Original Article

J Babol Univ Med Sci
Vol 20, Issu 2; Feb 2018. P:56-63

Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) for Typing Pseudomonas Aeruginosa Isolated from Urine Samples of Different Patients

H.E. Lashgarian (PhD)[1], A. Marzban (PhD)[2], M. Estaji (MSc)[3], M. Gholami (MSc)[4], H. Masoumi Asl (PhD)[5], J. Raheb(PhD) [3*]

1.Hepatitis Research Center, Lorestan University of Medical Sciences, Khorramabad, I.R.Iran
2.Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, I.R.Iran
3.Molecular Medicine Department, Medical Institute, National Institute of Genetics and Biotechnology, Tehran, I.R.Iran
4.Department of Microbiology, Khorramabad Branch, Islamic Azad University, Khorramabad, I.R.Iran
5.Infectious Disease Research Center, Iran University of Medical Sciences, Tehran, I.R.Iran

J Babol Univ Med Sci; 20(2); Feb 2018; PP:56-63
Received: Jul 11th 2017, Revised: Oct 15th 2017, Accepted: Nov 29th 2017.

ABSTRACT

BACKGROUND AND OBJECTIVE: *Pseudomonas aeruginosa* is considered as one of the important causes of urinary infections in hospitals. The aim of the current study is the genetic typing for the number of bacterial strains isolated from patients using MLVA technique.

METHODS: In this study, 70 isolates were collected from different hospitals located in Tehran city. First, DNA extraction was conducted for genotyping analysis by MLVA method. Subsequently, VNTR sequences located in several genes of bacterial genomes such as MS-214, MS-215, MS-217, MS-222, MS-223, MS-142 and MS-173 were amplified by specific primers using PCR technique. After confirming the PCR amplification using electrophoresis and visualization of their bonds on agarose gel, relationship evolutionary graph for the different strains was constructed based on MLVA technique.

FINDINGS: After the electrophoresis of PCR products and determination of VNTR copy-numbers, 70 strains were classified as 39 types and genetic evolutionary tree was also constructed based on VNTRs Data. According to the MST algorithm, 70 clinical strains divided into 11 clonal complexes which these criteria is interpreted as genetic distance based on the difference of VNTR copy numbers for each group.

CONCLUSION: The present study showed that MLVA could be helpful for typing clinical strains of *P. aeruginosa*. The results also showed that this method had great potential to differentiate those strains with high phenotypic similarity.

KEY WORDS: *Pseudomonas Aeruginosa, Genotyping, Urinary infection, MLVA, VNTR*.

Please cite this article as follows:

Lashgarian HE, Marzban A, Estaji M, Gholami M, Masoumi Asl H, Raheb J. Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) for Typing Pseudomonas Aeruginosa Isolated from Urine Samples of Different Patients. J Babol Univ Med Sci. 2018;20(2):56-63.

•Corresponding Author; J. Raheb(Phd)
Address: Department of Molecular Medicine, National Research Institute for Genetic Engineering and Biotechnology, Tehran, I.R.Iran.
Tel: +98 21 8896 4792
E-mail: jam@nigeb.ac.ir

[DOR: 20.1001.1.15614107.1396.20.2.8.4]
[DOI: 10.18869/acadpub.jbums.20.2.56]

Introduction

Pseudomonas is one of the bacterial species that has a high metabolic ability. This bacterium includes various species, both non-pathogenic and pathogenic. This bacterium is also found throughout the hospital environment in wet tanks such as foods, picked flowers, dishwashers, toilets, floor washer, respiratory equipment, and even disinfectant solutions (1, 2). Today, this bacterium is one of the bacteria that cause the spread of nosocomial infections, especially urinary tract infections. Pseudomonas aeruginosa is the most common type of pseudomonas which causes many problems in hospitals (3, 4).

