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ORIGINAL ARTICLE

Comparison of loop-mediated isothermal amplification, multiplex PCR, and REP- PCR techniques for identification of carbapenem-resistant *Acinetobacter baumannii* clinical isolates

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ABSTRACT

Background and Objectives: *Acinetobacter baumannii*, an opportunistic pathogen, is related to hospital-acquired infections and increased mortality. This study aimed to develop the loop-mediated isothermal amplification (LAMP) test for the fast-detecting of *A. baumannii* isolates as well as determining genetic relatedness for these isolates via the REP-PCR technique.

Materials and Methods: LAMP primers and multiplex PCR primers were designed for recognizing *A. baumannii* isolates harboring the bla_{SHV-1}, bla_{PER-1}, bla_{TEM-1}, AMPC, *qnr*, and *aac (6)-1* genes, were collected (October 2020 to February 2021) from Shahid Motahari Hospital, Tehran, Iran. Combination disc test (CDT) results were used to assess the phenotypic identification of isolates from ESBL producers. The sensitivity of the LAMP method was evaluated using a range of serial dilutions of genomic DNA. Results were compared between the LAMP technique, and multiplex PCR. The genetic diversity of clinical isolates was determined by REP-PCR.

Results: Among one hundred *A. baumannii* samples and based on the combined disc test, 56% of isolates were ESBL producers. The sensitivity of the LAMP technique for the identification of *A. baumannii* was 4.06 ng/µl whilst the multiplex PCR was (16.2 ng/µl). Regarding multiplex PCR, (68%) of the isolates were bla_{SHV-1} positive, (40%) bla_{PER-1}, (85%) *aac (6́)-1*, AMPC (67%), bla_{TEM-1} (63%), and (15%) *qnr* respectively. While in LAMP, (69%) of isolates were bla_{SHV-1} positive, (86%) *aac (6')-1*, and (20%) *qnr*. The results of AMPC, bla_{TEM-1}, and bla_{PER-1} genes showed 100% compatibility between multiplex PCR and LAMP assays. The results of REP-PCR indicated there were 17 clones, clone A at 14% was the most prevalent of the isolates.

Conclusion: Wherever equipment and financial constraints are crucial, the LAMP test offers a better and more potent detection rate for the identification of *A. baumannii* isolates than multiplex PCR. Furthermore, the genetic diversity of *A. baumannii* in these clinical isolates showed frequent commonality of genotypes.

Keywords: *Acinetobacter baumannii*; Antibiotic resistance; Loop-mediated isothermal amplification (LAMP); Multiplex polymerase chain reaction; Repetitive extragenic palindromic polymerase chain reaction

INTRODUCTION

Acinetobacter baumannii is a major opportunistic pathogen. It is widely spread in hospitals, especially among immune-compromised, burnt, and ICU (intensive care unit). Different studies showed that the patients with burn injuries are especially susceptible to *A. baumannii* infection during hospitalization

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(1, 2). After *Pseudomonas aeruginosa*, *A. baumannii* has emerged as a significant contributor to high morbidity and mortality rates, as well as a financial burden due to its ability to survive in a wide range of environmental conditions and its capacity to develop or acquire a variety of mechanisms to combat different antimicrobial compounds, which has resulted in rising costs for healthcare systems globally. The mortality rate of this bacterium reached 60% in vulnerable patients (3, 4). *A. baumannii* is a Gram-negative coccobacillus, nonmotile, catalase-positive, oxidase-negative, and aerobic bacterium. It is one of the ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (5). Many hospital-associated infections, including bacteremia, urinary tract infections, pneumonia, meningitis, and wound infections, are linked to *A. baumannii*. These infections are more prevalent in patients who have invasive care procedures like monitoring devices, mechanical ventilation, or urinary catheter (6). In the latest period, the spread of infections with multidrug-resistant *A. baumannii* has increased. The most distinguishing of this pathogen is the ability to evolve mechanisms of resistance against beta-lactam antibiotics. Carbapenem-resistant genes of *A. baumannii* are found on the plasmids, mobile genetic elements, transposons, and integrons which makes the transmission of those genes easy (7). *A. baumannii* possesses various resistance mechanisms against carbapenems which include enzymatic inactivation, increasing the efflux pump for the antibiotics, outer membrane porin, and the alteration of target. Enzymatic degradation of carbapenems is the most important carbapenem resistance mechanism in *A. baumannii*. Carbapenem-hydrolyzing class D β-lactamases known as oxacillinases are more commonly seen in *A. baumannii* than class metallo-β-lactamases (8, 9).

Extended-spectrum beta-lactamases (ESBLs) includes three Ambler Classes A, B, D. The ESBL-producing strains are mutant, it has the ability to digest beta-lactamases which is inhibited by tazobactam and clavulanate. ESBL members in many Gram-negative bacteria are bla_{SHV}, bla_{TEM}, bla_{PER}, and bla_{VEB}, often found on the plasmids. SHV and TEM are one of the most common genes in the world (10).

