5. Discussion

*Staphylococcus aureus* is one of the major pathogens associated with nosocomial infections may also be community acquired throughout the world (Martin and Henry, 2008). The disease conditions caused by *Staphylococcus aureus* are insignificant in terms of morbidity and mortality in developing countries as compared to other infectious disease such as malaria, tuberculosis and HIV infections (Nickerson, 2009). β lactam group antibiotics are usually used to treat *S. aureus* infections but strains resistant to multiple drugs including methicillin have emerged. This situation poses therapeutic challenge (Yah et al., 2007; Muralidharan et al., 2009). The surveillance of *S. aureus* strains and determination of their susceptibility pattern is therefore, quite essential beside the measures to drug resistance (Masterton et al., 2002). Approximately 20% healthy people are carriers and about 60% as intermediate carriers (Moreillon et al., 2005). Constant surveillance of the organism in the community as well as hospitalized patients, determination of antibiotic susceptibility of circulating strains, their epidemiology etc. would help in managing *S. aureus* infections both MRSA and MSSA infections in an effective manner.

In the present study, quite high prevalence of MRSA (45%) has been observed by MRSA detection kit. Of these (32%) were multi drug resistant (MDR) isolates in antibiotic culture sensitivity assay. The majority isolates were resistant to different antibiotic group: β lactam and macrolide group, oxazolidinone, sulfonamide group and tetracycline. Strains by antibiotic disc diffusion method (Sharma, 2014, Patil et al., 2013). The MRSA detection kit method was found more sensitive as compare to disc diffusion method as additional 13% strains were detected by kit. MRSA strains are difficult to detect because of the heterogeneity among MRSA stains. Most isolates were resistant to conventionally used drugs against *S. aureus*. Infections caused by MRSA have been reported from various parts of India Anupurda et al., 2003; Muraudharan et al., 2009. These workers have recorded prevalence rates of 59%, 54% and 40.60% respectively. However, other researchers have reported lower prevalence rates of 23.6% (Majumber et al., 2001) and 31.1% (Rajaduraiyan, 2006). The lower prevalence rates of MRSA strains could be attributed to several factors, such as; efficiency of infection control practices in healthcare facilities and the type of antibiotics that are effective for control of MRSA infections in each hospital, as their usages varies from hospital to hospital. Also, the distribution of MRSA in a hospital setting varies from unit to unit, for example, surgical unit accounted for 80% of MRSA isolates (Srinivasan et al., 2009). However, such unit wise data were not available in the present study because the
isolates studied were recovered from out patients as well as indoor patients at IGMC, Shimla, Himachal Pradesh. The acquisition of multi-drugs resistance by MRSA is a serious concern in the hospitals. Such multi drug resistant isolates may be regarded as superbugs, which are difficult to treat. The susceptibility patterns of MRSA and MSSA strains to antibiotics however, differ significantly (Vidhani et al., 2001). Our study reports the prevalence of methicillin as well as vancomycin resistance of MRSA as revealed by amplification of mec-A and van –A genes in the PCR assay since the acquisition of resistance to these antibiotics is due to the presence of gene van-(A, B, C) and mec-A respectively in Enterococcus and S. aureus (Khan et al., 2007).

