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ORIGINAL ARTICLE

Fluoroquinolone-resistant and extended-spectrum β-lactamase-producing *Escherichia coli* from the milk of cows with clinical mastitis in Southern Taiwan

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Background/Purpose: *Escherichia coli* is a common pathogen to cause clinical and subclinical mastitis in cows. A total of 57 *E. coli* isolates from raw milk from cows were characterized genetically and biochemically.
Methods: Extended-spectrum β-lactamase (ESBL) genes, the mechanism for fluoroquinolone resistance, and variations in virulence genes and genomes of these *E. coli* isolates were investigated by the antimicrobial susceptibility test, simplex and multiplex polymerase chain reaction (PCR), and pulsed-field gel electrophoresis (PFGE).
Results: All *E. coli* isolates were resistant to cloxacillin (100%) and to a lesser extent (50%) to tetracycline, neomycin, gentamycin, ampicillin, ceftriaxone, cefotaxime (CTX), and ceftazidime (CAZ). Nearly 70% of the isolates were resistant to at least two antimicrobials and 28.1% carried AmpA and AmpC genes simultaneously. The predominant *bla* gene was bla_{TEM}, followed by bla_{CMY}, bla_{CTX}, bla_{SHV}, and bla_{DHA}. Among the six (10.5%) ESBL-producing *E. coli* carrying $bla_{CTX\text{-}M15}$, $bla_{CTX\text{-}M55}$, or $bla_{CTX\text{-}M14}$, two isolates 31 of ST410 in the ST23 complex and 58 of ST167 in the ST10 complex were also resistant to ciprofloxacin, enrofloxacin, and levofloxacin, with mutations at codon 83 from serine to leucine and codon 87 from aspartic acid to asparagine in GyrA and at codon 80 from serine to isoleucine in ParC. These isolates were genetically diverse in pulsotype analysis, lacked toxin genes of human

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pathogenic *E. coli* and carried mostly the prevalent virulence genes *fimH*, *papGII*, and *α-hemolysin*.
Conclusion: Lacking virulence genes examined, genetic diverse *E. coli* isolates are unrelated to human pathogenic *E. coli*. Enhancing sanitation in milk processing and transportation is needed to eliminate multidrug-resistant (MDR), fluoroquinolone-resistant, and ESBL-producing *E. coli* isolates.

Introduction

Mastitis in cows and goats affects milk quality, human safety, and the income of the farming industry worldwide. Cases of subclinical mastitis are much higher than those of clinical mastitis. The predominant pathogens causing mastitis are Gram-negative bacteria (25–30%), coagulase-negative *Staphylococcus* (20%), *Staphylococcus aureus* and *Escherichia coli* (5–10% each), *Streptococcus* and *Enterococcus* (2–5%), and other species (< 2%).[1–5] In Taiwan, the predominant pathogens causing mastitis in dairy cows are *E. coli* (16.8%), *Staphylococcus* spp. (23.3%), *Streptococcus* spp. (14.5%), *Enterococcus* spp. (20.1%), and other Gram-negative versus Gram-positive (19.2% vs. 3.8%) pathogens.[6] As normal intestinal flora of the intestine, *E. coli* produces vitamins B and K and inhibits the growth of pathogens in the intestine,[7] and becomes diverse genetic groups and opportunistic pathogens to humans by incorporating different pathogenicity islands with virulence genes, for example, enterotoxigenic *E. coli* (ETEC) with genes for heat-stable toxin and heat-labile toxin,[8] enterohemorrhagic *E. coli* (EHEC) with genes for Shiga-like toxin I and II,[9] and enteropathogenic *E. coli* (EPEC) with genes for bundle-forming pilus.[10] The emergence of multidrug-resistant (MDR) bacteria and the transmission of these bacteria from foods to humans have become serious public health problems in treating their infection.[11,12]

β-lactamase can hydrolyze the β-lactam rings of expanded-spectrum cephalosporins (except cephamycins and carbapenems) and monobactams and then inactivates these compounds. Application of third-generation cephalosporins increases extended-spectrum β-lactamase (ESBL) *Enterobacteriaceae*, which are relatively high in food animals and reveal a high genetic diversity,[13,14] indicating that farm animals are a reservoir of ESBL-producing bacteria which can infect humans. Recently, ESBL-producing *E. coli* has been observed in economically important animals in Europe[15,16] and Asia,[17] in meat, fish, and raw milk,[18,19] and in healthy dairy cattle and retail meat in the USA.[20,21] Furthermore, fluoroquinolone- and carbapenem-resistant *E. coli* isolates have been reported in humans. Here, we investigated antimicrobial susceptibility, ESBL-producing genes, mutations in GyrA and ParC for fluoroquinolone resistance, and differences in virulence genes and genomes for mastitis-associated *E. coli* isolates.

