5. Discussion

*Staphylococcus aureus* has emerged as an important human pathogen world over and is responsible for a wide variety of clinical conditions. Although, infections due to this organism are trivial in terms of morbidity and mortality as compared to other infectious diseases like tuberculosis, malaria, acquired immunodeficiency syndrome (AIDS) etc. particularly in the developing countries like India but the impact of *S. aureus* infections in hospital settings and in the community cannot be underestimated. Thus, this organism has emerged as a world pathogen. Since 1960, 80% of *S. aureus* strains have developed resistance to penicillin (Lowy, 2003). Soon after the antibiotic methicillin was introduced into clinical use in the year 1961 for treating infections due to penicillin resistant *S. aureus* strains, the resistance to methicillin appeared throughout the world (Nickerson, 2009). Not only the MRSA strains have adapted to hospital environment, but MSSA strains are also of great clinical significance although, the latter are considered to be less virulent in hospital settings as compared to the MRSA (Vidhani *et al.*, 2001). Although, several phenotypic methods such as serotyping and biotyping have been used for phenotyping these strains, the screening of *S. aureus* strains for their susceptibility to different antibiotics (antibiotyping) has proved a good epidemiological tool. Most studies on this aspect throughout the world have revealed that most *S. aureus* strains were resistant to penicillin, whereas only a few incidences of resistance to other antimicrobial agents have been reported (Stephan *et al.*, 2001; Lowy, 2003). In the present study, 86.6% *S. aureus* isolates were found resistant to penicillin and 44/135 (32.6%) isolates were resistant to methicillin. Also, all the methicillin resistant *S. aureus* (MRSA) were resistant to multiple antibiotics (MDRs). An isolate which was resistant to two or more antibiotic groups/classes up to third generation was used as the criterion for an isolate to be a multidrug resistant (MDR). The multiple resistance was also observed among MSSA isolates though to a very small extent, only 7/91 (7.7%) were recorded as MDR-MSSA. These isolates were recovered from patients at IGMC Shimla, in the state of Himachal Pradesh. Interestingly, three isolates, one from pus and two from blood were resistant to all the 20 different antibiotics used in the antibiotic culture sensitivity assay and nine isolates all from pus were resistant to most 15-18 antibiotics. The antibiotics used in the assay belonged to 11 different antibiotic groups/classes. MRSA strains in general, are extensively more resistant to most commercially available antibiotics as compared to MSSA. Parveen *et al.*, (2011) have also made similar observations.
The prevalence of the resistant strains was as follows: penicillin resistant strains recorded were (86.66%) followed by co-trimoxazole (70%), ampicillin (69.66%), azithromycin (68.14%), chloramphenicol and linezolid (65.18%) each, erythromycin (54.81%) and vancomycin (53.33%). The reason for such high prevalence of strains resistant to these antibiotics can be attributed to indiscriminate use of these antibiotics for treating *S. aureus* infections in this geographical region. The resistance was also observed against other antibiotics but to a lesser extent. The percentage of strains resistant to other antibiotics was lower and ranged from 20.0% - 40.7% with amikacin at (40.74%) followed by gentamicin (32.59%), oxacillin (33.33%), clindamycin (31.0%), amoxyclyve (25.92%) and cephalothin (20.0%). The prevalence of 86.6% penicillin resistant strains observed in the present study is slightly lower than that reported by Duran *et al.*, 2012. These workers have reported a prevalence of 92.8% penicillin resistant strains in Turkey. Earlier, ciprofloxacin was considered as alternative therapy for treating MRSA infections but rapid emergence of resistance to this antibiotic too in *S. aureus* strains has been reported (Blumperg *et al.*, 1991). The MRSA strains, particularly hospital-acquired strains are often resistant to a number of antibiotics including beta-lactams therefore vancomycin has been considered as the only option for the treatment of MRSA (Fitzgerald *et al.*, 2001). We have recorded marked percentage of vancomycin resistant strains (51.9%) in the present study (Table 4.2). This observation is of clinical significance because this antibiotic is considered as the only therapeutic options for the treatment of MRSA and MSSA infections due to the emergence of ciprofloxacin resistant *S. aureus* strains. Further, such a high percentage of vancomycin resistant MRSA strains in our study are consistent with a CDC report from USA (CDC, 2005). It is further interesting to note that vancomycin intermediate *S. aureus* (VISA) have also appeared in this part of country which is evident from the fact that we recorded 21/135 (15.5%) VISA strains. Majority of the VISA strains 15/21 (71%) belonged to MRSA group while remaining (29%) 6/21 were MSSA. The emergence of intermediate resistance is of particular interest in that such strains can spread to the community causing difficulty in treating MSSA infections. The resistance to vancomycin has also been reported from other several countries (Tenover and Goering, 2009). Ours is perhaps the first report regarding the prevalence of vancomycin resistant and vancomycin intermediate *S. aureus* (VRSA and VISA) in Himachal Pradesh. Tiwari and Sen (2006) have reported VRSA strains from the Uttar Pradesh. Several reasons can be assigned to such high prevalence of MRSA and MSSA strains in the state of Himachal Pradesh such as
indiscriminate use of antibiotics, lack of awareness among healthcare workers, increased frequency and interaction with tourists and visitors from other states carrying *S. aureus* infections, lack of appropriate strategies to effectively control these infections. The present study thus, provides the basic information regarding a high prevalence (32%) of MDR-MRSA and emergence of MDR-MSSA (7.7%) in the state of Himachal Pradesh.