Pseudomonas (gram-negative basil) is sometimes bent and in most cases is right bar. This bacterium is capable of moving due to the presence of polar flagella. In relation to pathogenesis or pathogenicity, Pseudomonas have a wide range of invasive mechanisms, including organism components, toxins, and enzymes that play an important role in the pathogenesis of microorganisms. Pseudomonas is resistant to most antibiotics. Even in sensitive microorganisms, resistance can spread through a therapeutic period by inducing antibiotic inactivating enzymes (such as β-lactamases) or transferring resistance-mediated plasmid from a resistant organism to sensitive one (5).

Based on the principles of microbiology, urinary tract infection occurs when pathogenic microorganisms are found in the urine or the urethra or kidney or prostate. Generally, a wide range of microorganisms can cause urinary tract infection. But the most common agents are gram negative bacteria such as Escherichia coli, Pseudomonas, Klebsiella and Serratia (6). Recently, methods have been developed based on molecular techniques to typing the disease causative microorganisms.

Among these methods, those which based on the use of restriction enzymes can be mentioned such as PFGE, AFLP, RFLP and Southern sputtering methods. These methods, in addition to their great benefits, each have their own problems. For example, the PFGE technique requires a lot of skill, special equipment, expensive endonuclease, and much time should be spent doing the technique (7).

The AFLP technique is very time consuming and costly due to the stage of adding the adapter (8). One of the simple, fast, affordable, and reliable techniques for the characterization of microorganisms is the MLVA technique. This MLVA technique has been developed to typing bacteria such as Salmonella, Listeria monocytogenes and Escherichia coli (9). The basis of the MLVA technique is the identification of the variable number tandem repeat (VNTRs) in specific locuses on the genome of microorganisms (10). In the MLVA technique after selecting the desired locus and designing the primer for them, and extracting the desired strains DNA, the proliferation of the sequences containing the VNTR is performed by PCR.

The product obtained from PCR is sequenced and the number of replicates is calculated. Given that in the genome of the creatures there is a series of sequential repetitive sequences called VNTRs, which structurally and base containing are similar. The only difference in the different types is the number of tandem repeats from a VNTR (11).

In the Pseudomonas genus, repeated sequences have been identified, which in this study several of them were MS-213, MS-214, MS-215, MS-217, MS-222, MS-223, MS-142 and MS-173 that can be used to typing strains. These sequences, which have different replications in different strains of Pseudomonas aeruginosa, are an appropriate means for categorizing Pseudomonas aeruginosa subspecies from different patients or from different locations.

Considering that the typing of pathogenic strains of the hospital can provide very useful information for designing an effective therapeutic approach to physicians and medical staff, this study was conducted with the aim of reaching a clear and reliable pattern. Therefore, in this study, the Pseudomonas aeruginosa bacterial strains isolated from urine specimens of patients with urinary tract infection, based on sequential tandem repeats were investigated by MLVA technique.

Methods

Sample collection: In this study, urine specimens were collected from patients in different units such as emergency department, ICU, CCU, women, men, children and outpatients. After transferring the specimens to the laboratory, all of them were cultured on a Norint Agar culture medium. These specimens were examined for isolation of Pseudomonas aeruginosa strains in the next step. After confirming the samples containing Pseudomonas aeruginosa strains by common methods and with specific laboratory kits, pure cultures were prepared on nutrient

[DOR: 20.1001.1.15614107.1396.20.2.8.4]
[DOI: 10.18869/acadpub.jbums.20.2.56]

agar culture medium and were kept in the refrigerator for a while until the experimental stages.

DNA extraction: DNA was extracted using the G-spin Genomic DNA Extraction Kit (Germany, Roche). First, the bacteria cultured in the nutrient broth and after 24 hours of incubating at 35 °C, 1 ml of bacterial culture was removed and sterilized in vials and were centrifuged at 8000 rpm for 15 minutes. Then the supernatant was discarded, and then 50 µl of the pre-buffer solution and 3 µl of lysozyme were added to the cellular deposition and incubated at 37°C. Subsequently, 250 µl of buffer G solution and 250 µl of the binding buffer were added and each time mixed well.

