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RESEARCH ARTICLE

Molecular Identification of *Acinetobacter baumannii* Isolated from Clinical Samples in Iraq by Using Multiplex PCR

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ABSTRACT

Acinetobacter baumannii is one of the most clinically significant pathogens that cause severe nosocomial infections. Rapid identification of this bacterial species is essential so that proper treatment can be provided and outbreaks can be controlled. In this study, a multiplex PCR was used to identify *A. baumannii*. Twenty clinical isolates of *A. baumannii* and three each of Gram + ve (*Staphylococcus aureus*, *Bacillus licheniformis*, *Micrococcus yunnanensis*) and Gram − ve (*Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) bacterial isolates were subjected to singleplex and multiplex PCR reactions using three sets of species-specific primers (P-Ab-ITS-F, P-Ab-ITS-R; OXA-51-like-F, OXA-51-like-R; sp2-F, sp4-R). Additionally, 24 *Acinetobacter* spp., including *A. baumannii* genomes, were retrieved from the National Center for Biotechnology Information GenBank database and aligned with each primer using the Blast program. The complementary bases of the primers with the bacterial genome were determined, and their melting temperatures were calculated using the Promega Tm calculator. Multiplex PCR successfully amplified three amplicons in all the tested *A. baumannii* isolates; however, it did not amplify other bacterial species. Furthermore, in comparison to other *Acinetobacter spp*., there were significant differences in the mean annealing temperatures of the primer sets for *A. baumannii* ($p < 0.01$). Taken together, multiplex PCR can be considered a relatively simple, rapid, and specific method for the identification of *A. baumannii*. Moreover, it is feasible in most laboratories and is essential for clinicians, the control of outbreaks, and epidemiological studies.

Keywords: *Acinetobacter baumannii*, $bla_{\text{OXA-51-like}}$ gene, *gyrB* gene, intergenic spacer (ITS) region, multiplex PCR

Introduction

Acinetobacter baumannii is an emerging nosocomial pathogen worldwide. This bacterial species causes many infections, including ventilator-associated pneumonia, secondary meningitis, bacteremia, and urinary tract infections.[1,2] In addition, *A. baumannii* exhibits multidrug resistance (MDR), extensive drug resistance (XDR), and pan-drug resistance (PDR), acquired by mutations or acquisition of plasmids or transposons.[3,4] Furthermore, it can survive in different environments owing to biofilm production.[5] According to the World Health Organization (WHO), *A. baumannii* is a critical pathogen threatening human life and urgently requires new antibiotics.[6,7]

The accurate identification of *Acinetobacter* spp. is essential for assessing their frequency in clinical samples and their role as human pathogens.[8] However,

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currently, identifying the *Acinetobacter* genus at the species level remains challenging. The six species that comprise the *A. calcoaceticus–A. baumannii* complex (ACB complex) *A. calcoaceticus* (genomic species 1), *A. baumannii* (genomic species 2), *A. pittii* (genomic species 3), *A. nosocomialis* (genomic species 13TU), *A. seifertii,* and *A. lactucae*- are closely related genetically, making it complex to distinguish them phenotypically.[9]

Ribotyping is a method used to identify *A. baumannii* with promising results.[10] However, this method is time-consuming and complex.[11] Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a relatively new technology used to identify *Acinetobacter* spp. that generally shows promising results.[12] Nonetheless, this technology can also misidentify some strains of *Acinetobacter* due to the type of apparatus used and its database.[13]

The development of molecular methods for bacterial identification has permitted precise inter- and intra-species determination.[5] DNA–DNA hybridization techniques have been used to identify the *Acinetobacter* genus; however, this technique is inappropriate for routine laboratory use.[14] *Acinetobacter* spp. can also be determined by restriction analysis of ribosomal DNA genes,[15] the region located between the 16S-23S rRNA genes, the intergenic spacer (ITS),[11] and the *recA* gene.[16] However, these primers complement regions found in other bacterial genera; therefore, amplicons from other bacterial species are erroneously amplified.[17]

Three research groups have developed specific primers for identifying *A. baumannii*. The first group used the ITS region between the 16S-23S rRNA and *recA* genes in a multiplex PCR reaction to identify 22 reference strains and 238 clinical isolates of the genus *Acinetobacter*. Of these, 83 *A. baumannii* isolates were identified using this method with 100% specificity.[18] In contrast, a second research group used $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, and integrase I genes using a multiplex PCR. The $bla_{\text{OXA-51-like}}$ gene was identified in 141 *A. baumannii* isolates but not in other *Acinetobacter* spp.[19] The last group used a PCR-based method using differences in the *gyrB* gene to identify *A. baumannii* and *Acinetobacter* genomic sp. 13TU. They used 118 clinical and referenced *Acinetobacter* spp. and correctly identified 31 *A. baumannii* using that method.[20]

In this study, clinical isolates of *A. baumannii* were identified using a combination of three primer pairs (P-Ab-ITS-F, P-Ab-ITS-R), (OXA-51-like-F, OXA-51-like-R), and (sp2-F, sp4-R) in a multiplex-based PCR method to amplify the ITS region between the 16S-23S rRNA, $bla_{\text{OXA-51-like}}$, and *gyrB* genes.

