Antimicrobial susceptibility profiles, presence of integrons and associated cassette genes among *Acinetobacter baumannii* isolates from Southern part of Iran, Shiraz

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Abstract

Background

Acinetobacter baumannii is a global concern to cause the health-care-associated infections, due to multidrug resistance against available commercially antimicrobial agents. Regarding this, the present study was conducted to determine the antimicrobial susceptibility of *A.baumannii* isolates from clinical specimens in Shiraz, and explore the possible relationship of susceptibility patterns with the presence of integrons and related gene cassettes.

Methods

A.baumannii isolates were collected, and their susceptibility to various antibiotics was tested using Kirby-Bauer disk diffusion method. Also, molecular analyses were performed to detect the presence of OXA-51 like gene, class I, II and III integrons, and associated gene cassettes.

Results

Majority of isolates demonstrated resistance to imipenem(99.4%),piperacilin(98.2%),gentamycin (98.2%) meropenem (97.7%)ceftazidime(95.4%)amikacin(95.4%) and trimethoprim-sulfamethoxazole (90.8%). All strains showed multidrug-resistance to most of the tested antibiotics. The distribution analysis of integrons genes showed that 90.2%, 72.4% and 12.1% of isolates carried the intI 1, intI2 and intI3 genes, respectively. Moreover, two types of prevalent gene cassettes including *aad* and *dfr* were detected in Class 1 integron-carrying strains.

Conclusions

The current study showed the high prevalence of *A.baumannii* isolates harboring integrons in our investigated medical center, which may propel distribution of multidrug resistance event. The different types of gene cassette arrays in the present study spotlight the remarkable role of geographical issues in MDR isolates dissemination. This subject could attribute to choose appropriate therapeutic interventions in different areas. Obtained data highlighted the necessity for continuous surveillance to prevent distribution of multidrug resistance among *A.baumannii* strains in Iran.

Background

Infections caused by *Acinetobacter baumannii* are growing concerns in microbiology sciences which cause life-threatening infections involving various organs. (1) *A.baumannii* is rapidly developing resistance mechanisms to antibiotics. Extensive changes in resistance profiles especially against carbapenemes which are the drug of choice to treat and Nosocomial control the relevant infections of *A.baumannii* resulted in high mortality rate and economic burden, worldwide. Hence, Multi-drug resistant isolates of *A.baumannii* is a global problem in patients particularly in intensive care units (2). Various mechanisms are employed to resist against different antimicrobial agents. It is known that three classes of integrons (class 1, 2 and 3) have an important role in

dissemination of antimicrobial resistance genes leading to emerge the multi- drug resistant phenotypes. Integrons incorporate to a specific site of gene sequences contributing to antibiotics resistance in gram negative bacteria such as *A.baumannii* (1–4) .

Indeed, gene cassettes, containing antibiotic resistance genes, are associated with MDR patterns in *A.baumannii* resulted in outbreaks and therapeutic failures in healthcare setting(5). So, assessment of antimicrobial susceptibility profiles and detection the gene cassettes in clinical isolates of *A.baumannii* in each geographical zone present the importance of conducting epidemiological studies for effective treatment and advantageous control of MDR and PDR species of *A.baumannii* in hospital outbreaks(6).The latest epidemiological study in this area of Iran was conducted almost ten years ago which had determined the antimicrobial activity of conventional antibiotics against the isolates and existence of integrons. In this regard, the aim of the present study was new evaluation to explore the antimicrobial susceptibility patterns, presence of integrons and associated gene cassettes among *A.baumannii* isolates obtained from hospitalized patients in southern part of Iran.

