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Genotypic identification of *Pseudomonas aeruginosa* strains isolated from patients with urinary tract infection

Mohammad Estaji[a], Mohsen Tabasi[b], Fatemah Sadeghpour Heravi[c], Jamil Kheirvari Khezerloo[d], Amin Radmanesh[b], Jamshid Raheb[a], Mohammad Reza Ghadirzadeh[b], Azardokht Tabatabaei[e,*]

[a] Molecular Medicine Department, Medical Institute, National Institute of Genetics and Biotechnology, Tehran, Iran
[b] Legal Medicine Research Center, Legal Medicine Organization, Tehran, Iran
[c] Pertussis Reference Laboratory, Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran
[d] Young Researchers and Elite Club, Islamic Azad University, Tehran Medical Sciences Branch, Tehran, Iran
[e] Research Center of Pediatrics Infectious Diseases, Iran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background: Numerous nosocomial infections including urinary tract infection (UTI) have been reported to be linked to *Pseudomonas aeruginosa (P. aeruginosa)*. This bacterium is one of the most common pathogen colonized in the urinary tract. The main purpose of this study was to evaluated the presence of antibiotic resistance genes and also the most frequent genotype patterns of *P. aeruginosa* in the patients with UTI hospitalized in different wards of hospitals.

Materials and methods: In this study, 70 strains of *P. aeruginosa* isolated of urine samples from the patients with UTI were assessed. The isolated strains were genotyped using Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) method. We have also analyzed the presence of *TEM* and *SHV* resistant genes in the isolates.

Results: A total of 70 *P. aeruginosa* strains was isolated from the UTI patients. Based on MLVA method, 61 various genotypes of *P. aeruginosa* were identified which grouped into two main clusters and 4 sub-clusters. Moreover, approximately 80% and 70% of isolated strains carried the *TEM* and *SHV* resistance genes, respectively.

Conclusion: Our findings showed that the majority of patients hospitalized in different wards of hospitals have experienced the urinary tract infection caused by *P. aeruginosa*. According to the genotyping results, a high diversity of the *P. aeruginosa* population was observed in the patients with UTI. Our results can provide a better understanding of the *P. aeruginosa* genotype distribution and epidemiology of infection, which can be applied as basic data for future antibiotic therapies.

1. Introduction

Urinary tract infection occurs when the bacterial pathogens attack the kidney, ureter, bladder, prostate and the urethra [1]. Detection of greater than 10^5 bacterial pathogens per milliliter of the clean-catch urine sample is regarded as the definite diagnosis of UTI. Bacteriuria (the presence of bacteria in the urine) has been detected in approximately 10–20% of the patients who use urethral catheters. Bacteriuria is mainly caused by *E. coli, Proteus* spp., *Pseudomonas* spp., *Klebsiella* spp., and *Serratia* spp.

Also, bacteremia caused by gram-negative bacteria emerges in 1–2% of cases as the complex consequence of catheter-related UTI [2]. In contrast, about 6 million non-catheterized patients particularly women experienced acute urinary tract infection in the United States which can be deteriorated by sexual activity in females.

A diverse range of studies asserted the high prevalence of bacteremia in Iranian hospitals located in different cities such as Sari (12.6%) [3], Hamadan (34.2%) [4], and Tabriz (13.2%) [5]. Gram-negative bacilli are the most common cause of UTI among which *E. coli* is responsible for 80% of UTI in the patients without urologic disorders or kidney stones. Other bacilli, including *Proteus, Klebsiella, Enterobacter, Serratia,* and *Pseudomonas* play a key role in complex nosocomial infections, which are primarily associated with relapsing infections accompanied by the obstructions or urinary tract stones [1]. Based on the previous study, 5% of complex nosocomial infections were attributed to *P. aeruginosa*. *Pseudomonas* species can contaminate hospital wards,

* Corresponding author at: Research Center of Pediatric Infectious Diseases, Iran University of Medical Sciences, Hazrat Rasul Hospital, Niayesh Street, Satarkhan Avenue, Tehran 14455, Iran.
E-mail address: azardokht_tabatabaei@yahoo.com (A. Tabatabaei).

