Title	Prevalence and characterization of extended-spectrum beta-lactamase-producing Escherichia coli and mcr-1 gene- positive E. coli in domestic and imported chickens in Japan
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Prevalence and characterization of extended-spectrum beta- lactamase-producing Escherichia coli and mcr-1 gene-positive E. coli in domestic and imported chickens in Japan

（基質特異性拡張型βラクタマーゼ産生性大腸菌とコリスチン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）

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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 Enterbacteriaceae. 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*$_{\text{CTX-M 1, 2, 8/25, 9}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$) 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*$_{\text{CTX-M}}$ (91%) including [*bla*$_{\text{CTX-M-2}}$ (45%), *bla*$_{\text{CTX-M-1}}$ (34%), *bla*$_{\text{CTX-M-9}}$ (9.5%), *bla*$_{\text{CTX-M-8}}$ (1.9%)] followed by *bla*$_{\text{TEM}}$ (36%) and *bla*$_{\text{SHV}}$ (15%) in ESBL-Ec from DC whereas *bla*$_{\text{CTX-M}}$ (100%) including [*bla*$_{\text{CTX-M-2}}$ (53%), *bla*$_{\text{CTX-M-8}}$ (43%), *bla*$_{\text{CTX-M-1}}$(3.3%)] followed by *bla*$_{\text{TEM}}$ (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-positve 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 diarrheagenic *E. coli* (DEC) such as *eaeA* (*E. coli*-attaching and effacing), *bfp*A (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 (*pap*EF, *pap*C, *sfa/foc*DE and *afa*BC), toxins (*hly*A and *cnf*), siderophores (*fyu*A and *iroN*), protections and invasions (*tra*T 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 can 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* were positive for ExPEC associated genes such as *hlyA, cnf, sfa/focDE, afaBC, usp* and *ibeA* examined. Overall, it can 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.