Methods

Bacterial isolates

All clinical strains examined in this study were isolated over a 3month period-between July and September 2016- and submitted by clinical microbiology laboratory of the Namazi hospital and Prof. Alborzi Microbiology Research Center in Shiraz. Collected samples included sputum, blood, urine, throat swab, ulcer, endotracheal tube, biopsy of the lung, abdomen and axillary, eye and nasal discharge. Totally, 181 specimens were collected. Clinical samples of the patients were cultured on Blood Agar and Mckongey Agar and incubated for 16-18 h in 37°c.After performing initial differential tests and, if suspected to be *Acinetobacter* spp, they were transferred to the sterile tubes containing the TSB medium. All bacteria were stored at -70°c.It should be noted that the identification and confirmation of *Acinetobacter* spp at the species level are not possible using conventional biochemical tests in clinical laboratories. In order to identify the isolates; the bacterial specific ribosomal gene replication should be used.

However, for initial diagnosis of *Acinetobacter*, morphological and biochemical tests were performed, which include gram staining and observation of gram negative cocco bacillus under a microscope, culture on an agar medium, and observation of small to medium convex, white or gray and non-hemolytic colonies on the TSI environment and observation of the growth pattern of ALK/ALK and H2S negative. In addition, the bacterium had a positive catalase activity, negative oxidase activity, non-motile, indole negative, production of acid in OF(Oxidative fermentative) test, MR(-)VP(-) in Voges–Proskauer test, negative for nitrate and positive for citrate(7).

Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of the isolates were performed using Kirby-Bauer disk diffusion method as was essentially described by the Clinical and Laboratory Standards Institute (CLSI)(8).To prepare inoculum suspension, all the isolates were sub cultured from freezer stocks on sheep blood agar plates. After 18 to 24 h of incubation at 37°C on BA, the colonies were harvested to prepare a suspension. Bacterial isolates were

suspended in sterile 0.85% saline to a turbidity adjusted to 0.5 McFarland standard equivalents to 1.5×10^8 CFU/ml.

Ten antibiotic discs were placed on Muller-Hinton Agar inoculated by bacterial suspension formerly. Plates were incubated for 24h at 37°C. *A.baummanii* (ATCC 19606) was considered as the control strain. After incubation, the inhibition zone diameters were measured and the isolates were classified as Susceptible(S), Intermediate (I) and Resistant(R) based on manufacture data sheet. The antibiotic discs were as follows:Trimethoprim-Sulfamethoxazole(1.25/23.75µg),Amikacin(30µg),Gentamicin(10µg),Ampicillin-Sulbactam(10 /10 µg),Pipracillin(100µg),Imipenem(10 µg),Ceftazidime(30 µg),Cefepime(30 µg),Tetracyclin(30 µg),Meropenem(10 µg), and Polymyxin B(300 units). All discs were purchased from Mast group Ltd, UK.

Polymerase Chain Reaction (PCR)

The isolates were identified as *A.baumannii* on the basis of their molecular characteristics. Definitive diagnosis of *A.baumannii* isolates at a species level was confirmed by polymerase chain reaction (PCR), using the 16S r RNA-specific primers for *A.baumannii*OXA-51 like (F: 5'- TAA TGC TTT GAT CGG CCT TG-3') and (R: CTTCGTGGATTCGACTTCAT) (350 bp)(9) The PCR conditions were as follows: Initial denaturation at 95°C for 5 min,30cycles of 95°C for 60s, 50°C for 50s and 72°C for50s, followed by an elongation step at 72°C for 60s . (Total volume:12.5 µlit)The PCR products were visualized by agarose gel Electrophoresis (1.5 % agarose gel).

Molecular Characterization of class 1, 2 and 3 Integrons in *Acinetobacter baumannii*

PCR amplification of class 1,2 and 3 integrons was done with the set of primers described by Goldstein(10)with some modifications in temperature program.(Total volume:12.5µlit)

Primer sequences are shown in Table1.Amplified products were visualized by gel Electrophoresis, as mentioned previously.

Detection of class 1 integron gene cassette amplicons

To detect the gene cassettes, we amplified variable regions of type 1 integron, using primers described by Levesque etAl. (11).

