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論　文　名	Detection and characterization of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolated from retail raw foods and children with diarrhea in Khanh Hoa province, Vietnam (ベトナム、カンホア県での市販の生食品と小児下痢症患者における基質特異性拡張型ベータラクタマーゼ(ESBL)産生大腸菌の検出と細菌学的特性)
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論文要旨

Introduction

Antimicrobial resistance (AMR) has become one of the most serious threats to global health. Misuse of antimicrobials in humans and animals may accelerate the emergence of AMR bacteria, which is especially critical in low-resource countries such as Vietnam. β-lactam antibiotics are intensively used for the treatment of bacterial infections. Among them, cephalosporins (3^{rd}, 4^{th}, and 5^{th} generations) are classified as “Highest Priority”. However, resistance to these important antibiotics has been increasingly recognized worldwide. Various mechanisms of resistance to β-lactams have been described, but the production of extended-spectrum β-lactamases (ESBLs) is specifically concerned. The increase of ESBL-producers such as *Escherichia coli* has rendered many important antibiotics ineffective. Since the rise of multidrug-resistant (MDR) bacteria with the ability to produce ESBLs or carbapenemases, colistin has been re-classified as a critical last-resort antibiotic to treat life-threatening infections. However, the emergence of mobile colistin resistance (encoded by *mcr* genes) has threatened the treatment options for those infections. Recently, the extent of *mcr* genes in ESBL-producing bacteria has been gradually recognized worldwide. Such a

conjunction of *mcr* and ESBL genes is worried due to the possibility of limitation for therapeutic options. Vietnam, where use of antibiotics is poorly managed, is one of the countries facing a growing threat of AMR with thousands of deaths annually due to MDR infections. The spread of ESBL or carbapenemase-producing bacteria are challenging healthcare settings, especially for severe infections. Therefore, it is necessary to detect, track and prevent AMR bacteria both inside and outside healthcare settings. In the Central Vietnam, ESBL-producing bacteria such as *E. coli*, especially those from children with diarrhea have not been studied sufficiently. Thus, close surveillance to identify sources and reservoirs of ESBL and *mcr* genes are needed to contribute to the One Health approach to combat AMR in Vietnam. This study was aimed to detect and characterize ESBL-producing *E. coli* (ESBL-*Ec*) isolated from retail raw foods and children less than five years of age with acute diarrhea at a local area in Central Vietnam, Khanh Hoa province in terms of their trends of resistance, characteristics such as ESBL genotypes, virulence genes profiles, phylogenetic groups, serotypes and the occurrence of *mcr* genes for better understanding of their molecular epidemiology.

Chapter 1. Isolation and antimicrobial resistance of ESBL-producing *E. coli* from retail raw foods and children with diarrhea in Khanh Hoa province, Vietnam

The prevalence of ESBL-*Ec* from chicken, pork, fish and shrimp was 66.4, 55.4, 42.0 and 19.6%, respectively. Prevalence of ESBL-*Ec* was statistically significant ($p<0.001$) between chicken and fish, chicken and shrimp, pork and shrimp and fish and shrimp. Besides, ESBL-*Ec* was also found in 45.9% of *E. coli* strains isolated from patients with diarrhea. The reason why high prevalence of ESBL-*Ec* was detected in animal-originated foods was not clear. However, the intensive use of antibiotics in food-producing animals might be contributing to such predominance. Most of the food isolates were resistant to tetracycline (TET) (96.6%), chloramphenicol (CHL) (88.0%), sulfamethoxazole/trimethoprim (SXT) (81.7%), nalidixic acid (NAL) (77.9%), streptomycin (STR) (64.4%), ciprofloxacin (CIP) (59.1%), kanamycin (KAN) (58.2%), gentamicin (GEN) (51.9%), ceftazidime (CAZ) (46.6%), colistin (CST) (31.3%), fosfomycin (FOF) (12.0%) and cefoxitin (FOX) (4.8%). Noticeably, colistin resistance was found in 53.2, 31.8, 21.0, and 8.5% in ESBL-*Ec* isolated from chicken, shrimp, pork and fish, respectively. Whereas ESBL-*Ec* isolated from patients resistant mainly to SXT (84.0%), NAL (80.0%), STR (56.3%), TET (54.2%), CAZ (53.1%), CIP (47.0%), FOX (40.6%), GEN (37.5%), KAN (26.0%) and CHL (9.4%). MDR was observed in high number of ESBL-*Ec* isolated from all sources such as fish (100%), chicken (98.7%), pork (93.5%), patients (91.7%) and shrimp (86.4%). It was found that a significant