Resistance to aminoglycosides is common in *A. baumannii* strains. By interacting with the 30S bacterial ribosome, aminoglycosides generate abnormal proteins that cause the death of the bacterial cell. Aminoglycoside enzyme modifying (AMEs) is one of three main pathways that lead to resistance to aminoglycosides. In this mechanism, the ability to bind aminoglycosides is reduced, target site modulation via 16S rRNA by weakeaing the binding of 16S rRNA to aminoglycoside, and changes in efflux transport systems by a depression in intramembrane transfer, or over expression of efflux pumps (11, 12). The main mechanism of *A. baumannii* is the modification of enzymatic AMEs which may be splitted into three subclasses: aminoglycoside N-acetyltransferases (AACs) including [aaC(6')-Ib, aaC(6')-Ih, aaC(3)-I, aaC(3)-II], O-nucleotidyl transferases (ANTs), O-phosphotransferases (APHs). Whereas, the nomenclature of these enzymes is based on the modification of aminoglycoside in a specified position. *aac* (3′)-II and *aac(6′)-Ib* are prevalent genes of enzymatic modification in Gram-negative bacteria. The aminoglycoside enzyme modifying (AME) genes are often located upon mobile genetic elements, resulting in a wide transition of these genes among other origins (11, 13).

Another resistance mechanism that *A. baumannii* possesses is fluoroquinolone resistance. Fluoroquinolones are antibacterial substances that are often used to treat Gram-negative and Gram-positive bacterial infections. Due to its widespread usage, fluoroquinolone resistance has increased around the world. The mechanism of resistance by the appearance of qnr genes is the most prevalent of quinolone resistance. Resistance to quinolones in *A. baumannii* is due to mutations that occur in the quinolone resistance-determining regions of gyrase and topoisomerase genes (14).

Molecular epidemiological studies are important to control the prevalence of multidrug-resistant (MDR) *A. baumannii* infections. Repetitive extragenic palindromic (REP-PCR) is a suitable method for molecular epidemiological study in laboratories, and determine genetic relatedness among isolates within the hospital (15). For the purpose of detecting resistance, several phenotypic tests have advanced, but they often call for skilled personnel and other prerequisites (16). Additionally, conventional techniques for identifying *A. baumannii* have a number of flaws, including the need for costly PCR needs and several exhausting days of identification. In the last decades, loop-mediated isothermal amplification (LAMP) colorimetric assay was advanced as a fast, sensitive,

and highly specific assay. LAMP is more stable and resistant to DNA amplification inhibitors. This made it a good and suitable method to diagnose the carbapenem resistance in *A. baumannii* isolates in healthcare facilities, which is important to manage infection control (17). This study aims to develop LAMP method for the quick *A. baumannii* identification clinical isolates harboring $bla_{\text{SHV-1}}$, $bla_{\text{PER-1}}$, $bla_{\text{TEM-1}}$, AMPC, *qnr*, and *aac (6)-1* genes, and examining the suitability of LAMP and multiplex PCR method to diagnose *A. baumannii* isolates. To comprehend the epidemiology of *A. baumannii* isolates discovered in Tehran, Iran's Shahid Motahari Hospital, researchers are also looking at genetic diversity. Where gives us the ability to enhance treatment approaches required to stop the spread of *A. baumannii* infection.

MATERIALS AND METHODS

Bacterial isolates and identification. From October 2020 to February 2021, one hundred clinical specimens were obtained, from various clinical specimens (sputum, urine, catheter, respiratory tract, blood, cerebrospinal fluid (CSF), trachea, and wounds) from the patients in Shahid Motahari Hospital in Tehran, Iran.

Identification of bacterial isolates. The specimens were detected as *A. baumannii* by standard biochemical tests, such as oxidase, citrate, catalase, methyl red, and indole production. Then, the recognition confirmed of *A. baumannii* by the detection of $bla_{\text{OXA-51-like}}$ gene using PCR assay. Specific primers (Table 1) were used under optimal amplification conditions as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 93°C for 45 s, annealing at 60°C for 60 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using disc diffusion (Kirby -Bauer) method on Mueller-Hinton agar. Discs (Padten Teb Tehran-Iran) containing cefotaxime (CTX) (30 µg), ceftazidime (CAZ) (30 µg), imipenem (IMP) (10 µg), ciprofloxacin (CIP), (5 µg), gentamicin (GM) (10 µg), amikacin (AK) (30 µg), cefepime (FEP) (30 µg), tetracycline (30 µg) meropenem (10 micrograms), polymyxin B (10 micrograms), and trimethoprim/sulfamethoxazole (TS) (1.25/23.75 µg) were put on the inoculated plates and the cultures were incubated at 37°C overnight. The isolates were classified as sensitive, intermediate, or resistant based on Clinical Laboratory Standards Institute guidelines (CLSI 2021).