In the next step, the broken cell product was loaded onto the columns and was carried out for 13 minutes with 13000 rpm centrifuges. After washing the column, 100 µl of the buffer elution buffer was poured directly onto the membrane of the column. The resulting solution was centrifuged at 13000 rpm for 1 minute. The extracted DNA was kept in a refrigerator for electrophoresis and amplification by PCR, after being determined by a spectrophotometer (Merck, Germany).

DNA amplification by PCR method: After genomic DNA extraction, the amplification of the genes and sequences by their specific primers (Table 1) was performed by PCR method. These primers were extracted and used from studies conducted by other researchers (13, 12).

For this purpose, MgCl2, dNTp and buffer of this reaction were prepared as master mix from Sina Clone Company of Iran. A mixture of this reaction, consisting of 7 µL PCR Master mix, 0.5 µl Primer Forward, 0.5 µl Primer Reverse, 16 µl ddH2O and 1 µL Template DNA, was prepared using a 40-cycle thermal cycling consisting of a first denaturation step at 95 °C for 10 minutes, then the denaturation cycle at 94 °C for 30 seconds, the annealing step at 60°C for 30 seconds, the extension stage at 72°C for 30 seconds, and at Finally, a Final Extension was at 72°C for 5 minutes (12).

Table1. Primer sequence used for genes and repeat regions of the gene for amplification by PCR method (12)

Gene	Primer	(bp) Size	Gene	Primer	Size(bp)
MS-213	F-TGGCGTACTCCGAGCTGATG R-CTGGGCAAGTGTTGGTGGATC	103	MS-222	F-TGCAGTTCTGCGAGGAAGGCG R-AGAGGTGCTTAACGACGGAT	101
MS-214	F-CCATCATCCTCCTACTGGGTT R-AAACGCTGTTCGCCAACCTCTA	115	MS-223	F-TGAGCTGATCGCCTACTGG R-TTGGCAATATGCCGGTTCGC	106
MS-215	F-CTGTACAACGCCGAGCCGTA R-GACGAAACCCGTCGCGAACA	129	MS-142	F-GTGGGGCGAAGGAGTGAG R-AGCAGTGCCAGTTGATGTTG	115
MS-217	F-GAACAGCGTCTTTTCCTCGC R-TTCTGGCTGTCGCGACTGAT	109	MS-173	F-CTGCAGTTCGCGCAAGTC R-ATTTCAGCCAGCGTTACCAA	243

Electrophoresis: Electrophoresis was performed on an agarose gel with 1% concentration to confirm the PCR product. For this purpose, 1% agarose gel was prepared in buffer (x 0.5) TBE, and after heating and cooling, 5 ml of Ethidium bromide solution (Qiagen, USA) was added and poured into the mold. To load the gel, 5 µl of the PCR product with 1 µl of dye loading was mixed and was poured into special wells. The size marker of 100 bp plus was used to determine the size of the PCR product. In the next step, the electrophoresis voltage was adjusted to 80 and, after completion of the electrophoresis; the gels were placed on the Gel Documentation UV (Upland, USA) and photographed.

Draw an evolutionary link diagram and determine the strain type: To draw the evolution chart, the data is entered into the software available at http: //mlva.u-psud. fr/mlvav4/genotyping, and the resulting dendrogram for typing the isolates based on the stratified coefficient and the UPGMA algorithm. For this purpose, strains with 80% or more than 80% of the same repeat count (based on the difference in 2 loci VNTR (DLV)) were placed in one strain and other strains were placed in different strains.

Results

Isolation of strains: Among 70 strains isolated from patients with UTI, 44 isolates (63%) were from female patients and 19 were from male patients (27%). Among 70 isolates, 37 strains were from the ICU (52.9%), 3 strains were from the CCU (4.3%), 3 strains were from the men's (4.3%), 4 strains were from the women's department (7.7%), 5%), 3 strains were from

[DOR: 20.1001.1.15614107.1396.20.2.8.4]
[DOI: 10.18869/acadpub.jbums.20.2.56]

the emergency department (4.3%), 4 strains were from the pediatrics (5.7%) and 16 strains were (22.8%) from outpatients referring to the laboratories (Table 2). Strain Typing: After amplification of the genes by PCR, electrophoresis on the agarose gel was performed for the products of each of the 8 genes (Fig. 1). The bonds produced on the gels representing the size of each of the sequences were analyzed by Gene Tools software by comparison with the 100 bp size marker (ZR 100 bp DNA Marker ™).

