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論文名	<p>Prevalence and characterization of extended-spectrum beta-lactamase-producing <i>Escherichia coli</i> and <i>mcr-1</i> gene-positive <i>E. coli</i> in domestic and imported chickens in Japan</p> <p>（基質特異性拡張型βラクタマーゼ産生性大腸菌とコロistin耐性遺伝子陽性大腸菌の国産と輸入鶏肉における汚染状況と分離株の性状解析）</p>	
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論文要旨

Introduction

Emergence of antimicrobial resistance (AMR) bacteria, in particular 3rd generation cephalosporins (β -lactam antibiotic), fluoroquinolones, and polymixins (colistin), is a serious global problem. There are several mechanisms by which bacteria acquire resistance against antimicrobials. Enzymatic cleavage using β -lactamase enzyme is one of the major mechanisms to acquire resistance against β -lactam antibiotics. Extended-spectrum β -lactamases (ESBLs) can hydrolyse clinically important 3rd and 4th generation cephalosporin and thus, emergence and spread of ESBLs-producing bacteria are serious concern. ESBLs can be divided into three groups such as TEM, SHV and CTX-M. TEM and SHV were prevalent in 1980s and 1990s and mostly involved in hospital infection caused by ESBL-producing Enterobacteriaceae. After introduction of 3rd generation of cephalosporins, CTX-M type has become dominant and has substituted TEM and SHV. It is interesting to note that CTX-M type ESBL-producing Enterobacteriaceae is mostly *E. coli* and associated with community infections. Many studies on ESBL-producing *E. coli* (ESBL-Ec) from both clinical

and food-producing animal have been published worldwide in recent years. Animal-based foods have been demonstrated as a significant reservoir for ESBL-Ec whereas meat is considered to be an important source of contamination with ESBL-Ec. Besides 3rd generation of cephalosporin, a polymyxin antibiotic (colistin: CST) is also used widely in poultry production as growth promoter and for therapeutic purpose. Although CST has serious side effects, it is considered a last-resort antimicrobial and can be used to treat patients with serious infections caused by multidrug resistant (MDR) bacteria such as New Delhi metallo- β -lactamase-1-producing Enterobacteriaceae. CST resistance conferred by *mcr-1* gene is recent emerging issue since *mcr-1* gene located in plasmid and can be transferred which is revealed by recent study in China. Although *E. coli* is known to be a commensal in animal intestine including human, some can cause intestinal and extra-intestinal infection such as diarrhea, urinary tract infection (UTI), septicemia and neonatal meningitis. Emergence of AMR in intestinal and extra-intestinal pathogenic *E. coli* (ExPEC) has become a major public health concern. Among various meats, the chance of contamination of chicken with fecal bacteria especially *E. coli* appears to be higher because of its processing protocol. Therefore, retail chicken might have more chance to be contaminated with ESBL-Ec and *mcr-1* gene-positive *E. coli*. There are few reports regarding prevalence of ESBL-Ec and *mcr-1* gene-positive *E. coli* in domestic and imported retail chicken in Japan. In addition, there is no comprehensive study on AMR pattern and prevalence of virulence genes in ESBL-Ec and *mcr-1* gene-positive *E. coli* in retail chicken in Japan. Therefore, in this study, prevalence of ESBL-Ec and *mcr-1* gene-positive *E. coli* in domestic and imported retail chicken was examined and the isolates were further characterized for antimicrobial resistant profile, virulence gene profile and so on.

