

# Analyzing pmrA and pmrB genes in *Acinetobacter baumannii* resistant to colistin in Shahid Rajai Shiraz, Iran Hospital by PCR: First report in Iran

Shahriar Sepahvand<sup>1</sup>, Monir Doudi<sup>\*2</sup>, Mohammad Ali Davarpanah<sup>3</sup>, Abbas Bahador<sup>4</sup> and Mehraoosh Ahmadi<sup>5</sup>

<sup>1</sup>Department of Microbiology, Biology of Faculty, Falavarjan Branch, Islamic Azad University, Isfahan, Iran

<sup>2</sup>Department of Microbiology, Biology of Faculty, Falavarjan Branch, Islamic Azad University, Isfahan, Iran

<sup>3</sup>Shiraz HIV/AIDS Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>4</sup>Department of Microbiology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran

<sup>5</sup>Shiraz Trauma Center, Shiraz University of Medical Sciences, Shiraz, Iran

**Abstract:** *Acinetobacter baumannii* is known as a worldwide emerging nosocomial infections and it is classified as one of the six dangerous microorganisms by Diseases Society of America. Multi drug-resistant strains of *A. baumannii* have been reported in recent decades, which may be a result of the high use of antimicrobial agents. Colistin is the last form of treatment against this organism. The presence of pmrA and pmrB genes in *A. baumannii* causes the resistance of this organism against Colistin. This cross-sectional study was performed on 100 samples of *A. baumannii* isolated from ulcer, urinary, respiratory, blood of patients admitted to the intensive care unit of Shahid Rajai Shiraz hospital within a 12-month period. The diagnosis was performed by microscopic and biochemical testing using microgen kits. Determining Colistin resistance was carried out by Diffusion Disc, Colistin antibiotic disc of MAST- England and E-test. The analysis of genes pmrA and pmrB genes was done by PCR. 100 *A. baumannii* samples were diagnosed out of which using diffusion disk 94 cases were sensitive to Colistin and 6 cases were resistant to it. The E-test results in resistant samples presented an MIC equal to 64 micrograms per milliliter. The PCR results in sensitive and resistant to Colistin samples presented the existence of pmrA and pmrB genes. The results indicated the presence of pmrA and pmrB genes that are the main reason of *A. baumannii* resistance against the last line of treatment of this organism to Colistin.

**Keywords:** *Acinetobacter baumannii*; Multi drug-resistant; E-test; PCR

## INTRODUCTION

The history of the genus *Acinetobacter* can be traced back to the early twentieth century i.e. in 1911 when the Dutch microbiologist, Beijerinck described an organism named *Micrococcus calcoaceticus* which was isolated from the soil by the enrichment in the minimal medium containing calcium acetate (Henriksen 1973).

In the past two decades, the genus *Acinetobacter* and especially the *A. baumannii* species is one of the most pathogenic bacteria emerged in our times which has been emphasized very much (Joly-Guillou, 2005; Murray and Hostenenthal, 2008). Its emergence is important because it adapts with a wide range of antibiotics, it has high transformation and capable of long-term stability in any environment (Doughari *et al.*, 2011). *A. baumannii* is a Gram-negative pathogen that often causes nosocomial infections such as bacteremia, pneumonia, meningitis and urinary tract infections. *Acinetobacter baumannii* is known as a worldwide emerging nosocomial infections and it is classified as one of the six dangerous microorganisms by Diseases Society of America. *A. baumannii* is a non-fermentative, gram-negative, non-motile, aerobic,

negative oxidase and catalase coco bacillus bacteria. After the enriched culturing many *Acinetobacter* can be retrieved from all samples collected from the soil or water because it is believed that environmental microorganisms are the most representatives of this genus (Boucher *et al.*, 2009). It was assumed that a strain of *A. baumannii* in Kuwait is involved in crude oil decomposition because it was isolated from the oil-contaminated desert (Obuekwe *et al.*, 2009).

*Acinetobacters* were also known as a part of the microbial flora of the human skin. More than 43% of the population has them on the skin and mucous membranes. In Germany in a study conducted on *Acinetobacter spp.* Carriage rate on the skin of healthy people *A. Iwoffii* with 58% of all isolates form had the highest retrieved population (Seifert *et al.*, 1997). Similar findings were reported by the United Kingdom. In a study of 192 healthy people they found that *Acinetobacter* carriage rate on the skin was almost 44%.