Table 2. Frequency of isolated bacteria from different parts of hospitals

Origin of isolation	Number of isolates	Percentage isolate from total
ICU	37	52.9
CCU	3	4.3
MEN	3	4.3
WOMEN	4	5.7
EMERGENCY	3	4.3
CHILDREN	4	5.7
O.P.D	16	22.8

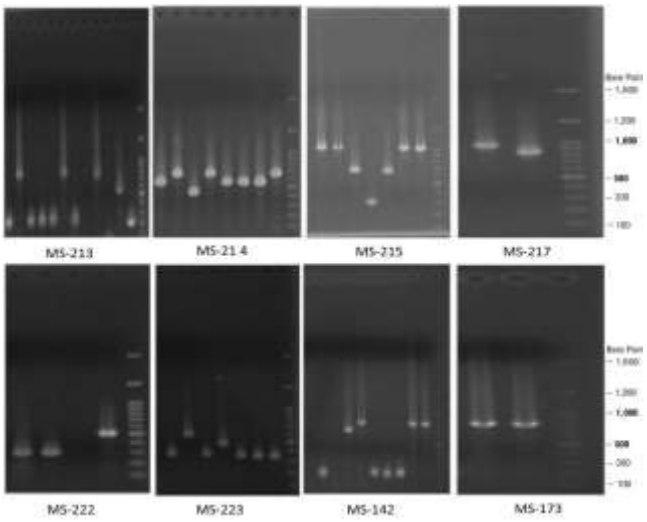

Figure 1. Gel electrophoresis obtained from the PCR product of genes used for strain analysis and typing

After sequencing and examining the frequency of repetitions in 70 strains, 39 types were obtained. Of these types, the highest number belonged to type 1 with 10 strains. After that, types 2 and 6 each included 5 strains. In the sequel, types 3 and 4 included 3 strains and 7 to 14 types included 2 strains. Finally, there were other types that included only one strain (Table 3). Figure 2 shows the genetic evolution tree of 70 isolated strains, which drawn by UPGMA software. In this phylogeny tree, the relationship between different strains was based on the similarity in their repetitions in a branch. The length of each branch also shows the difference in the number of repetitions in different branches (14).

Figure 3 shows the MST (Minimum spanning tree) pattern derived from the MLVA analysis for the desired strains. In this figure, 70 strains classified into 39 types are presented as a clone based on the number of strains that were categorized. In total, the MST pattern obtained in this study consists of 11 clonal complexes (CC). This concept is based on the relationship between the numbers of repetitions that have been considered as comparisons in each category. The distance between each CC represents markers that are common in each clone. And, if the similarity between the indices was more, the distance between the clones is closer to each other (15).

The MST pattern usually gives us a better understanding of the typing of strains that cause the creation of a common attribute. Because it indicates the differences and common indicators as clonal complex simultaneously (16).

Table 3. Determination of strains of Pseudomonas aeruginosa strains. The 70 selected strains are classified into 39 types, strains are based on similarity in the number of replicates and their affinity within a single type

Typing Number	Strains	Typing Number	Strains
1	2,3,9,22,44,49,50,52,8,59	21	31
2	11,13,15,19,21	22	30
3	46,53,54	23	64
4	6,10,34	24	47
5	4,14,20	25	12
6	35,36,37,39,48	26	61
7	5,7	27	28
8	25,27	28	23
9	38,56	29	45
10	24,65	30	16
11	18,63	31	62
12	41,42	32	43
13	32,33	33	51
14	66,67	34	60
15	40	35	17
16	1	36	69
17	55	37	8
18	57	38	70
19	26	39	68
20	29	-	-

[DOR: 20.1001.1.15614107.1396.20.2.8.4]
[DOI: 10.18869/acadpub.jbums.20.2.56]

