforts for preparing *Pseudomonas* phages for patients. Subsequently, the field has now required great appraisal in therapy against diseases of man and animals.

Smith and Huggins (1982) and workers (Smith and Huggins, 1983; Smith and Huggins, 1987) in Britain, carried out a series of excellent, well controlled studies on the use of phages in systemic *E. coli* infections in mice and then in diarrhea in young calves and pigs. This phage treatment was found more effective than using antibiotic such as tetracycline, streptomycin, ampicillin and trimethoprim / sulfafurazole.

Cislo et al. (1987) performed an experiment to visualize the effect of phage treatment on suppurative skin infections and advocated use of phage lysate for recovery from infection.

Slopek et al. (1987) treated purulent mastitis and suppurative infection caused by *S. aureus* and *E. coli* with 93% success rate by using bacteriophages.

Bogovazova et al. (1991) evaluated the safety and efficacy of *Klebsiella* phages. Pharmacokinetic and toxicological studies using intramuscular, intraperitoneal or intravenous administration of phages were carried out in mice and guinea pigs. The phage preparation was reported to be nontoxic and effective in treating *Klebsiella* infections as manifested by marked clinical improvements and bacterial clearance in the phage treated patients. Bogovazova et al. (1992) conducted similar studies and also reported immunological properties of *Klebsiella* phage lysate.

Soothill (1992) performed a study on use of phages for treatment of experimentally induced infection in mice and suggested therapy to be effective.

Levin and Bull (1996) carried out a detailed analysis of the tissue-phage distribution study which can be helpful in assessing the parameters involved in efficacy of antibiotics. They have gone on to perform interesting animal studies and conclude that phage therapy is well worth study.

Alisky et al. (1998) reviewed the literature from 1966-1996 that dealt with the therapeutic use of phages against infections like suppurative wound infections gastroenteritis, sepsis, dermatitis, empyema and pneumonia; pathogens included *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Shigella* and *Salmonella* species. They administered phage orally, topically or systemically for treating wide variety of antibiotic resistant pathogens in animal models (mice, guinea pigs and livestock) and reported a demonstrable efficacy against *E. coli*, *Acinetobacter*, *Pseudomonas* and *Staphylococcus* species. Conclusion was drawn that phages are promising for treating antibiotic resistant pathogens.

Barrow et al. (1998) used a lytic bacteriophage to prevent septicemia and meningitis like infection in chicken and calves caused by *E. coli*. In newly borne colostrum deprived calves given the *E. coli* orally, i/m inoculation of phage, delayed appearance of the bacterium in the blood and lengthened life-span. With all these findings, it was concluded that phage therapy has a considerable potential for treating bacterial diseases.

Carlton (1999) described that phage therapy was successful in treating variety of bacterial infections in the past and has again become popular particularly in view of emerging antibiotic resistance.

Kudva et al. (1999) studied biocontrol of *E. coli* 0157 with 0157 specific bacteriophage and reported that these 0157:H7 infecting phages effectively killed EDL 932, but only aerobically thus these phages were inappropriate for use in the anaerobic gut. They also stated that virulent 0157 antigen specific phages could play a role in bio control of *E. coli* 0157:H7 in animals.

Weber-Dabrowska et al. (2001) reported the recovery rate of 85.9% when specific bacteriophages were used for the treatment of suppurative bacterial infections caused by multidrug-resistant bacteria of different species.

Summers (2001) documented that there are good reasons to believe in effective phage therapy in some circumstances. On the basis of laboratory and animal studies and exploiting understandings of phage biology, he suggested that phages might be useful as antibacterial agents in various conditions.

Benson (2002) successfully treated *Pseudomonas aeruginosa* mastitis in 7 cows in USA by using bacteriophages.

Bull et al. (2002) developed quantitative microbiological procedures to explore the therapeutic potential of phages *in vitro* (the Phage Replication Assay or PRA) as well as *in vivo*, using the *E. coli* in mouse model. Results depicted that application of phages reduced the mortality.

Huff et al. (2002) conducted three studies to determine the efficacy of aerosol administration of phage to prevent on *E. coli* respiratory infection in broiler chickens. Results indicated that phage treatment did not provide complete protection; however, in all 3 studies mortality was significantly decreased, indicating that aerosol spray of phages might be practical for administration of bacteriophage and provide an alternative to the use of antibiotics in poultry production.

Goode et al. (2003) worked on reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic phages and reported that by increasing the number of phage particles applied (increased phage titer) it was possible to eliminate not only *Salmonella enterica* serovar Enteritidis or

Campylobacter jejuni but also other *Salmonella* strains which were resistant to antibiotics.

Huff et al. (2003b) conducted an experiment to determine the efficacy of multiple Vs single intramuscular injection of phages for treating a severe *E. coli* respiratory infection in broiler chickens. The results suggested that phages can be an effective treatment when administered early in the experimental *E. coli* respiratory disease and early multiple treatments are better than a single treatment. The efficacy of phage treatment diminished as it was delayed, with no difference between single or multiple treatments.

Mathur et al. (2003) reported phage therapy as an important alternative to antibiotics in the current era of multidrug resistant pathogens. They used phages topically, orally and systemically and success rate found in these studies was 80-95%. They also demonstrated significant efficacy of phages against *E. coli*, *Acinetobacter* spp. *Pseudomonas* spp. and *Staphylococcus aureus* and concluded phages therapy as an important alternative to antibiotics for treating multidrug resistant pathogens.

Chibani-Chennoufi et al. (2004) made comparison of *in vitro* and *in vivo* bacteriolytic activity of 4 *E. coli* phages in mice, when administered orally. For this, four T-4 coliphages with broad host range were used. The minimal oral dose for consistent fecal recovery was as low as 10^3 pfu/ml of drinking water and when introduced into mice, bacterial colonies were lysed. Likewise, an *in vitro* phage susceptible *E. coli* strains freshly inoculated into axenic mice was lysed *in vivo* by an orally applied phage. In contrast, the normal *E. coli* gut flora of conventional mice was only minimally affected by oral phage application despite the fact that *in vitro* majority of the murine intestinal *E. coli* strains were vulnerable to the phage preparation.

In similar studies, Johnson (2004) reported the effect of phage therapy for controlling *E. coli* 157:H7.

O'Flynn et al. (2004) performed a study on evaluation of a cocktail of 3 bacteriophages for biocontrol of *E. coli* 0157:H7. The study detailed the exploitation of phages as biocontrol agent to eliminate the pathogens *E. coli* 0157:H7. A cocktail of 3 phages were evaluated for their ability to lyse the bacterium *in vitro* and *in vivo*

and results indicated that the phage cocktail completely eliminated *E. coli* 0157:H7 from the beef meat surface in seven of nine cases.

Sajjad et al. (2004) performed a trial to treat an experimental *E. coli* infection by using coliphage lysate in broiler chicken. They assessed phage activity *in vitro* and *in vivo* against *E. coli* in chicken. The results indicated a good protection level using coliphages, hence it was concluded that phages might be used effectively as good alternatives of antibiotic resistant bacteria.

Shasha et al. (2004) also documented about the antibacterial action of phages and suggested that phages are proved antibacterial agents.

Tanji et al. (2004) and same group of workers (Tanji et al., 2005) conducted a trial on therapeutic use of phage cocktail for controlling *E. coli* 0157:H7 in mice and reported that a three-phage cocktail worked effectively *in vitro* (aerobically and anaerobically) but was unable to clear mice of *E. coli* during *in vivo* studies.

Brussow (2005) performed an experimental trial on chicken, mice and calves using phages. He found phage therapy to be effective against *E. coli* in these species when *in vivo* studies were made.

Huff et al. (2006) evaluated the influence of phage titer on the treatment of colibacillosis in broiler chicken.

Capparelli et al. (2007) described that bacteriophage (M(Sa)) against *Staphylococcus aureus* protected mice when *S. aureus* is inoculated into mice along with M(Sa) phage as phage completely eliminate all the bacteria *in vivo* and *in vitro* and hence workers suggested that phage M(Sa) can be used in controlling *S. aureus* infections.

Viscardi et al. (2008) isolated and characterized two novel coli phages with proven potential in controlling antibiotic-resistant and pathogenic EHEC and EPEC strains of *Escherichia coli*.

Li and Zhang (2014) isolated and characterized phage SPW depicting strong lytic activity against mastitis causing *Staphylococcus aureus*, from the wastewater of dairy farm. Phage SPW showed resistance to chloroform and isopropanol, has wide host range and was stable at broad range of pH and temperature.

Longping and Zhiying (2014) also carried out isolation and characterization of Anti-*Staphylococcus aureus* SPW phage against bovine mastitis.

Sarhan et al. (2016) conducted one study to isolate and identify the bacteriophage with anti-*Staphylococcal* activity from sewage water samples. They investigated the *in vitro* antibacterial activity of phage and antibiotics both and reported that *S. aureus* isolates which were resistant to antibiotic Penicillin G, methicillin and ceftiofur showed sensitivity to isolated phage. However, other factors need to be considered while using phage therapy to achieve the maximum favorable results.

Moller et al. (2019) suggested that coherent preparation of phage cocktails will be more effective in treating staphylococcal infections either alone by phage combinations or in combination with antibiotics.

Oduor et al. (2020) reported that due to variable host range among different *Staphylococcus* strains, phages Stab 20, 21, 22, and 23 could be used in cocktail preparations for therapeutic applications or for bio-control of bacterial infections.

These results suggested that the use of a phage cocktail containing phages that used different receptors might have prevented the emergence of phage resistant bacterial mutants and these phages multiplied inside the host.

2.5 Phage Therapy against mastitis

Chilamban and Rawat (2004) isolated 13 *S. aureus* phages and used successfully for treatment of mastitis in mice model.

Sridevi (2005) worked on isolation, characterization and therapeutic efficacy of bacteriophage against *Streptococcus agalactiae* associated with ruminant mastitis in IVRI, Bareilly.

Gill et al. (2006a) showed that bovine whey proteins inhibited the interaction of *Staphylococcus aureus* and bacteriophage K and reduced the expected success of phages in mastitis cases clinically.

Gill et al. (2006b) documented the efficacy of bacteriophage therapy in treating subclinical *S. aureus* induced mastitis in lactating dairy cattle where an increase in the SCC of treated udders along with a cure rate of 16.7% was recorded.

Basdew and Laing (2011) evaluated the use of bacteriophages as an alternative therapy to antibiotics in USA and suggested that phages can be the next avenue in controlling bovine mastitis.

Gupta and Prasad (2011) documented efficacy of polyvalent bacteriophage P-27/HP to control multidrug resistant *S. aureus* associated with human infections.

Tiwari et al. (2013) presented a compilation of results of bacteriophage therapy in treating *E. coli* infections in animal models, meningitis in mouse model, respiratory infections in chicken and diarrhea in calves. Workers stated that phage therapy in treating mastitis caused by *S. aureus* has variable results as in some studies intramammary infusion of bacteriophage into *S. aureus* infected quarters of lactating cow did not show significant protection, hence they advised to explore further research on therapeutic applications of bacteriophages in treating bacterial infections associated with clinical and subclinical mastitis.

Mishra et al. (2014) assessed bacteriolytic potential of bacteriophages against multi-drug resistant *S. aureus* induced mastitis in goats.

Basdew and Laing (2014) conducted a study on six *Staphylococcus aureus* phage isolates and suggested that bacteriophages can be successfully used to control *Staphylococcus aureus*, associated with bovine mastitis as out of six, three phages sabp-p1, sabp-p2 and sabp-p3, showed considerable bacteriolytic activity during *in vitro* lytic assays and hence can be employed for *in vivo* applications to treat mastitis-infected dairy cattle.

Ndlela et al. (2016) performed a study for biological control of *S. aureus* induced bovine mastitis in dairy cows using bacteriophages & documented that the *S. aureus* isolates from mastitis positive animals showed resistance to penicillin, methicillin, vancomycin, amoxicillin/flucloxacillin, erythromycin, streptomycin, trimethoprim and ampicillin but were sensitive to three strains of bacteriophages.

Porter et al. (2016) investigated the potential use of bacteriophage in preventing *E. coli* mastitis on dairies and *in vitro* results showed bactericidal activity of bacteriophage in raw milk and mammary tissue culture systems.

Saglam et al. (2017) determined the role of *Staphylococcus* in the formation of subclinical mastitis in cows and isolated phages against *S. aureus* strains obtained

from cases of sub-clinical mastitis and phages showed *in vitro* lytic activity against the field isolates of *S. aureus*.

Ganaie et al. (2018) isolated and characterized two lytic phages SAJK-IND and MSP against *Staphylococcus aureus* and documented that phage SAJK-IND exhibit 100% lytic activity while phage MSP showed 40% lytic activity against a number of *Staphylococcus aureus* strains isolated from mastitis milk samples.

Manohar et al. (2018) performed a study on isolation and tested *in vivo* efficacy of *Escherichia coli* phage named myPSH1131, which has broad host range.

Manohar et al. (2019) isolated bacteriophages from sewage water samples from Tamil Nadu, India and showed the promising bacteriolytic ability against *E. coli*, *K. pneumoniae*, and *Enterobacter* species, in *in vitro* phage killing assay.

Geng et al. (2020) evaluated success of phage therapy in the treatment of *Staphylococcus aureus*-induced mastitis in mice.

Titze et al. (2020) evaluated the lytic efficacy of bacteriophage mixture containing three phages, STA1.ST29, EB1.ST11, and EB1.ST27 against *S. aureus* isolates. The significant reduction in the *S. aureus* bacterial density indicated the potential therapeutic potential of phage therapy.



Materials

and

Methods

The present study describe the methodology for collection, isolation and preliminary characterization of major bacteria (*S. aureus/E. coli*) from milk samples of CM/SCM cases and isolation of specific phages from various goshalas/farms as part of a search for phages which might prove useful as anti-bacterial and biocontrol agents.

3.1. Place of study

The present study was conducted at the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, (DUVASU), Mathura, Uttar Pradesh, India. The samples of milk from cases of clinical and sub-clinical mastitis were collected for screening of mastitis and for bacterial isolation, while samples of animal wastewater (cows and buffaloes), fecal pellet (goats) and semidry feces (poultry) were collected from different locations and subjected to isolation of bacteriophages.

3.2. Permission to conduct the study

For sample collection permission was taken from DUVASU, Mathura as per University rules. Ethical approval was not required to conduct this research work.

3.3. Materials

3.3.1. Source of milk samples

Milk samples were collected during the period of June, 2017 to Sept, 2019 from cases of clinical/sub-clinical mastitis of lactating cows/buffalos from Teaching Veterinary Clinical Complex (TVCC), DUVASU, Mathura; Instructional Livestock Farm Complex (ILFC) farms, DUVASU, Mathura; Ambulatory visits/clinical camps and from different Goshalas (Surabhi Goshala, Vrindavan; Mataji, Rangili Mahal and Shriji Gopi Goshala Barsana; Bhaktivedanta Goshala, Vrindavan; Hassanand Goshala, Vrindavan; Panchayati Goshala, Vrindavan; Samarpan Goshala and Jai Gurudev Goshala, Mathura) in and around Mathura, U.P. India.

Table 3.1: Details of different locations for collection of milk samples

| Sl. No. | Source | Location | Total no. of animals/cases | Sample collected | |
|---------|--------------------------------------|--|----------------------------|------------------|----|
| | | | | C* | B* |
| 1. | TVCC | DUVASU, Mathura | 160 | 83 | 77 |
| 2. | ILFC farm | DUVASU, Mathura | 123 | 92 | 31 |
| 3. | Ambulatory visits and Clinical camps | Farah | 2 | 2 | 0 |
| | | Vrindavan | 13 | 12 | 1 |
| | | Mathura | 8 | 4 | 4 |
| 4. | Goshalas | Surabhi Goshala (Akshaypatra temple), Vrindavan | 17 | 17 | 0 |
| | | Bhaktivedanta Goshala (Eskcon temple), Vrindavan | 12 | 12 | 0 |
| | | Hassanand Goshala, Vrindavan | 88 | 88 | 0 |
| | | Panchayati Goshala, Vrindavan | 36 | 36 | 0 |
| | | Samarpan Goshala, | 10 | 10 | 0 |
| | | Jai Gurudev Goshala, Mathura | 62 | 62 | 0 |
| | | Mataji Goshala, Barsana | 212 | 212 | 0 |
| | | Rangili Mahal, Barsana | 17 | 17 | 0 |
| | | Shriji Gopi Goshala, Barsana | 5 | 5 | 0 |

*C=Cattle; B=Buffalo

3.3.2. Source of samples for bacteriophages

The samples of animal waste disposal constituting various body excretions of different species of animals (Cows, buffaloes, goats, sheep and poultry) were collected in sufficient amount (100-250 ml) from ILFC farms, DUVASU, Mathura; TVCC, DUVASU, Mathura; Goat farm unit, DUVASU, Mathura; Sheep unit, Aurangabad Mathura; Poultry farm unit, DUVASU, Mathura and different Goshalas (Surabhi Goshala, Vrindavan; Mataji, Rangili Mahal and Shriji Gopi Goshala Barsana; Bhaktivedanta Goshala, Vrindavan; Hassanand Goshala, Vrindavan;

Panchayati Goshala, Vrindavan; Samarpan Goshala; Pt Deen Dayal Upadhyaya Kamdhenu Goshala, Farah and Jai Gurudev Goshala, Mathura). For this purpose 50 ml disposable syringe and 30 cm long, sterile disposable pipette, pipette bulb and multipurpose sample containers were used.

Table 3.2: Details of different locations for collection of suitable samples as source of phage

| Sl. No. | Source | Location | Species# | No. of Sample collected |
|---------|-------------------|--|-----------------|-------------------------|
| 1. | TVCC | DUVASU, Mathura | C/B/S/G/ D/H | 5 |
| 2. | ILFC farm | DUVASU, Mathura | C/B | 9 (6+3) |
| 3. | Poultry farm unit | DUVASU, Mathura | P | 3 |
| 4. | Goat farm unit | DUVASU, Mathura | G | 3 |
| 5. | Sheep unit | Aurangabad, Mathura | S | 2 |
| 6. | Goshalas | Surabhi Goshala (Akshaypatra temple), Vrindavan | C | 3 |
| | | Bhaktivedanta Goshala (Eskcon temple), Vrindavan | C | 3 |
| | | Hassanand Goshala, Vrindavan | C | 3 |
| | | Panchayati Goshala, Vrindavan | C | 3 |
| | | Samarpan Goshala, | C | 3 |
| | | Jai Gurudev Goshala, Mathura | C | 3 |
| | | Mataji Goshala, Barsana | C | 3 |
| | | Rangili Mahal, Barsana | C | 3 |
| | | Shriji Gopi Goshala, Barsana | C | 3 |
| | | Pt Deen Dayal Upadhyaya Kamdhenu Goshala, Farah | C | 3 |

#C=Cattle; B=Buffalo; S=Sheep; G=Goat; H=Horse; D=Dog; P=Poultry-chicken

3.3.3 Millipore syringe filters: APD 0.22µm Millex GV disposable syringe filters were used for the preparation of samples for bacteriophage isolation.

3.3.4 Standard Host bacteria

Clinical (field) isolates of bacteria obtained from cases of CM in the study were used as the primary indicator host for the isolation, propagation and characterization of phages. The known isolates of *S. aureus* (MTCC No. 96), *S. epidermidis* (MTCC No. 435), *E. coli* (MTCC No. 443) and *Bacillus cereus* (ATCC No. 10876) were used for testing host range of phage isolates. Bacterial strains used for study were procured from the Microbial Type Culture Collection and Gene Bank (MTCC) CSIR-Institute of Microbial Technology, Chandigarh and from cultures stock of Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, DUVASU, Mathura (U.P.). All the isolates were preserved as 25% glycerol stock in the department and were maintained by serial subculture at regular intervals.

3.3.5 Laboratory media

A. For Bacteriophage Isolation and Characterization

Following dehydrated defined media (HiMedia Laboratories Limited, Mumbai) were used for the preparation of culture media for phage isolation in the present investigation.

1. Luria Bertani agar (LBA)
2. Luria Bertani HiVeg broth (LB)
3. NZCYM Growth Agar
4. NZCYM Growth Top agar
5. LB Growth Top agar
6. LB Growth agar for lambda
7. Deca strength nutrient broth (DSNB)

All the media were prepared as per the instructions given by manufacturer. The media prepared were first tested for sterility and stored at refrigeration temperature till use. Composition and detail preparation of media is mentioned in appendix.

B. For Isolation and Identification of Bacteria

Following dehydrated defined media (HiMedia Laboratories Limited, Mumbai) were used for the preparation of culture media for isolation, identification, characterization and maintenance and preservation of bacterial isolates

1. Nutrient Agar (NA)
2. Nutrient Broth (NB)
3. MacConkey Agar (MLA)
4. Eosine Methylene Blue Agar (EMB)
5. Mannitol Salt agar (MSA)
6. HiCrome *E. coli* Agar
7. Simmons Citrate Agar
8. Muller-Hinton Agar (MHA)
9. 10% Sugar solution
10. Peptone water

All the media were prepared as per the instructions given by manufacturer. The media prepared were first tested for sterility and stored at refrigeration temperature till use. Composition and detail preparation of media is mentioned in appendix.

3.3.6. Buffers and Reagents

The recipes for buffers/reagents used in this study are mentioned in the Appendix.

3.3.7. Chemicals and Reagents

For conducting different studies/tests for identification and biochemical characterization, following ANLAR/Guaranteed grade chemicals and reagents were used procured from reputed firms.

1. Newman's Stain Solution, modified (Sigma-Aldrich, St. Louis, Missouri, USA)
2. CMT Reagent (Alkyl aryl sulfonate with bromocresol purple) (GADVASU, Ludhiana)
3. Magnesium sulphate Heptahydrate ($MgSO_4 \cdot 7H_2O$) (Sigma-Aldrich, St. Louis, Missouri, USA)

4. Chloroform solution (Sigma-Aldrich, St. Louis, Missouri, USA)
5. Andrade's Reagent (HiMedia laboratories Limited, Mumbai)
6. Glycerol (87% w/v) (GeNei, India)
7. Sodium Chloride (Merck, India)
8. Hydrogen peroxide (Qualigens, India)

3.3.8. Antibiotic discs

Commercially available antibiotic discs (HiMedia laboratories Limited, Mumbai) were used to test the sensitivity of the bacterial isolates under investigation. The details of the twenty commonly used antimicrobials belonging to ten classes used in present study is mentioned in Table 3.3

Table 3.3: Details of antimicrobial discs used in the study

| Sl. No. | Class of Antimicrobial | Name of Antimicrobial | Symbol | Conc./disc |
|---------|---------------------------------|-----------------------|--------|------------|
| 1. | β-Lactams | Amoxicillin | Am | 10 µg |
| 2. | | Ampicillin | A | 10 µg |
| 3. | | Ampicillin/Sulbactam | AS | 10/10 µg |
| 4. | | Methicillin | M | 5 µg |
| 5. | | Penicillin-G | P | 10 units |
| 6. | Aminoglycosides | Amikacin | AK | 30 µg |
| 7. | | Gentamicin | GEN | 10 µg |
| 8. | | Kanamycin | K | 30 µg |
| 9. | | Streptomycin | S | 10 µg |
| 10. | Phenicol | Chloremphenicol | C | 30 µg |
| 11. | Floroquinolones | Ciprofloxacin | CIP | 5 µg |
| 12. | | Enrofloxacin | En | 10 µg |
| 13. | | Gatifloxacin | GF | 5 µg |
| 14. | Quinolone | Norfloxacin | NX | 10 µg |
| 15. | Cephalosporin | Cefotaxime | CE | 30µg |
| 16. | | Ceftriaxone | CTR | 30 µg |
| 17. | Trimethoprim - Sulfamethoxazole | Cotrimoxazole | CoT | 25 µg |
| 18. | Macrolide | Erythromycin | E | 15 µg |
| 19. | Tetracycline | Tetracycline | TE | 30 µg |
| 20. | Glycopeptide | Vancomycin | VA | 10 µg |

3.3.9. Glassware, Plasticware and Rubberware

The glasswares used during this study were procured from Corning (USA) and Borosil (India). All the glasswares were properly washed, dried, wrapped and sterilized at 160 °C for 1 hour in Hot air oven before use, as and when required.

Pre-sterilized polystyrene plates, centrifuge tubes and test tubes, pipettes, microtips (0.5-10 µl, 200 µl, and 1000 µl) and petri-plates were procured from Tarson (India), HiMedia (India), Axiva (India), Axygen (India), GeNie (India), Millex (Millipore) and Eppendorf (Germany) were used. All plastic wares were washed with distilled water, dried and sterilized by autoclaving at 121 °C for 15 min at 15 lbs as and when needed.

3.3.10. Instruments

1. Centrifuge
 - a. Refrigerated centrifuge CPR-30 (Remi Equipments, India)
 - b. RM12C micro centrifuge (Remi Equipments, India)
 - c. Table-top centrifuge (Remi Equipments, India)
 - d. Refrigerated benchtop centrifuge (Genetix Biotech Asia Pvt Ltd, Germany)
2. Incubator (Vaiometra, Sonar, India)
3. Water Bath with shaker (Remi Equipments, India)
4. Autoclave (Sonar, India)
5. Hot Air Oven (Sonar, India)
6. Freezer (-20°C) (Voltas, India)
7. Refrigerator (Videocon, India)
8. Weighing balance (Kern, Germany)
9. Vortex mixer (Remi Equipments, India)
10. Micro pipettes of various volumes (0.5 µL-1000 µL) (Eppendorf, Germany)
11. Magnetic stirrer (Scientech, India)
12. pH System 361 (Systronics, India)
13. Bio-safety cabinet A-II (Esco,)
14. Compound light microscope (Metzer, India)

3.4. Methods

3.4.1. Isolation and characterization of *S. aureus* and *E. coli* from milk samples of mastitis cases of cattle and buffaloes

3.4.1.1. Identification of cases of clinical and sub-clinical mastitis of cattle and buffalo

In present study, a total of seven hundred seventy lactating animals (cows and buffaloes) at ILFC, DUVASU, Mathura; ambulatory visits/clinical camps; TVCC, DUVASU, Mathura; and various Goshalas in and around Mathura, U.P. were identified and selected for screening of their animal-wise composite milk samples for presence of sub-clinical and clinical mastitis. The clinical mastitis cases were identified on the basis of signs and symptoms viz. inflammation of teat/udder, reduced and poor quality milk; flakes or blood in milk while cases of sub-clinical mastitis were suspected on the basis of history of decreased milk yield and/or compromised milk quality. Further, the SCM was confirmed by CMT and SCC.

3.4.1.2. Collection of milk samples

Seven hundred seventy (770) milk samples were aseptically collected from lactating cows and buffaloes for screening of sub-clinical and clinical mastitis. Out of 770 lactating cows and buffaloes, 657 were cows while 113 were buffaloes. Amongst 657 cows, 601 cows were screened for SCM while 56 showed symptoms of CM. Amid 113 buffaloes, 64 were screened for sub-clinical mastitis while 49 showed symptoms of clinical mastitis. Among 657 selected lactating cows, 92 cows were from ILFC, DUVASU, Mathura; 18 were from ambulatory visits/clinical camps; 83 were from TVCC, DUVASU, Mathura and 464 were from various Goshalas in and around Mathura, U.P. India. Likewise, among 113 buffaloes screened for sub-clinical and clinical mastitis, 31 were from ILFC, DUVASU, Mathura, 05 were from ambulatory visits/clinical camps and 77 were from TVCC, DUVASU, Mathura (Fig. 3.1).

The milk samples were collected from affected quarter in case of clinical mastitis and from each quarter (of same animal and combined as one composite milk sample from one animal in case of SCM) after proper washing and cleaning of udder and teat surface, thereafter swabbed with cotton soaked in 70 % alcohol and allowed to dry. First few streams of milk from teat were discarded and then from each quarter



Fig. 3.1: Milk samples collected from different Goshalas



Fig. 3.2: Screening of milk samples by California mastitis test (CMT)

10-15 ml of milk was collected in sterilized multipurpose sample container (HiMedia Laboratories Pvt Ltd, Mumbai, India India) and used for screening of sub-clinical and clinical mastitis either on the same day of collection or kept at 4 °C to process on the next day of collection.

3.4.1.3. Initial screening of milk by CMT and SCC

All collected milk samples were subjected to indirect test like CMT and direct test like SCC. Condition of udder and milk quality both were observed. The clinical examination of udder was conducted by observation and palpation for presence of any swelling, pain, erythema, hotness, firmness and fibrosis. Milk samples were examined for the appearance, consistency, smell, pigmentation, viscosity, presence of any flakes/clots or blood.

3.4.1.3.1. California Mastitis test (CMT)

CMT test was performed as per the method described previously by Abdel-Rady and Sayed (2009) with some modification. To perform the test, 2 ml of milk sample from each animal was placed in the respective cups of CMT Paddle and equal quantity of CMT reagent (CMT reagent- alkyl aryl sulfonate with bromcresol purple) was added in 1:1 ratio. After gentle mixing, mixture was allowed to swirl in gentle circular manner on horizontal plane for approximately 10-15 seconds and slightly tilted to read the results as mentioned below in Table 3.4 and Fig. 3.2.

Table 3.4: Result interpretation criteria for CMT

| S. No. | Visible observation | Grade | Interpretation |
|---------------|--|--------------|----------------------------|
| 1. | No change in color, consistency, mixture appears liquid | 0 | Negative or in traces |
| 2. | Gel formation begins but disappear upon continuous swirling and yellow color | 1 | Slightly positive, acidity |
| 3. | Gel formation after mixing which is stable gel | 2 | Positive |
| 4. | Thick gel formation starts immediately after mixing and upon swirling gel tends to adhere at one place | 3 | Strongly positive |

3.4.1.3.2. Somatic cell count (SCC)

Somatic cell count was performed by using Modified Newman's stain technique as per method of Hegde et al. (2013) with some modifications. In this technique, 1 sq cm area was marked on the clean grease free glass slide and 10 µl of milk was smeared in 1 cm² marked area on the glass slide. Smear is allowed to air dry. Smear was flooded with Modified Newman's stain for 1 minute, after 1 min stain was removed by tilting the slide under running tap water for 30 seconds (sec). Then, the slide is allowed to air dry and somatic cells were observed under microscope using oil immersion lens.