Chapter 1. Isolation and characterization of ESBL-producing *E. coli* in domestic and imported retail chicken

A total of 106 chicken including 56 produced domestically and 50 imported from abroad (Brazil [n=36], USA [n=8], Thailand [n=6]) were purchased from 9 supermarkets in Osaka prefecture, Japan between August and December 2015. ESBL producers were identified by double disk diffusion method using cefotaxime (CTX), ceftazidime (CAZ), CTX/clavulanic acid and CAZ/clavulanic acid and confirmed as *E. coli* by biochemical test. The result showed that prevalence of ESBL-Ec was 77% (43/56) in domestic chicken (DC) and 52% (26/50) in imported chicken (IC) [72% (26/36), 0% (0/6) and 0% (0/8) in samples from Brazil, Thailand and USA, respectively]. A total of 111 ESBL-Ec was isolated from 43 DC whereas 51 ESBL-Ec were isolated from 26 IC from Brazil. To see whether there was difference in ESBL genotypes between isolates of DC and IC, ESBL genotyping (*bla*_{CTX-M 1, 2, 8/25, 9}, *bla*_{TEM} and *bla*_{SHV}) was done. If more than one isolate from the same chicken carried

similar ESBL genes, only representative isolate was selected for further analysis. Subsequently, 53 out of 111 ESBL-Ec isolates from DC and 30 out of 51 from chicken from Brazil were selected. Analysis of ESBL genotype exhibited that ESBL genotypes were mainly *bla*_{CTX-M} (91%) including [*bla*_{CTX-M-2} (45%), *bla*_{CTX-M-1} (34%), *bla*_{CTX-M-9} (9.5%), *bla*_{CTX-M-8} (1.9%)] followed by *bla*_{TEM} (36%) and *bla*_{SHV} (15%) in ESBL-Ec from DC whereas *bla*_{CTX-M} (100%) including [*bla*_{CTX-M-2} (53%), *bla*_{CTX-M-8} (43%), *bla*_{CTX-M-1} (3.3%)] followed by *bla*_{TEM} (20%) were detected in ESBL-Ec from IC. Antimicrobial susceptibility testing analysis to other antimicrobials showed that most of the tested ESBL-Ec from DC were mostly resistant to tetracycline (83%) followed by streptomycin (70%) and nalidixic acid (62%) whereas most of the tested ESBL-Ec of IC were resistant to streptomycin (77%) followed by nalidixic acid (63%), tetracycline (57%). Notably, extensive MDR, which is defined to be resistant against at least five classes of antimicrobials, was detected in 60% and 70% in ESBL-Ec isolated from DC and IC, respectively. To see the clonal relationship among these isolates, pulsed-field gel electrophoresis (PFGE) was carried out with *Xba*I digestion. PFGE analysis showed that 53 ESBL-Ec from DC and 30 from IC generated 44 and 26 pulsotypes, respectively, suggesting high level of genetic diversity. Taken together, it can be concluded that both DC and IC from Brazil are highly contaminated with ESBL-Ec with high diversity at genetic level regarding ESBL groups as well as clonality along with extensive multidrug resistance.

Chapter 2. Isolation and characterization of *mcr-1* gene-positive *E. coli* in domestic and imported retail chicken

Plasmid mediated CST resistance is also recent emerging issue and thus investigation for the prevalence of *mcr-1* gene-positive *E. coli* in DC and IC is also of paramount importance. Therefore, retail chickens were investigated for the prevalence of *mcr-1* gene. The results showed that prevalence of *mcr-1* gene-positive samples was 55% (31/56) in DC while that was 22% (11/50) including 22% (8/36), 33% (2/6) and 13% (1/8) from Brazil, Thailand and the USA, respectively. Subsequently, *mcr-1* gene-positive *E. coli* was isolated from *mcr-1* gene-positive retail chicken meats. A total of 30 and 10 *mcr-1* gene-positive *E. coli* was successfully isolated from DC and IC, respectively and subsequent analysis was done. Furthermore, minimum inhibitory concentration for CST was determined to be 4-8 µg/ml for *mcr-1* gene-positive isolates from both DC and IC. Antimicrobial susceptibility testing analysis to other antimicrobials showed that most of the isolates from DC were resistant to tetracycline (63%) followed by kanamycin (50%), sulfamethoxazole/trimethoprim (50%) whereas most of the isolates from IC were resistant to ampicillin (80%) followed by streptomycin (70%), kanamycin (70%), ciprofloxacin (70%). Notably, extensive MDR was detected in 33% and 40% isolates from DC and IC, respectively. PFGE analysis showed that 30 and 10 *mcr-1* gene-positive *E. coli* isolates from DC and IC generated

25 and 9 pulsotypes, respectively suggesting that high level of genetic diversity. Taken together, it can be concluded that both DC and IC showed higher prevalence of *mcr-1* gene-positive *E. coli* with genetic diversity regarding clonality along with extensively MDR.

