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Molecular characteristics of *Escherichia coli* from bulk tank milk in Korea

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The authors declare no conflicts of interest.

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ABSTRACT

Background: *Escherichia coli*, which causes subclinical or clinical mastitis in cattle, is responsible for transmitting antimicrobial resistance via human consumption of raw milk or raw milk products.
Objectives: The objective of this study was to investigate the molecular characteristics of 183 *E. coli* from bulk tank milk of five different dairy factories in Korea.
Methods: The molecular characteristics of *E. coli* such as serogroup, virulence, antimicrobial resistance, and integron genes were detected using polymerase chain reaction and antimicrobial susceptibility were tested using the disk diffusion test.
Results: In the distribution of phylogenetic groups, group D was the most prevalent (59.6%) and followed by group B1 (25.1%). The most predominant serogroup was O173 (15.3%), and a total of 46 different serotypes were detected. The virulence gene found most often was *fimH* (73.2%), and *stx1*, *fimH*, *incC*, *fyuA*, and *iutA* genes were significantly higher in isolates of phylogenetic group B1 compared to phylogenetic groups A, B2, and D ($p < 0.05$). Among 64 *E. coli* isolates that showed resistance to at least one antimicrobial, the highest resistance rate was observed for tetracyclines (37.5%). All 18 integron-positive *E. coli* carried the integron class I (*int1*) gene, and three different gene cassette arrangements, *dfrA12+aadA2* (2 isolates), *aac(6′)-Ib+aac(6′)-Ib-cr+aadA4* (2 isolates), and *dfrA17+aadA5* (1 isolate) were detected.
Conclusions: These data suggest that the *E. coli* from bulk tank milk can be an indicator for dissemination of antimicrobial resistance and virulence factors via cross-contamination.

Keywords: Bulk tank milk; antimicrobial resistance; integron; virulence; *Escherichia coli*

INTRODUCTION

Escherichia coli can be found in animals' digestive systems as commensal bacteria and are easily observed in the environment [1]. Therefore, *E. coli* can quickly contaminate the bovine mammary gland and cause clinical mastitis [2]. Although the efficacy of antimicrobial treatment for *E. coli* mastitis is reported as limited, broad-spectrum antimicrobials are commonly used in farms [3].

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Recently, the spreading of antimicrobial-resistant *E. coli* are recognized more and concerns are increasing in humans and animals [1,4]. In particular, the continuous use of antimicrobials for the treatment and prevention of bovine mastitis has contributed to the emergence and sustenance of antimicrobial-resistant *E. coli* by genetic mutation or horizontal gene transfer [5]. Antimicrobial resistance genes are carried on plasmids, transposons, or integrons that can be vectors that transfer these genes to the same or another bacterial species, and gene transfer can potentially be a threat to public health [6].

The pathogenic trait of *E. coli* isolated from bulk milk can also play an important role in human health risk. *E. coli* strains are commonly classified into phylogenetic groups A, B1, B2, and D [7]. Groups B2 and D are associated with extraintestinal infections and regarded as more invasive strains [8]. Virulence factors also usually responsible for the ability to infect a host, and exchange of them can happen via horizontal gene transfer. Moreover, virulence factors contributing to iron uptake can provide antimicrobial resistance to host immunological defenses [9]. Although the characteristics of antimicrobial-resistant *E. coli* from bovine mastitic milk have been described in South Korea [5,10], those in bulk tanks which can transfer antimicrobial resistance asymptomatically have not been fully described. Therefore, this study aimed to determine the characteristics of *E. coli* isolated from bulk tank milk to provide important consideration for the management of dairy herds.

MATERIALS AND METHODS

Sample collection
A total of 1160 batches of bunk tank milk were collected from 290 dairy farms of five different dairy factories operated by three companies in Korea. Each 50 mL milk sample was aseptically collected twice, in summer (July 2019) and winter (December 2019), and sent to the laboratory under 4°C conditions.