Sequencing of class 1 integron gene cassette

The variable region of type 1 integron amplicons yielded from the previous step was sequenced in Macrogene(Korea) and nucleotide sequence alignment and comparisons were carried out using BLAST (Basic Local Alignment Search Tool) on the NCBI (National Center for Biotechnology Information). (https://www.blast.ncbi.nlm.nih.gov).

Statistical analysis

Descriptive analysis of data was done; also, statistical analyses were performed by Student's t-test, Fisher's exact test and chi- using SPSS version 18.P <0.05 was considered as statistically significant.

Results

A total of 174 isolates were included in our study. Isolates were collected from 112 (64.3%) male and 62 (35.7%) female with an average age of 51 years (Mean ±SD:51 ±26). Most of the isolates were recovered from patients in the age range between 61 and 70 years. The isolates mostly were obtained from ICU (50.9%, n: 87) followed by internal wards (27.01%, n: 47) and surgery wards (6.89%, n: 12).The most frequent specimen was respiratory secretion (27.5%, n: 48) followed by endotracheal tubes (21.8%, n: 38) and blood (16.6%, n: 29).

According to the PCR mapping results using forward and reverse primers of integrons (class 1, 2, 3), 90.2% of the isolates (n:157) were positive for Int I gene(Figure 1-A), 72.4% (n:126) for Int II gene(Figure 1-B) and 12.1% (n:21) for IntIII gene(Figure 1-C) .Based on statistical analysis, we found that there was a significant correlation between age and sex of the patients with the presence of intI2; intI2gene was merely detected in males(P=0.001) and younger patients(P=0.018). Moreover, a significant correlation was found between the resistance of isolates to Ampicillin-Sulbactam with the existence of intI1 (P=0.003), to Gentamycin and Ceftazidime with intI2 (p=0.05 and 0.02, respectively) and also, to Ceftazidime, Tetracyclin and Cefipime with intI3 (p=0.02, 0.05 and 0.02, respectively).

Results of antimicrobial susceptibility testing in this study showed that Polymixin B was the most effective antimicrobial agent against *A.baumanii* isolates. Besides, the lowest level of susceptibility was among more than 90% of the isolates which were resistant to Imipenem (99.4%), Piperacillin (98.2%), Gentamycin (98.2%), Meropenem (97.7%), Ceftazidime (95.4%), Amikacin (95.4%), and Trimethoprim-Sulfamethoxazole (90.8%).Association between the existence of integron and antibiotic resistance in *Acinetobacter* isolates shown in Table 2.

Statistical analysis showed that there was a significant correlation between antibiotic susceptibility patterns of Cotrimoxazole, Ceftazidime, and Amikacin and sex of patients. So, the resistance to three mentioned antibiotics was significantly higher in males (respectively P=0.027, 0.01 and 0.01). Also, a comparison of antimicrobial susceptibility based on age using ANOVA demonstrated that there was a significant correlation between age and susceptibility to Tetracyclin, exclusively (P= 0.01).

All of the isolates obtained from patients in this study were considered as MDR based on the definition given by Magiorakos et al. (12). MDR phenotype of clinical isolates for classes 1, 2, and 3 Integrons shown in Table 3.

Amplification of variable region of Int class 1 produced 7 different sizes of gene cassettes which were contributed to int I: ranged in size from 500 -1500bp. Several amplicons with different lengths were selected and analyzed by sequencing and data were compared in GeneBank. The results demonstrated that three separate isolates with size of 1500bp carried two different types of class 1 integron gene cassettes including *aadA2* and *dfrA12*.Data shown in Table 4.

Also, our results indicated that isolates which have gene cassettes of the same size almost presented similar antimicrobial resistance pattern (supplementary Table 1).