The milk samples containing number of somatic cells (leukocytes and/or epithelial cells) between 3-5 lakh cells per ml (3×10^5 - 5×10^5) were considered positive for sub-clinical mastitis and SCC above 5 lakh cells per ml of milk indicated mastitic milk.

3.4.1.4. Isolation and characterization of bacteria *S. aureus* and *E. coli* from milk samples

Milk samples suspected for SCM found positive for CMT and SCC and each milk sample collected from cases of CM were directly streaked over nutrient agar and incubated at 37°C for 24-48 h for primary isolation of target bacteria.

S. aureus: Nutrient agar was observed for characteristic yellow, round and smooth colony of *Staphylococcus* spp. and the culture was microscopically examined to detect presence of gram positive cocci in bunches. All the milk samples showing no growth over nutrient agar were incubated till 96 h and in absence of any detectable growth, they were discontinued from the experiment.

E. coli: Along with nutrient agar every milk sample was also streaked directly over MacConkey agar (MLA) and incubated at 37°C for 24-48 h to screen the milk sample for the presence of *E. coli*. Pink color umbonate medium size colonies surrounded by pink halo were presumed for *E. coli*.

3.2.1.5 Characterization based on-Cultural, morphological & biochemical characteristics

S. aureus: For cultural characterization, milk samples showing yellow colonies over nutrient agar were sub-cultured over selective media. For *S. aureus*, MSA media

selective for *Staphylococcus* and differential for mannitol fermenting and mannitol non-fermenting *Staphylococcus* was used. *S. aureus* is mannitol fermenting bacteria. Thus, plates showing non-fermenting red colonies were discontinued from the experiment and yellow colonies causing yellow discoloration of media indicating fermentation of mannitol were picked up. Colonial characteristics, pigment production and Gram's staining features were observed after incubation.

After cultural characterization, Gram's staining and motility testing was performed to confirm the morphology of bacteria and Gram positive non-motile cocci present in bunches were selected for further biochemical characterization. The isolates were further characterized as per the scheme of Cowan and Steel (1974). The bacteria identified as staphylococci were subjected to catalase and coagulase test. Catalase positive staphylococci were tested by coagulase test. Coagulase test was performed to differentiate between coagulase positive *S. aureus* and coagulase negative Staphylococci (CNS). Coagulase positive isolates of *S. aureus* were selected for phage isolation studies.

E. coli: Pink colonies on MacConkey agar were sub-cultured over selective media. Selective media, eosine methylene blue (EMB) and HiCrome *E. coli* agar were used for the growth of *E. coli*. Small colonies depicting metallic sheen over EMB and blue colored small colonies over HiCrome *E. coli* agar were selected for Gram's staining and motility testing. Gram negative motile rods were subjected to biochemical characterization like indole, methyl red, voges proskauer, sugar fermentation tests against glucose, lactose and maltose and citrate utilization test to observe the IMViC pattern and for biochemical characterization of isolates.

Pure isolates of bacteria were transferred onto nutrient agar slants in duplicate. After incubation and morphological checking for purity, slants were assigned a number and stored at 4°C.

3.4.2. Detection, Isolation and Characterization of the lytic bacteriophages from samples of animal wastes

3.4.2.1. Collection of samples of wastewater from disposal tanks

A total of fifty five samples of wastewater/fecal material were collected from different locations (cattle/buffalo/sheep/goat/poultry farms). Among 55, 33 from Goshalas (6 each from Hassanand Goshala, Jai Gurudev Gaushala, and Panchayati

Goshala; 3 each from Akshaypatra-Surabhi Goshala, Rangili Mahal & Shriji Gopi Goshala, Mataji Goshala, Eskcon-Bhaktivedanta Goshala, and Pt Deen Dayal Dham Kamdhenu Goshala); 9 were from ILFC Farm (6 samples from cattle and 3 samples from buffalo); 5 from TVCC, DUVASU (3 from large animal OPD and 2 from small animal OPD); 3 samples each from University Goat Unit and Poultry farm DUVASU, Mathura; and 2 from sheep flock, Aurangabad. Approximately 100-250 ml of representative samples from each of the livestock farms, Goshala were collected taking utmost precautions so as to ensure that the sample will provide sufficient population of phages. For this, sampling was done at different locations of the livestock and poultry farm/Goshala/TVCC clinics, DUVASU, Mathura. Sterilized 50 ml disposable syringe and modified pipetting device, fitted with rubber tube, rubber bulb/hand pumped device to aspirate the fluid and multipurpose sample collection container was used for collecting the fluid samples. From goats and poultry farm, fecal pellet and semi-dry fecal droppings were collected, mixed properly with 100 ml of LB and centrifuged at 3000 rpm for 15 minutes. Supernatant was collected for further processing and phage enrichment protocol.

3.4.2.2. Enrichment of Bacteriophage

The collected wastewater samples were processed for enrichment of phages against field (clinical) isolate of both the (*S. aureus* and *E. coli*) bacteria obtained from milk samples, separately as per the method of Rehman et al. (2016).

To increase number of *S. aureus* phages, enrichment was done by using deca strength nutrient broth (DSNB) medium. Collected wastewater samples were initially centrifuged at 3000 rpm for 15 min to settle down the large sized particles and supernatant was used for phage enrichment. About 1 ml of 16-18 hr incubated pure broth culture of *S. aureus* and 5 ml of DSNB was added into 45 ml of each of supernatant of (as a source of phage) wastewater sample and incubated overnight at 37 °C. To enhance number of *E. coli* phages, enrichment was done by using Luria Bertani HiVeg broth (LB) medium. About 1 ml of 16-18 hr incubated pure broth culture of *E. coli* and 5 ml of LB broth was added into 45 ml of each of centrifuged supernatant wastewater sample and incubated overnight at 37 °C.

Next day, after incubation, bacteria-wastewater-nutrient medium suspension was centrifuged at 6000 rpm at 4°C for 30 min by using refrigerated centrifuge. Pellet

was discarded and supernatant was filtered through 0.22µm pore size syringe filters (Millex GV) into sterile tubes. This bacteria free phage enriched filtrate was named as *S. aureus* bacteria free filtrate (SBFF) and kept at 4°C until the use. Similarly, to obtain BFF against *E. coli* next day, after incubation, bacteria-wastewater-nutrient medium suspension was centrifuged at 6000 rpm at 4°C for 30 min by using refrigerated centrifuge. Pellet was discarded and supernatant was filtered through 0.22µm pore size syringe filters (Millex GV) into sterile tubes. This bacteria free phage enriched filtrate was named as *E. coli* bacteria free filtrate (EBFF) and kept at 4°C until use. Both SBFF & EBFF were tested for presence of any lytic activity against respective bacteria.

3.4.2.3. Detection of Bacteriophage in the samples (BFF)

Presence of bacteriophages in SBFF and EBFF was determined by turbidity reduction method and spot inoculation/streaking over the bacterial lawn.

a) Turbidity reduction method

Turbidity reduction method was employed as a rapid tool for preliminary detection of lytic activity of phages. The procedure as described by Tiwari et al. (2010) was employed so as to detect the presence of phage by observing any lytic activity against indicator clinical isolates of *S. aureus* and *E. coli* obtained in laboratory from milk samples and also tested against standard known bacterial isolates (*S. aureus* MTCC No. 96; *S. epidermidis* MTCC No. 435; *E. coli*-MTCC No. 443).

For turbidity reduction technique, 3 ml of sterilized LB broth was taken, 300 µl of six hour old culture of *E. coli* bacteria (matched with McFarland Nephelometer tube 0.5 resembling to 1.5×10^8 cfu/ml) was taken into this broth and 100 µl of EBFF obtained from samples of wastewater collected from various sources was added. One bacterial control was also kept in which 100µl of normal saline solution (NSS) was added along with 300 µl of six hour old culture of *E. coli* bacteria into 3 ml of LB broth. Control and test sample of each bacteria were incubated at 37 °C and result was recorded after 24 and 48 hrs. They were observed for reduction in turbidity of test samples as compared to control tube (Fig. 3.3).

Likewise, for assessment of lytic activity of phage against *S. aureus*, 3 ml of sterilized NB broth was taken, 300 µl of six hr old culture of *S. aureus* bacteria was

taken into this broth and 100 µl of SBFF obtained from samples of wastewater collected from various sources was added. One bacterial control was also kept in which instead of 100 µl of SBFF, 100 µl of NSS was added into 3 ml of NB along with 300 µl of 6 hr old *S. aureus* bacterial culture. Control and test sample of each bacteria were incubated at 37°C and result was recorded after 24 and 48 hrs. They were observed for clearance of turbidity in test samples as compared to control tube.

The test was done twice for each sample by using bacterial suspensions of each bacterial isolates of *S. aureus* and *E. coli* using NB and LB broth, respectively and results were recorded before the wastewater sample is adjudged negative for the presence of phage.

b) Phage streaking/spot inoculation method

SBFF and EBFF were also tested by spot inoculation method for detection of lytic activity in terms of zone of bacterial lysis (plaque formation) over the bacterial lawn. The test was performed as per the method given by Goodridge et al. (2003) with some modification. For this, briefly, 100 µl of 6 hr old bacterial cultures of each clinical isolate was added into 2.5 ml of molten soft top agar (LB Growth Top Agar for *E. coli* phages and NZCYM Growth Top Agar for *S. aureus* phages) held at 45°C in a water bath, mixed properly and poured over basal agar plate containing 10 ml of LB growth agar and NZCYM growth agar for *E. coli* and *S. aureus* phages, respectively. After 10 min, 10 µl of BFF to be tested was spotted or streaked over the respective bacterial lawn of different bacterial isolates. After absorption of added BFF, plates were incubated in upside down position at 37 °C for 24 hrs. Next day they were examined for presence of plaques on the spot over bacterial lawn.

3.4.2.4 . Isolation of phage by double agar layer (DAL) method

The DAL method as described by Adams (1959) was employed. The agar gels used were 10 ml of basal growth agar (1.5%) dispensed into 100 mm sterile Petri dishes and 2.5 ml of soft top agar (0.7%) stored in sterile glass test tube (LB Growth Top Agar for *E. coli* phages and NZCYM Growth Top Agar for *S. aureus* phages). For the isolation of phage from the BFF, six hr old bacterial culture of *S. aureus* and *E. coli* were taken and tested separately. The concentration of bacteria was adjusted to 1.5×10^8 cfu/ml. NB and LB broth were used for *S. aureus* and *E. coli*, respectively (Fig. 3.4).

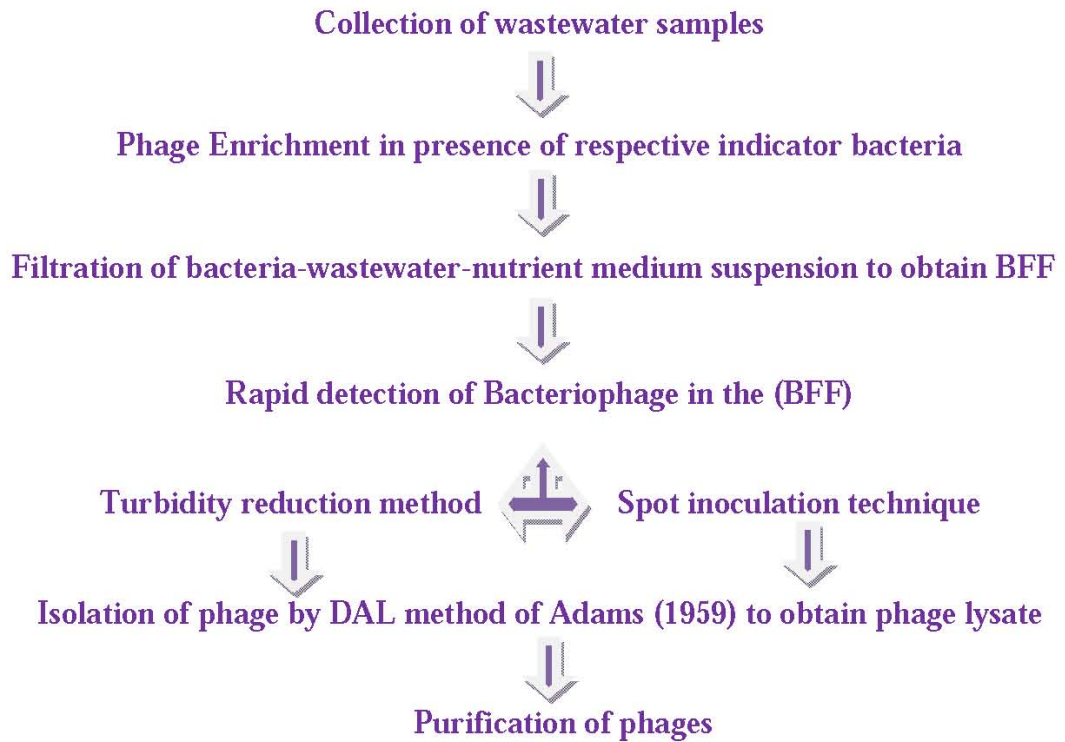


Fig. 3.3 Flow-chart depicting isolation and purification of phages

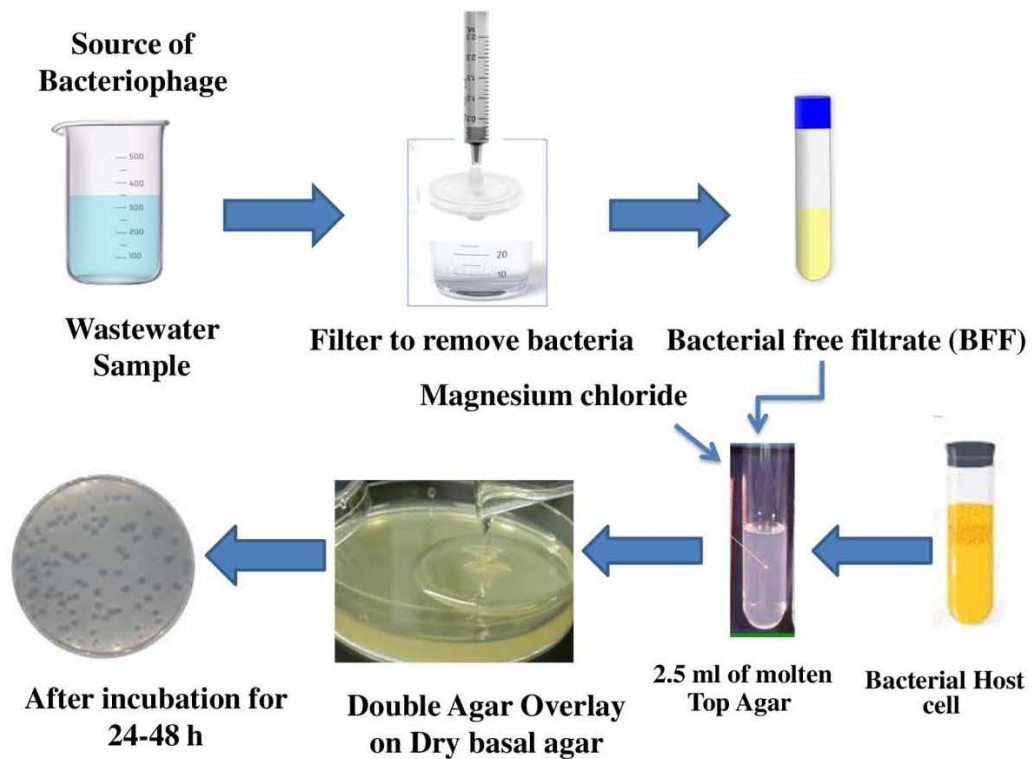


Fig. 3.4: Sequential steps of double agar overlay (DAL) method

In a sterile vial 100 µl of BFF and 300 µl of six hour old bacterial culture were mixed and 10 µl of 2.4 g/lit $MgCl_2 \cdot 7H_2O$ was added to enhance the adsorption of phage over bacterial surface. Thereafter, it was kept in incubator for 10-15 min, and then added into test tube containing 2.5 ml of molten soft agar held at 45°C in water bath/hot plate. The mixture was swirled, poured over LB growth agar and NZCYM growth agar plates for *E. coli* and *S. aureus*, respectively. Plates were allowed to solidify and then incubated at 37°C upto 48 hrs. Plates were observed at 6 h, 12 h, 24 h, and 48 h intervals for developments of plaques and plaque morphology was recorded for characterization.

3.4.2.4 .1. Purification of phages:

Phages were purified by three consecutive single plaque isolation cycle and propagated on the corresponding bacterial host strain. Individual plaques were picked up by using sterile Pasteur pipette or with the help of sterile micro-tip of 1 ml to cut the circular area of plaque and transferred into 300 µl of 12-18 hr old bacterial culture of indicator bacteria and incubated at 37 °C for 24 hrs. Next day, bacteria-phage suspension was filtered through 0.22 µm pore size syringe filters, and obtained phage lysate filtrate was again spot inoculated on Luria Bertani agar (LBA) or NA plate preinoculated with indicator (*E. coli* and *S. aureus*) bacteria. The plaques produced were passaged similarly for 3 consecutive times to obtain purified coliphage lysate containing EcD and Staphylococcal phage lysate containing SaD. The phage strains were designated by the laboratory identification number of Department of Veterinary Microbiology, DUVASU, Mathura as SaD (*Staphylococcus aureus* phage isolated in DUVASU) for *Staphylococcus aureus* phages and EcD (*E. coli* phage isolated in DUVASU) for *E. coli* phages of the bacterial isolates and subjected to characterization. In brief, the procedure of isolation and purification of phage is depicted in Figure 3.3.

3.4.2.5. Characterization of SaD and EcD based upon plaque morphology and type of plaque, host range, effect of pH, effect of temperature and effect of chloroform

3.4.2.5.1 Characterization based on plaque morphology and type

Morphology of plaque was described by the method of Ellis and Winters (1969). Recovered phage isolates were characterized by size of the plaque, by

diffused edge or defined round edge and boundaries of plaques. Measurements of plaque morphology were taken displaying the variation among plaques of the separate morphologies. The types of plaques were also noted as small pin-point (less than 2 mm), medium sized (2 mm) or large sized (larger than 2 mm), clear, opaque or diffused type plaques.

Electron microscopy: For morphological characterization of phage, scanning electron microscopy (SEM) was conducted (though electron microscopy was not part of the current study) to observe morphology of *E. coli* host specific (EcD) phage, as facility of Transmission electron microscopy (TEM) was not available during the time of study.

3.4.2.5.2 Host Range determination

Host range of phage isolates was determined by using the method of Goodridge et al. (2003) with slight modification. To predict the lytic activity, recovered phage isolates were tested against bacteria namely *S. aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, *E. coli*, *Bacillus cereus* and *Klebsiella pneumonia* maintained at Department of Veterinary Microbiology, DUVASU, Mathura. To assess the lytic effect of phage isolates SaD and EcD against Coagulase negative Staphylococci (CNS), standard known isolate of *S. epidermidis* (MTCC No. 435) was used.

In order to determine the host range of isolated phages, lytic activity of purified phage was tested against standard culture of *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*, *B. cereus* and *K. pneumonia* by spot inoculation method. Each bacterial culture was inoculated into NB and incubated at 37 °C for 6 hrs. Six hr old pure broth culture of each target bacteria was mixed to 2.5 ml (0.5 %) top molten agar maintained at 45 °C and poured over the NA plate and allowed to solidify at room temperature for 10-15 min. Volume of 10 µl of purified phage lysate (10^8 pfu/ml) was aseptically placed on the dried surface of agar. Plates were inverted and incubated at 37 °C for 24 hrs. After incubation period, sensitivity of target bacteria against the phage lysate was observed by appearance of plaques formed at the spot where phage lysate was deposited over the bacterial lawn.

3.2.2.5.3 Effect of Temperature

Viability of phage in terms of % activity was studied at 4°C, 22°C, 37°C and 70°C for 60 min exposure by the method of Litt and Jaroni (2017) and Svab et al. (2018) with some modification. 0.1 ml of phage suspension was mixed with 4.9 ml of NB in test tubes. 5 ml was distributed as 1 ml into five test tubes. The test tubes containing 1 ml mix were placed in water bath at temperature range of 22°C, 37°C, 70°C for 1 hr and 1 tube was kept at 4°C for 1 hr. Phage concentration (titer) before and after treatment with 1 hr exposure to heat was adjudged by DAL method (Adams, 1959). After 1 hr, 0.1 ml of exposed phage suspension was mixed with 0.3 ml of 16-18 hr old respective bacterial (*S. aureus* and *E. coli*) culture, mixed in between palm and added into 2.5 ml of soft top agar and plating was done as per DAL method of Adams (1959), media plates were inverted and incubated at 37°C for 24 hrs and observed for presence of plaques. Any reduction in phage titer and viability of phage was observed and compared with exposure at 4°C which acted as control.

3.2.2.5.4 Effect of pH

Effect of pH (3, 5, 7, 9 and 11) was seen on phage titer and viability of phages by using the method of Litt and Jaroni (2017) and Svab et al. (2018) with some modification. For this, 0.1 ml of phage suspension was mixed with 4.9 ml of nutrient broth, kept at various pH of 3, 5, 7, 9 and 11 and all five tubes were incubated at 37°C for 1 hr before assessment of effect of pH. The pH in test tubes were adjusted using 1M hydrochloric acid (HCL) and 1M sodium hydroxide (NaOH) solution and confirmed by using pH System 361 (Systronics). After 1 hr, 0.1 ml of exposed phage suspension was mixed with 0.3 ml of 16-18 hr old respective bacterial (*S. aureus/E. coli*) culture, mixed and added into 2.5 ml of soft top agar and plating was done as per DAL method of Adams (1959). Reduction in phage titer and/or viability of phages was adjudged as marker due to change in pH of the medium.

3.2.2.5.5 Effect of chloroform

Sensitivity of phages to chloroform was tested by following the method of Chow and Rouf (1983) with some modifications. Sensitivity of phages to chloroform was determined by mixing 0.05 ml (50 µl) of chloroform (10 %) in a test tube with 1 ml (2×10^3) phage suspension. To another tube of phage suspension, 0.05 ml of physiological saline/normal saline was added and kept as control. The mixture was

properly mixed for approximately 10 min and centrifuged at 3000 rpm for 5-7 min. After centrifugation, 0.1 ml from top most layer of liquid present in test tube was taken for plaque assay (as per DAL method of Adams, 1959) to visualize the effect of chloroform treatment on the viability of phages.

3.4.3. Assessment of *in vitro* antibiotic sensitivity pattern and *in vitro* Phage sensitivity testing against bacterial isolates

3.4.3.1. Antibiotic sensitivity test

All the bacterial isolates were subjected to antibiotic sensitivity test using standard antibiotic disc by the method described by Bauer et al. (1966). Each culture was inoculated into sterilized NB and incubated at 37 °C for 18-24 hrs. Pure broth culture of each bacterial isolate (*S. aureus*/*CNS*/*E. coli*) was spread plated on the surface of MHA media plates and allowed to dry at room temperature for 5-10 min. Antibiotic discs were placed by maintaining distance of 24 mm in between centre of any two disc and plate is incubated in inverted position at 37°C for 18-24 hrs. Antibiogram of bacterial isolates was recorded against twenty antimicrobials / antibiotics (Ampicillin (AMP), Amoxicillin, Ampicillin/Sulbactam (A/S), Amikacin (AK), Erythromycin (E), Gentamicin (Gen), Kanamycin (K), Methicillin (MET), Norfloxacin (Nx), Penicillin-G (P-G), Ciprofloxacin (CIP), Chloramphenicol (C), Streptomycin (S), Tetracycline (T), Enrofloxacin (En), Vancomycin (Va), Ceftriaxone (CTR), Cefotaxime (CTX), Cotrimoxazole (Co-T), Gatifloxacin (GAT)) to know the pattern of drug susceptibility/resistance as per CLSI, (2012). The zone of inhibition (diameter in mm) of each antibiotic disc was then correlated with sensitivity as per standard chart supplied along with disc and results was recorded in terms of either sensitive, intermediate or resistant bacteria to the respective antibiotics.

3.4.3.2. *In-vitro* phage sensitivity against bacterial isolates

3.4.3.2.1. Titration of phage lysate to standardize the PFU

i. Phage lysate preparation

High titer phage lysate stocks were prepared by the soft agar plate method (Adams, 1959). Phage stock (100 µl) was mixed with 300 µl of respective host bacterial culture either of *S. aureus* or *E. coli* (6 hour old) which had bacterial concentration of 1.5×10^8 to 9×10^8 cfu/ml; 10 µl of 2.4 g/lit. of $MgCl_2$ was added and kept for 10-15 min in incubator. The suspension was then mixed into 2.5 ml of molten

soft agar kept at 45°C and plated. Plates were incubated up to 48 h at 37°C. Confluently lysed plates were selected and the top agar was scrapped with 5 ml of LB and/or NB broth. The scrappings were pooled and 2-3 drops of chloroform was added to this and kept for 10 min and then centrifuged at 5000 rpm for 20 min in refrigerated centrifuge. Agar debris was settled down and supernatant was filtered through a 0.22µm syringe filter. This lysate was stored in sterile plastic vials at 4°C. Further, titration of lysate was done by using dilution method against respective (*S. aureus* and *E. coli*) bacterial host.

ii. Titration

Titration was done by preparing tenfold serial dilution of the phage lysate. For titration, 10⁻¹ to 10⁻⁹ dilutions were made in normal saline solution (NSS) and each dilution was subjected to plaque formation by DAL method (Adams, 1959). For this, 100 µl of each dilution from 10⁻¹ to 10⁻⁹ was mixed with 300 µl of overnight incubated pure broth culture of (*S. aureus* and *E. coli*) bacteria in separate sterile tubes. This suspension was mixed gently and kept at 37°C for 10-15 min. To each tube, about 2.5 ml of molten soft top agar was added at 45°C, mixed and poured over the plate of NA media. Plates were incubated at 37°C for 24 hrs and number of plaques were counted next day in units of pfu/ml. Mean titer of one morphological type of phage isolates was calculated (in PFU/ml) and presented in Table-4.23. Phage titer was determined in terms of plaque forming units (PFU/ml) with the help of formula given below:

$$\text{PFU /ml} = \text{No. of plaques} \times \text{Dilution factor}$$

3.4.3.2.2. Phage sensitivity testing

The sensitivity of the bacterial isolates to bacteriophages was assessed by observing and counting the plaques over bacterial lawn and/or by visual reduction in bacterial colony.

i. By Plaque formation

In vitro lytic activity of (SaD₁, SaD₂, SaD₃) phage isolates against 85 *S. aureus* and *E. coli* phage (EcD₁, EcD₂ and EcD₃) isolates against 121 *E. coli* bacterial isolates of target host bacteria was tested by spot inoculation method of Park et al. (2000).

Each bacterial culture was inoculated into sterile NB and LB broth and incubated at 37°C for 6 hrs. After 6 hrs, pure broth culture of each target bacteria (*S. aureus*/*E. coli*) was swabbed/ spread plated onto the NA and LBA plate and a loopful (10 ul) of each purified phage stock lysate was aseptically placed on the dried superficial surface of agar and allowed to be absorbed at room temperature for 15 min. Plates were inverted and incubated at 37°C for 24 hrs. After incubation at 37°C, the sensitivity of target bacteria against all the phage stock lysate was observed by formation of number of plaques at the spot and pattern of bacterial lysis. The result is recorded by observing number of plaques and pattern of bacterial lysis and compared as uniform volume of 10 ul was used for testing *in vitro* lytic activity of all phage isolates under test. Number of plaque formed (less than 15, 15- 100, more than 100) along with pattern of lysis (light or confluent lysis) on the spot of phage lysate over bacterial lawn were considered as parameters to compare the efficacy of *in vitro* lytic activity of various phage strains recovered from the animal wastewater samples. Result of *in vitro* lytic activity of various phages against bacterial isolates was summarized in Table-4.24 and Table-4.25 in terms of percent lytic efficacy.

ii. Reduction in number of bacterial colonies:

This test was performed as per the method of Sajjad et al. (2004) with slight modification. In this method, the concentration of six hour old host bacterial culture was kept constant (1.5×10^8 cfu/ml) while phages were used in 10-fold serial dilution. Both were mixed and processed as per the method of Adams (1959) and then observed for any reduction in number of bacterial colonies and observation was recorded.

A decorative border composed of black and grey floral and butterfly motifs. The border features intricate scrollwork, leaves, and three butterflies with detailed wing patterns, arranged in a roughly rectangular shape around the central text.

Results

4.1 Isolation and characterization of the major bacteria (*Staphylococcus aureus* and *E. coli*) from milk samples of mastitis cases of cows and buffaloes**4.1.1 Identification of cases of clinical and sub-clinical mastitis of cows and buffaloes**

A total of seven hundred seventy lactating cows and buffaloes were selected for screening of animal-wise composite milk samples for the presence of sub-clinical and clinical mastitis. Out of 770 lactating animals, 657 were cows, among which 601 were screened for SCM and 56 for CM. Among these 601 lactating cows screened for SCM, 459 were from various Goshalas in and around Mathura, U.P. India; 92 cows were from Instructional Livestock Farm Complex (ILFC) and 40 were from Teaching Veterinary Clinical Complex (TVCC), DUVASU, Mathura, U.P., India; and 12 were from ambulatory visits and clinical camps (Table 4.1).

Out of fifty six lactating cows suspected for CM, forty three were from TVCC, DUVASU, Mathura; six were from ambulatory visits and clinical camps; five were from various Goshalas in and around Mathura; and two were from ILFC, DUVASU, Mathura, U.P., India.

Amongst 770 lactating animals, a total of 113 were buffaloes, out of which 64 were screened for SCM and 49 for CM. Among 64, 30 each were from ILFC Farm and TVCC, DUVASU, Mathura, U.P., India; and 4 from ambulatory visits and clinical camps. Among 49 buffaloes studied for CM, 47 were from TVCC, DUVASU, Mathura; and one each from ambulatory visits/clinical camps, and ILFC Farm, DUVASU, Mathura, U.P., India. No buffalo samples were collected from any of Goshalas (Fig. 4.1).

4.1.2 Collection of milk samples aseptically from respective animals

A total of seven hundred seventy (770) milk samples were aseptically collected after proper washing and cleaning of udder and teat surface from lactating cows and buffaloes. First few streams of milk from teat were discarded and then from each quarter 10-15 ml of mid-stream milk was collected in sterilized multipurpose sample container for screening of SCM.

4.1.3 Initial screening of milk by California mastitis test (CMT) and somatic cell count (SCC)

In the present study, all the collected composite milk samples from lactating cows and buffaloes were screened for SCM by performing CMT and SCC.