Methods

Mastitis-associated bacterial species

A total of 412 milk samples from cows with clinical or subclinical mastitis were collected from 13 farms in Yunlin, Chiayi, and Tainan in Southern Taiwan based on the California mastitis test of microcolloid formation[22] and these samples were directly plated on Eosin Methylene Blue (EMB) agar to screen for *E. coli*. Bacterial colonies were further identified as *E. coli* by API 20E and polymerase chain reaction (PCR) amplification of the *E. coli*-specific gene *mdhR*. Hemolysin types of each isolate were determined by blood agar. Informed consent was obtained from all farm owners prior to the start of this study. The use of all bacteria was supervised by the Biological Security Committee of the National Chiayi University, Chiayi, Taiwan in accordance with the laws of Taiwan. The milk samples were collected by county governors based on the guidelines of Animal Use Protocol, the Institutional Animal Care and Use Committee, National Chiayi University.

Antimicrobial susceptibility test

Antimicrobial susceptibility to ampicillin, cefepime, cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone, ciprofloxacin, cloxacillin, enrofloxacin, ertapenem, gentamicin, levofloxacin, meropenem, neomycin, and tetracycline was determined by the disc diffusion method and the Clinical and Laboratory Standards Institute standard.[23] ESBL isolates were confirmed by determining their resistance to CAZ, CTX, CAZ/clavulanic acid, and CTX/clavulanic acid. *E. coli* ATCC 25922 was used as the reference strain.

PCR detection of genes for β-lactamase, virulence factors, and pathogenic *E. coli*

The primers used to amplify five β-lactamase genes, 12 virulence genes of uropathogenic *E. coli*, and 10 toxin genes of other pathogenic *E. coli* are listed in Table 1.[24–29] Three multiplex PCR sets were developed. Multiplex PCR I was used to detect genes bla_{SHV}, $bla_{CTX\text{-}M3}$, $bla_{CTX\text{-}M14}$, bla_{TEM}, and bla_{DHA}. Twelve virulence genes were amplified by multiplex PCR II for *hlyA*, *usp*, *sat*, *fyuA*, *ironN*, *iutA*, and *iucD* and multiplex PCR III for *fimH*,

Table 1 Primer sequences and PCR product sizes of genes for antimicrobial resistance and virulence factors