Bacterial identification
The isolation and identification of *E. coli* were performed following the standard microbiological protocols published by the Ministry of Food and Drug Safety (2018) [11]. Briefly, 1 mL of each milk sample was inoculated in 9 mL mEC broth medium (Merck, Darmstadt, Germany). After incubation at 37°C for 24 h, a loopful of medium (~10 μL) was streaked onto MacConkey agar (BD Biosciences, NJ, USA) and incubated at 37°C for 24 h. The confirmation of *E. coli* was performed using polymerase chain reaction (PCR) to detect *malB*, as described previously [12]. If two isolates of the same origin showed the same antimicrobial resistance patterns, only one isolate was randomly chosen and included in this study.

Phylogenetic groups and serogrouping
All 183 *E. coli* isolates included in this study were categorized by the phylogenetic group and serogrouping with PCR-based typing, as described previously by Clermont et al. [7] and Iguchi et al. [13], respectively.

Antimicrobial susceptibility testing
Based on the Clinical and Laboratory Standards Institute guidelines [14], all *E. coli* isolates were investigated for antimicrobial resistance using the disk diffusion test with the following disks (BD Biosciences): ampicillin (AM; 10 μg), amoxicillin-clavulanate (AMC; 20 μg), chloramphenicol (C; 30 μg), ceftazidime (CAZ; 30 μg), cefadroxil (CDX; 30 μg),

cephalothin (CF; 30 μg), ciprofloxacin (CIP; 5 μg), colistin (CL; 10 μg), cefotaxime (CTX; 30 μg), cefuroxime (CXM; 30 μg), cefazoline (CZ; 30 μg), cefepime (FEP; 30 μg), cefoxitin (FOX; 30 μg), gentamicin (GM; 10 μg), imipenem (IPM; 10 μg), nalidixic acid (NA; 30 μg), trimethoprim/sulfamethoxazole (SXT; 1.25 μg), and tetracycline (TE; 30 μg). *E. coli* ATCC 25922 was used as the quality control. Multidrug resistance (MDR) was defined as acquired resistance to at least one agent in three or more antimicrobial classes.

Detection of virulence, antimicrobial resistance, and integron genes

The detection of virulence, antimicrobial resistance, and integron genes was performed using PCR with the primers listed in **Supplementary Table 1**. The target virulence genes included in this study are *eaeA*, *stx1*, *stx2*, *hly*, *fimH*, *iucC*, *fyuA*, and *iutA*. The antimicrobial resistance determinants were genes conferring resistance to β-lactamase (bla_{OXA}, bla_{TEM}, bla_{SHV}, and bla_{CTX}), aminoglycosides [*aac(6′)-Ib*, *aac(3)-II*, and *ant(2″)-I*], tetracyclines (*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, and *tetG*), sulfonamides (*sul1* and *sul2*), and chloramphenicol (*catA1* and *cml1*). In particular, β-lactamase and integron cassette gene amplicons were sequenced with an automatic sequencer (Cosmogenetech, Seoul, Korea) and compared to those in GenBank using the Basic Local Alignment Search Tool program available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST) and Resfinder 4.0 (https://cge.cbs.dtu.dk/services/ResFinder/).

Statistical analysis

Statistical analyses were performed using SPSS version 25 (IBM Corp., USA). The prevalence of antimicrobial resistance and the distribution of virulence and antimicrobial genes of isolates by factories were compared using χ^2 test. When the expected count for χ^2 test is < 5, which can lead to inaccurate interpretation, Fisher's exact test was used as an alternative method, and $p < 0.05$ was considered statically significant [15].

RESULTS

Distribution of phylogenetic groups, serogroups, and virulence genes of *E. coli* isolates

The distribution of phylogenetic groups and virulence genes of 183 *E. coli* isolates are shown in **Table 1**. Phylogenetic group D was the most prevalent (109 isolates; 59.6%), followed by group B1 (46 isolates; 25.1%), group A (23 isolates; 12.6%), and group B2 (5 isolates; 2.7%). One hundred fifty-seven (85.8%) isolates were classified into 46 different serotypes, with 26 isolates remaining ungrouped. The most predominant serogroup was O173 (28 isolates; 15.3%), which all belong to phylogenetic group D. The virulence gene found most often was *fimH* (134 isolates; 73.2%), followed by *eaeA* (37 isolates; 20.2%), *iucC* (26 isolates; 14.2%), *fyuA* (9 isolates; 4.9%), *iutA* (6 isolates; 3.3%), and *stx1* (3 isolates; 1.6%). However, *fimH* was the most prevalent virulence gene in phylogenetic groups B1, B2, and D. In phylogenetic group A, only 1 (4.3%) of 23 isolates carried *fimH*. Interestingly, *stx1*, *fimH*, *incC*, *fyuA*, and *iutA* genes were significantly higher in isolates of phylogenetic group B1 than in phylogenetic groups A, B2, and D ($p < 0.05$). All isolates did not carry the *stx2* and *hly* genes.