Since the phenotypic methods such as antibiotyping, serotyping, biotyping, toxin estimation, level of coagulase production etc. are growth dependent as well as time consuming, molecular typing of *S. aureus* strains has been considered as accurate method as compared to phenotypic markers. Two specific proteins, Coagulase (*coa*) and protein A (*spa*) have been most widely used markers for molecular typing because they contain highly polymorphic repeat units (Frenay *et al*., 1996; Walker *et al*., 1998). PCR-RFLP studies of these genes were found to be quite useful in typing *S. aureus* strains and have proven absolute typeability, reproducibility and good discriminatory power (Wichelhaus *et al*., 2001). Nucleic acid sequencing is the direct method for establishing the identity of the amplified product and recognizing DNA sequence polymorphism. This is the most sensitive method and can differentiate between *S. aureus* strains of different origins with precision and accuracy however, this method is time consuming and costly.

In the present study we characterized the MRSA and MSSA strains by amplifying selective segments of coagulase (*coa*) and *spa* gene in order to study the restriction fragment length polymorphism and to determine the nucleotide sequence homology with the published sequences of various *S. aureus* strains. The sequences of these genes were submitted to the National Centre for Biotechnology Information (NCBI) and have been assigned accession numbers which authenticates the identity of the amplicons of these genes. Coagulase production is an important phenotypic characteristic of *S. aureus* which is associated with virulence. Expression of this gene is thought to enhance bacterial growth and promote infection (Baba *et al*., 2002). Different methods of serotyping of *S. aureus* coagulase have been developed, but unfortunately many strains still cannot be typed by this method. Variations in Staphylocoagulase have been noticed as the differences in the antigenicity, have been observed on the basis of which SCs have been classified into 10 serotypes by inhibition test of their clotting activity using type-specific antibodies against each serotype (Kanemitsu *et al*., 2001). The gene responsible for coagulase production (*coa* gene) by different strains has been shown to exhibit variations on PCR
amplification. SCs are composed of 6 fundamental segments: signal sequence at N-terminus, D1 region, D2 region, central region, 27 amino-acid repeat regions and C terminal sequence of 5 amino acids. SC binds to prothrombin via the D1, which contains N-terminal prothrombin-activating domain, and the D2 region (Friedrich et al., 2003). Watanabe et al., (2005) determined the nucleotide sequences of SC genes (coa) of 10 distantly related serotypes. The structural comparison of the deduced amino acid sequences of all the coa genes showed that D1 and the D2 regions were rather diverse, whereas the central regions were relatively conserved. Since identities of both nucleotide and amino acid in the D2 regions were higher than those in the D1 regions. These workers suggested that the D1 region might be responsible for the antibody recognition site for type specific antiserum.