The availability of sensitive and specific methods for the accurate detection of antibiotic resistance in S. aureus strains has become an important tool in clinical diagnosis. Since phenotypic typing methods can not discriminate accurately and are highly dependent on growth conditions the use of molecular typing is essential (Sinsimer et al., 2005). The commonly used methods for detection of methicillin resistance rely on modified culture conditions to enhance the expression of resistance. The mec-A gene, a structural determinant that encodes a protein PBP2a is considered as one of the useful molecular markers of putative methicillin resistance in S. aureus (Felten et al., 2002). The methicillin resistance primarily results from the overexpression of PBP2a protein, a penicillin binding protein having low affinity to β-lactam antibiotics (Perez et al., 2001). Following the emergence of methicillin resistant strains, the antibiotic vancomycin was considered to be the alternative therapeutic option for the treatment of these strains. However, several reports have appeared from different parts of the world which point out at the emergence of vancomycin resistant S. aureus (VRSA) strains. Initially, vancomycin-intermediate S. aureus (VISA) strains were reported from Japan in 1996 and in the following year from United States. The resistance was believed to be due to the thickened cell wall in which many vancomycin molecules were trapped (Hiramatsu et al., 1997). The trapped molecules clogged the peptidoglycan meshwork which acts as physical barrier for further incoming vancomycin molecules (Cui et al., 2006). S. aureus is armed with virulence genes some of which have shown to play important role in pathogenesis of S. aureus infections (Foster, 2005). The presence of Panton-Valentine Leukocidin (pvl) gene in MRSA strains is one such gene which has been linked to CA-MRSA in different parts of the world (Vandenesh et al., 2003). This gene encodes for a pore-forming cytotoxin which is responsible for tissue necrosis and leukocyte destruction. The virulence genes are frequently present in CA-MRSA and have
been shown to be associated with various virulence factors, such as \textit{pvl} stable markers of CA-MRSA cases worldwide (Lina \textit{et al.}, 1999; Kilic \textit{et al.}, 2006). In fact, CA-MRSA has been shown to be more virulent as compared to HA-MRSA (DeLeo \textit{et al.}, 2010). Both SCCmec typing and detection of the \textit{pvl} locus are useful tools for the molecular characterization of HA- and CA-MRSA isolates. In order to find the relatedness among different MRSA strains, quick and reliable typing methods are required for adopting suitable control measures for timely implementation. In the present study, we describe the amplification of selective segments of \textit{mec-A}, \textit{van-A} and \textit{pvl} genes of MRSA isolates which were recovered from blood, pus, urine and catheter associated infections. Further, the amplicons of these genes were sequenced for their nucleotides in order to find the variability among these isolates as well as to determine the sequence homology to the standard strains whose sequences have been published. The sequences of the amplicons of \textit{mec-A-H} and \textit{pvl} genes from three isolates, one each from blood (isolate no. 64), urine (isolate no. 135), and pus (isolate no. 97) were submitted to National Centre for Biotechnology Information (NCBI) and have been assigned accession numbers which authenticates the specificity of the products amplified in the PCR assays.

In the PCR assays, we observed amplicon of \textit{mec-A hypervariable} region with expected band size of the 550 ±50 base pairs (bps) which is similar to that reported by Awadalla \textit{et al.}, 2010 in Egypt using the same primer pair as we utilized in the assay to amplify the \textit{mec-A-H} region. These primers were selected from Staphylococcus Chromosome Cassette (SCC) of \textit{S. aureus} and matched with the sequence of SCC IVa of standard strain R-99 (Forward primer- nucleotide no. 6691 to 6708 and reverse primer-nucleotide no. 7261 to 7141) completely without any mismatch. The specified numbering is in accordance with the sequence of SCC IVa (accession no. KF23420.1). Out of 44 isolate studied, the amplification of \textit{mec-A-H} was achieved in 40 isolates. The failure to achieve amplification in the remainder four might be due several factors such as, modification of normal PBPs genes, overexpression of normal PBPs, or overproduction of staphylococcal \(\beta\)-lactamases (Chambers \textit{et al.}, 1997). Another reason for not achieving amplification in four isolates could be that these isolates did not contain SCC type IVa. This is further supported by the fact that three of these did not carry \textit{pvl} gene also as amplification of this gene was also not achieved in these isolates. The BLAST analysis of nucleotide sequence of \textit{mec-A-H} gene amplicon of \textit{S. aureus} recovered from blood (isolate-64), urine (isolate-135) and pus (isolate-97) showed 98%, 97% and 99% homologies respectively to \textit{S. aureus} strains CMF 3119 (accession no. HF 569105.1), M299 (accession no. HM 030721.1) and \textit{S. aureus} strain M1
mec type IV Staphylococcus aureus cassette (SCC) sequences respectively. The nucleotide sequence analysis of mec-A-H gene amplicon revealed that MRSA isolates studied were of SCCmec type IV, the predominant cassette type present in CA-MRSA. Similar observation has been made by Christine and co-workers, 2011. CA-MRSA isolates usually have been defined as containing SCCmec type IV cassettes, expressing pvl (Mouse et al., 2009). Further, in present study, the nucleotide sequence homology among the MRSA isolates of different origins was used for determining their predicted amino acids sequences by Expasy translate tool analysis. The major amino acid substitutions were found in all the isolates of pus, urine and blood origin. On comparative analysis of the predicted amino acid sequences of mec type IV Staphylococcus aureus cassette (SCC) with that of standard strain M29, major amino acid substitutions were observed at positions 66, 85, 98, 122, 145 and 161. The amino acid substitution might have resulted due to mutations in the genomic DNA. The observed substitutions might be linked to the antibiotic resistance as well as to virulence of the strain which contribute to the pathogenesis of infections due to these isolates. However, further studies are required for understanding the role of SCC of S. aureus in drug resistance. Since the SCC type IV carries other antibiotic resistance genes and which are transmissible to other organisms. Amplifying the whole SCC mecIV would help further in providing valuable information in this regard.

