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Frequency of Adhesive Virulence Factors in Carbapenemase-producing *Acinetobacter baumannii* Isolated from Clinical Samples in West of Iran

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ABSTRACT

Acinetobacter baumannii is a significant opportunistic pathogen which causes severe infections related to catheters and ventilator. Adhesive Virulence Factors (VFs) are effective in *Acinetobacter baumannii* adherence and pathogenicity. The aim of this study is to evaluate frequency of adhesive virulence factors in carbapenemase-producing *A. baumannii*. In total, 104 *Acinetobacter baumannii* were collected from teaching hospitals of Kermanshah, Iran during March 2011-2013. All the isolates were tested for antimicrobial susceptibility by Kirby-Bauer disk diffusion method. Carbapenemase-producing isolates were identified, DNA of isolates were extracted by boiling and were investigated for the presence of adhesive virulence factors by PCR. Among 50 carbapenemase-producing isolates, frequency of *fim*H and *csg*A genes obtained 30(60%) and 27(54%), respectively. The 20(40%) isolates carried both of *fim*H and *csg*A but 13(26%) carried non of these two genes. None of these isolates presented genes codifying for other different adhesive virulence factors include fimbriae Dr (*afa/dra*BC), fimbriae S (*sfa/foc*DE), fimbriae P (*pap*), capsule (*kps*MT) and fibronecting receptor (*fnb*). Adhesive virulence factors are responsible for pathogenesis of bacteria. As adhesive VFs, fimbriae type I (*fim*H) and curli fiber (*csg*A) are participated in adherence and biofilm formation and give bacteria, the ability to be hidden of host immune system and then causing infections more than 50% prevalence of *fim*H and *csg*A genes among 7 adhesive VFs studied in this study shows, that may cause significant relationship between the presence of *fim*H and *csg*A genes and *A. baumannii* infections.

Key words: *Acinetobacter baumannii*, virulence factors, carbapenemase-producing, adhesive

INTRODUCTION

Acinetobacter is a ubiquitous aerobic gram-negative cocobacillus that found extensively in natural environment (Kanafani and Kanj, 2013a). This organism can survive on different surfaces

for months (Hsueh *et al*., 2002). Between 30 different species, *Acinetobacter baumannii* is the most frequently reported in the clinical literature (Lolans *et al*., 2006; Abdel-El-Haleem, 2003). This organism is the major cause of sever infections including nosocomial, urinary tract, meningitis and wound infections in hospitals, especially in Intensive Care Unit (ICU) (Srinivasan *et al*., 2009). Current or prior intensive care, female gender, old age, diabetes mellitus, mechanical ventilation and septic shock increases mortality in patients with *Acinetobacter* infections (Kanafani and Kanj, 2013a, b; Hsueh *et al*., 2002; Lolans *et al*., 2006; Abdel-El-Haleem, 2003; Srinivasan *et al*., 2009).

Heavy use of antibiotics has contributed to the problem of resistance *A. baumannii* isolates (Moniri *et al*., 2010). Carbapenems are the treating choice for *A. baumannii* infections but resistance have been observed in these isolates recently (Kanafani and Kanj, 2013b). Therefore, treating MDR *A. baumannii* infections is an ongoing challenge worldwide. The resistance mechanism in carbapenem-resistant *A. baumannii* isolates is mostly producing Carbapenemase (Park *et al*., 2009a).

Prevalence of Virulence Factors (VF) contributed to pathogenesis in bacteria (Doughari *et al*., 2011). Virulence factors help bacteria to colonize on the epithelium, evade and inhibit the host's immune response through biofilm formation and obtain nutrition from the host (Connell *et al*., 1996; Barnhart and Chapman, 2006). VFs are adhesive and non-adhesive. Non-adhesives factors are sidrophores, serum resistance and island. Adhesive virulence factors are divided in to two subgroup; Fimbrial VF which colonization is related to this subgroup: P fimbriae (pap genes), S (*sfalfoc*DE), Dr antigen family (*afaldra*BC), type I fimbriae (*fim*H) and non-fimbrial VF: curli fibers (*csg*A); fibronectin receptor (*fnb*); polysaccharide coatings as group II capsules (*kps*MT) (Braun and Vidotto, 2004). Identification of virulence factors in *A. baumannii* is a key to fighting this pathogen but no data are available regarding adhesive virulence factors of *A. baumannii* in Iran. The aim of the present study is the evaluation of frequency distribution of adhesive virulence factors in carbapenemase-producing *Acinetobacter baumannii* isolated from clinical samples in Kermanshah, Iran.