number of ESBL-*Ec* isolates were multiple resistant phenotypes, but AMR patterns of ESBL-*Ec* strains were significantly diverse and most of the patterns were different from foods and diarrheal patients. These results suggested that MDR ESBL-*Ec* could disseminate via retail raw foods and children with diarrhea in Vietnam.

Chapter 2. Molecular characterization of ESBL-producing *E. coli* from retail raw foods and children with diarrhea in Khanh Hoa province, Vietnam

In this chapter, ESBL-*Ec* isolated from both foods and diarrheal patients were characterized for their ESBL genotype and virulence gene profile. The results indicated that the CTX-M type was remarkably dominant in ESBL-*Ec* isolated from retail raw foods (99.5%) and diarrheal patients (96.9%). Among CTX-M types, the CTX-M-1 and CTX-M-9 groups were popular in ESBL-*Ec* isolates. However, the CTX-M-1 group (71.6%) was more predominant in ESBL-*Ec* from foods than those from patients, whereas the CTX-M-9 group (51.0%) was more common in ESBL-*Ec* from patients than those from foods. Novelty, the CTX-M-2 (3.9%) and CTX-M-8/25 (1.3%) were detected in chicken isolates. This is the first detection of CTX-M-2 and -8/25 groups in *E. coli* from chicken in Vietnam. The majority of ESBL-*Ec* isolated from foods belonged to phylogenetic groups A and B1, whilst D and B2 were predominant in ESBL-*Ec* isolated from diarrheal patients. MDR was more prevalent in ESBL-*Ec* groups A (99.1%) and B1 (96%) than D (91.4%) and B2 (81%). Virulence gene profile analysis showed that the *astA* gene (29.3%) encoding heat-stable enterotoxin 1 (EAST1) was the most prevalent virulence gene in ESBL-*Ec* isolated from retail raw foods. Besides, the *eaeA* (6.5%) and *cdt* (5.2%) genes encoding intimin and cytolethal distending toxin (CDT) respectively, were also detected in ESBL-*Ec* isolated from chicken. Moreover, all *cdt* gene-positive *E. coli* (CTEC) produced a biologically active CDT, indicating that CDT might be potentially virulent. This finding also implied chicken as an important reservoir of CTEC in Vietnam. In ESBL-*Ec* isolated from patients, only *afaD-3* (31.2%) and *astA* (16.1%) genes were identified. Furthermore, the *afaD-3* positive strains showed adherence activity suggesting their potential association with acute diarrhea in children less than five years of age in the Central Vietnam. Most of the ESBL-*Ec* harboring *afaD-3* gene belonged to Og86 (29%), Og1 (25.8%) and OgGp7 (25.8%) genotypes. This is the first description of *cdt* and *afaD-3* genes in *E. coli* isolated in Vietnam.

Chapter 3. Occurrence of *mcr* genes in ESBL-producing *E. coli* isolated from retail raw foods and children with diarrhea in Khanh Hoa province, Vietnam

The aim of this chapter was to investigate occurrence of mobile colistin resistance (*mcr*) genes (*mcr-1* to *mcr-8*) in ESBL-*Ec* isolated from raw foods retailed in markets and supermarkets and children with acute diarrhea at a local province in the Central