4.1.3.1 Results of CMT

Based upon visible observation, grade of 0, 1, 2 and 3 were provided for milk samples and considered as negative, slightly positive, positive and strongly positive, respectively. In the current study, samples attaining score of 1, 2 and 3 were recorded as positive (Fig. 4.2). Out of 601 cows, 198 were found positive for SCM by CMT (Fig. 4.3). The prevalence of SCM in lactating cows was found to be 32.94% by CMT (Table 4.2). Out of 459 cows screened from various Goshalas, 166 (36.16%) were found positive for SCM, while out of 90 cows screened from ILFC, DUVASU, Mathura, 12 (13.33%) were positive; among 12 cows from ambulatory visits and clinical camps, 5 (41.67%) were positive; and out of 40 cows from TVCC, DUVASU, Mathura, 15 (37.50%) were found positive for SCM. The detailed results of 166 positive cows for SCM among various Goshalas are presented in Table 4.3 and Fig. 4.4

Out of 64 buffaloes screened for CMT, 15 (23.43%) were found positive for SCM by CMT. Out of 30 milk samples from TVCC, DUVASU, 8 were positive (26.6%); out of 30 samples from ILFC, DUVASU, Mathura, 6 were positive (20%) and among 4 milk samples obtained from buffaloes during ambulatory visits and clinical camps, one (25%) was found positive for the presence of SCM (Table 4.4; Fig. 4.5).

4.1.3.2 Results of SCC

Besides performing CMT, all the collected composite milk samples from lactating cows and buffaloes were subjected to somatic cell count (SCC) for indirect estimation of SCM.

Somatic cell count was performed using Modified Newman's stain technique as per method of Hegde et al. (2013) with some modifications. The milk samples containing number of somatic cells (leukocytes and/or epithelial cells) between 3-5 lac cells per ml (3×10^5 - 5×10^5) were considered as positive for sub-clinical mastitis and SCC above 5 lac cells per ml of milk were indicated as mastitic milk,

Table 4.1: Number of milk samples collected from cows and buffaloes for the study of sub-clinical mastitis and clinical mastitis

| S. No. | Place of sample collection | Number of cows | | Number of buffaloes | |
|--------|------------------------------|-----------------------|-------------------|-----------------------|-------------------|
| | | Sub-clinical mastitis | Clinical mastitis | Sub-clinical mastitis | Clinical mastitis |
| 1. | ILFC Farm, DUVASU | 90 | 2 | 30 | 1 |
| 2. | Ambulatory and Clinical camp | 12 | 6 | 4 | 1 |
| 3. | TVCC, DUVASU | 40 | 43 | 30 | 47 |
| 4. | Goshalas | 459 | 5 | 0 | 0 |
| | Total | 601 | 56 | 64 | 49 |
| | 770 | 657 | | 113 | |

Table 4.2: Details of milk samples collected from cows for the study of sub-clinical mastitis by California Mastitis Test (CMT)

| S. No. | Place of sample collection | Cows screened by CMT | Cows positive by CMT | % of CMT positive |
|---------------|--------------------------------------|-----------------------------|-----------------------------|--------------------------|
| 1. | ILFC Farm, DUVASU | 90 | 12 | 13.33 |
| 2. | Ambulatory and Clinical camps | 12 | 5 | 41.67 |
| 3. | TVCC, DUVASU | 40 | 15 | 37.50 |
| 4. | Goshalas | 459 | 166 | 36.16 |
| | Total | 601 | 198 | 32.94 |

Table 4.3: Details of milk samples collected from cows of various Goshalas for the study of sub-clinical mastitis by California Mastitis Test (CMT)

| S. No. | Name of Goshala | Cows screened by CMT | Cows positive by CMT | % of CMT positive |
|---------------|---|-----------------------------|-----------------------------|--------------------------|
| 1. | Akshaypatra Goshala | 17 | 3 | 17.64 |
| 2. | Mataji Goshala | 212 | 92 | 43.39 |
| 3. | Rangili Mahal (17) + Shriji Gopi (5) Goshalas | 22 | 1 | 4.54 |
| 4. | Eskcon (Bhaktivedanta) Goshala | 12 | 6 | 50 |
| 5. | Jai Gurudev Goshala | 62 | 29 | 46.77 |
| 6. | Hassanand Goshala | 88 | 25 | 28.41 |
| 7. | Panchayati Goshala | 36 | 5 | 13.88 |
| 8. | Samarpan Goshala | 10 | 5 | 50 |
| | Total | 459 | 166 | 36.16 |

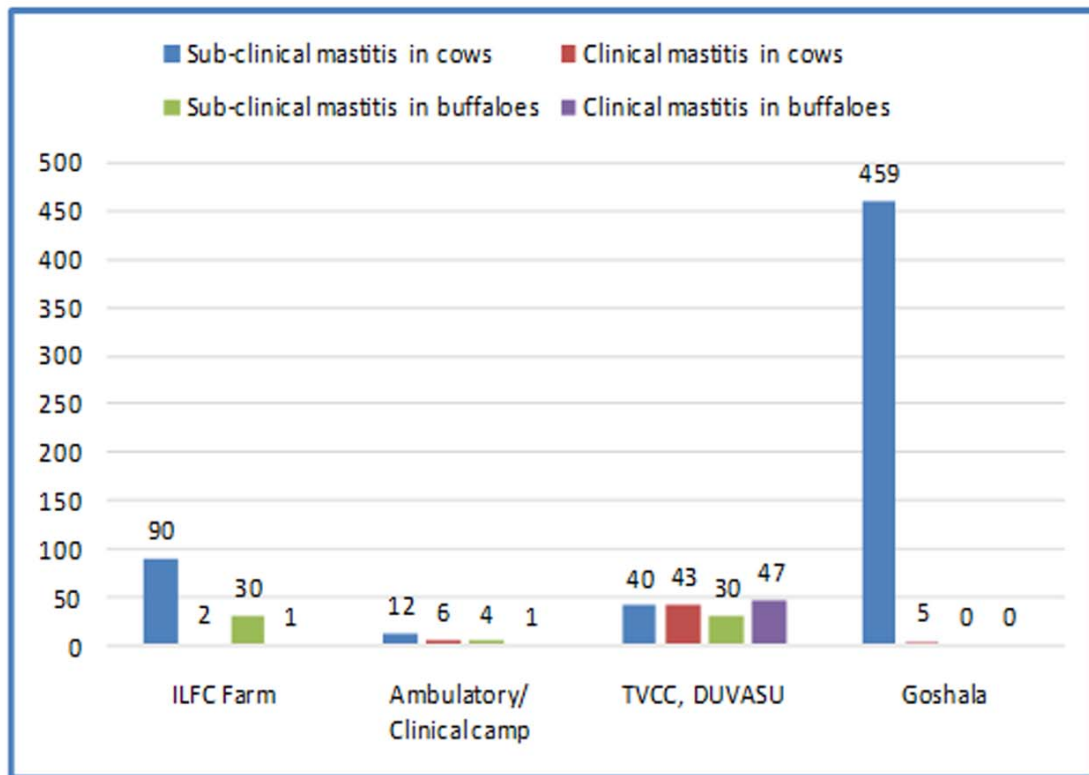


Fig. 4.1: Number of milk samples collected from cows and buffaloes for the screening of sub-clinical mastitis and clinical mastitis

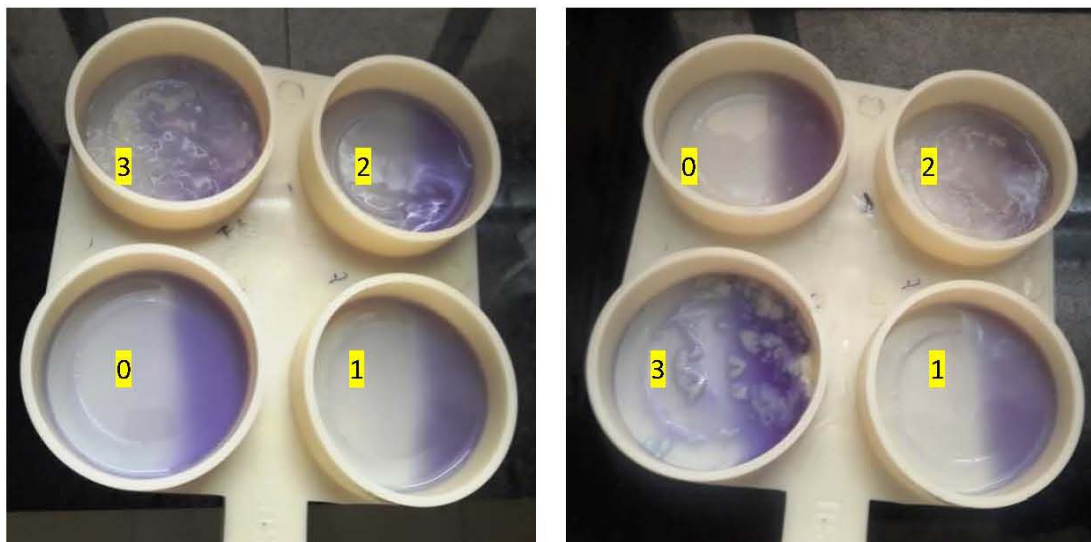


Fig. 4.2: Results of California mastitis test (CMT) - Score 0, 1, 2 and 3

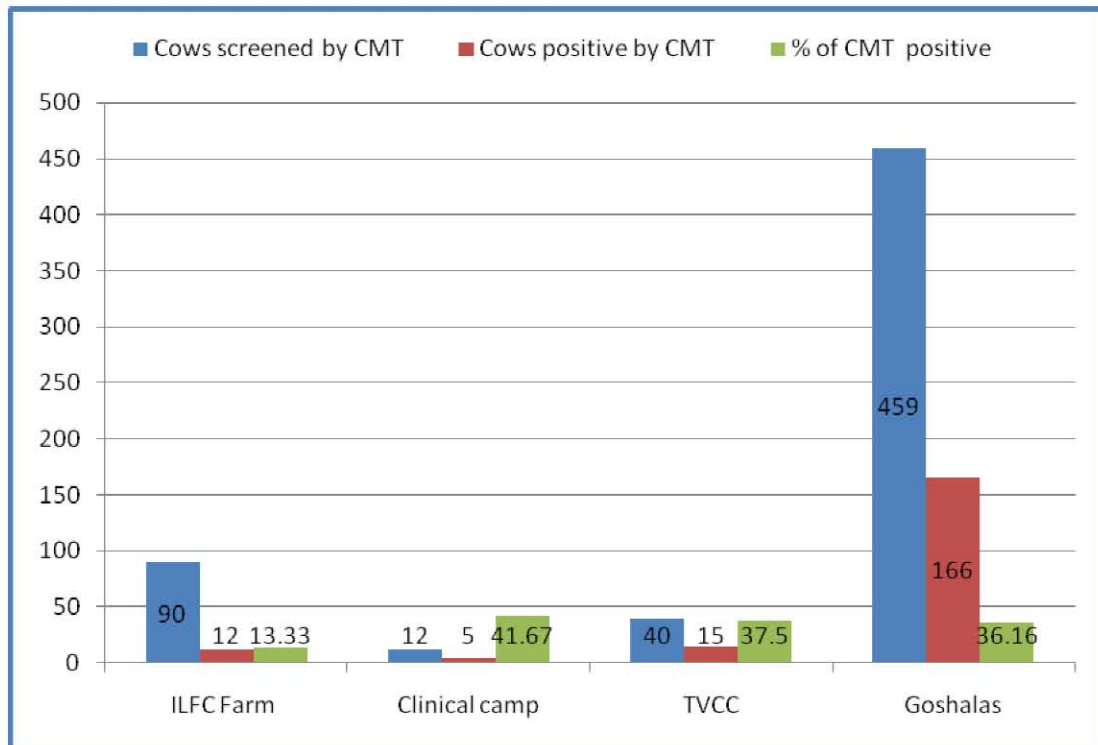


Fig. 4.3: Percentage of milk samples found positive by California Mastitis Test (CMT) for detecting sub-clinical mastitis (SCM) in cows

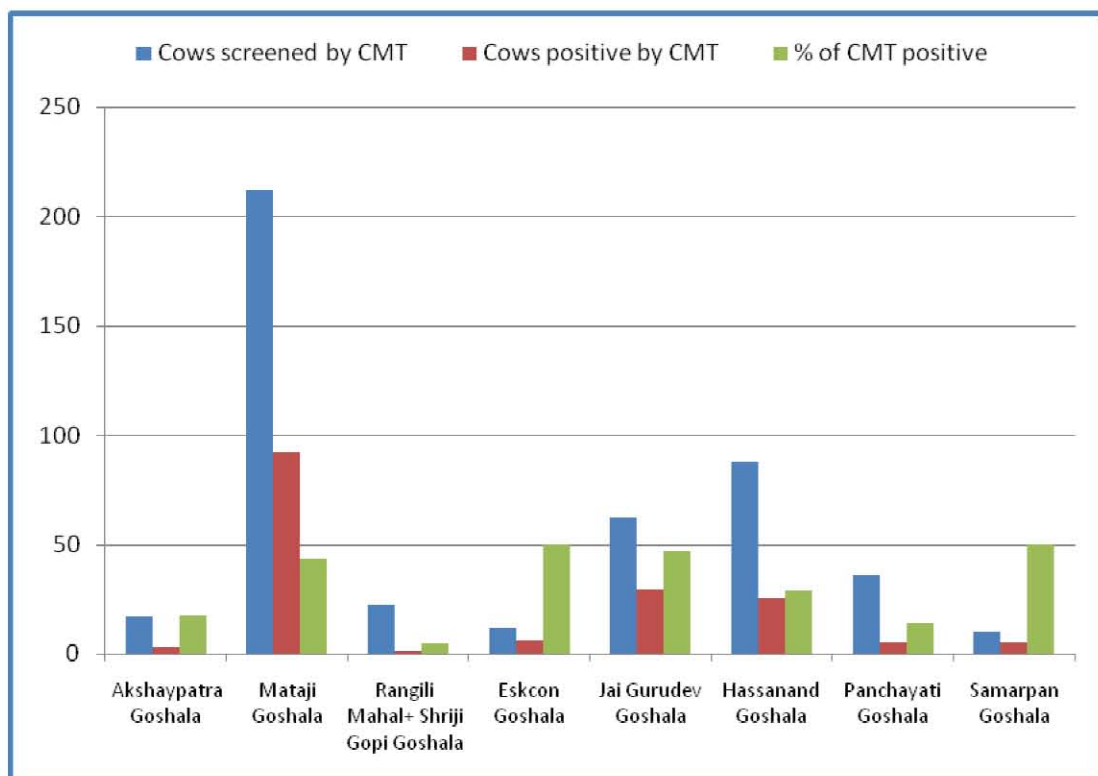


Fig. 4.4: Percentage of milk samples found positive by California Mastitis Test (CMT) for detecting sub-clinical mastitis (SCM) in cows in Goshalas

Table 4.4: Details of milk samples collected from buffaloes for the study of sub-clinical mastitis by California Mastitis Test (CMT)

| S. No. | Place of sample collection | Buffaloes screened by CMT | Buffaloes positive by CMT | % of CMT positive |
|--------|-------------------------------|---------------------------|---------------------------|-------------------|
| 1. | ILFC Farm, DUVASU | 30 | 6 | 20 |
| 2. | Ambulatory and Clinical camps | 4 | 1 | 25 |
| 3. | TVCC, DUVASU | 30 | 8 | 26.6 |
| | Total | 64 | 15 | 23.43 |

Table 4.5: Details of milk samples collected from cows for the study of sub-clinical mastitis by somatic cell count (SCC)

| S. No. | Place of sample collection | Cows screened by SCC | Cows positive by SCC | % of SCC positive |
|--------|-------------------------------|----------------------|----------------------|-------------------|
| 1. | ILFC Farm, DUVASU | 90 | 27 | 30 |
| 2. | Ambulatory and Clinical camps | 12 | 5 | 41.67 |
| 3. | TVCC, DUVASU | 40 | 16 | 40 |
| 4. | Goshalas | 459 | 258 | 56.21 |
| | Total | 601 | 306 | 50.9 |

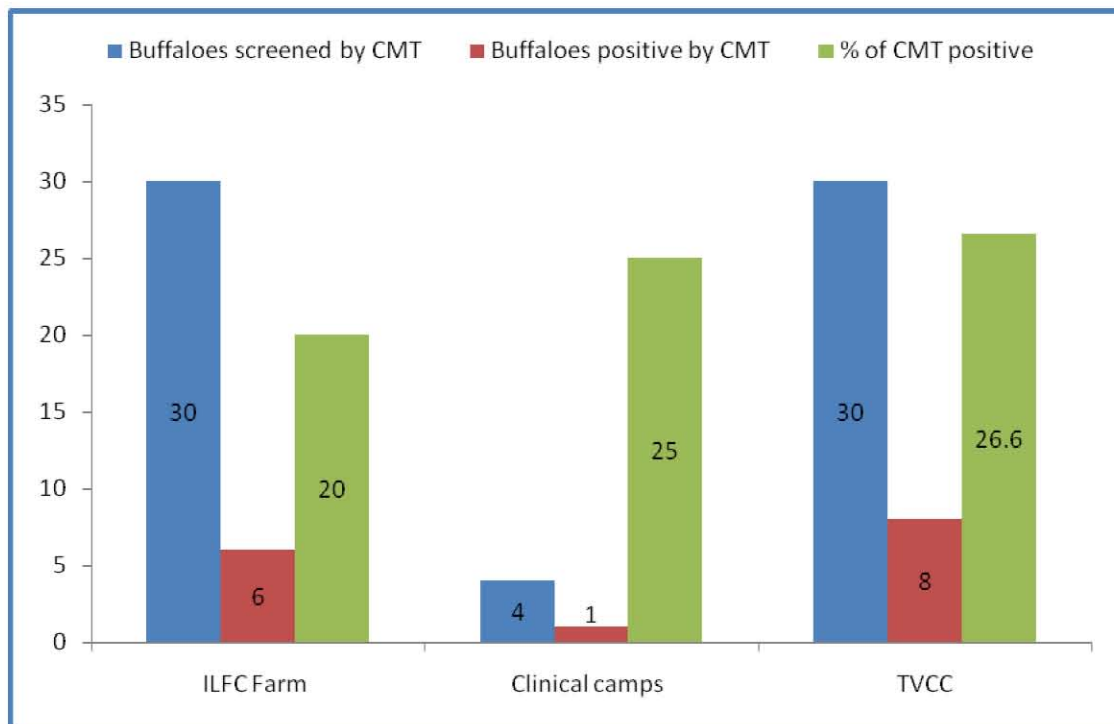


Fig. 4.5: Percentage of milk samples found positive by California Mastitis Test (CMT) for testing sub-clinical mastitis (SCM) in buffaloes

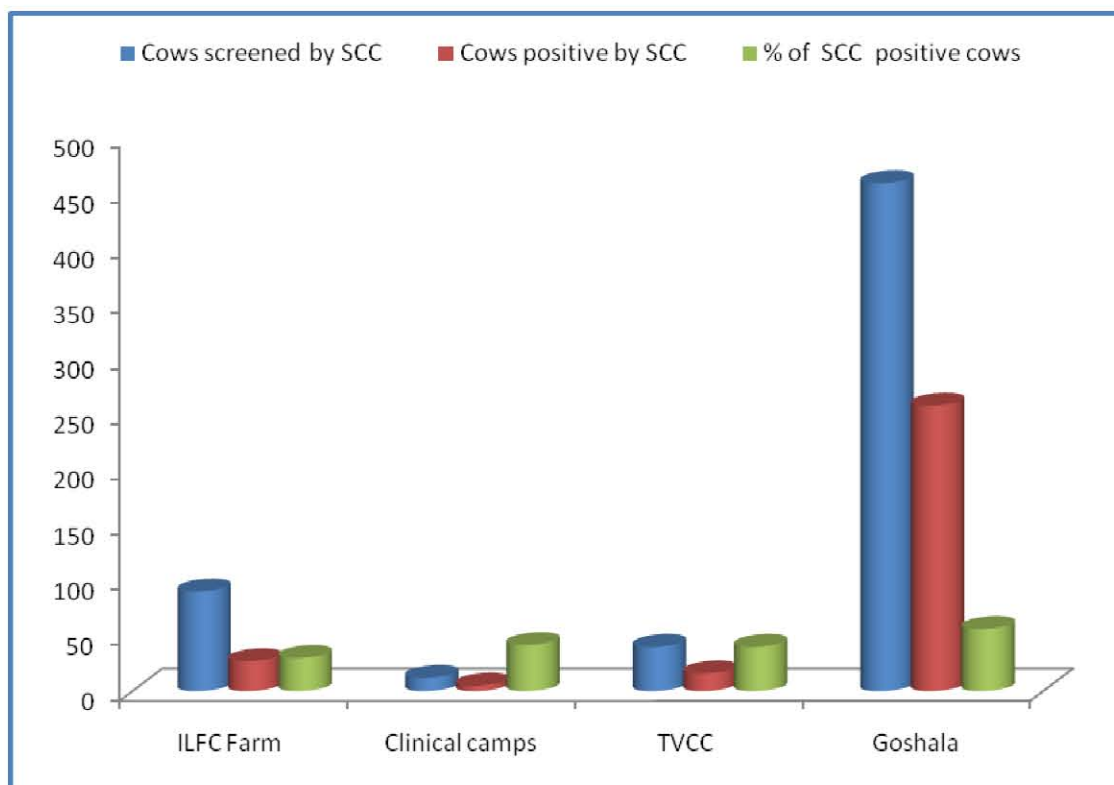


Fig. 4.6: Percentage of milk samples found positive by somatic cell count (SCC) for testing sub-clinical mastitis (SCM) in cows

while samples with less than 3 lac cells per ml of milk were recorded as negative. But irrespective of the results of SCC all the milk samples were subjected to bacterial isolation.

In the current study, milk samples reported as positive for SCM showed range of SCC as minimum somatic cell count of 3.5×10^5 to maximum of 4.8×10^5 in case of cow milk samples, while a minimum of 3.12×10^5 to maximum of 3.8×10^5 SCC was recorded in case of buffalo milk samples. Out of 601 cows, 306 were found positive for SCM by SCC. The prevalence of SCM in lactating cows was found to be 50.9% (Table 4.5). Out of 459 cows screened from various Goshalas, 258 (56.21%) were found positive for SCM, while out of 90 cows screened from ILFC, DUVASU, Mathura, 27 (30%) were positive; among 12 cows from ambulatory visits and clinical camps, 5 (41.67%) were positive; and out of 40 cows from TVCC, DUVASU, Mathura, 16 (40%) were found positive for SCM (Fig. 4.6). The detailed results of 258 positive cows for SCM among various Goshalas are presented in Table 4.6 and Fig. 4.7.

Out of 64 buffaloes screened for SCM, 19 (29.68%) were found positive by SCC. Out of 30 milk samples from TVCC, DUVASU, 10 were found positive (33.3%); out of 30 samples from ILFC, DUVASU, Mathura, 8 were positive (26.6%) and among 4 milk samples obtained from buffaloes during ambulatory visits and clinical camps, one (25%) was found positive for the presence of SCM (Table 4.7; Fig. 4.8).

4.1.4 Isolation of bacteria (*S. aureus* and *E. coli*) from milk samples of SCM and CM of cows and buffaloes

All the milk samples of selected lactating cows and buffaloes were screened for SCM and each milk sample collected from cases of CM were directly streaked over nutrient agar and MacConkey agar, inverted and incubated at 37°C for 24-48 h for primary isolation of *S. aureus* and *E. coli* bacteria from milk samples, respectively.

Out of 601 lactating cows screened for SCM, 7 (1.16%) *S. aureus*, 263 (43.76%) isolates of CNS and 107 (17.80%) isolates of *E. coli* were recovered (Table 4.8 and Table 4.9). Out of 56 lactating cows tested for clinical mastitis (CM), 45 *S. aureus* isolates (82.14%) and 4 (7.14%) isolates of *E. coli* were isolated (Table 4.11).

Out of 601 milk samples of lactating cows screened for SCM, no bacteria were isolated from 224 (37.27%) milk samples; and out of 56 lactating cows tested for CM, 7 (12.50%) milk samples were negative for any bacterial growth. The prevalence of CM in lactating cows was found to be 87.5%.

Among 657 cows tested for SCM and CM, 426 cows were found positive for bacterial growth (64.84%) and 231 (35.15%) were found negative as no bacterial isolation was observed. In the present study, out of 426 positive cows, a total of 52 *S. aureus* (12.20%), 263 (61.73%) isolates of CNS and 111 *E. coli* (26.05%) isolates were obtained.

Among 64 lactating buffaloes screened for SCM, eight isolates of CNS (18.75%), 4 isolates of *S. aureus* and 3 (4.68%) isolates of *E. coli* were recovered (Table 4.10). Out of 49 lactating buffaloes tested for clinical mastitis, 29 (59.18%) *S. aureus* isolates and 7 (14.28%) isolates of *E. coli* were isolated (Table 4.11). The prevalence of CM in lactating buffaloes was found to be 73.47%.

Out of 64 milk samples of lactating buffaloes screened for SCM, 49 (76.56%) samples did not reveal any bacterial growth and out of 49 lactating buffaloes tested for CM, no bacteria could be isolated from 13 (26.53%) milk samples of buffaloes. In total, among 113 buffaloes tested for SCM and CM, 51 (45.13%) buffaloes were positive while 62 (54.87%) were negative for bacterial isolation. Therefore, out of 51 positive buffaloes, a total of 33 *S. aureus* (64.71%), 8 CNS (15.69%) and 10 *E. coli* (19.61%) isolates were obtained.

Overall, from 770 lactating cows and buffaloes, 477 (61.94%) animals were positive for bacterial growth and no bacteria could be isolated from 293 (38.06%) animals. In the present study, a total of 85 *S. aureus*, 271 Coagulase negative Staphylococci (CNS) and 121 *E. coli* isolates were recovered.

4.1.5 Cultural, morphological and biochemical characterization of bacteria (*S. aureus* and *E. coli*) from milk samples

All the milk samples inoculated over nutrient agar and MacConkey agar were incubated at 37°C for 24-48 h and observed for cultural characteristics of *S. aureus* and *E. coli* bacterial isolates.

