The Journal of Veterinary Medical Science

FULL PAPER

Public Health

Prevalence and characteristics of extended-spectrum β-lactamase-producing *Escherichia coli* in domestic and imported chicken meats in Japan

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ABSTRACT. The purpose of this study was to investigate the prevalence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* (ESBL-Ec) in retail chicken meats in Japan. Fifty-six domestic and 50 imported (Brazil, n=36; United States, n=8; Thailand, n=6) chicken meat samples were analyzed. The 162 ESBL-Ec included 111 from 43 (77%) domestic samples and 51 from 26 (52%) Brazilian samples. Fifty-three and 30 of 111 and 51 ESBL-Ec from domestic and Brazilian chickens, respectively, were selected for ESBL genotyping. The $bla_{CTX\text{-}M}$ (91%), bla_{TEM} (36%) and bla_{SHV} (15%) genes were detected in ESBL-Ec isolated from domestic chickens, whereas $bla_{CTX\text{-}M}$ (100%) and bla_{TEM} (20%) were detected in ESBL-Ec isolated from imported chickens. Among the $bla_{CTX\text{-}M}$ group, $bla_{CTX\text{-}M\text{-}2}$ (45%) and $bla_{CTX\text{-}M\text{-}1}$ (34%) were prevalent in domestic chicken isolates, whereas $bla_{CTX\text{-}M\text{-}2}$ (53%) and $bla_{CTX\text{-}M\text{-}8}$ (43%) were prevalent in imported chicken isolates. Domestic chicken isolates were mostly resistant to tetracycline (83%), followed by streptomycin (70%) and nalidixic acid (62%). Imported chicken isolates were resistant to streptomycin (77%), followed by nalidixic acid (63%) and tetracycline (57%). Notably, extensive multidrug resistance was detected in 60% (32/53) and 70% (21/30) ESBL-Ec from domestic and imported chickens, respectively. Virulence genes associated with diarrheagenic and extra-intestinal pathogenic *E. coli* were detected in ESBL-Ec isolated from domestic and imported chickens. These data suggest that ESBL-Ec in retail chicken meats could be a potential reservoir for antimicrobial resistance determinants and that some are potentially harmful to humans.

KEY WORDS: domestic chicken, *E. coli*, ESBL, imported chicken, virulence genes

J. Vet. Med. Sci.
80(3): 510–517, 2018
doi: 10.1292/jvms.17-0708

Received: 24 December 2017
Accepted: 22 January 2018
Published online in J-STAGE: 12 February 2018

Use of antimicrobials in food animals is a public health concern because antimicrobial resistant (AMR) bacteria can emerge and be transmitted to humans through consumption and handling of foods of animal origin [35]. AMR bacteria may spread across borders via trade and travel and are therefore considered to be a global problem [13].

Bacteria can acquire antimicrobial resistance by several mechanisms including enzymatic inactivation of drugs, target alteration, and active efflux. Enzymatic cleavage using β-lactamase is one of the major mechanisms of acquired resistance against β-lactam antibiotics. Emergence and spread of extended-spectrum β-lactamase (ESBL)-producing bacteria are a serious concern because ESBLs can hydrolyze clinically important third- and fourth-generation cephalosporins. ESBLs can be divided into three groups: TEM, SHV, and CTX-M [36]. TEM and SHV were prevalent in the 1980s and 1990s and were mostly involved in hospital infections caused by ESBL-producing Enterobacteriaceae. After introduction of third-generation of cephalosporins, the CTX-M type has become dominant and has supplanted TEM and SHV. The CTX-M type ESBL-producing Enterobacteriaceae is mostly *Escherichia coli* and is often associated with community infections [36].

There are several variants of CTX-M type ESBLs. The $bla_{CTX\text{-}M}$ gene is broadly grouped into five subgroups ($bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}9}$, and $bla_{CTX\text{-}M\text{-}25}$) on the basis of amino acid sequence homology [4]. The $bla_{CTX\text{-}M\text{-}15}$ member of $bla_{CTX\text{-}M\text{-}1}$ group is the most dominant globally [27]. However, in Japan, $bla_{CTX\text{-}M\text{-}9}$ predominates, followed by $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}1}$ [6]. On the other hand, South America features a wide distribution of $bla_{CTX\text{-}M\text{-}2}$ as well as $bla_{CTX\text{-}M\text{-}8}$, which are rare in

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other continents [27]. As a matter of growing concern, ESBL-producing *E. coli* (ESBL-Ec) are often resistant to other classes of antimicrobials such as fluoroquinolones and aminoglycosides [15].