Prevalence of antimicrobial-resistant *E. coli* isolates

The prevalence of antimicrobial-resistant *E. coli* isolates by factories is shown in **Table 2**. Among 64 *E. coli* isolates that showed resistance to at least one antimicrobial, the highest resistance rate was observed in TE (37.5%), followed by CF (35.9%), AM (34.4%), GM (26.6%), CL

Table 1. Distribution of phylogenetic groups and virulence genes of 183 *E. coli* isolates from bulk tank milk of five dairy factories

Phylogenetic group	No. (%) of isolates	No. (%) of isolates with each virulence gene								O Serotypes (No. of isolates)
		eaeA	*stx1*	*stx2*	*hly*	*fimH*	*iucC*	*fyuA*	*iutA*	
Group A	23 (12.6)	0 (0)[a]	0 (0)[a,b]	0 (0)	0 (0)	1 (4.3)[a]	0 (0)[a]	0 (0)[a,b]	0 (0)[a,b]	O3 (1), O6 (1), O10 (1), O29 (1), O60 (1), O86 (1), O88 (5), O98 (2), O109 (4), O117 (1), O127 (1), O156 (1), O158 (1), O165 (1), O179 (1)
Group B1	46 (25.1)	7 (15.2)[a,b]	3 (6.5)[b]	0 (0)	0 (0)	44 (95.7)[b]	19 (41.3)[b]	9 (19.6)[b]	6 (13.0)[b]	O3 (1), O10 (2), O11 (3), O15 (2), O26 (1), O39 (1), O102 (3), O109 (3), O112 (1), O117 (1), O142 (1), O147 (1), O158 (3), O170 (1), O174 (2), O177 (1), Ogp8 (1)*, ONT (18)[†]
Group B2	5 (2.7)	0 (0)[a,b]	0 (0)[a,b]	0 (0)	0 (0)	4 (80.0)[b,c]	1 (20.0)[a,b]	0 (0)[a,b]	0 (0)[a,b]	O44 (2), O99 (2), O126 (1)
Group D	109 (59.6)	30 (27.5)[b]	0 (0)[a]	0 (0)	0 (0)	85 (78.0)[c]	6 (5.5)[a]	0 (0)[a]	0 (0)[a]	O3 (4), O8 (1), O10 (3), O11 (2), O15 (12), O25 (1), O28 (1), O38 (1), O51 (3), O52 (1), O55 (1), O60 (1), O74 (1), O76 (2), O80 (1), O88 (13), O95 (1), O96 (6), O98 (4), O100 (1), O102 (1), O109 (4), O112 (1), O125 (1), O137 (1), O153 (3), O158 (2), O173 (28), ONT (8)
Total	183	37 (20.2)	3 (1.6)	0 (0)	0 (0)	134 (73.2)	26 (14.2)	9 (4.9)	6 (3.3)	

Values within a column not having the same superscript letter differ significantly ($p < 0.05$).
*O107 or O117; [†]Not determined.

Table 2. Distribution of 64 antimicrobial-resistant *E. coli* isolates from bulk tank milk of five dairy factories

