Original Article

Evaluate the frequency distribution of nonadhesive virulence factors in carbapenemase-producing *Acinetobacter baumannii* isolated from clinical samples in Kermanshah

Parviz Mohajeri, Saba Sharbati[1], Abbas Farahani[2], Zhaleh Rezaei[1]

Department of Microbiology, School of Medicine, [1]Department of Pharmacology, Student Research Committee, [2]Department of Microbiology, Student Research Committee, Kermanshah University of Medical Sciences, Kermanshah, Iran

Address for correspondence:
Dr. Parviz Mohajeri, Associate Professor of Medical Bacteriology, Department of Microbiology, School of Medicine, Kermanshah University of Medical Sciences, Shirudi Shahid Blvd, Daneshgah Street, Postal Code 67148-69914, Kermanshah, Iran. E-mail: p_mohajeri@yahoo.com

Abstract

Background: *Acinetobacter baumannii* which is a Gram-negative bacterium can cause several different infections. The appearance of carbapenemase-producing *A. baumannii* in recent years has made the treatment process more difficult. The identification of virulence factors (VFs), such as nonadhesives in *A. baumannii,* helps to fight against related infections. **Materials and Methods:** A total of 104 samples from teaching hospitals in Kermanshah, Iran, were collected during a 24 months period (2011-2013). Sample identification was first carried out by biochemical tests, and then their susceptibility to carbapenems was determined using the Kirby–Bauer method. For confirmation of carbapenemase-producing *A. baumannii*, polymerase chain reaction (PCR) was done for carbapenemase-encoding genes. In addition, the frequency of nonadhesive VFs in carbapenemase-producing isolates was determined by PCR. **Results:** There were 50 isolates that were identified as carbapenemase-producing *A. baumannii*. The PCR results showed; 40 isolates (80%) for *traT*, 17 isolates (34%) for *cvaC*, and 8 isolates (16%) for *iutA,* and these encode serum resistance, colicin V and aerobactin, respectively. No significant correlation was observed between these three genes. **Conclusions:** The mechanism of *A. baumannii* virulence has always been in question. The role of VFs has also been recognized in other Gram-negative bacteria. According to the prevalence of *traT, cvaC and iutA,* as nonadhesive VFs, we can suggest that they could be the main mechanism of carbapenemase-producing *A. baumannii* pathogenesis.

Key words: *Acinetobacter baumannii*, carbapenemase-producing, nonadhesive, virulence factor

INTRODUCTION

Acinetobacter baumannii is an aerobic, nonfermentative Gram negative Coccobacillus that can survive for prolonged periods in the environment and can cause nosocomial infections, (urinary tract and wound infections).[1-3] Capability to live under critical circumstances makes it a major pathogen in hospitals.[4] Extensive use of antimicrobial agents in clinical cases has contributed to the appearance of resistant *A. baumannii* strains.[5] The antimicrobial agent

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recommended against *A. baumannii* infections are usually carbapenems. Increasing resistance to carbapenems in *A. baumannii* infections in the past decade[1] is creating therapeutic challenges.[6] One of the characteristics of pathogenic bacteria is that they contain virulence factors (VFs).[7] The bacterial VFs include adhesive and nonadhesive types. Adhesive VFs can attach by methods such as fimbriae. Nonadhesive VFs include: Diverse siderophores, such as; yersiniabactin (*fyuA*), aerobactin (*iutA),* invasins (*IbeA*), serum resistance (*traT*), and colicin V production (*cvaC*).[8] The purposes of this work was to detect the genes codifying nonadhesive VFs, and to evaluate the frequency distribution of nonadhesive VFs in carbapenemase-producing *A. baumannii* isolated from clinical samples.

MATERIALS AND METHODS

Bacterial collection

One-hundred four samples were isolated from patients hospitalized in three hospitals affiliated with Kermanshah University of Medical Sciences, Iran during March 2011-March 2013.