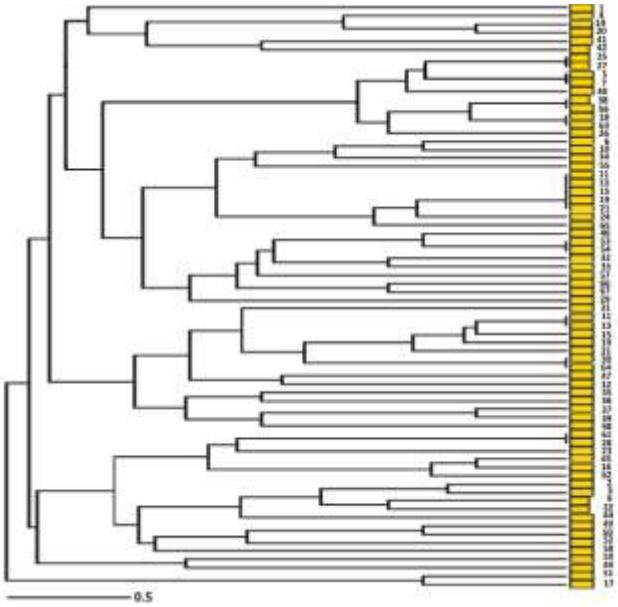

Figure 2. Diagram derived from the evolutionary diagram obtained from sequential repeat sequences from Pseudomonas aeruginosa strains isolated from patients with urinary tract infection.

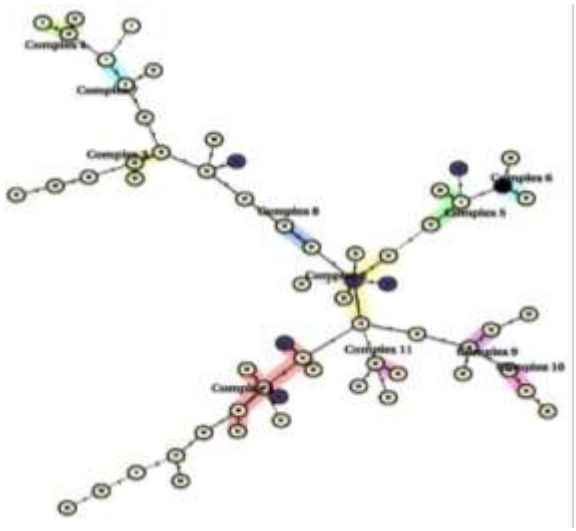

Figure 3. MST pattern obtained from clinical isolates causing urinary tract infection in different patients. The colonel complexes observed in this figure represents the relationship between several common markers (the colored part) and the distance between them showing the difference in the number of repetitions.

Discussion

In this study, 70 samples from different strains of Pseudomonas aeruginosa were isolated from a wide range of patients, including those admitted to the ICU and CCU units to outpatients. Although these strains were similar in nature to the biochemical characteristics and analyzes, 39 strains were classified in the MLVA analysis. This number of strains obtained from Pseudomonas aeruginosa strains indicates the high accuracy of this method in differentiating the differences in these strains.

These differences, which led to the creation of different types in the MLVA method, showed that they were not detectable by conventional biochemical methods. Urinary tract infections (UTIs) are one of the three most common causes in the community, especially at the hospital level. Urinary tract infection caused by pathogenic bacteria after respiratory infection is the most common type of infection in patients admitted to different parts of hospitals (17). Patients with UTI are very diverse, and a wide range of bacterial strains have been identified and reported for different characteristics and pathogens (19, 18).

Due to the high variation in the pathogenic strains that come from patients from different parts of the world, which often carry antibiotic resistance genes, researchers are seeking more practical and precise solutions to differentiate these close to each other strains. Because diagnostic methods for high volume of clinical specimens, in addition to being time consuming, will cost a lot, as a result of the importance of techniques such as MLVA, have become more commonplace to all therapists.