Commensal *E. coli* can be an important reservoir of AMR genes that may spread to pathogenic bacteria [3]. In addition, some *E. coli* are also pathogenic to humans and animals [2]. Pathogenic *E. coli* can be divided into two groups-diarrheagenic *E. coli* (DEC) and extra-intestinal pathogenic *E. coli* (ExPEC), which can cause urinary tract infection (UTI) and septicemia [12, 21]. DEC includes enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) based on the virulence genes present, which include *bfp* (EPEC), *eae* (EPEC/EHEC), *invE* (EIEC), *elt* and *est* (ETEC), *stx* (EHEC), *eagg* (EAEC), and *daaD* (DAEC). Among ExPECs, uropathogenic *E. coli* (UPEC) is one of the most important pathogens causing community-acquired UTI [12], and AMR UPEC has become a major public health concern [14].

Consumption of chicken has been rapidly growing globally including Japan. For food safety, it is important to continuously monitor the contamination of chicken meat by AMR bacteria and their possession of virulence genes in retail chicken meats sold in Japan. Antimicrobials are widely used as a growth promoter in the production of livestock including poultry, which has resulted in the emergence of AMR bacteria. These AMR bacteria could enter the food chain, resulting in a major impact on public health [22]. Animal-based foods, particularly meat, have been demonstrated to be a significant reservoir for ESBL-Ec [16]. Many studies on ESBL-Ec from clinical settings and from food-producing animals have been published worldwide in recent years [10]. There are a few reports regarding the prevalence of ESBL-Ec in retail chickens in Japan, but there are no comprehensive and comparative studies on AMR pattern and virulence gene profiles in ESBL-Ec in retail chicken meats sold in Japan.

Therefore, in this study, the prevalence of ESBL-Ec in domestic and imported chicken meats sold in supermarkets in Japan was examined and ESBL-Ec isolates were further characterized for ESBL genotypes, antimicrobial resistance, and virulence gene profiles.

MATERIALS AND METHODS

Chicken and bacterial isolates

A total of 106 retail chicken meats including 56 produced domestically and 50 imported from Brazil (n=36), the United States (n=8), and Thailand (n=6) were purchased from nine supermarkets in Izumisano City, Osaka, Japan, from August to December 2015. Twenty-five grams of each sample was stomached in 225 m*l* of buffered peptone water (Oxoid Ltd., Basingstoke, U.K.). After incubation at 37°C for 16 ± 2 hr, a loopful of enriched culture was streaked onto MacConkey agar (Eiken Chemical, Co., Ltd., Tokyo, Japan) containing 1 mg/*l* cefotaxime (CTX; Nihon Becton Dickinson, Tokyo, Japan). Three dark pinkish to reddish *E. coli*-like colonies were collected from each sample and identified as *E. coli* by biochemical tests, including tests for sugar fermentation on triple sugar iron agar, ability to reduce lysine, produce indole, and motility by lysine indole motility test, Simmon's citrate test, and Voges-Proskauer test following the standard protocols [31].

ESBL phenotyping and genotyping

E. coli isolates were phenotypically screened for ESBL production by the double-disc synergy test using CTX and ceftazidime (CAZ) with or without clavulanic acid (CA) as recommended by the Clinical and Laboratory Standards Institute (CLSI) [9]. An isolate was considered as an ESBL-producer when there was a ≥5 mm increase in the zone of inhibition with CTX or CAZ disk with CA in comparison to CTX or CAZ alone. ESBL gene grouping ($bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8/25}}$, $bla_{\text{CTX-M-9}}$, bla_{TEM} and bla_{SHV}) was carried out using multiplex PCR [25]. Furthermore, $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-25}}$ were differentiated by amplification and sequencing of $bla_{\text{CTX-M-8/25}}$ [33]. Sequencing reactions were performed by the chain termination method with the BigDye Terminator v1.1 cycle sequencing kit (Thermo Fisher Scientific, Foster City, CA, U.S.A.). Nucleotide sequences were determined using an ABI PRISM 3130-Avant Genetic Analyzer (Thermo Fisher Scientific).

Pulsed-field gel electrophoresis (PFGE)

To examine the clonal relationship among the ESBL-Ec isolates, PFGE analysis was carried out according to the Pulse Net USA protocol (https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf) with slight modifications. Briefly, genomic DNA of the isolates was digested with 30 U of *Xba*I (TaKaRa Bio Inc., Kusatsu, Japan) at 37°C for 2 hr. *Xba*I-digested *Salmonella enterica* serotype Braenderup H9812 was used as a molecular size marker. The digested DNA was electrophoresed on 1.0% pulsed-field certified agarose (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.) in 0.5X TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) buffer at 6 V/cm^2, with a switch time of 6.76–35.38 sec for 27 hr using a CHEF Mapper (Bio-Rad Laboratories Inc.). The gel was stained with ethidium bromide and de-stained with distilled water. The DNA fingerprint patterns were photographed with UV gel Doc (Bio-Rad Laboratories, Inc.) and analyzed by Fingerprinting II software (Bio-Rad Laboratories Inc.). Dendrogram analysis was performed using the unweighted-pair group method with arithmetic mean (UPGMA) analysis and the band-based (Dice correlation coefficient) option.

