Review Article

APALM

Prevalent Clones of Methicillin Resistant Staphylococcus Aureus Strains in Pakistan by Various Typing Techniques

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ABSTRACT

Staphylococcus aureus (S.aureus) is an important pathogen that causes infections in both hospital and community settings. The resistant to methicillin in S. aureus emerged in 1960s and since then the prevalence of Methicillin resistant S.aureus (MRSA) has been increasing globally. The increasing incidence of MRSA infections demands a quick and reliable characterization of isolates and investigation of the clonal spread of clones in hospitals as well as in the community to generate information that helps health care professionals to implement appropriate measures to control these infections. To investigate the MRSA outbreaks, evaluation of transmission of MRSA strains and their evolution, bacterial typing techniques are important tools. The continuous surveillance and monitoring of the presence of MRSA in community and hospital settings is important to make a clear understanding of the dynamics of the spread of MRSA to assist in controlling its dissemination.

In this review the various typing techniques that have been used to type MRSA isolates from Pakistan have been discussed. This review also focuses on the various clones of MRSA that are reported in Pakistani community as well as in hospitalized patients.

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Introduction

Staphylococcus aureus (S. aureus) is the cause of infections, both in hospitals and community settings. With the passage of time it is exhibiting more virulence and acquiring resistance to antibiotics like methicillin and vancomycin. New strains of S. aureus with different virulence and antibiotic resistance profiles are emerging. Knowledge about the difference in S. aureus genome and factors that control these differences can better help us to control infections caused by S. aureus.[1] The difference in S. aureus genome can be recognized by using a reliable typing method which can also help us in introducing better treatment strategies.

S. aureus infections are difficult to treat due to their acquisition of different antibiotic resistant determinants. In 1942 penicillin resistant S. aureus was recognized in both hospital and community. In 1961 methicillin was introduced, but soon S. aureus strains became resistant to methicillin by producing altered penicillin binding protein.[2] MRSA was once limited to hospitals called as hospital acquired MRSA but in mid 1990s a second type of MRSA known as community acquired MRSA (CA-MRSA) was isolated, that has greatly changed the epidemiology of MRSA.[3][4]According to Center for disease control and prevention (CDC), MRSA infections diagnosed in an outpatient setting or within 48 hours of admission to hospital with no history of infection, any medical devices or indwelling catheters will be categorized as CA-MRSA infection. MRSA infection in persons, who have recent contact with a hospital or health care facilities, has undergone an invasive procedure within a previous year or are immunocompromised, should be taken as hospital acquired MRSA infections.

The increasing prevalence of MRSA around the world is becoming a serious problem for clinicians and a threat to public health. Studies have reported that the prevalence of MRSA in USA has increased from 6% in 1998 to 50% in the year 2002.The data from Europe and Asia also show increased prevalence of MRSA infections.[5] The same is the case with CA- MRSA. In various countries 50% of outpatient Staphylococcal infections are caused by CA-MRSA.[6] The prevalence of MRSA in Pakistan is found to be 42% by Hafiz et al. (2002).

Clinicians and epidemiologists can discriminate S. aureus isolates by using various typing techniques. The typing systems are usually used for the investigation of outbreaks of nosocomial infection, can aid in clinical treatment of a patient, allowing for discrimination between recurrent and successive infections and in broader context can contribute to the understanding of the epidemiology of infections.[8]

The aim of this review is to report all the clonal types of MRSA circulating in Pakistani community and hospitalized patients reported by using various typing techniques. This review describes the epidemiology of various clones of MRSA in Pakistan in order to have an understanding of various clonal types common in this region.

Phage Typing

The concept of using bacteriophages for the typing of Staphylococci was first developed in the 1940s.[11] Phage typing was the method of choice for characterizing the MRSA outbreak strains in the past.[12]The strains are classified according to the susceptibility to the set of selected phages. An internationally accepted set of 23 phages is used for the typing of human strains of S. aureus. The discriminatory power of Phage typing is greater than capsular typing and zymotyping. [11] Phage typing has been reported to be valuable in the identification of known epidemic strains among endemic strains and is preferred as a first line approach in epidemiological investigation of MRSA strains.[13] Some of the disadvantages of Phage typing includes that it is time consuming, technically demanding and lack reproducibility. Phage typing has been confined to the larger laboratories because it can be done more efficiently on large batches. In phage typing high proportion of MRSA isolates remains non-typeable.[11]The biologically active phages are maintained and available only at the reference laboratories.[13]