In contrast, in patients admitted to the hospital, carrying rate of the genus representatives can reach 75% (Berlau *et al.*, 1999). *A. baumannii* is the most important hospital genus type. *A. baumannii* forms colonies in hospitals on the skin and mucous membranes of humans and medical

\*Corresponding author: e-mail: doudi@yahoo.com

equipment, such as faucet, sink knee, detergents, respiratory equipment, mattresses, charts and tables, pillows, bed linen, blankets, door handles, telephones, steel hand wheels, trash cans and computers (Berlau *et al.*, 1999). It is believed that the main factors involved in sustainability of *A. baumannii* are in hospitals and they are resistant to disinfectants and have the capacity to survive in dry conditions (Berlau *et al.*, 1999).

One of the most interesting features of *A. baumannii* is that it can easily acquire resistance to antibiotics. Mutation, acquisition of new genes, regulation of available gene expression (if they have gene-degrading enzyme or drug or diffusion pumps), or losing them, as in purines, can increase the resistance of these strains. The resistance of these creatures can affect any medicine that has clinical use. Due to fast acquisition of genes with resistance against different classes of antibiotic, several drugs were excluded from the options of *A. baumannii* infection treatment including penicillin, cephalosporin, amino glycosides, quinolone and tetracycline (Oh *et al.*, 2013). Polymyxins are a group of polypeptide Cation Antibiotics formed of 5 different combinations (polymyxin A-E) discovered in 1947 (Storm *et al.*, 1947). Colistin (also known as E polymyxin) is the first antibiotic discovered in Japan in 1949 (Kumazawa *et al.*, 2002).

Polymyxins are a group of polypeptide Cation Antibiotics formed of a Deca-Peptide molecule with positive charge connected to fatty acid chains, which might be 6-methyl-Octanoic acid or 6-methyl-pentanoic acid. The main difference between Polymyxin B and E molecules is in amino acid components (Falagas and Kasiakou, 2005). Polymyxin B Cationic molecules and Colistin compete with each other and exchange Ca<sup>2+</sup> and Mg<sup>2+</sup> ions that usually stabilize outer membrane Lipopolysaccharide molecule of gram-negative bacteria. The molecules are formed of a multi cationic peptide ring that contains ten amino acids and a fatty acid side chain. Both of these compounds have specific antibacterial properties, they target bacterial cell wall and disrupt membrane permeability and lead to cell death. Polymyxins only perform bactericidal activity through connecting into the bacterial cell membranes and disrupting their permeability, which leads to the leakage of the components inside the cell. Polymyxins also have anti-endotoxin activity (Cooperstock 1974).

There is relatively little research about *A. baumannii* resistant to Colistin (Adams *et al.*, 2009). Right now there are two main hypotheses on *A. baumannii* resistance to Colistin. The first hypothesis is related to LPS loss proposed by Moffatt *et al.* and Henry *et al.* (2010 and 2012). They first understood lipid biosynthesis a gene *lpxA*, *lpxC* or *lpxD* inactivation the result of which was the lack of complete production of LPS in *A. baumannii*.

The strains that lost LPS became resistant to Colistin. In analyzing ISAbal1 sequence entry (Gen Bank accession number JF309050) in both *lpxA* and *lpxC* genes observed the complete loss of LPS and high level of resistance to Colistin (Moffatt *et al.*, 2011). Following the complete loss of LPS, the biosynthesis expression and vital transformations associated with surface composition and structure of the bacteria are changed in *A. baumannii* (Henry *et al.*, 2012). In colistin resistant strains reduced negative charge is a reason for fewer tendencies to colistin (Rachel *et al.*, 2011). The second resistance hypothesis is through PmrAB system proposed by Adams *et al.* in (2009). By comparing the DNA sequence encoding genes Pmr Band PmrA between sensitive and resistant strains against Colistin they found that *A. baumannii* resistance to Colistin is associated with mutations in Pmr Band PmrA genes (Park *et al.*, 2011).

