수의학석사 학위논문

The difference on the third generation cephalosporin resistance in *Escherichia coli* from raw milk depending on the use of ceftiofur

Ceftiofur 의 사용에 따른 국내 원유 분리 ***Escherichia coli*** 의 3 세대 세팔로스포린계 항생제에 대한 내성 차이

2018년 8월

서울대학교 대학원
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A dissertation submitted to the faculty of the
Graduate School of Seoul National University
in partial fulfillment of the requirements for
the degree of Master in Veterinary Microbiology

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이 논문을 수의학 석사 학위논문으로 제출함
2018 년 5 월

서울대학교 대학원
수의학과 수의미생물학 전공
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김재희의 석사 학위 논문을 인준함
2018 년 6 월

위 원 장 황 철 용 (인)
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Abstract

The antimicrobial resistance to the third-generation cephalosporins (3G-Ceph) in gram negative bacteria is an emerging concern worldwide. The prevalence of 3G-Ceph resistant bacteria in livestock has also increased in the past a few years in Korea. It is important to elucidate the relatedness between the use of 3G-Ceph and the increased antimicrobial resistance to these antimicrobials in local farms. The current study was performed to evaluate the effect of 3G-Ceph (ceftiofur) use on the increase of antimicrobial resistance by comparing the antimicrobial resistant rates between 3G-Ceph (ceftiofur) using and non-using farms. A total of 160 *Escherichia coli* (123 from using and 37 from non-using farms) were

isolated from raw milk. The antimicrobial resistant rate to 3G-Ceph was significantly higher in 3G-Ceph using farms (9.8%) than in non-using farms (2.7%). The MICs of Ceftriaxone, Cefotaxime were measured as less than 4 ug/ml in isolates from non-using farms while 64-1024 ug/ml from using farms. All the resistant isolates from using farms harbored a CTX-M gene, whereas none from non-using farms. Contaminated raw milk can be a vehicle for transmission of antimicrobial resistant bacteria from dairy to humans. The current study indicates the overuse of 3G-Ceph in dairy farms could increase the risk of transmission of 3G-Ceph resistant bacteria to a human community. Therefore, prudent use of this category of antimicrobials in dairy farms is warranted to prevent the dissemination of 3G-Ceph bacteria from animals to humans.

Keywords: Third generation cephalosporin, antimicrobial resistance, *Escherichia coli*, raw milk, dairy cow, CTX-M

Student number: 2016 - 27924

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Abbreviations

ATCC	American type culture collection
CLSI	Clinical and laboratory standards institute
ESBL	Extended-spectrum beta-lactamases
MDR	Multidrug resistance
MICs	Minimal inhibitory concentrations
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
3G-Ceph.	Third generation Cephalosporin

I. Introduction

Third generation cephalosporins are a class of β-lactam antibiotics that are often used to treat human infections by gram-negative bacteria, particularly *Escherichia coli*. The increasing incidence of human infections caused by third-generation cephalosporin-resistant *E.coli* is public health concern worldwide. CTX-M type extended-spectrum β-lactamases (ESBLs) are plasmid-mediated ESBLs which are the major cause of transferable resistance to third generation cephalosporins in gram negative bacteria. According to the recent studies, these *E.coli* strains, and their antibiotic resistance genes, can spread from food-producing animals to humans through the food-chain [1-4].

Contaminated raw milk can transfer zoonotic pathogens from animals to humans [5]. Bovine mastitis is an inflammatory disease of udder tissue that causes significant economic loss and increases the use of antimicrobial agents in dairy industry [6]. Mastitis is associated with a variety of bacteria, and gram-negative bacteria such as *E. coli* are considered to be important agents of environmental-related bovine mastitis [7, 8]. *E.coli* is the cause of toxic mastitis and is associated with acute or chronic disease, leading to high rates of death and culling of cattle [9].

Antimicrobial therapy is used for prevention and control of mastitis, but unfortunately treatment failure is common because of antimicrobial resistance

[10]. Various antimicrobial agents have been approved for the treatment of bovine mastitis in Korea, such as cephalosporins, tetracyclines, sulfonamides, penicillins, aminoglycosides, fluoroquinolones, and macrolides [11]. In particular, the domestic sales volume of cephalosporins have increased gradually over the past five years (2011-2015) [11]. Especially in 2014 and 2015, sales volume reached about 10 tons, and among them, the ceftiofur (3G-Ceph) accounts for about 90% of total sales. The third and fourth-generation cephalosporins, grouped in the critically important antimicrobials, have been used in the treatment of severe infection with multidrug-resistant gram-negative bacteria [12]. Thus, the increase in the use of 3G-Ceph in animals has raised concerns about the resistance on farms and the potential for spread of antibiotic-resistant bacterial strains to humans.

