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การแพร่กระจายของเชื้อ *Acinetobacter baumannii* ที่ดื้อต่อยา colistin ในโรงพยาบาศิริราช

CLONAL SPREAD OF COLISTIN RESISTANCE IN *Acinetobacter baumannii* IN SIRIRAJ HOSPITAL

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บทคัดย่อ

เพื่อศึกษาการระบาดและแพร่กระจายของเชื้อ *Acinetobacter baumannii* ที่ดื้อต่อยา colistin ในโรงพยาบาลศิริราช เชื้อ A. *baumannii* ที่แยกได้จากผู้ป่วยในโรงพยาบาลศิริราชในช่วงระหว่างปี พ.ศ.2553 ถึง 2555 จำนวน 200 ตัวอย่าง ได้นำมาทำการทดสอบความไวของเชื้อต่อยา colistin ด้วยวิธี *microdilution* ตามหลักเกณฑ์ของ CLSI 2011 ต่อมาได้ทำการจัดกลุ่มทางพันธุกรรมของเชื้อด้วยวิธี PCR-randomly amplified polymorphic DNA (RAPD) ซึ่งเป็นวิธีระดับโมเลกุล หลังจากนั้นยีน *pmrCAB* ของเชื้อที่ดื้อต่อยา colistin ได้ถูกเพิ่มจำนวนและอ่านลำดับของนิวคลีโอไทด์ เพื่อนำข้อมูลที่ได้มาเปรียบเทียบกลุ่มพันธุกรรมของเชื้อ และเปรียบเทียบผลที่ได้กับผล RAPD ว่าให้ผลที่สอดคล้องกันหรือไม่ คณะผู้วิจัยได้พบเชื้อที่ดื้อต่อยา colistin ทั้งหมดร้อยละ 7.5 ซึ่งสามารถแบ่งระดับความดื้อได้เป็น 3 ระดับ คือ ระดับสูง, กลาง และ ต่ำ นอกจากนี้ยังพบว่าเชื้อทั้งหมดสามารถแบ่งกลุ่มทางพันธุกรรมได้ 16 แบบ แต่เชื้อที่ดื้อต่อยา colistin ส่วนมากอยู่ในแบบที่ 1 และถึงแม้ว่าเชื้อเหล่านี้จะอยู่ในแบบที่ 1 เหมือนกัน แต่ผู้วิจัยกลับพบว่าเชื้อเหล่านี้มีลำดับของนิวคลีโอไทด์ของยีนที่แตกต่างกัน โดยที่เชื้อหมายเลข 2, 6, 7, 8 และ R017นั้นมีลำดับของนิวคลีโอไทด์ที่คล้ายคลึงกัน แต่แตกต่างจากเชื้อหมายเลข 1, 3, 4 และ R055 ซึ่งเชื้อเหล่านี้ก็มีลำดับนิวคลีโอไทด์ต่างกันเพียงเล็กน้อย จากผลการศึกษาพบว่าเชื้อ A. *baumannii* สายพันธุ์ที่พบในโรงพยาบาลศิริราชมีความสัมพันธ์กันในลักษณะที่มักพบเป็นกลุ่มพันธุกรรมเดียวกัน อย่างไรก็ตามสายพันธุ์ที่มีลำดับนิวคลีโอไทด์ของ *pmrCAB* และค่า MIC ของเชื้อต่อยา colistin ที่แตกต่างกันนั้นสามารถพบได้ในเชื้อที่อยู่ในกลุ่มพันธุกรรมเดียวกัน ซึ่งความแตกต่างที่เกิดขึ้นนั้นอาจมีสาเหตุมาจากสภาวะในการคัดเลือกสายพันธุ์ของเชื้อที่ต่างกัน ภายในกลุ่ม RAPD type I ด้วยกันนั้น มีความเป็นไปได้ว่าเชื้อหมายเลข 2, 6, 7, 8 และ R017 อาจแยกเป็นกลุ่มพันธุกรรมย่อย ในขณะที่เชื้อหมายเลข 1, 3, 4 และ R055 นั้นก็พบเป็นกลุ่มพันธุกรรม ย่อยอีกกลุ่มหนึ่ง การศึกษานี้ชี้ให้เห็นว่าลำดับเบสการกลายพันธุ์ใน pmrCAB สามารถใช้เป็นเครื่องติดตามการดื้อต่อยา colistin ของเชื้อได้อีกด้วย