***S. aureus*:** Colonial characteristics, pigment production and Gram's staining features were observed and recorded after incubation. Milk samples showing round, smooth,

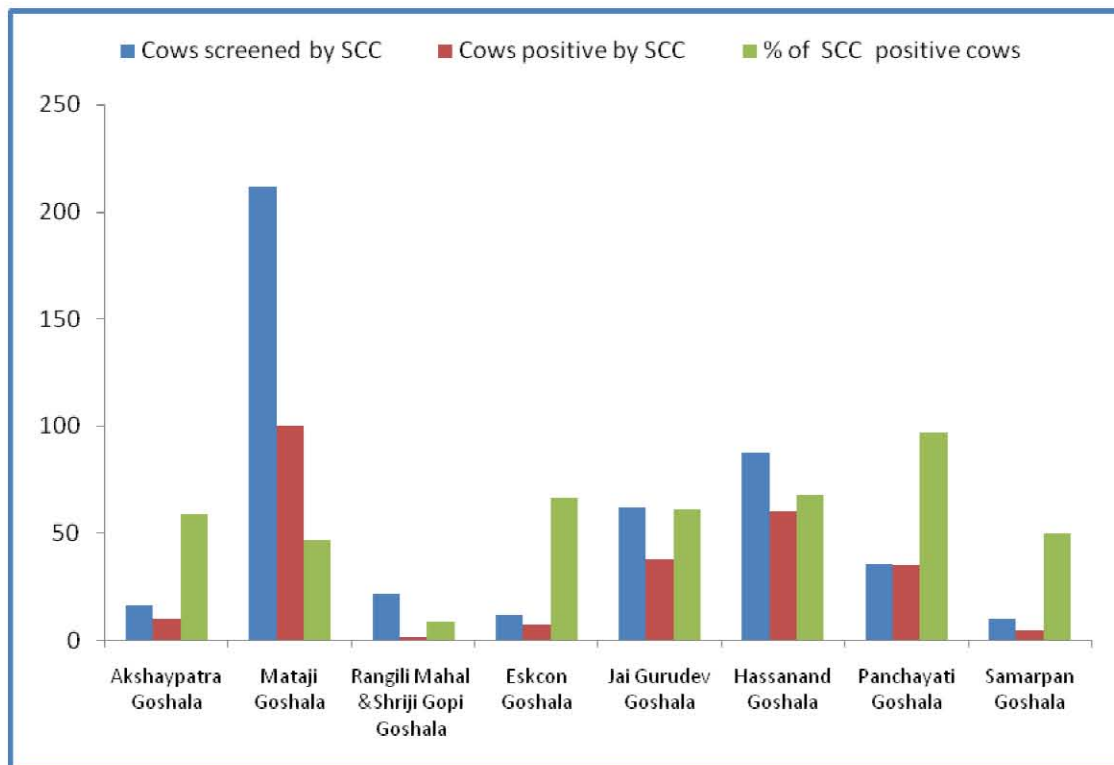


Fig. 4.7: Percentage of milk samples found positive by somatic cell count (SCC) for detecting sub-clinical mastitis (SCM) in cows in Goshalas

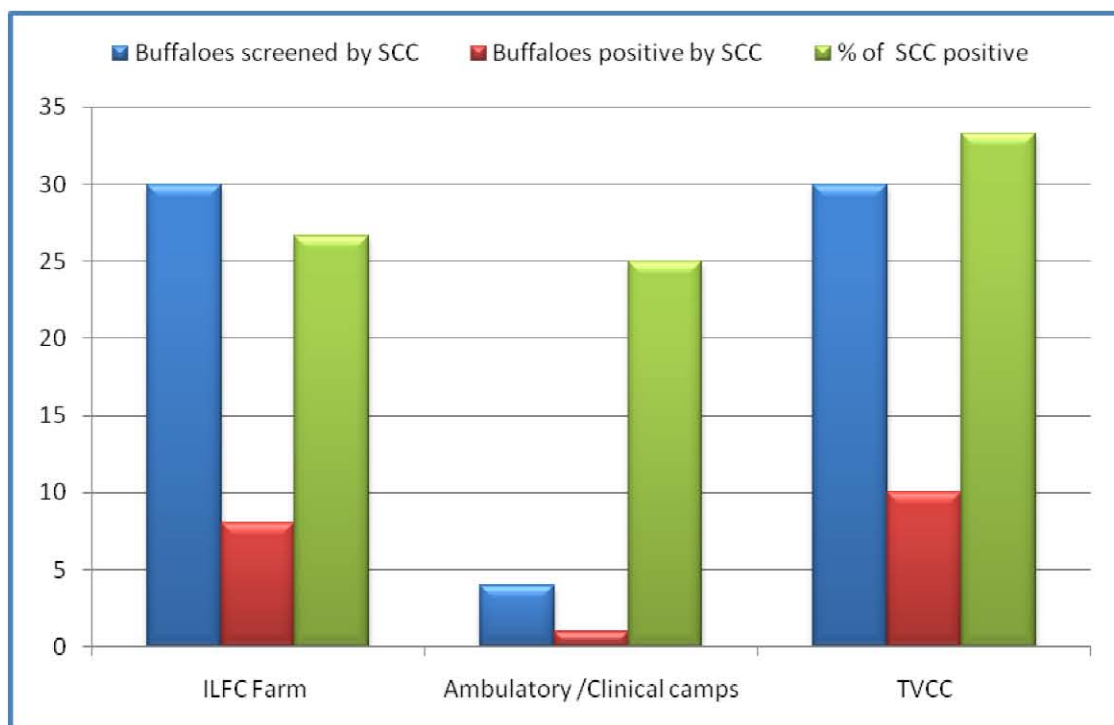


Fig. 4.8: Percentage of milk samples found positive by somatic cell count (SCC) for testing sub-clinical mastitis (SCM) in buffaloes

Table 4.6: Details of milk samples collected from cows of various Goshalas for the study of sub-clinical mastitis by somatic cell count (SCC)

| S. No. | Place of sample collection | Cows screened by SCC | Cows positive by SCC | % of SCC positive |
|---------------|--|-----------------------------|-----------------------------|--------------------------|
| 1. | Akshaypatra Goshala | 17 | 10 | 58/82 |
| 2. | Mataji Goshala | 212 | 100 | 47.16 |
| 3. | Rangili Mahal (17) + Shriji Gopi (5) Goshalas | 22 | 2 | 9.01 |
| 4. | Eskcon Goshala | 12 | 8 | 66.67 |
| 5. | Jai Gurudev Goshala | 62 | 38 | 61.29 |
| 6. | Hassanand Goshala | 88 | 60 | 68.18 |
| 7. | Panchayati Goshala | 36 | 35 | 97.22 |
| 8. | Samarpan Goshala | 10 | 5 | 50 |
| | Total | 459 | 258 | 56.21 |

Table 4.7: Details of milk samples collected from buffaloes for the study of sub-clinical mastitis by somatic cell count (SCC)

| S. No. | Place of sample collection | Buffaloes screened by SCC | Buffaloes positive by SCC | % of SCC positive |
|--------|-------------------------------|---------------------------|---------------------------|-------------------|
| 1. | ILFC Farm, DUVASU | 30 | 8 | 26.6 |
| 2. | Ambulatory and Clinical camps | 4 | 1 | 25 |
| 3. | TVCC, DUVASU | 30 | 10 | 33.3 |
| | Total | 64 | 19 | 29.68 |

Table 4.8: Number of milk samples from cows found positive for isolation of *Staphylococcus aureus*, coagulase negative Staphylococci (CNS) and *Escherichia coli* bacteria from sub-clinical mastitis

| S. No. | Place of sample collection | No. of cows | Positive for <i>S. aureus</i> / CNS isolates | Percentage of positive cows | Positive for <i>E. coli</i> isolates | Percentage of positive cows |
|--------|------------------------------|-------------|--|-----------------------------|--------------------------------------|-----------------------------|
| 1. | ILFC Farm, DUVASU | 90 | 29 CNS | 32.22 | 5 | 5.55 |
| 2. | Ambulatory and Clinical camp | 12 | 6 CNS | 50 | 2 | 16.66 |
| 3. | TVCC, DUVASU | 40 | 16 CNS | 40 | 2 | 5 |
| 4. | Goshalas | 459 | 212 CNS / 7 <i>S. aureus</i> | 47.71 | 98 | 21.35 |
| | Total | 601 | 263 CNS / 7 <i>S. aureus</i> | 44.92 | 107 | 17.80 |

Table 4.9: Number of milk samples from cows of various Goshalas found positive for isolation of *Staphylococcus aureus*, coagulase negative Staphylococci (CNS) and *Escherichia coli* bacteria from sub-clinical mastitis

| S. No. | Place of sample collection | No. of cows | Positive for <i>S. aureus</i> / CNS isolates | Percentage of positive cows | Positive for <i>E. coli</i> isolates | Percentage of positive cows |
|--------|---|-------------|--|-----------------------------|--------------------------------------|-----------------------------|
| 1. | Akshaypatra Goshala | 17 | 10 CNS | 58.8 | 0 | 0 |
| 2. | Mataji Goshala | 212 | 65 CNS | 30.66 | 35 | 16.5 |
| 3. | Rangili Mahal (17) + Shriji Gopi (5) Goshalas | 22 | 2 CNS | 9.52 | 0 | 0 |
| 4. | Eskcon Goshala | 12 | 5 CNS / 3 <i>S. aureus</i> | 66.67 | 8 | 66.67 |
| 8. | Jai Gurudev Goshala | 62 | 38 CNS | 61.29 | 4 | 6.45 |
| 9. | Hassanand Goshala | 88 | 56 CNS / 3 <i>S. aureus</i> | 67.0 | 32 | 36.36 |
| 10. | Panchayati Goshala | 36 | 35 CNS / 1 <i>S. aureus</i> | 97.22 | 16 | 44.44 |
| 11. | Samarpan Goshala | 10 | 2 | 20 | 3 | 30 |
| | Total | 459 | 212 CNS / 7 <i>S. aureus</i> | 47.71 | 98 | 21.35 |

Table 4.10: Number of milk samples from buffaloes found positive for isolation of *Staphylococcus aureus*, coagulase negative Staphylococci (CNS) and *Escherichia coli* bacteria from sub-clinical mastitis

| S. No. | Place of sample collection | No. of buffaloes | Positive for <i>S. aureus</i> /CNS | Percentage of positive | Positive for <i>E. coli</i> | Percentage of positive |
|--------|-------------------------------|------------------|---------------------------------------|------------------------|-----------------------------|------------------------|
| 1. | ILFC Farm, DUVASU | 30 | 6 CNS | 20 | 0 | 0 |
| 2. | Ambulatory and Clinical camps | 4 | 0 | 0 | 1 | 25 |
| 3. | TVCC, DUVASU | 30 | 4 <i>S. aureus</i> / 2 CNS | 20 | 2 | 6.66 |
| | Total | 64 | 8 CNS / 4 <i>S. aureus</i> | 18.75 | 3 | 4.68 |

Table 4.11: Milk samples from cows and buffaloes found positive for isolation of *Staphylococcus aureus* and *Escherichia coli* bacteria from clinical mastitis

| S. No. | Place of sample collection | No. of cows | Positive for <i>S. aureus</i> (%) | Positive for <i>E. coli</i> (%) | No. of buffaloes | Positive for <i>S. aureus</i> (%) | Positive for <i>E. coli</i> (%) |
|--------|-------------------------------|-------------|-----------------------------------|---------------------------------|------------------|-----------------------------------|---------------------------------|
| 1. | ILFC Farm, DUVASU | 2 | 2 (100) | 0 (0) | 1 | 1 (100) | 0 (0) |
| 2. | Ambulatory and Clinical camps | 6 | 4 (66.6) | 0 (0) | 1 | 1 (100) | 0 (0) |
| 3. | TVCC, DUVASU | 43 | 34 (79.1) | 4 | 47 | 27 (57.44) | 7 (14.89) |
| 4. | Eskcon Goshala | 2 | 2 (100) | 0 (0) | 0 | 0 | 0 |
| 5. | Hassanand Goshala | 3 | 3 (100) | 0 (0) | 0 | 0 | 0 |
| | Total | 56 | 45 (82.14) | 4 (7.14) | 49 | 29 (59.18) | 7 (14.28) |

Number shown in bracket indicates percentage

raised, glistening, and yellow colonies over nutrient agar were sub-cultured onto Mannitol salt agar (MSA). Circular (2–3 mm in diameter), smooth, and opaque characteristic golden yellow colonies causing yellow discoloration of media were obtained after 24–48 h of incubation (Fig. 4.9). This is characteristic cultural feature of *S. aureus*, which was confirmed by Gram's staining for typical morphological character. Mannitol fermenting yellow colonies showed Gram positive cocci in bunches upon Gram staining (Fig. 4.10), which were non-motile upon motility testing. Culture plates failed to produce yellow colonies and yellow discoloration of media were discontinued from the experiment as detection of bacteria other than *S. aureus* and *E. coli* was beyond the objectives of the study.

After cultural characterization, Gram's staining and motility testing, Gram positive non-motile cocci present in bunches were subjected to further biochemical characterization by standard procedure in the laboratory. Catalase and coagulase tests were performed and results showed all 356 *Staphylococcus* isolates were catalase positive (Fig. 4.11), on the other hand 74 *S. aureus* isolates recovered from milk samples of CM and 11 *S. aureus* isolates recovered from milk samples of SCM were coagulase positive (in total 85 *S. aureus* isolates) and rest 271 isolates of *Staphylococcus* spp. were coagulase negative Staphylococci and regarded as CNS, mostly isolated from milk samples of SCM.

E. coli: Milk samples inoculated over MacConkey agar were incubated at 37°C for 24–48 h and observed for the presence of lactose fermenting (Fig. 4.12) and non-fermenting colonies. Pink colored lactose fermenting circular colonies were further streaked over Eosin Methylene Blue agar and HiChrome *E. coli* agar, incubated at 37°C for 24 h and tested by Gram staining and motility testing. Gram staining and motility testing revealed Gram negative motile rods present in single form. The isolates showing black-magenta colored small dry colonies over EMB agar with characteristic metallic sheen (Fig. 4.13) and bluish-green colored small colonies over HiChrome *E. coli* agar were recorded as *E. coli* (Fig. 4.14). These strains were further confirmed by biochemical characterization by performing for indole, methyl red, Voges Proskauer (VP), citrate and sugar fermentation tests (1% glucose, 1% lactose and 1% maltose sugar). Results of biochemical reaction showed that 121 isolates were indole positive, methyl red positive, VP negative, citrate negative and ferment all three sugars.

Pure isolates of bacteria were transferred onto nutrient agar slants (Fig. 4.15) in duplicate, were assigned a number and stored at 4 °C.

4.2 Collection of samples of wastewater from disposal tanks from different locations

4.2.1 Rapid detection of bacteriophage by turbidity reduction method and streaking over the bacterial lawn and isolation of bacteriophage

Approximately 100-250 ml of 55 wastewater samples were collected for detection and isolation of bacteriophages (Fig. 4.16). From goats and poultry farms, fecal pellet and semi-dry fecal droppings, respectively were collected and processed. Details of wastewater sample collection from different locations were given in Table 4.12 and Fig. 4.17.

High efficacy of recovery depends on initial concentration of phage in sample and to enhance the concentration, enrichment protocol was adopted (Fig. 4.18) followed by filtration to have bacterial free filtrate (BFF) comprised of target phages (Fig. 4.19).

All the samples were subjected for the isolation of bacteriophage following a systematic approach. First, rapid detection of lytic activity of phage in wastewater was assessed by turbidity reduction method (Tiwari et al., 2010), secondly by spot inoculation (SP) method (Goodridge et al., 2003) and finally isolation of phages was done using Double Agar Layer (DAL) method (Adams, 1959).

The comparative results of lytic activity shown by various samples against bacteria by turbidity reduction method, spot inoculation method and DAL method were shown in Table 4.13.

Results of initial screening by the turbidity reduction method indicated that possibility of recovery of phages was maximum from wastewater samples collected from TVCC, DUVASU (100%), followed by samples of Goshalas (90.9%) and ILFC farm (88.8%) as compared to goat fecal pellet (66.6%). No reduction in turbidity was detected from sheep fecal pellet (0% positive sample), and poultry farm fecal droppings (0% positive sample). All five samples from TVCC, DUVASU were positive. In 90.9% positive samples of Goshala, among 33, 30 samples were positive (6 each from Hassanand Goshala, Jai Gurudev Gaushala and Panchayati Goshala; 3 each from Akshaypatra Goshala, Rangili Mahal & Shriji Gopi Goshala, Mataji

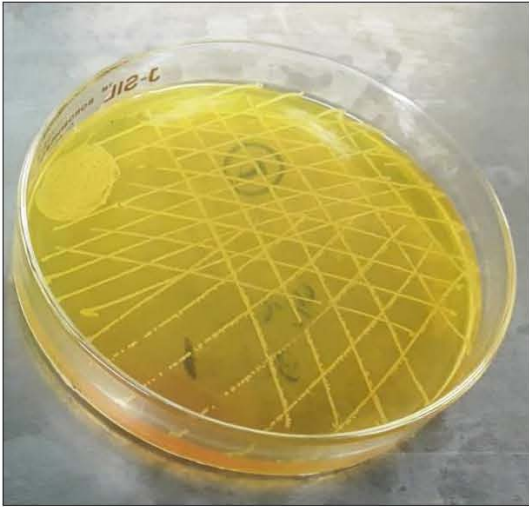


Fig. 4.9: Round yellow colonies of *Staphylococcus aureus* over Mannitol Salt Agar

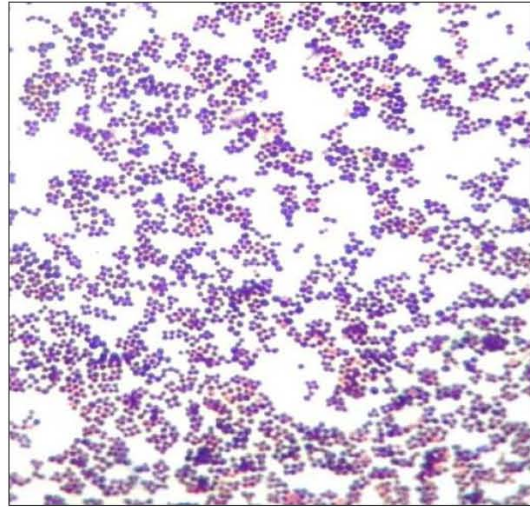


Fig. 4.10: Gram positive cocci in bunches. Gram Staining, 100X objective



Fig. 4.11: Catalase positive isolates of *Staphylococcus aureus*



Fig. 4.12: Pink lactose fermenting colonies of *E. coli* over MacConkey agar

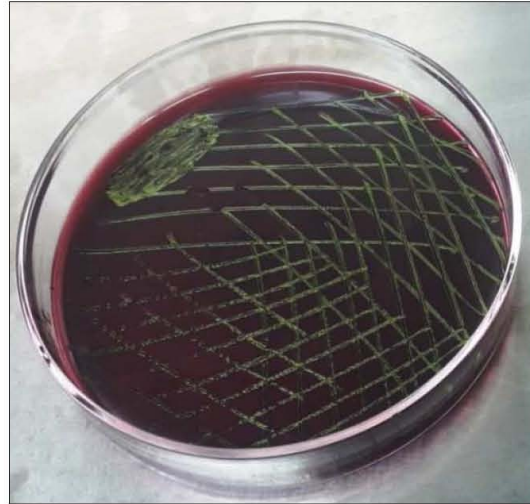


Fig. 4.13: Characteristic metallic sheen of *E. coli* over EMB agar

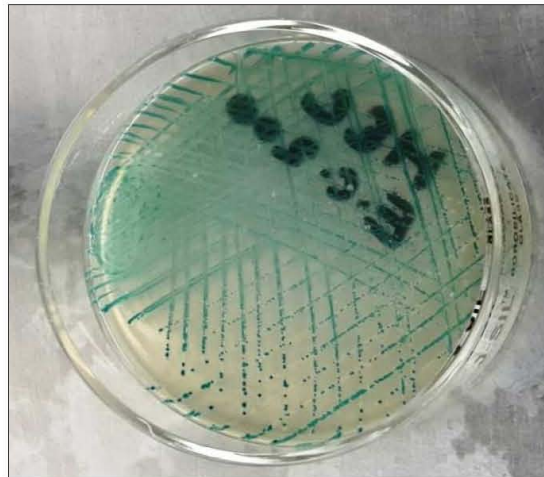


Fig. 4.14: Typical blue-green colonies of *E. coli* over HiChrome *E. coli* agar



Fig. 4.15: Growth of *Staphylococcus aureus* over Nutrient Agar Slant

Table 4.12 Details of animal wastewater samples collected from different locations

| S. No. | Place of sample collection | Number of wastewater samples collected |
|---------------|-----------------------------------|---|
| 1. | ILFC Farm, DUVASU | 9 (Cattle shed-6, Buffalo shed-3) |
| 2. | TVCC, DUVASU | 5 |
| 3. | Goshalas | 33 |
| 4. | Goat Farm, DUVASU | 3 |
| 5. | Sheep Flock, Aurangabad | 2 |
| 6. | Poultry Farm, DUVASU | 3 |
| | Total | 55 |



Fig. 4.16: Collection of wastewater sample from ILFC farm, DUVASU

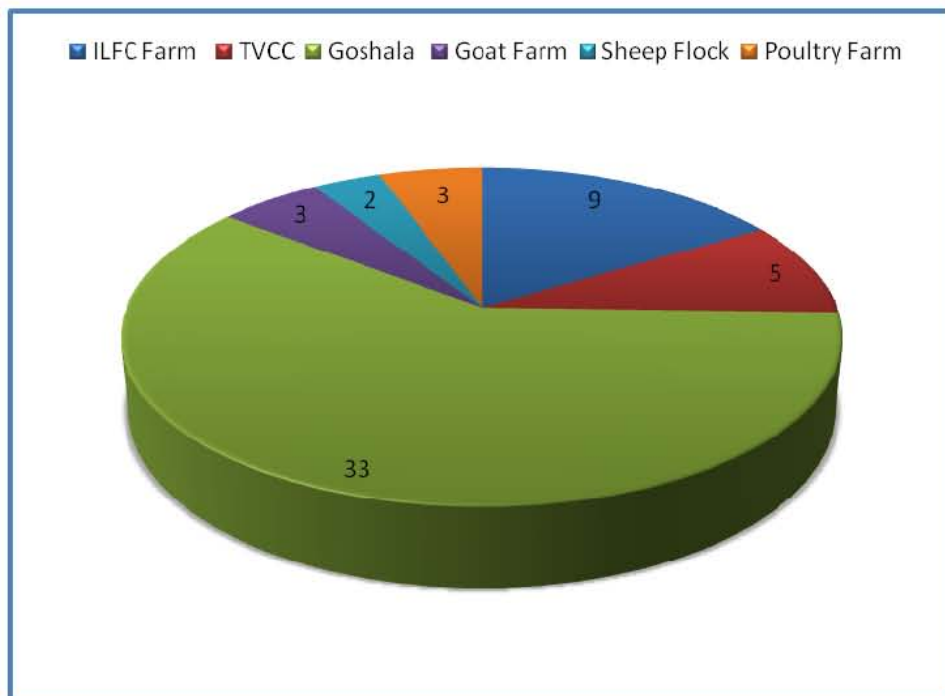


Fig. 4.17: Details of animal wastewater samples collected from different locations



Fig. 4.18: Enrichment of bacteriophage from sample of animal wastewater



Fig. 4.19: Preparation of bacterial free filtrate (BFF)

Table 4.13: Wastewater samples showing lytic activity by turbidity reduction method, spot inoculation method and double agar layer (DAL) method

| S. No. | Source of samples | Total number of wastewater samples collected | Positive by turbidity reduction method (%) | Positive by spot inoculation test (%) | Positive by DAL method (%) |
|--------|----------------------|--|--|---------------------------------------|----------------------------|
| 1. | ILFC Farm, DUVASU | 9 (Cattle-6, Buffalo-3) | 8 (88.8%) | 6 (75%) | 6 (75%) |
| 2. | TVCC, DUVASU | 5 | 5 (100%) | 3 (60%) | 3 (60%) |
| 3. | Goshalas | 33 | 30 (90.9%) | 20 (60.6%) | 20 (60.6%) |
| 4. | Goat Farm, DUVASU | 3 | 2 (66.6%) | 1 (33.3%) | 1 (33.3%) |
| 5. | Sheep Flock, DUVASU | 2 | 0 | 0 | 0 |
| 6. | Poultry Farm, DUVASU | 3 | 0 | 0 | 0 |
| | Total | 55 | 45 (81.8 %) | 30 (54.5%) | 30 (54.5%) |

Number shown in bracket indicates percentage.

Goshala, and Eskcon Goshala) and from Pt Deen Dayal Dham Kamdhenu Goshala, no sample showed reduction in turbidity. Among 9 samples collected from ILFC, DUVASU, Mathura, 8 samples (6 from cattle and 2 from buffalo wastewater) were found positive (88.8%). From three samples collected from goats, two were positive (66.6%) (Fig. 4.20).

In the present study, among fifty five, forty five (81.8%) samples shown turbidity reduction method as a rapid technique for detection of presence of phages (Fig. 4.21).

Spot inoculation or streak plate method was then performed on all samples and development of plaques on surface of solid media was adjudged as indication of lytic activity and among 55, 30 samples were found positive. As a result, maximum recovery was observed from cattle and buffalo wastewater from ILFC Farm (75%), followed by cattle wastes from Goshalas (60.6%), and from TVCC (60%), DUVASU, Mathura. Only 33.3% of positive results were obtained from goat faecal samples (Fig. 4.22).

Comparison of results of turbidity reduction method with that of streak plate or spot inoculation method indicated that number of samples showing lytic activity reduced from 81.8% to 54.5% when studied by streak plate method. This is possibly because assessment of plaque formation was difficult due to irregular plaque morphology and volume of BFF used in spot inoculation method (10 μ l) was ten times lesser than the turbidity reduction method (100 μ l) (Fig. 4.23).

Further comparison of SP method with DAL method showed cent percent correlation as all the samples processed by SP method (54.5%) were also positive by DAL method (54.5%) and used finally for obtaining pure culture of specific phages i.e. on *S. aureus* and *E. coli*.

Those samples which failed to induce lysis of bacteria in turbidity reduction method also did not show plaque formation by streak plate method. Every sample that showed lytic activity on the bacterial lawn by streak plate method was finally processed for isolation of phages by DAL method.

In the present study, primary isolation was done using two most common causative agents (clinical field strain) of bovine mastitis namely *S. aureus* and *E. coli* isolated from cases of CM and number of phages specific for *S. aureus* and *E. coli* were shown in Table 4.14.

A systematic approach was followed in present study for the recovery of phages so as to obtain pure phage isolated on the basis of single plaque morphology.

Wastewater samples found positive by rapid detection techniques of turbidity reduction method and streak plate method were subjected to DAL method. Out of 55 samples tested, a total of 51 phage isolates were obtained. Total number of phage strains recovered against *S. aureus* were 21 while 30 in case of *E. coli*. More recovery of *E. coli* phages may be because of abundant presence of host bacteria *E. coli* in soil and water as an environmental pathogen.

4.2 Characterization of phage isolates

Phage isolates were further subjected to physical and biological characterization based on plaque morphology, chloroform, temperature effect, pH effect and host range studies. Scanning electron microscopy was conducted for morphological characterization, though it was beyond the part of study.

4.2.1 Plaque morphology

Ellis and Winters (1969) reported that plaque morphology is one of the foremost criteria for characterization. In the present study, phages were categorized by plaque size, consistency of plaques and were assigned the designation. Plaque types ranged in size with small plaque categorized as being under 2 mm, medium plaque being 2 mm, and large plaque being more than 2 mm. Variation also occurred in the plaque consistency as clear and opaque or diffused plaques (Table 4.15, Fig. 4.24a and Fig.4.24b).

Media plated were observed from 6 h-48 h for appearance of plaques. Results revealed that *E. coli* supported the growth of more phages as compared to *S. aureus* as indicated by recovery of 30 *E. coli* host specific (EcD) phage as against 21 *S. aureus* host specific phages (SaD). Once, plaques begin to appear after 6 h, their number increased with the time of incubation upto 48 h. The size, morphology and edges of plaques vary as per respective phage isolate type and on the basis of plaque morphology, phages were categorized into different types. As per plaque morphology,

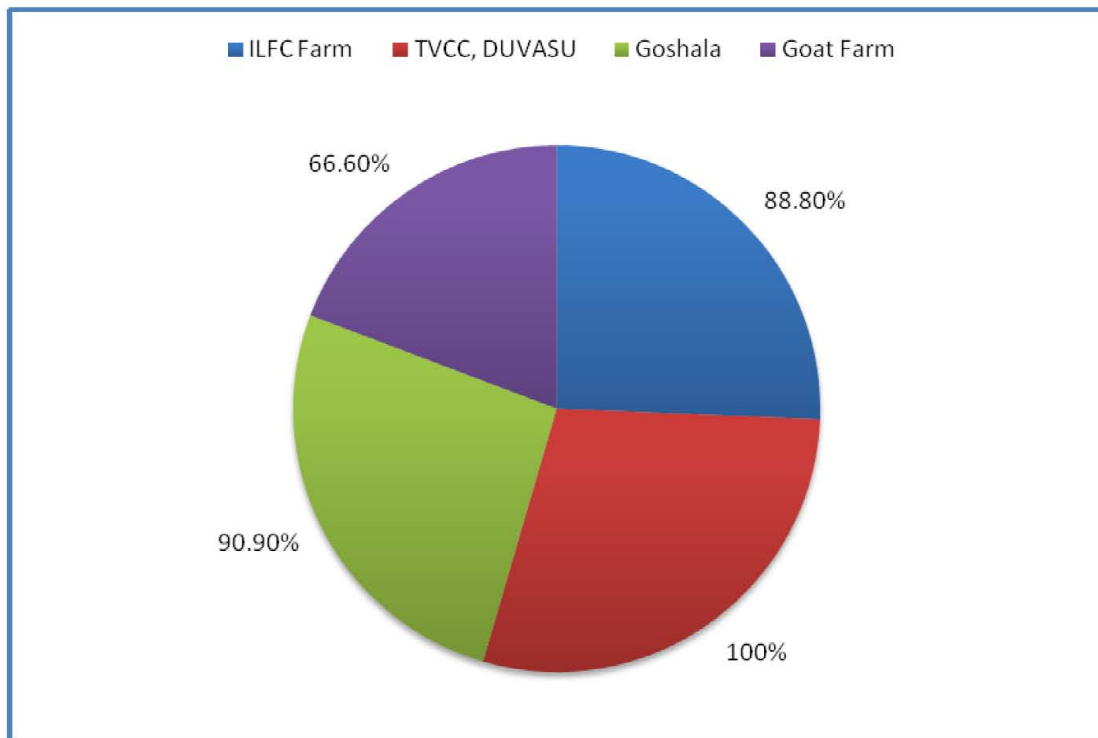


Fig. 4.20: Percentage of wastewater samples from different locations showing lytic activity by turbidity reduction method

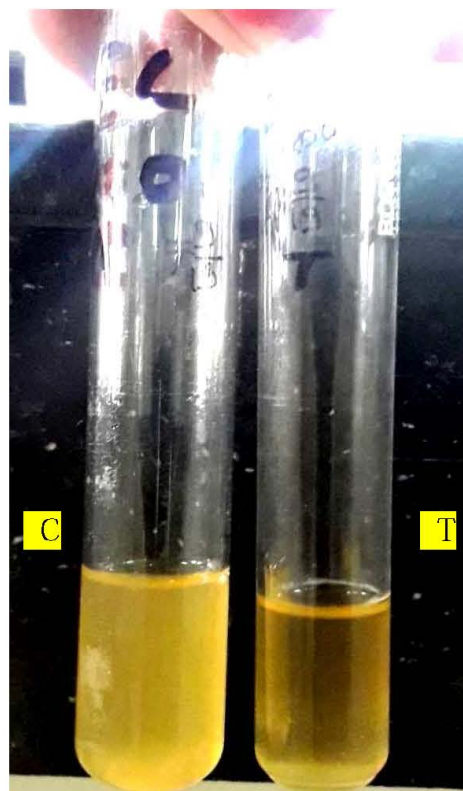


Fig. 4.21: Turbidity reduction test - Control-Left tube and Test-Right tube

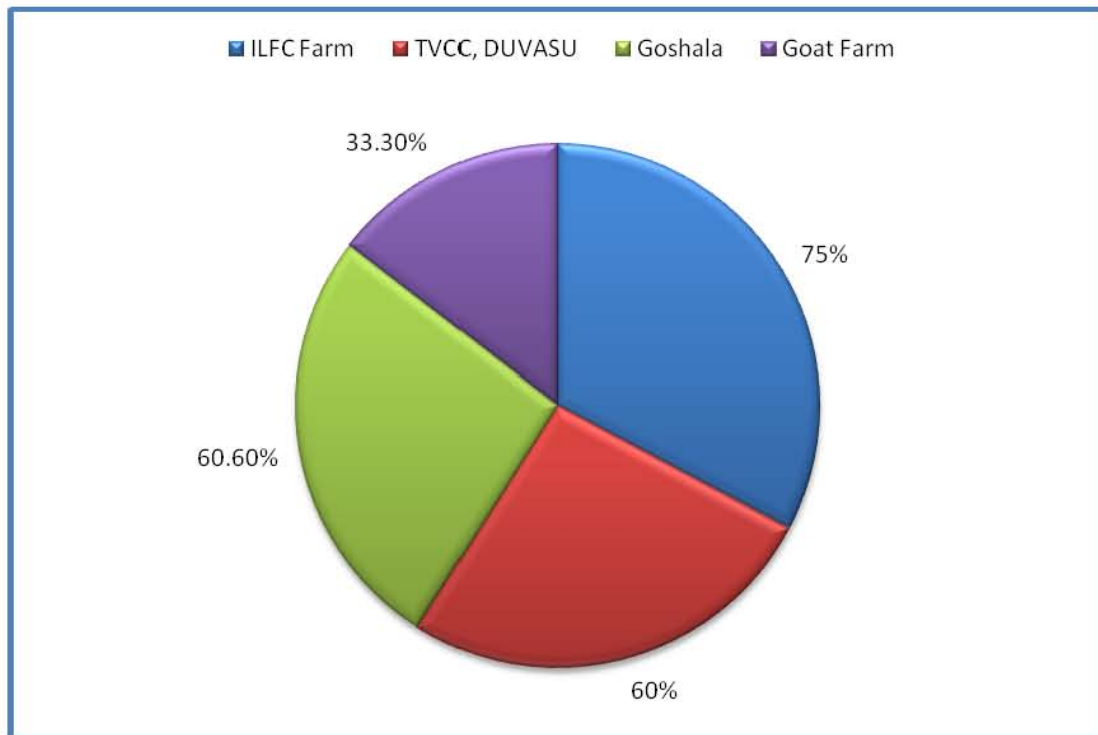


Fig. 4.22: Percentage of wastewater samples from different locations showing lytic activity by spot inoculation method

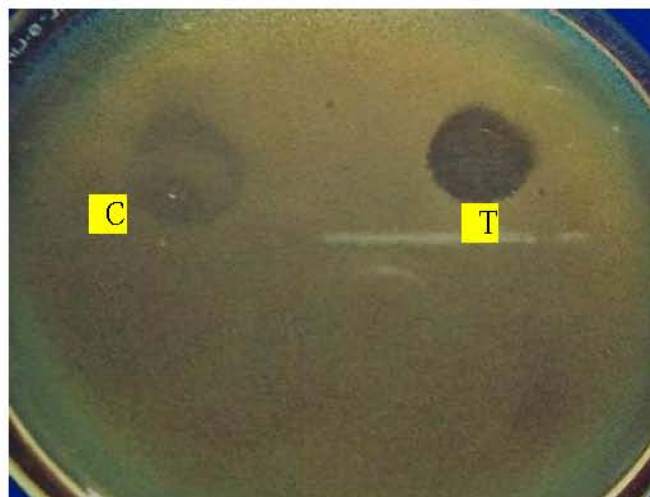


Fig. 4.23: Spot inoculation method - Control (C) and Test (T)

Table 4.14: Number of phages isolated against *Staphylococcus aureus* and *Escherichia coli* by DAL method

| S. No. | Source of samples | No. of samples selected* | No. of phages isolated on bacteria | | Total no. of phages isolated |
|--------|-------------------|--------------------------|------------------------------------|----------------------|------------------------------|
| | | | <i>S. aureus</i> phage | <i>E. coli</i> phage | |
| 1. | ILFC Farm, DUVASU | 6 | 5 | 6 | 11 |
| 2. | TVCC, DUVASU | 3 | - | 3 | 3 |
| 3. | Goshalas | 20 | 15 | 20 | 35 |
| 4. | Goat Farm, DUVASU | 1 | 1 | 1 | 2 |
| | Total | 30 | 21 | 30 | 51 |

* Selected on the basis of spot inoculation method

Table 4.15: Differentiation of phage isolates by plaque morphology

| S. No. | Phage type and designation | Designation on the basis of plaque morphology | Plaque morphology | No. of isolates/ Total no. of isolates | Percentage (%) |
|--------|---|---|-------------------------------------|---|----------------|
| 1. | Phages isolated on <i>S. aureus</i> (SaD) | SaD ₁ | Small-pin headed clear plaque | 15/21 | 71.4 |
| | | SaD ₂ | Small diffused/opaque plaque | 4/21 | 19.0 |
| | | SaD ₃ | Large clear plaque | 2/21 | 9.5 |
| 2. | Phages isolated on <i>E. coli</i> (EcD) | EcD ₁ | Medium sized and clear round plaque | 24/30 | 80.0 |
| | | EcD ₂ | Small sized and clear plaques | 4/30 | 13.3 |
| | | EcD ₃ | Large sized and clear round plaques | 2/30 | 6.66 |

Number shown in bracket indicates percentage.