Identification and antimicrobial susceptibility testing

The strains were identified as *A. baumannii* by conventional biochemical tests and API 20NE kit (version 6.0, bio-Mérieux, France).[9] Susceptibility to carbapenem was performed according to the standard Clinical and Laboratory Standards Institute guidelines to check their resistance to carbapenems using imipenem (10 μg) and meropenem (10 μg) (MAST, Merseyside, UK).[10] Carbapenem-resistant isolates were chosen for identifying nonadhesive VFs frequency. *Escherichia coli* MK1 and MK2 were used for *ibeA* and *fyuA* as a positive control. For VF *traT, cvaC and iutA* one amplified a polymerase chain reaction (PCR) product of each gene sent to Sinaclon Company for sequencing and the results were used as the positive control after nBlast alignment.

Polymerase chain reaction

Bacterial DNA was released from whole microorganisms by boiling .Bacteria were harvested from 1 ml of an overnight broth culture, suspended in 200 μl of sterile water, and incubated at 100°C for 10 min. Following centrifugation of the lysate, a 150 μl sample of the supernatant was stored at −20°C as a template DNA stock.[11] The primers used in this study are shown in Table 1.[8] Each reaction mixture (15 μl) contained 2 μl of DNA, 0.5 mM of each primer, 1 U of Taq DNA polymerase (Sinaclon), the four deoxynucleoside triphosphates (each at 200 μM) and 1.5 mM $MgCl_2$. PCR was performed under predenaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, at each specific annealing temperature [Table 1] for 30 s, and 72°C for 45 s ending with a final extension step at 72°C for 5 min. The PCR product was run and visualized on 1.5% agarose gels then were stained with ethidium bromide. PCR screening was done for the carbapenemase-encoding genes including *bla*OXA-23-like and *bla*OXA-24-like. PCR analysis was performed using the primers described previously.[12]

Statistical analysis

A statistical comparison of the frequencies of nonadhesive VFs in carbapenemase-producing *A. baumannii* isolates was conducted by Chi-squared test (variables were analyzed by Chi-square test). $P = 0.05$ was considered as statistically significant.

RESULTS

One-hundred four *A. baumannii* specimens were collected from three hospitals in Kermanshah (Iran). Fifty isolates were resistant to imipenem and meropenem and also positive for one of the carbapenemase-encoding genes (*bla*OXA-23-like, *bla*OXA-24-like); these isolates were detected as carbapenemase-producing isolates for future analysis. The carbapenemase resistant bacteria were

Table 1: Primers to VFs used in the polymerase chain reaction

Gene	VF	Name of primer	Sequence (5'-3')	Amplified DNA (bp)	Reaction conditions
cvaC	Colicin V	ColV-Cf	CAC ACA CAA ACG GGA GCT GTT	680	Annealing at 64
		ColV-Cr	CTT CCC GCA GCA TAG TTC CAT		
fyuA	Yersiniabactin	FyuA f	TGA TTA ACC CCG CGA CGG GAA	785	Annealing at 59
		FyuA r	CGC AGT AGG CAC GAT GTT GTA		
ibeA	Invasin	ibe10 f	AGG CAG GTG TGC GCC GCG TAC	170	Annealing at 65
		ibe10 r	TGG TGC TCC GGC AAA CCA TGC		
iutA	Aerobactin	AerJ f	GGC TGG ACA TCA TGG GAA CTG G	300	Annealing at 57
		AerJ r	CGT CGG GAA CGG GTA GAA TCG		
PAI	Pathogenicity-associated island	RPAi f	GGA CAT CCT GTT ACA GCG CGC A	930	Annealing at 61
		RPAi r	TCG CCA CCA ATC ACA GCC GAA C		
traT	Serum resistance	TraT f	GGT GTG GTG CGA TGA GCA CAG	290	Annealing at 60
		TraT r	CAC GGT TCA GCC ATC CCT GAG		