Chapter 3. Virulence gene profile of ESBL-producing *E. coli* and *mcr-1* gene-positive *E. coli* isolated from domestic and imported retail chicken

Phylogenetic analyses provide better understanding pathogenicity of the strains. On the basis of phylogenetic analyses, *E. coli* strains are characterized into four phylogenetic lineages (A, B1, B2, and D). Most commensal *E. coli* belong to lineage A and B1 and virulent strains belong mainly to lineage B2 and D. So, first to see phylogenetic groups of isolates of ESBL-Ec and *mcr-1* gene-positive *E. coli* under study, phylogenetic analysis was done using multiplex PCR (*chuA*, *yjaA* and TSPE4.C2). Results suggested that almost half of the ESBL-Ec from both DC and IC belonged to B2 /D phylogenetic groups but *mcr-1* gene-positive *E. coli* isolates mainly belonged to A/B1. Next virulence gene profiles were investigated for ESBL-Ec of DC (n=53) and IC (n=30) and *mcr-1* gene-positive *E. coli* of DC (n=30) and IC (n=10). Virulence genes analyzed includes several genes associated with diarrhegenic *E. coli* (DEC) such as *eaeA* (*E. coli*-attaching and effacing), *bfpA* (bundle-forming pilus), *elt* (heat-labile enterotoxin), *est* (heat-stable enterotoxin), *eagg* (plasmid of enteroaggregative *E. coli*), *astA* (enteroaggregative *E. coli* heat-stable enterotoxin 1), *stx* (Shiga toxin), *invE* (Invasin of EIEC), *daaD* (fimbriae adhesion) and *cdt* (cytolethal distending toxin) and extra-intestinal pathogenic *E. coli* (ExPEC) such as genes related to adhesion (*papEF*, *papC*, *sfa/focDE* and *afaBC*), toxins (*hlyA* and *cnf*), siderophores (*fyuA* and *iroN*), protections and invasions (*traT* and *kpsMT*) and miscellaneous genes (PAI, *usp* and *ibeA*). The results showed that among ESBL-Ec strains prevalence of *astA* gene was higher in isolates of IC (47%) compared to that of DC (23%). Similar trend was also observed in *mcr-1* gene-positive *E. coli* that 60% isolates from IC possessed *astA* gene in comparison to 6.7% isolates from DC. Furthermore, *cdtB* and *eaeA* genes were detected in one ESBL-Ec and 2 *mcr-1* gene-positive *E. coli* from IC, respectively. The *cdt* genes in ESBL-Ec were identified as *cdt IV* which was biologically active by cell culture assay. However, none of the ESBL-Ec or *mcr-1* gene-positive *E. coli* from DC was positive for *cdtB* and *eaeA* genes. Thus, it might be said that IC had higher prevalence of potentially pathogenic *E. coli* possessing diarrheic genes. On the other hand, none of the ESBL-Ec or *mcr-1* gene-positive *E. coli* was positive for *bfpA*, *elt*, *est*, *stx1*, *stx2*, *invE*, *eagg* and *daaD* genes irrespective of origin of chicken. In prevalence study of virulence genes associated with ExPEC, *traT*, which is associated with invasion and protection, showed highest prevalence in all the tested isolates irrespective of the origin. Among ESBL-Ec from DC and IC, *traT* prevalence was 60% and 97%, respectively. On the other hand, in *mcr-1*