In the present study, we have not sequenced the entire coa gene and spa gene because we selected primer pairs from the polymorphic regions of these genes for molecular typing of the strains. Earlier workers have also used this system for typing MRSA strains (Hooky et al., 1998; Janwithayanuchit et al., 2006; Kumar et al., 2008). Although, the sequencing of entire genes could provide valuable data in terms of variability in nucleotide sequences in various domains or regions such as those responsible for antigenicity and other functions. Such an endeavour is technically quite demanding and timeconsuming. The purpose of amplifying the polymorphic regions aimed at typing of MRSA and MSSA strains. The amplicons of coa gene on digestion with HaeII generated different restriction patterns which could differentiate MRSA from MSSA.

We achieved amplification of coa gene in 36 out 40 MRSA isolates and 18 of the 20 MSSA isolates studied. In the PCR assay we used the same primer pair as has been used by several workers (Hooky et al., 1998; Janwithayanuchit et al., 2006; Kumar et al., 2008). On Blast-2 analysis of the primer sequences reveled that they matched with the staphylocoagulase precursor of standard strain SA-40 with the nucleotide number as follows: Forward primer- nucleotide no. 248469 to 24847 and reverse primer- 230704 to 230686. Variations in the amplicon sizes of both MRSA and MSSA isolates were observed. This variability has been observed due to polymorphism of the coa gene amplicons which varied in size ranging from ~600-~820 bps in MRSA strains whereas it was smaller in case of MSSA strains and ranged from ~500~600 bps. Three different band sizes (~600 bps, ~700bps and ~750bps) were visible in MRSA isolates (Fig. 4.3). Four MRSA isolates (one originating from urine and three from pus) and among MSSA
(one each originating from catheter associated infection and blood) were not amplified in the PCR assays. The failure to achieve amplification of coa gene amplicons in such instances could be due to the fact that their blood clotting or coagulase activity was specified by genes other than the coa gene (Vandenseh et. al. 1994). Variations in the sequence of coa gene where the primers bind could be another possibility for not achieving the amplification.

The polymorphism of MRSA and MSSA isolates is further evident from the restriction patterns obtained after the digestion of the amplified product with HaeII. Five distinct restriction patterns; ~475 bps and ~225 (pattern-I), ~550 bps and ~260 bps (Pattern-II), ~500 bps and ~250 bps (Pattern-III), ~450 bps, ~250 bps (Pattern-IV) and ~560 bps and ~260 bps (V) of MRSA isolates (Fig. 4.4) were demonstrable in the present study. The present investigation of the coa gene amplicon on digestion with HaeII thus, reflects genotypic variability among the strains recovered from the blood, urine and pus of patients in Himachal Pradesh. The blood isolates had pattern-I. The isolates of urine origin showed pattern-III while the pus isolates exhibited different patterns. Although, the patterns were not distinct from origin point of view but the strains could be epidemiologically related. It is difficult, to correlate the exact clinical history of the patients and time of occurrence of the episodes. PCR-RFLP of coa amplicons of MRSA strains has extensively been used for screening them for epidemiological purposes by various workers (Mitani et al., 2005). Janwithayanuchit et al., (2006) in Thailand has reported different genotypes by AluI digestion of the coa gene amplicons and suggested that the genotyping coa gene with various methods such as antibiotyping, PCR- RFLP patterns etc. can be used to trace their origin for epidemiological purpose during the phase of an outbreak. Kobayashi et al., (1995) observed different restriction patterns using AluI digestion of coa gene of MRSA and MSSA isolates which could distinguish between the MRSA and MSSA as they were divided into 6 and 12 restriction fragment length polymorphism (RFLP) patterns, respectively, whereas five patterns were commonly detected in MRSA and MSSA. MRSA isolates that showed a particular RFLP pattern were considered to be predominant in the hospital.