Vietnam. All 96 ESBL-*Ec* isolated from patients were negative for *mcr* genes, but 65 out of the 208 (31.3%) ESBL-*Ec* isolated from foods were positive for *mcr* genes including *mcr-1* (26.9%), *mcr-3* (0.5%) and both *mcr-1* and *mcr*-3 genes (3.9%). Particularly, there was higher prevalence of *mcr-1* in ESBL-*Ec* isolates from chicken (53.2%) in comparison to those from shrimp (22.7%), pork (11.3%), and fish (6.4%). Both *mcr-1* and *mcr-3* genes were detected in ESBL-*Ec* isolates from shrimp (9.1%), pork (8.1%) and fish (2.1%) but not from chicken. This is the first detection of *mcr-3* gene in *E. coli* isolated from fish and shrimp in Vietnam. The 65 *mcr* gene-positive ESBL-*Ec* were MDR and resistant to colistin with the MICs of 4-8 μg/mL. PFGE analysis and O-genotyping revealed diverse genotypes implying particular clonal dissemination of *mcr*-harboring ESBL-*Ec* among retail raw foods in the Central Vietnam might be very limited. S1-PFGE and Southern hybridization illustrated that the *mcr-1* and *mcr-3* genes were located on either chromosomes or plasmids. However, the size of plasmids harboring *mcr* genes was varying from 30 to 390 kb. Noteworthy, co-location of CTX-M-1 with *mcr-1* or *mcr-3* genes on the same plasmid or at least on the similar-size of plasmids was identified. A conjugation experiment indicated that the *mcr-1* or *mcr-3* gene was horizontally transferable. Two *mcr* genes existed individually or concurrently in ESBL-*Ec* isolates from retail raw foods, which might further complicate the antimicrobial-resistant situation in Vietnam, and is a possible health risk for humans.

Conclusions

This study revealed that MDR ESBL-*Ec*, some of which were potentially pathogenic to humans, were disseminated among diarrheal children and retail raw foods in the Central Vietnam. Especially, the CTX-M-type ESBLs were predominant in the ESBL-*Ec* strains. Noteworthy, a high prevalence of transferrable *mcr-1*- and/or *mcr-3*-harboring ESBL-*Ec* among retail raw foods was observed, which could contribute to the wide dissemination of *mcr* genes to bacteria in the environment, animals and humans. Moreover, co-existence of *mcr* genes with the genes encoding CTX-M enzymes in a bacterial cell was found in ESBL-*Ec*. Such conjunction may further exacerbate the co-occurrence and transmission of the drug-resistance genes into wider population, which may result in increased human health risk.

審査結果の要旨

薬剤耐性の問題は世界で最も重要な健康課題の１つとなっている。薬剤耐性菌の出現は人や家畜への抗菌薬の不適切使用が影響し、ベトナムのような開発途上国では特に深刻な問題となっている。基質特異性拡張型βラクタマーゼ（ESBL）産生菌が出現し、世界中に

広がりを見せている。ESBL 産生菌は第 3 世代から第 5 世代の β ラクタム薬を無効とし、特に、多剤耐性の ESBL 産生菌やカルバペネマーゼ産生菌の場合選択できる治療薬がなく、コリスチンが最後の塞となっていた。しかしながら、近年、伝達性コリスチン耐性（*mcr*）遺伝子を持つプラスミドが同定され、ESBL 産生菌の中にも *mcr* 遺伝子が見出され医療現場に大きな問題となっている。

ベトナムにおいては、これらの薬剤耐性菌は重症患者の治療手段を奪い特に重要である。それゆえ、病院内外における ESBL 産生菌やカルバペネマーゼ産生菌をモニタリングし、これら耐性菌の拡散を食い止めることが重要である。ベトナム中部においては小児下痢症における ESBL 産生大腸菌との関係は明らかにされていない。それゆえ、ワンヘルスの観点から ESBL や *mcr* 遺伝子の食品や人における分布を調べることは重要である。本研究では、ベトナム中部に位置するカンホア県における市販の様々な生食品と急性下痢症を呈した 5 歳以下の小児下痢便から ESBL 産生大腸菌を分離し、分離菌の薬剤感受性、ESBL 遺伝子型、病原因子プロファイル、パイロジェネティックグループ（PG）、O 血清遺伝子型および *mcr* 遺伝子の有無などの細菌学的性状を詳細に解析し、分子疫学的特徴を明らかとすることを目的とした。