Variables	No. (%) antimicrobial-resistant isolates					Total
	Factory A-1	Factory B-1	Factory C-1	Factory C-2	Factory C-3	
No. of isolates	40	41	36	29	37	183
No. (%) of antimicrobial-resistant isolates	21 (52.5)	13 (31.7)	15 (41.7)	6 (20.7)	9 (24.3)	64 (35.0)
AM	4 (19.0)[a,b,c]	6 (46.2)[c,d]	0 (0)[a]	6 (100)[d]	6 (66.7)[b,c,d]	22 (34.4)
AMC	2 (9.5)	1 (7.7)	1 (6.7)	1 (16.7)	0 (0)	5 (7.8)
TE	4 (19.0)[a]	8 (61.5)[b]	2 (13.3)[a]	4 (66.7)[b]	6 (66.7)[b]	24 (37.5)
SXT	1 (4.8)[a,b]	4 (30.8)[a]	0 (0)[b]	0 (0)[a,b]	0 (0)[a,b]	5 (7.8)
Na	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
IPM	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CIP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
GM	1 (4.8)[a]	4 (30.8)[b]	0 (0)[a]	6 (100)[c]	6 (66.7)[b,c]	17 (26.6)
C	0 (0)[a]	7 (53.8)[b]	2 (13.3)[a]	0 (0)[a]	0 (0)[a]	9 (14.1)
CL	6 (28.6)[a,b]	0 (0)[a]	5 (33.3)[b]	0 (0)[a,b]	3 (33.3)[a,b]	14 (21.9)
CZ	2 (9.5)	4 (30.8)	1 (6.7)	0 (0)	0 (0)	7 (10.9)
CF	11 (52.4)[a]	5 (38.5)[a,b]	7 (46.7)[a,b]	0 (0)[b,c]	0 (0)[c]	23 (35.9)
CDX	2 (9.5)	0 (0)	1 (6.7)	0 (0)	0 (0)	3 (4.7)
FOX	1 (4.8)	0 (0)	1 (6.7)	0 (0)	0 (0)	2 (3.1)
CXM	1 (4.8)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.6)
CTX	1 (4.8)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.6)
CAZ	0 (0)	0 (0)	3 (20.0)	0 (0)	0 (0)	3 (4.7)
FEP	1 (4.8)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.6)

Values within a column not having the same superscript letter differ significantly ($p < 0.05$).
AM, ampicillin; AMC, Amoxicillin-clavulanate; TE, Tetracycline; SXT, Trimethoprim sulfamethoxazole; Na, Nalidixic acid; IPM, Imipenem; CIP, Ciprofloxacin; GM, Gentamicin; C, Chloramphenicol; CL, Colistin; CZ, Cefazolin; CF, Cephalothin; CDX, Cefadroxil; FOX, Cefoxitin; CXM, Cefuroxime; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime.

(21.9%), C (14.1%), and CZ (10.9%). None of the isolates were resistant to NA, IPM, and CIP. In particular, the prevalence of resistance to AM, TE, SXT, GM, C, CL, and CF showed significant differences among the five dairy factories ($p < 0.05$, χ^2 test). None of the 14 isolates from factory C-1 showed resistance to AM and GM. In contrast, all six antimicrobial-resistant isolates from factory C-2 showed resistance to AM and GM. Seven isolates (53.8%) from factory B-1 showed high resistance to C. In contrast, no isolates from factories A-1, C-2, and C-3 were resistant to C. Isolates from factories C-2 and C-3 did not show resistance to CF, but isolates from other factories showed relatively high resistance from 38.5% to 52.4%. Isolates from factories B-1, C-2, and C-3 also showed significantly higher resistance to TE than factories A-1 and C-1. The prevalence of antimicrobial-resistant *E. coli* isolates by the phylogenetic group was analyzed, but there were no significant differences among groups (data not shown).

Table 3. Distribution of multidrug resistance of 64 antimicrobial-resistant *E. coli* isolates

No. of antimicrobial-resistant classes	No. of isolates (%)					
	Factory A-1 (n = 21)	Factory B-1 (n = 13)	Factory C-1 (n = 15)	Factory C-2 (n = 6)	Factory C-3 (n = 9)	Total (n = 64)
1	13 (61.9)	4 (30.8)	8 (53.3)	1 (16.7)	3 (33.3)	29 (45.3)
2	8 (38.1)[a]	4 (30.8)[a,b]	7 (46.7)[a]	3 (50.0)[a]	0 (0)[b]	22 (34.4)
3	1 (4.8)[a]	1 (7.7)[a,b]	0 (0)[a]	2 (33.3)[b,c]	6 (66.7)[c]	10 (15.6)
4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5	1 (4.8)	2 (15.4)	0 (0)	0 (0)	0 (0)	3 (4.7)
6	0 (0)	2 (15.4)	0 (0)	0 (0)	0 (0)	2 (3.1)
No. (%) of MDR*	2 (9.5)[a]	5 (38.5)[b]	0 (0)[a]	2 (33.3)[b]	6 (66.7)[b]	15 (23.4)

Values within a column not having the same superscript letter differ significantly (*p* < 0.05).
*Multidrug resistance was defined as the acquired resistance to at least one agent in three or more antimicrobial classes.