Determination of antimicrobial susceptibility

Antimicrobial susceptibility of the ESBL-Ec isolates was tested by the disk diffusion method [8] using commercially available discs (Nihon Becton Dickinson) against 14 antimicrobials belonging to 10 antimicrobial classes. The antimicrobial classes were considered according to CLSI guidelines [8]. They included penicillins (ampicillin [AMP]), cephems (cefoxitin [FOX], CTX,

Table 1. Isolation of ESBL-producing *E. coli* (ESBL-Ec) from retail chicken meats

Origin	Country of origin	No. of *E. coli* positive samples	No. (%) of ESBL-Ec positive samples	Total no. of ESBL-Ec isolates
Domestic (n=56)	Japan (56)	54	43 (77)	111
Imported (n=50)	Brazil (36)	36	26 (72)	51
	U.S.A. (8)	5	0 (0)	0
	Thailand (6)	1	0 (0)	0
Sub total		42	26 (52)	51
Total		138	69 (65)	162

CAZ), carbapenem (imipenem [IPM]), aminoglycosides (gentamicin [GEN], kanamycin [KAN], streptomycin [STR]), quinolone (nalidixic acid [NAL]), fluoroquinolone (ciprofloxacin [CIP]), tetracycline (tetracycline [TET]), phenicol (chloramphenicol [CHL]), fosfomycin (fosfomycin [FOF], and sulphonamides/dihydrofolate reductase (sulfamethoxazole-trimethoprim [SXT]). In brief, ESBL-Ec kept as glycerol stocks at −80°C were sub-cultured on Luria-Bertani agar and three to five colonies were collected and suspended in 5 m*l* of sterilized saline. The suspension was adjusted to achieve a turbidity equivalent to 0.5 McFarland standards. Sterile cotton swabs were used to prepare an evenly distributed bacterial lawn on Mueller-Hinton agar plates. Antimicrobial discs were placed on each bacterial lawn. The inhibition zone of each antimicrobial agent was analyzed after 16 to 18 hr of incubation at 37°C. Results were interpreted according to the CLSI guidelines [9]. *E. coli* strain ATCC 29522 was used as a control strain in the susceptibility test. Multidrug resistance (MDR) was defined as resistance to at least one antimicrobial agent from three or more antimicrobial classes [5].

Phylogenetic characterization

The phylogenetic grouping of ESBL-Ec was determined using a multiplex PCR as previously described [7].

Detection of virulence genes for DEC and ExPEC in ESBL-producing E. coli

ESBL-Ec isolated from chicken meats were analyzed for the presence of virulence genes associated with DEC including *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). ESBL-Ec were also analyzed for the virulence genes associated with ExPEC, which included *pap*EF, *pap*C, *sfa/foc*DE, and *afa*BC (adhesion), *hly*A and *cnf* (toxins), *fyu*A and *iron* (siderophores), *tra*T and *kpsMT* (protection and invasion), and PAI, *usp* and *ibeA* (others). Specific DNA probes for selected genes associated with DEC and ExPEC were prepared by PCR using primers and conditions as described previously [31, 34]. The *cnf* gene was amplified with Cnfcom2-U (5′-TCCAGTATGGGGATCAG-3′) and Cnfcom2-D (5′-GCATCTACTATGAAGTGG-3′) primers. The samples were subjected to an initial denaturation step at 94°C for 3 min followed by 30 cycles of amplification, each cycle consisting of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min. A final extension step at 72°C for 3 min was included. The PCR product was purified using a Wizard SV gel and PCR clean-up system (Promega Corporation, Madison, WI, U.S.A.), and labeled by random priming method using MultiPrime DNA Labeling System (GE Healthcare, Little Chalfont, U.K.) with [α-^{32}P]-dCTP (37 Bq/mmole) (Perkin Elmer, Boston, MA, U.S.A.). Virulence genes were detected by colony hybridization assay as described previously [20].

RESULTS

Isolation of ESBL-producing E. coli

E. coli-like colonies on MacConkey agar with CTX were isolated from 54 of 56 domestic chicken meats and from all 36 imported chicken meats from Brazil. On the other hand, *E. coli*-like colonies were isolated from 5 of 8 and 1 of 6 chicken meats from the US and Thailand, respectively (Table 1). Three colonies were selected from each sample and confirmed as *E. coli* by biochemical tests. One hundred and eleven *E. coli* from 43 domestic and 51 *E. coli* from 26 imported chicken meats from Brazil were identified to be ESBL producers by phenotypic analysis (Table 1). However, none of the *E. coli* isolated from the US and Thai chicken meats were ESBL producers.