第 1 章では市販鶏肉、豚肉、魚およびエビにおける ESBL 産生大腸菌の汚染状況を解析した。その結果、それぞれ 66.4%、55.4%、42.0%、19.6%の検体から ESBL 産生大腸菌を分離した。また、45.9%の小児下痢症患者便からも ESBL 産生大腸菌を分離した。食品分離株の多くは薬剤耐性を示しテトラサイクリンに対して 96.6%と最も高い薬剤耐性率を示し、次いでクロラムフェニコールに 88.0%、ST 合剤に 81.7%であった。特筆すべき点は、*mcr* 遺伝子が鶏由来の ESBL 産生大腸菌の 53.2%、エビ由来で 31.8%が陽性となった。一方、小児下痢便から分離された ESBL 産生大腸菌は、84.0%が ST 合剤に耐性で、次いでナリジクス酸が 80.0%、ストレプトマイシンが 56.3%と食品由来株とは異なる傾向を示した。また、様々な種類の検体から分離された ESBL 産生大腸菌の多くが多剤耐性であったが、その耐性プロファイルには多様性があった。

第 2 章では、第 1 章で食品及び患者から分離した ESBL 産生大腸菌の細菌学的性状について詳細に解析した。その結果、ESBL の遺伝子型では CTX-M 型が最も多く、特に CTX-M-1 と CTX-M-9 が食品及び患者由来株でそれぞれ 99.5%、96.9%であった。今回鶏肉由来の ESBL 産生大腸菌で CTX-M-2 と CTX-M-8/25 がベトナムで初めて検出された。食品由来の多くの株は PG が A と B1 であったのに対し、患者由来株の多くは D と B2 であった。また、病原因子では *astA* 遺伝子が最も多く検出され 29.3%、次いで *eae* 遺伝子と *cdt* 遺伝子がそれぞれ 6.5%、5.2%で検出された。また、患者由来株でのみ細胞接着に関わる *afaD-3* 遺伝子が検出され、細胞への接着が確認された。*afaD-3* 及び *cdt* 遺伝子陽性菌の分離はベトナムで初めての報告となった。

第 3 章では ESBL 産生大腸菌中の *mcr* 遺伝子の分布について調べた。その結果、全ての患者由来株で検出されず、食品由来株の 31.3%で検出された。8 種類の *mcr* 遺伝子の中で *mcr-1* と *mcr-3* が検出され、検出率はそれぞれ 40.8%と 4.4%であった。また、鶏由来株が最も検出率が高く 53.2%で *mcr-1* であった。*mcr-1* と *mcr-3* 遺伝子の両方が検出されたのはエビ由来株で最も多く 9.1%であった。ベトナムにおいて *mcr-3* 遺伝子が初めてエビと魚由

来株で検出された。また、*mcr* 遺伝子陽性の 65 株の全てが多剤耐性菌であった。PFGE と O 血清遺伝子型解析から *mcr* 遺伝子陽性 ESBL 産生大腸菌には多様性が示された。*mcr* 遺伝子や ESBL 遺伝子はプラスミドや染色体上に検出され、両遺伝子が同一のプラスミド上に検出される例もあった。また、プラスミド性の *mcr* 遺伝子は他の大腸菌にも接合伝達されることが示された。

以上の結果は、人への病原性を示す可能性のある大腸菌を含む多剤耐性 ESBL 産生大腸菌がベトナム中部の生食品や下痢症患者の間にかなり拡がっていること、ESBL の遺伝子型は CTX-M 型が主流であることおよび *mcr* 遺伝子陽性の ESBL 産生大腸菌も高頻度に市販の生食品に拡がっていることを示した。ワンヘルスの観点から今後の薬剤耐性菌対策を講じる上において有益な多剤耐性 ESBL 産生大腸菌の分子疫学情報を提供するものであり、本研究成果は獣医学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、本論文の審査及び学力確認の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。