Identification of ESBL-producing isolates (phenotypic methods). ESBL-producing isolates were detected by the combination disc test (CDT). Discs containing ceftazidime (30 µg), and ceftazidime/clavulanate (30.10 µg) were put on Mueller-Hinton agar medium (Merck, Germany) with appropriate distance between two discs on the plate. The diameter of inhibitory zone around the ceftazidime (30 µg) and ceftazidime (30 µg) +clavulanate (10 µg) were measured. The samples that had an increase in resistant area diameter of ≥5mm around the disc of ceftazidime (30 µg) +clavulanate (10 µg) were identified as strains producing ESBL.

Bacterial DNA extraction. Genomic DNA from *A. baumannii* was extracted based on instructions from the manufacturer (DNA extraction kit, Bioneer, Korea) kit. Then, we measured the concentration, and purity of DNA using NanoDrop instrument. Then, the extracted bacterial DNA was stored at -20°C until the PCR test is performed.

Design of primers for the multiplex PCR. Forward and reverse primers for multiplex PCR were designed: First, we obtained the complete sequence of the entire target gene with accession number from the NCBI site (http://www.ncbi.nlm.nih.gov/gene/). Once we had the gene sequences in their entirety, we fed them Oligo Analyzer program. We then use an in silico PCR website to score the primer sets. Primer3Plus is used for primer design as well. Our custom primers turned out to be effective (Table 1).

Multiplex PCR reaction. Amplicon kit (Amplicon, Odense, Denmark) was used in multiplex PCR as instructed by the supplier. Six preliminary primers were used to characterize the six genes of 100 isolates. Multiplex PCR mixture components contain 12.5 µL 1× Master Mix (Amplicon, Odense, Denmark), 1 µL (10 µM) each of forward and reverse primer, 2 µL (40pg) of genomic DNA, distilled water to obtain the volume of 25 µl. An amplification program was carried out for ($bla_{\text{SHV-1}}$, AMPC, $bla_{\text{PER-1}}$) genes under the optimal conditions: initial

Table 1. Primer sequences used in this study

Primers	Sequence (5′→3′)	Amplified fragment (pb)	GenBank accession number
PF- bla_{SHV-1}	CTGGCGCGCCGATGAACGCT	576	CP128773.1
PR- bla_{SHV-1}	GTCGCCCTGCTTGGCCCGAAT		
PF- bla_{PER-1}	ATGAATGTCATTATAAAAGC	933	CP050386.1
PR- bla_{PER-1}	AATTTGGGCTTAGGGCAGAA		
PF-*aac (6')-1*	AGGAGTACGCGGAATAGAC	229	CP130711.1
PR-*aac (6')-1*	TGTACACGGCTGGACCA		
PF- *qnr*	ATGCCTGGAAAAATGCGAGC	196	CP129872.1
PR- *qnr*	GACTCCTTCGAGGTTGACCC		
PF- bla_{TEM-1}	ATGAGTATTCAACATTTCCG	861	VYVC01000076.1
PR- bla_{TEM-1}	TACCAATGCTTAATCAGTGA		
PF-AMPC	CTCGATGCCCCAGCATATGG	150	KC118541.2
PR-AMPC	CATGGTATTTACTTGATAGA		
PF- bla_{OXA-51}	TTGCTCGTGCTTCGACCGAG	164	NG_049788.1
PR-bla_{OXA-51}	AGCATCGCCTAGGGTCATGT		

denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 93°C for 45 s, annealing at 56°C for 60 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The amplifications were carried out for (*aac (6)-1, qnr,* bla_{TEM-1}) genes with the stander conditions: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 93°C for 45 s, annealing at 57°C for 60 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. in TC1000-G Thermal Cycler (DLAB Scientific CO., China). The amplicons of multiplex PCR products were analyzed in 1.5% agarose gel, stained with safe stain (SinaClon, Tehran, Iran). A PCR ruler (100 bp) (SMOBIO, Taiwan) was used as the molecular-size marker for explanation of PCR results, and were observed by UV transillumination (Kiagene Fanavar, Iran). *A. baumannii* ATCC 19606 was used as a positive control strain.

Primer design for LAMP assay. The complete sequences for six genes were downloaded from National Center for Biotechnology Information (NCBI) database. These sequences were analyzed with the LAMP primers design software "Primer Explorer V5" software (http://primerexplorer.jp/e/) specific primers were automatically designed. Primers used for the LAMP test were an outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP) [FIP (F1c + F2), backward inner primer (BIP) BIP (B1c + B2)], and additional loop primers (loop F and loop B) (LF, LB). Then, for ensuring specificity the primers, the primer sequences were validated using Basic Local Alignment Search Tool-National Center for Biotechnology Information (http://www.ncbi.nlm.gov/BLAST) (Table 2).