Fig. 4.24a: Control: No plaques over Luria Bertani agar



Fig. 4.24b: Plaques of bacteriophage on lawn of *E. coli* over Luria Bertani agar (LBA)

phages isolated on *S. aureus* (SaD) were designated as; SaD₁ with small-pin headed clear plaque, SaD₂ with small diffused/opaque plaque and SaD₃ with Large clear plaque; and phages isolated *E. coli* (EcD) were designated as; EcD₁ with medium sized and clear plaque, EcD₂ with Small sized and clear plaque and EcD₃ with Large sized and clear round plaque (Fig.4.25-Fig.4.30).

For each prime host, three different phage isolates were obtained on the basis of plaque morphology. In the present study, large batches (1000 ml) of soft top agar (0.7%) were made at one time, so that entire batch would have uniform gel strength. Hence, the phage isolates differentiated based on plaque morphology were all different from each other.

Electron microscopy: For morphological characterization of phage, scanning electron microscopy (SEM) was conducted to observe morphology of *E. coli* host specific phage, as facility of Transmission electron microscopy (TEM) was not available during the time of study. The electron microscopy of EcD₂ after negative staining showed presence of hexagonal head and long non-contractile tail, which indicated that isolated phage may belong to *Siphoviridae* family. Electron micrograph of EcD₂ has been presented in Fig. 4.31. Further studies including TEM and genomic characterization are desired to correlate with the host-range studies and physico-chemical method of characterization.

4.2.2 Effect of temperature

All the phage isolates were investigated for their temperature sensitivity based on ability of phage isolates to form plaque when exposed to temperatures of 4°C, 22°C, 37°C and 70°C for 60 min. For this, 0.1 ml of phage suspension was mixed with 4.9 ml of nutrient broth in test tubes. Volume of 5 ml was distributed as 1 ml into test tubes. The test tubes containing 1 ml mix were placed in water bath at temperature ranges of 22°C, 37°C, 70°C for 60 min and 1 tube was kept at 4°C for 60 min. Phage concentrations (titer) before and after treatment with 60 min exposure to heat were adjudged by DAL method. After 1 hr, 0.1 ml of exposed phage suspension was mixed with 0.3 ml of 16-18 hr old respective bacterial (*S. aureus* and *E. coli*) culture, gently mixed and added into 2.5 ml of soft top agar and plating was done as per DAL method, plates were inverted and incubated at 37°C for 24 h and observed for presence or absence or any reduction in number of plaques.

Any reduction in phage titer and viability of phage were observed and compared with exposure at 4°C, which acted as control. The effects of temperature on viability of phages are mentioned in Table 4.16.

Results presented in Table 4.16 indicated that the phage isolates EcD₁, EcD₂ and EcD₃ remain viable after treating at 70°C for 60 min but there was distinct reduction in the % viability (33.33% EcD₁ remain viable and among EcD₂ and EcD₃ 50% remain viable), and SaD₁, SaD₂ and SaD₃ phage isolates failed to show any viability and all three types were inactivated when exposed to temperature of 70°C for 60 min. When exposed to 4°C for 60 min, all phage isolates SaD₁, SaD₂, SaD₃, EcD₁, EcD₂ and EcD₃ remain viable and stable, which showed that they can be stored at refrigeration temperature of 4°C safely for longer duration of time. When exposed to 37°C for 60 min, all phage isolates SaD₁, SaD₂, SaD₃, EcD₁, EcD₂ and EcD₃ maintained viability and were stable, indicating that all phage isolates under study maintain their bacterial infectivity and viability at incubation temperature of 37°C. Temperature of 22°C for 60 min, inactivated all SaD₂ type phages and marked reduction in the % viability of 50% was shown by SaD₃ types; however, rest all phage isolates SaD₁, EcD₁, EcD₂ and EcD₃ remain viable and stable.

4.2.3 Effect of pH

In order to characterize the phage, pH study was conducted to see the effect of variable pH seen on phage titer and viability of phages using the method of Litt and Jaroni (2017) and Svab et al. (2018) with some modification. Here, *S. aureus* and *E. coli* phage isolates were tested for their viability at different pH i.e. 3, 5, 7, 9 and 11 for 60 min. For this, 0.1 ml of phage suspension was mixed with 4.9 ml of nutrient broth, kept at various pH of 3, 5, 7, 9 and 11 and all five tubes were incubated at 37 °C for 1 h before assessment of effect of pH. The pH in test tube was adjusted by using 1M hydrochloric acid (HCl) and 1M sodium hydroxide (NaOH) solution. After 1 h, 0.1 ml of exposed phage suspension was mixed with 0.3 ml of 16-18 hr old respective bacterial (*S. aureus* and *E. coli*) culture, mixed and added into 2.5 ml of soft top agar and plating was done as per DAL method. The effects of different pH on phage isolates were presented in the Table 4.17.

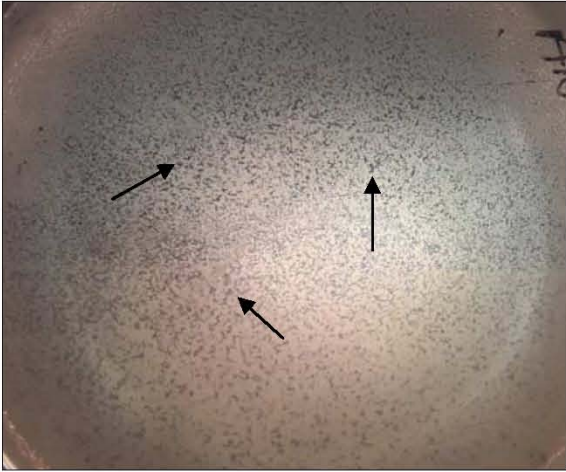


Fig. 4.25: Small pin-headed clear plaques on bacterial lawn (SaD₁)



Fig. 4.26: Small diffused and opaque plaques on bacterial lawn (SaD₂)



Fig. 4.27: Large clear plaques (SaD₃)

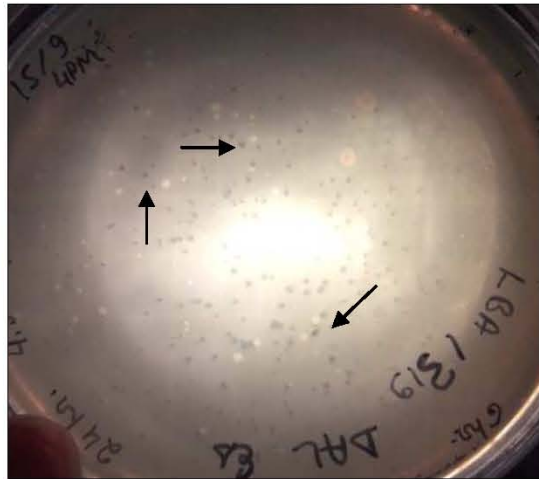


Fig. 4.28: Medium sized clear round plaques on bacterial lawn (EcD₁)

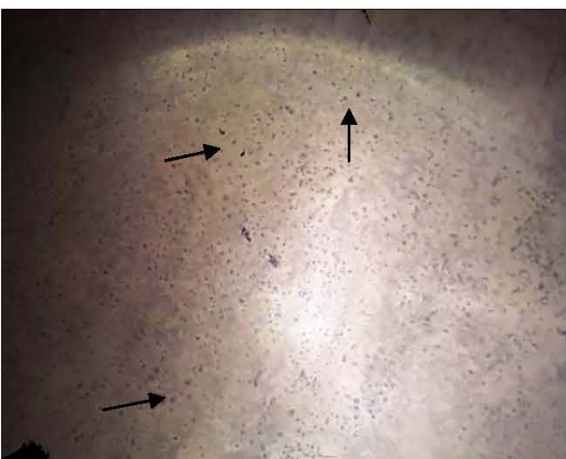


Fig. 4.29: Small sized clear plaques on bacterial lawn (EcD₂)

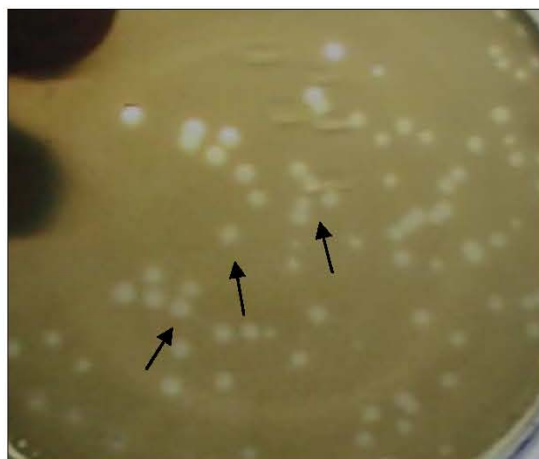


Fig. 4.30: Large sized clear round plaques on bacterial lawn (EcD₃)

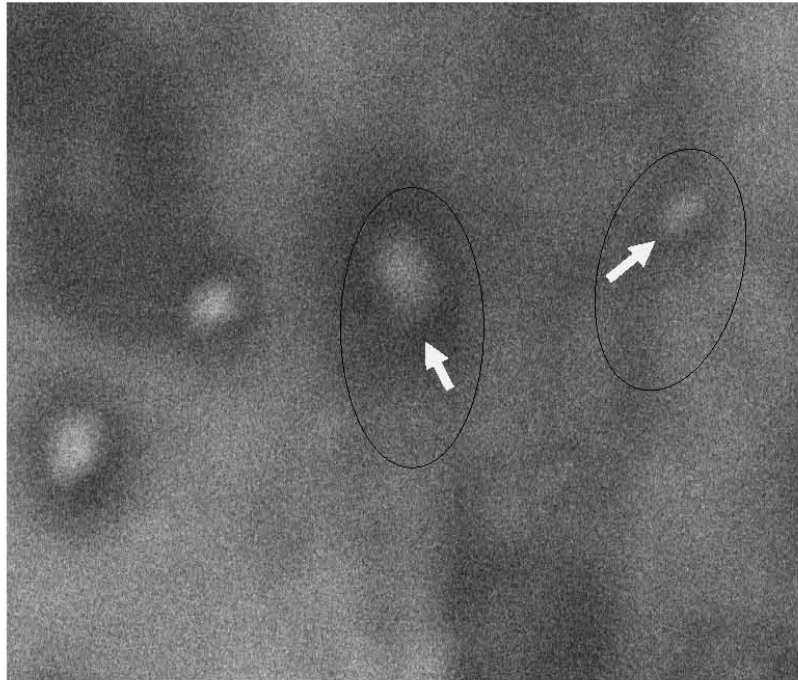


Fig. 4.31: Scanning Electron microscopic image (SEM) depicting hexagonal head and long tail of phage

Table 4.16 Effect of temperature on viability of phage isolates

| S. No. | Phage tested | No. of isolates tested | Viability of the phages at various temperature for 60 min (% activity) | | | |
|--------|------------------|------------------------|--|----------------------|--------------------|----------------------|
| | | | 4°C | 22°C | 37°C | 70°C |
| 1. | SaD ₁ | 15 | 15 (100) Stable | 15 (100) Stable | 15 (100) Stable | 0 (0) Inactivated |
| 2. | SaD ₂ | 4 | 4 (100) Stable | 2 (50) Reduced | 4 (100) Stable | 0 (0) Inactivated |
| 3. | SaD ₃ | 2 | 2 (100) Stable | 0 (0) Inactivated | 2 (100) Stable | 0 (0) Inactivated |
| 4. | EcD ₁ | 24 | 24 (100) Stable | 24 (100) Stable | 24 (100) Stable | 8 (33.33) Reduced |
| 5. | EcD ₂ | 4 | 4 (100) Stable | 4 (100) Stable | 4 (100) Stable | 2 (50) Reduced |
| 6. | EcD ₃ | 2 | 2 (100) Stable | 2 (100) Stable | 2 (100) Stable | 1 (50) Reduced |

Number shown in bracket indicates percentage

Observations indicated no significant effect due to pH on viability of phage isolates. The isolates appear to be stable to the pH range of 5 to 9, but inactivation was evident at the very low (pH 3) and very high pH (pH 11) levels.

4.2.4 Effect of chloroform

Sensitivity of phages to chloroform was tested by following the method of Chow and Rouf (1983) with some modifications to visualize the effect of chloroform treatment on the viability of phages. The effect of chloroform on phage isolates was recorded in terms of percent viability as shown in the Table 4.18.

Results showed that EcD₁ and EcD₂ maintain 100 % viability and activity after their exposure to chloroform; whereas, EcD₃ (50%) and ScD₁ (66.6%) demonstrated reduction in percent viability. Phage isolates SaD₂ and ScD₃ were inactivated and no plaques were produced after chloroform treatment. Results as presented in Table 4.18 revealed better % viability by Coliphages as compared to Staphylococcal phages.

4.2.5 Host range determination of phage isolates

Phage isolates which were initially isolated using *S. aureus* and *E. coli* were also tested for lytic activity against standard strain of bacteria viz. *Staphylococcus aureus*, *S. epidermidis*, *Bacillus spp.*, *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* maintained in the laboratory. Number of phage isolates that showed susceptibility to these bacteria were shown in Table 4.19.

Phages were tested against *S. aureus*, *S. epidermidis*, *Bacillus spp.*, *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella spp.* Results indicated that phage isolates either produced plaques or showed reduction in bacterial colony number against other bacterial genera. The host range of SaD phage and EcD phage was determined with selected strains of 6 different bacterial isolates maintained in laboratory. Phage isolate SaD showed lytic activity only against *S. aureus* strains, and not against any of five other bacteria. Few EcD₁ type phage isolates showed lytic activity against *Pseudomonas aeruginosa* and *Klebsiella spp.* also besides *E. coli* but not against *S. aureus*, *S. epidermidis* and *Bacillus spp.*

In the present study, phage isolate EcD₁ had wide hosts range as compared to other phage strains as it showed lytic activity against all 3 bacterial genera tested.

Phage isolates SaD₁, SaD₂ and SaD₃, isolated during the study were narrow range phages as they demonstrated lytic activity only against *S. aureus*.

4.3 *In vitro* antibiotic sensitivity test and *in vitro* anti-bacterial potential of Phage

4.3.1 Antibiotic sensitivity pattern of bacteria isolated from SCM and CM

The bacteria were also subjected to Antibiotic Sensitivity test (AST), to know the bacterial sensitivity for various antibiotics or in other words to know the percentage of antibiotic resistant bacteria against different antibiotics. Now-a-days, antibiotic resistance is developing world wide and a major cause of delayed recovery from mastitis, which led to subsequent economic losses.

Each bacterial culture was inoculated into sterilized NB and incubated at 37 °C for 18-24 h. After incubation, turbidity of culture was matched with McFarland Nephelometer tube 0.5 (resembling to 1.5×10^8 cfu/ml) and pure broth culture of each bacterial isolate was spread on the surface of Muller Hinton agar plates and allowed to dry at room temperature for 5-10 min. Antibiotic discs were placed and plates were incubated in inverted position at 37 °C for 18-24 h. The results of antibiotic sensitivity testing are described in Table 4.20.

Antibiotic sensitivity test of all 85 isolates of *S. aureus* obtained from milk samples of SCM and CM were conducted against selected 20 antimicrobial discs (Table 3.3, provided in chapter 3), namely, Ampicillin (AMP), Amoxycillin (AMC), Ampicillin/Sulbactam (A/S), Amikacin (AK), Erythromycin (E), Gentamicin (GEN), Kanamycin (K), Methicillin (MET), Norfloxacin (NX), Penicillin-G (P), Ciprofloxacin (CIP), Chloramphenicol (C), Streptomycin (S), Tetracycline (T), Enrofloxacin (En), Vancomycin (VA), Ceftriaxone (CTR), Cefotaxime (CTX), Cotrimoxazole (CoT) and Gatifloxacin (GAT). Their zone of diameter was measured by using Zone-reader scale, compared with the provided standard chart and results are expressed in the form of S (Number of bacterial isolates found sensitive to particular drug), I (Number of bacterial isolates found intermediately sensitive to particular drug) and R (Number of bacterial isolates found resistant to particular drug) as mentioned in Table 4.20. Results indicated that *S. aureus* bacteria isolated from milk samples showed resistance to one or more than one antibiotic.

Against Ampicillin, 47 (55.29%) out of 85 bacterial strains showed sensitivity, 23 showed intermediate sensitivity, while 15 (17.64%) were found resistant; against

Table 4.17 Effect of pH on viability of phage isolates

| S. No. | Phage tested | No. of isolates tested | No. of phage strains viable at different pH (% viability) | | | | |
|--------|------------------|------------------------|---|----------|----------|-----------|-------|
| | | | pH:3 | pH:5 | pH:7 | pH:9 | pH:11 |
| 1. | SaD ₁ | 15 | 0 (0) | 15 (100) | 15 (100) | 8 (53.33) | 0 (0) |
| 2. | SaD ₂ | 4 | 0 (0) | 2 (50) | 4 (100) | 1 (25) | 0 (0) |
| 3. | SaD ₃ | 2 | 0 (0) | 2 (100) | 2 (100) | 1 (25) | 0 (0) |
| 4. | EcD ₁ | 24 | 0 (0) | 24 (100) | 24 (100) | 24 (100) | 0 (0) |
| 5. | EcD ₂ | 4 | 0 (0) | 4 (100) | 4 (100) | 2 (50) | 0 (0) |
| 6. | EcD ₃ | 2 | 0 (0) | 2 (100) | 2 (100) | 2 (100) | 0 (0) |

Number shown in bracket indicates percentage.

Table 4.18: Effect of chloroform on viability of phage isolates

| S. No. | Phage tested | No. of isolates tested | Stability of the phages to chloroform treatment | % viability |
|--------|------------------|------------------------|---|-------------|
| 1. | SaD ₁ | 15 | 10 | 66.67 |
| 2. | SaD ₂ | 4 | 0 | 0 |
| 3. | SaD ₃ | 2 | 0 | 0 |
| 4. | EcD ₁ | 24 | 24 | 100 |
| 5. | EcD ₂ | 4 | 4 | 100 |
| 6. | EcD ₃ | 2 | 1 | 50 |

Table 4.19: Host range studies on phage isolates

| S. No. | Phage tested | No. of isolates tested for each host | Host range of phage strains against bacterial hosts | | | | | |
|--------|------------------|--------------------------------------|---|-----------------------|---------------------|----------------------|------------------------------|----------------|
| | | | <i>S. aureus</i> | <i>S. epidermidis</i> | <i>Bacillus spp</i> | <i>P. aeruginosa</i> | <i>Klebsiella pneumoniae</i> | <i>E. coli</i> |
| 1 | SaD ₁ | 15 | 15 | 0 | 0 | 0 | 0 | 0 |
| 2 | SaD ₂ | 4 | 4 | 0 | 0 | 0 | 0 | 0 |
| 3 | SaD ₃ | 2 | 2 | 0 | 0 | 0 | 0 | 0 |
| 4 | EcD ₁ | 24 | 0 | 0 | 0 | 20 | 15 | 24 |
| 5 | EcD ₂ | 4 | 0 | 0 | 0 | 0 | 2 | 4 |
| 6 | EcD ₃ | 2 | 0 | 0 | 0 | 0 | 0 | 2 |

Table 4.20 Results of antibiotic sensitivity testing of various isolates of *Staphylococcus aureus*

| Name of microorganism | Sensitivity pattern | Antibiotic discs used | | | | | | | | | | | | | | | | | | | |
|--|---------------------|-----------------------|-----|-----|----|----|-----|----|-----|----|----|-----|----|----|----|----|----|-----|-----|------|-----|
| | | AMP | AMC | A/S | AK | E | GEN | K | MET | NX | P | CIP | C | S | T | En | VA | CTR | CTX | Co-T | GAT |
| <i>Staphylococcus aureus</i> (N=85) | S | 47 | 54 | 84 | 27 | 20 | 23 | 0 | 0 | 2 | 3 | 31 | 6 | 3 | 72 | 77 | 0 | 73 | 69 | 0 | 69 |
| | I | 23 | 24 | 0 | 18 | 16 | 15 | 11 | 1 | 35 | 17 | 40 | 31 | 11 | 11 | 8 | 0 | 11 | 10 | 2 | 11 |
| | R | 15 | 7 | 1 | 40 | 49 | 47 | 74 | 84 | 48 | 65 | 14 | 48 | 71 | 2 | 0 | 85 | 1 | 6 | 83 | 5 |

N= Total number of isolates tested; S: Sensitive; I: Intermediate; R: Resistance

AMP – Ampicillin, AMC - Amoxycillin, A/S - Ampicillin/Sulbactam, AK - Amikacin, E - Erythromycin, Gen - Gentamicin, K - Kanamycin, MET - Methicillin, Nx - Norfloxacin, P - Penicillin-G, CIP - Ciprofloxacin, C - Chloramphenicol, S - Streptomycin, T - Tetracycline, En - Enrofloxacin, Va - Vancomycin, CTR - Ceftriaxone, CTX - Cefotaxime, Co-T - Cotrimoxazole, GAT - Gatifloxacin

Amoxicillin 54 (63.53%) showed sensitivity, 24 showed intermediate sensitivity and 7 (8.23%) were found resistant. Against Ampicillin/Sulbactam, 84 (98.82%) out of 85 bacterial strains were sensitive while 1 (1.17%) isolate remain resistant; against Amikacin, 27 (31.76%) out of 85 bacterial strains showed sensitivity, 18 showed intermediate sensitivity while 40 (47.06%) were found resistant. Against Erythromycin, 20 (23.53%) strains were found sensitive, 16 were intermediately sensitive and 49 (57.64%) were found resistant; against Gentamicin 23 (27.06%) out of 85 bacterial strains showed sensitivity, 15 showed intermediate sensitivity while 47 (55.29%) were found resistant; against Kanamycin no sensitive bacterial strains were observed, 11 were intermediately sensitive, while 74 (87.06%) were resistant. Likewise, against Methicillin no bacterial strain showed sensitivity, 1 showed intermediate sensitivity and 84 (98.82%) were found resistant; against Norfloxacin 2 (2.35%) out of 85 bacterial strains showed sensitivity, 35 showed intermediate sensitivity while 48 (56.47%) were found resistant. Against Penicillin-G, only 3 (3.52%) strains were found sensitive, 17 were intermediately sensitive and 65 (76.47%) were found resistant. Against Ciprofloxacin, 31 (36.47%) bacterial strains showed sensitivity, 40 showed intermediate sensitivity while 14 (16.47%) were found resistant; against Chloramphenicol, 6 (7.06%) strains were found sensitive, 31 were intermediately sensitive and 48 (56.47%) were found resistant; and against Streptomycin, 3 (3.52%) out of 85 bacterial strains showed sensitivity, 11 showed intermediate sensitivity while 71 (83.52%) were found resistant. Against tetracycline, 72 (84.70%) strains showed sensitivity, 11 showed intermediate sensitivity and 2 (2.35%) showed resistance. Against Enrofloxacin, 77 (90.58%) strains were found sensitive, 8 were intermediately sensitive and no resistant strains were noticed. Against Vancomycin, no sensitive strains were observed and all 85 (100%) strains were found resistant.

Against Ceftriaxone, 73 (85.88%) out of 85 strains showed sensitivity, 11 showed intermediate susceptibility while 1 (1.17%) showed resistance. Against Cefotaxime, 69 (81.17%) were found sensitive, 10 intermediately sensitive and 6 (7.06%) were resistant. Against Cotrimoxazole, no sensitive bacterial strains were observed, 2 were intermediately sensitive, while 83 (97.64%) were resistant; and at last, against Gatifloxacin 69 (81.17%) strains out of 85 were sensitive, 11 intermediately sensitive and 5 (5.889%) were resistant. Among tested 20 drugs, out of

85 stains, no strain was found sensitive to Kanamycin, Methicillin and Cotrimoxazole (Fig.4.32).

Antibiotic sensitivity test of 271 isolates of CNS obtained from milk samples of SCM and CM were also conducted against selected 20 antimicrobial discs as mentioned earlier. Their zone of diameter was measured, compared with the provided standard chart and results are expressed in form of S, I and R as mentioned in Table 4.21. Results indicated that CNS bacteria isolated from milk samples were resistant to many antibiotics.

