Antibiotic Resistance Gene Expression of Multi-Drug Resistant Gram-Negative Bacteria Isolated in a Military Hospital of Vietnam

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Abstract

Hospital-acquired infection is one of the main causes for prolong treatment and risk of death. In this study, nine multidrug resistant strains were isolated from patient’s samples. The antimicrobial susceptibility testing was performed to confirm the strong resistance of nine strains with β-lactam, carbapenem and colistin. The expression of resistance genes was examined using reverse transcription PCR to identify 14 genes associated with drug resistance and the genotype matched the phenotype. Two carbapenem resistance genes (blaNDM-1 and blaKPC-2) and one colistin resistance gene (mcr-1.1) were found in this study, indicating a likely shared origin of these resistance genes and the need of understanding the processes of drug resistance development, transmission and spread.
Keywords
Hospital-Acquired Infection; Antibiotic Resistance; Resistance Gene Expression; Carbapenem; Colistin

Abbreviations
HAI: Hospital-Acquired Infection; XDR: Extensively Drug Resistant; MDR: Multiple Drug Resistance; PDR: Pan Drug Resistant; MLST: Multi-Locus Sequence Typing

Introduction
Hospital-Acquired Infection (HAI) is one of the top challenges and concerns in Vietnam as well as around the world. HAI can be considered as a disease caused by the medical care in the hospital, because the patient only infected after 48 hours stay in hospital \[1-17\]. HAI is also a difficult problem even in developed countries. Statistics show that the rate of HAI is about 5-15\% in developed countries and up to 14.8-19.1\% in developing countries \[18,19\]. In Vietnam, some previous researches showed the HAI proportion was about 23.4-29.5\% \[20-31\].

Factors causing infection can be caused by bacteria, viruses, fungi, etc. In which, factors due to bacteria account for the highest proportion with 92\%, fungi and others are less than 10\%. Bacterial infection can separate to two groups, such as the endogenous bacteria group infection that caused by the bacteria available in the patient's body (bacteria reside in the skin, mucous membranes of the nose, throat, or gastrointestinal tract) and exogenous bacteria are living outside the body in hospital environment \[32-35\].

Nosocomial infections affect bodily functions, psychology and in some cases impair quality of life. Opportunistic infections are also one of the leading causes of death. In addition, hospital infections increase the patient's hospital stay, cost more money for the treatment and also cause economic loss to society due to the inability of the patient to work during the hospital stay. The bacteria that cause opportunistic infections for patients have a high risk of infecting their caregivers, health care workers and other patients \[11,35\]. HAI also contributes to the creation of several antibiotic resistant bacteria. The central hospitals have a higher rate of infection than the baseline hospital. The extensive using of antibiotics causes to promote the growth of antibiotic-resistant bacteria \[4\]. In addition to the above factors, immunodeficiency patients such as cancer diseases, diabetes, HIV, etc., environmental factors of the hospital also increase the patient's infection \[5\]. HAI's caused by MDR and XDR Enterobacteriaceae present a clinical challenge because even the most effective antibiotics are no longer effective. Over time, the
emergence of resistant bacteria with any antibiotic and the spread in the community or in hospitals contributes to the dispersion of resistant strains into the environment [28]. Higher prevalence of MDR and XDR strains can prolong the treatment with more expensive medical interventions and risk of mortality [36,37]. In recent years, many studies have been indicated that the prevalence rate of isolation of MDR and XDR for all species within the Enterobacteriaceae family is high, approximately from 65.7% to 92.2% in hospital wards [28,38]. The undeniable role of the Enterobacteriaceae in the HAIs and their high antibiotics resistance, therefore, there are the infection control, surveillance and management of antibiotic use remain critically important to all clinical practitioners [28].

In this study, we focused on nine multidrug-resistant HAI that were detected and isolated in 103 Military Hospital with the goal of determining the multidrug resistance genotype though the molecular analysis and relating between the current situation of hospital infections and antibiotic resistance of common bacteria strains in hospital infection at Hospital 103.

**Methodology**

**Bacterial Strains**

Pathogenic bacteria strains were isolated from blood, pleural fluid, sputum, urine or wound specimens of inpatients. Identification and antimicrobial susceptibility testing of bacterial isolates were performed with the VITEK 2 system (bioMérieux, USA). The multidrug resistant isolations were stored in cryotubes and thawed and cultured on blood agar and BHI (Brain Heart Broth Infusion) broth for further study.