Phage Typing of S. aureus Isolates From Pakistan

Only one study was found regarding phage typing of S. aureus isolates from clinical specimen and skin of healthy and normal individuals in Karachi, Pakistan.110 S. aureus isolates were collected from clinical samples mostly from osteomylitis, septic arthritis and wound infection. The phage typing of these S.aureus isolates was done by using 8 phages including: 3A, 44A, 68, 71, 77, 187, 285A and Twort. Among the 110 clinical S. aureus isolates 16 isolates belong to Phage type 68, 26 isolates showed phage type 44A and 6 isolates showed phage type 187. The number of un-typeable isolates was 62.The 42 S. aureus isolates were collected from the skin of normal healthy and hospitalized individuals. Among 42 isolates 20 isolates showed phages type 71, 77, 187 and 3A. 22 S. aureus isolates were un-typeable. The phage type 187 isolated from the skin of normal and hospitalized individuals showed relationship with the phage type 187 isolated from osteomylitis specimens. Which shows the epidemiological importance of this phage types.[14]

Pulsed Field Gel Electrophoresis (PFGE)

In Pulsed field gel electrophoresis involves the microorganism is lysed in situ and the chromosomal DNA is digested by restriction endonuclease enzymes.[14]The DNA fragments are then moved on agarose gel, as the DNA is negatively charged because of presence of phosphate backbone, under an electric field it will migrate towards anode and finally separated in different size fragments.[15] DNA restriction pattern of isolates is then compared to determine their relatedness.[14] Restriction enzymes commonly employed in PFGE e.g EcoRI, Hind III, recognized the sequences that are dispersed around a typical 2-4Mb bacterial chromosome and therefore these fragments are difficult to be accurately discriminated by gel electrophoresis. The large fragments especially of size 40-50kb produce by these restriction enzymes cannot be properly separated by conventional gel electrophoresis. Various alternative electrophoretic approaches that are based on the principal of periodic reorientation of electric field relative to the direction of gel have been proposed. The two most commonly used such approaches are :

- Field inversion gel electrophoresis (FIGE)
- Contour-clamped homogeneous electric field electrophoresis electrophoresis (CHEF).

CHEF is used most commonly in laboratories. In case of S. aureus the most sui restriction enzymes are SmaI, Csp I, Sst II, Sgr AI. Sma I is the enzyme of choice for PFGE analysis of S.aureus.The live stock isolates of S. aureus that belong to the MLST type ST398 cannot be typed by PFGE because of presence of restriction site methylation. These isolates can be type by using different enzyme like Cfr9I.[16]

PFGE has many of the features of an ideal typing method and it is also regarded as the gold standard for MRSA. In case of PFGE all the isolates are typeable. Discriminatory ability of PFGE is also high. It is time consuming technique i.e. results are not available for several days. PFGE is also expensive technique. Handling of PFGE is quite laborious. Slight differences in running conditions can alter the distance travelled by each band that results in complicating the comparison of isolates run on different gels.[11] One of the drawbacks of PFGE is that the interpretation of results is little. Some workers have reported that the stability of PFGE is not sufficient for reliable application in long term epidemiological surveillance due to long evolutionary history of pandemic clones.[13] It is often difficult to compare results between laboratories.[17]

Prevalent PFGE Types in Pakistan

Zafar et al., 2011 performed PFGE typing of MRSA isolates collected from Pakistani's hospitals in Karachi, Pakistan. 126 MRSA isolates collected from the year 2006-2007 includes Community onset MRSA(CO-MRSA) (n=19) and hospital onset MRSA(HO-MRSA) (n=107)were typed by PFGE. In addition to these MRSA isolates, isolates (n=39) collected in year 1997 were also typed by PFGE. Visual analysis of PFGE finger prints were performed according to the criteria proposed by Tenover et al. (1995). [14] 39 MRSA isolates collected in year 1997 showed 2 PFGE pulsotypes including pulsotype A (n=28) and Pulsotype B (n=11).These PFGE pulsotypes showed 8 subtypes. The 126 MRSA isolates typed by PFGE classified into 9 pulsotypes and 33 subtypes. The predominant pulsotypes showed were type A (n=22) and type B (n=11). The less common pulsotypes were C, D, E and F. The 48 isolates showed subtypes A1-A15 and 27 isolates showed subtypes B1-B10. Some isolates showed fewer subtypes including C1 and C2, D1, E1-E4 and F. Pulsotypes G-I had no subtypes. In this study, the CO-MRSA isolates showed five PFGE pulsotypes including A, B, E, H and I. The HO-MRSA isolates showed six PFGE pulsotypes A, B, C, D, F and G. The subtypes A16 and A17 observed in MRSA isolates collected in 1997 were not observed in MRSA isolates collected in year 2006-2007 that showed changing occurring in molecular subtypes. The A and B pulsotypes showed that they are the local predominant epidemic strains in Pakistan.