Materials and methods

Study design

This study was divided into experimental and theoretical parts (Fig. 1).

Bacterial isolates

Acinetobacter baumannii isolates were kindly provided by Mr. Sajjad Abudl Wahab, Institute of

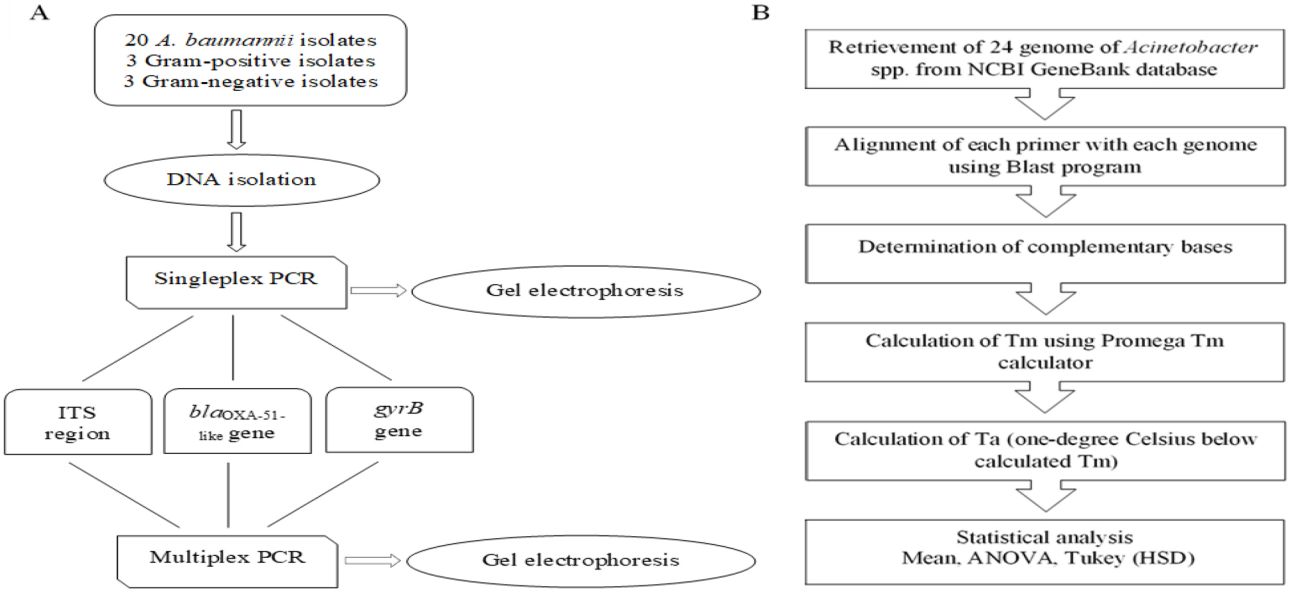

Fig. 1. Study design. A: Experimental part, B: Theoretical part.

Table 1. Primer sets used for multiplex PCR reaction to amplify three genes in *A. baumannii*.

Primer	Sequence (5'—3')	Ta* (°C)	Amplicon size (bp)	Reference
P-Ab-ITS-F	CATTATCACGGTAATTAGTG	58	208	18
P-Ab-ITS-R	AGAGCACTGTGCACTTAAG			
OXA-51-like-F	TAATGCTTTGATCGGCCTTG	60	353	19
OXA-51-like-R	TGGATTGCACTTCATCTTGG			
sp2-F	GTTCCTGATCCGAAATTCTCG	60	490	20
sp4-R	AACGGAGCTTGTCAGGGTTA			

*Ta: Annealing temperature.

Genetic Engineering and Biotechnology for Postgraduate Studies, Baghdad University. Gram-positive (*Staphylococcus aureus*, *Bacillus licheniformis*, *Micrococcus yunnanensis*) and gram-negative isolates (*Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) were kindly provided by Mr. Arif Abbas Ghiab, College of Biotechnology, Al-Nahrain University. All isolates were identified using 16S rRNA followed by sequencing and Blast program analysis. The isolates were inoculated into Brain Heart Infusion broth and incubated overnight at 37 °C with shaking. For long-term storage, 1 ml of the culture was mixed with 1 ml of 60% glycerol and stored at −20 °C.