gene-positive *E. coli* from DC and IC, *traT* prevalence was 80% and 50%, respectively. The ExPEC genes *PapEF* and *papC* are part of *pap* gene cluster, which is associated with adhesion, an important factor of pathogenesis of UTI. The prevalence of *pap* genes was 19% and 13% in ESBL-Ec from DC and IC, respectively. Interestingly, none of the *mcr-1* gene-positive *E. coli* isolated from DC carried *pap* genes whereas 20% isolates from IC were positive for *pap* genes. Prevalence of other ExPEC associated genes varied among *E. coli* isolates analyzed in this study. However, none of the ESBL-Ec or *mcr-1* gene-positive *E. coli* was positive for ExPEC associated genes such as *hlyA*, *cnf*, *sfa/focDE*, *afaBC*, *usp* and *ibeA* examined. Overall, it might be said that isolates from IC carried more virulence genes in comparison to that from DC.

ESBL-Ec and *mcr-1* gene-positive *E. coli* isolates examined in this study were extensively MDR at high level and also carried potential virulence genes. Thus, the isolates carrying both virulence and antimicrobial resistance genes might cause human illness.

Conclusions

ESBL-Ec and *mcr-1* gene-positive *E. coli* isolated from domestic and imported retail chicken could be a potential reservoir for antimicrobial resistance determinants and virulence genes. Thus, chicken contaminated with ESBL-Ec and *mcr-1* gene-positive *E. coli* might have potential to cause human illness and requires a continuous monitoring.

審査結果の要旨

基質特異性拡張型βラクタマーゼ（ESBL）は、様々な種類のβラクタム系薬を分解することから、ESBL産生菌は医療現場において脅威となっている。ESBLは現在、TEM、SHVとCTX-Mの3つに分類されている。1980から1990年代は、TEMとSHVが院内でしかも腸内細菌科菌群が主に流行に関わっていた。しかし、2000年代に入ると第3世代セフェムの特許が切れたため価格が低下したことで、家畜の生産現場でも多くのセフェム系抗菌薬が使用されるようになり、流行の中心はCTX-M型へ、院内から市中へ、腸内細菌科菌群から大腸菌へと変化した。畜産現場でセフェム系抗菌薬が多用された結果、食肉、特に鶏肉におけるESBL産生大腸菌汚染、特にCTX-M型のESBL産生大腸菌の流行が大きな問題となっている。開発途上国を中心に、CTX-M型のESBL産生大腸菌の健康保菌者も多数報告されている。一方、2015年にプラスミド媒介性のコリスチン耐性菌が中国で見つかり世界を震撼させた。コリスチンはニューデリーメタロプロテアーゼ産生菌など治療が困難な多剤耐性菌の最後の砦として臨床現場で使われて

いる抗菌薬である。また、コリスチン耐性 (*mcr-I*) 遺伝子がプラスミド上に見つかったことは、コリスチン耐性が容易に菌株間のみならず菌種間を超えて拡散する可能性がある。以上より、我が国の市販鶏肉における **ESBL** 産生大腸菌やコリスチン耐性 (*mcr-I* 遺伝子陽性) 大腸菌の汚染率や保有する病原遺伝子を調べることは極めて重要である。

第1章では、市販の国産鶏肉 **56** 検体及び輸入鶏肉 **50** 検体の合計 **106** 検体を対象に、**ESBL** 産生大腸菌の汚染率について調べた。その結果、国産鶏肉の **77%** から、輸入鶏肉の **52%** から **ESBL** 産生大腸菌を検出された。輸入鶏肉は、ブラジル産が **36** 検体、米産が **8** 検体、タイ産が **6** 検体で、検出された全てがブラジル産であり、陽性率は **72%** と国産鶏肉に匹敵する割合であった。国産鶏肉 **43** 検体及びブラジル産鶏肉 **26** 検体から、それぞれ **111** 株と **51** 株の **ESBL** 産生大腸菌を分離した。国産鶏肉由来の **ESBL** 産生大腸菌は **91%** が **CTX-M** 型陽性、**36%** が **TEM** 型陽性、**15%** が **SHV** 陽性であったのに対し、輸入鶏肉由来の **ESBL** 産生大腸菌は **CTX-M** 型は **100%**、**TEM** 型が **20%** で陽性であった。**CTX-M** の中でも、**CTX-M-2** は国産、輸入に共通して多数検出されたが、国産鶏肉由来株では、**CTX-M-1** 型が、輸入鶏肉では **CTX-M-8** 型の検出率が高かった。国産鶏肉由来株では、テトラサイクリン耐性が **83%**、ストレプトマイシン耐性が **70%**、ナリジクス酸耐性が **62%** であったのに対し、輸入鶏肉由来株ではストレプトマイシン耐性が **77%**、ナリジクス酸耐性が **63%**、テトラサイクリン耐性が **57%** であった。一方、多剤耐性菌の割合は国産及び輸入鶏肉由来株でそれぞれ **60%** と **70%** であった。以上の結果より、国産、輸入鶏肉ともかなりの割合で多剤耐性の **ESBL** 産生大腸菌で汚染されていることが明らかとなった。