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foods, plates, toilets, and even disinfecting agents. *P. aeruginosa* is the most frequent species causing complicated clinical challenges [6]. In the last decades, *P. aeruginosa* is considered as a significant opportunistic nosocomial pathogen, which is responsible for 11–23% of nosocomial infections in the patients with cystic fibrosis, burned patients or those with compromised immune system [7–9].

Due to a growing number of antibiotic-resistant bacteria particularly in *P. aeruginosa* in recent years, traditional antibiotics have become less effective to treat the *P. aeruginosa* infections [9]. Furthermore, the diversity of genotypes and molecular characteristics of the bacterial strains has augmented the barriers ahead of the therapeutic strategies.

To characterize the most frequent strains of *P. aeruginosa* in Iranian patients with UTI, we investigated the genotypic properties of *P. aeruginosa* strains isolated from urine specimens using MLVA as a simple, rapid, and cost-effective method [8]. In addition, the presence of *TEM* and *SHV* resistance genes have been analyzed in this study.

2. Materials and methods

2.1. Bacterial isolation

In this study, 70 urine samples were collected from different hospitals in Tehran, Iran. *P. aeruginosa* was isolated using the conventional microbiology methods in urine samples. Following the transferring of the samples and patient's history to the research laboratory, to confirm the bacterial infection, several diagnostic tests were performed including bacterial culture on differentiating media, and gram staining. Additionally, specific tests for *Pseudomonadaceae* family were conducted; such as Catalase and Oxidase tests, ability of growing on TSI and MR/VP, Simmons Citrate agar media, gas, H2S, and endol production, and amino acid reduction tests.

Accordingly, *P. aeruginosa* infection was verified in all the collected specimens. The confirmed samples were then cultured on the transport media for further analytical experiments. For the long-term use, the bacterial colonies were grown on the Blood Agar Medium (Merck, Germany) and diluted in the storing media (eg. BHI or TSB containing 15% Glycerol; from Merck, Germany) and kept in −70 °C.

2.2. DNA extraction

Bacterial DNA was extracted from all the infected and control samples using the G-Spin Genomic DNA Extraction Kit (Roche, Germany) according to the manufacturer's instructions.

2.3. Primer design and MLVA

In order to identify different variants of *P. aeruginosa* strains in the clinical samples, the PCR experiments based on MLVA were designed to amplify the different variable number tandem repeat (VNTR) in the bacterial genome. These regions have been reported to cover highly polymorph regions which allow the identification of various species. The VNTR regions selected in this study were MS-213, MS-214, MS-215, MS-217, MS-222, MS-223, MS-142, and MS-173 loci as well-established VNTR regions. Two genes reported to be associated with the resistance pattern (*TEM* and *SHV*) were also amplified by conventional PCR. *Klebsiella pneumoniae* ATCC700603 (*SHV* positive) and *Klebsiella pneumoniae* 7881 (*TEM* positive) as described previously were used as positive control samples in this study [10].

The primer pairs were designed using pick primer tool from NCBI database (https://www.ncbi.nlm.nih.gov/).The primers characteristics are listed in the Table 1. A mixture of PCR components were first provided including 7 µl of PCR Master Mix 2x (SinaClon, Iran), 0.5 µl of each forward and reverse primer, 1 µg of template DNA, and ddH2O to the total volume of 25 µl per reaction. The thermal reactions were performed using the ABI Thermal Cycler device (Applied Biosystems) as follows: the primary denaturation for 5 min at 95 °C; followed by 40 cycles as 30 s for 94 °C, 30 s at annealing temperature (Tables 1 and 2), and 30 s at 72 °C; and the final extension phase for 5 min at 72 °C. The PCR products corresponding to eight loci were sequenced and were used as a control for alignment of other clinical samples.

All the PCR products were then visualized by electrophoresis on a 1% agarose gel. To determine the repeat units of each locus, the number of TRs is obtained by subtracting the size of flanking regions (offset size) from the PCR product size, which is then divided to the distinction of the repeat size.

2.4. Data analysis

In order to cluster the MLVA profiles, the Bionumerics 7.6 software was applied using the UPGMA (Unweighted Pair Group Method with Arithmetic mean) method. Similarity coefficient of Pearson's correlation and the Minimum Spanning Tree [7] were also created using BioNumerics. The dendrogram showed the strain dissimilarity based on the UPGMA algorithm. The Hunter-Gaston diversity index was used to determine the polymorphism index of the individual or combined VNTR loci. Accordingly, the strains showing 80% or more similarity (based on 2 distinct VNTR loci (DLV)) were assigned to a single strain.