DNA extraction and PCR amplification

DNA was extracted from the bacterial isolates using the Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) according to the manufacturer's instructions. The concentration and purity were estimated using NanoDrop 2000C (Thermo Fisher Scientific, USA) and stored at −20 °C for further use. Using single and multiplex PCR, genes were amplified using three sets of primers (Table 1).

Each primer set was used in a single reaction to amplify a specific region of *A. baumannii* genome. After this, three primer sets were combined for bacterial identification using a multiplex PCR-based assay. First, a single PCR reaction was accomplished using template DNA (100 ng) in a total reaction volume of 25 μl containing 1X GoTaq® G2 Green Master Mix (GoTaq® G2 DNA polymerase, 200 μM of each dNTP, 1.5 mM $MgCl_2$, pH 8.5, and yellow as well as blue dyes) [Promega, USA] and 0.4 μM of each forward and reverse primers. The PCR program included an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C or 60 °C (depending on the primer set) for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 5 min. Then, multiplex PCR was performed by mixing three primer sets (P-Ab-ITS-F, P-Ab-ITS-R; OXA-51-like-F, OXA-51-like-R; sp2-F, sp2-R) with the same components and reaction conditions, except for annealing temperature of 56 °C. The identity of *A. baumannii* was further confirmed by sequencing the *gyrB* gene using the Sanger method (Macrogen, Korea).

Calculating annealing temperature

Genome sequences of the 24 *Acinetobacter* spp. were retrieved and aligned with each primer using the BLAST program on the NCBI website. The complementary bases of the primers with the genome of the isolates were determined, and their melting temperatures were calculated using the Promega Tm calculator (https://worldwide.promega.com/resources/tools/biomath/tm-calculator/). The annealing temperature was one degree below the lowest Tm for each primer set.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences version 26 (SPSS-V.26) [IBM, USA]. Analysis of variance was used to compare the mean of the annealing temperatures of the primers. Furthermore, a Tukey honestly significant difference (HSD) post hoc test was used to compare the significance of the mean annealing temperature of the primers that have annealed to the genome of *A. baumannii* compared to other *Acinetobacter* genomes.

Results

Amplification of ITS region

The DNA of 20 *A. baumannii*, 3 Gram-positive, and 3 Gram-negative bacterial isolates was subjected to PCR using specific primers (P-Ab-ITS-F and P-Ab-ITS-R) to amplify the ITS region, located between the 16S and 23S rRNA genes. Amplicons with a molecular size of 208 bp were observed in 20 *A. baumannii* isolates. On the other hand, there were no amplified bands were observed for other species of Gram-positive and Gram-negative bacteria which can be an indicator that the primer set are species specific (Fig. 2).

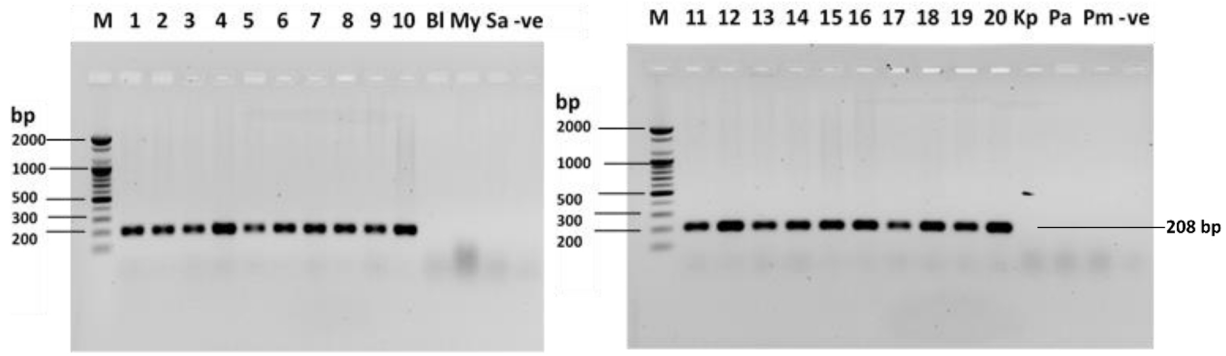

Fig. 2. Amplification of ITS region using primer pair (P-Ab-ITS-F and P-Ab-ITS-R). Lanes 1–20: *A. baumannii* isolates, Bl: *B. licheniformis*, MY: *M. yunnanensis*, Sa: *S. aureus*, Kp: *K. pneumoniae*, Pa: *P. aeruginosa*, Pm: *P. mirabilis*, −ve: negative control, M: 100 bp DNA ladder. Gel electrophoresis was done on 1% agarose at 5 V/cm for 90 min.