คำสำคัญ : การดื้อต่อยา colistin, ยีน *pmrCAB*, RAPD

Abstract

amplified polymorphic DNA (RAPD) technique. After that, the pmrCAB_genes were amplified and sequenced. We found that 7.5% of isolates were colistin resistant (15/200). These could be categorized based on MIC into three levels of resistance: high, moderate, and low levels of resistance. Sixteen RAPD types were detected. The majority of colistin-resistant isolates were in RAPD type I. However, the sequencing of pmrCAB operon showed sequence differences in the same RAPD type. Isolate no. 2, 6, 7, 8, and R017 have similar sequences which are significantly different from those of isolate no. 1, 3, 4, and R055. Additionally, the member of each sequence group showed small sequence differences among them.

The A. baumannii isolates which were found at a hospital in Thailand were molecular epidemiology studied. Furthermore the resistance nature identified bacterium was also investigated. The results indicated that the nosocomial strains in the hospital were highly clonal in nature. However, the strains with different pmrCAB sequence and MIC of colistin could develop within the same clone depending on selective pressure upon them. In the same RAPD type I, the isolate no. 2, 6, 7, 8, and R017 were possibly the same subclone while isolate no. 1, 3, 4, and R055 existed as another subclone. The mutations in studied genes may be useful markers for tracking the colistin resistance development.

Keywords : Colistin resistance, pmrCAB operon, RAPD

Introduction

Acinetobacter baumannii is an opportunistic gram-negative pathogen associated with nosocomial infections. *A. baumannii* is resistant to various kinds of antibiotics, such as β-lactams, fluoroquinolones, tetracyclines and aminoglycosides (3, 4, 6, 15, 19). Multi-drug resistant (MDR) *A. baumannii* has emerged and quickly became a critical public health problem worldwide. This organism has ability to survive and persist in environment, especially in hospital environment that has heavy antibiotic uses. Only a few antibiotic choices are still effective against MDR isolates. Then, an old antibiotic, colistin, is brought back and now being used widely, although this drug can generate nephrotoxicity and neurotoxicity in treated patients (7, 10-12). However, colistin-resistant isolates have been recently reported (16, 17).

Colistin is a cationic anti-microbial polypeptide, which binds to the negative charges of lipid A, the endotoxic part of lipopolysaccharide (LPS), and displaces divalent cations such as calcium and magnesium that stabilize the LPS layer, leading to the instability of the bacterial outer membrane. Consequently, the drug can insert itself into the cell membrane, causing an increase in permeability of cell membrane, leakage of intracellular molecules and bacterial cell death (7, 9, 10). One of the most concerned problems on antibiotic-resistant *A. baumannii* is its capability to resist to colistin as this drug is arguably the last effective choice against the bacteria. The resistance mechanism to colistin of *A. baumannii* was linked to PmrA-PmrB two-component regulatory system that is also responsible for lipid A modification. The PmrA-PmrB two-component system is a signal transduction cascade

containing PmrB sensor kinase and PmrA response regulator. This system works by the activation of PmrB sensor kinase causing PmrB autophosphorylation and then the phosphate was transferred to PmrA response regulator. The activated PmrA stimulates the transcription of *pmrC*, the phosphoethanolamine transferase gene, resulting in modification of lipid A (2, 8) by the addition of phosphoethanolamine residues to the lipid A molecules. This addition takes place at the negative-charged phosphates thus eliminating their negative charges. The mutations in *pmrAB* have been reported to reduce the affinity of colistin binding. Increase of *pmrA* expression was resulted from the mutation in *pmrA* gene that constitutively activates its expression. Furthermore, the colistin-resistant strains were reverted to colistin-susceptible strains when *pmrB* in colistin-resistant strains were deleted suggesting that the overexpression of *pmrAB* system is associated with colistin resistance (1, 2).