MATERIALS AND METHODS

Collection of bacterial samples: The bacterial samples were isolated from urine (n = 3), blood (n = 32) and sputum (n = 69) of hospitalized patients in different wards of 3 hospitals (Imam Reza, Imam Khomayni, Taleghani) affiliated with Kermanshah University of Medical Sciences, Iran during March 2011-2013. In total, 104 isolates were idendified as *Acinetobacter baumannii*. Carbapenemase-producing isolates were chosen for identifying adhesive virulence factors frequency. The strains were stored in glycerol Trypticase Soy Broth (TSB) at -20°C.

Bacterial identification and antimicrobial susceptibility testing: Identification of the isolates as *Acinetobacter baumannii* was carried out by conventional biochemical tests and confirmed by API 20NE kit (bio-Mérieux, Marcy L'Etoile, France) (Bosshard *et al*., 2006). Susceptibility to Carbapenem was assessed by the Kirby-Bauer method according to the CLSI guidelines to check their susceptibilities to imipenem (10 μg) and meropenem (10 μg) (MAST, Merseyside, UK) (CLSI, 2006).

Amplification of virulence genes by PCR: DNA was extracted from the isolates by boiling method (Mohajeri *et al*., 2013). The primer sequences and expected sizes of amplicons for each PCR assay are described in Table 1 (Braun and Vidotto, 2004).

Table 1: Primers to virulence factors genes amplification

Gene	Virulence factor	Primer	Sequence (5′-3′)	Amplified DNA (bp)	Reaction conditions
afa/*dra*BC	Dr fimbriae	afa1	GCT GGG CAG CAA ACT GAT AAC TCT C	794	Annealing at 70
		afa2	CAT CAA GCT GTT TGT TCG TCC GCC G		
*pap*C	P fimbriae	pap1	GAC GGC TGT ACT GCA GGG TGT GGC G	328	Annealing at 68
		pap2	ATA TCC TTT CTG CAG GGA TGC AAT A		
sfa/*foc*DE	S fimbriae	sfa1	CTC CGG AGA ACT GGG TGC ATC TTA C	410	Annealing at 63
		sfa2	CGG AGG AGT AAT TAC AAA CCT GGC A		
*fim*H	Type 1fimbriae	FimH f	TGC AGA ACG GAT AAG CCG TGG	508	Annealing at 58
		FimH r	GCA GTC ACC TGC CCT CCG GTA		
*csg*A	Curli fiber	M464	ACT CTG ACT TGA CTA TTA CC	200	Annealing at 48
		M465 R	AGA TGC AGT CTG GTC AAC		
*kps*MT II	Capsule	kpsII f	GCG CAT TTG CTG ATA CTG TTG	272	Annealing at 57
		kpsII r	CAT CCA GAC GAT AAG CAT GAG CA		
Fnb	Fibronectin	Fbn F1	GGT AAC CAG TCA TTC GAG	207	Annealing at 46
	receptor	Fbn R1	TGG CAC ACT GTC GAA GTC		

PCR was carried out in a total volume of 15 μL containing 2 μL of template DNA, 0.5 mM of each of the primers, the four deoxynucleoside triphosphates (each at 200 μM), PCR buffer 10x, 1.5 mM of $MgCl_2$ and 1 U of Taq DNA polymerase (Sinaclon). PCR amplifications were performed with the following amplification scheme; first denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 sec, at each specific annealing temperature (Table 1) for 30 sec and elongation at 72°C for 45 sec. The amplification was finished with an extension program at 72°C for 5 min in a thermal cycler PCR (BioRad C1000).

The amplified DNA was electrophoresed in 1.5% agarose, stained by ethidium bromide and images were obtained by gel documentation (BioRad XR+) system.

The 100-bp ladder (Sinaclon) was used as standard for determining molecular mass of PCR products.

The strains *E. coli* MK1 (*afaldra*BC), *E. coli* MK2 (*pap*C and *sfalfoc*DE), *E. coli* ATCC 25922 (*kps*MT) *Staphylococcus aureus* ATCC 25923 (*fnb*) were utilized as positive controls for VF. For VF *Fim*H and *Csg*A one amplified PCR product of each gene sent to Sinaclon company for sequencing and the results used as positive control after Nblast.