Distribution of MDR *E. coli* isolates

The distribution of MDR *E. coli* isolates by factories is shown in **Table 3**. Fifteen isolates (23.4%) of the 64 antimicrobial-resistant *E. coli* were categorized as MDR. In particular, the number of MDR isolates from factory C-3 (6 isolates; 66.7%) was significantly higher than that in factories A-1 (2 isolates; 9.5%) and C-1 (0%; *p* < 0.05). However, the MDR against five and six antimicrobial classes was higher in isolates (15.4% each) from factory B-1, although there was no significant difference among factories.

Distribution of antimicrobial resistance genes of *E. coli* isolates

The distribution of antimicrobial resistance genes of 64 antimicrobial-resistant *E. coli* is shown in **Table 4**. In the distribution of β-lactamase genes, the *bla*$_{OXA}$ gene alone and both *bla*$_{TEM}$ and *bla*$_{OXA}$ genes were detected in 4 (6.3%) and 19 (29.7%) isolates, respectively. In particular, the isolates from factories B-1, C-2, and C-3 showed a significantly high prevalence of *bla*$_{TEM}$ and *bla*$_{OXA}$ genes compared to that from factories A-1 and C-1 (*p* < 0.05). The other β-lactamase genes, such as *bla*$_{CTX-M}$ and *bla*$_{SHV}$, were not detected in all resistant isolates. In the antimicrobial resistance genes encoding for aminoglycoside-modifying enzymes, although both *aac(6′)-Ib* and *aac(3)-II* genes were detected in isolates from factories A-1 and B-1, the distribution of single *aac(3)-II* gene showed significantly high isolates from factory C-3 compared to other factories (*p* < 0.05). Among TE resistance genes, the *tetB* gene (18 isolates;

Table 4. Distribution of antimicrobial resistance genes of 64 antimicrobial-resistant *E. coli* isolates from bulk tank milk

Antimicrobial class	Antimicrobial resistance gene	No. (%) of isolates that carried the target gene					
		Factory A-1 (n = 21)	Factory B-1 (n = 13)	Factory C-1 (n = 15)	Factory C-2 (n = 6)	Factory C-3 (n = 9)	Total (n = 64)
β-lactamases	*bla*$_{OXA}$	3 (14.3)	0 (0)	1 (6.7)	0 (0)	0 (0)	4 (6.3)
	bla$_{CTX-M}$	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	bla$_{SHV}$	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	bla$_{TEM}$ + *bla*$_{OXA}$	1 (4.8)[a]	6 (46.2)[b]	0 (0)[a]	6 (100)[b]	6 (66.7)[b]	19 (29.7)
Aminoglycoside-modifying enzymes	*aac(3)-II*	0 (0)[a]	1 (7.7)[a]	0 (0)[a]	0 (0)[a,b]	6 (66.7)[b]	7 (10.9)
	ant(2″)-I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	aac(6′)-Ib + *aac(3)-II*	1 (4.8)	3 (23.1)	0 (0)	0 (0)	0 (0)	4 (6.3)
Tetracycline	*tetA*	1 (4.8)	3 (23.1)	0 (0)	0 (0)	0 (0)	4 (6.3)
	tetB	2 (9.5)[a]	4 (30.8)[a,b]	1 (6.7)[a]	4 (66.7)[b]	6 (66.7)[b]	17 (26.6)
	tetC	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	tetD	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	tetE	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	tetB + *tetG*	0 (0)	0 (0)	1 (6.7)	0 (0)	0 (0)	1 (1.6)
Sulfonamide	*sul1*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	sul2	0 (0)	2 (15.4)	0 (0)	0 (0)	0 (0)	2 (3.1)
	sul1 + *sul2*	1 (4.8)	2 (15.4)	0 (0)	0 (0)	0 (0)	3 (4.7)
Chloramphenicol	*catA1*	0 (0)	2 (15.4)	0 (0)	0 (0)	0 (0)	2 (3.1)
	cml1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Values within a column not having the same superscript letter differ significantly (*p* < 0.05).