LAMP reaction and product revelation. Executing the interaction was done in a 25 µl for 60 minutes at 65ºC. The mixture containing 0.2 µM each of outer primers F3 and B3, 1.6µM each of forward and reverse inner primers (FIP and BIP), and 0.4 µM every forward and reverse loop primers (LF and LB), 1× Isothermal Amplification Buffer (New England Biolabs, Hitchin, UK) and 6 mM $MgSO_4$, 1.4 mM of dNTPs, 320U/ml of the Bst DNA polymerase (New England Biolabs, Massachusetts, USA), and 13.3 µl Distilled water (DW). Finally, 1 µl of DNA extract (20 pg) was added to the tube. Positive control and negative control (distilled water) were included in LAMP assay. The reaction was carried out in a final volume of 25 µl in an Eppendorf tube in a HM100-Pro instrument (DLAB Scientific CO., China). The color shift of the strains may be seen with the unaided eye or under UV transillumination (Kiagene Fanavar, Iran). To detect color-change reaction, 1 µl of safe stain was added to each 25 µl LAMP-reaction mixture to initiating the reactions. The positive isolates for investigation are yellow in color, whereas the negative strains are orange (with no color change).

Table 2. The Sequence of primers used for LAMP assay in this study

Type Primers	Sequence (5′→3′)	Reference
aac(6')-1		This study
F3	CGCAAGAGTCCGTCACTC	
B3	GAACCAGCTTGGTTCCCAA	
FIP	TCCCCGCTTCCAAGAGCAACTGCAATGCTGAATGGAGAGC	
BIP	GAAACCGATCCAGGAGTACGCGGCCTTTGCCCAGTTGTGAT	
F2	TGCAATGCTGAATGGAGAGC	
F1c	TCCCCGCTTCCAAGAGCAAC	
B2	GCCTTTGCCCAGTTGTGAT	
B1c	GAAACCGATCCAGGAGTACGCG	
Qnr		This study
F3	GGGCGAGAAAAGGTCATT	
B3	ACCAACCAGAAATTATCAACC	
FIP	TAGACTGGGACTGCAGAAAGCCCGCTTGGGAAAAAGACA	
BIP	CAGGAACTTGCGCGACGTATCAATTTCAGCATTACCGAAAC	
F2	CCGCTTGGGAAAAAGACA	
F1c	TAGACTGGGACTGCAGAAAGC	
B2	CAATTTCAGCATTACCGAAAC	
B1c	CAGGAACTTGCGCGACGTAT	
bla_{SHV-1}		This study
F3	CGGCGACAACGTCACC	
B3	TCAACGGTCCGGCGAC	
FIP	ATGCTGGCCGGGGTAGTGGGCCTTGACCGCTGGGA	
BIP	GACCCTGCGCAAGCTGCTCGTCCACCATCCACTGCA	
F2	GCCTTGACCGCTGGGA	
F1c	ATGCTGGCCGGGGTAGTGG	
B2	CGTCCACCATCCACTGCA	
B1c	GACCCTGCGCAAGCTGCT	
bla_{PER-1}		This study
F3	TCGCAGGCTTTGTTATGGAA	
B3	GACTCGGCTGAGTCTTTCAC	
FIP	ATGTGCGACCACAGTACCAGCGTCGAAACCACCACAGGAC	
BIP	CGGGAAAACTGCGGCCACTAAAAAACAGCAACCAGCAAGG	
F2	GTCGAAACCACCACAGGAC	
F1c	ATGTGCGACCACAGTACCAGC	
B2	AAAAACAGCAACCAGCAAGG	
B1c	CGGGAAAACTGCGGCCACTA	
bla_{TEM-1}		This study
F3	CGGCATTTTGCCTTCCTGT	
B3	CGACCGAGTTGCTCTTGC	
FIP	ACTCGTGCACCCAACTGATCTTTTTGCTCACCCAGAAACGC	
BIP	ATCCTTGAGAGTTTTCGCCCCGGGGATAATACCGCACCACAT	
F2	TTTGCTCACCCAGAAACGC	
F1c	ACTCGTGCACCCAACTGATCTT	
B2	GGGATAATACCGCACCACAT	
B1c	ATCCTTGAGAGTTTTCGCCCCG	
AmpC		This study
F3	TTCAGGATAAAAAAGCCGTAA	

Table 2. Continuing...