第2章では、コリスチン耐性大腸菌の国産及び輸入鶏肉の汚染状況について解析した。国産鶏肉の **53%** から、輸入鶏肉では **22%** からコリスチン耐性遺伝子が検出され、それぞれから **30** 株と **11** 株を分離した。輸入肉の内訳は、ブラジル産 **36** 検体中 **8** 検体、タイ産 **6** 検体中 **2** 検体、米産 **8** 検体中 **1** 検体で、合計 **10** 株の *mcr-I* 遺伝子陽性大腸菌を分離した。国産鶏肉由来株の **63%** がテトラサイクリン耐性、**50%** がそれぞれカナマイシン耐性と **ST** 合剤耐性を示し、輸入鶏肉由来株では **80%** がアンピシリン耐性、**70%** がそれぞれストレプトマイシン耐性、カナマイシン耐性、シプロフロキサシン耐性を示した。多剤耐性菌は国産、輸入鶏肉由来株でそれぞれ **33%** と **40%** であった。以上の結果より国産、輸入鶏肉とも高率で多剤耐性 *mcr-I* 遺伝子陽性大腸菌で汚染されていることが明らかとなった。

第3章では、第1章、2章で分離した薬剤耐性大腸菌がヒトへの病原性を示す可能性を調べることを目的に、パイロジェニと病原遺伝子の分布を解析した。その結果、**A**、**B1**、**B2** と **D** の4種で分類されるパイロジェニ解析で、由来に関わらず約 **50%** の **ESBL** 産生大腸菌がヒトに腸管外感染症を引き起こす可能性のある **B2/D** であった。一方、*mcr-I* 陽性大腸菌のほとんどは非病原性の **A/B1** に属した。次に、病原遺伝子プロファイル調べた結果、輸入及び国産鶏肉由来 **ESBL** 産生大腸菌では *astA* 遺伝子がそれぞれ **47%** と **23%** で陽性であった。*mcr-I* 陽性大腸菌においても輸入及び国産鶏肉由来で *astA* 遺伝子が **60%** と **6.7%** で陽性であった。*cdtB* 遺伝子や *eae* 遺伝子も **ESBL** 産生大腸菌 **1** 株と *mcr-I* 陽性大腸菌

2株で検出されたが、全て輸入鶏肉由来株であった。さらに、腸管外感染症に関わる様々な病原遺伝子が検出され、特に侵入性に関わる *traT* 遺伝子は国産・輸入に関わらず ESBL 産生大腸菌や *mcr-I* 陽性大腸菌において 50% から 97% の高い陽性率を示した。

以上の結果は、我が国で市販されている鶏肉は ESBL 産生大腸菌や *mcr-I* 陽性大腸菌で高率に汚染されており、それらの多くが多剤耐性であり、一部の菌で、特に輸入鶏肉由来株では高率に、腸管内病原性大腸菌や腸管外病原性大腸菌が保持する病原遺伝子を保持していることを明らかとした。これらの研究成果は獣医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されると考えられる。従って、本論文の審査ならびに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。