At Siriraj hospital, the colistin-resistant *A. buamannii* isolates have been detected at the alarming rate. Therefore, we investigated the outbreak of colistin resistance in *A. baumannii* from patients in Siriraj hospital by using molecular techniques. The typing of colistin resistant *A. baumannii* isolates were determined by PCR-randomly amplified polymorphic DNA (RAPD). Then, the RAPD results waere compared with the sequences of *pmrCAB* operon to find the nature of the resistance whether it exists as the major clone or can develop as sporadic events. The results of this study can be used to plan the preventive measure against the spreading of colistin resistance strains in the hospital.

Objective:

To investigate the molecular epidemiology and the resistance nature of colistin resistant *Acinetobacter baumannii* isolated in Siriraj hospital.

Methods

Two hundred isolates of *A. baumannii* collected from patients in Siriraj Hospital during 2010 to 2012 were used in this study. Antibiotic susceptibility test (AST) of all isolates was determined by broth microdilution method according to the CLSI 2011 guideline (5). Inoculums were prepared by suspension of isolated colonies from overnight culture in sterile saline and adjusted to 0.5 McFarland standard (about 10^8 CFU/mL) and then diluted to 10^7 CFU/mL. Within 15 minutes after adjusting the turbidity of inoculums, the 5 µL of inoculums were added to 96-well plate that contained in each well 100 µL of sequential dilutions of colistin as listed in table 4.2. The 96-well plate was incubated at 35±2 °C for 20-24 hours. Minimal inhibitory concentrations (MICs) were determined by observing the lowest concentration of antimicrobial agents that inhibited bacterial growth. Finally, the results were recorded and interpreted according to CLSI 2011 guideline. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used as quality control strains.

PCR-randomly amplified polymorphic DNA (RAPD) was performed to type all of *A. baumannii* isolates. The reaction volume was 20 µL, which contained 1X of supplied PCR buffer with $MgCl_2$, 0.2 mM dNTPs, 1.0 U of *Taq* DNA polymerase (StrataGene®, USA), 5 ng/ µL of genomic DNA templates and 2 pmol/µL of R003 primer (18). The primer sequence is 5'-CCTTGACGCA-3'.

Then PCR-RAPD was performed with the following condition: initial denaturation at 94°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 10 seconds, annealing at 36°C for 30 seconds and extension at 72°C for 1 minute, with final extension at 72°C for 2 minutes. The low annealing temperature was to allow the random binding of primer across the genome. The PCR products were analyzed by electrophoresis in 1% agarose gel containing 1X Tris-Borate-EDTA (TBE) buffer. 1 kb plus DNA ladder was used as a marker. The amplification product of *A. baumannii* ATCC 19606 were ran in parallel with samples as an internal control. Visualized bands were compared among the isolates, and the RAPD profile was subsequently generated.

The last step, *pmrCAB* operon were amplified and sequenced in clinical resistance isolates (1, 2, 3, 4, 6, 7, 8, R017, and R055 stains). The sequencing primers used in this study were listed in table 1. The sequencing primers: promoter-pmrC-F, pmrC2-F, pmrC3-R, pmrC4-R, pmrA-F, pmrA-R, pmrB2-F, pmrB3-F, and pmrB3-R, were used for isolate no. 1, 3, and 4 while the operons of isolate no. 2, 6, 7, 8, R017 and R055 were sequenced by using promoter-pmrC-F, pmrC-R, pmrC2-F, pmrC2-R, pmrA-F, pmrA-R, pmrB-F, pmrB2-F, and pmrB2-R primers. The Sanger dideoxynucleotide phosphate sequencing provided by First Base Laboratories was used in DNA sequencing. The sequencing results were analyzed by using Bioedit software, and aligned with the reference sequences of *pmrCAB* operon in the database by using Genious Version5.4.5.

