RAPID DISCRIMINATION AMONG METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATES USING VARIABLE NUMBER TANDEM REPEAT ANALYSIS IN A SAMPLE OF IRAQI PATIENTS AND

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ABSTRACT

There is so little information available in Iraq about genetic variability in methicillin resistant *Staphylococcus aureus* (MRSA), so current study aimed to use six tandem repeat loci of multilocus variable number tandem repeat (MLVA) typing to discriminate among MRSA, so to achieve the aim of this study, six loci, *clfA, clfB, sdrC, spa, sspa* and *sav1078* were selected for multiplex PCR. The PCR product were subjected to capillary electrophoresis by using ABI-Genetic analyzer, then the data were analyzed by using GeneMapper™ Software 5. Fragment sizes were converted into repeats number. The total number of repeats are used to generate allelic profile. The allelic profile used to draw the minimum spanning tree and dendrogram , all the 85 MRSA isolates are clustered into 54 MLVA type.

Key words: Multiplex PCR, minimum spanning tree, capillary electrophoresis, repeat number.

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تحليل العدد المتغاير للتكرار الترادفي في بين بكتيريا المكورات العنقودية الذهبية المقاومة للميثيسيلين باستخدام التميز السريع عينات من مرضى عراقيين وبيئات المستشفيات

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المستخلص

هناك معلومات قليلة جداً متوفرة في العراق حول التغاير الوراثي في بكتيريا المكورات العنقودية الذهبية (MRSA)، لذا هدفت الدراسة الحالية الى استخدام ستة من مواقع التكرار الترادفي من تنميط العدد المتغاير للتكرار الترادفي متعدد المواقع (MLVA) لغرض التميز بين بكتيريا المكورات العنقودية الذهبية المقاومة للميثيسيلين، لذا لغرض تحقيق الهدف من هذه الدراسة تم اختيار ستة مواقع (*clfA, clfB, sdrC, spa, sspa* and *sav1078*) لتفاعل البلمرة المتسلسل المتعدد. تم اخضاع ناتج البلمرة الى الترحيل الكهربائي الشعري باستخدام المحلل الجيني–ABI، وبعد ذلك حُللت البيانات بواسطة برنامج GeneMapper™ Software 5. تم تحويل حجم القطع الى عدد التكرار. استخدم العدد الكلي للتكرار لانشاء الملف الاليلي الخاص بهذه المواقع. استخدم الملف الاليلي لغرض رسم الحد الأدنى من شجرة الامتداد والمخطط الشجري، ولوحظ تجمع الخمسة وثمانون عزلة الى أربعة وخمسون نمط MLVA.

الكلمات الافتتاحية : تفاعل البلمرة المتسلسل المتعدد، الحد الأدنى من شجرة الامتداد، الترحيل الكهربائي الشعري، عدد التكرار.

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INTRODUCTION

Staphylococcus aureus is linked nosocomial and community-acquired disease and it is considered an important bacterial pathogen (6). Endocarditis, pneumonia, deep abscess formation, bacteremia and variety of clinical syndrome can be caused by *S. aureus*, this bacteria appear to be widespread and is more often than not produce clinical symptoms. Carriers may transfer the pathogen infecting individuals who may develop disease. In the past few years methicillin resistant *S. aureus* bacteria has been rapidly emerged due to excessive usage of methicillin within hospitals worldwide (27, 22, 25). Unambiguous characterization of *S. aureus* isolates is needed to control MRSA infections through studying the impact of measures and understanding the population biology of *S. aureus*. Analysis of *S. aureus* requires many techniques of typing such as phage typing (29), ribotyping (15), random amplified polymorphic DNA analysis (28), sequence analysis of 16S–23S rDNA spacer regions (14), amplified fragment length polymorphism (13) and *SSCmec* typing (17). Pulse Field Gel Electrophoresis (PFGE) (16), Multilocus Sequence Typing (MLST) (5) and spa-sequence typing (10) are the most used typing techniques worldwide. Recently a technique known MLVA which is multiple-locus variable-number tandem repeat analysis that knowing by its ability to typing bacterial pathogens in large number (4, 7, 8, 11, 19, 20, 21). Epidemiological studies has been done through creation a DNA profiles by using the differences in the sequence numbers of short tandem repeat that knowing as VNTR. Many MLVA schemes of *S. aureus* bacteria have been made and utilized to type this bacterial pathogen. Advances in molecular typing was done through genome sequencing projects by analysis of VNTR loci that's localized in the genomes of prokaryotes and eukaryotes. At the same locus, the repeat number units different from one strain to another and can be investigated by technique of PCR by using specific flanking primers. Several VNTR loci have been indicated by genome sequencing of *S. aureus*, these include *spa*, *clfA*, *clfB*, *sdr* and *sspA* (24). In Iraq, there is so little information available about genetic variability in MRSA regionally, so current study aimed to use six tandem repeat loci (*sdrC*, *spa*, *clfB*, *clfA*, *sspA* and *sav1078*) of MLVA typing to discriminate among MRSA.