B3	CACTTGTATACGTCGCAAG
FIP	ATCCACCAGCTGTAGCGGTAGCAGTACCATTTTTGAACTAGG
BIP	ATCTCTTTTGATGACACACCCGTGAAGAAGATTAACTTGGTCAAT
F2	GCAGTACCATTTTTGAACTAGG
F1c	ATCCACCAGCTGTAGCGGTA
B2	TGAAGAAGATTAACTTGGTCAAT
B1c	ATCTCTTTTGATGACACACCCG

Determine the sensitivity of LAMP and multiplex PCR assays for detection of *Acinetobacter baumannii*. The sensitivity of LAMP and multiplex PCR was determined with genome concentrations. These concentrations were measured using a Nanodrop instrument. Then, bacterial DNA was diluted by a set of serial dilutions (10-fold). The first concentration of DNA was determined as 130 ng/µl.

Specificity of LAMP and multiplex PCR assays. To evaluate the specificity of LAMP and multiplex PCR assay was detected by testing *A. baumannii* (N= 30) bacterial strains (Fig. 1), and non-*Acinetobacter* species (N = 7) (*Staphylococcus aureus* ATCC *25923*, *Pseudomonas aeruginosa*, *Salmonella spp.*, *E. coli* O157:H7, *Listeria monocitogenes*, *Klebsiella pneumoniae* ATCC BAA 1705 and *Enterobacter aerogenes*), were used by designed methods.

Determining genetic relatedness of *A. baumannii* isolates using repetitive extragenic palindromic PCR (REP-PCR). The genetic relatedness was determined using repetitive extragenic palindromic PCR (REP-PCR) was performed in a final volume of 25 µL; 2.5 µL 10× PCR buffer, 20 mM dNTP, 0.5 µL $MgCl_2$ (3mM), 2 U (0,5 µL) of Taq DNA polymerase (Amplicon, Odense, Denmark), 25pM of each forward and reverse primers (reverse: 5′-III GCGCCGICATCAGGC-3 and forward: 5-ACGTCTTATCAGGCCTAC-3), 3 µL of genomic DNA, and 1/16 µL of distilled water. Initial denaturation at 95°C for 10 minutes was followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute followed by a final extension phase at 72°C for 16 minutes. Amplification products were electrophoresed on 1.5% agarose gel (Pishgam Iran), and, then, PyElph 1.4 software was used to generate the phylogenetic tree. PyElph, a software tool that automatically extracts data from gel images, computes the molecular weights of the analyzed molecules, and generates phylogenetic trees based on information available in a gel image. Thus, that software tool can be used successfully for molecular biology and genetics studies.

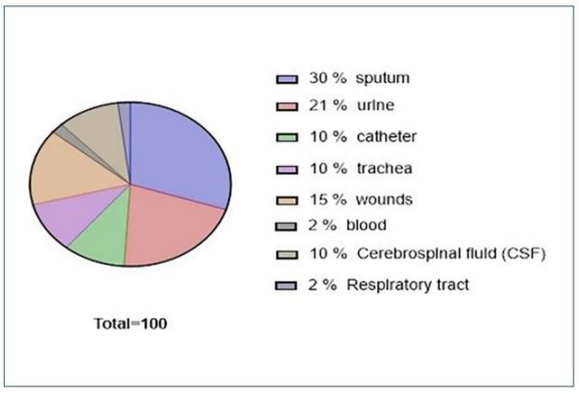

Fig. 1. Frequency distribution of *A. baumannii* isolated from patients according to the kind of clinical samples.

RESULTS

Characteristics of *A. baumannii* samples. One hundred isolates of *A. baumannii* positive for $bla_{\text{OXA-51-like}}$ gene were confirmed by PCR. Out of 100 *A. baumannii* isolates obtained from different clinical specimens, including sputum (30. %), urine (21%), respiratory tract (10%), trachea (10%), a catheter (10%), blood (2%), wounds (15%), and cerebrospinal fluid (CSF) (2%). These clinical specimens were collected from males (80%) and females (20%). The frequency distribution of *A. baumannii* isolates based on the sample is represented in Fig. 1. The categories age of patients were: 0-25 years (n = 10%), 25-50 years (n = 50%), and 50-75 years (n=40%).

Antimicrobial susceptibility testing. Out of 100 *A. baumannii* isolates, the rate of resistance was as follows: (100%) ceftazidime, (87%) ciprofloxacin,

(84%) amikacin, (87%) tetracycline, (97%) cefotaxime, (94%) cefepime, (100%) imipenem, (81%) trimethoprim /sulfamethoxazole, (85%) gentamicin, and (95%) meropenem. While the highest rate of susceptibility was found for polymyxin B (100%), ceftazidime and imipenem showed high level of resistance (Fig. 2).

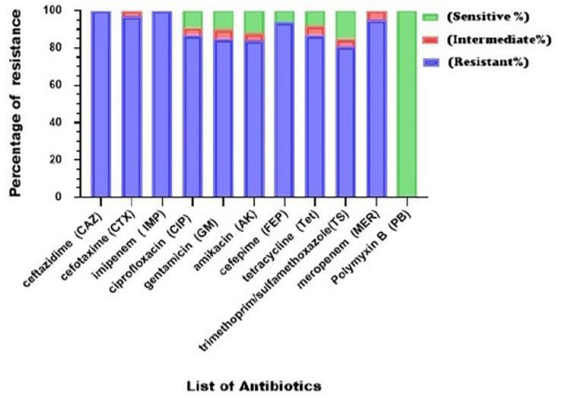

Fig. 2. The percentage of non-susceptibility of *A. baumannii* to various antibiotic classes.