The nucleotide sequence analysis of MRSA isolates of different origins (Isolate no.-64-blood, 128-urine, 97-pus) in the present study revealed homology ranging from 88% to 99% of these isolates to the standard strains as mentioned in the results (section 4.5). On alignment of the nucleotide sequence of coa gene segment, the variations of different origins were observed (Fig.
Similarly, nucleotide sequence homology ranging from 89% to 96% were observed between the MSSA isolates of different origins (Isolate no.-96-blood; 81- urine, 75-pus) with the standard strains and were thus closely related to them. The variations were also observed in the nucleotide sequences among the MSSA isolates of different origins (Fig. 4.19 and Fig. 4.20.). The variations in the nucleotide sequences have been reflected in the changes in predicted amino acid sequences. Comparative analysis of the predicted amino acid sequences of MRSA and MSSA isolates using Translate tool revealed substitutions at positions 50, 57, 58, 59, 62, 71, 72, 73, 84, 85, 86, 108, 112, 113, 121, 130, 131, 132, 156, 157, 158 and 159 (Fig. 4.40). Most of the substitutions were seen in MRSA isolates as compared to MSSA isolates. Among MRSA isolates, maximum substitutions were observed in the isolate of urine origin (isolate-128). The substitutions in both the MRSA and MSSA isolates might be associated with the resistance to antibiotics and/or increased virulence of the isolates. Thus, these variations might be linked to the pathogenesis of infections due to these isolates. However, further studies are required before arriving at a definite conclusion.

Staphylococcal protein-A is a peptidoglycan-bound surface protein of the cell wall of S. aureus (Lee et al., 2004) and is encoded by 2 kb spa gene. This protein is one of the virulence factors which is involved in the initial stages of MRSA and MSSA infections. The C-terminal end of the protein includes a sequence required for binding with the cell wall. The Fc-binding region is localised at the N-terminal segment. The corresponding part of the spa gene comprises five 160 bps repeats. The spa gene includes a polymorphic sequence known as x-region which consists of a variable number of tandemly repeated 24 bps units (Frenay et al., 1996). Polymorphism of x-region of spa is widely used for genotyping of S. aureus. This method has discriminatory power which allows the recognition of small differences among genetically related strains, therefore, is very useful tool in the epidemiological investigations. In the present study, we amplified this region and achieved amplicons which were variable in size and ranging from ~150~250 bps in 32 out of 40 MRSA isolates (80%) and 14 out of 20 MSSA isolates (70%). We utilised the same primer pair as was used by Kurlenda et al., 2010. The primer pair was derived from IgG binding protein precursor (Forward primer- nucleotide no. 92883 to 92900 and reverse primer- 92746 to 92729) of standard strain SA-40. The reason for not achieving amplification in some MRSA and MSSA isolates could be due to deletion of IgG binding site particularly in the C region as has also been reported by Baum et al., 2009. The size of the ampicons of spa (x-region) in MRSA
isolates was larger as compared to MSSA isolates. Adsida et al., 2006 have reported upto 5% frequency of S. aureus without spa gene expression. However, Strommenger et al., 2008 found 99.8% strains of S. aureus typeable by spa gene typing. We observed single band on electrophoresis of amplicons of spa (x-region) of S. aureus isolates originating from blood, pus and urine. Shakeri et al., (2010) however, reported two bands of spa (x-region) but these workers amplified longer segment of this gene where as the amplicon size in the present case varied only between ~150~250 bps. Also, these workers correlated the length of the amplicon size to the origin of isolation e.g. the spa gene amplicon of isolates recovered from urinary tract were longer as compared to others.

The digestion of PCR products of x-region of spa gene with EcoRII generated single type of restriction pattern in which two bands, ~200 bps to ~150 bps (Fig.4.22) were demonstrable in MRSA isolates. However, a single band of variable size ~300 bps, ~200 bps were also seen in many isolates which suggests that either these isolates differed in the restriction site for EcoRII or the PCR products were not digested with this restriction enzyme.

Our results showed that the length of the amplicons of spa (x-region) in strains recovered from blood was more than in these recovered from urine and pus in case of MRSA, but in case of MSSA the length of amplicons was more in isolates of urine origin. In the present study, we recorded two tandem repeats in all the isolates sequenced except the MSSA isolate of urine origin which had three 24 bps tandem repeats. Different workers have reported variable number of tandem repeats in the x-region of spa gene ranging from 2 to 16 (Walker et al., 1998; Mehndiratta et al., 2009). Kuzma et al., (2005) found 2-11 tandem repeats in MSSA strains recovered from mastitis in cows in Poland. Some workers also, have tried to correlate the number of repeats with the virulence level of strains. For example Vimercati et al., 2006 presumed that the number of repeated units below seven correlated with decreased virulence the strains. A correlation between the number of tandem repeats and the strain’s ability to cause epidemic has also been suggested by Frenay et al., 1994. These workers believed that strains with more than 7 repeats were epidemic strains. On the contrary, Kurlenda et. al., 2010 did not find any correlation with the x- region polymorphism and virulence of MRSA and MSSA strains. Multiple repetitions in the x-region reflect a longer protein-A domain that binds to Fc fragment of IgG (Frenay et. al., 1994). Further studies are required with regard to the number of 24 bps
tandem repeats among the human and animal strains of this geographical region in order to determine their genetic relatedness and epidemiological significance.