MATERIALS AND METHODS

Isolates and phenotypic identifications

Eighty five isolates of MRSA were obtained from previous study. The *S. aureus* isolates were re-cultured on Mannitol salt agar medium and confirmed by using BHIA then incubated overnight at 37C°. Positive isolates for Mannitol salt agar where selected for testing by Gram stain, catalase, coagulase and oxidase test (18). MRSA confirmed by using HiCrome MeReSa Agar Base medium (26).

Molecular techniques

DNA extraction: Genomic DNA was extracted by using genomic DNA purification kit (Promega company/USA). The purity and concentration were measured by using Quantus (Promega/USA). The integrity of DNA samples were tested by gel electrophoresis.

PCR

PCR was used to confirm the isolates as reported by the previous study by using *nuc* and *mecA* genes (3, 30).

Multiplex PCR based multilocus variable number of tandem repeats (MLVA) assay

The multiplex PCR was used for MLVA typing by using six genes included *spa (*Protein A gene), *sdrC* (Ser-Asp-rich fibrinogen binding proteins), *clfA* (Clumping factor A), *clfB* (Clumping factor B), *sspa* (Serine protease V8) and *sav1078* (Hypothetical Protein). The fluorescent labeled primers used are given in the Table 1, *clfA*, *sdrC* and *sav1078* were multiplex separately from *clfB*, *spa* and *sspA*.

Table 1. The fluorescent labeled primers used in this study

Gene Name	Primer Sequence (5`→ 3`)	Dye	References
clfA (Clumping factor A)	F-GCATTTAATAACGGATCAGG R-TGAATTAGGCGGAACTACAT	FAM	21
clfB (Clumping factor B)	F-ATGGTGATTCAGCAGTAAATCC R-CATTATTTGGTGGTGTAACTCTT	VIC	21
sdrC (Ser-Asp-rich fibrinogen binding proteins)	F-ATGATTTCACACTTGATAATGGC R- GCTGTTTTATGCTGATCTTTAAC	VIC	21
spa (Protein)	F-AGCACCAAAAGAGGAAGACAA R-GTTTAACGACATGTACTCCGT	FAM	21
sspA (Serine protease V8)	F-ATCMATTTYGCMAAYGATGACCA R-TTGTCTGAATTATTGTTATCGCC	Ned	21
sav1078 (Hypothetical Protein)	F-GTGCATAATGGCTTACGAAT R-TGGGAGGAATTAATCATGTC	FAM	21

F- Forward
R- Reverse

A thermal cycler (BioRad, USA) were used to amplify PCR reactions. Reaction mixtures were established as follows: GoTaq®Green Master Mix (1X) (12.5µl) (promega company /USA), that composed of MgCl2, deoxynucleotides (dNTP), Taq DNA polymerase, reaction buffer, green and yellow dyes that used to observe progress during electrophoresis; various concentration of each utilized primers (10 pmol)(0.75µl), (50-80) ng/µl (2µl) of DNA template then free nuclease water was added to obtain the final volume (6µl). The products of PCR were separated by using agarose gels (2% agarose,1µ of ethidium bromide (10mg/ml),1X TAE), scanned, and analyzed on OWL Electrophoresis System (Thermo, USA). The mixture of reactions were taken through thermocycling conditions as shown in Table 2. The product of PCR were introduced to gene scan in ABI-genetic analyzer for size analysis. Gene scan peaks were analyzed using the Applied Biosystem GeneMapper™ program 5 to obtain the exact PCR product size.