Identification of ESBL-producing isolates (Phenotypic methods). Performing combined disc test (CDT) showed 56% of isolates were positive for the ESBLs. Among 56% of ESBL-producing isolates, bla_{SHV-1} positive isolates were predominating (41%) followed by AMPC (37%), bla_{TEM-1} (31%), and bla_{PER-1} (23%).

Determine the sensitivity of LAMP and multiplex PCR assay for detection of *A. baumannii.* The sensitivity of primers for detecting the target bla_{SHV-1}, bla_{PER-1}, bla_{TEM-1}, AMPC, *qnr*, and *aac (6)-1* genes by the usage of a set serial dilutions of genomic DNA of *A. baumannii* was determined. The last dilution of multiplex PCR was the positive dilution (16.2 ng/ µl) that was obtained as PCR sensitivity. While, (4.06 ng/µl) was the lowest dilution of the LAMP reaction that was obtained as LAMP sensitivity (Fig. 3 A and B). The multiplex PCR could detect bla_{SHV-1}, bla_{PER-1}, bla_{TEM-1}, AMPC, *qnr*, and *aac (6)-1* genes at 2 µl (40pg) of bacterial DNA. Whereas, the LAMP could detect these genes at 1 µl (20pg) of bacterial DNA.

The analytical specificity of LAMP assay. Through the use of *A. baumannii* and non-*Acinetobacter* species, the specificity of the LAMP technique was

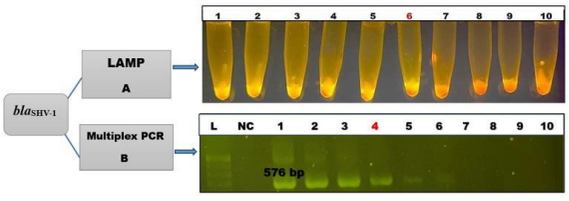

Fig. 3. The sensitivity of LAMP reaction and multiplex PCR for the identification of the bla_{SHV-1} gene. using different concentrations of bacterial Genomic DNA. LAMP assay (A), and multiplex PCR (B) were performed induplicate for each dilution. Tube and lane: NC: negative control, L: Ladder DNA 100 bp, 1: 130 ng/ µl, 2: 65 ng/ µl, 3:32.5 ng/ µl, 4: 16.2ng/ µl, 5: 8.1 ng/ µl, 6: 4.06 ng/ µl, 7: 2.03 ng/ µl, 8: 1.01 ng/ µl, 9: 0.50 ng/ µl,10: 0.25 ng/ µl. (A) The direct optical method for the detection of LAMP after coloration by added the safe stain to 25 µl of LAMP products; (B) Multiplex PCR products were detected by 1.5% agarose gel electrophoresis stained with safe stain.

discovered. The study's particular resistance genes amplified using both techniques were positive, but not for the non-*Acinetobacter* species that were evaluated. All *A. baumannii* (N = 30) were detected by multiplex-PCR, but all non-*Acinetobacter* isolates (N = 7) were negative in this reaction. In return, LAMP technique was specific for *A. baumannii* successfully amplified, while non-*Acinetobacter* isolates were not amplified which suggest the specificity of that LAMP assay for *A. baumannii.*

Comparison of LAMP and multiplex PCR in clinical samples. According to the LAMP assay, 86 percent of the 100 clinical isolates tested were positive for *aac (6')-1*, while it was 85% in multiplex PCR technique (Fig. 4). Sixty-nine strains (69%) were found to be positive for bla_{SHV-1} when tested using LAMP, whereas only (68%) were discovered using multiplex PCR. For the detection of *qnr*, 20 strains were determined positive for *qnr* by LAMP while only fifteen strains (15%) were found out by multiplex PCR. For AMPC (67%), bla_{PER-1} (40%), and bla_{TEM-1} (63%) genes were found positive by multiplex PCR and LAMP completely (Table 3). In general, LAMP method was more sensitive and had a higher diagnosis performance than multiplex PCR in clinical samples of *A. baumannii.*

Determining genetic relatedness of *A. baumannii* isolates using repetitive extragenic palindromic PCR (REP-PCR). The isolates of *A. baumannii* were

classified based on the banding patterns. The valuation of REP-PCR patterns showed 14% of the isolates were included in clone A, 13% in clone B, 10% in clone C, 8% in clone D, 6% in clone E, 5% in clone F, 3% in clone G, 2% in clone H, I and 1% in clone J, K, L, M, N, O, P, Q. (Fig. 5).