Genotypic characterization of ESBL-producing E. coli

The 111 ESBL-Ec isolated from domestic and 51 from imported chicken meats were subjected to ESBL genotyping viz. $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8/25}}$, $bla_{\text{CTX-M-9}}$), bla_{TEM} and bla_{SHV}. If more than one isolate from the same chicken meat sample carried identical ESBL genes, only one representative isolate was selected for further analysis. Subsequently, 53 of 111 ESBL-Ec isolates from domestic and 30 of 51 isolates from Brazilian chicken meats were selected (Table 2). Analysis of ESBL genotypes revealed that 91% of the ESBL-Ec isolates from domestic chicken meats carried $bla_{\text{CTX-M}}$ followed by 36% of bla_{TEM} and 15% of bla_{SHV}, whereas all ESBL-Ec isolates from imported chicken meats were positive for $bla_{\text{CTX-M}}$ (100%) and 20% isolates carried bla_{TEM}. However, none of the ESBL-Ec isolates from imported chicken meats were positive for bla_{SHV} (Table 2). Among the

Table 2. Prevalence of ESBL genotypes in ESBL-producing *E. coli* isolated from retail chicken meats

ESBL genotypes	No. of isolates (%)	
	Domestic (n=53)	Imported (n=30)
bla_{CTX-M}	48 (91)	30 (100)
$bla_{CTX-M-1}$	18 (34)	1 (3.3)
$bla_{CTX-M-2}$	24 (45)	16 (53)
$bla_{CTX-M-8}$	1 (1.9)	13 (43)
$bla_{CTX-M-9}$	5 (9.4)	0 (0)
bla_{TEM}	19 (36)	6 (20)
bla_{SHV}	8 (15)	0 (0)

Table 3. Antimicrobial resistance of ESBL-producing *E. coli* isolated from retail chicken meats

Antimicrobial agents	No. of resistant isolates (%)	
	Domestic (n=53)	Imported (n=30)
AMP[a)]	53 (100)	30 (100)
FOX[b)]	2 (3.7)	0 (0)
CTX[c)]	53 (100)	30 (100)
CAZ[d)]	10 (19)	1 (3.3)
IPM[e)]	0 (0)	0 (0)
STR [f)]	37 (70)	23 (77)
KAN[g)]	31 (58)	12 (40)
GEN[h)]	0 (0)	0 (0)
CIP[i)]	13 (25)	8 (27)
NAL[j)]	33 (62)	19 (63)
TET[k)]	44 (83)	17 (57)
CHL[l)]	18 (34)	6 (20)
FOS[m)]	2 (3.8)	0 (0)
SXT[n)]	27 (51)	15 (50)

a) ampicillin, b) cefoxitin, c) cefotaxime, d) ceftazidime, e) imipenem, f) streptomycin, g) kanamycin, h) gentamicin, i) ciprofloxacin, j) nalidixic acid, k) tetracycline, l) chloramphenicol, m) fosfomycin, n) sulfamethoxazole-trimethoprim.

bla_{CTX-M} group, $bla_{CTX-M-2}$ (45%) was most prevalent followed by $bla_{CTX-M-1}$ (34%) and $bla_{CTX-M-9}$ (9.5%) in ESBL-Ec domestic chicken isolates. Similarly, $bla_{CTX-M-2}$ (53%) was most prevalent and $bla_{CTX-M-8}$ (43%) was the second most prevalent in ESBL-Ec imported chicken isolates. In some cases, two or three combinations of bla_{CTX-M}, bla_{TEM}, and/or bla_{SHV} were detected (Table 2).

PFGE

Based on the sample information and ESBL genotype, 53 and 30 ESBL-Ec isolates from domestic and imported chicken meats, respectively, were selected and analyzed concerning their clonal relationship by PFGE. *Xba*I-digested genomic DNA of 53 ESBL-Ec isolates from domestic chicken meats and 30 isolates from imported chicken meats generated 44 and 26 pulsotypes, respectively, at a cut-off level of more than 80%, suggesting a high level of genetic diversity in the isolates analyzed. However, there was no clonal relationship between isolates from domestic and imported chicken meats, suggesting that there was no cross contamination. Most of the isolates from the same supermarket showed no clonal relationship, indicating that the spread of ESBL-Ec was not due to clonal expansion (data not shown).

Antimicrobial susceptibility

The 53 and 30 ESBL-Ec isolates from domestic and imported chicken meats, respectively, were analyzed for their antimicrobial susceptibilities. As expected, all the tested ESBL-Ec isolates displayed resistance to AMP and CTX (Table 3). On the other hand, all the isolates were susceptible to IPM and GEN. The ESBL-Ec isolates from domestic chicken meats were mainly resistant to TET (83%), STR (70%), NAL (62%), KAN (58%) and SXT (51%), whereas ESBL-Ec isolates from imported chicken meats were resistant to STR (77%), NAL (63%), TET (57%), SXT (50%) and KAN (40%) (Table 3). Notably, extensive MDR bacteria, which is defined as resistance to at least five classes of antimicrobials, was detected in 60 and 70% of domestic and imported chicken meats, respectively (Table 4).