The CTX-M β-lactamases that express resistance to expanded spectrum cephalosporins by hydrolyzing β-lactam antibiotics are predominant ESBLs worldwide [13-15]. A variety of CTX-M β-lactamases are present in food producing animals and animal products [16]. Several CTX-M enzymes have been reported in bovine milk samples in Japan, the United Kingdom, France, and Korea [17-20]. However, few studies have reported relatedness between the use of 3G-Ceph and ESBLs in Korea. The current study was performed to compare

and characterize ESBL-producing *E.coli* from raw milk between 3G-Ceph using and non-using farms.

II. Materials and methods

1. Sampling and bacterial isolation

A total of 490 raw milk samples were collected from cows that were raised in 20 ceftiofur using (n =240) and 20 non-using farms (n=250) in Gyeonggi province in Korea from February to September in 2017. We call ceftiofur using farms as CT and non-using farms as NT, and CT2 and CT3, CT14 and CT15 farms are adjacent each other. From each cow, 50 ml of raw milk was collected in a sterilized tube and kept at 4°C until processed. In enrichment process, EC broth (Oxoid, England) was used as enrichment media. One ml of samples were inoculated 9 ml of EC broth and incubated 37°C, 18 hr. After incubation, the samples were streaked on the ECC agar (CHROMagar^{TM}ECC, Paris, France) and incubated 24 hr and only blue colonies were picked and inoculated on 5% sheep blood containing agar plates (Hangang, Anyang, Republic of Korea). These inoculated plates were incubated at 37°C for 24 hr. Only one *E.coli* isolate per sample was selected for further analysis. For confirmation of *E.coli*, PCR targeting the 16S ribosomal RNA region was used, as described previously in table 1 [21].

2. Identification and antimicrobial susceptibility tests of 3G-Ceph resistant *E. coli*

For all isolated *E.coli*, standard disk diffusion tests were performed according to the Clinical and Laboratory Standards Institute (CLSI) guideline [22] to identify 3G-Ceph resistant *E.coli* isolates and to determine their antimicrobial resistance profiles. Antimicrobial susceptibility tests were performed using the following antimicrobial disks (Becton Dickinson and Company, New Jersey, USA) : Ampicillin (AM), Amoxicillin/clavulanic acid 2:1 (AmC), Chloramphenicol (C), Ciprofloxacin (CIP), Imipenem (IPM), Sulfonamides (G), Trimethoprim/sulfamethoxazole (SXT), Tetracycline (TE), Cefazolin (CZ), Cefoxitin (FOX), Ceftazidime (CAZ), Cefotaxime (CTX), Cefixime (CFM), Ceftriaxone (CRO), and Cefepime (FEP). Among these, CAZ, CTX, CFM and CRO are 3G-Cephs. *E.coli* ATCC 25922 was used in the tests as a control strain.

3. MICs of 3G-Cephs (Ceftiofur, Ceftriaxone, Cefotaxime)

MICs of Ceftiofur (CEF) , CRO and CTX (Sigma, St Louis, MO, USA) for 3G-Ceph-resistant *E.coli* were determined by the broth microdilution method according to the CLSI guideline [22]. *E.coli* ATCC 25922 was used as a reference strain.

4. Detection of bla$_{CTX-M}$ genes

3G-Ceph-resistant *E.coli* isolates were screened by PCR to detect *bla*$_{CTX-M}$ genes. We prepared DNA template and PCR amplification was performed with primers as described previously [23, 37, 38]. The primers are listed in Table 1.

5. Pulsed-Field Gel Electrophoresis (PFGE)

3G-Ceph-resistant *E.coli* isolates were analyzed by standard pulsed-field gel electrophoresis (PFGE) using Xba1 (New England Biolabs, Ipswich, MA, USA) with CHEF MAPPER (Bio-Rad, Hercules, CA, USA) [24]. BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze DNA restriction patterns. *E. coli* ATCC 25922 was used as a reference strain.