3. Results

3.1. Study population

In this study, we have investigated 70 *P. aeruginosa* isolates from urine samples of the patients with UTI. Infection prevalence was 63% in female and 27% in male patients isolated from different hospital wards including ICU (52.9%), CCU (4.3%), internal male (4.3%), internal female (5.7%), emergency (4.3%), pediatrics (5.7%), and outpatients (22.8%) referring to different medical labs. Furthermore, the isolated bacteria showed an age related distribution with 4.28% in children under 10 years, 20% in the patients ranging from 10 to 55 years, and 75.72% in the patients older than 55 years old.

3.2. MLVA analysis revealed diverse types of P. aeruginosa strains

The isolates were assessed by MLVA PCR regarding the amplification of eight distinct VNTR loci (Fig. 1). Noticeably, based on PCR result, all the isolates were positive for the repeat sequences. This analysis revealed a large diversity in the number of repeats among the different VNTR loci. Generally, our data revealed 61 various types of *P. aeruginosa* isolates divided into two main clusters and 4 sub-clusters, which are illustrated in the dendrogram (Fig. 2).

Interestingly, the cluster analysis of the MLVA profiles Minimum spanning tree algorithm portrayed a great value of this methodology in terms of discriminating *P. aeruginosa* isolates collected from urine specimens of patients with UTI (Fig. 3).

3.3. Isolated P. aeruginosa strains displayed antibiotic resistance genes

To detect the presence of *TEM* and *SHV* resistance *genes,* conventional PCR was performed on the isolated strains (Fig. 4). Subsequently, 80% and 70% of strains had *TEM and SHV* resistance genes, respectively. In addition, 61% of isolates had both *TEM* and *SHV* resistance genes concomitantly.

4. Discussion

P. aeruginosa causes many severe nosocomial infections in different wards of hospitals which proves the significant role of typing techniques to understand the hospital epidemiology pertinent to this species [11]. Strain identification of the various type of bacterial species is essential for the investigation of outbreaks and controlling the bacterial

Table 1
Primer characteristics of multiple VNTR loci used in the PCR reactions.

VNTR Locus	Primer pair	Product size (bp)	Tm* (°C)	HGDI# Index[b]	Reference
MS-213	F- TGGCGTACTCCGAGCTGATG	103	62	0.86	[12]
	R- CTGGGCAAGTGTTGGTGGATC		61.49		
MS-214	F- CCATCATCCTCCTACTGGGTT	115	58.59	0.81	[12]
	R- AAACGCTGTTCGCCAACCTCTA		62.75		
MS-215	F- CTGTACAACGCCGAGCCGTA	129	62.53	0.80	[12]
	R- GACGAAACCCGTCGCGAACA		63.59		
MS-217	F- GAACAGCGTCTTTTCCTCGC	109	59.84	0.79	[12]
	R- TTCTGGCTGTCGCGACTGAT		61.88		
MS-222	F- TGCAGTTCTGCGAGGAAGGCG	101	65.52	0.76	[12]
	R- AGAGGTGCTTAACGACGGAT		58.52		
MS-223	F- TGAGCTGATCGCCTACTGG	106	58.88	0.77	[12]
	R- TTGGCAATATGCCGGTTCGC		62.00		
MS-142	F- GTGGGGCGAAGGAGTGAG	115	59.73	0.68	[13]
	R- AGCAGTGCCAGTTGATGTTG		59.04		
MS-173	F- CTGCAGTTCGCGCAAGTC	243	59.83	0.82	[13]
	R- ATTTCAGCCAGCGTTACCAA		58.10		

* Tm: Melting Temperature.
\# HGDI: Hunter-Gaston discriminatory index.

Table 2
Primer characteristics of *TEM* and *SHV* used in the PCR reactions.