Phylogenetic characterization

On the basis of phylogenetic analyses, *E. coli* can be grouped into four phylogenetic lineages (A, B1, B2 and D). Most commensal *E. coli* belong to lineage A and B1 while virulent strains mainly belong to lineages B2 and D [32]. The results showed that 47 and 57% of the ESBL-Ec isolates from domestic and imported chicken meats, respectively, belonged to phylogenetic group B2/D. On the other hand, 53 and 43% of the ESBL-Ec isolates from domestic and imported chickens, respectively, were associated with the A/B1 lineage (Table 5).

Virulence gene profiles

The results of the virulence gene profiling are shown in Table 6. Of the 53 domestic and 30 imported chicken ESBL-Ec isolates, the prevalence of *astA* was higher in imported chicken meats (47%) than in domestic chicken meats (23%). The *cdtB* gene was detected in only one isolate from imported chicken meat, which also carried the *astA,* $bla_{CTX-M-2}$, and bla_{TEM} genes. However, none of the isolates were positive for *eaeA*, *bfpA*, *elt*, *est*, *stx1*, *stx2*, *invE, eagg* and *daaD* genes. On the other hand, concerning the virulence genes associated with ExPEC, *traT* displayed the highest prevalence in the domestic (60%) and imported (97%) chicken isolates (Table 6). The *pap* genes, which are associated with adhesion, were detected in 19 and 13% of the ESBL-Ec isolates from

Table 4. Multidrug resistance pattern in ESBL-producing *E. coli* isolated from retail chicken meats

Domestic			Imported		
NR[a)]	MDR pattern[b)]	NI[c)]	NR[a)]	MDR pattern[b)]	NI[c)]
8	AMP, CTX, STR, KAN, CIP, NAL, TET, CHL, SXT	7	8	AMP, CTX, STR, KAN, CIP, NAL, TET, CHL, SXT	1
	AMP, CTX, STR, KAN, NAL, TET, CHL, FOS, SXT	1		AMP, CTX, STR, CIP, NAL, TET, CHL, SXT	1
	AMP, CTX, STR, KAN, CIP, NAL, TET, FOS, SXT	1	7	AMP, CTX, KAN, CIP, NAL, CHL, SXT	1
7	AMP, CTX, CAZ, STR, KAN, NAL, TET, CHL, SXT	4		AMP, CTX, STR, KAN, CIP, NAL, TET, SXT	1
	AMP, CTX, STR, KAN, NAL, TET, CHL, SXT	3	6	AMP, CTX, STR, NAL, CIP, TET	3
	AMP, CTX, FOX, CAZ, STR, CIP, NAL, TET, SXT	1		AMP, CTX, STR, KAN, NAL, TET, SXT	1
	AMP, CTX, FOX, STR, NAL, TET, CHL, SXT	1		AMP, CTX, STR, KAN, CIP, NAL, TET	1
	AMP, CTX, STR, NAL, TET, CHL, SXT	1		AMP, CTX, STR, NAL, TET, SXT	1
	AMP, CTX, STR, KAN, CIP, NAL, TET, FOS	1		AMP, CTX, STR, NAL, TET, CHL	1
6	AMP, CTX, CAZ, STR, KAN, TET, CHL, SXT	1	5	AMP, CTX, STR, KAN, NAL, TET	1
	AMP, CTX, CAZ, STR, NAL, TET, SXT	1		AMP, CTX, STR, KAN, NAL, SXT	3
	AMP, CTX, STR, KAN, CIP, NAL, TET	1		AMP, CTX, KAN, TET, SXT	1
	AMP, CTX, STR, NAL, TET, SXT	3		AMP, CTX, STR, KAN, TET, CHL	1
	AMP, CTX, STR, CIP, NAL, TET	1		AMP, CTX, STR, NAL, SXT	2
5	AMP, CTX, STR, KAN, NAL, TET	3		AMP, CTX, STR, TET, SXT	2
	AMP, CTX, STR, KAN, TET, SXT	2	4	AMP, CTX, CAZ, NAL	1
4	AMP, CTX, STR, KAN, TET	3		AMP, CTX, STR, KAN, TET	1
	AMP, CTX, NAL, TET	2		AMP, CTX, STR, SXT	1
	AMP, CTX, KAN, TET	1		AMP, CTX, STR, TET	1
3	AMP, CTX, TET	5		AMP, CTX, STR, CHL	1
	AMP, CTX, CAZ, STR	1	3	AMP, CTX, NAL	1
	AMP, CTX, CAZ, KAN	2	2	AMP, CTX	3
	AMP, CTX, NAL	1			
2	AMP, CTX	5			
	AMP, CTX, CAZ	1			
Total		53	Total		30

