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学位授与の日付	平成２９年３月３１日
論文名	Prevalence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in Vietnamese healthy adults and impact of cephem antibiotics on mice intestinal colonization and emergence of multi-drug resistance （基質特異性拡張型βラクタマーゼ[ESBL]産生大腸菌のベトナムの成人における保菌状況とセフェム系抗菌薬投与がESBL 産生大腸菌のマウス腸管内定着と多剤耐性化に及ぼす影響）
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論文要旨

Introduction

β-lactam antibiotics are among the most important groups of antimicrobial agents, broadly used as human and veterinary medicine, and as prophylactics in animal food productions. However, resistance against β-lactam antibiotics has already been reported worldwide, and poses a serious challenge to treatment options for infections. The most common mechanism of resistance to β-lactam antibiotics involves the production of β-lactamases, the encoded genes of which lie on chromosome or plasmid. Extended-spectrum β-lactamases (ESBLs) can hydrolyze most β-lactam antibiotics such as penicillins, cephalosporins, and monobactams. Most ESBLs can be broadly divided into three groups: TEM, SHV, and CTX-M. Among them the CTX-M β-lactamases are the most widespread and frequently associated with *Escherichia coli* infections. The mobilization and dissemination of ESBL-genes, especially $bla_{\text{CTX-M}}$ genes, are mediated by conjugative plasmids and mobile genetic elements.

The emergence of multidrug resistant (MDR) bacteria including ESBL-producing *E. coli* has been increasingly recognized as a major threat for health worldwide. A fecal carriage of ESBL-producing *E. coli* is considered as the major source of ESBLs in both hospital and community settings. Like many other developing countries, in Vietnam, an alarming increase in the infection rate of MDR pathogens, including ESBL-producing *E. coli*, has been reported. Nonetheless, only limited comprehensive information is available

for the antimicrobial resistant *E. coli* strains isolated from healthy humans, which could be probable reservoir of the microbial populations acting as reservoirs for the virulence and antimicrobial resistance related genes, including ESBL-producing *E. coli* in Vietnam.

The indiscriminate use of antimicrobial agents can induce the selection pressure and lead to the emergence of the MDR bacteria. *In vitro* studies demonstrated that at sub-inhibitory concentrations some antimicrobial agents can produce genetic variability, including mutations and recombination, in bacteria. The impacts of antimicrobial agents to selectively promoting the colonization of ESBL-producing bacteria in an animal intestine have been considered important in recent studies. Until recently, only limited scientific studies have been carried out to examine the effects of antimicrobials, namely the third generation drugs, on the intestinal colonization of pathogenic *E. coli*. However, there is a lack in detail information regarding the impacts of antimicrobial agents influencing the intestinal colonization, changes in antimicrobial susceptibility profiles, and producing genetic variations, of colonized ESBL-producing *E. coli*, particularly in an animal model.

In this study, *E. coli* strains were attempted to be isolated from healthy adults living in Ho Chi Minh City, Vietnam and the isolates were characterized for their antimicrobial resistance, ESBL and virulence gene profiles. Furthermore, a mouse model of the intestinal colonization of ESBL-producing *E. coli* was established to examine the impacts of introducing cefoperazone (CFP), a third generation cephalosporin, on bacterial colonization, susceptibility to various antimicrobial agents, and to check CFP-mediated genetic changes in bacterial populations. Additionally, comparative whole-genome analysis between the parental strain and the representative daughter strains obtained from CFP treated mice were performed to understand the effect of the CFP on the genetic changes in the ESBL-producing *E. coli* genome

Chapter 1. Isolation of *E. coli* from healthy adults in Ho Chi Minh City, Vietnam, and characterization of their resistance to antimicrobials, including ESBL, and virulence genes profile

A total of 103 *E. coli* isolates were screened through identification at species level from 103 stool specimens of healthy adults in Ho Chi Minh City, Vietnam from March to November 2013. The antimicrobial susceptibility profile of the identified *E. coli* strains was examined. Most of the strains were resistant to antimicrobials, e.g., streptomycin (STR; 80.6%), tetracycline (67.0%), ampicillin (AMP; 65.0%), sulfamethoxazole-trimethoprim (SXT; 48.5%) and nalidixic acid (NAL; 43.7%), chloramphenicol (CHL; 34.0%), cefotaxime (CTX; 15.5%), ciprofloxacin (CIP; 15.5%), kanamycin (KAN; 12.6%), ceftazidime (CAZ; 10.7%), fosfomycin (4.9%), and gentamicin (GEN; 2.9%). However, all these *E. coli* strains were susceptible to imipenem. Interestingly, out of 103 strains, 74 (71.8%) and 43 (41.7%) showed resistance to more than 3 and 5 classes of antimicrobials, respectively. Furthermore, 10 *E. coli* strains were ESBL-producers and positive for $bla_{\text{CTX-M}}$ genes while five were additionally positive for bla_{TEM} genes. S1-nuclease treated pulsed-field gel electrophoresis (PFGE) analysis revealed that 7 and 3 out of these 10 *E. coli* strains carried the $bla_{\text{CTX-M}}$ genes on their large plasmid and chromosome, respectively. Virulence genes associated with diarrheagenic *E. coli* such as *astA*, EAF, *eaeA*, *elt*, and *eagg* were also detected in the ESBL-producing *E. coli*, and also some other strains showing antimicrobial resistance. These data suggest that *E. coli* strains in healthy adults, residing in Ho Chi Minh City, Vietnam, could act as reservoirs of AMR genes, including ESBL genes, and might contribute to the increased incidences of MDR infections in human.