Discussion

Clinical importance of *A.baumannii* arises from prompt global emergence of multi-drug resistant strains resulted in high rate of mortality and difficulties for health organization. Regarding the mentioned facts, epidemiological findings could be helpful to recognize the susceptibility profiles of *A.baumannii* cause infections in different

area. Current study evaluated the distribution of integrons and antimicrobial susceptibility patterns among *A.baumanii* isolates in south area of Iran. Obtained data showed a high percentage of MDR phenotypes and integrons existence in clinical isolates which implicate the importance of integrons in dissemination of antibiotic resistance genes in the environment (13, 14).

Our findings showed that a half of *A.baumannii* isolates (50.9 %) were obtained from hospitalized patients in the ICU wards. This result is in accordance with those of previous studies about the role of *A. baumannii* in ICU infections (15, 16).

Screening for MDR phenotype among *A. baumannii* isolates showed an alarming trend of increasing resistance to multiple antibiotics. Several studies have also shown emergence of MDR strains and it is likely due to improper use of antimicrobial agents, which limits therapeutic protocols (17-19).

Several cases of MDR *A. baumannii* have been reported from hospitals in the United Arab Emirates, Bahrain, Saudi Arabia, Palestine and Lebanon(12, 20).

The MDR frequency among tested isolates is similar to that stated previously in Poland (100%) (21), Greece (100%)(22) , China (93 %)(23, 24) and also, this is considerably higher than those reported in other recent reports from Thailand (21.1%) (25)and China (61.3%)(26). This aspect should be considered in the treatment of the relevant infections in order to prevent the inappropriate use of broad-spectrum antibiotics which could cause more implication during sickness.

It should be noted that resistant to Carbapenems including Imipeneme and Meropeneme had dramatically increased in comparison with previous study in Shiraz by Japoni et al. In accordance with our results, Carbapenem-resistant *A.baumanii* isolates were detected in a recent study in Shiraz by investigating Metallo-beta-lactamase (MβL) enzymes production(27).

In comparison with previous reports, our findings showed significantly higher resistance rates to Cephalosporins and Aminoglycosides agents. So, prescription of these antimicrobial groups should be reviewed in nosocomial infections management.

As the results shown, Polymixins (PMb) are the most effective antimicrobial agents in agreement with previous reports .Although the efficacy of Polymixins have declared in studies ,their prescription are confined because of its neurotoxic or nephrotoxic side effects(28, 29).Acquisition of foreign genetic elements such as integrons leads to emerge the resistant phenotypes. The spread of these mobile elements between species causes the extension of resistance in health-care settings. We found a remarkable increase in presence of class 1 and 2 integrons compared to the previous study in the same area. This trend is acceptable considering the changes in resistance patterns of isolates compared to the previous study. Also, our results are in consistence with the results of the other studies represented higher prevalence of integron class 1 compared to class 2 ,and rarely class 3 (30, 31).

Statistical analysis showed a significant association between the presence of integrons and resistance to antimicrobial agents including Ampicillin-Sulbactam, Cephalosporins, Gentamycin and Tetracyclin. So, it should be remembered that other mechanisms are involved in resistance to antibiotics in addition to integrons acquisition. (32, 33). The prevalence of class 1 integrons are higher than the rate found in other areas of country(34, 35). Since the presence of class I integron in the present study is significantly associated with

Ampicillin-Sulbactam resistance, the increase in resistance to this class of antibiotics is consistent with the increase in the presence of class I integron compared to the previous study(35).

Whereas none of the isolates in previous study harboring the class 3 integrons (35, 36), 12.1 % of our isolates contained this class of integron genes. As we found a significant correlation, existence the class 3 integron gene could be associated with increased resistance of our isolates to Cephalosporins. Indeed, prescription these group of antimicrobial agents should be given more attention considering the global resistant dissemination(37) Also, in accordance with a previous study in Iran By Japoni-Nejad, resistant to Aminoglycosides were reported.

Moreover, the acquisition of class 3 integron gene may be resulted in activated efflux pumps and resistance to Tetracyclin(38) .

Present study is also first investigation of the gene cassettes among isolates of *A. baumannii* in Shiraz.