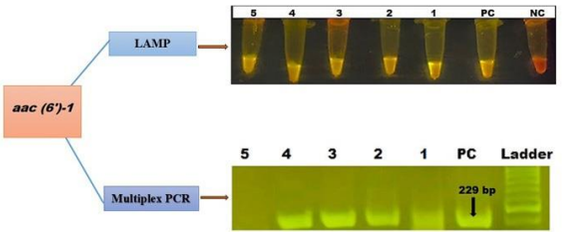

Fig. 4. Comparing between LAMP and Multiplex PCR assays for the Identification *aac (6')-1* gene of *A. baumannii* in clinical isolates. lane Ladder,100 bp DNA marker; lane PC, positive control; lane NC, negative control.

Table 3. Comparable between of LAMP and Multiplex PCR in Clinical isolates

Gene	Multiplex PCR		LAMP	
	Positive	Negative	Positive	Negative
aac (6')-1	85%	15%	86%	14%
bla_{SHV-1}	68%	32%	69%	31%
Qnr	15%	85%	20%	80%
AMPC	67%	33%	67%	33%
bla_{PER-1}	40%	60%	40%	60%
bla_{TEM-1}	63%	37%	63%	37%

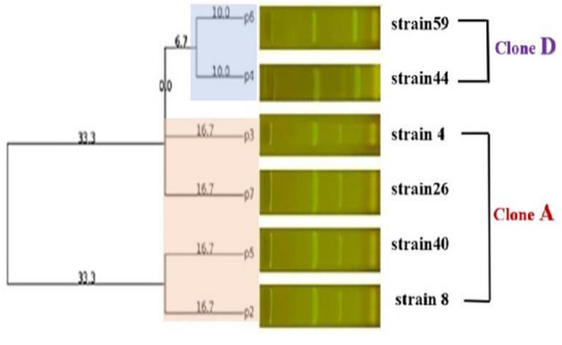

Fig. 5. Phylogenetic tree constructed by PyElph1.4 software based on REP-PCR amplifications of *A. baumannii* isolates.

DISCUSSION

A. baumannii has become increasingly resistant to most of the currently effective antibiotics, and it is a major concern in hospital environment. *A. baumannii* developed various resistance mechanisms, and is still developing new ways to acquire antibiotic resistance (18).

One of the noticeable, findings of this study, all *A. baumannii* isolates were positive for resistance to at least one antibiotic stands out. One hundred percent of the bacterial isolates tested grew rapidly in the presence of ceftazidime and imipenem, whereas trimethoprim/sulfamethoxazole showed the lowest level of resistance (81%), for polymyxin B all the isolates were susceptible. Therefore, these antibiotics remain the best therapeutic choices against *A. baumannii* infections which made treating infections caused by this pathogen very difficult. Similar results have been observed in the studies conducted in Iran and South Africa (19, 20). The antimicrobial resistance rate seems to be substantial and comparable to that of the present investigation. This implies that using antibiotics wisely and taking strict precautions are both required for treating and avoiding *A. baumannii* infections.

Extended-spectrum β-lactamases (ESBLs) production is one of the most significant resistance mechanisms of *A. baumannii* isolates. This study showed, 56% of isolates were ESBL producers. These results were compatible with another study in Iran which reported that 59% ESBL-producing of *A. baumannii* strains (21). However, another study further showed results lower than we obtained in our study (39%) (22). While in contrast to our study, the study carried out in 2018 in Tehran showed a higher occurrence for ESBL production 78% (23), this result is closer to those by other study showing 88% of isolates produce ESBL (24). The variance in results between these investigations may be the result of variations in the techniques used to identify the ESBL isolates. Because of the complexity and therapeutic restrictions of these isolates, the current study's high prevalence of *A. baumannii* ESBL-producing isolates raises concerns about potentially dangerous nosocomial infections in Iran. However, multiple factors such as different practices common in hospitals and an important reason is inappropriate antibiotic use contribute to the increase in the prevalence of resistant organisms globally. Our data highlight the requirement to establish an antimicrobial resistance monitoring system at local and national levels.

Molecular methods can show advantages above phenotypic methods that are time-consuming and affects negatively on the patient's health and on anti-

biotic resistance challenges (25). In our study, among all 100 *A. baumannii* strains tested by multiplex PCR assay 68%, 67%, 40%, 63%, and 15% of isolates carried the, $bla_{\text{SHV-1}}$, AMPC, $bla_{\text{PER-1}}$, $bla_{\text{TEM-1}}$, *qnr* genes respectively. The *acc (6')* is connected to high-level aminoglycoside resistance in *A. baumannii* strains where our results showed a high prevalence aminoglycoside-modifying gene (85%) *acc (6')-1* gene among the *A. baumannii* isolates. The outcome of the current study has a similarity with other studies from Iran and other countries (26-28). The result of earlier Indian investigation showed the prevalence of the bla_{SHV} gene was consistent with our results, but the prevalence of the bla_{TEM} gene was higher than our results (29). Quinolone resistance in *A. baumannii* is caused by the *qnr* gene, and it has become more common recently. The prevalence rate of the *qnr* gene in our study are lower than results of Mirnejad et al. in which 50% of *A. baumannii* isolates had the *qnr* gene (30).