Table 2. The program used in multiplex PCR analysis

Steps	Temperature	Time	Cycle No.
Initial denaturation	105C^{o}	5min.	1
Denaturation	94C^{o}	30sec.	30
Annealing	53C^{o} for (*clfA, sdrC* and *sav1078)* 61C^{o} for (*clfB, spa* and *sspA)*	30sec.	
Extension	72C^{o}, 2min.	2min.	
Final extension	72C^{o}	5min.	1
Hold	4C^{o},	~	1

VNTR calculations and MLVA Data analysis: The tandem repeat numbers for whole loci were calculated from the standard sequenced strain *S. aureus* NCTC8325 utilizing a program of Tandem Repeats Finder (http://tandem.bu.edu/trf.html) version 4.03 (2). PCR products from this strain were used as a reference for calculation of repeat numbers in the tested strains. The mathematical formula used for calculation of repeat numbers was as follows: size of PCR product minus size of the flanking region divided by the size of the repeat units.

Phylogenetic analysis: Tandem repeat numbers gained for every loci to each isolates were introduced into BioNumerics software: version 7.6.1 (https://applied-maths.com) for phylogenetic tree to be calculated. In the trees every circle act a particular genotype (MLVA) and the size of circle represents strain numbers in each particular genotype and the color of the circle represents the isolation source of each strain. Lines between the circles represent repeat numbers variation. The isolates varying at one locus are connected by bold lines and those differ at more than one locus are linked by thin dotted lines.

RESULTS AND DISCUSSION

Isolates and phenotypic identifications

All isolates showed typical *S.aureus* on Mannitol salt agar, positive coagulase, and catalase and negative oxidase test, in addition

to Gram positive grape clusters on microscopic examination. All isolates Identified as MRSA on HiCrome MeReSa Agar Base medium.

Molecular identification

All isolates showed positive reaction for amplification of *nuc* and *mecA* genes by identifying *S. aureus* (276bp) and MRSA (147bp) respectively.

Multilocus variable Number of Tandem Repeats (MLVA) Analysis of MRSA Isolates: The approach of MLVA was introduced to test these MRSA isolates as a cost-effective and speed method to determining their phylogenetic relationships. In the current study the fluorescent labeled primers were used for performance of capillary electrophoresis assay on ABI genetic analyzer as given in material and methods. ABI sequencer was used for analyzing of PCR products of MRSA isolates and this method of MLVA typing considered robust and more accurate in comparison to gel, this for its power to separate products PCR with little variations in its size through providing a delicate sizes for each PCR product. Chosen of VNTR was based on established MLVA protocols (9, 12). Six loci included *spa (*Protein A gene), *sdrC* (Ser-Asp-rich fibrinogen binding proteins), *clfA* (Clumping factor A), *clfB* (Clumping factor B), *sspa* (Serine protease V8) and *sav1078* (Hypothetical Protein) were chosen for the multiplex PCR for typing of MLVA. The chosen primers from 6 loci were multiplexed in two separate PCR reactions. *clfA*, *sdrC* and *sav1078* primers as showed in Figure 1 and Figure 2 were multiplexed together separately from *clfB*, *spa*, *sspa* in order to prevent band overlap and to avoid the fake band formation. Before capillary electrophoresis the amplified product were also checked on agarose gel stained with ethidium bromide in order to check the amplification.