In a study performed by Shabir et al., 2010.[18] 60 clinical MRSA isolates collected from hospitals in both India and Pakistan were typed by PFGE. 49 MRSA isolates were collected from Pakistan's hospitals and 11 MRSA isolates were collected from India's hospitals. 57 MRSA isolates that belonged to clonal complex 8 showed 24 PFGE profiles and 3 isolates that belonged to clonal complex 30 showed same PFGE profiles. PFGE efficiently distinguished between isolates of both countries.

Multi-locus Sequence Typing (MLST)

For typing of Neisserria meningitides MLST was first developed by Maiden et al. (1998) and since then this typing technique has been applied to man other pathogen.[17]

In MLST various numbers of loci, usually the house keeping gene's internal fragments are chosen that yields the specific sequence. Housekeeping genes are chosen because they are always present in specific specie and present the sufficient variation within the species.[9]The multiple markers or housekeeping genes that are chosen for typing should be physically scattered around the genome, This increases the discriminatory power of technique.[17] In case of MLST typing for S. aureus the seven loci that represent the housekeeping genes are chosen. These seven housekeeping genes are then amplified by Polymerase

chain reaction by using primers for conserved regions of these genes. The PCR products are then sequenced and the number describing the allele is assigned to each locus based on its sequence. In case of S. aureus, 30 alleles for each locus have been described this gives the possibility of over 20 billion different allelic profiles that can result in different strain types.[19]

The internal fragments of following genes are use for MLST typing of S. aureus:

1. Carbamate kinase (arcC),
2. Shikimate dehydrogenase (aroE),
3. Glycerol kinase (glpF),
4. Guanylate kinase (gmk),
5. Phosphate acetyltransferase (pta),
6. Triosephosphate isomerase (tpi) and
7. Acetyl coenzyme A acetyltransferase (yqiL)[20]

Advantages

In MLST typing each strain is defined by its allelic profile that consists of no more than seven numbers. The allelic profile of seven numbers is unambiguous and can be easily transmit over electronic media around the world. MLST data of various strain types exists on internet and can be accessible at the URL http://www.mlst.net.[19]

Disadvantages

Sequences in MLST evolve slowly and are highly conserved. MLST is not suitable for recent evolution events especially for short epidemiological studies. Sequencing of targeted genes also makes MLT expensive.[21]

Prevalent MLST clones in Pakistan

In a study performed by Zafar et al. 2011 for 19 CO-MRSA isolates and HO-MRSA belong to major PFGE pulsotypes collected in year 2006-2007 Karachi, Pakistan. Among the 18 CO-MRSA isolates the major sequence type observed was ST 8.12 isolates of CO-MRSA showed ST 8 sequence type. ST239 was represented by three CO-MRSA isolates. The remaining three isolates showed ST1, ST217 and ST1175. HO-MRSA isolates showed two sequence types, i.e. ST 239 represented by 14 isolates and ST8 represented by 11 isolates.

Shabir et al., 2010 performed MLST for MRSA isolates that belong to 14 major SIRU and PFGE profiles within CC8.Three STs were observed ST239, ST8, ST113 among isolates that belong to CC8. Among three MRSA isolates that belong to clonal complex CC30 one isolate was type by MLST that showed ST30.

In both studies ST239 was the major sequence type observed among hospital MRSA strains.