In this research, the performance of LAMP was validated and compared with multiplex PCR to assure that it could be used as an alternative diagnostic assay for rapid detection. This study applied LAMP as a novel molecular technique, rapid, sensitive, specific, and not difficult. It does not require expensive equipment special equipment or skilled person also the capability of LAMP technique to production 10^9 copies of amplified DNA in little time makes the interaction more sensitive than other methods. In LAMP reaction, four or six primers recognize six or eight regions on the target gene, therefore it a high specificity (31).

Based on our experiment, LAMP assay was found to be highly specific and non- *A. baumannii* species was not amplified. These results were compatible with preceding studies conducted by Chu et al. (32). LAMP had been described as a powerful amplification tool with high sensitivity in terms of DNA concentration. In the our study, the detection limit of LAMP assay for $bla_{\text{SHV-1}}$ primer was 4.06 ng/ µl, which was more sensitive than multiplex PCR (16.2ng/ µl), similar results were shown in previous study for identification of *Streptococcus pneumonia*, *Listeria monocyte* genes, and *Staphylococcus* strains. LAMP technique efficiency was 10- to 1,000-fold more senior compared with conventional PCR (33).

The importance of the study was that the LAMP assay was standardized for detection of the important genes of the *A. baumannii* while it was conducted for six distinct resistance genes, no cross-interaction between the six genes tested occurred. These results showed high efficiency when compared with the multiplex PCR results, the *aac (6')-1* and $bla_{\text{SHV-1}}$ genes could not be amplified from one clinical isolate. Then, the strain was analyzed for the presence of the *aac (6')-1*, $bla_{\text{SHV-1}}$ genes by LAMP method, which was a positive result in this case. For the *qnr* gene, the result that we got by the multiplex PCR was 15%. While LAMP assay detected 20 positive samples, 5 strains were positive results for the *qnr* gene that were not amplified by multiplex PCR. We believed that the low bacterial content and incorrect pathogen detection may be reason for the failure in amplifing these isolates. These results indicate that the sensitivity of multiplex PCR is not always satisfactory and that there may be issues with the diagnostic results. Finally, the LAMP technique used in this work has shown trustworthiness in its capacity to recognize various gene-groupings. In our research, DNA molecule amplification took place over a shorter period of time (65 min), compared to the multiplex PCR method. Thereby, providing time and effort while performing a LAMP test.

As regards the clonal relationships, in this study, 100 isolates of *A. baumannii* were assessed by the REP-PCR technique. In the current study, 17 genotypes were acquired which classified to A - Q. REP-PCR results showed 14% of the isolates were attributed in clone A, 13% in clone B, 10% in clone C, 8% in clone D, 6% in clone E, 5% in clone F, 3% in clone G, 2% in clone H, I and 1% in clone J, K, L, M, N, O, P, Q. clone A was the most prevalent (Fig. 5). The study by Nogbou et al., from March 2017 to April 2018 showed 4 clones among the isolates collected from Dr George Mukhari Academic Hospital. Clone A (69%) was the most prevalent. This indicates a high prevalence rate of *A. baumannii* in South Africa (34). 120 *A. baumannii* isolates, predominantly from ICUs and infectious illness wards, were divided into 9 genotypes in the research by Kian et al. in Iran (35). Understanding strain diversity is essential in promoting early detection of resistance and may reduce bacterial transmission in hospital settings. The significant degree of similarity among *A. baumannii* isolates found in metropolitan hospitals suggests that some strains may be spread by medical personnel, equipment, or other elements. Our results showed the genotype prevalence of these resistant isolates

in Shahid Motahari Hospital, Tehran, and based on these results, there are dangerous alarms about the ability of these bacteria to transport their resistance genes to other microorganisms.

CONCLUSION

LAMP technique is a powerful tool for the quick detection of pathogens in clinical samples, especially in resource-constrained countries. Based on the results of our study, the LAMP test is highly sensitive and has a great possibility in diagnostics microorganisms.

Using molecular methods including repetitive element sequence-based (REP-PCR) is of great importance to determine the provenance of infection and the genetic relationships between the microbial isolates, and classified strains in specific genotype groups, which are useful methods for genotyping purposes and access outbreaks at the local scale but the delicate interpretation of results should be done. Therefore, further endemic strains of the *A. baumannii* strain isolates inside the hospital may be discovered via a longer sampling period and continued molecular epidemiologic monitoring.

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