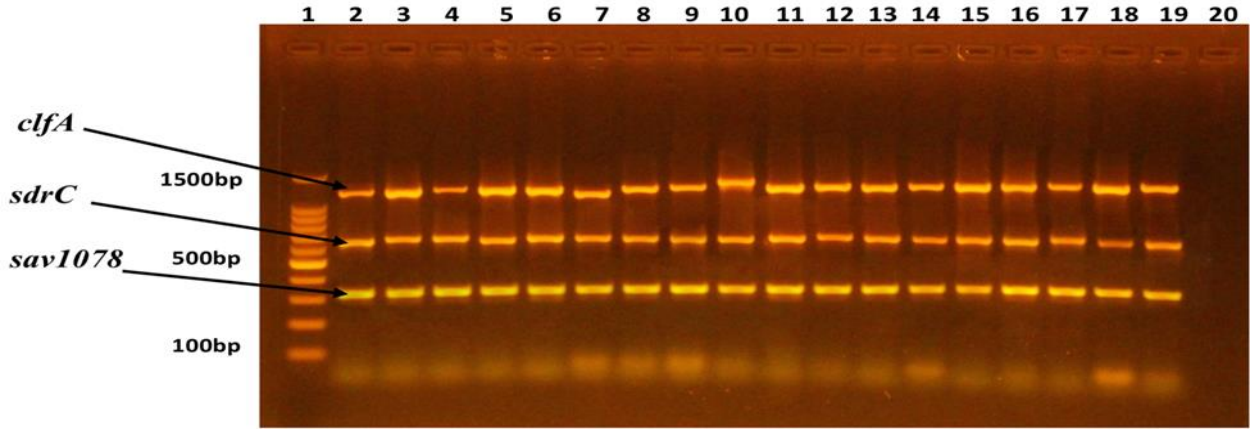

Figure 1. Gel electrophoresis of *clfA* (957bp), *sdrC*, (646bp) and *sav1078* (319bp), lane (1) represents the 100bp DNA ladder, lanes (2-19) represent the MRSA isolates and lane (20) represents negative control, which has been separated on 2% agarose gel (90 V, 1X TBE buffer) for 1hour and has been visualized under U.V. lights post EtBr staining

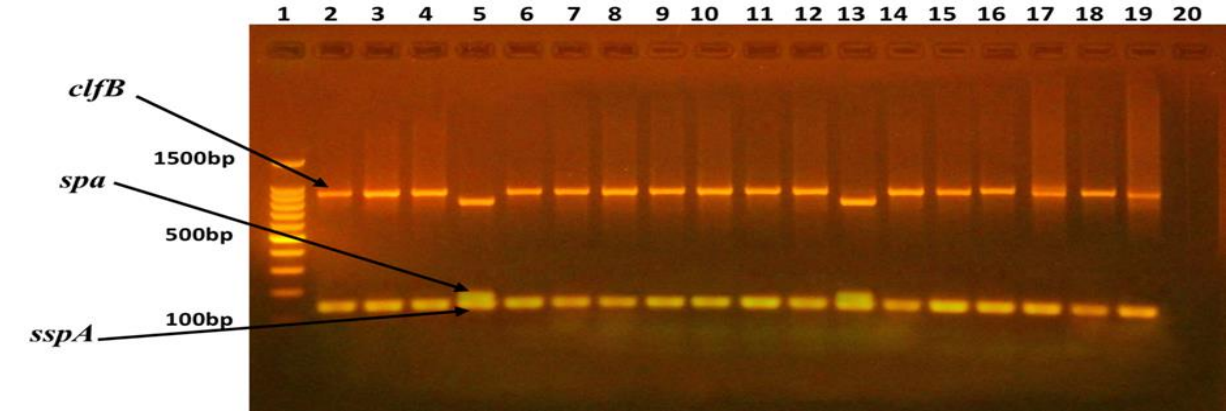

Figure 2. Gel electrophoresis of *clfB* (828bp), *spa*, (290bp) and *sspA* (155bp), lane (1) represents the 100bp DNA ladder, lanes (2-19) represent the MRSA isolates and lane (20) represents negative control, which has been separated on 2% agarose gel (90 V, 1X TBE buffer) for 1hour and has been visualized under U.V. lights post EtBr staining

PCR product obtained after two multiplex PCR were subjected to capillary electrophoresis by using ABI-genetic analyzer. The data obtained from capillary electrophoresis in the form of peaks were analyzed by using GeneMapper™ Software 5 as showed in Figure 3.