a) Number of antimicrobial resistance classes according to CLSI [8], b) AMP (ampicillin), FOX (cefoxitin), CTX (cefotaxime), CAZ (ceftazidime), STR (streptomycin), KAN (kanamycin), CIP (ciprofloxacin), NAL (nalidixic acid), TET (tetracycline), CHL (chloramphenicol), FOS (fosfomycin), SXT (sulfamethoxazole-trimethoprim), c) Number of isolates.

Table 5. Distribution of phylogenetic groups of ESBL-producing *E. coli* isolated from retail chicken meats

Phylogenetic group	No. of isolates (%) by origin	
	Domestic (n=53)	Imported (n=30)
A	11 (21)	7 (23)
B1	17 (32)	6 (20)
B2	0 (0)	1 (3.3)
D	25 (47)	16 (53)

Table 6. Prevalence of virulence genes in ESBL-producing *E. coli* isolated from retail chicken meats

Origin	Prevalence of virulence genes										
	Diarrheagenic genes (%)[a)]			Extra intestinal pathogenic *E. coli* (ExPEC) genes (%)[b)]							
	astA	*cdtB*	Others	*papEF*	*papC*	*fyuA*	*iroN*	*kpsMT*	*traT*	PAI	Others
Domestic (n=53)	12 (23)	0 (0)	0 (0)	10 (19)	10 (19)	6 (11)	26 (49)	5 (9)	32 (60)	9 (17)	0 (0)
Imported (n=30)	14 (47)	1 (3.3)	0 (0)	4 (13)	4 (13)	6 (20)	13 (43)	8 (27)	29 (97)	8 (27)	0 (0)

a) *astA* (enteroaggregative *E. coli* heat-stable enterotoxin 1), *cdtB* (cytolethal distending toxin), others [*eaeA* (*E. coli*-attaching and effacing), *bfpA* (bundle-forming pilus), *elt* (heat-labile enterotoxin), *est* (heat-stable enterotoxin), *eagg* (plasmid of enteroaggregative *E. coli*), *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2), *invE* (invasin of EIEC), *daaD* (fimbriae adhesion)], b) *papEF*, *papC* (pilus associated with pyelonephritis, P fimbriae, adhesion), *fyuA* (yersiniabactin receptor, siderophore), *iroN* (catecholate receptor, siderophore), *kpsMT* (capsular polysaccharide synthesis, protection and invasion), *traT* (serum survival associated, protection and invasion), PAI (pathogenicity-associated island marker), others [usp (uropathogenic-specific protein), *ibeA* (invasion of brain endothelium), *sfa*/*focDE* (S and F1C fimbriae, adhesion), *afaBC* (Dr antigen-specific adhesion operons), *hlyA* (hemolysin, toxin) and *cnf* (cytotoxic necrotizing factor, toxin)].

Table 7. Distribution of virulence gene profile of ESBL-producing *E. coli* isolated from retail chicken meats

Domestic		Imported	
Virulence gene profile[a)]	NI[b)]	Virulence gene profile[a)]	NI[b)]
papEF, papC, iroN, traT, PAI, *astA*	1	*papEF, papC, kpsMT, traT, astA*	3
papEF, papC, fyuA, iroN, traT, PAI	2	*papEF, papC, kpsMT, traT*	1
iroN, kpsMT, traT, astA	1	*fyuA, kpsMT, traT, astA*	1
papEF, papC, iroN, traT	1	*iroN, traT, astA, cdtB*	1
papEF, papC, astA	1	*iroN, kpsMT, traT,* PAI	1
papEF, papC, traT	2	*iroN, traT*, PAI, *astA*	3
iroN, traT, PAI	2	*fyuA, traT,* PAI, *astA*	1
traT, PAI, *astA*	2	*kpsMT, traT, astA*	2
iroN, traT, astA	3	*fyuA, iroN, traT,* PAI	2
fyuA, iroN	3	*iroN, traT,* PAI	1
kpsMT, traT	2	*iroN, traT, astA*	1
kpsMT, astA	1	*fyuA, traT*	2
kpsMT, PAI	1	*iroN, traT*	3
traT, astA	1	*traT, astA*	1
fyuA, traT	1	*astA*	1
traT, PAI	1	*traT*	6
papEF, papC	3		
iroN, traT	9		
astA	2		
traT	4		
iroN	4		
None	6		
Total	53	Total	30

a) *astA* (enteroaggregative *E. coli* heat-stable enterotoxin 1), *cdtB* (cytolethal distending toxin), *papEF*, *papC* (pilus associated with pyelonephritis, P fimbriae, adhesion), *fyuA* (yersiniabactin receptor, siderophore), *iroN* (catecholate receptor, siderophore), *kpsMT* (capsular polysaccharide synthesis, protection and invasion), *traT* (serum survival associated, protection and invasion), PAI (pathogenicity-associated island marker), b) Number of isolates.