III. Results

1. Isolation of 3G-Ceph resistant *E. coli* from raw milk

A total of 160 *E. coli* (32.7%) were isolated in 490 raw milk samples. Among the 160 *E.coli* isolates, 123 (76.9%) were from ceftiofur using farms, and the other 37 (23.1%) isolates were from non-using farms. Of the 160 isolates, 13 (8.1%) were confirmed as 3G-Ceph-resistant *E.coli*, among which 12 (9.8%) of 123 were from using farms and 1 (2.7%) of 37 were from non-using farms. Overall, *E.coli* isolation and 3G-Ceph resistance rates were higher in using farms than in non-using farms (Table 2).

2. Susceptibility of 3G-Ceph-resistant *E. coli* isolates to other antimicrobials

Disk diffusion tests revealed that all of 13 3G-Ceph resistant isolates were multidrug-resistant (MDR). *E.coli* isolates that exhibit resistance to more than three different classes of antibiotics were defined as MDR *E. coli* according to the previous terminology [25]. Of 13 3G-Ceph resistant isolates, all were also resistant to AM, TE, CZ, and AMC for only ceftiofur non-using farms (1/13, 7.7%), and C for only using farms (12/13, 92.3%) (Table3).

3. Determination of MICs

The MICs of 13 3G-Ceph-resistant *E.coli* isolates ranged from to 4 to ≥ 1024 μg/ml. Among them, 12 isolates, that came from ceftiofur-using farms showed high MICs of 3G-Ceph (≥ 1024 μg/ml for CEF, ≥ 512 μg/ml for CRO and ≥ 64 μg/ml for CTX). On the other hand, 1 isolate from ceftiofur-non using farms showed relatively low MICs of 3G-Ceph (32 μg/ml for CEF, 4 μg/ml for CRO and 4μg/ml for CTX) (Table 3).

4. Detection of bla_{CTX-M} genes

All of the resistant strains isolated from the ceftiofur-used farms have bla_{CTX-M} genes, but the resistant strains isolated from the non-used farms didn't have bla_{CTX-M} genes (Table 3). The isolates from CT2 and CT3 farms, which are adjacent each other, possess same group of bla_{CTX-M} genes, group 9 and also CT14-2 and CT15-5 possess same group of bla_{CTX-M} genes, group 1.

5. Genotyping of 3G-Ceph resistant *E. coli* by PFGE

10 types of PFGE patterns were revealed. Among them, patterns of CT3-7 and CT3-8, CT2-1 and CT2-3 as well as CT2-8 and CT3-4 are identical, respectively (Figure 1). Isolates from the same farms and adjacent farms, CT2 and CT3 farms showed the same PFGE patterns.

IV. Discussion.

Bovine mastitis is the most common and economically important disease caused by gram negative organisms, such as *E.coli* [7, 8]. Antimicrobial agents have been frequently used to treat infection of mammary in dairy cows [26, 27]. Although total antibiotic consumption in cattle has been declining in in Korea, the use of ceftiofur, a third-generation cephalosporin, has been increasing in veterinary medicine in Korea [28].

In this study, we investigated the third generation cephalosporin antibiotic resistance from dairy cows in Republic of Korea. Resistance rates to third-generation cephalosporins were higher in this study (8.1%) than in the previous study in Korea (4.0%) (2012 – 2015) [18], EU (0%, 0/280 *E. coli*) [29], Canada (0.8%, 3/394 *E. coli*) [8] and France (0.4%, 6/1,427 *E. coli* and *Klebsiella pneumoniae* isolates) [17]. Overall, 3G-Ceph resistance rates showed higher in using farms than in non-using farms in this study.

As a result of antimicrobial tests and detection of CTX-Ms in 13 *E. coli* resistant to third generation cephalosporin antibiotics, the strain isolated from the non-used farms showed a low MIC for the 3G-Ceph and did not have a resistant gene associated with 3G-Ceph. On the other hand, 12 resistant strains isolated from the used farms showed high MIC for the 3G-Ceph and were confirmed that they possessed resistant gene, CTX-Ms. As seen in the results, the isolates from

same farms and adjacent farms have same group of CTX-Ms, which means they exhibit >94% amino acid identity [30]. Furthermore, our results are consistent with previous studies of ESBL-producing *E. coli* strains reporting dissemination of *E. coli* isolates possessing bla_{CTX-M} genes of the CTX-M-1 or CTX-M-9 family among food animals from the Republic of Korea [31], China [32], and Europe [33-35].