In one study performed by Khatoon et al. 2013 S. aureus isolates collected from tertiary care hospitals in Karachi, Pakistan were typed by a new touchdown single run PCR protocol for MLST. Khatoon *et al*., 2013 develop a new protocol for MLST that is based on amplification of seven housekeeping genes in MLST typing by a single run in PCR. This protocol seems to be less time consuming than the conventional protocol for MLST typing. [20]

Staphylococcal Cassette Chromosome Mec (Scc Mec) Typing

MRSA carry the Mec A gene present in the SCC mec element that is responsible for methicillin resistant. It has been proposed that MSSA have acquired the SCC mec element and became the MRSA.[22]

SCC mec elements consists of following two components 1) ccr gene complex 2) mec gene complex, which is composed of mec A gene. Therefore SCC mec have been classified in different types based on various alleles types of ccr gene and mec gene in SCCmec typing of MRSA. SCC mec typing classifies Sccmec elements on the basis of their structural differences (Sccmec 1). Currently, various mec and ccr allotypes have been discovered among Scc mec elements, including almost eight types of SCC mec from type I to type III and along with various subtypes. Health care associated MRA strains contain Type I, type II and type III SCCmec types. Community associated MRSA strains contain type IV and type V types. A combination of two approaches like SCCmec typing along with MLST is recommended for reliable typing for multicentre surveillance, inter-hospital and international transmission and evolution of MRSA strains.[13]

Prevalent clones in Pakistan

Zafar et al., 2011 performed SCC mec typing for 126 MRSA isolates collected in year 2006-2007.102 MRSA isolates were collected from Agha khan hospital, Karachi province. 12 MRSA isolates from Sindh,8 isolates from Punjab and 4 from north-west frontier province, Pakistan. 39 MRSA isolates collected in year 1997 from Karachi, Pakistan. Among 126 MRSA isolates eight SCC mec types were observed. Among 126 MRSA isolates 78 isolates showed SCCmec type III or type III variant.38 isolates showed SCC mec type IV or type IV variant.9 MRSA isolates showed SCC mec type IV variant 1. One isolates showed unique type of SCC mec that lacked 342bp band and was named as SCC mec SCCmec type III variant. In community settings it was SCC mec type IVA and type IV. Only two types of SCCmec types including SCCmec type III variant I and SCC mec type type IV variant 2. The predominant SCC mec type among hospital setting was

IVA was observed in HO-MRSA isolates collected in year 1997.The MRSA isolates collected in year 2006 showed more diverse SCCmec types.

The SCCmec typing of MRSA isolates collected from different hospitals in Lahore was also performed by Romezza et al. 2010. 35 MRSA isolates collected from various hospitals in Lahore, Pakistan during the period February –May 2008. Among 35 MRSA isolates 21 MRSA isolates they showed SCCmec type IA, variant of SCCmec type I. SCC mec type IIC was observed in 14 MRSA isolates. SCCmec type IIC variant differs from SCC mec type II because it lacks amplification of 284bp and 209bp.

Among 60 MRSA isolates genotyped from India and Pakistan SCCmec type III and SCCmec type IV was observed in isolates from Pakistan. Among two Clonal complexes observed in Pakistan CC239 and CC30 the SCCmec type III and SCCmec type IV was observed. [18]

Multiple Locus Variable Number Tandem Repeat Analysis:

Most tandem repeats are small segments of DNA repeated one after another.

Number of tandem repeat sequences having different length, DNA sequences, and different copy numbers per genome has been identified in prokaryotes as well as in eukaryotes.[23] Mycobacterium tuberculosis, the first bacterium in which the tandem repeats were identified.[24] Later tandem repeats have also been found in Bacillus anthracia, Legionella pneumophille, Pseudomonas aeroginosa, Salmonella.[25]

Multiple locus variable number tandem repeat analysis is fast, easy to use, and highly sui typing method with low costs. It does not need exotic reagents and is therefore can be performed in any laboratory that is equipped with PCR machine. The data obtain from MLVA can be used in electronic databases.[26] MLVA is highly discriminatory as compared to PFGE, spa typing and MLST. [21][27]

MLVA scheme for typing of S. aureus was first described by Sabat et al. Then hardy et al., presented a set of seven loci named as Staphylococcal interspersed repeat unit typing and recently a slightly adapted version of Hardy's MLVA is presented by Ikawaty et al. 2008.[26] Pourcel *et al*. 2009. also used set of different VNTRs for S. aureus typing.

Staphylococcal Interspersed Repeat Unit(SIRU) Typing

Seven VNTRs have been identified in S. aureus genome and these VNTRs are named as staphylococcal interspersed repeat units (SIRUs) by Katherine J Hardy.