domestic and imported chicken meats, respectively. In addition, other ExPEC genes were also detected in domestic and imported chicken isolates (*iroN* 49 vs. 43%, PAI 17 vs. 27%, *fyuA* 11 vs. 20% and *kpsMT* 9 vs. 27%, respectively) (Table 6). However, none of the ESBL-Ec isolates was positive for *hlyA, cnf, sfa/focDE, afaBC, usp* and *ibeA* genes associated with ExPEC. DEC and ExPEC related genes were detected at a high rate in some of the domestic and imported chicken isolates (Table 7).

DISCUSSION

Food contaminated with ESBL-producing bacteria is a potential risk factor for their widespread dissemination in humans [24]. The present study was carried out to examine the prevalence of and characterize the ESBL-Ec isolates in domestic and imported retail chicken meats from August to December 2015 in Japan. The data showed that 77% of domestic and 52% of imported chicken meats (all from Brazil; 72% of the total imported meats) purchased from supermarkets in Izumisano City, Osaka, were contaminated with ESBL-Ec. Although chicken meats imported from three different countries were analyzed, ESBL-Ec was isolated from only Brazilian chickens. However, we could obtain only a limited number of chicken meats from the U.S. (n=8) and Thailand (n=6). Therefore, further study is required to understand the real picture of ESBL-Ec contamination in imported chicken meats from these two countries. Kawamura *et al.* [23] reported ESBL-Ec prevalence of 45 and 58% in domestic and imported (mainly from South America) retail chicken meats, respectively, collected in Aichi Prefecture, Japan, from January to October 2010. The prevalence of ESBL-Ec in the present study was higher than that reported previously [23]. This could be due to the different study locations in Japan or the different years of the studies.

Analysis of β-lactamase genes in this study revealed that most of the domestic chicken isolates carried $bla_{\text{CTX-M}}$ (91%) followed by bla_{TEM} (36%) and bla_{SHV} (15%). Hara *et al*. [17] reported that among ESBL-positive clinical *E. coli* strains, 94% of the isolates carried $bla_{\text{CTX-M}}$ but none carried bla_{TEM} and bla_{SHV}, indicating that $bla_{\text{CTX-M}}$ in clinical settings might have originated from animal foods. On the other hand, all the ESBL-Ec isolated from Brazilian chicken meats in this study carried $bla_{\text{CTX-M}}$ with a carriage of bla_{TEM} of 20% and no bla_{SHV} detected. Nogueira *et al*. [30] reported that $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} genes were detected in 77, 0.5, and 24% of clinical Enterobacteriaceae in Brazil, respectively. In this study, among CTX-M β-lactamase, most of the domestic chicken isolates carried $bla_{\text{CTX-M-2}}$ (45%) followed by $bla_{\text{CTX-M-1}}$ (34%) and $bla_{\text{CTX-M-9}}$ gene-groups (9.5%). These findings are also similar to those reported by Kawamura *et al* [23]. In Japan, $bla_{\text{CTX-M-2}}$ is common [19]. Thus, the higher prevalence of $bla_{\text{CTX-M-2}}$ in domestic chicken isolates may be relevant. However, a study conducted in Japan [6] demonstrated that among ESBL gene-positive *E. coli*, $bla_{\text{CTX-M-9}}$ (67%) was most prevalent followed by $bla_{\text{CTX-M-1}}$ (19%) and $bla_{\text{CTX-M-2}}$ (5.8%) genes in outpatients.

On the other hand, this study revealed that $bla_{CTX\text{-}M\text{-}2}$ (53%) and $bla_{CTX\text{-}M\text{-}8}$ (43%) were prevalent in the imported chicken isolates from Brazil. Dhanji *et al.* [11] also reported that in the U.K., $bla_{CTX\text{-}M\text{-}2}$ (54%) and $bla_{CTX\text{-}M\text{-}8}$ (46%) genes were prevalent in *E. coli* strains isolated from raw chicken imported from South America including Argentina, Brazil, and Chile. These data suggest that chickens produced in South America including Brazil might be contaminated with EBSL-Ec, in particular, $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}8}$, during production in Brazil but that cross-contamination during handling and processing does not occur in Japan. It is of interest to note that $bla_{CTX\text{-}M}$, bla_{TEM} and/or bla_{SHV} genes were detected more in domestic chicken meats than imported meats. Moreover, PFGE data revealed diverse DNA fingerprints in ESBL-Ec isolates from both domestic and imported chickens. This high genetic diversity indicated that the spread of ESBL-Ec is likely not due to the dissemination of particular clones but rather might be due to the spread of ESBL genes.