Table 5. Characterization of the 18 integron-positive *E. coli* isolates from bulk tank milk

Factory	Isolates	Integron gene	Cassette array for class I integrons	Other antimicrobial resistance genes	Antimicrobial resistance profiles
A-1	19-A1-025-1	*int1*	-	-	CF, CL
A-1	19-A1-025-2	*int1*	-	-	CF
A-1	19-A1-026-1	*int1*	-	-	CF
A-1	19-A1-027-2	*int1*	-	-	CF, CL
A-1	19-A1-041-1	*int1*	*dfrA12, aadA2*	*bla$_{OXA}$, sul1, sul2, aac(6′)-Ib, aac(3)-II*	CF, AM, AMC, SXT, GM
B-1	19-B1-002-2	*int1*	-	*bla$_{TEM}$, bla$_{OXA}$*	AM, C
B-1	19-B1-068-1	*int1*	*dfrA17, aadA5*	*bla$_{TEM}$, bla$_{OXA}$, sul1, sul2, catA1, aac(6′)-Ib, aac(3)-II, tetB*	AM, TE, SXT, GM, C
B-1	19-B1-107-1	*int1*	*aac(6′)-Ib3, aac(6′)-Ib-cr, aadA4*	*bla$_{TEM}$, bla$_{OXA}$, sul2, aac(3)-II, tetA*	CZ, CF, AM, TE, SXT, GM, C
B-1	19-B1-107-2	*int1*	*aac(6′)-Ib3, aac(6′)-Ib-cr, aadA4*	*bla$_{TEM}$, bla$_{OXA}$, sul2, aac(3)-II, tetA*	CZ, AM, TE, SXT, GM, C
B-1	19-B1-110-1	*int1*	-	*tetB*	CZ, CF, TE
B-1	19-B1-115-1	*int1*	*dfrA12, aadA2*	*bla$_{TEM}$, bla$_{OXA}$, sul1, sul2, aac(3)-II*	AM, SXT, GM
C-1	19-C1-008-2	*int1*	-	-	CF, C
C-1	19-C1-016-2	*int1*	-	*bla$_{OXA}$*	CZ, CF, FOX, AMC, CDX
C-1	19-C1-043-1	*int1*	-	-	CF
C-2	19-C2-061-1	*int1*	-	*bla$_{TEM}$, bla$_{OXA}$, aac(3)-II, tetB*	AM, TE, GM
C-2	19-C2-071-1	*int1*	-	*bla$_{TEM}$, bla$_{OXA}$, aac(3)-II, tetB*	AM, TE, GM
C-3	19-C3-098-1	*int1*	-	*bla$_{TEM}$, bla$_{OXA}$, aac(3)-II, tetB*	AM, TE, GM
C-3	19-C3-119-1	*int1*	-	*bla$_{TEM}$, bla$_{OXA}$, aac(3)-II, tetB*	AM, TE, GM

AM, ampicillin; AMC, amoxicillin-clavulanate; TE, tetracycline; SXT, trimethoprim sulfamethoxazole; GM, gentamicin; C, chloramphenicol; CL, colistin; CZ, cefazolin; CF, cephalothin; CDX, cefadroxil; FOX, cefoxitin.

28.1%) was predominant and showed significantly high isolates from factories C-2 and C-3 compared to that from factories A-1 and C-1 ($p < 0.05$).

Characterization of integron-positive *E. coli* isolates

The characterization of 18 integron-positive isolates (28.1%) among 64 antimicrobial-resistant *E. coli* are shown in **Table 5**. All integron-positive *E. coli* carried the integron class I gene (*int1*). Among them, five isolates showed the three different gene cassette arrangements, *dfrA12 + aadA2* (2 isolates), *aac(6′)-Ib3 + aac(6′)-Ib-cr + aadA4* (2 isolates), and *dfrA17 + aadA5* (1 isolate). Eleven (61.1%) of 18 isolates were also categorized as MDR. No isolates were positive for the integron class II gene (*int2*).