PCR Set	Gene	Primer	Nucleotide sequence (5′ → 3′)	PCR product size (bp)
Multiplex I[a]	Class A β-lactamase			
	bla_{CTX-M3}	F	AATCA CTGCG CCAGT TCACG CT	479
		R	GAACG TTTCG TCTCC CAGCT GT	
	$bla_{CTX-M14}$	F	TACCG CAGAT AATAC GCAGG TG	355
		R	CAGCG TAGGT TCAGT GCGAT CC	
	bla_{SHV}	F	AACGG AACTG AATGA GGCGC T	141
		R	TCCAC CATCC ACTGC AGCAG CT	
	bla_{TEM}	F	GAAGA TCAGT TGGGT GCACG AGT	520
		R	CAACT TTATC CGCCT CCATC CAGT	
	Class C β-lactamase			
	bla_{DHA}	F	AACTT TCACA GGTGT GCTGG GT	405
		R	CGTAC GCATA CTGGC TTTGC	
Simplex	bla_{CMY-2}	F	CTGAC AGCCT CTTTC TCCAC A	1100
		R	CTACG TAGCT GCCAA ATCCA C	
Multiplex II	*hlyA*	F	AACAA CGATA AGCAC TGTTC TGG	1177
		R	CCATA TAAGC GGTCA TTCCC G	
	fyuA	F	GGCTT TATCC TCTGG CCTT	878
		R	GAAAA CCCAG TCATC GGTGG	
	iroN	F	CTCTG GTGGT GGAAG CC	815
		R	TGTCG GTACA GGCGG TTC	
	usp	F	GGAAA ATGGT CGCTC AGTGG	992
		R	CTGTA GTGAA TCTCA TCGTG TAGTC	
	sat	F	TCAGA AGCTC AGCGA ATCAT TG	931
		R	CATTA TCACC AGTAA AACGC ACC	
	iutA	F	CACTC CGGTA CTCCA GTCA	688
		R	CCTCC AACCA GATGT TCTTC G	
	iucD	F	CCGGA GAAGC CTGAA ATATA TTCA	584
		R	CCGGA TTGTC ATATG CAGAC C	
Multiplex III	*fimH*	F	GTTTA TAATT CGAGA ACGGA TAAGC C	494
		R	GTGCA TAATT TGCCG TTAAT CCC	
	cnf1	F	TTCTT CTGTA CTTCC CCCAG	407
		R	TGAGC GGCAT CTACT ATGAA GT	
	kpsMTII	F	CATCA GACGA TAAGC ATGAG CA	270
		R	TGCGC ATTTG CTGAT ACTGT	
	papGII	F	GGGCC CCCAA GTAAC TC	188
		R	GGATG AGCGG GCCTT TG	
	traT	F	CATAA CCACG GTTCA GCCAT C	328
		R	TTGCA CTGGT CAGTT CCAC	
Specific PCR sets for EHEC[b]	*stx*1	F	CAGTT AATGT GGTGG CGAAG G	348
		R	CACCA GACAA TGTAA CCGCT G	
	*stx*2	F	ATCCT ATTCC CGGGA GTTTA CG	584
		R	GCGTC ATCGT ATACA CAGGA GC	
Specific PCR sets for EPEC[c]	*eae*	F	TCAAT GCAGT TCCGT TATCA GTT	482
		R	GTAAA GTCCG TTACC CCAAC CTG	
	bfp	F	GGAAG TCAAA TTCAT GGGGG TAT	300
		R	GGAAT CAGAC GCAGA CTGGT AGT	
Specific PCR sets for ETEC[c]	*Lt*	F	GCACA CGGAG CTCCT CAGTC	218
		R	TCCTT CATCC TTTCA ATGGC TTT	
	stII	F	AAAGG AGAGC TTCGT CACAT TTT	129
		R	AATGT CCGTC TTGCG TTAGG AC	
Specific PCR sets for EIEC[d]	*virF*	F	AGCTC AGGCA ATGAA ACTTT GAC	618
		R	TGGGC TTGAT ATTCC GATAA GTC	
	ipaH	F	CTCGG CACGT TTTAA TAGTC TGG	933
		R	GTGGA GAGCT GAAGT TTCTC TGC	
Specific PCR set for DAEC[d]	*daaE*	F	GAACG TTGGT TAATG TGGGG TAA	542
		R	TATTC ACCGG TCGGT TATCA GT	
Specific PCR set for EAggEC3[d]	*aafII*	F	CACAG GCAAC TGAAA TAAGT CTGG	378
		R	ATTCC CATGA TGTCA AGCAC TTC	

Table 1 (*continued*)

PCR Set	Gene	Primer	Nucleotide sequence (5′ → 3′)	PCR product size (bp)
Quinolone-resistant determining region	*gyrA*[e]	F	GCGCGGGCTGTGTTATAATT	519
		R	CCGTGCCGTCATAGTTATCAA	
	parC[f]	F	AAACCTGTTCAGCGCCGCATT	391
		R	GTGGTGCCGTTAAGCAAA	

[a] The PCR method was described by Chia et al[24] and primers for bla_{DHA} was designed in this study.
[b] The PCR method was described by Cebula et al.[25]
[c] The PCR method was described by Stacy-Phipps et al.[26]
[d] The method was described by Vidal et al.[27]
[e] The method was described by Cattoir et al.[28]
[f] The method was described by Wada et al.[29]
DAEC = diffuse adherent *Escherichia coli*; EAggEC3 = enteroaggregative *Escherichia coli*; EHEC = enterohemorrhagic *Escherichia coli*; EIEC = enteroinvasive *Escherichia coli*; EPEC = enteropathogenic *Escherichia coli*; ETEC = enterotoxigenic *Escherichia coli*.