**RNA Extraction and cDNA Synthesis**

Total RNA was extracted in 1 ml of an antibiotic-free BHI broth by TRIzol Reagent (Invitrogen) according to RNA isolation protocol [11]. Total RNA samples were measured concentration on a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Thermo Scientific) and then 1mg total RNA was used for cDNA synthesis by RevertAid First Strand cDNA Synthesis Kit following the Manufacturer’s instructions.

**Gene Amplification and ABI3500 Sequencing**

PCR was performed with 50 μL reaction mix including: 1X Dream Taq Buffer, 10 mM dNTP, 10 pmol / μL per primer (the list of primer sequences in Table 1, 2U Dream Taq DNA polymerase and 40 - 100 ng / μl DNA template; the optimized thermal cycle: 95°C / 5 min;
(95°C / 30s; 55°C / 30s; 72°C / 1 min) x 30 cycles; 72°C / 10 min. The annealing temperature may be change to suit each specific primer [1,-7,9,14,15,19,23,26,31,32,42,44,46].

The amplifier products of 16S rRNA, MLST genes (adk, gdh, mdh, metA, ppk and gcl) and positive PCR products of 3 resistance genes (blaNDM, blaKPC and mcr1) were purified and sent to 1st BASE Company (Singapore) for sequencing using ABI3500 system (Applied Biosystems, Thermo Fisher Scientific, US) following the manufacturer's instructions.

16S rRNA phylogeny and MLST Analysis

The sequences of the 16S rRNA genes were alignment with the GenBank reference sequences. Alignment was performed by the MUSCLE algorithm on the MEGA6.0 software [13]. The Best Model Test tool was also used to determine the most suitable evolutionary model before building phylogenetic trees. Phylogenetic tree was built following the of Maximum Likelihood method [33]. The allele numbers for MLST gene of each E. coli isolates were compared to the MLST database using MLST 2.0 (Multi-Locus Sequence Typing) tool (Center for Genomic Epidemiology) and the Sequence Type (ST) was determined by the combination of all seven alleles.

Identification of Carbapenem and Colistin Resistance Genes

To confirm of carbapenem and colistin resistance genes that tested from the reverse transcription PCR, the focused genes sequence reading results were analyzed on BioEdit 7.0 software. Three gene sequence including FN396876, AY034847 and KP347127 were used as reference genes of blaNDM1, blaKPC2 and mcr1.1 gene, respectively.

Results

Antimicrobial Susceptibility

The samples were taken from patients in three different departments. The HAI positive findings were found in nine patient specimens, including four blood samples, two urine samples, one pleural fluid, one sputum sample and a wound sample. The VITEK 2 system identified nine HAI strains isolated from patient material (Table 2).

Antibiotic susceptibility testing was performed on all nine strains to validate their multidrug resistance. They were all resistant to trimethoprim-sulfamethoxazole, ciprofloxacin and norfloxacin. Other antibiotics with lower resistance were cefotaxime (7/9; 77.78%), gentamicin...
(7/9; 77.78%), ampicillin (7/9; 77.78%), ceftazidime (6/9; 66.67%), imipenem (6/9; 66.67%), ertapenem (5/9; 55.56%), meropenem (5/9; 55.56%), fosfomycin (5/9; 55.56%), amoxicillin/clavulanate (4/9; 44.44%), piperacillin/tazobactam (6/9; 66.67%), cefepime (4/9; 44.44%), colistin (5/9; 55.56%), amikacin (3/9; 33.33%) and only one strain (PM13-009N) showed the resistance with nitrofurantoin.

16S rRNA Phylogeny and MLST Analysis

Using the 16S rRNA gene sequences and the GenBank reference sequences, the optimal substitution model was HKY+G+I and a phylogenetic tree was constructed (Fig. 1). The results were identical to VITEK 2's results, with more than 98 per cent bootstrap support. Furthermore, the data revealed that E. coli isolates were divided into two categories. According to the MLST database, they are of the following sequence types: ST457 (EC17-008M), ST405 (EC13-051M), ST354 (EC16-331M), ST46 (EC16-401M) and ST744 (EC16-510M) (Table 4) (Fig. 2).