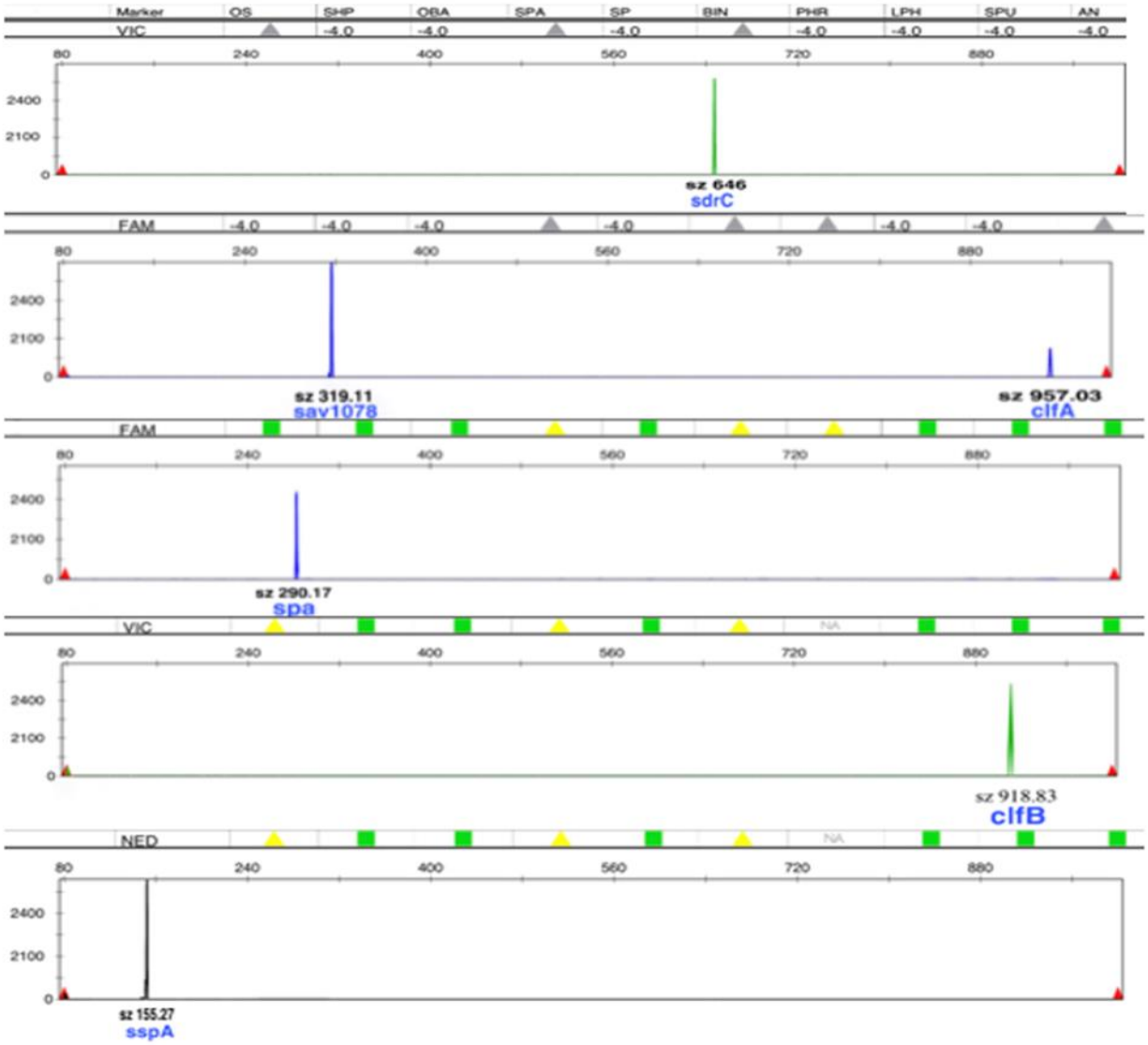

Figure 3. Capillary electrophoresis of *sdrC, clfA, sav1078, spa, clfB* and *sspA*. The results analyzed by GeneMapper™ Software 5