cnf1, *traT*, *kpsMTII*, and *papGII*. bla_{CMY-2} was identified by single PCR. A 50-μL PCR reagent included 5 μL of DNA template, 0.2 μM of each primer, 5 μL of 10X PCR reaction buffer, 200 μM of deoxyribonucleoside triphosphates (dNTPs), and 1.4 U Taq DNA polymerase. The PCR conditions were as follows: predenaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Toxin genes for pathogenic *E. coli* were amplified with previously described PCR conditions.[24–29] All PCR products were separated on 2% agarose gels at 50 V for 120 minutes. After ethidium bromide staining and UV illumination, gel images were recorded and analyzed.

Sequence analysis of quinolone-resistance determining regions of *gyrA*, *parC*, and bla_{CTX-M}

The quinolone-resistance determining regions of *gyrA* and *parC* genes were amplified from two fluoroquinolone resistant strains and sensitive strains with the PCR primers listed in Table 1. PCR products were purified and sequenced. The bla_{CTX-M} was amplified with the primer set in Table 1 and sequenced. The sequences were compared to the NCBI database by the Blast program.

Pulsed-field gel electrophoresis analysis

The PulseNet standardized laboratory pulsed-field gel electrophoresis (PFGE) protocol for molecular subtyping of *E. coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* was used to analyze the isolates.[30] A total of 10 U of *Xba*I was used for restriction digestion. Digested DNA fragments were separated by the CHEF-DRIII system (BioRad, Hercules, CA, USA) in 1% agarose gel with 0.5X TBE running buffer at 14°C in conditions of 6 V/cm, 120° separation angle, and switching time of 4～70 seconds for 18 hours and another run for 4 hours in the same condition, with only change of voltages to 4 V/cm. PFGE images were analyzed and a unique PFGE pattern was defined as one or two DNA bands differing between PFGE patterns of two isolates.

Results

Antimicrobial susceptibility of *E. coli*

E. coli from milk samples of the cows with clinical mastitis differed in prevalence of 13.8% (57/412) in total, 51.6% in Yunlin, 10.8% in Chiayi, and 4.1% in Tainan. Based on resistance to 1—12 antimicrobials, the number of antibiograms ranged from 13 in Yunlin to six in Chiayi and Tainan; however, the highest antimicrobial resistance was found in two isolates from Chiayi and Tainan (Table 2). Although all 57 isolates were resistant to cloxacillin, a drug used to treat *S. aureus*, a total of 33.3% of the isolates were susceptible to the remaining 11 antimicrobials, with a prevalence of 64.7% in Chiayi, 37.5% in Tainan, and 15.6% in Yunlin. Prevalence of the resistance to traditional antimicrobials was 51% for tetracycline, 42% for neomycin, and 19% for gentamicin.

Regarding resistance to beta-lactam antimicrobials, 54% isolates were resistant to ampicillin of the penicillin group, while < 25% isolates were resistant to CAZ, CTX, and ceftriaxone of the third-generation cephalosporins, and one isolate was resistant to cefepime (the 4th-generation cephalosporin). Furthermore, only six (10.7%) ESBL-producing isolates were identified and all revealed resistance to 7—11 antimicrobials (Table 2). ESBL-producing isolates VMEC31 and VMEC58 were also resistant to fluoroquinolones (ciprofloxacin, enrofloxacin, and levofloxacin) and revealed mutations at codon 83 from serine to leucine and codon 87 from aspartic acid to asparagine in GyrA, and at codon 80 from serine to isoleucine in ParC. These isolates reduced susceptibility to carbapenems.