PFGE analysis revealed clonal spreading of 3G-Ceph resistant bacteria in farms between CT2 and CT3 farms. As seen in the PFGE patterns of CT3-7 and CT3-8, CT2-1 and CT2-3 as well as CT2-8 and CT3-4, clonal spreading was observed between CT2 and CT3 farms and this means they are presumed to be derived from a common parent [36].

Contaminated raw milk can be a means of delivering antimicrobial resistant bacteria to humans [5]. The current study indicates the overuse of 3G-Ceph in dairy farms could increase the risk of transmission of 3G-Ceph resistant bacteria to a human community. Therefore, prudent use of this category of antimicrobials in dairy farms is warranted to prevent the dissemination of 3G-Ceph bacteria from animals to humans.

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Table 1. Sequences of primers

Target	Primers	Sequence (5′→3′)	Reference
E. coli	*E. coli* 16S rRNA	F: GGGAGTAAAGTTAATACCTTTGCTC	[21]
		R1: TTCCCGAAGGCACATTCT	
		R2: TTCCCGAAGGCACCAATC	
CTX-M	bla_{CTX-M}	Pan CTX-M F: TTTGCGATGTGCAGTACCAGTAA	[37]
		Pan CTX-M R: CGATATCGTTGGTGGTGCCATA	
	Group I	CTX-M1-F3: GACGATGTCACTGGCTGAGC	[23]
		CTX-M1-R2: AGCCGCCGACGCTAATACA	
	Group II	TOHO1-2F: GCGACCTGGTTAACTACAATCC	
		TOHO1-1R: CGGTAGTATTGCCCTTAAGCC	
	Group III	CTX-M825F: CGCTTTGCCATGTGCAGCACC	
		CTX-M825R: GCTCAGTACGATCGAGCC	
	Group IV	CTX-M914F: GCTGGAGAAAAGCAGCGGAG	
		CTX-M914R: GTAAGCTGACGCAACGTCTG	
	Group 9	M9 F: ATGGTGACAAAGAGAGTGCA	[38]
		M9 R: CCCTTCGGCGATGATTCTC	

Table 2. Proportions of 3G-Ceph resistant *E.coli* in the ceftiofur using and non-using farms

Farms	Number of *E.coli*/Samples	Number of 3G-Ceph resistant *E.coli*/Isolated *E.coli*
Using Farms (n =20)	123/240 (51.3%)	12/123 (9.8%)
Non-using Farms (n = 20)	37/250 (14.8%)	1/37 (2.7%)
Total (n = 40)	160/490 (32.7%)	13/160 (8.1%)

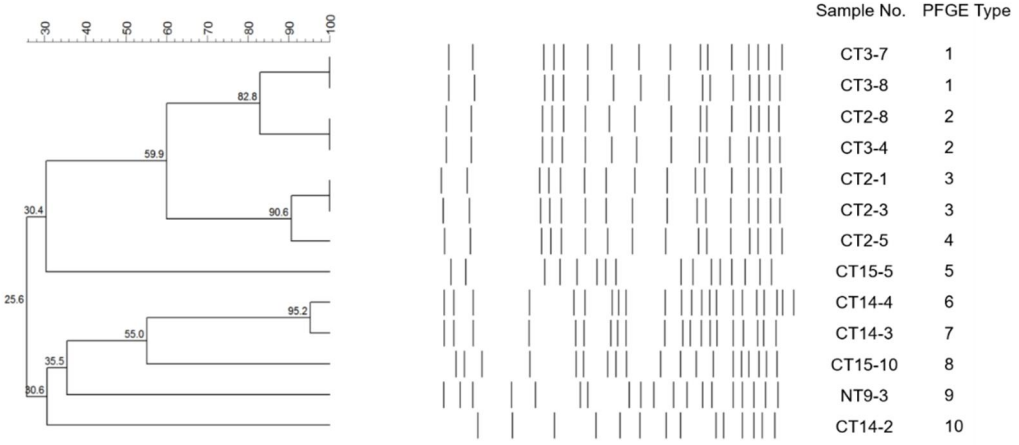

Figure 1. PFGE dendrogram of Xba-1 digested genomic DNA from 13 3G-Ceph resistant *E.coli* isolates. Patterns are shown with sample number and PFGE pulsotype