## MATERIALS AND METHODS

### Identification and isolation

This study was carried out during 12 months in the intensive care unit of Shahid Rajai Hospital in Shiraz. Sampling was performed from ulcers, urinary tract, blood, and respiratory secretions of patients admitted to ICU. The bacteria were cultured in Eosin Methylene Blue, blood agar and MacConkey agar media. After inoculation, the samples were placed on the relevant culture medium. The cultured plates were then incubated at 37°C for 18-24 hours. *A. baumannii* was recognized using the tests catalase, oxidase, TSI and motility; other biochemical tests were performed subsequently by a Microgen kit.

### ID-GN A microgen Kit

In this method, a single colony from the culture medium is used to prepare a bacterial suspension in 3MI saline according to McFarland 0.5 turbidity standard. The plastic strip over the kit was removed and 2 or 3 drops of the suspension (100µL) was added to the wells in each strip. Oil was dripped into the wells which were then covered by the plastic strip. The strips were then incubated at 37°C for 24 hours, and the necessary reagents were added to the wells. The results were obtained based on comparison of color formed in the wells with the Color Guide, and to find the diagnosis code, they were inserted in a report form, and ultimately the 4-digit code was entered in Microgen specific software.

### Antimicrobial susceptibility testing

The susceptibility testing of *A. baumannii* isolates to different antibiotics was carried out by the disc diffusion method (Modified Kirby-Bauer method) according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2011). The antibiotics included were grouped as follows: Meropenem, ceftazidime, Amikacin, ticarcilin, azeteronam, rifampin, tigecycline and colistin.

### Antibiogram

All suspensions and plates were numbered and *antibiogram* was performed on 100 purified samples through the following general procedure. To prepare a suspension of *A. Baumannii* with turbidity equal to that of half McFarland, a number of *A. baumannii* colonies were dissolved in some sterile normal saline to produce a suspension with half McFarland turbidity; a swab was entered in the suspension and its extra solution was squeezed, the swab was then streaked slowly in different directions on Mueller Hinton agar medium so that its entire surface was covered uniformly with the bacteria; after the surface was dried (not to be more than a quarter), antibiotics disks (MAST Co. England) were placed on the medium at least 2 cm apart from each other using sterile forceps; the plates were then incubated at 35 °C for 18-24 hours. Finally, the zone of inhibition was measured with a ruler and matched with CLSI tables for different antibiotics.

### E-test

To perform E-test, a suspension of *A. baumannii* was prepared equivalent to the half McFarland standard using sterile saline. A sterile swab was entered in the suspension and its extra solution was squeezed, the swab was then streaked slowly in different directions on Mueller Hinton agar medium so that its entire surface was covered uniformly with the bacteria. E-test strips were placed on the culture medium and the plates were incubated at 35°C for 18-24 hours. The confluence of inhibition zone with the strips shows the MIC number of colistin for inhibition of *A. baumannii*.

### PCR molecular test

In this study the presence of *prmA* and *prmB* genes in the following primers were used to analyze the molecules shown in table 1.

Acycle with a temperature of 94°C for 5 minutes as the initial denaturation. Three consecutive steps that repeated for 25 cycles: Denaturation step: 94°C for 1 minute. The connection: 60°C for 30 seconds. Elongation phase: 72°C for 30 seconds. A final peak of Elongation phase of: 72°C for 5 minutes. Once completed, the samples were kept at a temperature of 4°C until getting out of the rnocycler instrument. At Laboratory of Molecular Microbiology Department of Medical Sciences, Shiraz after the extraction of obtained DNA kept in the freezer at -20°C was used as a model for PCR testing. The volume of the reaction mixture per micro tubes was 25ml. Accordingly, Master Mix with the available number of samples was evaluated. For each sample, 22ml of Master Mix was added to the eppendorf intended for each sample. Then 3ml of the extracted DNA samples was added to the eppendorf and the samples were transferred to the rnocycler PCR devise to perform PCR steps based on the mentioned order. Once completed, the samples