The nucleotide sequence analysis of amplicons of spa gene of MRSA isolates of different origins (Isolate no.-64-blood, 128-urine, 97-pus) in the present study revealed their homology ranging from 92% to 97% to the standard strains as mentioned in the results (section 4.9). The variations in the nucleotide sequences among the MRSA isolates of different origins are presented through Fig. 4.29 and Fig. 4.30. Similarly, nucleotide sequence homology ranging from 91% to 100% were observed between the MSSA isolates of different origins (Isolate no.-96-blood, 81-urine, 75-pus) with the standard MSSA strains. The variations in the nucleotide sequences among the MSSA isolates of different origins in the present study have been depicted through Fig. 4.37 and Fig. 4.38. The major amino acids substitutions were found at position 84 and 87 as follows: K84D (96-blood MSSA) and G87R (96-blood MSSA) (Fig 4.42 and 4.43). These substitutions might be linked to the differences in virulence of the strains.

*S. aureus* produces a number of toxins which serve as virulence factors and contributed to the pathogenesis of infections due to this organism. The amount of toxin produced is related to virulence. *S. aureus* strains have potential to produce extracellular toxins as well as enterotoxins such as staphylococcal entrotoxins (SEs) which are classified as A, B, C1, C2, C3, D and E. Pimbley and Patel (1998) recovered *S. aureus* strains from clinical conditions with severe acute illness that rapidly resulted into multi organ failure, due to a toxin known as toxic shock syndrome toxin-1 (TSST-1). In the present study we amplified the segment of *tst* gene and achieved amplification in two out of 40 MRSA isolates (5%) in the state of Himachal Pradesh. The amplification was however not seen in 38 MRSA isolates and one most important reason could be the absence of the *tst* gene from the isolates. Since the information regarding exact clinical history of the patients from which the MRSA isolates were recovered is not available, it appears that among the 40 isolates tested only 2 could be from the cases of toxic shock syndrome or associated conditions such as septicemia. We used primer pair to amplify the segment of *tst* gene which were used by Johnson and Tyler (1993). Ghoban and co-workers (2006) recorded 7.5% positive *tst* in Libya in 2006 which were comparable to our observations in the present study. These workers also used the same set of primers in their study. Tsen *et al.*, 1998 recorded amplification of (4.8%) of 62 strains of *S. aureus* form clinical cases which is again quite similar
to our findings. On the contrary, Crass and Bergdool, 1986 found that 91.6% of strains produced tsst-1 alone or in combination with one or more staphylococcal enterotoxins. The young menstruating women who use high absorbency tampons constitute high risk group. Cases are also seen in men and non- menstruating woman (Davis et al., 1980). This toxin is encoded by the tst gene.