Table 1 Primers for amplification and sequencing of *pmrCAB* operon

Primer	Sequence
promoter-pmrC-F	5'-CCGTGTTGGTTTAATGGCTAA-3'
pmrC-R	5'-ATATTGCAGCAACGTGATCG-3'
pmrC2-F	5'-GGTAGCTTCATTTGCAGTGG-3'
pmrC2-R	5'-CAACACGATCACATGCACCT-3'
pmrC3-R	5'- CCGTTATTGACCCATTCCAC-3'
pmrC4-R	5'- CACATGCGCCTTTACAACC-3'
pmrA-F	5'-CCCAAGTTTGTTAAGTTTGCTG-3'
pmrA-R	5'-TCGACTTCTTGAAGTGCAACC-3'
pmrB-F	5'-AGAGCGAAGCTGGGTAAAGA-3'
pmrB-R	5'-GGGTCGTTTGGGCAATAAAT-3'
pmrB2-F	5'-ACGAACACCTGTGACTGCAT-3'
pmrB2-R	5'-GGTCATGCAAGTGCAGGTTT-3'
pmrB3-F	5'-GATGTGACCAGCAACACGAT-3'
pmrB3-R	5'-GCGAGGAGCACATTTCCTAA-3'
pmrB4-R	5'-TCAGCTCATCGGTACAGCAC-3'

Results

The MICs values derived from two hundred isolates of *A. baumannii* identified fifteen resistant isolates as show in the figure 1. The levels of resistance to colistin can be divided into three levels. Four isolates, 2, 6, 7, and 8, are high levels resistance (>128 µg/mL). R017, 1, 3, and 4 are moderate resistance (32-64 µg/mL), and seven isolates, R004, R041, R055, R069, R088, R090, and R188 are low levels resistance (4-8 µg/mL).

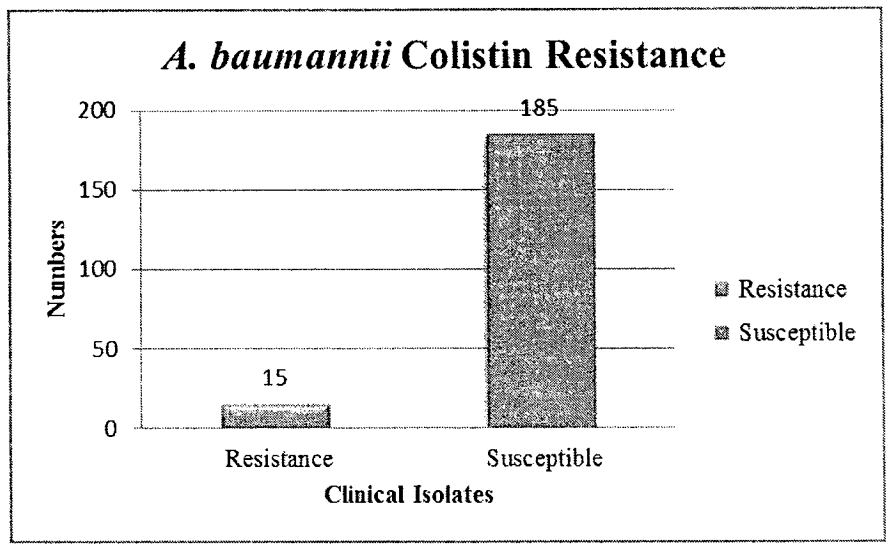

Figure 1 The conclusion of numbers of colistin susceptible and resistance *A. baumannii* clinical isolates.

Sixteen RAPD types were identified in this study (Figure 2). Most of the isolates were typed as RAPD type I (n=133, 66.5%). Thirty isolates were RAPD type II (15%). RAPD type III, and IV had 7 (3.5%), and 5 (2.5%) isolates, respectively. RAPD type V, VI, and VII each consisted of 4 isolates (2%) while 3 isolates (1.5%) were typed as RAPD type VIII. Both of RAPD type IX, and X had 2 isolates (1%). Lastly a single isolate was typed into RAPD type XI to XVI each. The resistance strains were found in only RAPD type I, II, V and VIII. Most of the resistance isolates were found to be RAPD type I which also represented the major type.