Vancomycin has been used as the main antimicrobial agent available to treat serious MRSA infections but unfortunately, decrease in susceptibility of S. aureus to this antibiotic as evidenced by isolation of vancomycin intermediate and resistant S. aureus strains has further complicated the therapeutic management of MRSA infections. The resistance to this antibiotic has been reported from many countries of the world (Benjamin et al., 2010). Low level and intermediate vancomycin resistance has also been reported from the neighbouring state Uttar Pradesh in north India (Tiwari and sen, 2006; Veer et al., 2010). We observed a band of ~400 to ~500bps of van-A gene amplicon using the same primer pair was utilized by other groups (Tiwari and sen, 2006 and Venubabu et al., 2011). These workers observed similar size amplicon. The primers were derived from putative VanA protein which matched with the nucleotide sequence of standard strain Enterococcus cecorum (accession no.AB663321.1) (Forward primer- nucleotide no. 6978 to 7002 and reverse primer- nucleotide no. 8007 to 7993). There is evidence in support of the fact that VRSA strains acquired vancomycin resistance genes, such as van A, from vancomycin-resistant enterococci (VRE) in US (Witney et al., 2005). Such transfer was first described by Clewell and co-
workers in 1985 which was mediated through responsive drug resistance plasmids and mobile genetic elements, transposons. Since both enterococcus spp. and S. aureus are implicated in the nosocomial infections, there exists greater opportunity for the exchange of genetic material in the hospital settings. We achieved amplification of van-A gene segment in 38 out of 44 isolates. The failure to amplify the van-A in six isolate could be due to the absence of van-A gene in the isolates examined. Such isolate have been described as van gene negative VRSA by Tewari and Sen (2006). However, other workers suggested that some other mechanisms might be operating for vancomycin resistance (Cui et al., 2006).