Figure 1: 16sRNA phylogenetic tree was built following the of maximum likelihood method.
**Antibiotic Resistance Genes and β-lactam Resistance Gene Group**

The results in Table 3 indicated that 14 antibiotic resistance gene families were discovered in nine study samples using the reverse transcription PCR technique. These genes were resistant to 12 different antibiotic classes. Antimicrobial resistance genes were expressed in the isolated isolates (n = 9) against blaTEM (6/9; 66.67%), SulII (6/9; 66.67%), blaCTX-M (5/9; 55.56%), blaNDM (5/9; 55.56%), SulI (5/9; 55.56%), aac(6')-Ib-cr (4/9; 44.44%), blaCMY (3/9; 33.33%), mcr1 (3/9; 33.33%), blaDHA (2/9; 22.22%), qnrB (2/9; 22.22%) and only one strains expressed blaKPC (EC17-008M), blaSHV (EC16-522D), qepA (PM13-009N) and rmtB (CF16-367M).

**Figure 2:** Reverse transcription PCR results. 16S rRNA was used as housekeeping gene.
Sanger Gene Sequencing

The emergence of one carbapenem resistance gene family (blaNDM and blaKPC) and one colistin resistance gene family (mcr-1.1) was indicated in the reverse transcription PCR screening findings (Table 4). Sanger sequencing was used to validate the presence of the resistance gene member.

The results also revealed that only the EC17-008M strain possesses the blaKPC-2 gene, whereas three E. coli strains (EC16-331M, EC16-401M and EC16-510M) possessed the mcr-1.1 gene. The blaNDM-1 gene was found in E. coli strains EC13-051M and others (Table 4). The sequencing findings revealed that the sample sequence genes were 100 per cent homologous to the reference sequence genes, confirming that the results were valid.

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<th>Product Size (bp)</th>
<th>Ref</th>
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**blaTEM_R**  CGTTCTCCATAGTGGCTGAC  
**blaSHV_F**  ATGCCTTAATACGCTGTTG  50   753  [31]
**blaSHV_R**  TGCTTTGTTATTCGCGCCAA  
**blaCMY_F**  TGGCCAGAAGCTGACCCGA  55   462  [44]
**blaCMY_R**  TTTCTCCTGAACGTGGCCTGCG  
**qnrB_F**  CCTGAGCGCAGCTGAATTT  53   409  [7]
**qnrB_R**  GTTTGCTGCTGCCAGTGCGA  
**qepA_F**  CGGCAGCGTGTGGCTGAGTCTTT  55   548  [26]
**qepA_R**  CGACAGGCGGCGAGCGAGGATGC  
**sulI_F**  CGGCATCGTCAACATAACCT  60   433  [6]
**sulI_R**  GCGATCGCAGTGAAAGTCCG  
**sulII_F**  CGGCATCGTCAACATAACCT  55   721  [23]
**sulII_R**  GCGATCGCAGTGAAAGTCCG  
**aac(6')-Ib-cr-F**  TTGCAGATGCTCTATAGTGGCTA  55   482  [14]
**aac(6')-Ib-cr-R**  CTCGAATGCTGGCGCTTGT  
**rmtB-F**  GCTTTCTGCGGGCGATGTAA  55   173  [5]
**rmtB-R**  ATGCAATGCGGGCGCTGAT  
**mcr1-F**  TGCCGTAATATACCCCCGCT  50   1726  [46]
**mcr1-R**  CCCACCCGCCCATATACGAA  

**Table 1:** Primers list.

| ID  | Department    | Sample   | Species | Collection year | Gender | AMP | ASC | TET | CTX | CIL | CLI | FEP | IMP | APM | ASK | GRI | CHF | NOC | FOF | PCH | NAT | VCT | STX | VIT |
|-----|---------------|----------|---------|----------------|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| PM11-0422 | Internal medicine | Urine | P. aeruginosa | 2013 | M | +16 | +16 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 |
| EVT1-4123 | Surgery | Blood | Z. colli | 2014 | M | +15 | +16 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 |
| EVT1-4124 | Surgery | Urine | Z. colli | 2014 | M | +15 | +16 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 |
| MRRP-4176 | Surgical | Blood | M. morganii | 2016 | M | +16 | +16 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 |
| RRRP-4177 | Infectious Diseases | Blood | Z. colli | 2016 | M | +16 | +16 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 |
| RRRP-4178 | Resuscitation | Blood | Z. colli | 2016 | M | +16 | +16 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 | +6 |

**Table 2:** The information and antibiotic susceptibility testing of nine isolates.


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**Table 3:** Antibiotic resistance genes screening results.

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**Table 4:** Sanger gene sequencing results.