Variations in *bla* genes

Among five *bla* genes encoding AmpA and AmpC lactamase, the prevalent *bla* genes in order were bla_{TEM} (47%), bla_{CMY} (40%), bla_{CTX} [$bla_{CTX-M3-like}$ (9%) and $bla_{CTX-M14-like}$ (4%)], bla_{SHV} (9%), and bla_{DHA} (2%) (Table 3). Over 70% of the isolates contained at least one *bla* gene, while the number of isolates without the *bla* gene was identical to those resistant only to cloxacillin in Yunlin and Tainan, not Chiayi (Table 3). The predominant *bla* genes were bla_{TEM} in 43.9%

Table 2 Prevalence of antimicrobial resistance in different counties

Resistance no.	Resistance (%)												ESBL		Yunlin	Chiayi	Tainan	Total, *n* (%)
	Penicillin		Cephalosporin				Fluoroquinolone			GEN	NEO	TET						
	CLO	AMP	CAZ	CTX	CRO	FEP	CIP	ENR	LVX				CTX	CAZ	*n* (%)	*n* (%)	*n* (%)	
1	R	S	S	S	S	S	S	S	S	S	S	S	−	−	5 (15.6)	11 (64.7)	3 (37.5)	19 (33.3)
2	R	S	S	S	S	S	S	S	S	S	S	R	−	−	1 (3.1)	0 (0.0)	0 (0.0)	1 (1.8)
	R	S	S	S	S	S	S	S	S	S	R	S	−	−	2 (6.3)	0 (0.0)	0 (0.0)	2 (3.5)
	R	R	S	S	S	S	S	S	S	S	S	S	−	−	3 (9.4)	1 (5.9)	0 (0.0)	4 (7.0)
3	R	S	S	S	S	S	S	S	S	S	R	R	−	−	2 (6.3)	1 (5.9)	0 (0.0)	3 (5.3)
	R	S	S	S	S	S	S	S	S	R	R	S	−	−	1 (3.1)	0 (0.0)	0 (0.0)	1 (1.8)
	R	R	S	S	S	S	S	S	S	S	S	R	−	−	6 (18.8)	1 (5.9)	0 (0.0)	7 (12.3)
	R	R	S	S	S	S	S	S	S	S	R	S	−	−	2 (6.3)	0 (0.0)	0 (0.0)	2 (3.5)
4	R	R	S	S	S	S	S	S	S	S	R	R	−	−	0 (0.0)	2 (11.8)	0 (0.0)	2 (3.5)
	R	R	S	R	S	S	S	S	S	S	S	R	−	−	0 (0.0)	0 (0.0)	1 (12.5)	1 (1.8)
5	R	R	S	R	S	S	S	S	S	S	R	R	−	−	3 (9.4)	0 (0.0)	1 (12.5)	4 (7.0)
	R	R	S	S	S	S	S	S	S	R	R	R	−	−	2 (6.3)	0 (0.0)	1 (12.5)	3 (5.3)
6	R	R	S	R	S	S	S	S	S	R	R	R	−	−	0 (0.0)	0 (0.0)	1 (12.5)	1 (1.8)
	R	R	S	R	R	S	S	S	S	S	R	R	−	−	1 (3.1)	0 (0.0)	0 (0.0)	1 (1.8)
7	R	R	S	R	R	S	S	S	S	R	R	R	+	−	1 (3.1)	0 (0.0)	0 (0.0)	1 (1.8)
8	R	R	R	R	R	S	S	S	S	R	R	R	+	+	3 (9.4)	0 (0.0)	0 (0.0)	3 (5.3)
11	R	R	R	R	R	S	R	R	R	R	R	R	+	+	0 (0.0)	0 (0.0)	1 (12.5)	1 (1.8)
	R	R	R	R	R	R	R	R	R	R	S	R	+	+	0 (0.0)	1 (5.9)	0 (0.0)	1 (1.8)
Total, *n* (%)	57 (100)	31 (54)	5 (9)	13 (23)	7 (12)	1 (2)	2 (4)	2 (4)	2 (4)	11 (19)	24 (42)	29 (51)	6 (11)	5 (9)	32 (100)	17 (100)	8 (100)	57 (100)

AMP = ampicillin; CAZ = ceftazidime; CLO = cloxacillin; CRO = ceftriaxone; CIP = ciprofloxacin; CTX = cefotaxime; ENR = enrofloxacin; ESBL = extended-spectrum β-lactamase; ETP = ertapenem; FEP = cefepime; GEN = gentamicin; LVX = levofloxacin; NEO = neomycin; TET = tetracycline.