Gene	Primer Pair	Repeat Size (bp)	Tm* (°C)	Reference
TEM	F- GAGTATTCAACATTTCCGTGTC	861	55.49	[14]
	R-TAATCAGTGAGGCACCTATCTC		56.79	
SHV	F- AAGATCCACTATCGCCAGCAG	231	59.93	[14]
	R- ATTCAGTTCCGTTTCCCAGCGG		63.39	

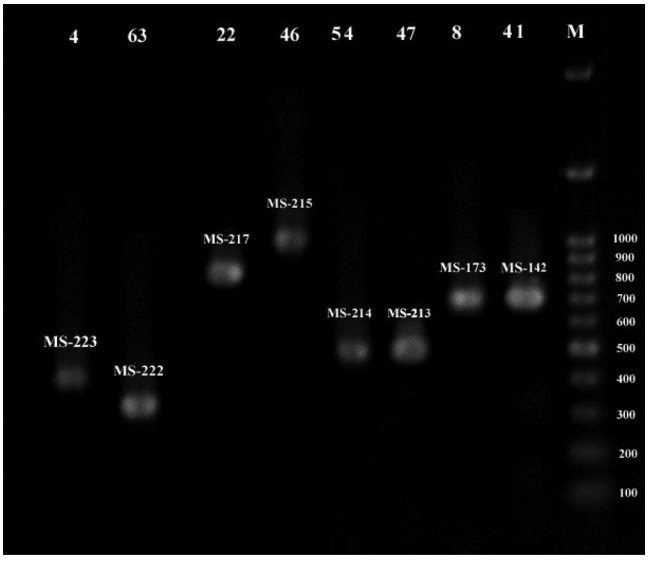

Fig. 1. Agarose gel electrophoresis of amplicons from 8 VNTRs. (M: 100 bp ladder, line 41 to 4 shows the VNTR Locus position).

infections [12].

Identification of various strains requires a good typing method that should be rapid, easy to use, inexpensive, reproducible and interpretable. Also, different typing methods should have the ability to differ endemic, epidemic and sporadic isolates [11]. Phenotypic and genotypic approaches are two different methods of typing [13].

However, the results of phenotypic approaches are varying and sometimes inconsistent from one study to another that may be due to the limitation of culture-based methods (Differences in culture condition, human errors in interpretation of phenotypic result). Also, phenotypic methods have less discriminatory power compared to genotypic approaches. It may explain the gradual replacement of phenotypic methods to genotypic techniques over the past two decades [14].

There are various genetic typing methods available with high discriminatory power. However, due to excellent discriminatory power of pulsed-field gel electrophoresis (PFGE), this method has been a gold standard method for genetic typing for many years. On the downside, this method is very expensive, time-consuming, labor-intensive and also suffers from inter-laboratory reproducibility [15].

Multilocus Variable Number Tandem Repeat Analysis (MLVA) has addressed some of the PFGE limitations. MLVA is based on polymorphic tandem repeat loci of bacterial species which has been successfully applied for epidemiological investigations. Compared to PFGE, MLVA is less expensive, faster, and easier to use/interpretation with high reproducibility and discriminatory power [16]. Also, Comparison of PFGE with MLVA in terms of concordance and discriminatory power showed relatively similar results in many studies [12,16,17].

There are six to sixteen VNTR markers reported for typing of *P. aeruginosa* [18]. Based on previous findings reducing the number of loci to eight do not have an impact on discriminatory power of MLVA [19].

In the present study, 70 *P. aeruginosa* strains were isolated from patients with UTI hospitalized in different wards on a hospital in Tehran, Iran. To characterize the isolated strains genetically, MLVA typing technique has been applied. MLVA is a cost-effective, easy, and high resolution technique, by which the results can be obtained within a few days which is faster than MLST and PFGE methods [20]. Our findings showed high diversity of *P. aeruginosa* population in the patients diagnosed with UTI. Moreover, isolated strains had *SHV* and *TEM* resistance genes in this group. Based on previous studies in Iran, the UTI mainly caused by *P. aeruginosa* is regarded as one of the frequent complications of hospitalized patients. Based on Askarian et al. in 1483 patients under surgery in Shiraz hospitals, the prevalence of UTI was 17.59% compared to other nosocomial infections [21]. In this study, *P.aeruginosa* was colonized in 35% of catheter-associated UTIs cases. In the assessment of MST pattern, 11 clonal complexes were identified in 39 isolates, among which only one isolate was collected from a non-hospitalized patient.