Chapter 2: Effects of orally administered CFP on intestinal colonization of the ESBL-producing *E. coli* in a mouse model

A mouse model examining intestinal colonization of the ESBL-producing E. coli strains isolated from healthy human was established. Among 12 tested E. coli strains,

ESBL-producing E. coli strain KC90 showed better intestinal colonization and was selected for further studies. The effect of CFP on the intestinal colonization, and susceptibility to antimicrobials of the ESBL-producing E. coli strain, along with the concurring genetic changes in the bacterium were studied. Four week-old male ICR mice (n = 7-8, for each group) were used and they received either sterile drinking water without (control group) or with CFP (low dose group; 50 μg/ml, and high dose group; 500 μg/ml) ad libitum from 3 days, prior to the oral administration of approximately 107 colony forming units (CFU) of the strain KC90 (day 0), till 60 days post infection. Prolonged shedding of E. coli in feces indicated intestinal colonization of the bacteria in mice. Significant differences ($P<0.001$) were observed in the number of ESBL-producing E. coli among three groups. In the control group, the mice were transiently colonized with the test strain for only 5 days. In the low dose group, colonization of the ESBL-producing E. coli persisted reaching from 104 to 105 CFU/g of feces in all mice. The bacterial colonization efficiency was highest, persisting from 108 to 109 CFU/g for 60 days in all mice, in the high dose group. These data suggest that CFP enhanced colonization of ESBL-producing E. coli in mice in a dose-dependent manner.

The genetic similarity of the cefotaxime resistant E. coli isolated from mice feces (presumptive daughter strains of E. coli strain KC90) was analyzed by PFGE with XbaI digestion. None of the analyzed strains from the control group showed changes in genetic fingerprints (0/12 isolates). However, 14.8% (9/61) strains from the low dose group showed one band difference, in comparison to the parental E. coli strain KC90, with differentiation into 3 pulsotypes. Interestingly, 75.6% (90/119) strains in high dose group showed extensive genetic changes with differentiation into 48 pulsotypes. These results imply that introduction of CFP induces genetic diversification, as observed in PFGE fingerprinting patterns of genomic DNA of the daughter E. coli strains isolated from mice feces, in a dose-dependent manner.

Additionally, antimicrobial susceptibility profiles of the daughter E. coli strains revealed that the daughter strains isolated from the low dose group had same MDR phenotype with the parental strain. However, a portion of the daughter E. coli strains isolated from the high dose group exhibited additional resistance to CAZ, GEN, KAN, STR, and /or NAL. By determination of minimum inhibitory concentration (MIC) of 4 representative daughter strains (B2, B3, B4, and B5) isolated from the high dose group, an increase of at least 4-fold MICs to the third generation cephalosporin compared to the parental strain was observed. Moreover, at least 8-fold increase in MIC for GEN and KAN was observed for strains B2 and B5. However, strain B5 unexpectedly became susceptible to SXT and CHL. These findings suggest that use of CFP increased MIC of not only the β-lactams but also other classes of antimicrobials among the ESBL-producing E. coli strains in mice.

Plasmid profiles of the parental strain E. coli KC90 and its representative daughter strains (B2-B5) were determined by PFGE with S1-nuclease digestion. The results revealed a high genetic variation in the plasmid profiles of the daughter strains. Moreover, the Southern hybridization of S1-PFGE for detection of antimicrobial resistance (AMR) genes showed large variation in the location of AMR genes in these daughter E. coli strains. This data clearly indicate the occurrence of various in vivo recombination events in the AMR-encoding plasmids under CFP pressure.

Chapter 3: Whole genome sequence analysis of the CFP induced extensive genetic changes in the ESBL-producing *E. coli in vivo*

To elucidate the causal mechanism of CFP induced alterations in DNA fingerprints, increase of MIC, and plasmid profile changes in the ESBL-producing *E. coli*, whole genome sequence of the parental strain *E. coli* KC90 (B1) and the representative daughter *E. coli* strains (B2-B5) from high dose group was analyzed.