The genetic transfer of virulence gene among the strains of the same bacterial species or between different species of bacteria is responsible for enhancing virulence and promoting emergence of new epidemics due to acquisition of virulence genes from external environment. The combination of drug resistance and virulence gene leads to emergence of superbug. We observed expected band size of 400-450 base pairs (bps) of pvl gene amplicon. The amplification was achieved in 38 out of 44 isolates. The failure to amplify the pvl gene segment in six isolates might be due to absences of pvl gene in these isolates. We used the same primer pairs as were utilized by Lina et al., 1999 to amplify this gene. Other workers have also observed the same amplicon size of pvl gene with these primers (Oaterry alli et al., 2013 and Anyanwu et al., 2013). These primers were derived from Panton-Valentine leukocidin chain S precursor and matched with the nucleotide sequence of standard strain SA-957 (accession no. CP003603.1) (Forward primer- nucleotide no. 1535189 to 1535219 and reverse primer-nucleotide no. 2444576 to 2444554). The BLAST analysis of nucleotide sequence of pvl gene amplicons of S. aureus recovered from blood (isolate-64) showed 99% sequence homology to S. aureus strain 7401 (accession no. AP 012341.1), strain FD SB11 (accession no. AB 678715.1) and S. aureus strain BJ-12 (accession no. AB 678712.1) Panton valentine leukocidin (LuckS-pv, LuckF-pv) nucleotide sequences, while sequences homologies of 94% and 92% homologies respectively were observed with urine isolate (no.-135) and pus isolate (no.97) with all the S. aureus strains 7401 (accession no. AP 012341.1), FD SB11 (accession no. AB 678715.1) and BJ-216 (accession no. AB 678717.1), Panton valentine leukocidin (LuckS-pv, LuckF-pv) gene nucleotide sequences. Utilizing the same primer pair, Mouse et al., (2009) amplified pvl gene and observed amplicons of 433-450 base pair fragments specific for lukS/F-PV and SCCmec sub type IV genes in multi drug resistant isolates of S. aureus which were identified as CA-MRSA by these workers on the basis of the presence of these marker genes. i.e., pvl and SCCmec type IV of CA-MRSA. Our findings
are similar to the observations of these workers. The nucleotide sequence homology of the strains of different origins revealed that they were closely related. These sequences were utilized for determining their predicted amino acid sequences by Expasy translate tool analysis. Major amino acid substitutions were recorded between the isolates of pus and urine origin. Similar analysis of pvl F and S on comparison to standard pvl positive strains FD SB11, revealed major amino acid substitution at positions 20, 21, 22, 40, 65, 76, 87 and 98. Majority substitutions were seen in pus isolates (no 97) as well as in case of urine isolate (no. 135). The amino acid substitution might have reason because of the replacement of nucleotides in genomic DNA due to mutations. Such substitutions might be linked to virulence of the isolates.

The MRSA strains of different origins contained mec SSC type IV cassettes as revealed by the amplification and sequencing of mec-A-H gene. This gene was amplified in 40/44 (90.90%) isolates. The MRSA strains having mecSCC type IV have thus been recorded in the state of Himachal Pradesh. In the present study, high proportions of the pvl positive MRSA isolates has been observed which is evident by amplification of pvl gene in 38/44 (86.36%) MRSA isolates. In the present study, we did not achieve amplification of both mec-A-H and pvl genes of three isolates which further reflects that these genes co-exist in the SCC type IV. This fact has been demonstrated by Shannon et al., 2007 as discussed earlier. Further three more isolates in which mec-A-H gene was amplified but the pvl gene was not which suggest that these isolates did not carry pvl gene. The combination of drug resistance genes and virulence genes leads to emergence of highly virulent strains which pose challenge in treating infections due to such strains. Also, the MRSA strains recovered from blood, urine and pus exhibited variability in nucleotide sequences of their amplicons. Such variation might play some role in the pathogenesis of MRSA infections.

RE analysis of PCR product is a preliminary screening method for epidemiological study of nosocomial infections caused by MRSA (Mitani et al., 2005). The digestion of the amplicon of van A with SmaI, revealed a single restriction pattern in which bands of ~300bps and ~200bps were observed. This enzyme was selected because it is four base cutter. However, a single band of variable size ~275bps to ~350bps (pus origin) were also seen which may represent either undigested DNAs of the amplicon or such isolates might have no SmaI restriction site for their amplicons. In case of pvl gene amplicons digestion, we found two distinct patterns. Pattern I: ~400bp to ~50bp and pattern II: ~375bp to ~100bp and ~50bp. The pattern I was seen in blood isolates while isolate of urine and pus origin exhibited
pattern II. The restriction pattern of \( pvl \) gene amplicons thus, reflected variability among the MRSA isolates of blood, urine and pus origins and might be useful for differentiations of strains. However, further studies are required to ascertain this.