For this reason, in recent years, MLVA-based typing, in which isolates are evaluated by the number of replicates in several genetic regions, for a number of important bacteria such as Bacillus anthraise, Staphylococcus aureus, Enterococcus faecium, Haemophilus influenzae, Bordetella pertosis and many others have been used (20). Another common technique that has been compared with MLVA in various studies is the PFGE technique. Johansson et al. analyzed 232 isolates of Pseudomonas aeruginosa isolated from cystic fibrosis patients by both methods. In this comparative study, 91% of the results were similar to each other. However, they emphasized that, despite the expensive and time-consuming PFGE, its accuracy is higher than that of MLVA (12).

Another study by Lee et al. concluded that for the analysis of Pseudomonas strains isolated from cystic fibrosis patients who became infected with chronic infections and pathogenic bacteria colonized in the lung, more precise molecular methods for strain differentiation should be applied (21). MLVA method, although is less accurate than techniques such as PFGE, but because each strain is described by a code that is associated with the number of replicates in the

[DOR: 20.1001.1.15614107.1396.20.2.8.4]
[DOI: 10.18869/acadpub.jbums.20.2.56]

VNTR, the analysis of a large number of samples is easily possible. In the present study, it has been shown that using MLVA technique can well differentiate strains from each other with high speed and precision, so that each of the 70 strains studied by this technique were analyzed. In the present study, among eight loci (MS-213, MS-214, MS-215, MS-217, MS-22, MS223, MS142, MS-173) selected for analysis, most of the strains have high-level of mentioned loci and only the abundance of the MS-173 locus was less than that found in other studies in the world (13, 9). The results of our study indicate that the locus used in this study had a high degree of differentiation. Our data showed clonal release of Pseudomonas aeruginosa genotypes in hospitals in important areas such as ICU. The high prevalence of Pseudomonas aeruginosa in investigated hospitals is likely to be a result of a single-strand transmission of patients with clonal release. The results of the study showed that most patients, including patients admitted to ICU or outpatients are prone to urinary tract infection, so in this study, all clinical specimens were identified from different strains of pathogenic strains. In addition, the results of strain typing by the new MLVA technique showed that this method can act with greater speed and accuracy in identifying the strain origin in addition to lower cost and this indicates the relationship between the source of isolated strains.

Acknowledgments

Hereby, we would like to thank the Center for Pediatric Infectious Diseases, Iran University of Medical Sciences, for the donation of patient samples and collaboration with Genetic Engineering and Biotechnology Research Institute.

[DOR: 20.1001.1.15614107.1396.20.2.8.4]
[DOI: 10.18869/acadpub.jbums.20.2.56]

References

1.Moore NM, Flaws ML. Epidemiology and pathogenesis of Pseudomonas aeruginosa infections. Clin Lab Sci. 2011;24(1):43-6.

2.Ebrahimipour G, Moradi A, Karkhane M, Marzban A. Antibiotics and heavy metal resistance of three strains of pseudomonas aeruginosa isolated from different ecological areas. Med Lab J. 2015;8(4):55-60.

3.Strateva T, Mitov I. Contribution of an arsenal of virulence factors to pathogenesis of Pseudomonas aeruginosa infections. Ann Microbiol. 2011;61(4):717-32.

4.Karkhane M, Pourhoseingholi MA, Kimiia Z, Mortazavi SM, Torkabad MRA, Aghdam SKH, Marzban A, Zali MR. Attitudes toward nosocomial infections associated mortality at intensive care units, and evaluation of the risk factors. Arch Clin Infect Dis. 2016;11(2):e22504.

5.Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev. 2009;22(4):582-610.

6.Hooton TM, Bradley SF, Cardenas DD, Colgan R, Geerlings SE, Rice JC, et al. Diagnosis, prevention, and treatment of catheter-associated urinary tract infection in adults: 2009 international clinical practice guidelines from the infectious diseases society of america. Clin Infect Dis. 2010;50(5):625-63.

7.Goering RV. Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. Infect Genet Evol. 2010;10(7):866-75.

8.Naze F, Jouen E, Randriamahazo R, Simac C, Laurent P, Blériot A, et al. Pseudomonas aeruginosa outbreak linked to mineral water bottles in a neonatal intensive care unit: fast typing by use of high-resolution melting analysis of a variable-number tandem-repeat locus. J Clin Microbiol. 2010;48(9):3146-52.