Table 3 Prevalence of different beta-lactamase genes in three counties

No. of resistant gene	AmpA *bla*				AmpC *bla*		ESBL		County			Total, *n* (%)
	TEM	SHV	CTX-M-3	CTX-M-14	DHA	CMY	CAZ	CTX	Yunlin	Chiayi	Tainan	
0	−	−	−	−	−	−	−	−	5 (15.6)	8 (47.1)	3 (37.5)	16 (28.1)
1	+	−	−	−	−	−	−	−	8 (25.0)	3 (17.6)	2 (25.0)	13 (22.8)
	−	+	−	−	−	−	−	−	1 (3.1)	2 (11.8)	0	3 (5.3)
	−	−	−	+	−	−	−	−	1 (3.1)	0	0	1 (1.8)
	−	−	−	−	+	−	−	−	1 (3.1)	0	0	1 (1.8)
	−	−	−	−	−	+	−	−	4 (12.5)	3 (17.6)	0	7 (12.3)
2	+	−	−	−	−	+	−	−	7 (21.9)	0	1 (12.5)	8 (14.0)
	−	−	+	−	−	+	+	+	0	1 (5.9)	0	1 (1.8)
	−	−	−	+	−	+	−	+	1 (3.1)	0	0	1 (1.8)
3	+	+	−	−	−	+	−	−	1 (3.1)	0	1 (12.5)	2 (3.5)
	+	−	+	−	−	+	+	+	3 (9.4)	0	1 (12.5)	4 (7.0)
Total, *n* (%)	27 (47)	5 (9)	5 (9)	2 (4)	1 (2)	23 (40)	5 (9)	6 (11)	32	17	8	57 (100)

CAZ = ceftazidime; CMY = ; CTX = cefotaxime; DHA = ; ESBL = extended-spectrum β-lactamase; SHV = ; TEM = .

of isolates carrying one *bla* gene, bla_{TEM} and bla_{CMY} in 17.6% of isolates with two *bla* genes, and bla_{TEM}, $bla_{CTX\text{-}M3}$, and bla_{CMY} in 10.5% of isolates with three *bla* genes. The latter two groups all contained AmpC bla_{CMY} and at least one AmpA *bla* gene. Isolates with three *bla* genes were only observed in Yunlin and Tainan. In contrast to CAZ^{ESBL} and CTX^{ESBL} phenotypes of *E. coli* carrying $bla_{CTX\text{-}M3\text{-}like}$, *E. coli* with $bla_{CTX\text{-}M14\text{-}like}$ exhibited only a CTX^{ESBL} phenotype or non-ESBL phenotype. Sequence analysis of $bla_{CTX\text{-}M3}$-like genes determined $bla_{CTX\text{-}M55}$ for isolates 39, 42, 43, and 58 and $bla_{CTX\text{-}M15}$ for isolate 31.

Differences in virulence genes

Examination of hemolysin types found α-hemolysin in 96.7% (55/57) isolates and β- and γ-hemolysin in one isolate, respectively. PCR amplification of toxin genes only identified *eae* of EPEC in one isolate and lacked other toxin genes of EHEC, ETEC, enteroinvasive *E. coli* (EIEC), diffuse adherent *E. coli* (DAEC), and enteroaggregative *E. coli* (EAggEC). Among the other 13 virulence genes for urinary tract infection (UTI)-associated *E. coli* in humans, almost all isolates carried *fimH* encoding type 1 fimbriae and *papGII* encoding P fimbriae, and < 10% of isolates consisted of the *kpsMTII* encoding capsule and *cnf1* and *hlyA* encoding cytotoxin (Table 4). Both capsule and cytotoxin genes were only present in the isolates from Yunlin (14%, 8/57) and Chiayi (2%, 1/57) and were absent in Tainan, while both fimbriae and cytotoxin genes were only observed in the isolates from Yunlin (7%, 4/57) and Tainan (2%, 1/57). Furthermore, siderophore genes were present in < 40% of isolates and ranged from 10.5% (6/57) for *iroN*, encoding the aerobactin receptor, 40.3% (23/57) for *iutA* and 15.8% for *iucD*, encoding salmochelin, and 17.5% for *fyuA*, encoding the yersiniabactin receptor. However, almost all isolates with *iucD* carried *iutA*.

