学 位 論 文 の 要 旨

論文題目 **Molecular Pharmacological Studies on Multidrug-Resistant Bacteria: Analysis of Antimicrobial Resistance Mechanisms and Evaluation of Antimicrobial and Antivirulence Activities of Novel Plant Extracts**

（多剤耐性菌の分子薬理学的解析：抗菌剤耐性化機構の解析と新規植物抽出物の抗菌・抗病原性活性の評価）

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Since the first use of antibiotics in 1940s for the treatment of bacterial infection, they have been central in the modern healthcare. Antibiotics also gained a great interest, as their role have been extended from treating bacterial infections to prevent the infections, protect immunocompromised patients, and as growth promoters for animals. However, few years after the first use of antibiotics, resistant bacteria have been emerged and received worldwide attention. This problem become worse in the last few years because no new antibiotics in the pipeline. For instance, since the early 1960s, only four new classes of antibiotics have become commercially available. Therefore, this study was designated to develop strategies to overcome the antimicrobial resistance problem. First, through analysis, at the molecular level, the antimicrobial resistance mechanisms in bacteria. Second, through investigation the antimicrobial, antivirulence, and mode of action of novel plant extracts as a new effective approach for treatment of such bacteria.

In the first part of this study, I analyzed different antimicrobial resistance mechanisms in Gram-negative bacteria. First, I analyzed carbapenemase production in Gram-negative bacteria isolated from hospitalized patients in Egypt in 2014. A total of 128 Gram-negative isolates were tested phenotypically and genotypically for carbapenemase production. The most striking finding in this study that, 50.8% of the isolates harbored at least one carbapenemase. The most prevalent carbapenemases are OXA-48 and NDM-1, were being detected in 49.2%, and 47.7% of

carbapenemase-positive isolates, respectively. VIM was detected in only 26.2% of carbapenemase-positive isolates. This study also demonstrated high level of coexistence of different carbapenemases, being detected in 21.5% of carbapenemase-positive isolates. PFGE of carbapenemase-producing *K. pneumoniae* isolates revealed no clonal relationship among the isolates. This result clearly demonstrated that the genes coding for carbapenemase production are not restricted to specific clone(s). The high prevalence of carbapenemase-encoding genes and coexistence of different carbapenemases among Gram-negative bacteria in Egypt in this study is extremely worrisome, as carbapenems remained the last resort for treatment of multidrug-resistant bacteria. Thus, therapeutic options will be extensively limited and inevitably leading to higher mortality rates associated with bacterial infections.

Interestingly, during our screening of carbapenemase-producing Gram-negative bacteria, I discovered the co-occurrence of two highly resistant *K. pneumoniae* isolates in a six month-old infant, one isolate harbored *bla*$_{\text{NDM-4}}$ and the other one harbored *bla*$_{\text{NDM-5}}$. These finding is of great interest, as this co-infection may lead to therapeutic failure and death. For this reason, I fully characterize both bacterial isolates. Both isolates were phenotypically resistant to all the antimicrobials tested and harbored different antimicrobial resistance genes. Both isolates belonged to the same multilocus sequence typing (ST45), while they were genetically different by ERIC-PCR. The genetic environment of both genes was estimated and confirmed to be similar to that previously described in most NDM-1-positive enterobacterial isolates. Conjugation experiment, plasmid analysis and southern blotting confirmed that the *bla*$_{\text{NDM-4}}$ and *bla*$_{\text{NDM-5}}$ genes were located on a two different non-conjugative plasmids > 93 kb in size. Finally, PCR-based replicon typing method, revealed that *bla*$_{\text{NDM-4}}$-positive plasmid belonged to the L/M incompatibility group, while *bla*$_{\text{NDM-5}}$-positive plasmid was un-typeable. In conclusion, this study encouraged the relevant medical authorities to consider the prevalence of NDM enzymes within

the hospitals and the community.

Although, investigation of the molecular mechanisms of carbapenemase-producing Gram-negative bacteria have special importance, the investigation of other mechanisms is also imperative. Therefore, I also investigated the molecular level of antimicrobial resistance to different categories of antimicrobial resistance determinants including integrons, extended spectrum ß-lactamases (ESBL), AmpC ß-lactamases and plasmid mediated quinolone resistance genes among Gram-negative bacterial strains. The results were so surprising, where I recorded high prevalence of nearly all the antimicrobial resistance gene determinants in Egypt. A total of 128 non-duplicate Gram-negative isolates, which were tested previously for carbapenemase-encoding genes used in this study. The results showed that, class 1 integrons were detected in 51.6% (66/128) of the isolates, with six isolates harbored two different gene cassette profiles. Class 2 integrons were detected in 2.3% (3/128) of the isolates. ESBL-encoding genes were detected in 73.4% (94/128) of the bacterial isolates, with *bla*$_{\text{CTX-M-15}}$ (64.8%) was the most prevalent, followed by *bla*$_{\text{SHV-}}$ (46%). The AmpC, *bla*$_{\text{CMY-}}$, was detected in 8.6% (11/128) bacterial isolates with *bla*$_{\text{CMY-42}}$ was the most prevalent. The plasmid-mediated quinolone resistance gene, *qnr*, was detected in 58.6% (75/128) of the tested isolates and the *qnrS* was the most prevalent. The results presented in this study are striking, therefore this study renewed the interest in developing new antimicrobial agents, applying strict antibiotic supervision and effective infection control measures could help to overcome the antimicrobial resistance problem.