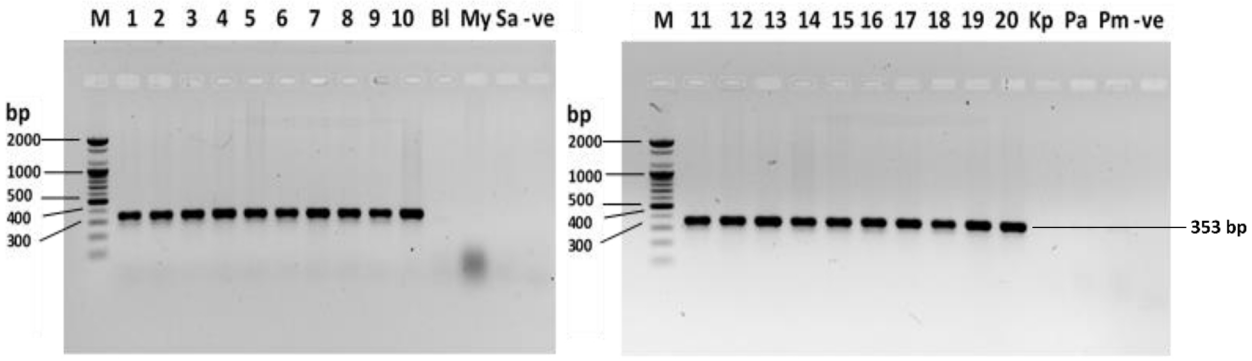

Fig. 3. Amplification of $bla_{OXA\text{-}51\text{-}like}$ carbapenemase gene using primer pair (OXA-51-like-F and OXA-51-like-R). Lanes 1–20: *A. baumannii* isolates, Bl: *B. licheniformis*, MY: *M. yunnanensis*, Sa: *S. aureus*, Kp: *K. pneumoniae*, Pa: *P. aeruginosa*, Pm: *P. mirabilis*, −ve: negative control, M: 100 bp DNA ladder. Gel electrophoresis was done on 1% agarose at 5 V/cm for 90 min.

Amplification of $bla_{OXA\text{-}51\text{-}like}$ carbapenemase gene

The DNA of 20 *A. baumannii*, 3 Gram-positive, and 3 Gram-negative bacterial isolates was exposed to PCR using specific primers (OXA-51-like-F and OXA-51-like-R). Amplicons with a molecular size of 353 bp were detected in 20 *A. baumannii* isolates. Conversely, there were no amplified bands were observed for other Gram-positive and Gram-negative bacteria indicating the specificity of the primers to *A. baumannii* isolates (Fig. 3).

Amplification of gyrB gene

The DNA of 20 *A. baumannii*, 3 Gram-positive, and 3 Gram-negative bacterial isolates was amplified using PCR with specific primers (sp2-F and sp4-R). Amplicons with a molecular size of 490 bp were observed in the case of 20 *A. baumannii* isolates. Nonetheless there were no amplification was observed for other Gram-positive and Gram-negative bacteria indicating the specificity of this primer set to *A. baumannii* isolates (Fig. 4).

Detection of A. baumannii using multiplex PCR

The DNA of 20 *A. baumannii*, 3 Gram-positive, and 3 Gram-negative bacterial isolates were subjected to multiplex PCR using three primer sets (P-Ab-ITS-F and P-Ab-ITS-R, OXA-51-like-F and OXA-51-like-R, and sp2-F and sp4-R). Three amplicons with molecular sizes of 208 bp, 353 bp, and 490 bp were noticed for 20 *A. baumannii* isolates. On the other hand, there were no amplified bands for other Gram-positive and Gram-negative bacteria which relatively can be considered an easy and accurate detection method for *A. baumannii* isolates (Fig. 5).