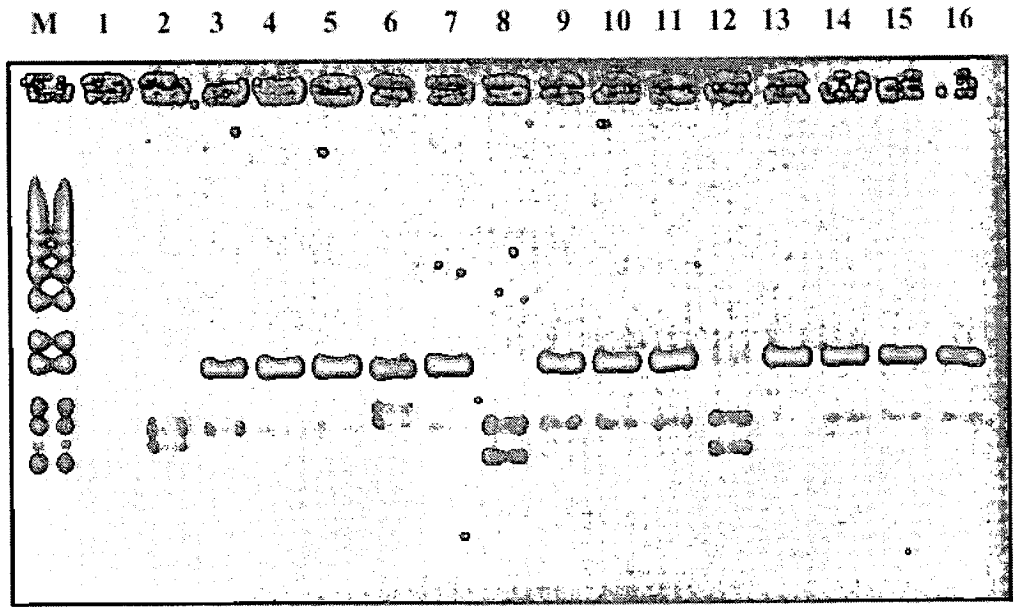

Figure 2 Example of RAPD banding pattern of clinical isolates in this study.
Lane M indicates 1 kb plus DNA ladder
Lane 1 indicates negative control
Lane 2 indicates RAPD type of *A. baumannii* ATCC 19606 act as positive control
Lane 3 to 16 indicates RAPD types of isolate no. R017, R018, R019, R020, R021, R022, R023, R024, R025, R026, R027, R028, R030, and B031

The sequences of *pmrCAB* operon of colistin resistance isolates were compared and aligned. Isolate no. 1, 3, and 4 showed identical of sequences while no. R055 differed at only one position in PmrB, which is threonine in isolate no. 1, 3, 4 but isoleusine (wild type) in R055. Isolated no. 2, 6, 7, 8 and R017 had almost identical *pmrCA* sequenced but had moderate difference in *pmrB* sequence.

In isolate no. 2 and 6, the unique amino acid changes, R125H and P233S, were found in PmrC and PmrB, respectively. Two unique amino acids change in PmrB, A142T, and T232I were only detected in isolate no. 7. Isolate no. 8 also had unique one amino acids change P170L in PmrB. In all, the PmrC, PmrA, PmrB sequences clearly divided the resistance strain into two sequence types: the isolate no. 2, 6, 7, 8, and R017; and the isolate no. 1, 3, 4, and R055 with minor differences among isolates in the same sequence type. The mutation results were summarized in table 2.

Table 2 The number of mutations found in resistance isolates. S and NS represent synonymous and non-synonymous mutations, respectively

Isolate	*pmrC*		*pmrA*		*pmrB*		MIC
	S	NS	S	NS	S	NS	(μg/ml)
1 ,3 ,4	120	66	65	5	105	25	32
R055	120	66	65	5	105	24	8
2, 6	13	5	4	-	7	3	
7	13	4	4	-	8	4	>128
8	13	4	4	-	7	3	
R017	13	4	4	-	8	2	32

