原　著

Detection of Extended Spectrum β-Lactamase producing *Escherichia coli*（ESBL *E.coli*）from chicken meat in Niigata Prefecture, Japan

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要旨　The extended-spectrum β-lactamases (ESBLs) are the enzymes which degrade oxyimino-cephalosporins such as cefotaxime and ceftazidime, and make the antibiotics ineffective. In the past decade, drug resistance derived from Extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL *E. coli*) has been increasing dramatically worldwide. The ESBLs genes are located on plasmids that can be easily transferred between and within bacterial species. It is indicated the linkage of ESBL *E. coli* from the food producing meats and the clinical samples. The presence of ESBL genes has been clearly documented in chickens. In this study, 27 raw chicken livers acquired from a supermarket in Niigata were used for detecting ESBL *E. coli*. *E. coli* was identified by DHL and chromogenic agar results as 19 of 27 (70.3%) samples. Antibiotic susceptibility test identified 6 of 19 (31.5%) ampicillin resistant *E. coli* samples and 1 of 19 (5.2%) ESBL *E. coli* sample. With genotyping result, an ESBL *E. coli* isolated in this study was confirmed as CTX-M2.

Introduction

Extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL *E. coli*) is specific bacteria which produces enzymes (extended-spectrum β-lactamases; ESBL) that can hydrolyze the beta-lactam ring of beta-lactams and carbapenems, compound of many common antibacterial (eg. oxyimino-cephalosporins such as cefotaxime and ceftazidime) making the antibiotics treatment ineffective[1)]. During the past decade, drug resistance in ESBL *E. coli* has increased dramatically worldwide. ESBL-producing *E.coli* can cause a wide range of infections, from urinary tract infections to severe blood poisoning. Infections with ESBL-producing *E. coli* most commonly hit the elderly, people who have recently been in hospital and people who have received antibiotic treatment[2)].

The ESBLs genes are located on plasmids that can be easily transferred between and within bacterial species. The main ESBL types are TEM, SHV, and CTX-M. Rates of CTX-M infections have increased during the last decade compared with rates of TEM and SHV infections[3)]. Some ESBL genes are mutant derivatives of established plasmid-mediated β-lactamases (e.g., $bla_{\text{TEM/SHV}}$), and others are mobilized from environmental bacteria (e.g., $bla_{\text{CTX-M}}$). The presence of ESBL genes has been clearly documented in food-producing animals, especially chickens in different countries[2),3),4),5),6),7)]. Recent surveys of broiler chickens in Great Britain and Sweeden found $bla_{\text{CTX-M-1}}$ is a high prevalent ESBL gene[7),8)]. Genetic analysis showed that the predominant ESBL genes in chicken meat and human rectal swab specimens were identical. These genes were also frequently found in human blood culture isolates[5)], indicating the linkage of ESBL *E.*

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coli from the meats and the clinical samples[9].

In Shizuoka Prefecture, Japan, β-lactamase-producing *E. coli* from farm animals including chickens was surveyed, and the prevalence of CTX-M-type ESBL was estimated to be 13.6% (9.1% for CTX-M-2 and 4.5% for CTX-M-14) in broiler farms[10].

Aiming to add information on the occurrence of ESBL *E. coli* in Japan, this study surveyed for ESBL *E.coli* in chicken meat obtained from markets in Niigata Prefecture.

Material and methods

Sample selection and Preparation

During 15th of October to 20th of November 2013, 27 raw chicken livers were acquired from a supermarket in Niigata Prefecture, Japan. Samples were diluted at a ratio of 1 to 5 in normal saline. Samples were digested by stomacher and the broth (liquid part) was separated into a Petri dish.

Isolation and confirmation of *E.coli*

From the chicken liver broth obtained, 100µl aliquots were plated onto DHL (Deoxycholate Hydrogen Sulfide Lactose (EIKEN CHEMICAL Co., Ltd. Tochigi, Japan) agar and incubated aerobically at 42ºC overnight to allow *E. coli* growth. Pink colour non-mucoid colonies were collected from DHL agar and streaked onto chromogenic agar called Tricolor (Elmex Ltd, Tokyo, Japan) and incubated at 37ºC overnight. Blue colour colonies on chromogenic agar were confirmed the isolated organism as *E. coli*. Blue color colonies were re-streaked on nutrient agar for Antibiotic susceptibility test.

Antibiotic susceptibility test

The antibiotic susceptibilities of the *E. coli* isolates were determined by the Antibiotic susceptibility test method. The Clinical Laboratory Standards Institute (CLSI) guidelines were followed with regards to inoculum standardization, medium and incubation conditions. Bacterial suspension was prepared according to the 0.5 Mcfarland turbidity standard. Fifty microliters of suspension was spread evenly on Mueller Hinton (BD Difco, Sparks MD, U.S.A) agar. All *E. coli* isolates from the samples were tested for resistance against ampicillin (AM, 10 µg), ceftazidime (CTZ, 30 µg), ceftazidime/clavulanic acid (CTZ/CVA, 30 µg), cefotaxime (CTX, 30 µg), cefotaxime/clavulanic acid (CTX/CVA, 30 µg). Production of ESBL was confirmed, when an increase of the inhibition zone around cefotaxime and/or ceftazidime disks containing clavulanic acid exceed more than 5 mm compare to the disks without clavulanic acid.

Confirmation of *E. coli* by biochemical tests

Biochemical tests; H2S production, Esculin, Phenyl pyruvic acid, Indole, Voges-proskauer, Citrate, Lysine decarboxylase test, Arginine, Ornithine, o-Nitrophenyl-D-galactopyranoside, urease, Malonate, Adonitol, Inositol, Raffinose, Rhamnose, Sorbitol, Sucrose, Mannitol and Arabinose were performed as confirmatory tests of *E. coli*. After biochemical confirmation, ESBL *E. coli* samples were subjected to PCR analysis for the detection of ESBL genes.

Genotyping of ESBL *E. coli* (PCR)

The most prevalent ESBL gene is CTX-M and found most frequently among chicken meat isolates. Therefore the primer sets which detect CTX-M1 (forward; GCTGTTGTTAGGAAGTGTGC and reverse; CCATTGCCCGAGGTGAAG), CTX-M9 (forward; GCAGATAATACGCAGGTG and reverse; CGGCGTGGTGGTGTCTCT)[11] and CTX-M2 (forward; ACGCTACCCCTGCTATTT and reverse; CCTTTCCGCCTTCTGCTC)[12] ESBL groups were used for genotyping. ESBL *E. coli* DNA was extracted from organisms using boiling method. The target sizes were, CTX-M1: 516bp, CTX-M2: 779-780, CTX-M9: 393bp. PCR conditions were 2 min of initial denaturation at 94° C, followed by 35 cycles at 94° C for 1 min, 55° C for 1 min, and 72° C for 1 min 30 s and final extension at 72° C for 5 min. The PCR amplicons were electrophoresed in 2% agarose gel.

Results

A total of 27 fresh chicken liver samples were included in this study. DHL and chromogenic agar results were initially identified 19 (70.3%) samples containing *E. coli*. ABST identified 6 (31.5%) Ampicillin resistant *E. coli* samples and 1 (5.2%) ESBL *E. coli* sample. Ninety eight isolated *E. coli* colonies showed inhibition zones for CTZ, CTZ/CVA, CTX, and CTX/CVA. However some of them did not show inhibition zones for ampicillin. The isolated ESBL *E. coli* was ampicillin resistant and the difference of the inhibition

zone with cefotaxime disks containing clavulanic acid exceeded 14 mm compared to the disks without clavulanic acid (Table 1).

Table 1 Antibiotic susceptibility tests (ABST) of 7 samples showing lack of growth inhibition to 10 μg Ampicillin (AM) and its susceptibility for Cefotaxime (CTX) and Cefotaxime/Clavulanic Acid (CTX/CVA). Differences of growth inhibition using 30 μg CTX and 30 μg CTX/CVA is showed determining the ESBL *E. coli* (*) found in this study.

Sample No.	Inhibition zone diameter (mm)			Difference (mm)
	AM	CTX	CTX/CVA	CTX/CVA – CTX
2-1	0	33	33	0
3-5	0	31	32	1
4-19	0	33	33	0
6-30	0	39	39	0
7-37*	**0**	**19**	**33**	**14**
8-2	0	38	39	1
20-2	0	33	33	0

E. coli samples were re-confirmed by biochemical tests. All isolated *E. coli* strains showed positive results for O-Nitrophenyl-β-D-Galactopyranoside, Rhamnose, manitol and Arabinose tests and negative results for H2S production, Esculin, Phenyl pyruvic acid, Vogas-proskauer, Citrate, Arginine, Urease, Malonate and Inositol tests. ESBL *E. coli* showed positive results for Indol, Lysine decarboxylate test, O-Nitrophenyl-D-galactopyranoside, Raffinose, Rhamnose, Sorbitol, Sucrose, Manitol and Arabinose tests and negative results for H2S production, Esculin, Phenyl pyruvic acid, Vogas-proskauer, Citrate, Arginine, Ornithine, urease, Malonate, Adonitol, Inositol tests. Isolated *E. coli* strains and ESBL *E. coli* showed typical biochemical patterns. The PCR targeted CTX-M genotype group was conducted. DNA target sizes were, CTX-M1: 516bp, CTX-M2: 779-780, CTX-M9: 393bp. According to the CTX-M PCR results the sample was CTX- M1 and CTX- M9 negative (Figure 1). Under the CTX- M2 ESBL group, approximately 800 bp in size PCR product was observed. CTX-M2 DNA target size is 779-780 bp, therefore isolated ESBL *E. coli* genotype was confirmed as CTX- M2 (Figure 1).

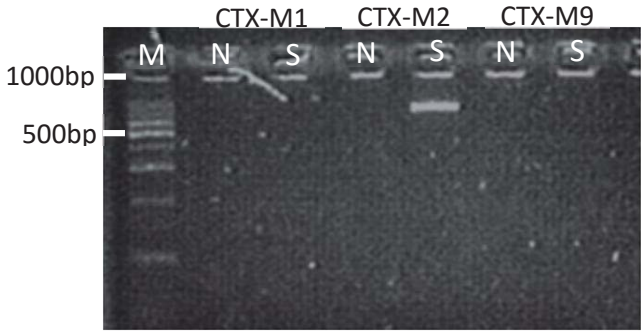

Figure 1 PCR targeting CTX-M group; CTX-M1, CTX-M2 and CTX-M9 using an ESBL *E. coli* isolated from chicken meat.
M: DNA marker (100bp ladder), N: Negative control, S: ESBL *E. coli* sample

Discussion

Contamination of food with ESBL *E. coli* has been reported worldwide [2), 3), 7), 8), 13)] however in Japan only limited data have been documented so far [10), 11), 12)]. In the present survey, ESBL *E. coli* occurrence was confirmed from chicken meat acquired in Niigata. All strains analyzed were confirmed as *E. coli* through biochemical tests; moreover the antibiotic resistant *E. coli* strains presented different biochemical patterns.

In this study, chicken samples were directly cultured and used for the antibiotic resistance tests with only one ESBL *E. coli* identified (Table 1). It is expected that the ESBL *E. coli* prevalence may be low in Niigata prefecture, and to determine the prevalence of ESBL *E. coli* Niigata, an enrichment culture and selective culture containing antibiotics might be useful for the improvement of the yield of *E. coli* in the samples, as recommended for scarce or non-dominant type of microorganisms [14)].

Confirmed ESBL *E. coli* has been confirmed as genotype CTX-M2. In further studies, DNA sequencing method can be used to determine the sequence of the amplified region and give more information on the antibiotic resistance genotype found in this work. In Japan, ESBL *E. coli* have been detected in clinical samples[15)]. Previous studies in Netherland demonstrated that the ESBL genes in the human intestinal *E. coli* population are derived from chicken meat [5), 13)], however this type of study has not yet done in Niigata. Therefore there is a potential link between ESBL *E. coli* genes in chicken meat and human samples [16)].

For further studies, it is needed to search more evidences on the linkage of ESBL *E. coli* from the meats and the clinical samples. Therefore it is important to examine the clinical samples and increase the sample number from the environment.

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