Comparison of primer annealing temperature

The possibility of the three primer sets to detect other *Acinetobacter* spp. was theoretically tested by comparing the annealing temperature of the three primers to the genome of *A. baumannii* (CP043953) with other *Acinetobacter* spp. The mean annealing temperature for all primers was calculated for each species and compared with *A. baumannii*. The highest

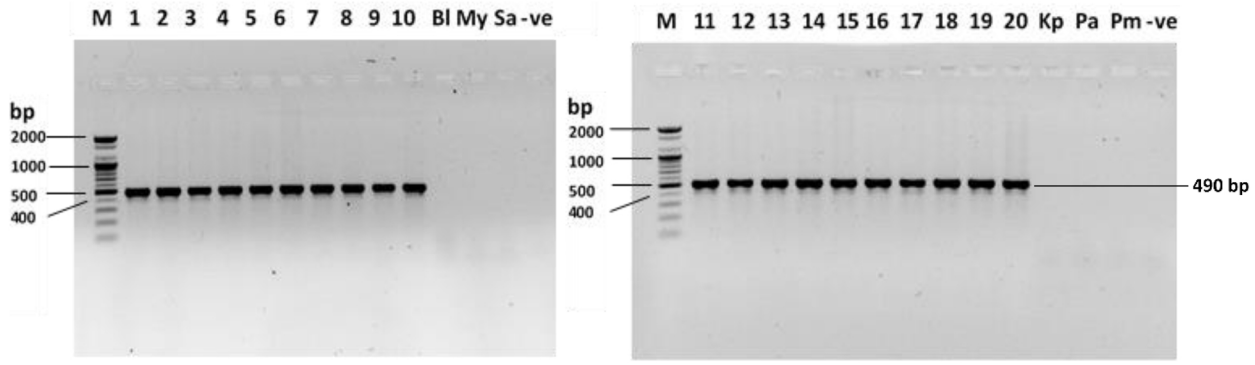

Fig. 4. Amplification of *gyrB* gene using primer pair (sp2-F and sp4-R). Lanes 1–20: *A. baumannii* isolates, Bl: *B. licheniformis*, MY: *M. yunnanensis*, Sa: *S. aureus*, Kp: *K. pneumoniae*, Pa: *P. aeruginosa*, Pm: *P. mirabilis*, −ve: negative control, M: 100 bp DNA ladder. Gel electrophoresis was done on 1% agarose at 5 V/cm for 90 min.

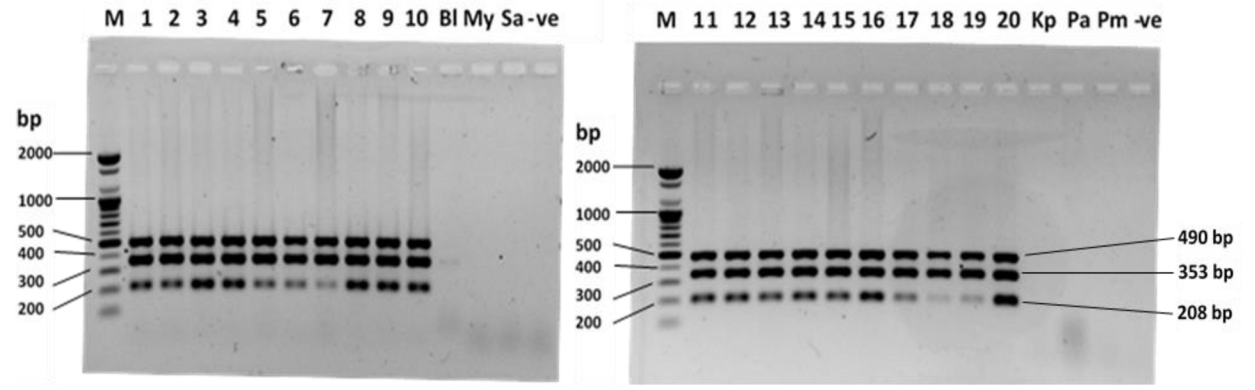

Fig. 5. Detection of *A. baumannii* using multiplex PCR with three primer sets (P-Ab-ITS-F and P-Ab-ITS-R; OXA-51-like-F and OXA-51-like-R; sp2-F and sp4-R): *A. baumannii* isolates, Bl: *B. licheniformis*, MY: *M. yunnanensis*, Sa: *S. aureus*, Kp: *K. pneumoniae*, Pa: *P. aeruginosa*, Pm: *P. mirabilis*, −ve: negative control, M: 100 bp DNA ladder. Gel electrophoresis was done on 1% agarose at 5 V/cm for 90 min.

mean annealing temperature was observed for *A. baumannii* (59.67 ˚C), followed by *A. pittii* (CP002177) (45 ˚C) and the lowest was for *A. towneri* (CP071766) (38.33 ˚C). Additionally, the mean difference in the annealing temperatures of the primers for *A. baumannii* differed significantly from that of other species ($p < 0.01$) (Fig. 6, Table 2).