RESULTS

A total of 104 clinical isolates of *A. baumannii* were collected from 3 hospitals in Kermanshah (Iran). The results showed 50 isolates were resistance to imipenem and meropenem as Carbapenemase-producing isolates. Distribution of sputum, blood and urine among these carbapenemase-producing isolates was 38 (76%), 11 (22%) and 1 (2%), respectively. The isolates were predominate in men 35 (70%) than in women 15 (30%). Distribuation of carbapenemase-producing *A. baumannii* according to age is shown in Fig. 1.

Diagnosis of adhesive virulence factors was carried out by PCR assay. Figure 2a-b demonstrates the presence of *Fim*H and *Csg*A and positive controls for the other VFs. The presence of fimbriae type I (*Fim*H) and *curli* fiber (*Csg*A) was confirmed while, the genes *pap*C, *afaldra*, *sfa*, KpsMT and *fnb* codifying for the fimbriae P, Dr, S, capsule and fibronectin receptor, respectively, were not detected in this study. No. virulence factor was found in 13 isolates (26%). Frequencies of *Fim*H and *Csg*A were 30 isolates (60%) and 27 isolates (54%), respectively showed in Table 2. The result

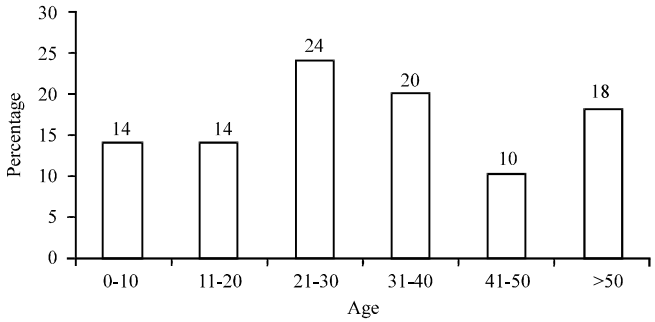

Fig. 1: Distribuation of carbapenemase-producing *A. baumannii* according to age

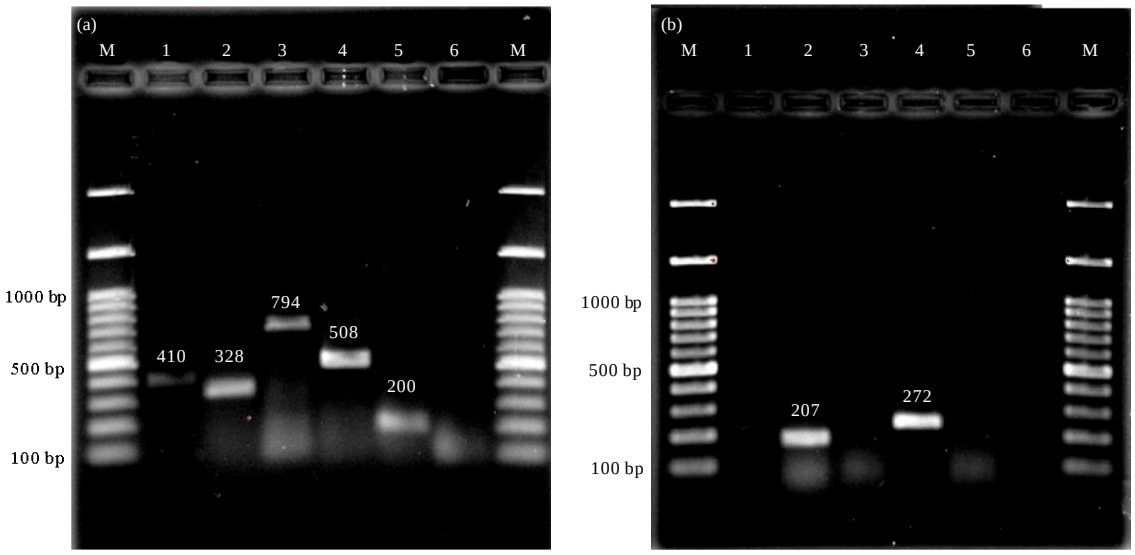

Fig. 2(a-b): Patterns of agarose gel electrophoresis showing PCR amplification products for the adhesive VFs genes. Lane M: DNA molecular size marker (100-bp ladder; Sinaclon), (a) Fimbrial VFs, Lane 1: *sfalfoc*DE, Lane 2: *pap*, Lane 3: *afaldra*BC, Lane 4: *fim*H, Lane 5: *Csg*A, Lane 6: Negative control and (b) Non fimbrial Vfs, Lane 2: fnb, Lane 4: kpsMT, Lane 3 and 5: Negative control