DISCUSSION

Although contamination of *E. coli* in raw milk is at low level, its transmission via human consumption, such as raw milk or raw milk products, could be a source of both enteric disease and antimicrobial resistance [1]. In this study, the characteristics of 183 *E. coli* isolates from bulk tank milk of five different dairy factories were investigated. The phylogenetic characterization of *E. coli* is an important tool to improve the understanding of the relationship between strains and disease [16]. *E. coli* is commonly classified into four major phylogenetic groups A, B1, B2, and D [8,17,18]. Group D was the most prevalent (59.6%), followed by B1 (25.1%). Previous studies have reported that the intestinal pathogenic *E. coli* mostly belong to groups A, B1, and D [17]. In contrast, the most virulent extraintestinal pathogenic strains belong to phylogenetic groups B2 and D [19]. In Korea, the study of the phylogenetic group of *E. coli* from milk samples was only reported by Tark et al. [5]. *E. coli* isolates from bovine mastitic milk were mainly assigned to phylogenetic group A (60.0%), which was different from the results of this study. However, the distribution of phylogenetic group D in *E. coli* isolates from dairy products was continuously reported in Korea (20.0%; Tark et al. [5]), China (21.7%; Ali et al. [20]), and Iran (28.6%; Jamali et al. [21]). Moreover, *E. coli* isolates from bovine mastitis in China were mainly categorized as phylogenetic group B1 (58.6%; Liu et al. [22]). *E. coli* isolates from a dairy farm

in the United States were also predominantly phylogenetic group B1 (63.9%; Son et al. [23]). The correlation between the phylogenetic group and pathogenic trait of *E. coli* in bovine mastitis should be further investigated.

In the study, the mannose-specific adhesion type 1 fimbriae gene, *fimH*, among eight virulence profiles (*eaeA*, *stx1*, *stx2*, *hly*, *fimH*, *iucC*, *fyuA*, and *iutA* genes), was the most prevalent virulence gene (73.2%). Other studies also reported that *fimH* was the most widespread virulence gene of *E. coli* isolates from the subclinical mastitis milk samples (93%) in Egypt [24] and clinical mastitis milk samples (84.2%) in Taiwan [25]. The *fimH* gene was associated with the initial colonization of tissues, and uropathogenic *E. coli* strains were reported to express genes by more than 90% [24]. In contrast, only one isolate (4.3%) from phylogenetic group A, considered commensal, carried the *fimH* gene in this study. Intimin plays a role in the attachment of bacterial cells to host cells [26]. In this study, 37 (20.2%) isolates carried the intimin gene, *eaeA*, and isolates in phylogenetic group D were significantly higher than in group A ($p <$ 0.05). However, *stx1*, *fimH*, *incC*, *fyuA*, and *iutA* genes were significantly higher in isolates of phylogenetic group B1 than that of phylogenetic groups A, B2, and D ($p < 0.05$). Shiga toxin, encoded by *stx1* and *stx2*, is one of the most potent virulence factors in *E. coli* and is associated with hemolytic uremic syndrome and neurological disorder [17,27]. Aerobactin synthesis genes, *iucC* and *iutA*, are conserved in virulent strains [28]. The yersiniabactin receptor gene, *fyuA*, is correlated with other virulence factors contributing to urinary tract infection [29]. Although Ombarak et al. [17] also reported that *E. coli* isolates belonged to phylogenetic group B1 in raw milk showing a high prevalence of virulence genes, *E. coli* isolates were mostly classified into phylogenetic group B1 (63.5%). Only 3.8% belonged to phylogenetic group D. Group D was the most prevalent, but *E. coli* belonging to group B1 carried more virulence genes. Therefore, further studies about the link between the virulence of a strain and its phylogenetic group are needed to better understand the disease pathogenesis of *E. coli* isolated from dairy products.

Previous studies reported various serotypes in *E. coli* isolates from bulk tank milk or raw milk [30-32]. In this study, 183 *E. coli* isolates were classified into 46 serotypes, and the most prevalent type was O173 (28 isolates; 15.3%). Although Linnerborg et al. [33] reported that O173 is grouped as enteroinvasive *E. coli*, the virulence of O173 was rarely reported. The O173 isolates in this study did not showed any typical virulence factors and various phylogenetic groups and diverse distribution of virulence genes were observed.