Bacteriophage typing is an important tool for epidemiological investigation of MRSA strains. Particularly to determine endemic and epidemic strains. Strain typing is an integral part of epidemiological surveillance and infection control in hospitals. For over 24 years, the National Staphylococcal Phage Typing Centre in India has been using this method for typing \( S. aureus \) strains prevalent in different parts of the country. In the present study, using 23 international sets of bacteriophage, 86.48% of MRSA strains could be typed; only a 13.5% of the strains could not be typed by this method. Higher non typeability percentage have been recorded by others Witte \textit{et al.}, 1979 reported 20% and Mendhiratta \textit{et al.}, (2010) found 26.34% \( S. aureus \) strains as non-typeable. In the present study the most common phage group was mixed phage group comprising of (46.87%) strains. In phage group I, only 3.13% strains were typed. The MRSA strains of this group are associated with hospital acquired and endemic infections. We recorded 37.50% MRSA strains in phage group III. Strains carrying antibiotic resistance genes usually belong to this phage group. Similar observations have been made using tetracycline and streptomycin resistance (Ramani and Jayakar 1980). Only 12.50% strains in the present study belonged to phage group II and none of the strain belong to group V. Wide variation in the incidence of MRSA strains of different phage group has been reported by various workers from different geographical locations (Bhat \textit{et al.}, 1990). In India and other developing countries non-typeability of \( S. aureus \) strains is a major problem with the available sets of bacteriophages (Dugid 1989). Non-typeability can be reduced by using routine test dilution strength and heat shock treatment before phage typing of \( S. aureus \). Bacterium can alter its phage restricting activity, which is unlikely to occur at 37\(^\circ\)C (Rajwade \textit{et al.}, 1985). The bacteriophage typing is more sensitive than serotyping of bacteria (Rennie \textit{et al.}, 1978). This method is recommended as first line of approach in epidemiological investigation of MRSA strains, although PFGE remains the gold standard for characterization of outbreak strains (Mehndiratta and Bhalla, 2012). Although, bacteriophage typing is a sensitive method but it is cumbersome and time consuming as it requires propagation, standardization and maintenance of the phages (Paul-satyseela \textit{et al.}, 2011).

Despite the use of antibiotics for treating \( S. aureus \) infections recent improvements of medical services, MDR \( S. aureus \) is recognized as a major cause of nosocomial infections. This organism results in significant morbidity and mortality rates in nosocomial infections.
High levels of resistance to antibiotics have been associated in most instances with plasmids (Bhakta et al., 2003; Daini et al., 2006; Diep et al., 2006). Plasmids play a significant role in the biology of enterococci. They represent an immense reservoir of genetic variability and contribute to genetic exchange between bacteria (Norman et al., 2009). Plasmid profile analysis has been shown to be a good epidemiological tool in investigating epidemics or outbreaks of resistant strains (Mayer, 1988). The ability to detect and classify plasmids based on their phylogenetic relationship would provide an essential tool for investigating their distribution among bacteria and to elucidate their significance in the host cell, such as their role in dissemination of antimicrobial resistance. A simple method for plasmid detection would be a very useful tool to trace antibiotic resistance plasmids in a clinical setting, for epidemiological surveillance. In the present study, we detected a single plasmid less than 2kb. This finding is consistent with others (Diep et al. 2006, Uchechi and Erinma 2007 and Akinjogunla and Enabulele 2010). The detection of antibiotic resistance plasmids could be due to over zealous desire to treat every S. aureus infection (Daini et al., 2006). But in the present instance, the plasmid we observed has not been characterized for its association with antibiotic resistance or virulence. Further studies are required on this aspect. Also, studies on plasmid profiling of the isolates over a longer period of time are also required to know the exact profile of the plasmid encountered in MRSA strains in the shimla region.

Future projections

The studies can be extended further in order to establish the clonal lineage of the MRSA strains prevalent in the state of Himachal Pradesh. Also, further characterization of
SCCmec type IV is also required on *ccr* and chromosomal cassette. Other housekeeping genes and more virulence gene can be characterized for correlating virulence and antibiotic resistance. Also, the plasmid profiles of more isolates can be obtained to study antibiotic resistance elaborately. This would help in better understanding the mechanism of drug resistance and pathogenesis of MRSA infections and their epidemiology. The implementation of more efficient and advanced methods for strain typing such as MLST, SLST and Pulse filed gel electrophoresis (PFGE) can be applied for strain difference. Constant surveillance and regular monitoring of the HA-MRSA and CA-MRSA is also required for effective management of MRSA infections.