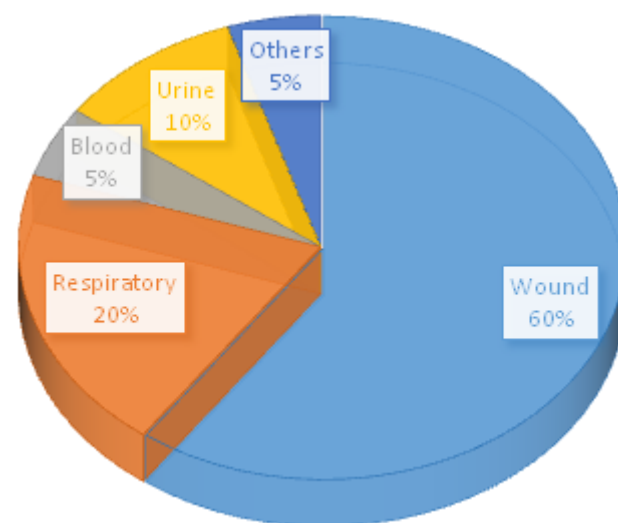
were stored in the freezer at -20°C until the analysis of products.

### PCR products analysis

PCR products were taken out of the freezer and analyzed. For this purpose, using Agarose buffer powder TAE, 1.5% gel was produced and then the 15ml per 100ml of solution ethidium bromide was added for staining DNA and poured into gel preparation tray. Then after cooling and coagulating of the gel (at 55°C) the wells were created by markup (charging). After cooling and coagulating the gel the charger was taken out and placed in the electrophoresis tank tray containing TAE buffer. Then 10ml of the PCR product was added to the well. Electrophoresis tank was connected to the power generator and the electric current density of 100m was set. After about an hour, the gel was removed from the tank and evaluated under UV radiation using a UV trans illuminator and the bands were observed on the screen and by comparing the size of the band with the marker the result was interpreted.

## RESULTS

All 100 isolates identified as *A. baumannii* in this study had the same biochemical pattern. *A. baumannii* produced pale pink mucoid colonies on blood agar (without hemolysis and pigment) and MacConkey agar media. At the end of this study, 100 strains of *A. baumannii* were recovered in Shahid Rajai Hospital using microscopy and biochemical tests and by eliminating unrelated and repeated isolates. Most isolates were isolated from ulcers shown in and (fig. 1).



**Fig. 1:** Percentage Sources of the *A. baumannii* isolates

Regarding the resistance of *A. baumannii* to colistin in Shahid Rajai Hospital in Shiraz, 6 and 94 isolates were resistant and sensitive to colistin, respectively. The MIC number of colistin-resistant *A. baumannii* in E-test was more than 64µg/mL.

**Table 1:** Sequences of primers used to determine the presence of *pmrA* and *pmrB* genes

Primer	Primer sequences	References
<i>pmrA</i> -F5'	ATGACAAAAATCTTGATGATTGAAGAT-3'	25
<i>pmrA</i> -R5	CCATCATAGGCAATCCTAAATCCA-3	25
<i>pmrB</i> -F5	GAACAGCTGAGCACCCCTTTAA-3	25
<i>pmrB</i> -R5	ACAGGTGGAACCAGCAAATG-3	25
16S rRNA-F	5-TCAGCTCGTGTCTGAGATG-3	26
16S rRNA-R5	CGTAAGGGCCATGATG-3	26

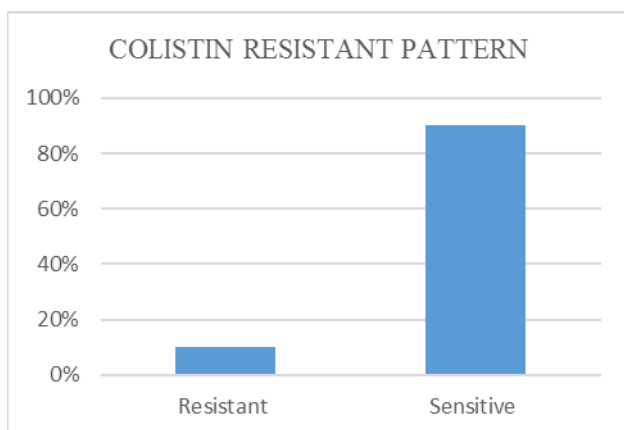
**Table 2:** The pattern of drug resistance against colistin in *A. baumannii* isolates in Shahid Rajai Hospital in Shiraz by E-test