9.Vu-Thien H, Corbineau G, Hormigos K, Fauroux B, Corvol H, Clément A, et al. Multiple-locus variable-number tandem-repeat analysis for longitudinal survey of sources of Pseudomonas aeruginosa infection in cystic fibrosis patients. J Clin Microbiol. 2007;45(10):3175-83.

10.Dass SC, Abu-Ghannam N, Antony-Babu S, Cummins EJ. Ecology and molecular typing of L. monocytogenes in a processing plant for cold-smoked salmon in the Republic of Ireland. Food Res Int. 2010;43(5):1529-36.

11.Larsson J, Torpdahl M, Petersen R, Sørensen G, Lindsted B, Nielsen E. Development of a new nomenclature for Salmonella Typhimurium multilocus variable number of tandem repeats analysis (MLVA). Eurosurveillance (Online Edition). 2009;14(15).

12.Johansson E, Welinder-Olsson C, Gilljam M, Pourcel C, Lindblad A. Genotyping of Pseudomonas aeruginosa reveals high diversity, stability over time and good outcome of eradication. J Cystic Fibrosis. 2015;14(3):353-60.

13.Onteniente L, Brisse S, Tassios PT, Vergnaud G. Evaluation of the polymorphisms associated with tandem repeats for Pseudomonas aeruginosa strain typing. J Clin Microbiol. 2003;41(11):4991-7.

14.Beranek A, Mikula C, Rabold P, Arnhold D, Berghold C, Lederer I, et al. Multiple-locus variable-number tandem repeat analysis for subtyping of Salmonella enterica subsp. enterica serovar Enteritidis. Int J Med Microbiol. 2009;299(1):43-51.

15.Santos AS, Tilburg JJ, Botelho A, Barahona MJ, Núncio MS, Nabuurs-Franssen MH, et al. Genotypic diversity of clinical Coxiella burnetii isolates from Portugal based on MST and MLVA typing. Int J Med Microbiol. 2012;302(6):253-6.

16.Prendergast D, O'Grady D, Fanning S, Cormican M, Delappe N, Egan J, et al. Application of multiple locus variable number of tandem repeat analysis (MLVA), phage typing and antimicrobial susceptibility testing to subtype Salmonella enterica serovar Typhimurium isolated from pig farms, pork slaughterhouses and meat producing plants in Ireland. Food Microbiol. 2011;28(5):1087-94.

17.Farrell D, Morrissey I, De Rubeis D, Robbins M, Felmingham D. A UK multicentre study of the antimicrobial susceptibility of bacterial pathogens causing urinary tract infection. J Infect. 2003;46(2):94-100.

[DOR: 20.1001.1.15614107.1396.20.2.8.4]
[DOI: 10.18869/acadpub.jbums.20.2.56]

18.Glover M, Moreira CG, Sperandio V, Zimmern P. Recurrent urinary tract infections in healthy and nonpregnant women. Urolog Sci. 2014;25(1):1-8.

19.Leclercq SY, Sullivan MJ, Ipe DS, Smith JP, Cripps AW, Ulett GC. Pathogenesis of Streptococcus urinary tract infection depends on bacterial strain and β-hemolysin/cytolysin that mediates cytotoxicity, cytokine synthesis, inflammation and virulence. Sci Rep. 2016;6.

20.Elberse KE, Nunes S, Sá-Leão R, van der Heide HG, Schouls LM. Multiple-locus variable number tandem repeat analysis for Streptococcus pneumoniae: comparison with PFGE and MLST. PloS One. 2011;6(5):19668.

21.Lee TW, Brownlee KG, Conway SP, Denton M, Littlewood JM. Evaluation of a new definition for chronic Pseudomonas aeruginosa infection in cystic fibrosis patients. J Cyst Fibros. 2003;2(1):29-34.

[DOR: 20.1001.1.15614107.1396.20.2.8.4]

[DOI: 10.18869/acadpub.jbums.20.2.56]