Discussion

The emergence of MDR *A. baumannii* has been increasing and related with higher mortality from nosocomial infections during the past few years. Moreover, some isolates has been reported to resist to the polymyxin drugs, especially colistin which is the last effective choice to treat these bacteria, leading to treatment failure. In this study, we collected 200 *A. baumannii* isolates from various kinds of clinical samples. The majority (92.5%) of the isolates was still susceptible to colistin with 7.5% that are colistin resistant. This percentage may seem to be low, but when considering the number of patients inflicted by these bacteria, this is an alarming proportion. Moreover, the colistin resistant isolates had three levels of resistance, 26.7%, 26.7% and 46.6% were high, moderate, and low levels of resistance, respectively, based on standard broth microdilution method. Even with the low level resistance, the resistant strains could potentially lead to morbidity of the patients, as the adjusted dosage of colistin is limited due to its toxicity. The levels of resistance reflect the difference of resistance mechanisms or different positions of mutation in the involving genes. Thus, we studied the genetic background in all isolates to determine the relation of colistin resistance isolates in Siriraj hospital by using molecular method, RAPD typing. RAPD is a PCR-based analysis that used the short primer with low annealing temperature to generate the PCR fingerprints. This technique categorizes the genetic of organisms by comparing banding pattern, and can be used to investigate local epidemiology. The advantages of RAPD are the ease to perform, less time consuming, cheap, and easy to interpretation of data. However, the positive control is required in every run to confirm the accuracy of the banding pattern. *A. baumannii* ATCC 19606 was used as positive control in this study.

The several RAPD types of isolates were detected. The major types are RAPD type I (66.5%), and II (15%). The members of RAPD type I composed of isolates with all levels of resistance, whereas only the low levels of colistin resistance isolates were exhibited in the members of RAPD type II, V, and VIII. This result suggested that *A. baumannii* which isolated from patients in Siriraj hospital have various genetic backgrounds, although the prominent type was RAPD type I which represented the

endemic clone of the hospital. However, the limitation of RAPD method lies on its low discriminatory power. Hence, the members of RAPD type I could consist of isolates with heterogenous genetic backgrounds which can in turn affect the heterogeneity of phenotypes as shown by various levels of resistance in this study. If we use another technique that has high discriminatory power such as multi-locus sequence typing (MLST), the result may show distinct types of isolates typing.

The results of *pmrCAB* operon sequencing suggested that there were two main sequence types among resistance isolates. These two types might develop as subclones of RAPD type I upon different circumstances on the selection by drug usage. The *pmrCAB* of isolate no. 1, 3, 4 and R055 had identical sequence except one position in *pmrB* resulting in the amino acid substitution from threonine to isoleucine at position 13 in isolate no. 1, 3, 4. Interestingly, the MIC of colistin of isolate no. 1, 3, 4 was 32 μg/ml comparing to 8 μg/ml of isolate no. R055 suggesting that mutation in *pmrB* might have a large role in the resistance. This finding was consistent with the previous reports of finding the mutations in *pmrB* of the clinical isolates. The threonine at position 13 is in the unknown functional domain. Mutation at this position might cause the conformation change of PmrB, leading to the enhancement of PmrB activation (2, 14). Nonetheless, this hypothesis needs to be further proved by site-directed mutagenesis of wild-type *pmrB*.

Another sequence type was represented by isolate no. 2, 6, 7, 8 and R017. Isolate no. 2 and 6 had an exactly identical sequence indicating that they were the same clone. Again, the main difference among the isolates lies in *pmrB*, supporting the hypothesis that PmrB plays a major role in the resistance. However, the effect of mutations in this case is more complicated. All of the strains contained the common mutations A138T and A444V in PmrB, which could originally made them diverse from the other sequence type. Additionally, the isolate no. 2, 7, 8 each had their own unique base substitutions in PmrB. As the MICs of colistin in all three isolates were > 128 μg/ml, it still could not be directly concluded that these unique mutations affected the resistance. The molecular structure of *A. buamannii* PmrB has to be deduced in order to study the roles of these residues in folding and the activity of the protein. Nevertheless, the possibility of additional resistance mechanisms besides lipid A modification still cannot be ruled out in all cases.

As the mutants of *pmrCAB* operon were reported to lower the strains fitness (13), it is suggested that the resistance strains might not tend to establish the clones. Instead, the sporadic resistance emergence with diverse mutations could be preferentially observed. However, our data suggested the clonal nature of the resistance. Additionally, our preliminary results demonstrated that the fitness of the resistance strains were not significantly different from wild type strain in liquid culture (data not shown). It is possible that multiple mutations found in *pmrCA* and even in *pmrB* are to complement the resistance mutation and thus restoring the fitness of the protein. If it is the case, it posts a serious concern on the ability of the resistance strains to spread and persist as the hospital clone. However, more data has to be collected including patient data and the typing data from higher discriminatory power typing method to support this speculation.