Antibiotic	Breakpoint (µg/mL)	Resistant (%)	Intermediate (%)	Sensitive (%)	MIC (µg/mL)
Colistin	Sensitive ≤ 2	0	0	94	≤ 1
	Resistant ≥ 4	6	0	0	≥ 64

**Table 3:** The drug resistance pattern of other antibiotics

Antibiotic	TIC	CAZ	CTX	ATM	IMP	IMP+EDTA	MEM	DOR	TGS	RA	AN
MIC (µg/ml)	>256	>256	>256	>32	>32	<1	>32	>32	1	2	4

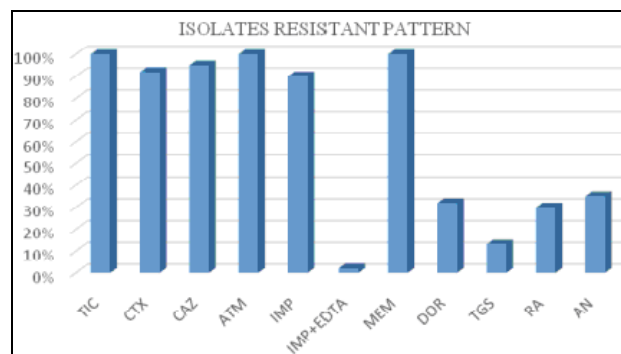
The isolated strains presented considerable resistance to Colistin given that Colistin is the last treatment line for multi drug-resistant *A. baumannii* and there are few reports of resistance to Colistin in Iran and other countries. In analyzing *A. baumannii* in Shahid Rajai Shiraz hospital 6 samples were resistant to Colistin and 94 samples were sensitive to it. In E-test MIC of *A. baumannii* resistant to Colistin was more than 67 micro liters per milliliter shown in (table 2) and (fig. 2).



**Fig. 2:** Percent resistance pattern of *A. baumannii* Against colistin

Using Antibiogram and E-test method to test the Resistant of *A. baumannii* isolates to different antibiotics, according to the Clinical Laboratory Standards Institute's (CLSI) we found that; The highest Resistant of *A. baumannii* was for TIC, CAZ, CTX, ATM and MEM (100%, 95%, 92%, 100% and 100% of isolates). Followed by IMP (90% of isolates); Then DOR, AN, RA and TGS (32%, 35%, 30% and 13%). The Lowest Resistant of *A. baumannii* was for colistin and IMP+EDTA (6% and

2%). Isolated strains in (table 3) and (fig. 3) showed high resistance against carbapenem antibiotics.



**Fig. 3:** Percent resistance pattern of *A. baumannii* isolates to different antibiotics

**The results of genes *pmrA* and *pmrB* by technique PCR**

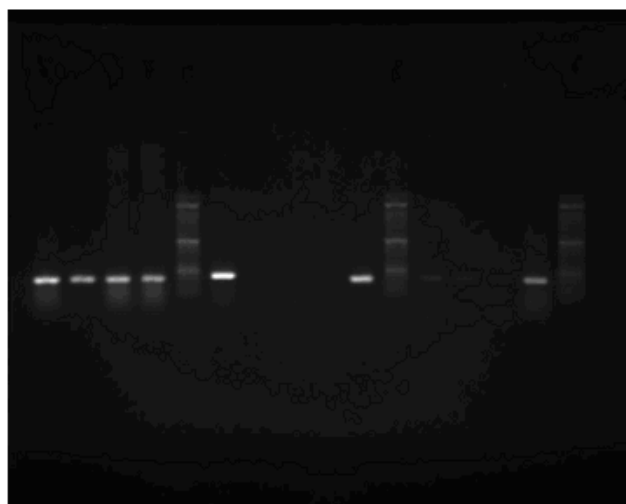
Using specific primers *pmrA* and *pmrB* Presence or absence of this gene in isolates were tested by PCR. The results were as follows: In all samples sensitive and resistant against colistin existence *pmrA* and *pmrB* Genes (fig. 4).