According to the PCR method 7 different sizes of integron class 1gene cassettes were detected. Sequencing method for identification the types of gene cassettes indicates two different types of class I integron gene cassettes which both of them previously reported: *aadA2* and *dfrA12.*

DfrA12 is related to expression of Dihydrofolate reductase gene which is contributed to resistance to Trimethoprim. In this study 90.8% of isolates were resistant to Cotrimoxazole (Trimethoprim- Sulfamethoxazole). These gene cassette previously reported in studies in Iran and other countries(31, 39).

AadA2 is related to expression of Aminoglycoside adenylyl transferase gene which is responsible for resistant to Aminoglycoside antibiotics (in this study: Amikacin and Gentamycin).Our results determined that 98.2% and 95.4% of isolates were resistant to Gentamycin and Amikacin respectively. This gene cassette is reported in other region in Iran(31, 40, 41)and other countries(18, 42).

Considering our results, resistant isolates to Trimethoprim- Sulfamethoxazole contained the *DfrA12* gene cassettes. Also, the presence of *AadA2* gene cassettes is in consistent with resistance to Gentamycin and Amikacin. So, it is worth noting to declare that there is a significant association between the presence of gene cassette and a reduced susceptibility to antibiotics.

Conclusion

Acinetobacter baumannii is an emerging pathogen caused life-threatening nosocomial infections, worldwide. Multidrug-resistant (MDR) *A. baumannii* has considered as a major problem in healthcare settings which poses limitations for therapeutic options in infected patients. Despite the data limitation, Tigecyclin is the most potent antimicrobial agents with considerable activity against MDR *A.baumannii* species(43).Moreover, emerging the resistant phenotypes to drug choices such as Tigecyclin has created new challenges in treatment (44–46).Finally, it should be remembered that following the guidelines to prevent the transmission of *Acinetobacter* species in hospital settings and explore to discovery the novel therapeutic agents could be helpful to overcome such nosocomial infections.

Abbreviations

CLSI: Clinical and laboratory standards institute; DNA: Deoxy-ribonucleic acid; PDR: Pun Drug Resistance; MDR: Multi-drug resistance; PCR; Polymerase chain reaction; XDR: Extensive-drug resistance

Declarations

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Author Contribution

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information flies.

Ethics approval and consent to participate

An ethical approval for conducting this study was taken from Yasuj University of medical science Research Council (Reference code IR.YUMS.REC.1395.52). Data used in this retrospective study were the anonymised routine microbiology laboratory results
originating from Nemazi Hospital. These data were devoid of any patients 'identifying information.

Consent for publication

All data used in this study were the anonymised microbiological data devoid of patients' identification and personal information. Thus obtaining consent for publication is not applicable for this study. All authors consented for the
publication of this research.

Competing interests

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published and agreed to be accountable for all aspects of the work. Furthermore The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table 1: Primers Applied for PCR amplification

gene	Sequence (5′ ⟶ 3′)	F/R	Product size(bp)
Oxa51 like	TAATGCTTTGATCGGCCTTG	Forward	350
	CTTCGTGGATTCGACTTCAT	Reverse	
Int I1	CCTCCCGCACGATGATC	Forward	280
	TCCACGCATCGTCAGGC	Reverse	
Int I 2	TTATTGCTGGGATTAGGC	Forward	233
	ACGGCTACCCTCTGTTATC	Reverse	
Int I3	AGTGGGTGGCGAATGAGTG	Forward	600
	TGTTCTTGTATCGGCAGGTG	Reverse	

Table 2: Association between the existence of integrons and antibiotic resistance in 174 clinical isolates of *A.baumanii.*