Table 3. Profiles of 3G-Ceph resistant isolates

Antibiotics usage	Isolate	MIC(μg/ml)			Antimicrobial resistance	*bla*$_{CTX-M}$	PFGE type
		CEF	CRO	CTX			
Non-using	NT9-3	32	4	4	AM, AMC, G, SXT, TE, CZ, FOX, CAZ, CTX, CFM	-	9
Using	CT2-1	1024	512	64	AM, C, TE, CZ, CTX, CFM, CRO	CTX-M$_{G9}$	3
	CT2-3	1024	512	256	AM, C, TE, CZ, CTX, CFM, CRO	CTX-M$_{G9}$	3
	CT2-5	1024	512	256	AM, C, TE, CZ, CTX, CFM, CRO	CTX-M$_{G9}$	4
	CT2-8	1024	512	256	AM, C, TE, CZ, FOX, CTX, CFM, CRO	CTX-M$_{G9}$	2
	CT3-4	1024	512	128	AM, C, CIP, TE, CZ, CTX, CFM, CRO	CTX-M$_{G9}$	2
	CT3-7	1024	512	256	AM, C, TE, CZ, CTX, CFM, CRO	CTX-M$_{G9}$	1
	CT3-8	1024	512	256	AM, C, TE, CZ, CTX, CFM, CRO	CTX-M$_{G9}$	1
	CT14-2	1024	≥1024	1024	AM, C, G, SXT, TE, CZ, CTX, CFM, CRO, FEP	CTX-M$_{G1}$	10
	CT14-3	1024	1024	256	AM, C, G, SXT, TE, CZ, CTX, CRO, FEP	CTX-M$_{G9}$	7
	CT14-4	1024	≥1024	512	AM, C, G, SXT, TE, CZ, CTX, CRO, FEP	CTX-M$_{G9}$	6
	CT15-5	1024	≥1024	512	AM, C, G, SXT, TE, CZ, CTX, CFM, CRO, FEP	CTX-M$_{G1}$	5
	CT15-10	≥1024	512	128	AM, C, G, SXT, TE, CZ, CTX, CRO	CTX-M$_{G9}$	8

국문초록

Ceftiofur 의 사용에 따른 국내 원유 분리 *Escherichia coli* 의 3 세대 세팔로스포린계 항생제에 대한 내성차이

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그람 음성 세균의 3 세대 세팔로스포린계 항생제에 대한 항생제 내성은 전 세계적으로 문제가 되고 있다. 우리나라의 가축에서도 이러한 3 세대 세팔로스포린계 항생제에 내성을 가지는 세균이 몇 년 동안 증가해왔다. 따라서 지역 농장에서의 3 세대 세팔로스포린계 항생제의 사용과 이로 인한 항생제 내성 사이의 관련성을 밝히는 것이 중요하다. 본 연구는 3 세대 세팔로스포린계 항생제 중 우리나라 가축에서 가장 많은 사용량을 차지하는 ceftiofur 를 사용한 농장와 사용하지 않은 농장 간의 항생제 내성 비율을 비교하여 3 세대 세팔로스포린계 항생제의 사용이 항생제 내성 증가에 미치는 영향을 평가하기 위해 수행되었다. 원유에서 총 160 개의 *Escherichia coli* (ceftiofur 사용농장에서 123 개 및 사용하지 않은 농장에서

37 개) 가 분리되었다. 3 세대 세팔로스포린계에 대한 항생제 내성률은 ceftiofur 를 사용하지 않은 농장 (2.7 %)보다 사용한 농장 (9.8 %)에서 더 높았다. Ceftriaxone, Cefotaxime 의 MIC 값은 항생제 비사용 농장에서 분리된 균의 경우 4 ug/ml 미만인 반면, 항생제 사용 농장에서 분리된 균의 경우에는 64-1024 ug/ml 으로 측정되었다. 또한 항생제 사용농장에서 분리된 모든 3 세대 세팔로스포린계 내성 균주는 CTX-M 유전자를 가지고 있는 반면, 항생제 비사용 농장에서는 가지고 있지 않았다. 오염 된 원유는 사람에게 항생제 내성 박테리아를 전달하는 수단이 될 수 있다. 젖소 농장에서 3 세대 세팔로스포린계 항생제의 과용은 동물 뿐 아니라 사람에게도 내성 세균이 전파 될 가능성이 있으므로, 그 사용을 신중하게 해야 할 것이다.

주요어: 3 세대 세팔로스포린계 항생제, 항생제 내성, *Escherichia coli*, 우유, 젖소, CTX-M

학 번: 2016-27924