The results of the reviews *pmrA* and *pmrB* genes by PCR method Shows that most of strains Carriers *pmrA* and *pmrB* genes. As shown in (fig. 5) 70% of the strains carriers' *pmrA* Gene and 30% of the strains carriers *pmrB* Gene.

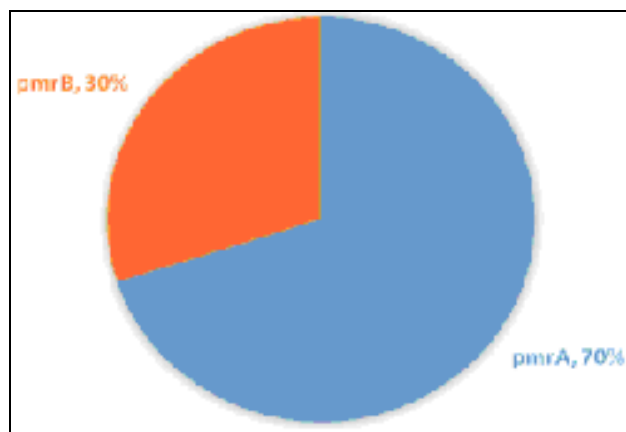
**DISCUSSION**

Antibiotic resistance exists among the bacteria causing the primary infections. This serious problem (eg, resistance to penicillin and macrolide in *Streptococcus*

*pneumonia*, pathogens producing ESBL of urinary tract are only a few cases) has impressed the situation in our hospitals and limited health care, which can be infectious. In our study, the largest number of *Acinetobacter baumannii* was associated with the patients in the intensive care unit. Recently a list of organisms that cause infectious resistance among the hospitals have been identified by Infectious Diseases Society of America. These organisms are members of a notorious summarized list of ESKAPE, which presents *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae sp* (Boucher et al., 2009).



**Fig. 4:** Analyzing pmrA and pmrB Genes in *A. baumannii* Resistant to Colistin with PCR: Ladder 50 bp, PmrA genesize 175bp, pmrB gene size 145bp, 16s gene size 151bp



**Fig. 5:** Percentage pmrA and pmrB genes

The situation is very serious, particularly in gram-negative members. There is a new therapeutic alternative for gram-negative organisms, even if these options are considered as the "last hope" for a long time (e.g., vancomycin), they will eventually destroy. In this study *A. baumannii* resistance to Colistin as the last line of

treatment for this organism was observed. In the study conducted the intensive care unit of Shahid Rajai Shiraz hospital a large number of patients who were in special care units were infected with *Acinetobacter baumannii* that the most ulcer samples were isolated from these patients and the Antibiogram and E. test showed that they have 100% resistance against many antibiotics and they were just sensitive against Colistin that out of 100 isolates, 6 isolates were resistant to Colistin and since there is no new drugs on the way as an alternative to the existing drugs for this disease, and there is no vaccine against infection with this organism, the only way to relieve the effects of infection is to control their spread. In the analyses conducted on the isolated samples of Shahid Rajai Shiraz hospital, we concluded that there are *A. baumannii* with pmrA and pmrB genes in samples sensitive or resistant to Colistin. Adams et al. stressed that *A. baumannii* resistance against Colistin is through PmrAB system, which was first proposed in 2009 (Adams et al., 2009). By comparing the DNA sequence of genes coding PmrA and Pmr B between strains sensitive and resistant to Colistin found that *A. baumannii* resistance to Colistin is associated with mutations in pmrA and pmrB genes and the results indicate that the increased expression of PmrAB system is necessary *A. baumannii* resistance to Colistin.

## CONCLUSION

*Acinetobacter baumannii* resistant to colistin is expanding in IRAN and considered as critical risk for hospitalized patient. Because of the most of the resistant strains origin are from specific care unit, so, disinfecting of patients room and observing of sanitary principles by hospital personnel are important in resistant strains spreading and control. Most of the strains of this study are pmrA and pmrB genes carriers. It can be stated that these genes are involved in *A. baumannii* resistant to colistin, so, recognition of colistin resistance genes and resistance mechanisms control and treatment of this bacteria are important.