By analyzing the various types recognized in this study, we identified some levels of similarities among the isolates, which might be due to the infection transmission among the hospitalized patients. Moreover, Beta-lactamases genes including *TEM* and *SHV* genes were also detected in the isolated strains. Interestingly, isolated strains in the hospitalized patients expressed *TEM* and *SHV* genes more significantly than the outpatients group. In this context, out of 54 hospitalized

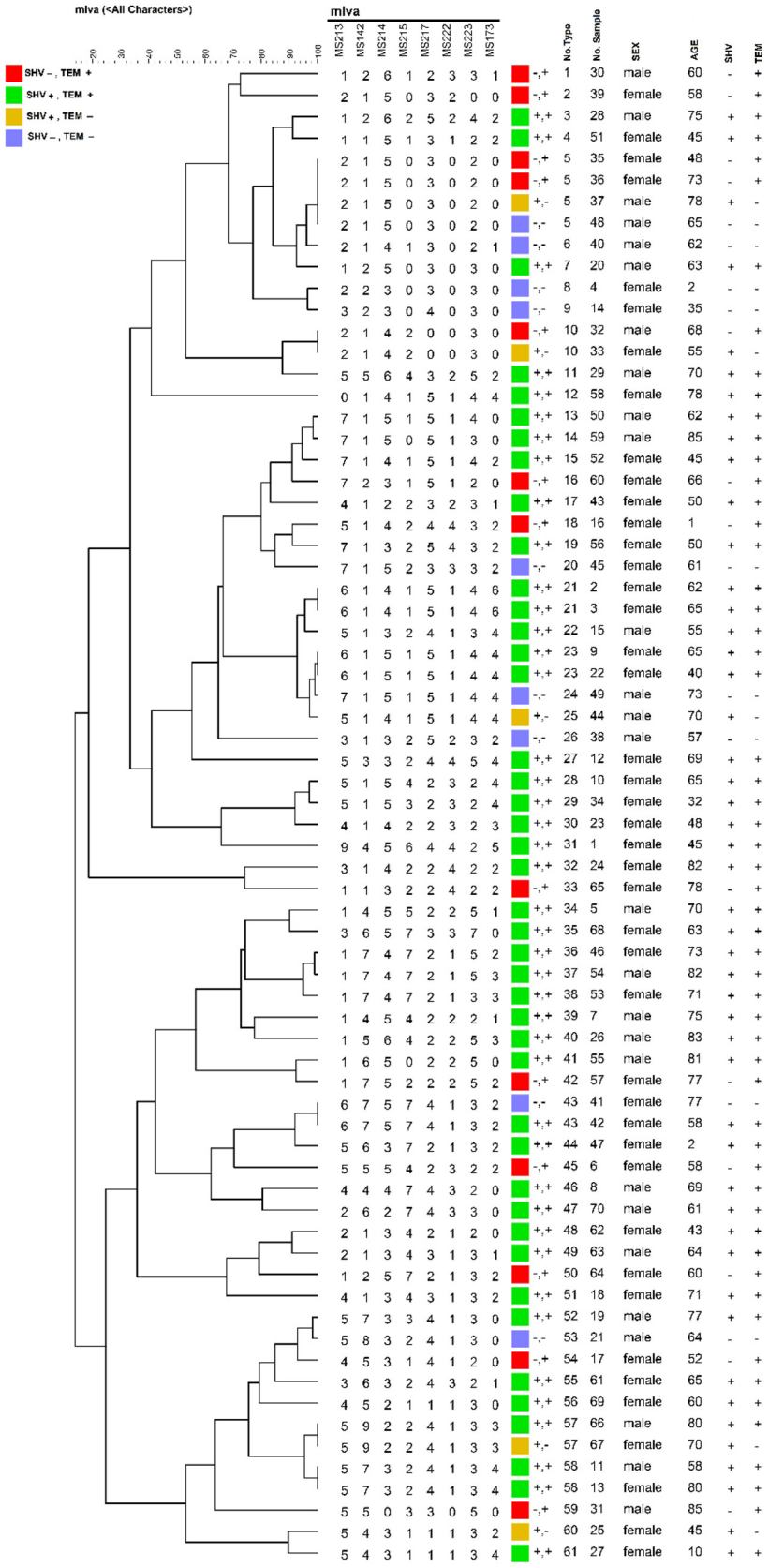

Fig. 2. Clustering analysis of MLVA results of 70 *P. aeruginosa* strains using UPGMA algorithm. The dendrograms illustrate the names and origin of isolates, presence of methicillin resistance genes, and their MLVA profiles.