The fragment sizes obtained for each locus for all the isolates were converted into the repeats number. For this purpose the sequence data for the standard strains NCTC8325were imported into the program of tandem repeats finder (http://tandem.bu.edu/trf.html), this program in Bioinformatics introduced by Gary Benson, Boston Version 4.0 (2). The software identified the repeat numbers present in the DNA sequences of the six loci *clfA*, *sdrC*, *clfB*, *spa*, *sspA* and *sav1078*. The size of the flanking regions was assessed by subtracting the repeat containing region from the total amplicon size. In this way we got the length of flaking region, length of VNTR region, and size of repeat unit from the tandem repeat finder version 4.0. Then this approach was used to calculate the repeat numbers found in the PCR amplified products obtained from other MRSA strains. Total repeat numbers were identified by using the following formula: In this way we got the number of VNTR for all the loci. The total number of repeats are used to generate allelic profile (1).

$$\text{Total repeat numbers} = \frac{\text{size of the PCR product} - \text{size of the flanking region}}{\text{size of the repeats unit}}$$

In order to draw the minimum spanning tree (Phylogenetic tree), the repeats obtained with five loci for each isolate were feed into the

Bionumeric program. The order of allelic profile fed into the Bionumeric was *clfA-sdrC-clfB-spa-sspA*. The gene *sav1078* was not used in clustering as all the isolates were similar in PCR product. Minimum spanning tree (Phylogenetic tree) of 85 bacterial isolates derived from VNTRs of *spa, sdrC, clfA, clfB,* and *sspA* loci using software provided by Bionumerics is shown as Figure 4. In the phylogenetic tree circle colors represents the sources isolation of each isolate and the size of circles are proportional to isolate numbers present in each node. The isolates of MRSA that differs by repeat at a one VNTR locus are linked with bold lines, in other hands the isolates differ at two or more VNTR loci are shown linked with dotted thin lines. All the 85 MRSA isolates are clustered into 54 MLVA type or genotype without applying cut off as shown in Figure 4. Arfat, (1) mentioned that 123 MRSA isolates clustered into 63 genotypes. It is clear that these isolates are closely linked as majority of MLVA type are connected with bold lines and it is also clear in the Figure 4 that most of the genotypes are originating from genotype 33 that is contain only one isolate which is isolates number 23. This distribution of strains from different hospitals into the same MLVA type suggests that there is no association between specific strains and hospitals. This might be due to the fact that these hospitals are not geographically isolated from each other and visits of patients between hospitals are common resulting in frequent exchange of strains between these closely linked hospitals. Bionumerics software use to generate minimum spanning tree by using provided MLVA profiles to know genetic relationships of different MLVA types (1).