Bacteriophage typing is one of the oldest techniques for studying the epidemiology of infectious agents. Compared to serotyping of bacteria, this technique is more sensitive (Rennie et al., 1978). Therefore, phage typing is recommended as firstline of approach in epidemiological investigations of MRSA and MSSA strains. During past few decades, the epidemiology of S. aureus has been continuously changing. In order to control the spread of S. aureus strains, it becomes essential to understand their epidemiology. Using international set of bacteriophages, in the present study, 20/37 isolates (54.05%) of MSSA strains were successfully typed. However, 17/37 (45.94%) isolates were non-typeable. In general, the percentage of non-typeable strains is high, but in a recent study, Mehdiratta et al., (2010) have reported a higher percentage of 39.0% among MRSA as non-typeable in India, as against 45.94% in the present study. Some workers have however, recorded lower proportion of non-typeable strains. Witte et al., (1979) have recorded 20% strains as non-typeable. Non-typeability of S. aureus strains is a major problem with the available sets of bacteriophages in India and other developing countries (Dugid, 1989). On analysis of the phage typing data, we observed that most of the MSSA (45.0%) strains from hospital setting in Himachal Pradesh belonged to phage group I. The MSSA strains in these group I are generally associated with hospital acquired and endemic infections (Rennie et al., 1978; Sanjay et al., 2012). The predominance of MSSA strains in hospital settings has also been reported by others (Usman et al., 1996; Samba and Gadba, 1993). We observed two strains in phage group III which is a quite interesting in that, MSSA strains generally do not fall in Phage group III. However, multidrug resistant strains particularly MRSA fall in this group (Udo et al., 2008). MRSA strains of this group are epidemic strains. We did not record any strain in phage group V. It is interesting to note that the phage type 81 in phage group NA lysed the MSSA strains which were resistant to penicillin but sensitive to a number of antibiotics. This observation is consistent with others (Kareiviene et al., 2006). All the nine MSSA strains typed in phage group I were sensitive to methicillin, oxacillin, novobocin and tetracycline. Similar observations have been made by Mahndiratta et al., 2010. Most strains in this group showed
resistance to teicoplanin, chloramphenicol, vancomycin, penicillin, ampicillin azithromycin and linezolid. The percentage of strains sensitive to these antibiotics was higher than 55% and ranged from 11.11% to 44.44%. These observations are consistent with the findings of others (Gupta et al., 1999). Strains in group II were resistant to penicillin. In the mixed phage group, all the three strains tested were resistant to penicillin, erythromycin ampicillin and amoxycillin. However, resistance was also seen against other antibiotics but to a lesser extent. In the non-typable group, all the 17 strains were susceptible to methicillin and cephalothin but the percentage of sensitive strains ranged from 47.05% to 88.23%. It is perhaps for this reason that MSSA infections are relatively easier to control as compared to MRSA strain. This fact is substantiated by observation of Jones et al., 1999 who reported that MSSA strains were more susceptible to antibiotics as compared to MRSA. Several other studies also support this view as discussed in chapter 2 under review of literature. Further, in the present study, phage types 52 and 79 were most predominant in MSSA strains. Although bacteriophage typing is very good technique but it has certain limitations such as; the typing technique is cumbersome, time consuming and requires intense efforts in propagation, standardization and maintenance of phages (Maneesh et al., 2011).

It may be concluded form the present study that MRSA and MSSA both (HA-MSSA as well as CA-MSSA) strains are prevalent in the state of Himachal Pradesh which could be differentiated on the basis of bacteriophage typing and nucleotide sequencing of amplicons of virulence genes (coa, spa and tst) of MRSA and MSSA isolates. However, other sensitive techniques such as pulse field gel electrophoresis (PFGE), Single-locus sequence typing (SLST), Multi-locus sequence typing (MLST) etc. can be utilized for typing these strains with precision and accuracy. The present study would be useful in understanding the molecular epidemiology and pathogenesis of MRSA and MSSA isolates. It might prove helpful in the better management of MRSA and MSSA infections in light of the therapeutic options based on the study in the state of Himachal Pradesh.

**Future projections**

The present study depicts a high prevalence of 32% of MDR MRSA and 7.7% MDR MSSA. Ours is perhaps the first report regarding the prevalence of vancomycin resistant strains (VRSA) as well as vancomycin intermediate resistant (VISA) strains among both MRSA and MSSA strains in the state of Himachal Pradesh. The prevalence of multidrug resistant strains is thus, quite alarming with regard to multiple drug resistant strains. After the development of resistance
to penicillin, methicillin and later on to ciprofloxacin, vancomycin was thought to be the magic drug to treat MDR *S. aureus* strains but resistance to this antibiotic has been observed in the state of Himachal Pradesh also. Three isolates (two from the blood and one from pus) were resistant to all the 20 antibiotics belonging to 11 different antibiotic classes while other nine isolates all from pus were resistant to most of the 15 to 18 antibiotics used in the assay. Such strains may be regarded as superbugs. The infections due to these organisms become difficult to treat. Majority of isolates were susceptible to novobiocin, teicoplanin, cephalothin and clindamycin. This study might be useful to the clinicians for designing effective strategies to treat MRSA and MSSA infections. It would also be useful in further preventing the spread of the infections particularly in the hospital settings in the state. Since due to indiscriminate use of the antibiotics in treating *S. aureus* infections, this organism acquires resistance to most of them, regular and continuous monitoring and surveillance of *S. aureus* strains in hospital settings and in communities covering different geographic locations in the state is therefore, warranted on urgent basis.