Against Ampicillin, 8 (2.95%) out of 271 bacterial strains showed sensitivity, 15 showed intermediate sensitivity while 248 (91.51%) were found resistant; against Amoxycillin, 5 (1.84%) showed sensitivity, 7 showed intermediate sensitivity and 259 (95.57%) were found resistant; against Ampicillin/Sulbactam, 54 (19.92%) out of 271 bacterial strains were sensitive, 38 were intermediately sensitive while 179 (66.05%) isolates were resistant; against Amikacin, 3 (1.10%) out of 271 bacterial strains showed sensitivity, 18 showed intermediate sensitivity while 250 (92.25%) were found resistant; against Erythromycin, 21 (7.74%) strains were found sensitive, 33 were intermediately sensitive and 217 (80.07%) were found resistant; against Gentamicin, 5 (1.84%) out of 271 bacterial strains showed sensitivity, 20 showed intermediate sensitivity while 244 (90.04%) were found resistant; against Kanamycin no intermediately sensitive bacterial strains were observed, 14 (5.16%) were sensitive, and 257 (94.83%) were resistant. Likewise, against Methicillin, no bacterial strains showed sensitivity, 85 showed intermediate sensitivity and 186 (68.63%) were found resistant; against Norfloxacin, 127 (46.86%) out of 271 bacterial strains showed sensitivity, no intermediate sensitive and 134 (49.44%) were found resistant. Against Penicillin-G, only 6 (2.21%) strains were found sensitive and 265 (97.79%) were resistant. Against Ciprofloxacin, 152 (56.08%) bacterial strains showed sensitivity, 11 showed intermediate sensitivity, and 108 (39.85%) were found resistant; against Chloramphenicol, 97 (35.79%) strains were found sensitive, 22 were intermediately sensitive and 152 (56.08%) were found resistant; against Streptomycin, 5 (1.84%) out of 271 bacterial strains showed sensitivity, no intermediate sensitive strain and 266 (98.15%) were found resistant. Against tetracycline, 124 (45.75%) strains showed sensitivity and 147 (54.24%) showed resistance. Against Enrofloxacin, 230 (84.87%) strains were found sensitive, 12 were intermediately sensitive and 29 (10.70%)

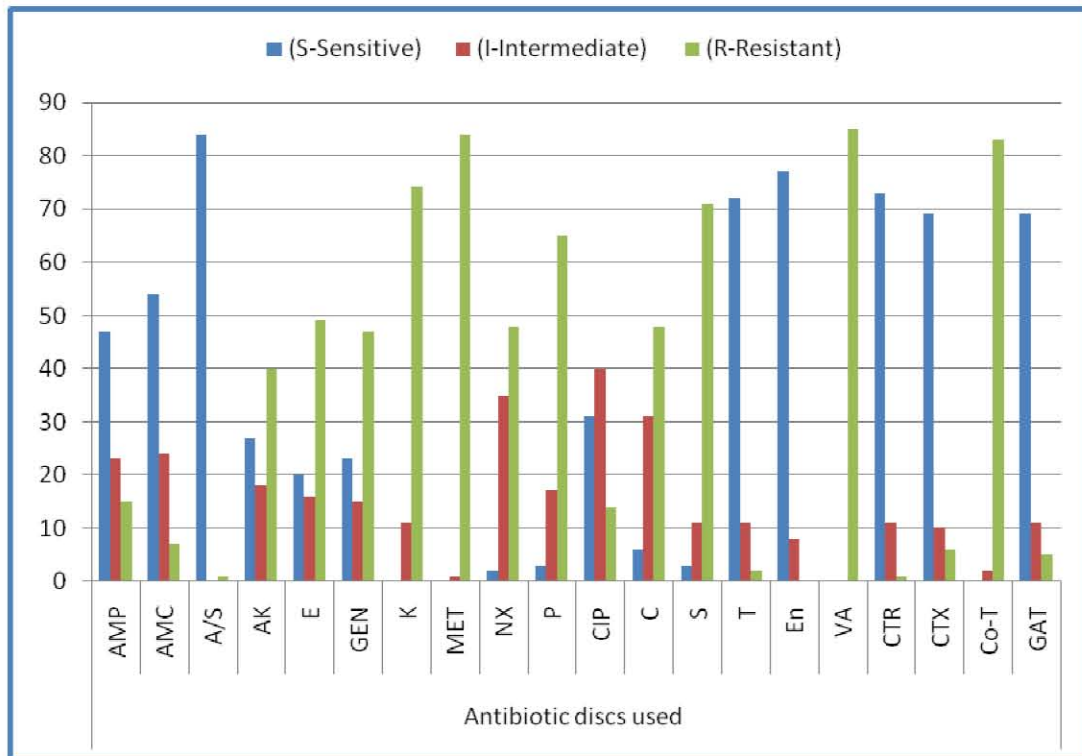


Fig. 4.32: Antibiogram of various isolates of *Staphylococcus aureus*

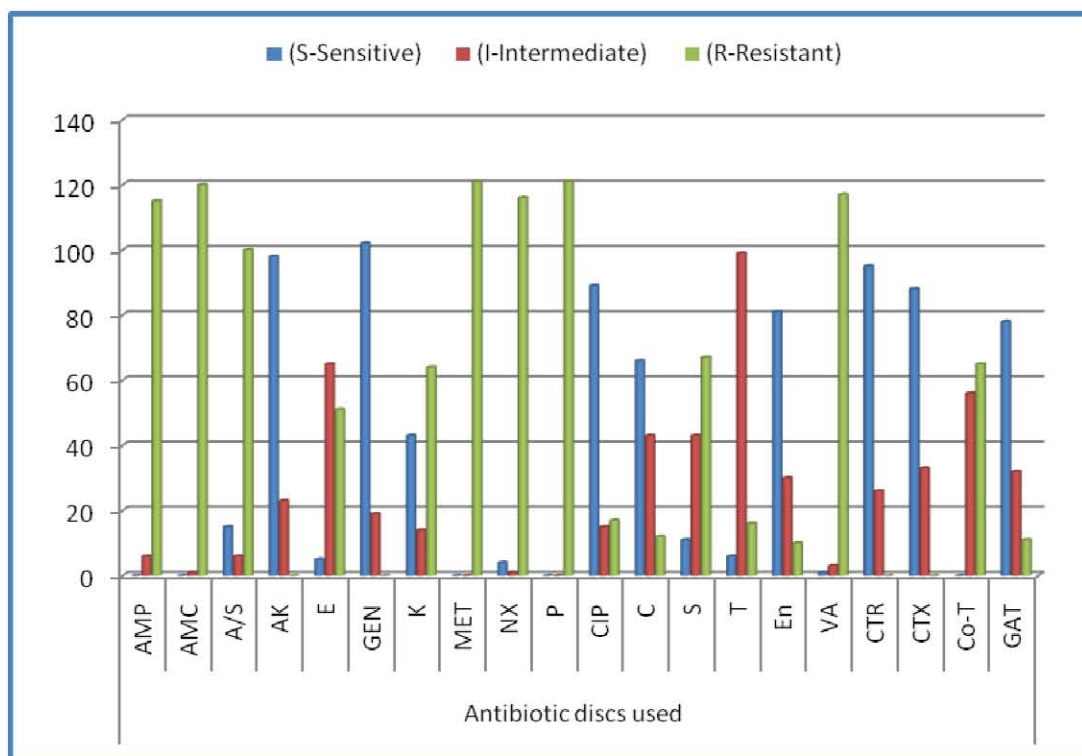


Fig. 4.33: Antibiogram of various isolates of *Escherichia coli*

Table 4.21 Results of antibiotic sensitivity testing of various isolates of coagulase negative Staphylococci (CNS)

| Name of microorganism | Sensitivity pattern | Antibiotic discs used | | | | | | | | | | | | | | | | | | | |
|-----------------------|---------------------|-----------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|-----|
| | | AMP | AMC | A/S | AK | E | GEN | K | MET | NX | P | CIP | C | S | T | En | VA | CTR | CTX | Co-T | GAT |
| CNS (N=271) | S | 8 | 5 | 54 | 3 | 21 | 5 | 14 | 0 | 127 | 6 | 152 | 97 | 5 | 124 | 230 | 13 | 44 | 49 | 0 | 140 |
| | I | 15 | 7 | 38 | 18 | 33 | 20 | 0 | 85 | 10 | 0 | 11 | 22 | 0 | 0 | 12 | 19 | 8 | 5 | 11 | 34 |
| | R | 248 | 259 | 179 | 250 | 217 | 244 | 257 | 186 | 134 | 265 | 108 | 152 | 266 | 147 | 29 | 239 | 219 | 217 | 260 | 97 |

N= Total number of isolates tested; S: Sensitive; I: Intermediate; R: Resistance

AMP – Ampicillin, AMC - Amoxycillin, A/S - Ampicillin/Sulbactam, AK - Amikacin, E - Erythromycin, Gen - Gentamicin, K - Kanamycin, MET - Methicillin, Nx - Norfloxacin, P - Penicillin-G, CIP - Ciprofloxacin, C - Chloramphenicol, S - Streptomycin, T - Tetracycline, En - Enrofloxacin, Va - Vancomycin, CTR - Ceftriaxone, CTX - Cefotaxime, Co-T - Cotrimoxazole, GAT - Gatifloxacin

resistant strains were noticed. Against Vancomycin, only 13 (4.79%) strains showed sensitivity, 19 were intermediately sensitive and 239 (88.19%) strains were found resistant.

Against Ceftriaxone, 44 (16.23%) out of 271 strains showed sensitivity, 8 showed intermediate susceptibility while 219 (80.81%) showed resistance. Against Cefotaxime, 49 (18.08%) were found sensitive, 5 intermediately sensitive and 217 (80.07%) were resistant. Against Cotrimoxazole, no sensitive bacterial strains were observed, 11 were intermediately sensitive, while 260 (95.94%) were resistant; and against Gatifloxacin, 140 (51.66%) strains out of 271 were sensitive, 34 intermediately sensitive and 97 (35.79%) were resistant. Among tested 20 drugs, out of 271, no strains were found clearly sensitive against Methicillin and Cotrimoxazole.

Alike *Staphylococcus aureus* and CNS, all 121 isolates of *E. coli* obtained from milk samples of SCM and CM of cows and buffaloes were subjected to antibiotic sensitivity testing against selected 20 antimicrobial discs as mentioned earlier. Their zone of diameter was measured using Zone-reader scale, compared with the provided standard chart and results are expressed in form of S (Number of bacterial isolates found sensitive to particular drug), I (Number of bacterial isolates found intermediately sensitive to particular drug) and R (Number of bacterial isolates found resistant to particular drug) as mentioned in Table 4.22. Results indicated that many bacteria isolated from milk were resistant to various antibiotics. Against Ampicillin, out of 121, no bacterial strains were found sensitive, 6 were intermediately sensitive and 115(95.04%) were found resistant. Similarly against Amoxicillin, no bacterial strains showed sensitivity, one showed intermediate sensitivity and 120 (99.17%) were found resistant. Against Ampicillin/Sulbactam, 15 (12.39%) out of 121 bacterial strains were sensitive, 6 showed intermediate sensitivity while 100 (82.64%) isolates remain resistant. Against Amikacin, 98 (80.99%) out of 121 bacterial strains showed sensitivity, 23 showed intermediate sensitivity while no strains were found resistant. Against Erythromycin, 5 (1.84%) strains were found sensitive, 65 were intermediately sensitive and 51 (42.14%) were found resistant. Against Gentamicin, 102 (84.29%) out of 121 bacterial strains showed sensitivity, 19 showed intermediate sensitivity while no strains were found resistant.

Against Kanamycin, 43 (35.53%) sensitive bacterial strains were observed, 14 were intermediately sensitive, while 64 (52.89%) were resistant; against Methicillin

not even single bacterial strain showed sensitivity or intermediate sensitivity and all 121 (100%) were found resistant. Against Norfloxacin, 4 (3.30%) out of 121 bacterial strains showed sensitivity, one showed intermediate sensitivity while 116 (95.86%) were found resistant. Against, Penicillin-G also, like Methicillin no strains were found sensitive or intermediately sensitive and all 121 (100%) were found resistant. Against Ciprofloxacin sensitivity tests, 89 (73.55%) bacterial strains showed sensitivity, 15 showed intermediate sensitivity while 17 (14.04%) were found resistant. Against Chloramphenicol, 66 (54.54%) strains were found sensitive, 43 were intermediately sensitive and 12 (9.91%) were found resistant. Against Streptomycin, 11 (9.09%) out of 121 bacterial strains showed sensitivity, 43 showed intermediate sensitivity while 67 (55.37%) were found resistant. Against tetracycline, 6 (4.95%) strains showed sensitivity, 99 (81.81%) showed intermediate sensitivity and 16 (13.22%) showed resistance. Against Enrofloxacin, 81 (66.94%) strains were found sensitive, 30 were intermediately sensitive and 10 (8.26%) resistant strains were noticed. Against Vancomycin, only one strain (0.83%) showed sensitivity, three were intermediately sensitive while rest 117 (96.69%) strains were found resistant. Against Ceftriaxone, 95 (78.51%) out of 121 strains showed sensitivity, 26 showed intermediate susceptibility while no strains showed resistance. Against Cefotaxime, 88 (72.72%) were found sensitive, 33 intermediately sensitive and no strains showed resistance. Against Cotrimoxazole, no sensitive bacterial strains were observed, 56 were intermediately sensitive, while 65 (53.71%) were resistant; and at the last, against Gatifloxacin, 78 (64.46%) strains out of 121 were sensitive, 32 intermediately sensitive and 11 (9.09%) were resistant. Among tested 20 drugs, out of 121, no strains were found clearly sensitive against Ampicillin, Amoxycillin, Penicillin, Methicillin and Cotrimoxazole (Fig. 4.33). All 121 bacterial strains showed complete resistance to Ampicillin, Amoxycillin, Penicillin, Methicillin and Cotrimoxazole.

In the current study also antibiotic resistant strains of *S. aureus* (resistant to Kanamycin, Methicillin, Vancomycin and Cotrimoxazole) and *E. coli* (resistant to Ampicillin, Amoxycillin, Penicillin, Methicillin and Cotrimoxazole) were recovered.

4.3.2 Preparation and titration of phage lysate

After characterization of the recovered phage isolates, these phages were subjected for titration to obtain high titer phage lysate preparation, to ensure maximum number of phages in lysate to trigger lytic activity even if used in single or

Table 4.22 Results of antibiotic sensitivity testing of various isolates of *Escherichia coli*

| Name of microorganism | Sensitivity pattern | Antibiotic discs used | | | | | | | | | | | | | | | | | | | |
|-----------------------------|---------------------|-----------------------|-----|-----|----|----|-----|----|-----|-----|-----|-----|----|----|----|----|-----|-----|-----|------|-----|
| | | AMP | AMC | A/S | AK | E | GEN | K | MET | NX | P | CIP | C | S | T | En | VA | CTR | CTX | Co-T | GAT |
| <i>E. coli</i> (N = 121) | S | 0 | 0 | 15 | 98 | 5 | 102 | 43 | 0 | 4 | 0 | 89 | 66 | 11 | 6 | 81 | 1 | 95 | 88 | 0 | 78 |
| | I | 6 | 1 | 6 | 23 | 65 | 19 | 14 | 0 | 1 | 0 | 15 | 43 | 43 | 99 | 30 | 3 | 26 | 33 | 56 | 32 |
| | R | 115 | 120 | 100 | 0 | 51 | 0 | 64 | 121 | 116 | 121 | 17 | 12 | 67 | 16 | 10 | 117 | 0 | 0 | 65 | 11 |

N= Total number of isolates tested; S: Sensitive; I: Intermediate; R: Resistance

AMP – Ampicillin, AMC - Amoxycillin, A/S - Ampicillin/Sulbactam, AK - Amikacin, E - Erythromycin, Gen - Gentamicin, K - Kanamycin, MET - Methicillin, Nx - Norfloxacin, P - Penicillin-G, CIP - Ciprofloxacin, C - Chloramphenicol, S - Streptomycin, T - Tetracycline, En - Enrofloxacin, Va - Vancomycin, CTR - Ceftriaxone, CTX - Cefotaxime, Co-T - Cotrimoxazole, GAT - Gatifloxacin

small dose (10 µl) for assessment of *in vitro* phage lytic activity and for phage therapy.

Lysate was prepared and titrated to know the concentration of phages in lysate. Titration was done by preparing ten-fold serial dilutions of the phage lysate (in order to have countable plaques number). For titration, 10^{-1} to 10^{-9} dilutions were made in NSS and each dilution was subjected to plaque formation by DAL method. For this, 100 µl of each dilution from 10^{-1} to 10^{-9} was mixed with 300 µl of overnight incubated pure broth culture of *S. aureus* and *E. coli* bacteria in separate sterile tubes. This suspension was mixed gently and kept at 37°C for 10-15 min. To each tube, about 2.5 ml of molten soft top agar was added at 45°C, mixed and poured over the plate of NA agar. Plates were incubated at 37°C for 24 h and number of plaques were counted next day and mean titer of one morphological type of phage isolates was calculated in units of pfu/ml. Mean phage titer was determined in terms of plaque forming units (pfu/ml) with the help of formula given below:

$$\text{pfu /ml} = \text{No. of plaques} \times \text{Dilution factor}$$

The mean phage titer in the prepared lysate for all 6 types of phage isolates were shown in Table 4.23. All 15 phage isolates of SaD₁ type showed mean titer of 3.6×10^9 pfu/ml, four phage isolates of SaD₂ type showed mean titer of 5.2×10^8 pfu/ml, two phage isolates of SaD₃ type showed mean titer of 2.4×10^9 pfu/ml.

Likewise, mean phage titer of EcD type phages was also calculated. All 24 phage isolates of EcD₁ type showed mean titer of 5.1×10^{11} pfu/ml, four phage isolates of EcD₂ type illustrated mean titer of 3×10^{12} pfu/ml and two phage isolates of EcD₃ type showed mean titer of 4×10^{10} pfu/ml. Results depicted that phage EcD₂ type has highest pfu/ml, followed by EcD₁ and EcD₃ type. Among SaD phage types, SaD₁ type has maximum pfu/ml, followed by SaD₃ type and SaD₂ type.

4.3.3 *In vitro* antibacterial activity of phages:

4.4.2 Effect of phage on bacterial isolates obtained from SCM and CM:

Bacterial strains isolated from SCM and CM cases of cows and buffaloes were tested for phage sensitivity either for plaques formation or reduction in number of bacterial colony. Phage isolates tested against respective host genera showed variation in plaque number and pattern of lysis. Number of plaques formed (15, 15-100 and more than 100) along with pattern of lysis (light or confluent lysis) over bacterial

lawn were considered as parameters to compare the efficacy of *in vitro* lytic activity of various phage strains recovered from the animal wastewater samples.

Phage isolates (SaD₁, SaD₂, SaD₃, EcD₁, EcD₂, and EcD₃) were tested against bacterial isolates of *S. aureus* (85) and *E. coli* (121) to assess the *in vitro* lytic activity as a means of phage sensitivity testing and results are presented in Tables 4.24 & Table 4.25 in terms of % lytic efficacy.

Phage therapy can be very effective in certain conditions and has some unique advantages over antibiotics. With the increasing incidences of antibiotic-resistant bacteria and a deficit in the development of new classes of antibiotics to counteract them, there is a need to investigate the use of phage in a range of infections. Clearly the time has come to look more carefully at the potential of phage therapy, both by strongly supporting new research and by scrutinizing the research already available.

Table 4.23: Mean titer (pfu/ml) of phage lysate

| S. No. | Phages used for titration | Mean titer of phage (pfu/ml) |
|---------------|----------------------------------|-------------------------------------|
| 1. | SaD₁ | 3.6×10^9 |
| 2. | SaD₂ | 5.2×10^8 |
| 3. | SaD₃ | 2.4×10^9 |
| 4. | EcD₁ | 5.1×10^{11} |
| 5. | EcD₂ | 3×10^{12} |
| 6. | EcD₃ | 4×10^{10} |

Table 4.24: *In vitro* lytic efficacy of the *Staphylococcus aureus* phages (SaD) against different isolates of *Staphylococcus aureus*

| S. No. | Phage isolates | Lytic efficacy against bacterial isolates (%) |
|---------------|------------------------|--|
| 1. | SaD₁ | 69/85 (81.17%) |
| 2. | SaD₂ | 64/85 (75.29%) |
| 3. | SaD₃ | 66/85 (78.64%) |

Table 4.25: *In vitro* lytic efficacy of the *E. coli* phages (EcD) against different isolates of *E. coli*

| S. No. | Phage isolates | Lytic efficacy against bacterial isolates (%) |
|---------------|------------------------|--|
| 1. | EcD₁ | 114/121 (94.21) |
| 2. | EcD₂ | 121/121 (100) |
| 3. | EcD₃ | 109/121 (90.08) |

A decorative border composed of black and grey floral and butterfly motifs. The border features intricate scrollwork, leaves, and three butterflies with detailed wing patterns, arranged in a rectangular frame around the central text.

Discussion

Agricultural livestock is the widely contributing sector to India's gross capital income. The cow is one of the remarkable domestic animals in the world, adapted to live in arid and semi-arid conditions. In India, cow is inherently associated with the culture, religion and social life of the most of the communities. Cow is used for production of milk, meat, as well as for transportation of both people and goods. However, under unhygienic conditions, the opportunistic micro organisms invade the udder through the teat canal and colonize around the teat duct and epithelial cells causing massive inflammation of udder resulting in cattle mastitis.

Mastitis causes economic and production losses by deteriorating milk quality, reducing production performance, carrying risk of transmission of communicable diseases, increased culling rate, cost of treatment, and sometimes mortality associated with per acute form of the disease (Batavani et al., 2007; Bardhan, 2013; Jeykumar et al., 2013; Sinha et al., 2014; Romero et al., 2018). Several groups of microbial organisms are accredited with the ability to produce both clinical and subclinical forms of the disease. Subclinical form of mastitis is more dangerous as compared to the clinical mastitis due to its ability to deteriorate milk quality to such a level that it cannot be detected grossly but will affect the overall quality. From early 19's a lot of studies have been conducted to draw the attention of animal owners and veterinarians in understanding the mechanism behind the occurrence of mastitis and responsible causative agents, so that proper preventive measures and therapeutic applications can be followed (Saini et al., 1994; Samanta and Prasad, 1998).

Current study aids significantly to the literature in describing the prevalence of sub-clinical mastitis and clinical mastitis in cows and buffaloes maintained at organized farms and goshalas, and from cases of ambulatory visits and clinical camps. Along with reporting prevalence, major associated bacteria (*S. aureus* and *E. coli*) were investigated, their antibiogram was studied and in lieu of the developing antimicrobial resistance, as an alternative therapeutic solution bacteriophages were isolated, characterized and tested for in vitro lytic activity against *S. aureus* and *E. coli*.

Several well established and economic conventional diagnostic techniques are available for the diagnosis of mastitis (Boerlin et al., 2003; Taponen et al., 2007; Hussain et al., 2012; Sanotharan et al., 2016). Current study utilized direct and indirect quick diagnostic tests like California mastitis test (CMT) and Somatic cell count (SCC) to ascertain the prevalence of SCM and CM. Our screening method coincides with earlier approaches employed by other workers for screening of lactating cows and buffaloes for subclinical mastitis and clinical mastitis by performing CMT and SCC (Hegde et al., 2013; Patil et al., 2015; Fahim et al., 2019). In current study, out of 601 cows, 198 were found positive for SCM by CMT. The prevalence of SCM in lactating cows was found to be 32.94% by CMT. Among 12 cows screened from ambulatory visits and clinical camps, 5 (41.67%) were positive for SCM; out of 40 cows from TVCC, DUVASU, Mathura, 15 (37.50%) were found positive; out of 459 cows screened from various Goshalas, 166 (36.16%) were found positive for SCM, while out of 90 cows screened from ILFC, DUVASU, Mathura, 12 (13.33%) were positive. Our results expressing prevalence of SCM of 32.94% by CMT in lactating cows were very similar to the results depicted in previous study conducted earlier in dairy herds at South Ethiopia, where out of 529 lactating cows, 172 (32.51%) were positive for SCM by CMT (Abebe et al., 2016). The results of current study were also in conformity to the results of study performed at Northern Ethiopia over 305 cows of dairy farms, where prevalence of subclinical mastitis was 33.8 %, very close to our findings.

However, our reported prevalence of 32.94% by CMT was comparatively lower than the results of other studies conducted in different countries. Our prevalence values were lesser than the prevalence of SCM of 43% among 152 lactating cows in Sri Lanka (Sanotharan et al., 2016), the prevalence of SCM of 50.4% in Western Rwanda (Mpatswenumugabo et al., 2017), 51.6% in Tanzania (Mdegela et al., 2009), prevalence of 52.75% from the study conducted on 400 lactating cows by CMT (Saglam et al., 2017), 87.9% in Uganda (Abrahmsen et al., 2012), prevalence of SCM of 73.1% in 395 cows in Kenya (Mbindyo et al., 2020) and 85.3% in Nigeria (Shittu et al., 2012). Difference in the values of prevalence emphasizing low prevalence values in India is a positive sign reflecting on the better animal management practices in Indian conditions broadly when compared to other regions and countries as described above in various studies.

Maximum prevalence of 41.67% was observed in cows from ambulatory visits and clinical camps, which is well understood as in rural areas hygienic conditions are not properly maintained and under such poor hygienic conditions, teat orifices are more prone to acquire infection from the soil, contaminated water or surroundings (Piessens et al., 2011; Iraguha et al., 2015). Animal looks apparently healthy and unless until milk production or quality of milk hampers, animal owners usually do not consider probability of mastitis, hence most of time cases of sub-clinical mastitis remain unnoticed (Burvenich et al., 2003; Petersson-Wolfe et al., 2010)). Minimum prevalence of 13.33% was noticed in cows screened from ILFC, DUVASU, Mathura. This is one of the reasons that animals are kept in organized farm and sound milking practices help in keeping the prevalence of mastitis low. These findings are somewhat similar to the results of Gogoi et al. (2017) who reported prevalence of 19.56% by CMT in North Lakhimpur town in Assam.

Likewise, Out of 64 buffaloes screened for CMT, 15 were found positive for SCM by CMT. The prevalence of SCM in lactating buffaloes was found to be 23.43% by CMT, where, TVCC, DUVASU showed 26.6%; ILFC, DUVASU, Mathura had 20% and ambulatory visits and clinical camps expressed 25% prevalence of SCM. In buffaloes, maximum prevalence of 26.6% was recorded in buffalo screened from clinics, which is accurately justified as these animals had history of abnormal milk production, hence were brought to clinics and upon testing of milk, occurrence of SCM was confirmed. Lowest prevalence of 20 % was seen in buffaloes of ILFC, DUVASU, Mathura as compared to other places as animals were properly maintained in the organized livestock farm following practices to prevent mastitis such as post-milking teat cleaning, hand milking instead of machine-milking, treatment of mastitis during non-lactating period and culling of chronically infected animals.

Besides performing CMT, all the collected composite milk samples from lactating cows and buffalos were subjected to somatic cell count (SCC) for indirect estimation of SCM. As bovine mastitis is characterized by inflammation of the udder and severity of inflammation is influenced by the pathogenic potential of causative agent and resultant host response to the bacteria, hence resident and recruited cells collectively play an important role in direct defense against local infection, which may turn to mastitis. In this regards, extensive neutrophil recruitment from the circulation to the lumen of the teat/udder and shedding of alveolar epithelial cells into

the milk, both as somatic cells are an indicator hallmark of the early immune response towards sub-clinical and subsequently clinical mastitis (Oviedo-Boyso et al., 2007; Carrillo-Casas and Miranda-Morales, 2012). For this obvious reason, the International Dairy Federation recommended that the diagnosis of mastitis should be based on the SCC and microbiological findings (Hegde et al., 2013). The acceptable cut-off values of SCC adopted by different countries vary as in the European Union, Australia and New Zealand penalty limit of SCC is 4.9×10^5 cells/ml while in Canada it is 5×10^5 , in the US 7.5×10^5 and in Sweden 2×10^5 cells/ml. As no standards are universally adopted in India, in our study value of 5×10^5 cells/ml was considered as SCC limit value to differentiate sub-clinical mastitis from clinical mastitis (Hegde et al., 2013). Estimation of somatic cell counts is described as an important indicator parameter in assessing the quality of milk as number of SCC usually remains below 2 lacs and value of 3.1×10^5 somatic cells/ml of milk can be considered as threshold limit to differentiate case of sub-clinical mastitis from normal milk (Harmon, 1994; Kayesh et al., 2014; Gogoi et al., 2017; Jadhav et al., 2018).

The prevalence of SCM in lactating cows was found to be 50.9% by SCC. In Goshalas, prevalence was 56.21% for SCM, followed by 41.67% in animals screened from ambulatory visits and clinical camps, 40% in cows from TVCC, DUVASU, Mathura, and 30% in cows screened from ILFC, DUVASU, Mathura. In lactating buffaloes screened for SCM, prevalence of SCM was 29.68% by SCC. Place-wise prevalence in lactating buffaloes was 33.3% in TVCC, DUVASU; 26.6% in ILFC, DUVASU, Mathura and 25% in ambulatory visits and clinical camps. The prevalence of SCM in lactating cows was found to be 50.9% and maximum (56.21%) was from Goshalas. The higher prevalence of 56.21% in Goshalas was expected and could be because in few of the Goshalas many animals were maintained under intensive management system with large herd size. Poor ventilation in stalls, no bedding material, damp dirty floors owe to presence of cow urine and dung, close contact because of overcrowding of animals were some critical factors responsible for high prevalence of SCM. Our findings are closely in agreement with earlier reports documenting high prevalence of mastitis of 52.4% by Giannechini et al. (2002) and 52.7% by Haltia et al. (2006). Higher somatic cell count of more than 2 lac cells/ml is an indication of either existing bacterial infection or is linked with increased susceptibility of animal towards clinical mastitis (Bradley and Green, 2005; Moroni et

al., 2006; Hussain et al., 2012). Researchers reported that udder injury/infection, multiple physiological and environmental conditions can be responsible for change in somatic cell counts and a significant rise in SCC is evident, therefore samples rendered positive by CMT and SCC must be confirmed by bacterial isolation (Dohoo and Meek, 1982; Harmon, 1994; Honkanen-Buzalski et al., 1994; Kayesh et al., 2014)

In present study prevalence of 30% and 26.6% in lactating cows and buffaloes, respectively for SCM reported from ILFC, DUVASU, Mathura was lowest as compared to other places under study possibly due to the adaptation of modern approaches for animal husbandry systems, proper cleanliness, appropriate nutrition, routine health checkups of animals and practice of milking mastitis animal in the last. Studies suggested that SCC assessment is more valuable in case of cows as compared to buffaloes (Joshi and Gokhale, 2006), however in buffaloes also there is difference in the SCC values of normal and mastitis milk (Dhakal, 2006; Sharif et al., 2007). Variations have been observed in the prevalence of mastitis in the current study and other scientific reports which may be probably due to agro-climatic geographical differences, animal parity, stage of lactation, kind of teat surface-flat or raised, disparity in management and husbandry practices in the area and lack of awareness in the farmers regarding long-term health effects and economic losses due to the mastitis (Burvenich et al., 2003).

The prevalence of CM in the current study was found to be 87.5% and 73.47% in lactating cows and buffaloes, respectively. The present finding is within the range of mastitis prevalence ranging from 19.56% to 87.9% recorded by most of recently published studies. Numerous studies have documented variable prevalence of mastitis; 19.56% (Gogoi et al., 2017), 21.1% (Katsande et al., 2013), 31-38% (Pitkala et al., 2004), 33.8 % in Northern Ethiopia (Haftu et al., 2012), 38.89% in Sudan (Hussein, 2012), 50.4% (Mpatswenumugabo et al., 2017), 52.4% (Giannechini et al., 2002), 52.7% (Haltia et al., 2006), 58.75% (Saglam et al., 2017), 59.2% (Abebe et al., 2016), 73.1% in Kenya (Mbindyo et al., 2020), 87.9% in Uganda (Abrahmsen et al., 2012) with significant economic losses associated with the disease. In our study, prevalence of clinical mastitis was higher than the sub-clinical mastitis. This finding is in contrast to what is reported by other workers who documented that subclinical mastitis was more common in the investigated samples than clinical mastitis (Kahir et al., 2008; Turkyilmaz et al., 2010; Abera et al., 2012; Amer et al., 2018).

Once all the milk samples were adjudged by CMT and SCC, further microbiological investigation and biochemical tests need to be performed for identifying the infectious and causative agent of mastitis for adopting accurate treatment strategy. Therefore, all the milk samples positive for sub-clinical mastitis and clinical mastitis as well as negative samples were subjected to microbiological evaluation for isolation and identification of causative agents. In majority, most of cases of sub-clinical and clinical mastitis are caused by reasonably small group of bacteria, mainly *Staphylococcus aureus*, *Streptococcus uberis*, *Mycoplasma spp*, *Escherichia coli* and coagulase negative staphylococci (Miranda-Morales et al., 2008; Abdel-Rady and Sayed, 2009).