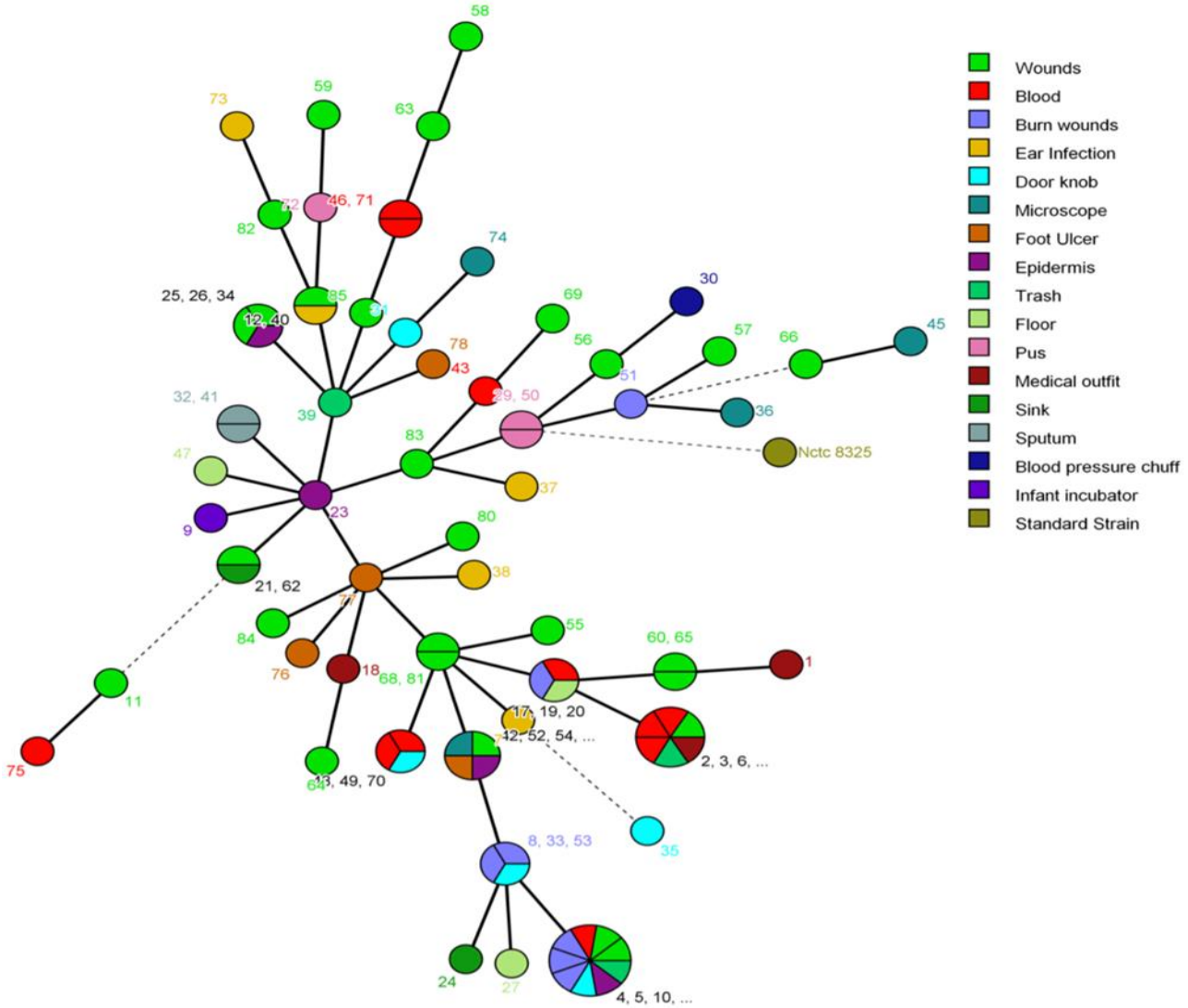

Figure 4. Minimum spanning tree of 85 MRSA isolates derived from VNTRs of *clfA, sdrC, clfB, spa* and *sspA* loci using Bionumerics software

The allelic profile was also used to draw the dendrogram.The dendrogram obtained from the MLVA types (genotypes) of the 85 MRSA isolates and one standard strains is shown in Figure 5. The dendrogram adaptation technique was UPGMA with (80%) a cluster cut-off. Sources of isolation, Strain names and time of isolation are also noticed in the Figure 5. All the 85 MRSA were clustered into 54 genotypes at 100% similarity (without cut-off) and clustered into 25 group when 80% cut-off technique was used. As mentioned by Sabat et al (23), the MLVA clusters gained by using a cluster cut-off of 88% never clustered isolates that were diagnosed as being unrelated by MLVA and spa typing. The isolates of MLVA groups defined by a cluster cut-off 88% were mostly clustered in the matching spa-CCs and widely in the matching MLVA-CCs. for epidemiological typing, MLVA groups identified with 88% cut-off value are the most appropriate in identifying outbreaks and detection transmissions routes as well as sources (23).