Discussion

A. baumannii is a pathogen associated with many nosocomial infections, such as septicemia, endocarditis, meningitis, urinary tract infections, wound infections, and pneumonia.[21] Hence, controlling outbreaks and treating bacterial infections require precise and rapid identification of *A. baumannii*.[22–24] Therefore, many research groups have used molecular techniques using species-specific primers for specific genes to identify bacterial isolates at the species level.

This study used three primer sets targeting three specific regions of the *A. baumannii* genome. The first set targeted the ITS region between the 16SrRNA and 23SrRNA genes. Single-plex PCR was used to amplify this region, and a 208 bp amplicon was observed for all tested *A. baumannii* isolates. On the other hand, it did not give any amplified band of the other six Gram-positive and Gram-negative bacterial isolates. This region is an excellent marker for identifying bacterial species and has high interspecies heterogeneity among various *Acinetobacter* spp., with a range of 48–92% identity. Additionally, using this region, the identity of the closest bacterial species within *A. calcoaceticus-A. baumannii* complex ranged from 86% to 92%. Furthermore, the primer set (P-Ab-ITSF and P-Ab-ITSB) are completely specific to *A. baumannii* with identity 100% while the identity of this primer set for other species within the complex ranges from 63–84%.[25] Therefore, many research groups have used this marker to identify *A. baumannii* isolates from various sources.[26,27]

The second set of primers was used to amplify part of $bla_{\text{OXA-51-like}}$ carbapenemase genes in *A. baumannii* isolates. A PCR reaction using this primer set for amplifying a region corresponding to 353 bp band

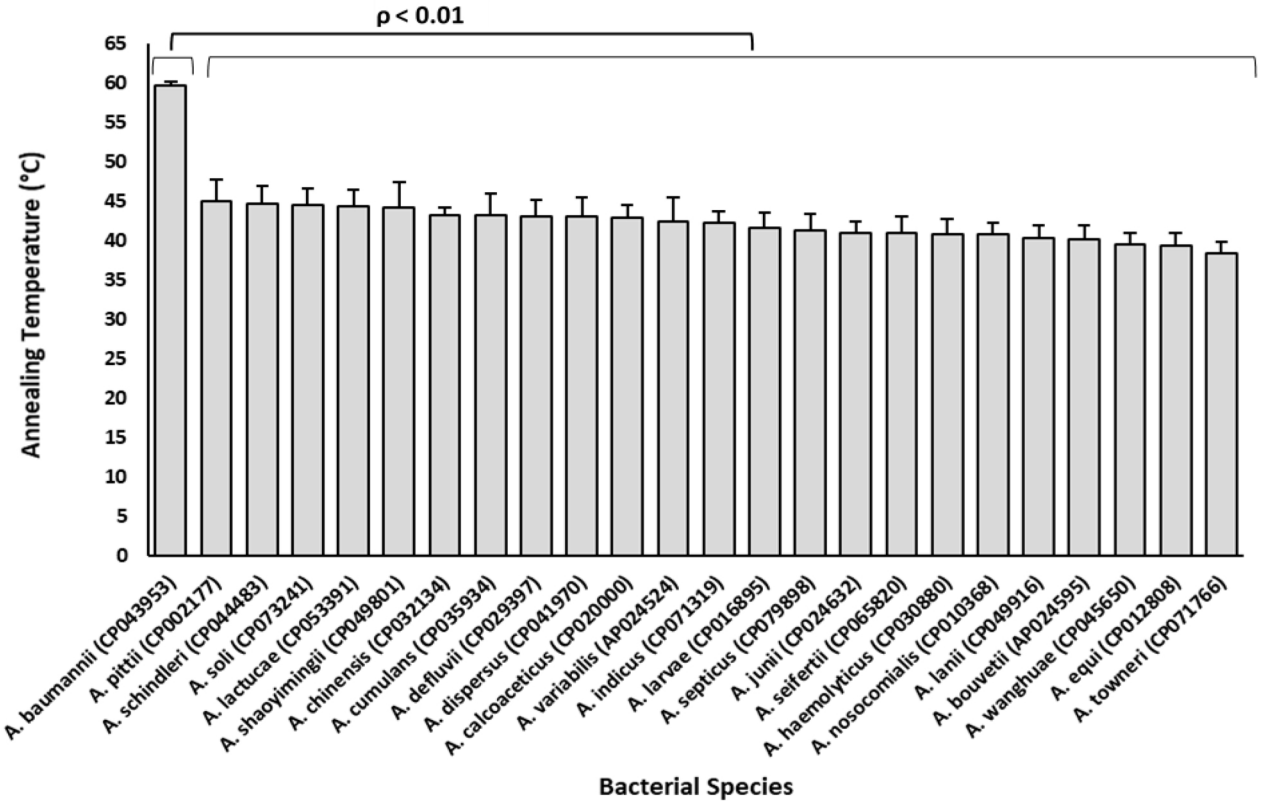

Fig. 6. The mean differences±standard error of primer annealing temperatures between *A. baumannii* and other *Acinetobacter* spp.