also showed there was not a significant correlation between *Fim*H and *Csg*A and the sex of patients (p-value>0.05) (Table 2). Twenty isolates (40%) were positive for both *Fim*H and *Csg*A that there was a significant correlation between these two genes (p-value<0.05).

DISCUSSION

Nowadays controlling infections caused by gram negative pathogen bacteria such as *Acinetobacter baumannii* and appearance of resistant isolates has become a clinical challenge (Braun and Vidotto, 2004). In the present study, of the 104 isolates of *Acinetobacter baumannii,* 50 (48%) carbapenemase producing isolates were recognized. Feizabadi *et al.* (2008) reported

Table 2: Distribution of *fim*H and *Csg*A according to sex

	Sex					
	Male		Female			
Virulence genes (%)	No.	%	No.	%	Total	p-value
Csg positive	19	-54.28	8	-53.33	27 -54	0.59
Csg negative	16	-45.72	7	-46.67	23 -46	
*Fim*H positive	22	-62.85	8	-53.33	30 -70	0.54
*Fim*H negative	13	-37.15	7	-46.67	20 -30	

resistance to carbapenems (imipenem and meropenem) as 50% in their study on 128 *Acinetobacter* isolates in Tehran, Iran, in 2008. Kheltabadi *et al.* (2009) reported 25% resistance to imipenem in their study on 60 clinical isolates of *Acinetobacter* in Kashan, Iran in 2008. Park *et al.* (2009b) reported 31.7 and 34.9% of resistance to imipenem and meropenem in their study on 63 isolates of *Acinetobacter* in South Korea in 2009. The resistance to carbapenems in our studied isolates of *Acinetobacter baumannii* is somewhat different from that in other studies, which can be attributed to geographical differences. Fifty carbapenemase producing isolates of *Acinetobacter baumannii* were studied in terms of 7 adhesive virulence factors. They had two adhesive virulence factors of fimbriae *csg*A and *fim*H in 27 (54%) and 30 (60%) of cases, respectively. Adhesive virulence factors are considered an important factor in adhesion, biofilm formation and survival of most bacteria and their virulence in human body (Doughari *et al.*, 2011). Compared to other gram-negative pathogens, relatively few virulence factors have been identified for *A. baumannii* (McConnell *et al.*, 2013). Therefore, it can be concluded that *csg*A and *fim*H are the main virulence factors of carbapenemase producing *Acinetobacter baumannii*, given the genes of *csg*A and *fim*H involved in forming biofilm (Ofek and Doyle, 1994; Sokurenko *et al.*, 1999). It can be expected that isolates bearing these genes have high capacity in causing biofilm-related infections especially pulmonary infections such as ventilator associated pneumonia. The existence of thin fimbriae in *A. baumannii* which are a major factor in adherence was described by Rosenberg *et al.* (1982) and Braun and Vidotto (2004). The presence of *fim*H in 60% of these samples may show their high capacity to adhere to surfaces. Braun studied 13 isolates of *Acinetobacter baumannii* isolated from urine for genotypic and phenotypic virulence factors. They concluded that 7 (53%) isolates were able to adhere phenotypically. Contrary to our study, none of Braun's isolates had relevant adhesive genes (Braun and Vidotto, 2004). It gave that most of the samples in our study were isolated from sputum, the difference between these two studies can be attributed to difference in the source of isolates and mechanism of adhesion in bacteria causing urinary tract infections with those of other infections. Studies show that 30% of *Acinetobacter baumannii* produce capsule (Joly-Guillou, 2005) while we did not find the relevant gene (*kps*MT). Therefore, it can be assumed that carbapenemase-producing *Acinetobacter baumannii* cannot produce capsule. Generally, it can be concluded that non-fimbriae genes may have less role in causing infections induced

by carpabenemase-producing *Acinetobacter baumannii* as compared to fimbriae genes. Furthermore, our study showed the high importance of two fimbriae virulence factors including fimbriae type I and curli fiber.

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