Phylogenetic analysis

PFGE analysis separated 57 isolates into 39 pulsotypes and nontypeable (Fig. 1 and Table 3). Clonal dissemination was only observed in pulsotypes 5, 16, 22, and nontypeable in Yunlin, and in pulsotypes 20, 25, 32, and nontypeable in Chiayi. These nontypeable isolates differed in their virulence genes. Pulsotypes of two fluoroquinolone-resistant strains were pulsotype 28 for isolate 31 and nontypeable for isolate 58.

Discussion

E. coli are important pathogenic sources for cow mastitis with a prevalence of 5–10% in South Eastern Ethiopia,[1] 15.9% for clinical mastitis in Estonia,[31] and 4.8% for subclinical mastitis.[32] In this study, genetically diverse *E. coli* isolates were the predominant pathogens from the milk of cows with clinical mastitis (Fig. 1), suggesting that these *E. coli* isolates may originate from the environment. Virulence factors, such as S and P fimbriae and CNF1, are important for the severity and persistence of mastitis.[33] Examining the virulence and toxin genes for disease in cattle, these *E. coli* isolates carried *fimH*, *papGII*, and *α-hemolysin* genes more prevalent than other virulence genes (> 80% vs. < 40%; Fig. 1, Table 3), implying that these three genes are important for clinical mastitis in cows and that these *E. coli* isolates was not associated with EPEC, EHEC, ETEC, EIEC, DAEC, and EAggEC pathogenic to humans.

Subtherapeutic antimicrobials are frequently used in animal feed, leading to an increase in antimicrobial-resistant bacteria in the animal. In feedlot cattle, given feed with subtherapeutic antimicrobials, the isolates increased resistance to sulfamethoxazole and chloramphenicol, and did not change other antimicrobial resistance patterns.[34] Recently, MDR *E. coli* isolates have emerged from cows with mastitis. Antimicrobial resistance of mastitis-associated *E. coli* isolates differs among countries from low resistance in Sweden and higher resistance in France.[35] Additionally, *E. coli* isolates from the feedlot cattle were less resistant to ampicillin (5.9–24.3%), kanamycin (l5.9%), streptomycin (5.9–15.6%), tetracycline (5.9–13.5%), susceptible to cefotaxime, ceftiofur, chloramphenicol, gentamicin, and nalidixic acid, and not ESBL producers,[32,34] while other research reported the isolates

Table 4 Prevalence of virulence genes in three counties

No. factor	Fimbriae	Capsule	Cytotoxin	Siderophore				Pulsotype	Number	County			Sum
	fimH + *papGII*	*kpsMTII*	*cnf1* + *hlyA*	*iroN*	*iutA*	*iucD*	*fyuA*			Yunlin	Chiayi	Tainan	
0	−	−	−	−	−	−	−	18;24/25/26/31;40	1;1/2/1/1/;1	1	5	1	7
2	−	−	+	−	−	−	−	1/22	1/2	3			17
	−	−	−	−	−	−	−	4/7/8/11/19/23;20/32/33;38	1/1/1/1/1/1; 2/3/1;1	6	6	1	
				−	+	+	−	ND	1			1	
3	+	−	−	−	+	−	−	8/14/16/17/21,29/ND;37	1/1/2/1/1;1/3;1	6	4	1	19
	+	−	−	−	−	−	+	3/ND;34	1/2;1	3		1	
	+	−	−	+	−	−	−	27;36	1;1		1	1	
	+	+	−	−	−	−	−	10/15	1/1	2			
4	+	−	+	−	−	−	−	20	1	1			3
	+	−	−	−	+	+	−	35	1			1	
5	+	+	−	−	−	−	+	3	1	1			8
	+	+	−	−	+	−	−	6	1	1			
	+	−	−	−	+	+	+	13	1	1			
	+	−	+	+	−	+	−	12	1	1			
	+	+	−	−	+	+	−	2/5	1/3	4			
6	+	−	−	+	+	+	+	39	1			1	3
	+	−	+	+	−	−	+	9	1	1			
	+	−	+	+	+	−	−	9	1	1			
7	+	+	−	+	+	+	+	28	1		1		1
Total	48 (84.2)	9 (15.8)	8 (14.0)	6 (10.5)	23 (40.3)	9 (15.8)	10 (17.5)			32	17	8	57

more resistant to ampicillin (100%), chloramphenicol (75.4%), gentamycin (54%), neomycin (43.2%), streptomycin (97.3%), sulfonamide (98.6%), and tetracycline (100%).[36] However, resistance to fluoroquinolones or cephalosporins is still uncommon among coliforms isolated from bovine mastitis. Although < 50% isolates were resistant to ampicillin, gentamycin, neomycin, and tetracycline, an ESBL producer was identified in this study (Table 3).