Table 2. The mean differences and significance of primer annealing temperatures between *A. baumannii* and other *Acinetobacter* spp.

Bacterial isolate*a	Bacterial isolate*b	Ta (˚C) Mean±Standard error	Mean difference (*a-*b)	Significance ($\rho < 0.01$)
A. baumannii (CP043953)*	*A. pittii* (CP002177)	45.00 ± 2.68	14.67	$\rho < 0.01$
	A. schindleri (CP044483)	44.67 ± 2.31	15.00	
	A. soli (CP073241)	44.50 ± 2.08	15.17	
	A. lactucae (CP053391)	44.33 ± 2.17	15.33	
	A. shaoyimingii (CP049801)	44.17 ± 3.26	15.50	
	A. chinensis (CP032134)	43.17 ± 1.05	16.50	
	A. cumulans (CP035934)	43.17 ± 2.79	16.50	
	A. defluvii (CP029397)	43.00 ± 2.13	16.67	
	A. dispersus (CP041970)	43.00 ± 2.52	16.67	
	A. calcoaceticus (CP020000)	42.83 ± 1.60	16.83	
	A. variabilis (AP024524)	42.33 ± 3.14	17.33	
	A. indicus (CP071319)	42.17 ± 1.56	17.50	
	A. larvae (CP016895)	41.67 ± 1.86	18.00	
	A. septicus (CP079898)	41.33 ± 2.09	18.33	
	A. junii (CP024632)	41.00 ± 1.37	18.67	
	A. seifertii (CP065820)	41.00 ± 2.11	18.67	
	A. haemolyticus (CP030880)	40.83 ± 1.83	18.83	
	A. nosocomialis (CP010368)	40.83 ± 1.45	18.83	
	A. lanii (CP049916)	40.33 ± 1.63	19.33	
	A. bouvetii (AP024595)	40.17 ± 1.70	19.50	
	A. wanghuae (CP045650)	39.50 ± 1.38	20.17	
	A. equi (CP012808)	39.33 ± 1.59	20.33	
	A. towneri (CP071766)	38.33 ± 1.52	21.33	

*Ta for this isolate is 59.67 ± 0.42 ˚C.

was tested in all 20 *A. baumannii* isolates. In contrast, there were no amplicon was detected in the other Gram-positive and Gram-negative bacterial isolates tested. This marker was found to be specific to *A. baumannii* isolates and has been used by many research groups to distinguish this species from other *Acinetobacter* spp.[28,29] Although several variants of *bla*OXA-51 genes have been determined, this primer set were designed to detect almost all *A. baumannii* variants with high specificity.[29]

The third primer set was used to amplify a 490 bp of the *gyrB* gene, which showed positive results in all tested *A. baumannii* isolates; however, it did not show any amplicon in the other Gram-positive and Gram-negative bacterial isolates tested. The *A. calcoaceticus-A. baumannii* complex comprised species that have been grouped together because they are phenotypically nearly similar and genetically closely related. Yet, there are substantial clinical and epidemiological differences among these species. The *gyrB* gene was used to identify *Acinetobacter* isolates to the species level. Its nucleotide and amino-acid sequences can be used for classification of *Acinetobacter* spp. Therefore, this marker was selected due to its effectiveness in identifying *A. baumannii* isolates from other closely related *Acinetobacter* spp. of the (*A. baumannii-A. calcoaceticus*) ACB complex.[30,31]

A multiplex PCR reaction was successfully performed by mixing the three primer sets in a single tube. Three amplicons of 208, 353, and 490 bp were seen in every agarose gel lane for all *A. baumannii* isolates; however, they failed to amplify the other Gram-positive and Gram-negative bacterial isolates. This reaction is easy to perform, confirms the bacterial species within a few hours, decreases the time and labor required for DNA sequencing, and can be performed quickly in many clinical laboratories.[31]