Our results indicated a heavy use of colistin as shown by collective mutations in *pmrCAB*. Each bacterial cell has ability to mutate to increase its chance to survive from natural selection posted on it. With a strong selection by antibiotic, eventually the mutations causing the resistance will inevitably emerge, even with the most effective drug. Proper use of antibiotics is a key to lower the selection and slow the bacterial evolution toward the resistance.

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References

1 Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, et al. (2009). Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. Antimicrob Agents Chemother, 53, 3628-34.

2 Arroyo LA, Herrera CM, Fernandez L, Hankins JV, Trent MS, Hancock RE. (2011). The *pmrCAB* operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. Antimicrob Agents Chemother, 55, 3743-51.

3 Bergogne-Bérézin E. (2001). The increasing role of *Acinetobacter* species as nosocomial pathogens. Curr Infect Dis Rep, 3, 440-444.

4 Bergogne-Bérézin E, Towner KJ. (1996). *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clin Microbiol Rev, 9, 148-65.

5 Clinical and Laboratory Standard Institute. Performance standards for antimicrobial susceptibility testing; Twenty-first information supplement. M100-S21. Wayne: CLSI; 2011.

6 Durante-Mangoni E, Zarrilli R. (2011). Global spread of drug-resistant *Acinetobacter baumannii*: molecular epidemiology and management of antimicrobial resistance. Future Microbiol, 6, 407-22.

7 Falagas ME, Kasiakou SK. (2005). Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. Clin Infect Dis, 40, 1333-41.

8 Gunn JS, Lim KB, Krueger J, Kim K, Guo L, Hackett M, et al. (1998). PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol Microbiol, 27, 1171-82.

9 Hancock RE, Chapple DS. (1999). Peptide antibiotics. Antimicrob Agents Chemother, 43, 1317-23.

10 Li J, Nation RL, Milne RW, Turnidge JD, Coulthard K. (2005). Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. Int J Antimicrob Agents, 25, 11-25.

11 Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, et al. (2006). Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. Lancet Infect Dis, 6, 589-601.

12 Lim LM, Ly N, Anderson D, Yang JC, Macander L, Jarkowski A 3rd, et al. (2010). Resurgence of colistin: a review of resistance, toxicity, pharmacodynamics, and dosing. Pharmacotherapy, 30, 1279-91.

13 López-Rojas R, Domínguez-Herrera J, McConnell MJ, Docobo-Peréz F, Smani Y, Fernández-Reyes M, et al. (2011). Impaired virulence and in vivo fitness of colistin-resistant *Acinetobacter baumannii*. J Infect Dis, 203, 545-8.

14 Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, et al. (2012). PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother, 56, 1019-30.

15 Peleg AY, Seifert H, Paterson DL. (2008). *Acinetobacter baumannii*: emergence of a successful pathogen. Clin Microbiol Rev, 21, 538-82.

16 Rodriguez CH, Bombicino K, Granados G, Nastro M, Vay C, Famiglietti A. (2009). Selection of colistin-resistant *Acinetobacter baumannii* isolates in postneurosurgical meningitis in an intensive care unit with high presence of heteroresistance to colistin. Diagn Microbiol Infect Dis, 65, 188-91.

17 Tan CH, Li J, Nation RL. (2007). Activity of colistin against heteroresistant *Acinetobacter baumannii* and emergence of resistance in an in vitro pharmacokinetic/ pharmaco-dynamic model. Antimicrob Agents Chemother, 51, 3413-5.

18 Thapa B, Tribuddharat C, Srifuengfung S, Dhiraputra C. (2010). High prevalence of bla(OXA)-23 in oligoclonal carbapenem-resistant *Acinetobacter baumannii* from Siriraj Hospital, Mahidol University, Bangkok, Thailand. Southeast Asian J Trop Med Public Health, 41, 625-35.

19 Villers D, Espaze E, Coste-Burel M, Giauffret F, Ninin E, Nicolas F, et al. (1998). Nosocomial *Acinetobacter baumannii* infections: microbiological and clinical epidemiology. Ann Intern Med, 129, 182-9.