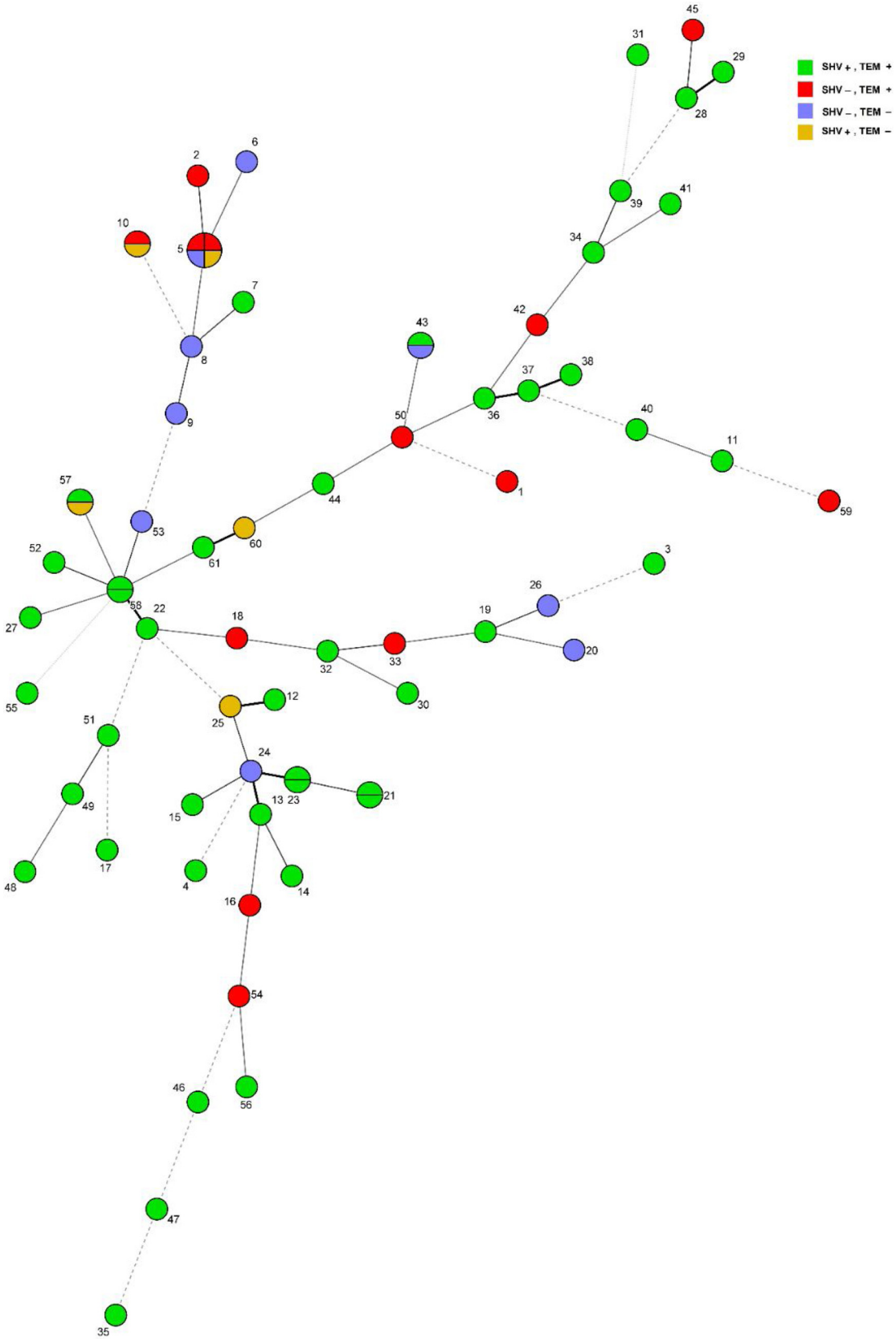

Fig. 3. MST of the 70 *P. aeruginosa* isolates typed by MLVA. The MLVA clustering was performed using a categorical coefficient. The circles with variable colors in the MST graph indicate the MLVA types and the number of a particular MLVA type directly correlates with its circle size.