Bioinformatics was also used to test the possible amplification of the three primer sets within other *Acinetobacter* spp. because of the limitation of this research resource and to ensure that the primer set did not amplify any other *Acinetobacter* spp. The results showed a significant difference between the annealing temperatures of the three primer sets within *A. baumannii* in comparison to the other *Acinetobacter* spp. The annealing temperature of the *A. pittii* (CP002177) was (14.76 ˚C) which was the closest annealing temperature to the *A. baumannii*; however, still there was a significant difference between the two species making the possibility of detecting this species is negligible using multiplex PCR with the three primer sets. The annealing temperature differences for other species compared to the annealing temperature of *A. baumannii* was ranged from 15 ˚C to 19.33 ˚C. The highest difference of the annealing temperature compared to *A. baumannii* annealing temperature was 19.5 ˚C in *A. bouvetii* (AP024595). Consequently, using a multiplex PCR with these three primer sets specifically amplifies the *A. baumannii* genome alone and cannot definitely amplify other *Acinetobacter* spp. even the most related ones.

Conclusion

Multiplex PCR is a relatively simple and reproducible technique, and the results can be obtained within a few hours. Consequently, it can be employed in most laboratories to identify bacterial species and understand their epidemiology. In this study, three primer sets (P-Ab-ITS-F, P-Ab-ITS-R; OXA-51-like-F, OXA-51-like-R; sp2-F, sp2-R) were mixed in a one-tube multiplex PCR reaction, which offered simplicity and specificity for identification of *A. baumannii* from other *Acinetobacter* spp. as well as from other bacterial genera. The results of this study using multiplex PCR were rapid and specific, and both infection control staff and clinicians could benefit from this method.

Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for republication, which is attached to the manuscript.
- No animal studies are presented in the manuscript.
- Authors signed on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee at Al-Nahrain University.

Authors' contribution statement

Y.I.M, I.A.A. and S.A.H. contributed in design study and perform the experiments. Y.I.M. and M.R.J. data analysis and interpret the results. Y.I.M. and A.M.A. prepared manuscript draft. All authors read and approved final version of the manuscript.

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التشخيص الجزيئي لبكتريا الراكدة البومانية المعزولة من العينات السريرية باستخدام تفاعل البولميريز المتسلسل المتعدد

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المستخلص

بكتريا الراكدة البومانية المعزولة سريرياً هي واحدة من مسببات الأمراض المهمة و التهابات المستشفيات الشديدة. يعد التشخيص السريع على هذه الأنواع البكتيرية أمرًا ضروريًا حتى يمكن توفير العلاج المناسب والسيطرة على تفشي المرض. في هذه الدراسة، تم استخدام تفاعل البوليمريزالمتسلسل المتعدد لتشخيص الراكدة البومانية. تم تشخيص عشرين عزلة سريرية من الراكدة البومانية وثلاث عزلات بكتيرية موجبة لصبغة كرام واخرى سالبة لصيغة كرام باستخدام تفاعل البوليمريزالمتسلسل المتعدد و ثلاثة مجموعات من البادئات الخاصة بالأنواع (P-Ab-ITS-F, P-Ab-ITS-R; OXA-like-F, OXA-51-like-R; sp2-F, sp4-R-51) بالإضافة إلى ذلك، تم الحصول 24 جينوم لانواع بكتريا الراكدة وبضمنها جينوم الراكدة البومانية من قاعدة بيانات بنك الجينات للمركز الوطني لمعلومات التقنيات الحيوية ومطابقتها للبادئات باستخدام برنامج Blast. تم تحديد القواعد التكميلية للبادئات مع الجينوم البكتيري، وحساب درجات حرارة ذوبانها باستخدام الآلة الحاسبة من شركة بروميكا. نجح تفاعل البوليمريزالمتسلسل المتعدد في تضخيم ثلاثة أمبليكونات في جميع عزلات الراكدة البومانية التي تم اختبارها؛ ومع ذلك، فإنه لم يتم الحصول على اي تضخيم مع الانواع البكتيرية الأخرى. بالاضافة الى ذلك، وبالمقارنة مع أنواع الراكدة الأخرى، كانت هناك اختلافات كبيرة في متوسط درجات حرارة الارتباط لمجموعة البادئات مع جينوم الراكة البومانية ($P>0.01$). يمكن اعتبار تفاعل البوليمريزالمتسلسل المتعدد طريقة بسيطة وسريعة نسبيًا لتحديد هوية الراكدة البومانية. بالاضافة الى ذلك، فهو ممكن في معظم المختبرات وهو ضروري للأطباء لمكافحة تفشي المرض وللدراسات الوبائية.

الكلمات المفتاحية: الراءدة البومانية، جينblaOXA-51-like ،جينgyrB ،منطقة الفاصل الجيني، تفاعل البولي،ريزالمتسلسل المتعدد.