VF: Virulence factors

isolated from 35 (70%) males to 15 (30%) females, and the distributions of carbapenemase-producing *A. baumannii,* according to age, are shown in Figure 1. Nonadhesive VFs were analyzed by a PCR assay. Products of the PCR assay are shown in Figure 2. Frequencies of nonadhesive VFs include: Serum resistance (*traT*), colicin V (*cvaC*), and aerobactin (*iutA*), were; 40 isolates (80%), 17 isolates (34%), and 8 isolates (16%), respectively. However, the genes *fyuA*, *ibeA*, and pathogenicity-associated islands (PAI), codifying for; yersiniabactin, invasion and PAIs, respectively, were not detected by means of a PCR with specific primers. There were 4 (8%) isolates that carried all three genes. However, no VF was found in 5 (10%) isolates. Eight (16%) isolates carried both *traT* and *cvaC* ($P = 0.27$), 6 (12%) isolates carried *traT* and *iutA* ($P = 0.18$), and 4 (8%) isolates carried *cvaC* and *iutA* ($P = 0.41$). There were no significant correlations found between these three genes. Distribution of *traT, cvaC* and *iutA* did not correlate with gender [Table 2].

DISCUSSION

Treatment of infections with opportunistic pathogens such as *A. baumannii* has been a great challenge. Carbapenems are the first-line of treatment of *Acinetobacter* infections, but nowadays resistance to them is reported.[8] In this study, 50 isolates (48%) were recognized as carbapenemase-producing phenotypes out of 104 clinical isolates of *A. baumannii*. 25-50% incidence of resistance to carbapenems is previously reported in Iran.[13,14] Park *et al.* reported resistance to imipenem and meropenem, 31.7% and 34.9% respectively from 63 isolates of *Acinetobacter* in South Korea.[15] Resistant rate to carbapenem in *A. baumannii* isolates investigated in this study is different compared to other studies, which may be due to geographical differences in isolates.

Table 2: Distribution of *traT, cvaC* and *iutA* according to sex

Virulence genes (%)	Sex		Total	*P*
	Male	Female		
traT positive	28 (80)	12 (80)	40 (80)	1.00
traT negative	7 (20)	3 (20)	10 (20)	
cvaC positive	14 (40)	3 (20)	17 (34)	0.21
cvaC negative	21 (60)	12 (80)	33 (66)	
iutA positive	6 (17.14)	2 (13.33)	8 (16)	1.00
iutA negative	29 (82.85)	13 (86.67)	42 (84)	

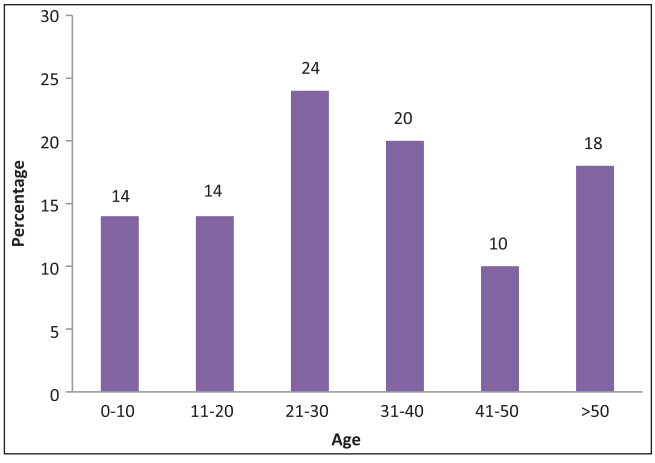

Figure 1: Distribution of carbapenemase-producing *Acinetobacter baumannii* according to the age of patients