The antibiotic susceptibility of the MSSA isolates was further correlated to bacteriophage groups of MSSA. These strains were susceptible to more number of antibiotics as compared to MRSA although only a small proportion of MSSA was found multidrug resistant. Since the bacteriophage typing is considered as first line approach in epidemiological investigations and is one of the old but time tested method of tracing the source of infection and spread of the etiological agent, continuous surveillance and monitoring of circulating field strains of *S. aureus* in different geographical locations in the state of Himachal Pradesh is therefore essentially required. Since this method has limitation in that a proportion of the strains are non type-able as we observed in the present study, this method can be used as adjunct to other precise and accurate methods such as PCR- RFLP of virulence genes and antibiotic resistance genes etc. Besides the bacteriophage typing of MRSA isolates, Patil, 2014 characterized the MRSA isolates by amplifying selective segments of *mecA*, *pvl* and *vanA* genes in our laboratory. These studies reflected that staphylococcal cassette chromosome (*SCCmec*) type IV. Epidemic strains of MRSA belonging to phage group III were also prevalent in the state according to the study. The *in vitro* expression of the virulence factors of MRSA and MSSA isolates in our laboratory such as lipases, proteases, hemolysins, coagulase and biofilm formation revealed that the former were better producers hence, more virulent as compared to the latter (Guleri, 2014). Taken together, the data obtained gives an insight into the MRSA and MSSA strains prevalent in the state of
Himachal Pradesh. These studies can be further extended to determine the toxin profiles of the strains particularly MRSA. The toxins include: exfoliative toxin, TSST-1 and Staphylococcal enterotoxins (SEs) as MRSA strains recovered from different geographical areas have shown to possess different toxin gene profiles and a strong correlation has been found between toxin gene profiles and HA-MRSA strains. Multiplex PCR is one of the useful technique that is useful for toxin detection in MRSA as well as to determine chromosomal diversity and evolutionary history of MRSA strains.

In the present investigations, selective segments of coagulase (coa) gene, Staphylococca protein A (spa) gene and toxic shock syndrome toxin (tst-1) have been amplified using primer pairs utilized by other workers earlier. The nucleotide sequences of the coa and spa genes of three isolates each of MRSA and MSSA which originated from blood, urine or pus were submitted to NCBI and the accession numbers were granted to the sequences. The variations in the nucleotide sequences among the MRSA as well as MSSA of different origins have been observed. Such variations might be linked to the pathogenesis of the infections due to these organisms. However, further studies are required to establish this. We could differentiate between MRSA and MSSA strains by PCR-RFLP of coa gene amplicons on digestion with HaeII restriction enzyme. Four restriction patterns among the MRSA strains and one pattern in the MSSA strain were observed. This method can thus, prove useful for molecular typing of S. aureus strains. Multi–locos sequence typing (MLST) is useful technique for studying clonal evolution of MRSA and seven housekeeping genes arcC, aroE, glpF, gmK, pta, tpi and yqiL can be targeted for this purpose.

Studies on the alternate therapies to treat the MDR S. aureus strains are of paramount importance which include natural compounds from bacteria, plants, animal, anti-virulence therapy and immune-prophylaxis as reviewed in the section 2.12 on the prevention and control of S. aureus infections. In our laboratory, studies on the evaluation of leaf extracts of Camellia sinensis (green tea) Murraya koenigii (curry plant) on MRSA and MSSA isolates have been conducted (Guleri, 2014). The results of the study are quite encouraging in light of the fact that these extracts had good inhibitory activity even against antibiotic resistant strains. The plant extracts offer a potential alternative therapy to prevent infections with MDR organisms. More studies have been carried out in our laboratory on determination of inhibitory activity of extracts of different plats on various bacterial and fungal pathogens. These studies suggest that the plant extracts can offer an alternative. However, further studies are required in this regard.