In the current study, from 477 positive animals (among total 770 animals), a total of 85 *S. aureus* (17.81%), 271 (56.81%) coagulase negative staphylococci (CNS) and 121 (25.37%) *E. coli* isolates were recovered from cases of SCM and CM. From buffaloes, a total of 33 *S. aureus* (64.71%), 8 CNS (15.69%) and 10 *E. coli* (19.61%) isolates were obtained, while from cows, a total of 52 *S. aureus* (12.20%), 263 (61.73%) isolates of CNS and 111 *E. coli* (26.05%) isolates were obtained. Our results are similar to earlier reports confirming presence of *S. aureus*, coagulase negative staphylococci and *E. coli* as causative agents of clinical and subclinical mastitis (Burvenich et al., 2003; Haltia et al., 2006; Hegde et al., 2013; Kayesh et al., 2014; Sanotharan et al., 2016; Saglam et al., 2017; Bolte et al., 2020). In the present study, CNS and *E. coli* both were isolated in higher numbers as compared to *S. aureus*, this finding coincides with other reports, which demonstrated that majority of mastitis cases were associated with environmental and minor bacteria than contagious pathogens (Kivaria and Noordhuizen 2007; Carrillo-Casas and Miranda-Morales, 2012; Al-Haddadi et al., 2020).

In the current study, coagulase negative Staphylococci, which were considered minor pathogens in earlier times, were the most frequently isolated bacteria. Moreover, recent trends of publication reflected that CNS strains are emerging as common pathogens associated with mastitis (Adwan et al., 2005; Zeryehun and Abera 2017). This findings of current study depicting more number of CNS bacteria than the *S. aureus* are similar to the earlier reports of available studies performed by various other researchers (Honkanen-Buzalski et al., 1994; Gentilini et al., 2002; Pitkala et al., 2004; Tenhagen et al., 2006; Bradley et al., 2007; Bal et al.,

2010; Nam et al., 2010; Viridis et al., 2010; Kaliwal et al., 2011; Martins et al., 2016; Mpatswenumugabo et al., 2017). In our study, predominant bacteria recovered from cases of subclinical mastitis were coagulase negative Staphylococci and this finding is in conformity with other reports illustrating high prevalence of subclinical mastitis with CNS as major bacteria, while *E. coli* was the chief pathogen associated with clinical mastitis (Kalmus et al., 2011; Zeryehun and Abera, 2017; Mbindyo et al., 2020). Available literature documented that among all types of CNS, *Staphylococcus epidermidis* were the most frequently occurring and markedly more prevailing CNS species in subclinical mastitis than in clinical mastitis (Waller et al., 2011; Al Tayyar et al., 2015). This is perhaps because of the reason that coagulase-negative staphylococci (CNS) get entry to the teat orifice from the skin of animal or milkers as many species of CNS are normal inhabitant of the skin and environment and if animal had once encountered CNS induced mastitis, such CNS bacteria may continue to present throughout the lactation period and cause persistent infection (Taponen et al., 2007; Thorberg et al., 2009; Piessens et al., 2011; Supre et al., 2011). However, our findings are contrary to some studies, which found that among the recovered bacterial isolates *S. aureus* (62.8-68.3%) were more than coagulase-negative staphylococci (7.4-35.6%) and *E. coli* (1.5%) isolates (Giannechini et al., 2002; Boerlin et al., 2003; Adwan et al., 2005; Sanotharan et al., 2016).

Isolation of 121 isolates of coliform bacteria *E. coli* ((25.37%) from milk samples indicated probability of contamination from animal environment, soil, cow urine, dung or water used for teat washing prior to milking (Iraguha et al., 2015; Gogoi et al., 2017; Fahim et al., 2019; Nuesch-Inderbinen et al., 2019; Yu et al., 2019). Our findings showing occurrence of 19.61% of *E. coli* from buffaloes coincides with the results of previous study conducted to screen 97 dairy farms in England and Wales for clinical and subclinical mastitis who reported that 19.8 % of bacterial strains obtained were *E. coli* (Bradley et al., 2007). However, another study showed occurrence of 38.88% of *E. coli*, which is slightly higher than what is reported in the current study (Gogoi et al., 2017).

Our findings revealed that *S. aureus* was the most frequently (64.71%) isolated bacteria from buffaloes and these finding corroborate with previous reports of other studies (Giannechini et al., 2002; Boerlin et al., 2003; Abebe et al., 2016; Rehman et al., 2016; Sanotharan et al., 2016). Our results showing presence of 85

isolates of *S. aureus* in total, are also similar to the findings of Bolte et al. (2020) who similarly isolated 85 isolates of *S. aureus* from Northern German dairy farms in their report. The high prevalence of *S. aureus* in this study could be associated with absence of hand cleaning in between cows and the invariable hand milking practice among the dairy herds. *S. aureus* and other contagious microorganisms are usually found on the udder or teat surface of infected cows and buffaloes and are the primary source of infection between uninfected and infected udder quarters, usually during milking. Although it is observed that milkers wash their hands before milking but this was done only before milking the first cow and not in between milking all the cows. Hence it is evident that the causative organisms could be transmitted easily from infected to uninfected udder quarters or from infected cows/buffaloes to uninfected lactating animal through the milkers hands.

Once mastitis is diagnosed the main challenge for the veterinarian or the producer is to treat the animals in such a way that it will not become an economic burden to the production system. Antibiotics are most popular and widely preferred treatment option. Effortless availability of antibiotics and their user-friendly route of application led to the indiscriminate use of antibiotics in mastitis suspected cases even without proper antibiotic susceptibility testing. Such medication practices followed for long time in past many decades have induced bacteria to express their defense system including inherent drug resistant genetic mechanism and resulted into emergence and gradual spread of antimicrobial resistant strains of mastitis bacteria. Such AMR strains of bacteria may be transmitted from milkers or environment to the lactating animals or vice-versa during animal handling and/or milking. Furthermore, the bacteria and their genes can also be transmitted during consumption of non-pasteurized milk to humans (Manie et al., 1999). Though it is conceived that success of antibacterial therapy in treating *Staphylococcus aureus* associated bovine mastitis depends not only on the therapeutic regimen but interplay of cow, pathogenic bacteria and environment all three components plays active role in the recovery of animal (Barkema et al., 2006). To assess the effects of various antibiotics, ABST was performed against all bacterial isolates of *S. aureus*, Coagulase negative Staphylococci and *E. coli*.

In the current study, all recovered isolates of *S. aureus* (85), Coagulase negative Staphylococci (271) and *E. coli* (121) were tested against selected 20

antimicrobial discs namely, ampicillin (AMP), amoxycillin (AMC), ampicillin/sulbactam (A/S), amikacin (AK), erythromycin (E), gentamicin (GEN), kanamycin (K), methicillin (MET), norfloxacin (NX), penicillin-G (P), ciprofloxacin (CIP), chloramphenicol (C), streptomycin (S), tetracycline (T), enrofloxacin (En), vancomycin (VA), ceftriaxone (CTR), cefotaxime (CTX), co-trimoxazole (CoT) and gatifloxacin (GAT). The results are recorded in the form of susceptible (S), intermediate (I) and resistant (R) bacterial isolates to particular drug as per the guidelines of new ISO 20776-1 standard, which is followed world-wide to express result of ABST (Rodloff et al., 2008). Microorganisms are called susceptible to a given antibiotic when it is inhibited *in vitro* by a concentration of particular drug associated with a high probability of therapeutic success. On the other hand, susceptibility of a bacterial strain to a given antibiotic is called to be intermediate when it is inhibited *in vitro* by a concentration of this drug but with an uncertain therapeutic effect, that means they should not be recommended for therapeutic use; and finally bacterial strains are considered to be resistant to a given antibiotic when they are inhibited *in vitro* by a concentration of this drug that is associated with a high likelihood of therapeutic failure, means such drugs are not effective, hence should not be used. Outcome of ABST indicated that bacteria isolated from milk samples showed variations in antibiotic susceptibility profiles as many isolates were susceptible to few drugs while some were resistant to various antibiotics, and similar type of antibiotic spectrum was reported by others and attributed to differences in the use of antimicrobials (Kalinska et al., 2017). The results were discussed with the available literature to compare the pattern of AMR among bacterial isolates (Tenhagen et al., 2006; Sumathi et al., 2008; Waller et al., 2011; Alekish et al., 2013; Beyene et al., 2017; Bhat et al., 2017; Al-Haddadi et al., 2020).

Against ampicillin, 55.29% bacterial strains of *S. aureus* showed susceptibility, while 17.64% were resistant; against amoxycillin 63.53% showed susceptibility and 7 8.23% were resistant. Against ampicillin/sulbactam, 98.82% bacterial strains were susceptible while 1.17% isolate remain resistant; against amikacin, 31.76% bacterial strains showed susceptibility, while 47.06% were resistant. Against erythromycin, 23.53% strains were found susceptible and 57.64% were resistant; against gentamicin 27.06% bacterial strains showed susceptibility while 55.29% were resistant; against kanamycin no susceptible bacterial strains were

observed, 87.06% were resistant. Likewise, against methicillin no bacterial strain showed susceptibility, 98.82% were resistant; against norfloxacin 2.35% bacterial strains showed susceptibility while 56.47% were resistant. Against penicillin-G, only 3.52% strains were found susceptible and 76.47% were resistant. Against ciprofloxacin, 36.47% bacterial strains showed susceptibility while 16.47% were resistant; against chloramphenicol 7.06% strains were susceptible and 56.47% were resistant; and against Streptomycin, 3.52% bacterial strains showed susceptibility while 83.52% were resistant. Against tetracycline, 84.70% strains showed susceptibility and 2.35% showed resistance. Against enrofloxacin, 90.58% strains were found susceptible and no resistant strains were noticed. Against vancomycin, no susceptible strains were observed and cent-percent strains were found resistant. Against ceftriaxone, 85.88% strains showed susceptibility, while 1.17% showed resistance. Against cefotaxime, 81.17% were found susceptible and 7.06% were resistant. Against co-trimoxazole, no susceptible bacterial strains were observed, while 97.64% were resistant; and at last, against gatifloxacin 81.17% strains were susceptible, and 5 (5.88%) were resistant. In current study, all 85 strains of *S. aureus* showed cent-percent resistance to 4 drugs i.e. kanamycin, methicillin, vancomycin and co-trimoxazole as not even single strain was found susceptible. More than 50% *S. aureus* strains were susceptible to 8 drugs namely ampicillin, amoxicillin, ampicillin/sulbactam, tetracycline, enrofloxacin, ceftriaxone, cefotaxime and gatifloxacin; more than 75% were susceptible to 6 drugs that is; to ampicillin/sulbactam, tetracycline, ceftriaxone, cefotaxime and gatifloxacin; and more than 90% to only 2 antibiotics i.e. ampicillin/sulbactam and enrofloxacin. Isolates were resistant to many antibiotics as evident from the results. More than 50% resistance was shown to 10 antibiotics namely; erythromycin, gentamicin, kanamycin, methicillin, norfloxacin, penicillin-G, chloramphenicol, streptomycin, vancomycin and co-trimoxazole. No antibiotic showed 100% susceptibility but 4 antibiotics were 100% resistant. Such escalating percentage of drug resistant strains of bacterial pathogens is an alarming call to the veterinarians, clinicians and researchers to be prepared with the solutions for treating bacterial infections and diseases. Comparative discussion on trend of susceptibility and/or resistance to previous studies is discussed after detailing the ABST results of CNS.

Results of ABST against CNS bacteria showed that against ampicillin, 2.95% bacterial strains showed susceptibility, while 91.51% were found resistant; against amoxicillin, 1.84% showed susceptibility, and 95.57% were found resistant; against ampicillin/sulbactam, 19.92% bacterial strains were susceptible, while 66.05% isolates were resistant; against amikacin, 1.10% bacterial strains showed susceptibility, while 92.25% were found resistant; against erythromycin, 7.74% strains were found susceptible, and 80.07% were found resistant; against gentamicin, 1.84% bacterial strains showed susceptibility, while 90.04% were found resistant; against kanamycin 5.16% were susceptible, and 94.83% were resistant. Against methicillin, no bacterial strains showed susceptibility, and 68.63% were found resistant; against norfloxacin, 46.86% bacterial strains showed susceptibility, and 49.44% were found resistant. Against penicillin-G, only 2.21% strains were found susceptible and 97.79% were resistant. Against ciprofloxacin, 56.08% bacterial strains showed susceptibility, and 39.85% were found resistant; against chloramphenicol, 35.79% strains were found susceptible, and 56.08% were found resistant; against streptomycin, 1.85% bacterial strains showed susceptibility, no intermediate susceptible strain and 98.15% were found resistant. Against tetracycline, 45.75% strains showed susceptibility and 54.24% showed resistance. Against enrofloxacin, 84.87% strains were found susceptible, and 10.70% resistant strains were noticed. Against vancomycin, only 4.79% strains showed susceptibility, and 88.19% strains were found resistant. Against ceftriaxone, 16.23% strains showed susceptibility, while 80.81% showed resistance. Against cefotaxime, 18.08% were found susceptible, and 80.07% were resistant. Against co-trimoxazole, no susceptible bacterial strains were observed, while 95.94% were resistant; and against gatifloxacin, 51.66% strains were susceptible, and 35.79% were resistant.

Coagulase negative Staphylococci (CNS), were generally considered as non-pathogenic bacteria due to their insignificant or little impact on udder health being part of normal microflora as per earlier studies, but recent reports suggested that CNS have become one of the leading pathogen isolated from cases of SCM, which may cause considerable herd problems in several countries. In current study, majority of CNS isolates shown more resistance to antimicrobials as compared to *S. aureus*. In current study, among tested 20 antibiotics, CNS showed more than 50% resistance to 16 antibiotics, namely; ampicillin, amoxicillin, ampicillin/sulbactam, amikacin,

erythromycin, gentamicin, kanamycin, methicillin, penicillin-G, chloramphenicol, streptomycin, tetracycline, vancomycin, ceftriaxone, cefotaxime and co-trimoxazole and 100 % resistance was recorded against methicillin and co-trimoxazole. Resistance to antimicrobial agents in bacteria may occur due to a spontaneous mutation or acquired via plasmids, transposons and integrons from other resistant bacteria. Results were compared with previous studies performed in India and other countries and results are variable though most of them indicated rising pattern in emergence of antibiotic resistant strains with the passing years (Martins et al., 2016; Rehman et al., 2016; Al-Haddadi et al., 2020).

Results of current study are similar to the reports of former study featuring the role of enrofloxacin in controlling the severity of the clinical mastitis caused by *Staphylococcus aureus* as all isolated *S. aureus* strains showed susceptibility to enrofloxacin (Attili et al., 2016). Our results are also in agreement that among fluoroquinolones, enrofloxacin were highly susceptible to CNS as we reported 84.87% and such high level of susceptibility of CNS to enrofloxacin (84.5%) has also been reported in previous studies (Bansal et al., 2015). Our findings are more or less consistent (80.07% resistance to erythromycin) with the findings of Alekish et al. (2013) who reported 92% resistance of CNS to macrolides such as erythromycin in northern Jordan.

Our results showed that only 3.52% *S. aureus* strains were susceptible to penicillin-G while 76.47% were found resistant and among CNS, only 2.21% strains were susceptible and 97.79% were resistant. These findings are in agreement to the findings of Bhatt et al. (2011), who documented that *S. aureus* strains showed highest resistance to penicillin. However, our results are in contrast to end results of studies of *in vitro* antimicrobial sensitivity testing done by earlier workers, which revealed that 52.1% *S. aureus* strains showed penicillin resistance while it was 32.0% by coagulase-negative staphylococci (Pitkala et al., 2004). Another study illustrated that coagulase-negative staphylococci showed 27.6% resistance to penicillin, 4.8% to erythromycin, while were sensitive to gentamicin, cephalothin and ampicillin-sulbactam (Gentilini et al., 2002). Our findings of 80.07% resistance of CNS to erythromycin, 90.04% resistance to gentamicin, 68.63% to methicillin and 56.08% to chloramphenicol were very similar to results reported in earlier studies that CNS showed 67.5% resistance to methicillin, 90% to gentamicin, 80% to erythromycin,

60% to tetracycline and 56% to chloramphenicol (Koksal et al., 2009). Other studies conducted few years back illustrated that CNS strains isolated from bovine mastitis showed highest susceptibility of 83.88% to ceftriaxone, and 79.41% to cefotaxime, 76.47% to methicillin, 73.52% to ciprofloxacin, 70.05% to erythromycin, 66.11% to amikacin, 42.94% to gentamycin, 36.76% to amoxicillin, 29.41% to ampicillin and 23.23% to penicillin (Kaliwal et al., 2011). These results are contrary to the findings of current study that reported higher rate of resistance against mentioned antibiotics. Our study is in close accordance to evidence based findings of earlier study, which emphasized that coagulase-negative staphylococci were resistant to many antibiotics such as they showed resistance of 73.2% to erythromycin, 70.2% to ampicillin, 58.3% to penicillin, 53.8% to gentamicin, 52.3% to tetracycline and 51.8% to vancomycin; but comparatively they are most resistant to penicillin group of drugs probably due to their extensive use in prevention and treatment of mastitis (Kenar et al., 2012; Bansal et al., 2015).

In current study, CNS isolates showed 54.24% resistance to tetracycline, which is nearly similar to acclaimed 60% resistance to tetracycline as reported previously by workers (Koksal et al., 2009). Moderate resistance (54.24%) shown by tetracyclines in the present study, is comparable with 52.3% as reported by Kenar et al. (2012), while is in contrast to Mahami et al.(2011), who reported 100% resistance to tetracycline.

Separate study showed that CNS isolates revealed 58% resistance to penicillin G and 48% to ampicillin (Bal et al., 2010), which is lesser than latest findings of our study. Our results are similar to the findings mentioned earlier that isolates of CNS were found highly resistant to ampicillin, amoxicillin, penicillin, ceftriaxone and erythromycin (Al Tayyar et al., 2015).

Result of current study is also in conformity to earlier findings which described that CNS isolates were resistant to most of antimicrobial agents tested namely, ampicillin, gentamicin, kanamycin and penicillin (Gentilini et al., 2002; Sumathi et al., 2008; Mahami et al., 2011; Bansal et al., 2015; Beyene et al., 2017; Martins et al., 2016). This could be logically justified looking into the growing pattern of antibiotic resistance among microbes due to indiscriminate use of antibiotics (Sharma et al., 2007; Ventola, 2015; Argaw, 2016).

Among *E. coli* isolates tested against ampicillin, no bacterial strains were found susceptible, 95.04% were resistant. Similarly against amoxicillin, no bacterial strains showed susceptibility, 99.17% were resistant. Against ampicillin/sulbactam, 12.39% bacterial strains were susceptible, while 82.64% isolates remain resistant. Against amikacin, 80.99% bacterial strains showed susceptibility, while no strains were resistant. Against erythromycin, 1.84% strains were susceptible, 42.14% were resistant. Against gentamicin, 84.29% bacterial strains showed susceptibility, while no strains were resistant. Against kanamycin, susceptible bacterial strains were 35.53%, while 52.89% were resistant; against methicillin not even single bacterial strain showed susceptibility or intermediate susceptibility and all strains were (100%) resistant. Against norfloxacin, 3.30% bacterial strains showed susceptibility, 95.86% were resistant. Against, penicillin-G also, like methicillin no strains were found susceptible or intermediately susceptible and all 121 (100%) *E. coli* strains were resistant. Against ciprofloxacin susceptibility tests, 73.55% bacterial strains showed susceptibility, while 14.04% were resistant. Against chloramphenicol, 54.54% strains were susceptible, and 9.91% were resistant. Against streptomycin, 9.09% bacterial strains showed susceptibility, 55.37% were resistant. Against tetracycline, 4.95% strains showed susceptibility, and 13.22% showed resistance. Against enrofloxacin, 66.94% strains were susceptible, and 8.26% were resistant strains. Against vancomycin, only one strain (0.83%) showed susceptibility, and 96.69% strains were resistant. Against ceftriaxone, 78.51% strains showed susceptibility, while no strains showed resistance. Against cefotaxime, 72.72% were susceptible, and no strains were resistant. Against co-trimoxazole, no susceptible bacterial strains were observed, while 53.71% were resistant; and against gatifloxacin, 64.46% strains were susceptible, and 9.09% were resistant. Condition is more worrisome for treating environmental bacteria *E. coli* induced mastitis as among tested 20 drugs, out of 121 *E. coli* strains, no strains were susceptible to ampicillin, amoxicillin, penicillin, methicillin and co-trimoxazole. All *E. coli* strains showed complete 100% resistance to ampicillin, amoxicillin, penicillin, methicillin and co-trimoxazole.

Result of ABST described in earlier studies showed that *E. coli* isolates were susceptible to most of antibiotics and few strains showed mild resistance to gentamicin (3.7 %), while resistance to ampicillin was 22 % and 14.6 % to tetracycline (Nuesch-Inderbinen et al., 2019). Our result is consistent with the above

mentioned study as we reported resistance of 13.22% to tetracycline, very close to 14.6 % resistance as described by the workers (Nuesch-Inderbinen et al., 2019), however in contrast to mentioned resistance of 22 % to ampicillin, current study described much higher resistant strains comprising 95.04%. Our results are in agreement with some former studies, which elaborated that *E. coli* isolates were resistant to most of the antibiotic drugs tested (Nam et al., 2009; Haftu et al., 2012).

Such comparative results are a clear indication of consistent increase in the number of antimicrobial resistant strains of bacteria with the passage of time. Findings of recent studies further confirmed that such drug resistant and multi-drug resistant (MDR) strains are increasing. Hence it is high time to search, test, validate and opt other alternative anti-bacterial therapeutic modalities.

In the current study, antibiotic resistant strains of *S. aureus* (resistant to kanamycin, methicillin, vancomycin and cotrimoxazole) and *E. coli* (resistant to ampicillin, amoxycillin, penicillin, methicillin and co-trimoxazole) were recovered. Until now, antibiotics have been widely used as the sole therapeutic agent in managing mastitis, but with the emergence of antibiotic resistant microorganisms, which occurred due to the unsupervised use of antibiotics, several other treatment options are being explored (Sandholm et al., 1990). Several therapeutic strategies like antibiotics, vaccines, bacteriocins, herbal therapy, bacteriophage therapy have been evaluated for efficacy in treating mastitis, but no single technique was found to be effective in controlling or treating the disease due to the variable response of etiological agents to the therapeutic techniques.

Nevertheless, considering the vast potential and enormous applications of phage therapy, current study included isolation, characterization and assessment of *in vitro* lytic activity of phages obtained from animal wastewater against clinical isolates of *S. aureus* and *E. coli* and results were truly encouraging to pave the way in search of some noble alternative anti-bacterial agents. Since discovery of phages, various workers have isolated phages from different environmental samples namely cow dung, sewage, wastewater from the collection tanks etc (Rehman et al., 2016). Phage subsists wherever bacteria exist and share common ecology with respective bacterial species (Goyal et al., 1987). A report from Kutter and Sulakvelidze (2005) has estimated that titer of phage was around 10^{30} and 10^{32} in biosphere and hence stated that phages are one of the most abundant entity presence in the environment.

In the present study various farms were visited frequently and wastewater samples were collected from different depths from the surface of the collection tank. Subsequently, these water samples were subjected for isolation of phages using initially *S. aureus* and *E. coli* as host bacteria. Most of the workers stressed upon choice of appropriate host bacterium for optimum recovery of phages. There are reports that support the use of either *S. aureus* and/or *E. coli* (Goodridge et al., 2003; Huff et al., 2003a; Muniesa et al., 2004; Han et al., 2013).

All the samples were subjected for the isolation of bacteriophage following a systematic approach. First, rapid detection of lytic activity of phage in wastewater was assessed by turbidity reduction method (Tiwari et al., 2010), secondly by spot inoculation (SP) method (Goodridge et al., 2003) and finally isolation of phages was done using DAL method as described by Adams (1959).

First step of rapid detection of lytic activity has been advocated by some workers (Parisi and Talbot, 1974). This technique helps to assess the possibility of phages that may be present in sample. Rapid technique is inexpensive and relatively simple. In the present study also the rapid detection by either one or both of the above mentioned methods was found much helpful for further processing of sample by DAL method. This has reduced the bulk of work and also highlighted importance of initial screening for successful isolation of phage.

Result of initial screening by the turbidity reduction method indicated that possibility of recovery of phages was maximum from cattle and buffalo waste water collected from TVCC, DUVASU (100%) Mathura, followed by various Goshalas (90.9%) and ILFC farm (88.8%) as compared to samples of goat waste (33.3%), sheep (0%), and poultry farm waste (50%). Spot inoculation or streak plate method was then performed on all samples and development of plaques on surface of solid media was adjudged as indication of lytic activity. Result of spot inoculation method revealed that maximum recovery was observed from cattle and buffalo wastewater from ILFC Farm (75%), followed by cattle wastes from Goshalas (60.6%), from TVCC DUVASU, Mathura (60%) and only 33.3% from goat samples. Similar observations of maximum phage recovery from cattle wastes were also reported by other workers (Dhillon et al., 1976; Klaus et al., 2003). However, findings of Havelaar et al. (1986) deviate from the current study as they reported greater number of phages in pig feces followed by chicken, sheep and calves. They did not find

appreciable number of phages in cow dung. Abundance of coliphages in pig feces was also reported in other studies (McLaughlin et al., 2006; Jamalludeen et al., 2007). In the present study 81.8% samples showed presence of phages, however maximum recovery was observed from cattle & buffalo wastes.

Comparison of results of Turbidity reduction method with that of streak plate method indicated that number of samples showing lytic activity reduced from 81.8 % to 54.5 % when studied by streak plate method. This is possibly because assessment of plaque formation was difficult due to irregular plaque morphology. As reported by Goyal et al. (1987) large volume of sample is required for phage propagation. Hence, failure of streak plate method to give cent percent recovery at par with that of turbidity reduction method may be attributed by less concentration of phages in the volume of 10 ul used for spot inoculation besides other factors.

Further comparison of SP method with DAL method showed cent percent correlation as all the samples processed by SP method were also positive by DAL method and used finally for obtaining pure culture of specific phage i.e. on *S. aureus* and *E. coli*.

High efficacy of recovery depends on initial concentration of phage in sample. Some workers stressed upon concentrating the sample by enrichment (Goyal et al., 1987; Grabow et al., 1998 and McLaughlin et al., 2006); ultrafiltration as well as density gradient ultracentrifugation (Divizia et al., 1989).

On this basis, it is strongly recommended that high concentration of phages is essential for primary isolation and done either by ultracentrifugation; enrichment of sample or density gradient centrifugation (Kwiatek et al., 2012; Rehman et al., 2016a).

Those samples which failed to induce lysis of bacteria in turbidity reduction method also did not show plaque formation by streak plate method. Every sample that showed lytic activity on the bacterial lawn by spot inoculation method was finally processed for isolation of phages by DAL method (Adams, 1959).

In the present study primary isolation was done for two most common causative agents of bovine mastitis namely *S. aureus* and *E. coli* causing mastitis. Phages have lytic activity against pathogenic *S. aureus* and *E. coli* isolated from mastitis milk. A systematic approach was followed in present study for the recovery

of phages so as to obtain pure phage isolated on the basis of single plaque morphology. Out of 55 samples tested, a total of 51 phage isolates were obtained; including 21 phage strains recovered against *S. aureus* while 30 against *E. coli*. Results revealed that *E. coli* supported the growth of more phages as compared to *S. aureus* as indicated by recovery of 30 *E. coli* host specific (EcD) phage as compared to twenty one *S. aureus* host specific phages (SaD). Our findings on recovery of phages against *S. aureus* support earlier reports of other workers (Ackermann and Nguyen, 1983; Brenner et al., 1999; Han et al., 2013). More recovery of *E. coli* phages may be because of seasonal variation and abundant presence of host bacteria *E. coli* in soil and water as an environmental pathogen (Stirm, 1968; Jothikumar et al., 2000; Goodridge et al., 2003; Begum et al., 2010; Manohar et al., 2018).

For confirmation of presence of phage in plaques, though electron microscopy was not part of the current study, but for morphological characterization of phage, scanning electron microscopy (SEM) was conducted as facility of Transmission electron microscopy (TEM) was not available at that time. Results based on negative staining of EcD₂ showed presence of hexagonal head and long non-contractile tail, which indicated that isolated phage may belong to *Siphoviridae* family. Our results are supported by similar findings reported earlier by other studies (Deghorain and Melderer, 2012; Melo et al., 2014; Litt and Jaroni, 2017; Moller et al., 2019). Further studies including TEM and genomic characterization are desired to elaborate more information.

These phage isolates were further subjected to physical and biological characterization based on plaque morphology, host range determination, chloroform, pH and temperature effect studies. Earlier similar approach was adopted by number of workers for characterization of phages (Anderson et al., 1966; Adeles, 1984; Ackermann et al., 1994; Ackermann, 2003). In light of this the present study is worth to set an appropriate methodology to proceed for isolation of phages from the waste water and also probably from other environmental samples. It is also suggested that more concentration of phages in the sample by enrichment improve recovery of phages.

Various workers have adopted different schemes for characterization of field isolates of bacteriophage (Romig and Brodetsky, 1961; Shimizu et al., 1970). By and large these workers have stressed upon characterization of isolates on the basis of

plaque morphology, host-range and physical parameters such as effect of temperature and pH on the propagation of phages (Lu et al., 2003). Pedroso and Martins (1995), however did not find any relationship between coliphage family and specific plaque morphology.

Plaque morphology is considered as one of the foremost criteria for characterization (Ellis and Winters, 1969). In the present study, phages were categorized by plaque size, consistency of plaques and were assigned the designation. Plaque types ranged in size with small plaque categorized as being smaller than 2 mm, medium plaque being 2 mm, and large plaque being larger than 2 mm. Variation also occurred in the plaque consistency as clear and opaque or diffused plaques.

For each prime host, three different phage isolates were obtained on the basis of plaque morphology. Variation in the plaque morphology indicated the difference in phage strain (Romig and Brodetsky, 1961; Colasito and Rogoff 1969). However, standardization of the soft agar medium, used for preparation of bacterial lawn in the plaque assay, became a variable which interfered with the isolation. It is also opined that the strength of gel, also affected the size of plaque. As the gel strength was lower, the larger the pore size, resulting in a large plaque. The stronger the gel strength, the pore size would be the smaller resulting in a smaller plaque. This indicated that the size of the plaque is not independent of the gel strength.