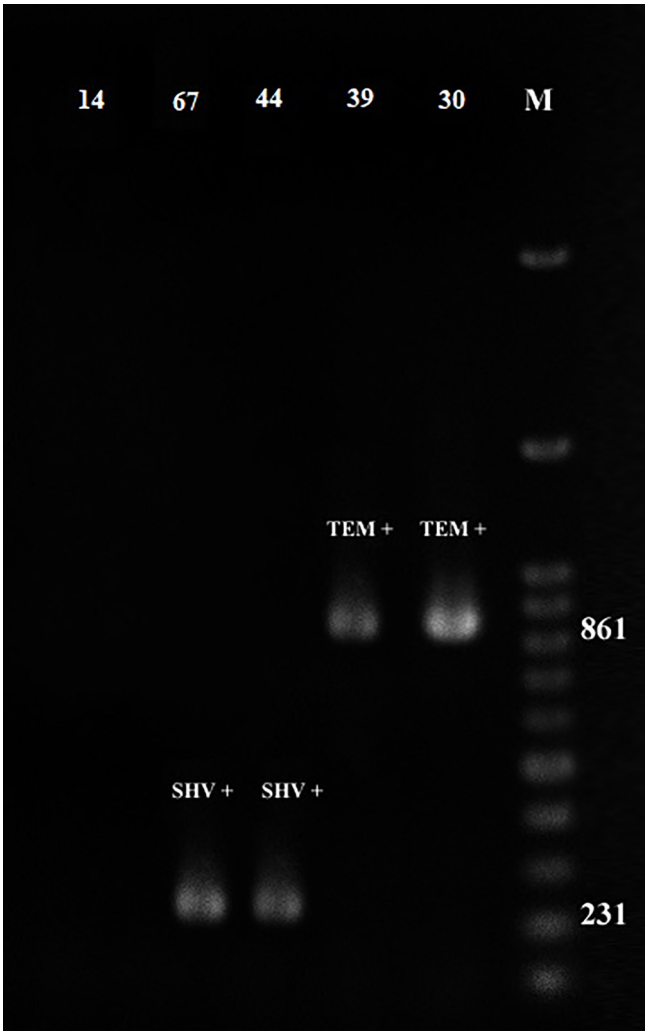

Fig. 4. Agarose gel electrophoresis of amplicons from *TEM* and *SHV* (M: ladder 100bp).

patients, 50 and 41 cases were infected with strains having *TEM* and *SHV* genes, respectively. In contrast, out of 16 outpatients, the *TEM* and *SHV* were expressed in 6 and 8 cases.

We have analyzed 8 loci (MS-213, MS-214, MS-215, MS-217, MS-22, MS223, MS142, and MS-173) which have been found in the majority of isolated strains. Nevertheless, the frequency of MS-173 was indicated to be slightly lower than other loci which was also consistent with other global reports [18,22].

In a relatively similar study, 82 *P. aeruginosa* strains producing beta-lactamase were evaluated using MLVA method. In this study, isolated strains showed a branch of 31 patients (MLVA 364), some branches including 13 cases (MLVA 255), 7 cases with MLVA 882, four cases with MLVA 404, as well as 9 other smaller branches pertinent to one or two patients [23].

The above information indicates the importance of regular observation of transmission and emergence of *P. aeruginosa* strains, which allow scientist to track the infection source and bacterial transmission pathways more accurately.

5. Conclusion

Our observations show that a high percentage of the patients hospitalized in different wards of hospital were mainly older than 55 and infected by *P.aeruginosa*-related UTI. The pathogenicity of this organism is also noticeable in the outpatients group. MLVA-based typing is a rapid and cost-effective method and the obtained results from this method are useful to identify the origins of the strains and the interconnection of different types.

More importantly, a study like this should not be limited to a short period, and continuous monitoring of the molecular epidemiology of *P. aeruginosa* is definitely required to detect any small changes in the epidemiology of *P. aeruginosa* infections during the time.

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