Class of antimicrobial agent	Antimicrobial agent	Resistant isolates (%)	Presence of integron 1 (p-value)	Presence of integron 2 (p-value)	Presence of integron 3 (p-value)
Cephalosporins	Cefepime	88.5	0.58	0.06	0.02
	Ceftazidime	95.4	0.56	0.02	0.02
Tetracyclines	Tetracycline	63.2	2.2	8.7	0.05
DHFR inhibitor/Sulfonamide	Trimethoprim/Sulfamethoxazole (Co-trimoxazole)	90.8	0.14	3.5	1.1
Penicillins/beta-lactamase inhibitor	Ampicillin/sulbactam	76.4	0.003	1.4	0.71
Polymyxins	Polymyxin B	0.57	0.1	2.6	0.12
Beta-lactams	Imipenem	99.4	0.1	0.38	0.12
	Meropenem	97.7	0.41	1.5	0.5
Broad-spectrum Beta-lactams	Piperacillin	98.2	1.92	2.3	0.37
Aminoglycoside	Gentamicin	98.2	1.92	0.05	0.37
	Amikacin	95.4	0.07	0.41	0.89

Table 3: MDR phenotype of *A.baumanii* clinical isolates for Int I, II and III.

	Antibiotics resistant pattern(number of antibiotics)	Number of isolates	Integron class 1	Integron class 2	Integron class 3
1	CPM/T/CAZ/PB/GM/PRL/TS/AK/MEM/ IMI(10)	1	1	0	0
2	CPM/T/SAM/CAZ/GM/PRL/TS/AK/MEM/ IMI(10)	56	51	38	8
3	CPM/SAM/CAZ/GM/PRL/TS/AK/MEM/ IMI(9)	54	48	46	7
4	CPM/T/CAZ/GM/PRL/TS/AK/MEM/ IMI(9)	17	16	10	1
5	T/SAM/CAZ/GM/PRL/TS/AK/MEM/ IMI(9)	6	6	4	0
6	CPM/T/SAM/CAZ/GM/PRL/AK/ MEM/IMI(9)	8	7	4	1
7	CPM/T/SAM/GM/PRL/TS/AK/MEM/ IMI(9)	4	4	3	0
8	CPM/T/SAM/CAZ/GM/AK/MEM/IMI(8)	1	1	0	0
9	CPM/T/CAZ/GM/PRL/AK/MEM/IMI(8)	3	2	1	9
10	T/CAZ/GM/PRL/TS/AK/MEM/IMI(8)	5	5	0	0
11	CPM/CAZ/GM/PRL/TS/AK/MEM/IMI(8)	4	3	2	1
12	CPM/T/SAM/CAZ/PRL/TS/MEM/IMI(8)	1	1	0	0
13	CPM/T/CAZ/GM/PRL/AK/MEM/IMI(8)	2	2	0	0
14	CPM/SAM/CAZ/GM/PRL/AK/MEM/ IMI(8)	1	1	0	1
15	CPM/T/CAZ/GM/PRL/TS/MEM/IMI(8)	1	1	0	0
16	T/CAZ/GM/PRL/AK/MEM/IMI(7)	4	4	0	1
17	T/GM/PRL/TS/AK/MEM/IMI(7)	1	1	1	1
18	CPM/SAM/CAZ/GM/PRL/TS/IMI(7)	1	1	1	0
19	CAZ/PRL/TS/AK/MEM/IMI(6)	1	1	1	0
20	T/SAM/CAZ/GM/PRL/TS(6)	1	1	1	0
21	CAZ/TS/MEM/IMI(4)	1	0	0	0

22	T/GM/PRL/AK/MRM(4)	1	1	1	0

Abreviations: AK:amikacin CAZ:ceftazidime CPM:cefepime GM:gentamicin IMI:imipenem MRP:meropenem PRL:piperacillin SAM:ampicillin-sulbactam T:tetracycline TS: trimethoprim- sulfamethoxazole

Table 4: Size of amplicons and gene cassettes associated with Int I.