## REFERENCES

- Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR and Bonomo RA (2009). Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two component system. *Antimicrob. Agents. Chemother.*, **53**: 3628-3634.
- Bartlett J (2009). Bad bugs, no drugs: No ESKAPE! An update from the infectious diseases society of America. *Clin. Infect. Dis.*, **48**: 1-12.
- Berlau J, Aucken H, Malnick H and Pitt T (1999). Distribution of acinetobacter species on skin of healthy humans. *Eur. J. Clin. Microbiol. Infect. Dis.*, **18**: 179-183.

- Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Obuekwe CO, Al-Jadi ZK and Al-Saleh ES (2009). Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon degraders from petroleum-contaminated Kuwait desert environment. *J. International Biodegradation*, **63**(3): 273-279.
- Cooperstock MS (1974). Inactivation of endotoxin by polymyxin B. *Antimicrob. Agents. Chemother.*, **6**: 422-425.
- Doughari HJ, Ndakidemi PA, Human IS and Benade S (2011). The ecology, biology and pathogenesis of *Acinetobacter* spp. An overview. *Microbes. Environ*, **26**: 101-112.
- Falagas ME and Kasiakou SK (2005). Colistin: The revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin. Infect Dis.*, **40**: 1333-1341.
- Henry R, Vithanage N, Harrison P, Seemann T, Coutts S, Moffatt JH, Nation RL, Li J, Harper M, Adler B and Boyce JD (2012). Colistin-resistant, lipopolysaccharide deficient *Acinetobacter baumannii* responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids and poly-b-1,6-N-acetylglucosamine. *Antimicrob. Agents. Chemother.*, **56**: 59-69.
- Henriksen SD (1973). *Moraxella*, *Acinetobacter* and the Mimosaceae. *Bacteriol. Rev.*, **37**: 522-561.
- Joly-Guillou ML (2005). Clinical impact and pathogenicity of *Acinetobacter*. *Clin. Microbiol. Infect*, **11**: 868-673.
- Kumazawa J and Yagisawa M (2002). The history of antibiotics: The Japanese story. *J. Infect Chemother.*, **8**(2): 125-133.
- Moffatt JH, Harper M, Harrison P, Hale JD, Vinogradov E, Seemann T, Henry R, Crane B, St Michael F, Cox AD, Adler B, Nation RL, Li J and Boyce JD (2010). Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob. Agents. Chemother.*, **54**(12):4971-4977.
- Murray CK and Hospenthal DR (2008). *Acinetobacter* infection in the ICU. *Crit. Care. Clin.*, **24**: 237-248.
- Moffatt JH, Marina H, Ben A, Roger LN, Li J and John DB (2011). Insertion sequence ISAbal1 is involved in colistin resistance and loss of lipopolysaccharide in *Acinetobacter baumannii*. *Antimicrob. Agents. Chemother.*, **55**: 3022-3024.
- Oh YJ, Song SH, Seung HB, Hak HL, In MH and Dong HO (2013). A case of fulminant community acquired *Acinetobacter baumannii* pneumonia in Korea. *Korean. J. Intern Med.*, **28**(4): 486-490.
- Rachel LS, Roger L, Nation, Stewart C, Jennifer HM, Marina H, Ben A, John DB, Ian L and Jian L (2011). Different surface charge of colistin-susceptible and-resistant *Acinetobacter baumannii* cells measured with zetapotential as a function of growth phase and colistin treatment. *J. Antimicrob. Chemother.*, **66**: 126-133.
- Park YK, Choi JY and Shin D (2011). Correlation between over expression and amino acid substitution of the PmrAB locus and colistin resistance in *Acinetobacter baumannii*. *Int. J. Antimicrob. Agents.*, **37**: 525-530.
- Seifert H, Dijkshoorn L, Gerner-Smidt P, Pelzer N, Tjernberg I and Vaneechoutte M (1997). Distribution of *Acinetobacter* species on human skin: Comparison of phenotypic and genotypic identification methods. *J. Clin. Microbiol.*, **35**: 2819-2825.
- Storm DR, Rosenthal KS and Swanson PE (1977). Polymyxin and related peptide antibiotics. *Annu. Rev. Biochem.*, **46**: 723-763.