The latest antimicrobial resistance mechanism comes to light is the discovering of the plasmid-mediated colistin resistance, encoded by the *mcr-1* gene that discovered few months ago in China. These finding warns loudly, it confirmed the horizontal transmission of colistin resistance, as well as vertical transmission, taking in account that colistin used as a last-resort for treatment of infections caused by carbapenemase-producing Enterobacteriaceae. Therefore, I

investigated the prevalence such resistance mechanism in 431 non-duplicate Gram-negative isolates collected from different sources and different countries. The *mcr-1* gene was detected in only one *E. coli* isolate (M165), which showed 100% similarity to the first discovered Chinese allele. *E. coli* M165 was isolated from a cow suffering from sub-clinical mastitis in November, 2014 from Egypt and showed phenotypic resistance to different antibiotics including colistin and harboured different antibiotics resistance genes. Multilocus sequence typing (MLST) revealed that *E. coli* M165 belonged to ST10. Conjugation experiment, plasmid analysis and southern blotting confirmed that the *mcr-1* gene was located on a non-conjugative plasmid > 93 kb in size. Our results confirmed the global emergence of such resistance and renewed the interest for the restriction of colistin use in agriculture and veterinary medicine fields

It is clear from the first part in this study that, the antimicrobial resistance, especially in Egypt, is a serious problem. Not only due to, the detection of high levels of antimicrobial resistant bacteria, but also due to the emergence of new antimicrobial resistance mechanisms. Therefore, in the second part of this study I tried to develop new natural antibacterial agents to overcome this problem. First, I estimated the antimicrobial and antivirulence activities of blueberry, raspberry and strawberry aqueous extracts. The extracts were dissolved to 20% (w/v) in distilled deionized water, filter sterilized before their testing. The total contents of phenolics, flavonoids, and proanthocyanidins in the extracts were measured with colorimetric methods, and were highest in strawberry, followed by raspberry and then blueberry. The minimum inhibitory concentrations and minimum bactericidal concentrations of the extracts were determined before and after neutralization to pH 7.03 ± 0.15 on 13 pathogenic bacteria. The antimicrobial assay revealed that, both Gram-positive and Gram-negative pathogenic bacteria were selectively inhibited by the non-neutralized berries, with blueberry was the best inhibitor, and *Vibrio* and *Listeria* were the most sensitive bacteria. After neutralization, only *Vibrio* and *Listeria* were

affected by blueberry, whereas the antimicrobial activities of raspberry and strawberry were eliminated. The antivirulence activities of the three berries were tested by studying the effects of sub-bactericidal concentrations of the three berry extracts on virulence gene expression in *V. cholerae*. The results revealed that the three berry effectively repressed the transcription of the *tcpA* gene. *ctxA* gene transcription was only repressed by raspberry, while the three berry extracts did not affect the transcription of *toxT*. The results presented in this study clearly demonstrate that, the three berry extracts exert potent antimicrobial effects and inhibit the expression of the virulence factors of *V. cholerae* that could be used effectively to combat bacterial infections.

In another study I also evaluated the antibacterial activities and mode of actions of commercially available *Aristotelia chilensis* (Maqui berry) and *Punica granatum* (Pomegranate) extracts against three multidrug-resistant bacteria causing severe infectious diseases, namely methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, and one drug-sensitive strain of *Escherichia coli.* The extracts were dissolved in ethanol, filter sterilized, and diluted in sterilized distilled deionized water to a concentration of 10 mg polyphenol/mL (with a 5% final concentration of ethanol). These extracts at a range of concentrations, were incubated with bacteria for up to 24 h, then serial dilutions were plated and colony-forming units were enumerated. The mechanism of action of both extracts was studied by testing their effects at high concentration on MRSA and *A. baumannii* by Transmission electron microscopy. Our results illustrated that both extracts induced broad spectrum antibacterial activity, decreasing bacterial cell counts to undetectable levels within 24 h. Transmission electron microscopy showed critical cell wall alterations (irregular shaped outer membrane displaying deformities and localized disintegrations), as well as intracellular changes (condensed cytoplasm containing mesosome-like structures and electron dense granules deposited inside the cells), in

the treated organisms. In conclusion, our findings demonstrated that both extracts induced broad-spectrum antibacterial activities based on the action of several of their constituents on different cellular targets. Both extracts may be a promising new natural antimicrobial agent to overcome drug resistance in bacteria.