Plasmids bearing *bla* genes often carry multiple resistant genes against aminoglycosides, chloramphenicol, sulfonamide, trimethoprim, and tetracycline [4]. As expected, ESBL-Ec isolates from both domestic and imported chicken meats were 100% resistant to β-lactam antibiotics including AMP and CTX. However, ESBL-Ec isolates from domestic chicken meats showed the highest resistance to TET (83%) followed by STR (70%) and NAL (62%), whereas isolates from imported chicken meats showed the highest resistant to STR (77%) followed by NAL (63%) and TET (57%). Ahmed *et al*. [1] also reported that *E. coli* isolates from retail chickens in Japan were resistant to AMP (74%) followed by STR (67%), spectinomycin (58%), KAN (55%), and TET (54%), indicating that the STR and TET resistance rates are relatively high in chicken meats. STR and TET are often used as a growth promoter for broilers. Thus, the use of STR and TET in broiler production might have contributed to resistance against these antimicrobials. It is unclear, however, why both imported and domestic chicken isolates showed high resistance rate to NAL. One possibility is that enrofloxacin is often used for the treatment of broilers in farms [18]. Since fluoroquinolone is an important antimicrobial that is often used for human patients, it is important to continuously monitor the prevalence of quinolone-resistant bacteria in chickens.

The characterization of the phylogeny of *E. coli* suggests their association with diseases and occurrence in the environment. *E. coli* are generally characterized into four phylogenetic lineages (A, B1, B2 and D). B2 and D are considered to be pathogenic and are generally associated with extraintestinal infections. In this study, almost half of the ESBL-Ec from both domestic and imported chicken meats belonged to B2 or D (Table 5). The presence of ESBL-Ec in pathogenic phylogeny might imply that these isolates could pose a risk to humans.

To examine whether ESBL-Ec isolated from domestic and imported chicken meats are potentially pathogenic, virulence gene profiling was carried out. The profiles indicated that the *astA* gene was more prevalent in imported chicken isolates (47%) than domestic isolates (23%) (Table 6). Although the importance of EAST1 (*astA* gene product) as a virulence factor remains ambiguous, several reports have described the association of *astA* gene-positive *E. coli* in various outbreaks with gastroenteritis globally [28, 29, 37], indicating that EAST1 could be an important virulence factor in gastroenteritis. Moreover, one isolate from an imported chicken meat carried the *cdtB* gene. The cell lysate from *cdtB*-positive ESBL-Ec caused cell distention in CHO cells, suggesting that biologically active CDT is produced (data not shown). This CDT-producing ESBL-Ec from imported chicken meat belonged to phylogenetic group B2. Overall these findings suggest that imported chicken meats had a higher prevalence of potentially pathogenic *E. coli* possessing genes related to DEC. In the present study, a slightly higher prevalence of *papEF/papC* gene associated with ExPEC was found in domestic chicken meats (19%) in comparison to imported chicken meats (13%) (Table 6). However, the prevalence of other ExPEC related-genes including *fyuA*, *iroN*, *kpsMT*, *traT* and PAI was higher in imported chicken isolates. Moreover, a high prevalence of DEC and ExPEC related genes were found in both domestic and imported chicken isolates indicating their potential to cause infections. In previous studies, virulence gene profiles were not evaluated for ESBL-Ec isolates [1, 17, 23]. In this study, we analyzed the ESBL-Ec prevalence in chicken meats and also performed an extensive analysis of the virulence gene profile for virulence genes associated with DEC and ExPEC. These findings suggest that ESBL-Ec from both domestic and imported chicken meats might be harmful to humans as a cause of gastroenteritis and extra-intestinal infection such as urinary tract infection [26].

In conclusion, the prevalence of ESBL-Ec from domestic and imported chicken meats, in particular from Brazil, was similar and most were extensively MDR. The ESBL genotype and AMR profile differed between the domestic and imported chicken isolates. Both domestic and imported chicken isolates carried virulence related genes for DEC and mostly for ExPEC. Thus, retail chicken meats sold in Japan might contribute to serious public health problem as a reservoir of extensively MDR and potentially pathogenic ESBL-Ec.

ACKNOWLEDGMENTS. We thank Dr. Rupak K. Bhadra (CSIR-Indian Institute of Chemical Biology, Kolkata, India) for the critical reading of the manuscript. This study was performed in partial fulfillment of the requirements of a PhD thesis for Azimun Nahar from Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan. Azimun Nahar and Jayedul Hassan were recipients of the Monbusho Scholarship for a PhD program from the Ministry of Science, Culture and Sports of Japan.

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