Fifty carbapenemase-producing isolates were examined in terms of having six nonadhesive VFs including *traT* (serum resistance), *cvaC* (colicin V), *iutA* (aerobactin), *ibeA*, (invasion), *fyuA* (yersiniabactin) and *PAI*. Frequency of *traT*, *cvaC* and *iutA* factors were 40 (80%), 17 (34%) and 8 (16%) isolates, respectively. VFs cause infection in most Gram negative pathogenic bacteria. Of these, nonadhesive VFs play a key role in bacterial survival in special conditions such as in human blood and exposure to serum and poor iron environments that cause diseases.[8] Compared to other Gram-negative pathogens, relatively few VFs have been identified for *A. baumannii*.[16] Serum resistance (*traT*) is one of the nonadhesive factors investigated in this study. Studies indicate that serum resistance is the ability of the bacterial cell to resist the lytic effects of serum and to invade and survive in the human blood stream. Doughari *et al.* (2012) studied 14 samples of *Acinetobacter* and indicated that 7 isolates (50%) had serum resistance phenotypically.[17] King *et al.* in the USA found that from 7 *A. baumannii* isolates, 3 isolates (43%) had serum resistance phenotypically.[18] The results obtained from carbapenemase-producing *A. baumannii* isolates in this study show that genotypic frequency of *traT* gene is higher than in other studies. Of course, this difference could be due to phenotypic and genotypic detection techniques for these VFs. There is also a direct relationship between serum resistance and biofilm formation, and hence they increase bacterial

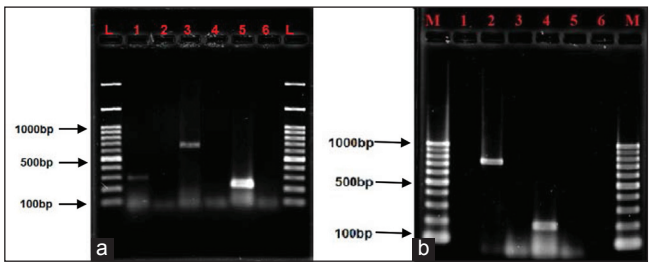

Figure 2: Patterns of agarose gel electrophoresis showing polymerase chain reaction amplification products for the nonadhesive virulence factors genes. Lane M, DNA molecular size marker (100 bp ladder; Sinaclon). (a) Lane 1: *traT* (290 bp); Lane 3: *cvaC* (680 bp); Lane 5: *iutA* (300 bp); Lane 2, 4, 6: Negative control. (b) Lane 2: *fyuA* (785 bp); Lane 4: *ibeA* (170 bp); Lane 3 and 5: Negative control

pathogenicity.[18] As a result, considering the existence of 80% *traT* gene in this study, more pathogenicity is expected for these isolates. Another VF described in *A. baumannii* is the production of siderophores by isolates growing under iron limiting conditions. These bacteria are capable to express high-affinity iron uptake systems composed by siderophores. In Braun study, all *A. baumannii* strains were able to grow under iron limiting conditions, and they concluded that this bacterium produces siderophores.[8] Unlike our study, the genes *iutA* and *fyuA* from iron uptake system of *E. coli* and *Yersinia* sp., respectively, were not present in the Braun studied isolates. Considering the frequency of *iutA* gene in this study,[17] it seems that *iutA* siderophore unlike *fyuA* in *Acinetobacters* isolated from sputum/tracheal secretion, is more important VF compared to urinary isolates. Another gene that was positive in this study is *cvaC,* which was observed in 34% of cases. cvaC, which encodes colicin V, have been proposed to confer enhanced virulence through their carriage of other specific VFs, including the aerobactin system and serum survival genes, such as *traT*.[19]

CONCLUSION

Carbapenemase-producing *A. baumannii* strains are among the most important agents of nosocomial infections in our hospital and worldwide. Three VFs of *traT*, *cvaC* and *iutA* with frequency of 80%, 34%, and 16%, respectively, revealing a role in the pathogenesis of these isolates and also may be have the most impact on existence for long time by carbapenemase-producing *A. baumannii* in environment of hospital. Frequent screening for these VFs may contribute to better infection control measures and to the investigate new treatment options.

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Conflicts of interest

There are no conflicts of interest.

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