Chromosomal DNA of the parental strain was determined to be 4,66,3738 bp in size, and harbored two plasmids of 136,569 bp (pKC90-L) and 61,067 bp (pKC90-S). The large plasmid possessed ~36.6-kb unique module, encoding genes for AMR to β-lactam

antibiotics (*bla*$_{CTX-M-14}$, *bla*$_{TEM}$), trimethoprim (*dfrA12*), sulfonamide (*sul3*), chloramphenicol (*clmA1*), aminoglycosides (*aac(3)-II*; *aadA1*; *aadA2*), and quinolone (*qnrS1*), and mobile-element proteins such as transposases and resolvases, while the small plasmid was identified as IncFII conjugative plasmid, without any antimicrobial resistance genes.

Comparative sequence analysis of the chromosomes between the parental and daughter strains revealed a difference in size of their genomes. The changes in DNA fingerprinting could be related to the observed deletion and or insertion of certain DNA segments in the chromosomal DNA. However, correlation between the deleted DNA segments and AMR determinants was not observed. Notably, one novel 14,612 bp region, encoding AMR genes (*bla*$_{CTX-M-14,}$ *qnr*S1) and mobile-genetic elements (IS*Ecp*1, IS*26*), was integrated into the chromosome of strain B3. This inserted region had a high sequence identity with part of the plasmid pKC90-L, suggesting that the mobilization of AMR genes via insertion sequences were induced under the pressure of CFP.

Additionally, comparative plasmid analyses between the parental and daughter strains revealed a high variation in plasmid size and the number of AMR genes among the daughter strains. This diversity could be due to the insertion, deletion, and recombination events. A region of ~39.8 kb, encoding tail proteins, fimbrial proteins, transposase, AMR determinants [*aac(3)-II*, *aadA1*, *aadA2*, *dfrA12*, *sul3*, and *clmA1*], found in the parental B3 strain, was not detected but a ~12.6 kb region carrying AMR genes (*qnrS1*, *bla*$_{CTX-M-14}$) and mobile genetic elements (IS26, IS*Ecp*1, IS903D), were present in two copies in daughter strain B3 (pB3-L). Similarly, a parental ~35.7 kb region, which harbored AMR determinants (*aadA1*, *aadA2*, *dfrA12*, *sul3*, and *clmA1*), was absent in the large plasmid, but a ~27.5 kb region with three copies of *qnrS1*, *bla*$_{CTX-M-14}$ was detected in strain B5 (pB5-L). On the other hand, the large plasmid in the daughter strains of B2 (pB2-L) and B4 (pB4-L) exhibited 100% nucleotide sequence identity with pKC90-L, and additionally, a ~16.1 kb region comprising the AMR determinants [*qnrS1*, *bla*$_{CTX-M-14}$, and *aac(3)-II*] and mobile genetic elements (IS26, IS*Ecp*1, IS903D) were inserted. Notably, the insertion region of all large plasmid in the daughter *E. coli* strains were flanked by insertion sequence IS*26*, suggesting that IS*26* could be involved in the mobilization of the resistance cassettes. Interestingly, AMR cassette [*aac(3)-II*, *aadA1*, *aadA2*, *dfrA12*, *sul3*, and *clmA1*] and mobile genetic elements from plasmid pKC90-L were observed to be introduced into a small conjugative plasmid in case of *E. coli* strain B3, resulting in the generation of new IncFII-conjugative plasmid (pB3-S). This recombination event is important because the conjugative IncFII plasmids are commonly involved in the spread of MDR determinants including ESBL genes.

Conclusions

This study illustrates that E. coli strains from healthy carriage in Ho Chi Minh City, Vietnam could act as potential reservoirs for AMR and virulence genes, and facilitate the dissemination of plasmid-mediated ESBL genes. An animal model for studying the effect of CFP on intestinal colonization of ESBL-producing E. coli was established in the present study. CFP enhanced colonization of ESBL-producing E. coli in mice in a dose-dependent manner. In addition, genetic changes of ESBL-producing E. coli were induced under the pressure of CFP. Comparative whole genome analysis revealed that CFP might stimulate recombination hotspots in the genome of the ESBL-producing E. coli strains, with changes in genetic regions responsible for recombination events, maintenance, and dissemination of antimicrobial determinants. Thus, this study demonstrates that antimicrobials can play a vital selective role contributing to not only the mobility of resistance genes, related to the dose of antimicrobial usage, but also other resistant genes, resulting in genetic divergence and multi-drug resistance. It can be recommended that the use of antimicrobial agents should be reduced not only for animals but also humans to prevent the alarming emergence of MDR strains.