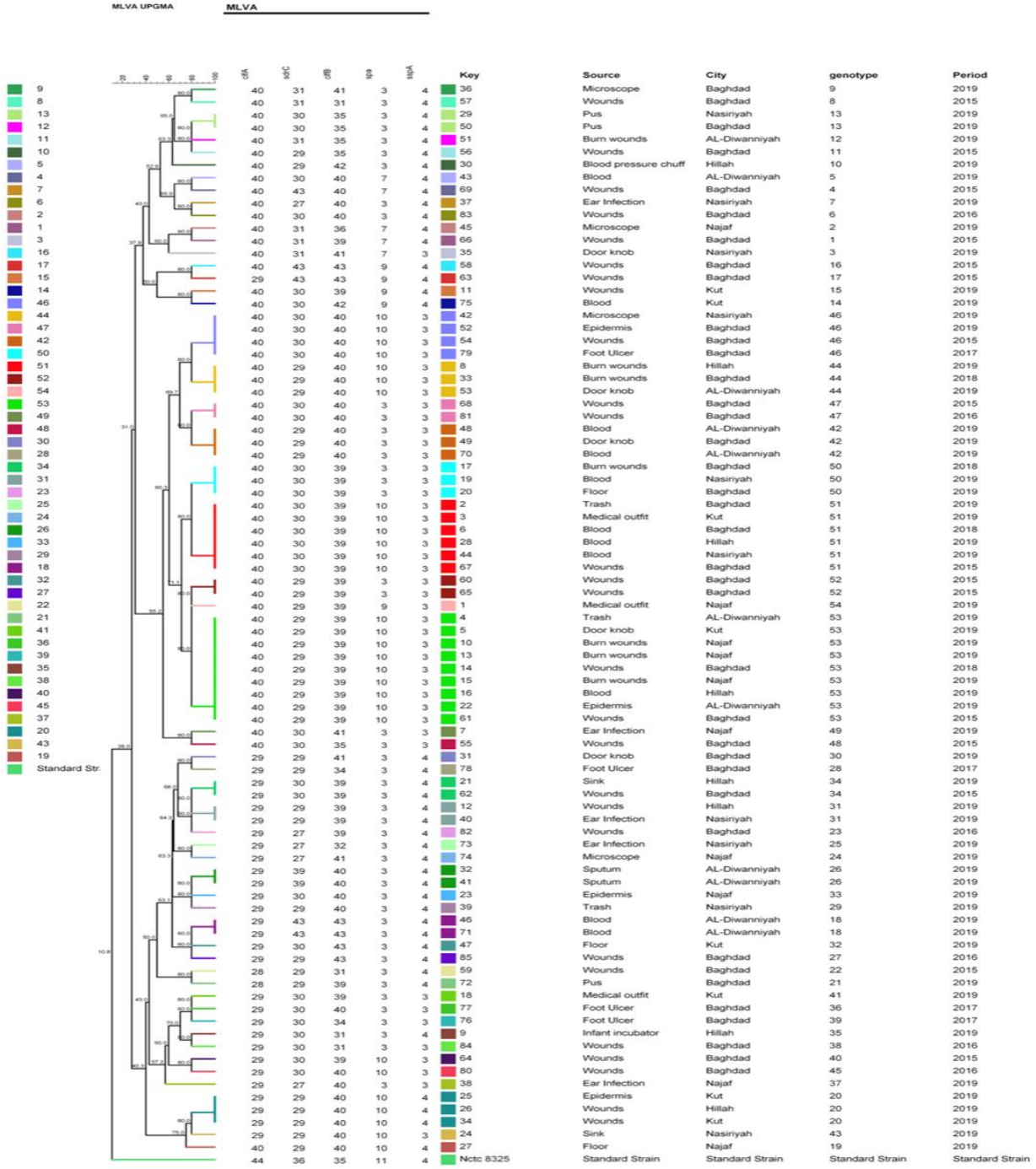

Figure 5. Dendrogram base on clustering with respect to MLVA types of MRSA Isolates represented by UPGMA. 54 haplotypes are shown at 100% similarity, which clustered into 25 groups when 80%cut off technique was used

CONCLUSION

Using of MLVA led to clustering of MRSA isolates rapidly and robustly for detection of localized outbreaks. MLVA typing may also give knowledge about the processes of evolutionary as different the repeat numbers at various loci considered evidence of which VNTR are prone for natural selection producing variation in higher levels. In current study, more variation in *clfB* and *sdrC* than in *sspa*, *spa* and *clfA* are observed. Additionally changing in the number of repeats was not needed stepwise but may have happened due to large jumps. Large jumps might be happened during replication of DNA because of deletions due to slipped strand mispairing or might be happened during replication as result of insertions and deletions.

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