CHAPTER V
SUMMARY AND CONCLUSIONS

Agriculture sector has leading role in Indian economy. In that livestock contributes 25.6% of total Agriculture GDP of India (Dash, 2017). Dairy industry is the major allied activity associated with agriculture. Dairying and related activities played a major role in strengthening the rural economy. But, the bacterial diseases are major threat to livestock along with other infectious diseases. Bacterial infections cause considerable economic losses through decreased production, costs in diagnosis and treatment. Staphylococci strains are impact pathogens in animals, as they possess properties to produce enzymes, toxins and intrinsic virulence factors. *Staphylococcus* spp. can cause invasion and tissue damage resulting in mastitis and other pyogenic infections. In preventive and therapeutic measures there is indiscriminate and inappropriate use of antibiotics, which has led to the increased selective pressure for the development of antibiotic resistance over a period of time.

Bacteriophages are a potential alternative for antibiotics in the treatment of bacterial infections. The use of phage therapy with host specific bacteriophages is cost effective and acceptable approach to control multidrug resistant bacteria (Sulakvelidze and Barrow, 2005). Bacteriophages are natural predators of bacteria and are ubiquitous in nature. The advantages of host specific bacteriophages over chemotherapy are that; they are self limiting and replicating only on the specific bacteria without affecting harmless flora in body. Considering these advantages host specific bacteriophages were isolated against MDR staphylococci.

This study was intended to investigate sewage water sources for the isolation and characterization of indigenous bacteriophages to assess their lytic activity against MDR staphylococci. Various samples obtained from TVCC clinical cases like milk, pus, lesion wash, nasal swab, ocular swab etc. were screened. A total of 30 Staphylococcal isolates were recovered from samples. Staphylococci have been identified and characterized on the basis of phenotypical properties like, colony morphology, staining characteristics, growth pattern, fermentation of various carbohydrates and production of enzyme like catalase. A total of 18 isolates were identified as *Staphylococcus aureus* according to their carbohydrate utilization.
patterns and biochemical profile. Identification of methicillin resistant *Staphylococcus aureus* was carried out using HiCrome™ MeReSa agar. Antibiotic susceptibility profile and E-test of bacteria was carried out to detect MDR isolates. Total 30 isolates showed resistance towards various antibiotics *viz.* Penicillin-G (100%), Methicillin (80%), Amoxicillin-sulbactam (60%), Ceftriaxone (100%), Cefoperazone (80%), Gentamicin (100%), Enrofloxacin (100%), Levofloxacin (80%), Oxytetracycline (100%) and Chloramphenicol (70%).

Molecular confirmation of bacteria was carried out with mPCR. Multiplex PCR with 4 gene specific primers (*16S rRNA, Nuc, Coa & mecA* gene) revealed that all 4 products were amplified by some of isolates.

Bacteriophages were isolated out using TVCC (Teaching Veterinary Clinical Complex) and CBF (Cattle breeding Farm) drainage water by plaque assay. Isolated phage was enriched by providing host bacteria in Tryptic Soya broth. Then phage was precipitated with Polyethylene glycol overnight and purified by dialysis. Concentration of purified phage in pfu/ml was estimated by double agar overlay method. Titre of enriched phage was $2.6 \times 10^9$ pfu/ml. For demonstration of replication cycle of phage one step growth curve experiment was carried out with 0.1 MOI. Scanning and Transmission electron microscopy of phage was carried out to determine morphology and for classification of phage. The details of morphology and dimensions in electron microscopy revealed that the phage was morphologically similar to phages of family *Podoviridae* according to ICTV.

Physico-chemical properties like, thermostability, pH stability, effect of UV light and 25% chloroform was determined by observing phage survival rate. For genetic characterization of phage, nucleic acid extraction was carried out. Extracted nucleic acid was treated with DNase-I, RNase-A and Mung bean nuclease to determine nucleic acid type. Gel electrophoresis of treated nucleic acid revealed that phage has dsDNA in its genome. Restriction enzyme analysis was carried out to detect restriction sites of phage. The phage contains restriction sites for *NdeI* & *HindIII*, while it was refractory to digestion by endonuclease like, *EcoRI* and *XhoI*. Phage protein profiling was carried out with SDS-PAGE and it revealed some major and minor structural proteins having size ranging from of 54 to 124 kDa.
Host range of phage was determined by checking susceptibility of *Staphylococcus* and non-*Staphylococcus* genera to the phages. Susceptibility of clinical staphylococcal isolates and MRSA isolates was also determined. It revealed that phage was specific to MRS only and could not infect non-*Staphylococcus* spp. Activity of phage on biofilm producing MRSA was also assessed by inducing biofilm formation with 10% dextrose and 0.9% NaCl, which revealed strong lytic activity of phage on biofilm formed by MRSA.

This study successfully isolated bacteriophages specific to MRSA from drainage samples. This also suggests a substantial ability of the bacteriophage to persist in sewage water environments, possibly replicating off a resident host bacteria population. *Staphylococcus aureus* specific phages appear to be naturally abundant enough to achieve phage titration. This study also examined the possibility that different strains of bacteriophages infecting same host bacteria were present in drainage water. Considering above findings following conclusions can be drawn from the present research work.

1. A total of 120 clinical isolates of *Staphylococcus spp.* were observed to be MDR with prevalence of more than 37%. All the MRSA isolates were found to be Vancomycin sensitive.
2. Multiplex PCR, growth on selective media and biochemical features confirmed the MRSA isolates and they possessed resistance gene *meca*.
3. Three bacteriophages shown lytic activity on MDR were isolated in this study based on plaque morphology. Medium sized plaque (3-5mm) forming phage was studied in detail & named vB_SaP-AZ2.
4. One step growth curve revealed a latent period of 90 min. with burst size of 93.
5. Electron microscopy study revealed that phage morphology was similar to that of *Podoviridae* (Head diameter- 86 nm, Non contractile tail length- 36 nm).
6. The stability of phage was observed up to 45°C and completely loss the activity at 65°C in 1 hr.
7. UV light exposure upto 1 hr did not reduce phage ability, pH stability of phage observed between 6 to 8 and 25% chloroform did not altered phage activity in 24 hrs.
Summary and Conclusions

8. Genetic nature of phage was observed to be linear dsDNA with restriction sites of NdeI and HindIII. Genome length of phage observed to be >15kbp and <20kbp.

9. Protein profile of phage revealed 3 major and 4 minor proteins. Major head protein was observed to be of 54 kDa.

10. Isolated phage shown lytic activity on 52.5% of clinical isolates of Staphylococcus spp. whereas it was effective against 73.33% of MRS.

11. Lytic activity was observed even on biofilm producing MRSA.

Promising effect of phage vB_Sap-AZ2 against MDR Staphylococci has raised the probable chances to utilization of these phages for biological control of bacterial infection including MRSA. It can be a potential candidate for phage therapy. An abundance of bacteriophages in the natural environment, in addition to the ease with which they can be isolated, make them a good contender for phage therapy. Isolated phages need to be further characterized for their clinical application; in particular, the protein segments of isolated phages need to be assessed for their antibacterial ability against MRSA.