審査結果の要旨

薬剤耐性菌の出現は、開発途上国のみならず先進国を含め世界的な脅威となっている。医療現場を始め畜水産現場における不適切な抗菌薬の使用が多剤耐性菌を生み出す温床となっていると考えられている。抗菌薬の中でもβラクタム系薬剤は医療現場で最も高頻度に使用されており、βラクタム系抗菌薬に対する耐性菌は細菌感染症の治療を困難にするなど問題である。近年、基質特異性拡張型βラクタマーゼ（ESBL）産生大腸菌が出現、拡散し医療現場において大きな問題となっている。

ESBL は、従来のβラクタマーゼ遺伝子が突然変異により分解可能な薬剤の種類を拡げ、第３世代セフェム系をも分解できるようになったβラクタマーゼのことである。ESBL をコードする遺伝子は大きく分けて TEM、SHV 及び CTX-M の３種類が知られている。1990 年代頃から第 3 世代セフェム系抗菌薬が多用されるようになり、過去 20 年間は CTX-M 型の ESBL 産生大腸菌が世界的に流行している。大腸菌はヒトをはじめ温血動物の腸内細菌叢を構成する１菌種であり、ESBL 遺伝子は多くの場合プラスミド上にコードされている。それゆえ、ESBL 遺伝子は菌から菌へと水平伝播し、ESBL 産生大腸菌は家畜や食品からヒトへと拡散する。それゆえ、健常人を含めヒトが容易に保菌者となることが問題視されている。

抗菌薬の使用と耐性菌出現の関係を解析する動物モデルは構築されておらず、不適切な抗菌薬使用がどのように耐性化に繋がるかの詳細な機構は明らかとなっていない。また、ベトナムでの健常者における薬剤耐性菌、特に ESBL 産生大腸菌の保有状況は十分に明らかとなっていない。本研究では、ベトナム、ホーチミンシティーの健康成人における ESBL を含む多剤耐性大腸菌の保菌状況を調べ、さらにマウスモデルを構築しセフェム系抗菌薬投与がどのように高度耐性化、多剤耐性化に影響を与えるかを調べることを目的とした。

第１章では、ベトナム南部の主要都市であるホーチミンシティーの 103 人の健康成人の糞便から大腸菌を分離し ESBL を含む 13 薬剤に対する薬剤感受性を解析した。その結果、99 株（96%）が１つ以上の薬剤に、74 株（72%）が異なる３クラス以上の薬剤に、43 株（42%）が異なる５クラス以上の薬剤に耐性を示した。さらに、10 株が CTX-M 型単独あるいは TEM 型との両陽性の ESBL 産生大腸菌で、3 株は ESBL 遺伝子が染色体上に、7 株はプラスミド上に存在することを明らかとした。また、多剤耐性を示した 18 株は何らかの下痢原性大腸菌の病原遺伝子を保持していることを明らかとした。

第 2 章では、抗菌薬投与がマウスへの定着、高度耐性化，多剤耐性化にどのような影響を及ぼすかを解析する為のマウスモデルを構築した。その結果、投与するセフェム系薬の濃度依存的にマウスへの定着菌数，定着期間が増大し、マウス糞便か

ら回収した ESBL 産生大腸菌が高度耐性化、多剤耐性化していること、また、PFGE によるDNAフィンガープリントも抗菌薬の濃度依存的に変異の割合が高くなることを明らかとした。高濃度のセフェム系薬投与マウスから回収した 4 株について、プラスミドプロファイル、薬剤耐性遺伝子の局在を解析した結果、プラスミドの大きさ、薬剤耐性遺伝子の局在も変化していることを明らかとした。

第 3 章では、マウスへ投与した親株とマウス糞便から回収した先の 4 株について全ゲノム解析を行い、高度耐性化、多剤耐性化の要因について解析した。その結果、トランスポゾン上にコードされた ESBL を含む薬剤耐性遺伝子カセットのコピー数の増加が高度耐性化、多剤耐性化に関わっていたことがわかった。さらに興味深いことに、マウスから回収した ESBL 産生大腸菌が新たな耐性能を獲得したが、これらの耐性遺伝子は既に親株に存在しており、転写，翻訳レベルで耐性化に関わっていた可能性が考えられた。

以上の結果は、ベトナムホーチミンシティーの健常成人が ESBL 産生大腸菌のリザーバーとなっている可能性を示し、抗菌薬投与が薬剤耐性菌出現に及ぼす影響を解析するためのマウスモデルの確立に成功したことを示すものである。このモデルを用いてセフェム系抗菌薬投与が抗菌薬の濃度依存的にマウスへの定着を増強し、高度耐性化、多剤耐性化に繋がることをゲノム解析に基づく遺伝子レベルで明らかにするなど、獣医公衆衛生学の分野のみならず医学の分野においても多大な貢献をすると考えられる。従って、最終試験の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。