Staphylococcal interspersed repeat unit typing was developed by Hardy in 2004.The seven sequenced S. aureus genome was analyzed for the presence of VNTRs. Total 26 tandem repeats were found out of which seven repeats units were found in seven sequenced genomes of S. aureus with high similarity of their flanking regions. These seven repeats were called as staphylococcal interspersed repeat units by hardy. Each SIRU locus in strains of S. aureus were having different number of repeats and therefore can use for S. aureus typing.

The seven sequenced genomes of S. aureus were analyzed to find the presence of VNTRs. Seven tandem repeats were found that were present in all seven sequenced S. aureus genome. These repeats then named by Hardy as Staphylococcal interspersed repeat units. These loci have highly conserved flanking regions and carry varying number of repeats hence can be an appropriate marker for typing S. aureus.

Four SIRU locus i.e. 16, 15, 21, and 13 occur only once in the S. aureus genome. These 4 VNTRs have similarity in their flanking regions.

Four loci i.e. SIRU locus 16, SIRU locus 15, SIRU locus 21, and SIRU locus 13 occur only once in the genome while the remaining three loci i.e. locus 5, locus 7, locus 1 occur at multiple sites on S. aureus genome. From the multiple sites SIRU loci only those were selected that showed high similarity of flanking region between different sequenced S. aureus genome.

Six of seven SIRU loci are present in the non coding region. One SIRU locus 21 is in the coding region of protein A gene.[28] Protein A is a cell wall anchored protein and has a possible role in S. aureus virulence.[24]

Advantages

SIRU typing is easy to perform, and results obtain are transpor . It has high discriminatory ability to the characterization of MRSA in an endemic setting. [29] It is faster and easier to perform technically. SIRU typing is cheaper to perform. [28] Moreover VNTRs of S. aureus are s under laboratory conditions.[26] Which makes the SIRU typing a reliable typing method.

Among seven SIRU loci, some five SIRU loci i.e. SIRU locus 1, SIRU locus 7, SIRU locus 13, SIRU locus 15 and SIRU locus 116 are conserved during evolution and SIRU locus 5 and SIRU locus 21 show high variability thus giving information about recent evolution. Therefore, by using SIRU typing different levels of evolution can be determined SIRU typing has been successfully used by Katherine J hardy to distinguish various endemic MRSA strains. The discriminatory powers of SIRU typing have been found to be 99% by Conceicao et al. 2009.[28]

Prevalent SIRU types in Pakistan

CC8 was the dominant clonal complex observed in both India and Pakistan. Within CC8 SIRU (Staphylococcal Interspersed repeat unit typing) and PFGE divided isolates into 10SIRU profiles and 24 PFGE profile. In SIRU typing the variation was observed in locus 1, 7 and 21predominantly that differ by a single repeat. Seven SIRU profiles were distinct to Pakistan among 10 SIRU profiles. The SIRU profile 2113722 was predominant in both India and Pakistan. The most common SIRU profile in Pakistan was 3113822 observed in a cluster of 11 isolates. [18]

Discussion

MRSA strains seems to be derived from a small number of strains and therefore belong to genetically restrict group as compared to MSSA strains which shows the significant genetic diversity. Because of low genetic diversity of MRSA strains, the typing technique with high discriminatory power should be employed for typing of MRSA strains. The knowledge about the clonal spreading within a hospital is important, that can help us to generate enough information to permit the implementation of appropriate measure for the control of these infections allowing for the containment of an outbreak. [8]

There is a need for having continuous surveillance and monitoring of the presence of MRSA in hospitals as well as in the community and a complete understanding about the dynamics of the spread of MRSA is important in order to control the dissemination of MRSA.[9] Therefore, future surveillance studies can play an important role in elucidating the various changes that are occurring in the clonal distribution of MRSA strains and in designing and evaluating interventions to control the spread of MRSA.[4]

The traditional typing methods for S. aureus have been replaced by molecular typing methods. All the properties that an ideal typing method should possess, molecular typing methods have that like good typeability, reproducibility, stability and discriminatory power. They also show convenience in terms of accessibility, ease of use and ease of interpretation.[10]

Conclusion

The MRSA is continuously evolving which can be a threat in future. The data about the clonal types circulating in Pakistan is not enough and further studies should also be done to report the MRSA clones circulating in the Pakistani community and even in animals too.

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Competing Interest

None declared

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