In the present study, large batches (1000 ml) of soft agar were made at one time, and the entire batch would have uniform gel strength. Hence, the phage isolates differentiated based on plaque morphology were all different from each other. Scientists have also reported that cations have influence on plaque morphology (Romig and Brodetsky, 1961; Havelaar and Hogeboom 1983). In this study $MgCl_2 \cdot 7H_2O$ was used in concentration of 2.4 g/litre as it promoted better adsorption of phage over the bacteria. Findings of current study are in consistent with the reports of Havelaar and Hogeboom (1983). Our findings are in contrast to that of Jothikumar et al. (2000) who did not observed favourable effect of addition of cations. Other workers have also characterized the phages based on plaque morphology (Anderson et al., 1966; Colasito and Rogoff, 1969; Sajjad et al., 2004; Jamalludeen et al., 2007).

After isolation and purification of phages their host range was determined and for this purpose phages were allowed to grow on different bacterial strains and their

ability to infect those bacteria was determined by observing the formation of plaques in case of each new hosts. Phage isolates which were initially isolated by using *S. aureus* (30) and *E. coli* (21) were also tested for lytic activity against other bacteria viz. *S. aureus*, *S. epidermidis*, *Bacillus spp.*, *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. Results indicated that phage isolates either produced plaques or showed reduction in bacterial colony number against other bacterial genera. It was found that most of our isolated phages showed narrow host range due to their ability to specifically infect only one type of bacteria as phage isolate SaD showed lytic activity only against *S. aureus* strains, and not against any of five other genera. These are in contrast to the findings of Synnott et al. (2009), who isolated and characterized *S. aureus* bacteriophages with wide host ranges along with potent lytic activity. Phage isolate EcD₁ had comparative wider hosts range as compared to other phage strains as EcD₁ type phage isolates showed lytic activity against *Pseudomonas aeruginosa* and *Klebsiella spp.* also besides *E. coli* but not against *S. aureus*, *S. epidermidis* and *Bacillus spp.* Our observations are in total conformity to the earlier reports of Bielke et al. (2007) who reported that phage host range is not always genera restricted and phages could be of wide host range. These observations are in partial conformity to earlier researchers who reported that narrow range phages are restricted to maximum of two bacterial species (Ibrahim, 1969; Bhimani and Freitas, 1990; Goodridge et al., 2003; Carey-Smith et al., 2006).

All the phage isolates were investigated for their temperature susceptibility based on ability of phage isolates to form plaque when exposed to temperature of 4°C, 22°C, 37 °C and 70°C for 60 min. Our findings are in partial conformity to those reported by Lu et al. (2003), who reported that phages get inactivated at 70°C and above 70°C. Our reports are also similar to previous literature which documented that 4°C was the minimum temperature at which phages could be stored without a significant reduction in their lytic and replication abilities (Basdew and Laing, 2014). The reduction of burst size at higher temperature is probably result of the effect of higher temperature on the metabolism of the host, because the bacterial growth rate is decreased between 45 and 51°C (Nishihara and Romig, 1964). Interpretation is complicated by the possibility that the metabolic reaction accelerated at the high temperature may continue at an increased rate. These findings of present study are in accordance to other workers (Bronfenbrenner, 1932; Rogers and Sarles. 1963; Phelps,

1967; Charles et al., 1970; Lamontagne and McDonald, 1972; Seeley and Primrose, 1980; Litt and Jaroni, 2017). Our findings are also in agreement to Rehman et al. (2016b), who reported that the lytic activity of bacteriophage isolated from the sewage water of dairy farm, against *staphylococcus aureus* causing mastitis was optimally highest at 37 °C temperature. Our results are also comparable with the findings of Oduor et al. (2020) who suggested that *Staphylococcus* phages were active below temperature of 40°C.

Besides, assessing thermo-tolerance many workers have conducted the pH study in order to characterize the phage (Anderson et al., 1966; Phelps, 1967). Here, *S. aureus* and *E. coli* phage isolates were tested for their viability at different pH i.e. 3, 5, 7, 9 and 11 pH for 60 min. Observations indicated no significant effect due to pH on viability of phage isolates. The isolates appear to be stable to the pH range pH 5 to pH 9 but inactivation is evident at the very low (pH 3) and very high pH levels (pH 11). These findings are in total confirmation with earlier reports of Colasito and Rogoff (1969) who described that phage viability was maximal between pH 5 and 9 and all phages were completely inactivated at pH values of 3 and 11. Similar findings have also been recorded by other workers who studied the effect of pH on viability of phages as important criteria for characterization of phages and suggested that the lytic activity of bacteriophage was highest at pH of 7.0 (Grilione and Carr, 1959; Rogers and Sarles, 1963; Phelps, 1967; Jamalludeen et al., 2007; Rehman et al., 2016b; Litt and Jaroni, 2017; Oduor et al., 2020). Our results are also consistent to the previous findings that isolated *Staphylococcus* phages showed tolerance to pH range of 5.4 to 9.4 but an increase in acidity or alkalinity both had negative impact on the viability of phage as phages were inactivated below the pH of 5.4 and above pH 9.4 (Oduor et al., 2020).

Our findings are contrary to Goyal et al. (1987) who suggested that pH would not have a major effect on phage survivability. Our findings does not support earlier findings that isolates showed optimal lytic ability only at pH 6 to 7 and above or below pH of 6 to 7, their activity would be reduced (Basdew and Laing, 2014) as we observed that isolates were stable to the pH range of 5 to 9 and inactivation was evident at the very low (pH 3) and very high pH (pH 11) levels. It is important to characterize phages based on physic-chemical parameters as stability of phages at

variable conditions is a prime necessity for their consistent and sustainable therapeutic efficacy and application under *in vitro* and *in vivo* both.

Therefore, all six types of phage isolates were characterized and their *in vitro* lytic activities were assessed. Among six types, four phages SaD₁, EcD₁, EcD₂ and EcD₃ remain viable and stable to a wide range of temperatures which indicates that these four types could withstand the hot climatic conditions of India. Similarly, all six types of phages were able to withstand the pH of mastitic milk which varied from 6.9 to 7.4 because of their stability over a wide range of pH (Hussain et al., 2012).

The phages also showed considerable survivability rates at chloroform treatments. Chloroform treatment of a phage suspension is necessary because it removes cells infected with lysogenic phages, eliminating any possibility of transfer of toxic/resistance genes (Carlton, 1999). Results depicted that EcD₁ and EcD₂ maintain 100 % viability and activity after their exposure to chloroform, these findings are similar to work done by others (Oduor et al., 2020). EcD₃ and ScD₁ demonstrated reduction in percent viability by 50% and 66.6%, respectively. Phage isolates SaD₂ and ScD₃ were inactivated and no plaques were produced after chloroform treatment. Our results are comparable to the findings of earlier workers, who had reported variation in the sensitivity of phage isolates to chloroform exposure as we also observed somewhat similar variation (Basdew and Laing, 2014) Our results revealed better % viability by coliphages as compared to staphylococcal phages.

All isolates demonstrated considerable long-term stabilities at 37°C and 4°C. Long-term stabilities are the essential requirement for any phage-preparation to be used therapeutically. The shelf life should be enough for the time needed for quality control, packaging and labelling, distribution to clinical sites and treatment. All the phage isolates qualified on these physio-chemical and stability parameters were tested further for their *in vitro* potential against bacterial isolates obtained from cases of SCM and CM of cows and buffaloes.

After characterization of the recovered phage isolates, these phages were subjected for titration to obtain high titer lysate preparation so as to ensure maximum number of phages in lysate to trigger lytic activity even if used in single or small dose for phage therapy. Lysate was prepared by using the method of Jothikumar et al.

(2000) and titrated to know the concentration of phages in lysate. Many other workers have prepared high titre phage lysate (Lu et al., 2003; Sajjad et al., 2004 and McLaughlin et al., 2006) so that it could be used for therapy.

The use of antibiotics is popular in our daily lives. The indiscriminate and improper use of these remarkable drugs is the most common cause of the emergence of drug resistant bacterial strains. This has created interest among scientists to develop alternatives for antibiotic therapy. Bacteriophage therapy is re-emerging as a possible alternative for the treatment of bacterial infections (Lorch, 1999) as evident from the results of current study.

The bacterial isolates obtained from SCM and CM were subjected to Antibiotic Susceptibility test (ABST) so as to know the bacterial susceptibility for various antibiotics or in other words to know the percentage of antibiotic resistant bacteria against different antibiotics. Now a day, antibiotic resistance is developing world wide and a major cause of delayed recovery and subsequent economic loss. Results indicated that many bacterial isolates were resistant to various antibiotics. Our findings are in conformity to results of previous workers, who had reported about the resistance of bacteria against many antibiotics such as streptomycin, ampicillin, tetracycline and trimethoprim/sulfafurazoles and also highlighted that they were phage susceptible (Smith and Huggins, 1982; Basdew and Laing, 2011; Sarhan et al., 2016).

In the current study antibiotic resistant strains of *E. coli* and *S. aureus* were recovered. These bacteria were tested for phage susceptibility and found susceptible to phage. These findings are in agreement to the observations made by several other workers (Ahmad, 2002; Chilamban and Rawat, 2004; Soothill et al., 2004; Ndlela et al., 2016; Porter et al., 2016).


Phage therapy can be very effective in certain condition and has some unique advantages over antibiotics. With the increasing incidence of antibiotic-resistant bacteria and a deficit in the development of new classes of antibiotics to counteract them, there is urgent need to investigate the use of phage in a range of infections. Clearly the time has come to look more carefully at the potential of phage therapy, both by strongly supporting new research and by scrutinizing the research already available.

Bacterial strains isolated from SCM and CM cases were tested for phage susceptibility (either for plaques or reduction in number of bacterial colony) also besides ABST and bacteria were found phage susceptible *in vitro*. These findings are in support to those reported by other researchers (Slopek et al., 1987; O’Flynn et al., 2004; Sajjad et al., 2004; Brussow, 2005; Titze et al., 2020).

Our findings are similar to many other workers who isolated and characterized anti-staphylococcal phages from farmyard slurry and effluents and also assessed their lytic activity against pathogenic *Staphylococcus aureus* associated with bovine infections (O’Flaherty et al., 2005; Garcia et al., 2009; Synnott et al., 2009; Mishra et al., 2011; Li and Zhang, 2014; Ganaie et al., 2018). Based on efficacy of phages, it is recommended to use such tested phages in form of teat dips or as teat washing solution as means of non-antibiotic prophylactic against staphylococcal bovine mastitis (Shasha et al., 2004; Longping and Zhiying, 2014; Sarhan et al., 2016; Saglam et al., 2017).

The literature revealed use of Staphylococcal phages and coliphages for successful treatment of many bacterial infections including experimental murine mastitis and sub-clinical and clinical bovine mastitis induced by *S. aureus* and *E. coli* and (Kudva et al., 1999; Bull et al., 2002; Huff et al., 2002; Goode et al., 2003; Gill et al., 2006b; Atterburry, 2007; Viscardi et al., 2008; Gupta and Prasad, 2011; Basdew and Laing, 2014; Porter et al., 2016; Manohar et al., 2019; Geng et al., 2020).

The first and mandatory step for developing a universal phage based therapeutic system is to search for the broad host range lytic phages isolated from an epidemiological area. Therefore, we assessed the lytic potential of the SaD and EcD phages against *S. aureus* and *E. coli* bacterial isolates. The results of the present study provided insight for using lytic bacteriophages for therapeutic interventions against multi-drug-resistant *S. aureus* and/or coliform induced infections including mastitis.

A decorative border composed of intricate black and white floral and vine patterns. The border is shaped like a rounded rectangle, with the top and bottom edges being more densely decorated with swirling vines and leaves. Three stylized butterflies are scattered within the border: one in the upper left, one in the lower right, and one in the lower center. The text is centered within this decorative frame.

Summary
and
Conclusions

CHAPTER-6

SUMMARY AND CONCLUSIONS

Mastitis deteriorates milk quality, reduces production performance and increases cost of treatment. Several groups of microbial organisms are responsible for clinical and subclinical forms of the disease. Subclinical form of mastitis is considered more dangerous as compared to the clinical mastitis due to its ability to deteriorate milk quality to such a level that it cannot be detected grossly but will affect the overall quality. The main challenge for the veterinarian is to treat the animals in such a way that it will not become an economic burden to the production system. Until now, antibiotics have been widely used as the sole therapeutic agent in managing mastitis, but with the emergence of antibiotic resistant microorganisms due to the unsupervised use of antibiotics, several other treatment options are being explored. Development of a universal therapeutic agent/technique that can be considered as a replacement of the antibiotic therapy to solve the emerging problem of antibiotic resistance is the need of this century. Therefore, the present study was conducted with the following objectives:

Objectives of the Study

1. Isolation and characterization of the major bacteria (*S. aureus/E. coli*) from milk samples of mastitis (SCM & CM) cases of cattle and buffaloes.
2. Detection, isolation and characterization of the lytic bacteriophages against these bacterial isolates, from samples of animal wastes.
3. Assessment of *in vitro* antibiotic sensitivity pattern and *in vitro* phage sensitivity testing against bacterial isolates.

Results are summarized as below:

1. A total of seven hundred seventy composite milk samples from lactating cows and buffaloes were screened, animal-wise for presence of sub-clinical and clinical mastitis from various Goshalas in and around Mathura, U.P. India.
2. Out of 770 lactating animals, 657 were cows, among which 601 were screened for SCM and 56 for CM and 113 were buffaloes, among them 64 were screened for SCM and 49 for CM.

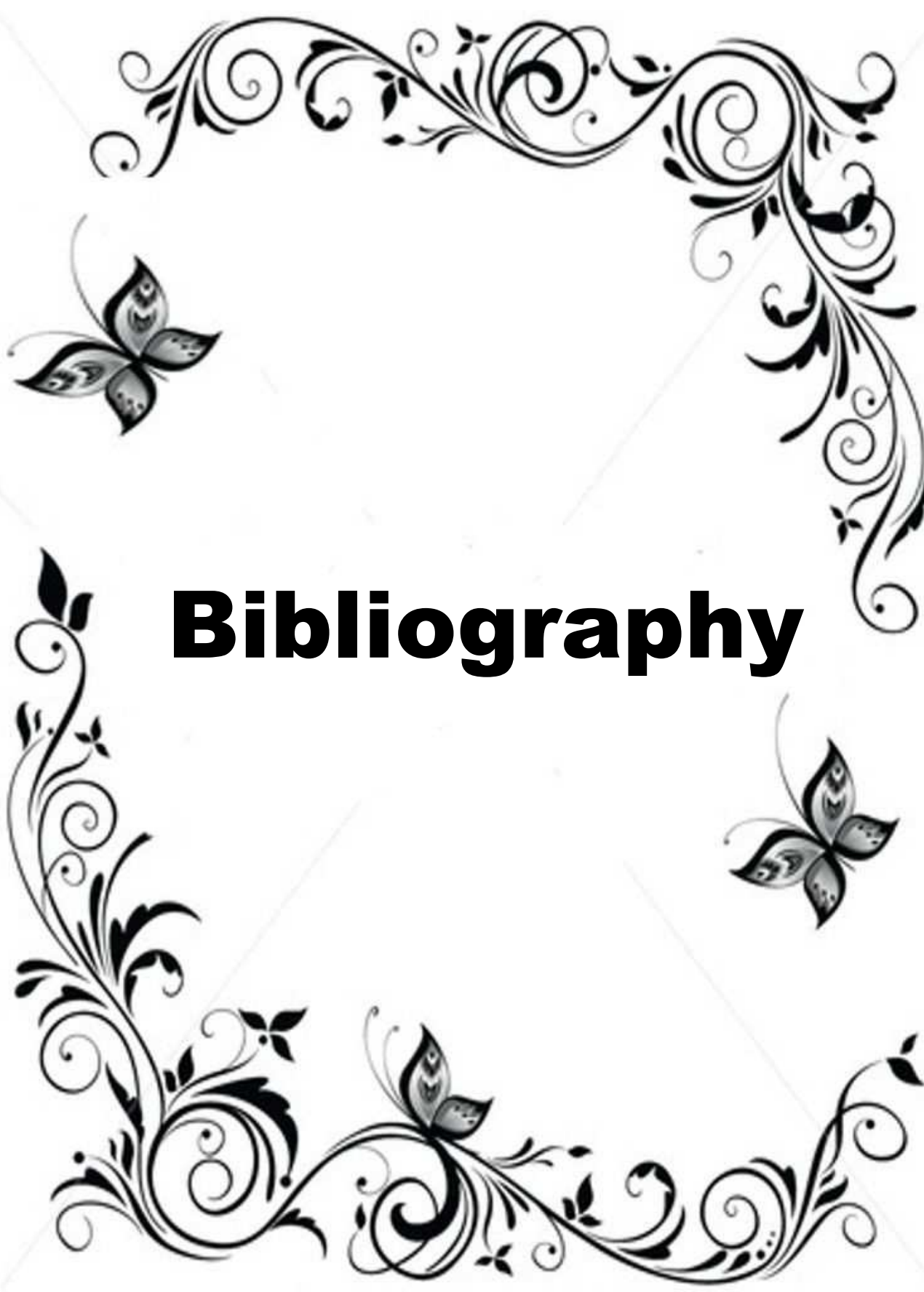
3. The prevalence of SCM in lactating cows was found to be 32.94% by CMT as out of 601 cows, 198 were found positive for SCM by CMT. Prevalence of SCM was 41.67% in cows from ambulatory visits and clinical camps followed by 37.50% from TVCC, DUVASU, Mathura, 36.16% from various Goshalas and 13.33% from ILFC, DUVASU, Mathura.
4. The prevalence of SCM in lactating buffaloes was found to be 23.43% by CMT as out of 64 buffaloes, 15 were found positive for SCM by CMT. The prevalence of 26.6% was recorded from TVCC, DUVASU, 25% from ambulatory visits/clinical camps and 20% from ILFC DUVASU Mathura. A comparative lower prevalence was observed in well managed dairy farm e.g. ILFC DUVASU Mathura.
5. Upon screening the milk samples by somatic cell count (SCC), out of 601 cows, 306 were found positive for SCM with the animal-wise prevalence of 50.9% in lactating cows, where prevalence of 56.21% was reported from various Goshalas, 41.67% from ambulatory visits/clinical camps, 40% from TVCC DUVASU, Mathura and 30% from ILFC DUVASU, Mathura for SCM.
6. Among buffaloes screened for SCM, prevalence of 29.68% was observed by SCC, including 33.3% from TVCC, DUVASU, 26.6% from ILFC, DUVASU, Mathura and 25% from ambulatory visits and clinical camps.
7. The prevalence of CM was found to be 87.5% in lactating cows and 73.47% in lactating buffaloes.
8. Overall, from 770 lactating cows and buffaloes, 477 (61.94%) animals were found positive for bacterial growth and no bacteria could be isolated from 293 (38.06%) animals. In the present study, a total of 85 *S. aureus*, 271 Coagulase negative Staphylococci (CNS) and 121 *E. coli* isolates were recovered.
9. For rapid detection and isolation of bacteriophages from animal wastewater, 55 samples of wastewater, fecal pellet and semi-dry fecal droppings were collected from different Goshalas, TVCC, DUVASU and ILFC, DUVASU, Mathura mainly comprised of animal wastes of cattle, buffaloes, goats, sheep and poultry.

10. Among 55 samples of wastewater, 45 (81.8%) samples were found positive by turbidity reduction method, while 30 (54.5%) samples were found positive by streak plate or spot inoculation method.
11. Results of turbidity reduction method indicated that possibility of recovery of phages was maximum from wastewater samples collected from TVCC, DUVASU (100%), followed by samples of Goshalas (90.9%) and ILFC farm (88.8%) as compared to goat fecal pellet (66.6%) and samples of sheep and poultry were found negative.
12. By spot inoculation method, maximum recovery of phages was observed from cattle and buffalo wastewater from ILFC Farm (75%), followed by cattle wastes from Goshalas (60.6%), and from TVCC (60%), DUVASU, Mathura. Only 33.3% of positive results were obtained from goat faecal samples.
13. Out of 55 samples tested, a total of 51 phage isolates were obtained. Total number of phage strains isolated against *S. aureus* were 21 while 30 in case of *E. coli* and were designated as *S. aureus* host specific phages (SaD) and *E. coli* host specific (EcD) phages, respectively.
14. As per plaque morphology, phages isolated on *S. aureus* (SaD) were designated as; SaD₁ with small-pin headed clear plaque, SaD₂ with small diffused/opaque plaque and SaD₃ with Large clear plaque; and phages isolated against *E. coli* (EcD) were designated as; EcD₁ with medium sized and clear plaque, EcD₂ with Small sized and clear plaque and EcD₃ with Large sized and clear round plaque. The electron microscopy of EcD₂ after negative staining showed presence of hexagonal head and long non-contractile tail, which indicated that isolated phage may belong to *Siphoviridae* family.
15. Thermo-tolerance studies showed that all phage isolates SaD₁, SaD₂, SaD₃, EcD₁, EcD₂ and EcD₃ maintained viability and were stable when exposed to 37°C for 60 min; however phage isolates EcD₁, EcD₂ and EcD₃ remain viable at 70°C for 60 min with a distinct reduction in the % viability (33.33% EcD₁ remain viable and among EcD₂ and EcD₃ 50% remain viable), and SaD₁, SaD₂ and SaD₃ phage isolates were inactivated.
16. When tested for effect of pH, all the isolates SaD₁, SaD₂, SaD₃, EcD₁, EcD₂ and EcD₃ appear to be stable to the pH range of 5 to 9, but inactivation was evident at very low (pH 3) and very high pH (pH 11) levels.

17. Sensitivity of phages to chloroform revealed better % viability by Coliphages as compared to Staphylococcal phages as only EcD₁ and EcD₂ maintained 100 % viability and activity after their exposure to chloroform.
18. Host range studies demonstrated that phage isolate EcD₁ had wide hosts range as compared to other phage strains as it showed lytic activity against *Pseudomonas aeruginosa* and *Klebsiella spp.* also besides *E. coli*, however phage isolates SaD₁, SaD₂ and SaD₃ were narrow range phages as they showed lytic activity only against *S. aureus*.
19. After isolation and characterization of the recovered phage isolates their high titer phage lysate was prepared to ensure maximum number of phages in lysate for assessment of *in vitro* phage lytic activity.
20. When all the bacterial isolates were tested for antibiotic susceptibility profile, isolates were resistant to many antibiotics. More than 50% resistance was shown to 10 antibiotics namely; erythromycin, gentamicin, kanamycin, methicillin, norfloxacin, penicillin-G, chloramphenicol, streptomycin, vancomycin and co-trimoxazole and all 85 strains of *S. aureus* showed even cent-percent resistance to 4 drugs i.e. kanamycin, methicillin, vancomycin and co-trimoxazole. However they showed more than 90% susceptibility to 2 antibiotics i.e. ampicillin/sulbactam and enrofloxacin.
21. Among coagulase negative staphylococci, isolates showed more than 50% resistance to 16 antibiotics, namely; ampicillin, amoxicillin, ampicillin/sulbactam, amikacin, erythromycin, gentamicin, kanamycin, methicillin, penicillin-G, chloramphenicol, streptomycin, tetracycline, vancomycin, ceftriaxone, cefotaxime and co-trimoxazole and 100 % resistance was recorded against methicillin and co-trimoxazole.
22. All *E. coli* strains showed complete 100% resistance to ampicillin, amoxicillin, penicillin, methicillin and co-trimoxazole.
23. In the current study, antibiotic resistant strains of *S. aureus* (resistant to kanamycin, methicillin, vancomycin and cotrimoxazole) and *E. coli* (resistant to ampicillin, amoxicillin, penicillin, methicillin and co-trimoxazole) were recovered.

24. Phage isolates (SaD₁, SaD₂, SaD₃, EcD₁, EcD₂, and EcD₃) were tested against bacterial isolates of *S. aureus* (85) and *E. coli* (121) to assess the *in vitro* lytic activity as a means of phage sensitivity testing and maximum lytic efficacy was shown by EcD₂ (100%), followed by EcD₁ (94.21%), EcD₃ (90.08%), SaD₁ (81.17%), SaD₃ (78.64%) and SaD₂ (75.29%). Coliphages were found more efficacious and can be explored for cocktail preparation in treating cases of SCM and CM.

In brief, conclusion from the study can be drawn that from the milk samples of CM/SCM cases *S. aureus* and *E. coli* were isolated as major bacteria and then specific phages were isolated and characterized from various goshala/farms against these major bacteria, which might prove useful as anti-bacterial and bio-control agents. This study provided first ever effort in Braj region (U.P.) focusing on investigation of the *in vitro* lytic strength of bacteriophages as natural antibacterial agents against *S. aureus* and *E. coli* involved in bovine mastitis.



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APPENDIX

1) Nutrient agar- (HiMedia, Laboratories Limited, Mumbai)

Composition:

| | |
|----------------|--------|
| Agar powder | 2 gm |
| Nutrient broth | 100 ml |

Use-Nutrient agar is used as a source of nutrition for the bacteria.

2) Nutrient broth- (HiMedia, Laboratories Limited, Mumbai)

| Ingredients | Grams / liter |
|---------------------------------|----------------------|
| Peptone | 5 |
| Sodium chloride | 5 |
| Beef extract | 1.5 |
| Yeast extract | 1.5 |
| Final pH (at 25 ⁰ C) | 7.4±0.2 |

Use- A general culture medium which may be used as enriched medium.

3) Mac Conkey Agar- (HiMedia, Laboratories Limited, Mumbai)

| Ingredients | Grams / liter |
|---------------------------------|----------------------|
| Peptic digest of animal tissue | 20.50 |
| Lactose | 10 |
| Sodium taurocholate | 5 |
| Neutral red | 0.04 |
| Agar | 20 |
| Final pH (at 25 ⁰ C) | 7.4±0.2 |

Use: For cultivation and differentiation of enteric bacteria, the omission of sodium chloride prevents the spreading of *Proteus* colonies.

4) E M B Agar- (HiMedia, Laboratories Limited, Mumbai)

| Ingredients | Grams / liter |
|--------------------------------|----------------------|
| Peptic digest of animal tissue | 10 |
| Dipotassium phosphate | 2 |
| Lactose | 5 |
| Sucrose | 5 |
| Eosin | 0.40 |
| Methylene blue | 0.065 |

| | |
|---------------------------------|---------|
| Agar | 13.50 |
| Final pH (at 25 ⁰ C) | 7.2±0.2 |

Use: Recommended for the isolation and differentiation of Gram negative enteric bacteria from clinical and non clinical specimens.

5) HiCrome *E. coli* Agar (HiMedia, Laboratories Limited, Mumbai)

6) Mannitol salt agar (HiMedia, Laboratories Limited, Mumbai)

| Ingredients | Grams / liter |
|---|----------------------|
| Peptone | 5.0 |
| Tryptone | 5.0 |
| HM Peptone B | 1.0 |
| Sodium chloride | 75.0 |
| Mannitol | 10.00 |
| Phenol red | 0.025 |
| Agar | 15.00 |
| Final P ^H at 25 ⁰ C | 7.4±0.2 |

Use: It is used for selective isolation of *Staphylococcus aureus*.

7) Simmons Citrate Agar- (HiMedia, Laboratories Limited, Mumbai)

| Ingredients | Grams / liter |
|---------------------------------|----------------------|
| Magnesium sulphate | 0.2 |
| Ammonium dihydrogen phosphate | 1.0 |
| Dipotassium phosphate | 1.0 |
| Sodium citrate | 2.0 |
| Sodium chloride | 5.0 |
| Bromo thymol blue | 0.08 |
| Agar | 15 |
| Final pH (at 25 ⁰ C) | 7.4±0.2 |

Use: For the differentiation of enteric pathogens by ability to utilize citrate.

8) Luria Bertani HiVeg Broth (HiMedia, Laboratories Limited, Mumbai)

| Ingredients | Grams / liter |
|--------------------|----------------------|
| HiVeg hydrolysate | 10.00 |
| Yeast extract | 5.00 |
| Sodium chloride | 10.00 |

Final pH (at 25°C) 7.5 ± 0.2

Use: Recommended as a selective medium.

9) LB Growth Agar for Lambda (HiMedia, Laboratories Limited, Mumbai)

| Ingredients | Grams / liter |
|--------------------------------------|----------------------|
| Tryptone | 10.00 |
| Yeast extract | 5.00 |
| Sodium chloride | 10.00 |
| MgSO ₄ .7H ₂ O | 2.00 |
| Agar | 15.00 |
| Final pH (at 25°C) | 7.5 ± 0.2 |

Use: Recommended as a selective medium for isolation of coliphages particularly lambda phage.

10) LB Growth Top Agar for Lambda (HiMedia, Laboratories Limited, Mumbai)

| Ingredients | Grams / liter |
|--------------------|----------------------|
| Tryptone | 10.00 |
| Yeast extract | 5.00 |
| Sodium chloride | 10.00 |
| Agar | 7.00 |

Use: Recommended as a selective medium for isolation of lambda phage.

11) NZCYM Growth Agar (HiMedia, Laboratories Limited, Mumbai)

| Ingredients | Grams / liter |
|---------------------------------------|----------------------|
| Casein enzymic hydrolysate (Tryptone) | 10.00 |
| Casein enzyme hydrolysate | 1.0 |
| Yeast extract | 5.0 |
| Sodium chloride | 5.0 |
| Magnesium sulphate heptahydrate | 2.0 |
| Agar | 15.0 |

Use: Recommended as a selective medium for isolation of phages against *Staphylococcus aureus*.

12) NZCYM Growth Top Agar (HiMedia, Laboratories Limited, Mumbai)

| Ingredients | Grams / liter |
|---------------------------------------|----------------------|
| Casein enzymic hydrolysate (Tryptone) | 10.00 |
| Casein enzyme hydrolysate | 1.0 |
| Yeast extract | 5.0 |
| Sodium chloride | 5.0 |
| Magnesium sulphate heptahydrate | 2.0 |
| Agar | 7.0 |

Use: Recommended as a selective medium for isolation of phages against *Staphylococcus aureus*.

13) Deca strength nutrient broth (DSNB)

Use: Recommended for enrichment of wastewater for phages against *Staphylococcus aureus*.

14) Muller-Hinton Agar

Use: Recommended for antibiotic sensitivity testing of bacterial isolates.

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