Pattern of gene cassettes associated with *Int* I	Number of isolates	Gene cassette
500bp	8	*aadA1*
700bp	17	*dfrA5, dfrA25*
750bp	1	*aadB*
1000bp	6	*aadA1, aadA2*
1200bp	16	*blaCARB-2*
1400bp	13	*aadB-catB3*
1500bp	3	*dfrA1–aadA1a*

Figures

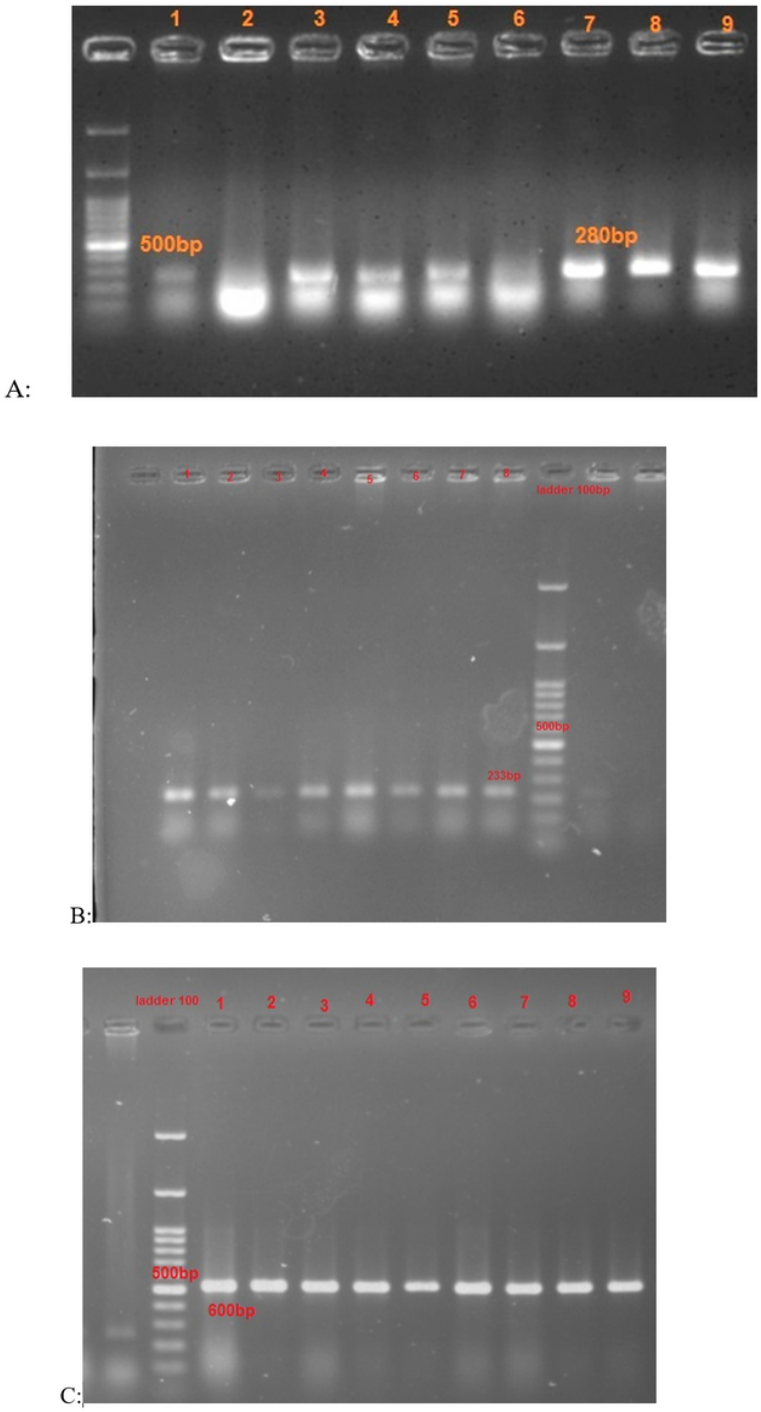

Figure 1

Agarose gel electrophoresis of PCR products of Integrons amplification;A: IntI 1(280 bp), B:IntI2(233 bp), C:IntI 3(600 bp).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx