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Detection of CTX-M Type ESBL Producing *Salmonella* in Retail Meat in Korea

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ABSTRACT - This study was performed to evaluate antimicrobial resistance of food-borne pathogens isolated from retail meat in Korea. A total of 157 samples of beef, pork, and chicken were collected and analyzed for *E. coli, Salmonella, Campylobacter*. Resistances to tetracycline were declined in accord with reduced usage of tetracycline in live stock production. *E. coli* stains from chicken meat had higher multi-drug resistance ratio than strains from other meat. One extended spectrum beta lactamase (ESBL) producing *E. coli* and two ESBL producing *Salmonella* were identified in this study. ESBL producing Salmonella strains were confirmed to carry CTX-M-1 type genes.

Key words: Antimicrobial resistance, Food-borne pathogen, ESBL, CTX-M, Meat

Acquisition of antimicrobial resistance by bacteria became crucial problem of human health care. Supervision for use of antimicrobials in animal production is also needed to fight against the advent of multi-drug resistance bacteria that threaten health of human race. Emergence of microorganism selectively resistant to antimicrobials has been attributed to the significant misuse and unnecessary overuse of antimicrobials for human and animal health. The mechanisms of antimicrobial resistance are enzymatic degradation to antimicrobials, alteration of bacterial enzymes that are antimicrobial targets, changes in membrane permeability to antimicrobials, efflux of antimicrobials outside bacteria cell and etc. Antibiotic resistance can be driven by singly or in combination with other resistance determinants. (Tenover, 2006) Antimicrobials are extensively used in animal husbandry and antimicrobial resistance of *E.coli* and *Salmonella* is widely reported in many countries of the world. (D. Wasyl et al., 2012; Chen et al., 2004; Bertrand et al., 2006; Boyle et al., 2010; Jouini et al., 2009; Ryu et al., 2012) Likewise, antimicrobial resistance would cause serious damage not only from clinical use of antimicrobial agents but antimicrobial resistance bacteria caused by overuse of antimicrobials in animal husbandry, resulting in possible threat to public health. Therefore, it is necessary to continuously conduct surveys and researches on antimicrobial resistance. South Korea has been implementing national resistance monitoring system on food-borne pathogens since 2003 and successfully using the data of system to bring about changes in national policies and practices of antimicrobial use in animal husbandry. The objective of study is to evaluate the resistance rate against antimicrobials and a genotype of food isolates of the food-borne pathogens and indicator bacteria from retail meats.

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Materials and Methods

Sampling and isolation

Meat samples including beef ($n = 52$), pork ($n = 54$), chicken ($n = 51$) were collected from retail stores in five provinces of Korea (Seoul/Kyeonggi Do, Chucheong Do, Gyeongsang Do, Jeolla Do and Gangwon Do) during a three-month-period of April 2012-July 2012.

Three major food-borne pathogens (*Salmonella* spp., *E. coli, Campylobacter* spp.) from the samples were isolated by using enrichment medium and selected medium in accordance with Korea Food Code (2012). For the isolation of *Salmonella,* samples of 25 g were added to buffered peptone water and incubated at 37°C for 24 hours. A 0.1 mL of enriched broth was transferred to 10 mL of Rappaport-Vassiliadis broth enrichment medium (Merck, Germany) and incubated at 42°C for 24 hours. The enriched broth was streaked to XLD agar (Difco, USA) and after 24 hours of incubation at 37°C typical colonies of *Salmonella* were examined. For isolation of *E. coli*, samples of 25 g were added to EC broth (Difco, USA), and incubated at 37°C for 24 hours. The enriched broth was streaked to EMB agar

(Difco, USA) and incubated at 37ºC for 24 hours. After incubation typical colonies of *E. coli* were examined. For detection of *Campylobacter* spp. 100 mL of Bolton broth (Oxoid, UK) was added to 25 g of samples in tissue culture flask with filter cap (Nunc, Denmark) and incubated for 4 hours at 37ºC and 48 hours at 42ºC under microaerophilic conditions created by gas pak (Campygen, Oxoid, UK). The enriched broth was streaked to modified Campy blood free agar (Oxoid, UK) and incubated for 48 hours at 37ºC micro-aerobically. Typical colonies of *Campylobacter* spp. were selected. At least 3 typical colonies if possible of each microbes were selected and identified biochemically using VITEK2 system (Biomeriux, France).

Antimicrobial susceptibility testing

Isolated colonies were diluted to bacteria concentration equivalent to a 0.5 McFarland with Muller Hinton Broth (Difco, USA) and spreaded onto Muller-Hinton Agar plates (Difco, USA) with a sterile swab. The agar plates were dried for 3-5 min and inoculated using a disc dispenser. After incubation at 35ºC for 16-18 h, the size of the inhibition zone was determined, according to Clinical and Laboratory Standard Institute (CLSI) guidelines. Antimicrobial resistance of vancomycin, oxacillin was determined after 24 hours incubation. MIC (Minimum Inhibition Concentration) of antimicrobial resistant bacteria of ESBL was determined by micro-dilution method using Vitek2 system with AST-GN27 card (Biomeriux, France). The selected antimicrobial resistant bacteria was applied on TSA agar plate and incubated for 24 h. Bacterial colonies were suspended in 3 mL of 0.45% NaCl to a turbidity equivalent to 0.6 McFarland. Bacterial suspension of 145 μL were diluted respectively with 3 mL 0.45% saline and loaded in antimicrobial susceptibility testing card. Minimum Inhibition Concentration (MIC) and antimicrobial resistance were determined, according to the CLSI guidelines in the Vitek2 system program. The strains suspected as ESBL through micro-dilution were identified by using cefpodoxime combination disc kit (Oxoid, UK).

Identification of antimicrobial resistance gene

Regarding identified MRSA, VRE and ESBL isolates, the final identification test was conducted by using PCR. Anti-microbial resistance gene and primers are shown in Table 1. After taking one loop of sub-cultured colonies, genes were extracted using Ultra Clean Microbial DNA Isolation kit (MO BIO Laboratories Inc., CA, USA) and used as PCR

Table 1. Primers used in identification of resistance gene

Type of resistance	name	size	sequence	reference
VRE	*van*A	732 bp	F 5'- GCATGGCAAGTCAGGTG -3' R 5'- GATTCCGTACTGCAGCCT -3'	Lim et al., (AEM,2006, 72:6544-6553)
	*van*B	635 bp	F 5'- ATGGGAAGCCGATAGTC -3' R 5'- GATTTCGTTCCTCGACC -3'	Dutka-Malen et al., (JCM,1995,33:24-27)
	*van*C-1	822 bp	F 5'- GGTATCAAGGAAACCTC -3' R 5'- CTTCCGCCATCATAGCT -3'	Dutka-Malen et al., (JCM,1995,33:24-27)
	*van*C-2,3	439 bp	F 5'- CTCCTACGATTCTCTTG -3' R 5'- CGAGCAAGACCTTTAAG -3'	Dutka-Malen et al., (JCM,1995,33:24-27)
MRSA	mec A	533 bp	F 5'- AAAATCGATGGTAAAGGTTGGC -3' R 5'- AGTTCTGCAGTACCGGATTTGC -3'	Taweeporn et al., (Southeast Asian J. Trop. Med. Public Health, 2002, 33(4); 758-763)
ESBL	TEM	1058 bp	F 5'- TCCGCTCATGAGACAATAACC -3' R 5'- ACGCTCAGTGGAACGAAAAC-3'	Carattoli et al., (J. Microbial Methods, 2005, 63: 219-228)
	SHV	1071 bp	F 5'- CGCCGGGTTATTCTTATTTG -3' R 5'- CCACGTTTATGGCGTTACCT -3'	Carattoli et al., (J. Microbial Methods, 2005, 63: 219-228)
	CTX-M1	782	F 5'- CCGTCACGCTGTTGTTAGG -3' R 5'- ACGGCTTTCTGCCTTAGGTT -3'	Carattoli et al., (J. Microbial Methods, 2005, 63: 219-228)
	CTX-M2	832	F 5'- CGACGCTACCCCTGCTATT -3' R 5'- CAGAAACCGTGGGTTACGAT -3'	Carattoli et al., (J. Microbial Methods, 2005, 63: 219-228)
	CTX-M8	601	F 5'- GGCGCTGGAGAAAAGCAG -3' R 5'- GGTTTTATCCCCGACAACC -3'	Carattoli et al., (J. Microbial Methods, 2005, 63: 219-228)
	CTX-M9	862	F 5'- CAAAGAGAGTGCAACGGATG -3' R 5'- CCTTCGGCGATGATTCTC -3'	Carattoli et al., (J. Microbial Methods, 2005, 63: 219-228)
	GES	903	F 5'- GTTAGACGGGCGTACAAAGATAAT -3' R 5'- TGTCCGTGCTCAGGATGAGT -3'	Carattoli et al., (J. Microbial Methods, 2005, 63: 219-228)
	PER	513	F 5'- CCTGACGATCTGGAACCTTT -3' R 5'- TGGTCCTGTGGTGGTTTC -3'	Carattoli et al., (J. Microbial Methods, 2005, 63: 219-228)

template. Five microliters of templates were mixed with PCR premix (AccuPower, BIONEER, Korea) and amplified using PCR system (Veriti, Applied Biosystems, Singapore). Denaturation was conducted at 94ºC for 30 seconds and annealing at 58ºC for one minute and extension at 72ºC for one minute. PCR amplification cycles were repeated for 35 times. PCR products were electrophoresed in 2% agarose gel for 30 min and identified the size.

Pulsed Field Gel Electrophoresis (PFGE) and rep-PCR

PFGE typing was carried according to PulseNet protocol and the results were analyzed with Bio Numerics program (Applied Math, Belgium) using PulseNet recommended parameters. Rep-PCR was performed using DiversiLab (Biomeriux, France) and relevant PCR kit.

Results

Isolations from retail meat

In total 157 meat samples, 61 *E.coli* and 5 *Salmonella* strains were isolated and no *Campylobacter* was found. For *E. coli* 27 strains were isolated form chicken, 23 from beef, and 11 from pork. For *Salmonella*, all five were isolated from chicken meat. The five isolated *Salmonella* were all classified as serogroup C (2 strains of *S.* Montevideo and 3 strains of *S*. Virchow).

Antimicrobial resistance profiles

Antimicrobial resistance of *E. coli* was shown in Table 2.

Table 3. Multi-drug resistance of *E.coli* from meat

Antimicrobials	No. of resistance isolates (%)			
	Beefs (n=23)	Porks (n=11)	Chickens (n=27)	Total (n=61)
No resistance detected	12(52.2)	4(36.4)	0(0.0)	16(26.2)
Resistance 1 CLSI subclasses	5(21.7)	2(18.2)	1(3.7)	8(13.1)
Resistance 2 CLSI subclasses	5(21.7)	2(18.2)	2(7.4)	9(14.8)
Resistance 3 CLSI subclasses	0(0.0)	1(9.1)	4(14.8)	5(8.2)
Resistance 4 CLSI subclasses	1(4.3)	1(9.1)	3(11.1)	5(8.2)
Resistance 5 CLSI subclasses	0(0.0)	1(9.1)	6(22.2)	7(11.5)
Resistance 6 CLSI subclasses	0(0.0)	0(0.0)	5(18.5)	5(8.2)
Resistance 7 CLSI subclasses	0(0.0)	0(0.0)	5(18.5)	5(8.2)
Resistance 8 CLSI subclasses	0(0.0)	0(0.0)	1(18.5)	1(1.6)

The highest resistance rate was detected for tetracycline with 50.8% and resistance rate of ampicillin and nalidixic acid were 47.5% and 44.3% respectively. No clinically important resistant bacteria against imipenem, amikacin and cefepime were found. Two strains that were resistant to cefotaxime were found and double disk diffusion test and micro-dilution test were performed. Antimicrobial resistance rate of chicken meat is higher than those of other meats (data not shown).

Table 2. Antimicrobial resistance rate of Enterobacteriacea isolated from meat

Antimicrobials	Number of isolates of *E. coli*			Number of isolates of *Salmonella*		
	Resistant	Intermediate	Susceptible	Resistant	Intermediate	Susceptible
Ampicillin (AMP)	29	1	31	3	0	2
Cefazolin (KZ)	3	15	43	3	0	2
Cefotaxime (CTX)	2	1	58	2	1	2
Cephalothin (KF)	4	8	49	3	0	2
Cefepime (FEP)	0	1	60	0	1	4
Cefoxitin (FOX)	0	1	60	0	0	5
Amoxillin/clavulanic acid (AMC)	2	0	59	0	1	4
Imipenem (IPM)	0	0	61	0	0	5
Nalidixic acid (NA)	27	1	33	5	0	0
Norfloxacin (NOR)	4	10	47	0	0	5
Enrofloxacin (ENR)	19	4	38	1	0	4
Ciprofloxacin (CIP)	9	11	41	0	0	5
Amikacin (AK)	0	0	61	0	0	5
Trimethoprim/sulfamethoxazole (SXT)	17	1	43	0	0	5
Gentamicin (CN)	6	0	55	1	0	4
Tetracycline (TE)	31	8	22	2	1	2
Chloramphenicol (C)	15	1	45	0	0	5
Streptomycin (S)	19	8	34	2	0	3

In case of multi-drug resistance, 26.6% strains are susceptible to all antimicrobials and 45.9% strains are resistant against more than three antimicrobials (Table 3). For *Salmonella*, of five isolates, two strains were resistant only against nalidixic acid, and one strain against four antimicrobials, two strains against six antimicrobials (Fig. 2). Because the two strains which were resistance to six antimicrobials showed resistant to cefotaxime also, micro-dilution test and double-disk diffusion ESBL confirming test were conducted .

To confirm ESBL phenotype, microdilution test was performed for the 4 strains that were assumed to be ESBL through disk diffusion test (2 *E. coli* strains and 2 *Salmonella* strains). One isolate of *E.coli* resistant to cefotaxime was identified as susceptible strains in minimal inhibitory concenteration determination test. As a result of combination disk test, one *E.coli* strain and two *Salmonella* strains were identified as ESBL strains, equivalent to the result of micro dilution test.

Identification of antimicrobial resistance gene

Existence of resistance gene were identified by PCR for three ESBL strains. All three ESBL producing strains were identified to carry CTX-M1 genes.

PFGE and rep-PCR analysis of *Salmonella* spp.

Results of PFGE and rep-PCR to analyze genetic correlation of *Salmonella* are shown in Fig. 1 and Fig. 2. Though the two strains of *S.* Montevideo (sample ID 99, 100) were determined to be different by PFGE analysis with genetic similarity below 95%, they showed high similarity of 99.3% by rep-PCR. Because they also had same resistance profile (only against nalidixic acid), they were thought to be closely related. Two strains (sample ID 140, 179) identified as ESBL were found to be different in rep-PCR pattern and PFGE pattern.

Discussion

Regarding *E.coli*, the highest resistance rate was seen for

Fig. 1. Result of rep-PCR analyzing *Salmonella* spp.

tetracycline, but it declined from 80% in 2003 to 50.8% in 2012 which was at the same level as 2011. In Korea, the decreased use of tetracycline in food animals has led to low rate of resistance. (724 t in 2003, 308 t in 2011, www.kahpa.or.kr) However, the study showed that one of the strains isolated from the poultry meat sample produced ESBL and was resistant against 8 antimicrobial agents such as fluoroquinolone and tetracycline, showing the possible spread of antimicrobial resistance. And 17 out of 18 strains of severe multi-drug resistance (in this study, we classified it as resistant to more than 5 antimicrobial agents) were isolated from the chicken sample, showing the fact that chicken meat carried relatively higher multi-drug resistance bacteria than that of other meats. All the isolated *Salmonella* had resistance against nalidixic acid and that seemed highly associated with the rise of quinolone-resistant *Salmonella* worldwide. (Piddock, 2002, Miriagou et al., 2004). And it was the first time to identify CTX-M type ESBL-producing *Salmonella* from retail meat in Korea. In recent years, ESBL-producing *Salmonella* from meats has dramatically risen across the world (Wasyl. et al., 2012; Chen. et al., 2011). Studies of ESBL-producing *Salmonella* from animals and human in Korea had been reported several times (Tamang et al., 2011, Yong et al., 2005, Lee et al., 2003), but it was first time to identify CTX-M type ESBL-producing *Salmonella* from retail meats. The two identified strains identified in this study belonged to CTX-M-1 group. Despite the small number of strains, it showed the same results of the previous studies such as Tamang, which

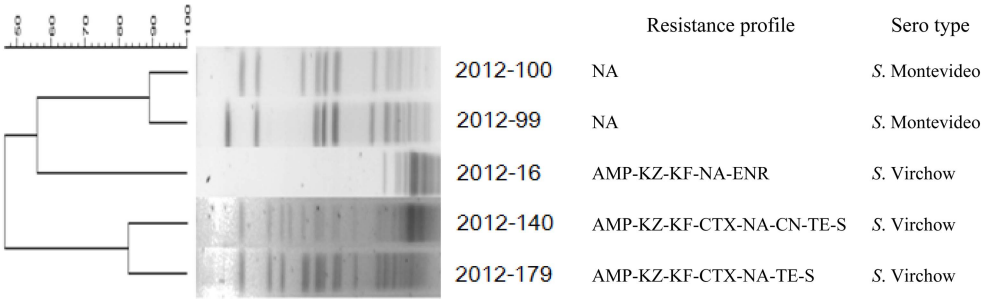

Fig. 2. Result of PFGE and antimicrobial resistance profile analyzing *Salmonella* spp.

identified most of 20 ESBL-producing *Salmonella* belonged to CTX-M-1 group. The two ESBL producing *S.* Virchow strains didn't seem to be single clone because they showed slightly different gene finger printing patterns and came from different places, slaughter houses and retailers. Two strains had resistance against 6 antimicrobial agents while maintained susceptibility to fluoroquinolone, showing that they were not affected by the recent prevalence of fluoroquinolone-resistance.

Campylobacter spp. was not found from all the samples in this study. That was quite different result from other study which showed contamination rate of 81.4% in chicken meat, 1.6% pork and 1.2% beef (Hong et al., 2007). It could be explained obligatory application of HACCP to chicken slaughter house in 2003 and recent increase of HACCP application. The number of livestock farms that applied HACCP was increased from 341 in 2010 to 6369 in 2012 (www.ihaccp.or.kr)

Conclusion

The decrease of tetracycline usage in producing live stock has induced to lower resistance rate of *E. coli* for tetracycline. However, this study showed that ESBL-producing *Salmonella* which had been seen only in human and animals in Korea were found in food meats and ESBL-producing *E.coli* was also found, identifying the possible spread of ESBL-resistant genes and strains to foods. In conclusion, it is necessary to maintain continuous surveillance on spread and prevalence of antimicrobial resistance through ongoing monitoring.

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