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<th>ID</th>
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<th>blaKPC-2 (AY034847)</th>
<th>mcr-1.1 (KP347127)</th>
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**Discussion**

The majority of the HAI in our research were connected to surgery, which is perfectly consistent with earlier findings where blood and surgical wound infections were regarded the

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main sources of HAI [16]. This demonstrates that postoperative infection is a huge challenge; these issues cause the wound to heal more slowly, the patient may require another surgery and the patient must be treated with antibiotics. This requires a longer therapy and raises the related expenditures [18]. Furthermore, the respiratory tract was found; the source of the respiratory HAI might be directly tied to the hospital's ventilation and air conditioning system [10]. Other investigations have found that MDR and XDR enterobacterial isolates are more prevalent in wound infections, particularly open and severe wounds and Burn critical care units than in other wards [2,3,28]. The problem is that these gram-negative bacteria strains frequently cause higher morbidity and death [29].

Antibiotic resistance in bacterial strains has been observed in a variety of antibiotics. The most prevalent is resistance to -lactam antibiotics such as ampicillin, amoxicillin/clavulanate, piperacillin/tazobactam, cefotaxime, ceftazidime and notably 4th generation antibiotics like cefepime [21]. Previous research [22,27,34] demonstrated high resistance to the antibiotics amikacin, gentamicin, ciprofloxacin, norfloxacin, fosfomycin, nitrofurantoin and trimethoprim-sulphamethoxazole. Furthermore, we discovered high resistance to carbapenem families such as ertapenem, imipenem, meropenem and polymyxin (colistin). In contrast, HAI isolates were shown to be very sensitive to the antibiotic nitrofurantoin. However, this antibiotic is highly toxic and is mostly used to treat urinary infections [30]. This implies a tough period for antibiotic-assisted HAI therapy.

Previous research has shown hydrolysis of the -lactam rings as the most prevalent antibiotic resistance mechanism for antibiotics with reported strong resistance; this might be regarded an explanation for isolates' significant resistance to ampicillin and other -lactam antibiotics. blaCTX-M, blaTEM and blaCMY were the most prevalent Extended Spectrum Beta-Lactamase (ESBL) members. Other bacterial resistance mechanisms may include antibiotic enzyme inactivation, modification of the target of action of antibiotics and decreased permeability of the bacterial membrane to the antibiotic and channels, which was also taken into account in our investigation [25]. The widespread presence of the sul, gene family or qnr and aac(6) gene family (particularly the aac(6')-Ib-cr gene) explains the high resistance to folate pathway antagonists (trimethoprim-sulphamethoxazole) and fluoroquinolone group (ciprofloxacin, norfloxacin) respectively. However, because of the limitation of tested resistance gene members, the resistance phenotype of EC13-051M and EC16-510M has not been explained yet [20,41].

While multi-drug resistant infections, particularly ESBL resistant strains, were becoming more widespread, the usage of carbapenem, an effective broad-spectrum antibiotic group, was increasing [24]. This resulted in a rise in carbapenem resistance, which was mostly mediated by carbapenemase expression [14,40]. The most common carbapenemase enzymes were NDM, KPC and OXA enzymes, which were encoded by the blaNDM, blaKPC and blaOXA gene
families, respectively [12]. The ultimate option for these instances was a polymyxin antibiotic containing colistin. Resistance was previously demonstrated in strains containing the mcr gene [8]. Both carbapenem (including ertapenem, imipenem and meropenem) and colistin resistant isolates were isolated in this investigation. We confirmed the expression of one of two genes as blaNDM-1 (5/6 isolates), blaKPC-2 (1/6 isolates) in six carbapenem resistant isolates and mcr-1.1 gene in all colistin resistance isolates (3/3 isolates), with the exception of MM16-367M strain due to intrinsic colistin resistance of M. morgani [39]. The presence of the same gene in multiple species and E. coli ST raised the possibility of a similar origin and horizontal transfer of these resistance genes. However, due to the large number of subjects, reaching a conclusion was still difficult.

**Conclusion**

Obviously, antibiotic resistance and other methods will continue to be a problem until the deeper knowledge of the processes of drug resistance development, transmission and spreading is obtained. Doctors can make more educated decisions about prescribing and utilizing antibiotics using this information. Furthermore, antibiotic resistance is an unavoidable result of incorrect antibiotic usage. Finding novel candidates with strong activity against both Gram-positive and Gram-negative infections is critical in the near future if we wish to reduce or reverse the current development of resistance.

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**Conflicts of Interest**

The authors declare that have no competing interest and not any conflict of interest.

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