In this study, 64 *E. coli* showed resistance to at least one antimicrobial, and the highest resistance rate was observed for TE (37.5%), followed by CF (35.9%) and AM (34.4%). Tark et al. [5] also reported that the most prevalent antimicrobial resistance in *E. coli* isolates from bovine mastitic milk in Korea was TE (23.3%), followed by AM (16.6%). Moreover, the distribution of antimicrobial resistance of *E. coli* and MDR also showed significant differences among the factories in this study. Isolates from factories B-1, C-2, and C-3 also showed significantly higher resistance to TE than that of A-1 and C-1. Also, isolates from factory C-1 did not show resistance to AM and GM, but all isolates from factory C-2 were resistant to AM and GM. Based on the antimicrobial consumption report in Korea, AM and TE are widely used antimicrobial agents for cattle in Korea [34]. However, the prevalence of antimicrobial resistance among factories showed significant differences because dairy factories allow producers to combine different biosecurity and sanitation practices, housing technologies, and feeding regimens, including the use of antimicrobials. In addition, isolates from factory B-1 showed high resistance to C, although C has been withdrawn from veterinary use in Korea in 1992 [10]. It may be because florfenicol can give resistance to C, as they have partially overlapped drug-binding sites [35].

The most prevalent antimicrobial resistance genes against β-lactamase class were bla_{TEM} + bla_{OXA} (29.7%), with a significant difference among factories ($p < 0.05$). Although bla_{CTX-M}, one of the most commonly detected β-lactamase genes [5,20], was not revealed in this study, the *TEM* gene has also been identified in clinical *E. coli* isolates from foods, humans, and healthy animals [36-38]. β-Lactamase-producing *E. coli* are resistant to most β-lactams and other antimicrobials, such as aminoglycosides, C, quinolones, sulfonamides, tetracyclines, and trimethoprim [6]. Therefore, monitoring of β-lactamase-producing *E. coli* by dairy factories is crucial to prevent the emergence and dissemination of MDR strains [5,38].

Among antimicrobial genes for tetracyclines, *tetB* (26.6%) was the most prevalent gene, followed by *tetA* (4 isolates, 6.3%) in this study. In particular, the *tetB* gene's distribution was significantly different among factories ($p < 0.05$). It is analogous with the findings of a study in Canada, which showed *tetB* (31.0%) and *tetA* (28.6%) as the most prevalent antimicrobial resistance genes of *E. coli* isolates from milk samples [21]. Abdus Sobur et al. [12] also reported that *E. coli* isolates from dairy farms and its environment carried *tetA* (80.51%) as the most prevalent antimicrobial resistance gene in Bangladesh.

In this study, 18 integron-positive isolates (28.1%) among 64 antimicrobial-resistant *E. coli* carried the *int1* gene, and 5 integron-positive isolates harbored *aadA* and/or *dfrA* gene cassettes. Moreover, 11 (61.1%) of 18 *int1*-positive isolates showed MDR. Previous studies reported that the *int1* gene is important for the transfer of resistance genes, and the *aadA* and *dfrA* genes, which are resistant to GM and SXT, respectively, are the most frequent gene cassettes in humans and animals in Korea [39]. In particular, Seo et al. [39] reported that isolates harboring *aadA* or *dfrA* showed higher antimicrobial resistance. Integrons have been continuously considered responsible for the emergence of MDR by enhancing gene expression and acquiring new gene cassettes [6,40].

The occurrence of antimicrobial-resistant bacterial infection can be problematic not only for the deterioration of milk quality management but also for limiting antimicrobial therapeutic choices in humans [21]. Although the characteristics of *E. coli* from bovine mastitic milk have been reported in the world [2,5], the results suggest that *E. coli* from bulk tank milk, which is not mastitic milk, can also become a reservoir to disseminate antimicrobial resistance and virulence factors via cross-contamination.

SUPPLEMENTARY MATERIALS

Supplementary Table 1
Primers used in this study

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REFERENCES

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