Beta-lactam and fluoroquinolone antimicrobials have been frequently used to treat bacterial infections in veterinary hospitals and farms. The use of a third-generation cephalosporin, ceftiofur, in dairy cows and cattle reduces the susceptibility to ceftriaxone in *E. coli* and does not have an effect on the maintenance and dissemination of bla_{CMY-2}-bearing plasmid.[37,38] However, the application of these cephalosporins may increase ESBL-producing *E. coli*

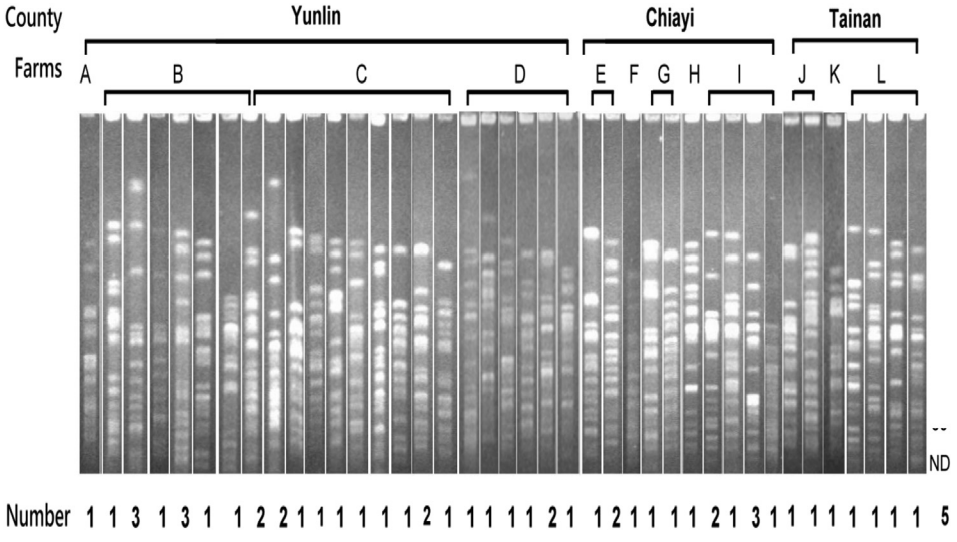

Figure 1. Pulsed-field gel electrophoresis analysis of clinical *Escherichia coli* isolates associated with cow mastitis.

Table 5 Characterization of *Escherichia coli* isolates from cattle worldwide

Country	Animal condition	Source	CTX-M type	Other *bla* genes	ESBL prevalence (%)	Refs
China	Healthy	Rectal	CTX-M14, CTX-M55			44
Switzerland	Healthy	Feces, Beef	CTX-M1, 14,15, 117	bla_{TEM}	13.7	14
		Milk	CTX-M1	bla_{TEM}		
		Feces	CTX-M9 group	bla_{TEM}	17.1	45
France	Healthy	Soil, feces	CTX-M1	bla_{TEM-71}		40
	Diarrhea, septicemia	Feces, Blood	CTX-M1 group (65.7%), CTX-M2 group (3.9%), CTX-M9 group (27%)	bla_{TEM} (3.4%)	100	46
Japan	Diseased		CTX-M2	bla_{TEM}, bla_{CMY-2}	2.8	43
	Healthy	Rectal	CTX-M		12.5	47
Korea	Healthy	Feces	CTX-M		0.2	48
		Feces, Milk	CTX-M14, CTX-M15, CTX-M32	bla_{TEM-1}		49
Ireland	Diseased		CTX-M2	bla_{CMY-2}		36
United Kingdom	Healthy	Feces	CTX-M1, 14, 15			39
		Milk	CTX-M15	bla_{TEM-1}		50
Taiwan	Clinical mastitis	Milk	CTX-M14, 15, 55	bla_{CMY}, bla_{TEM}, bla_{SHV}	11	This study

ESBL = extended-spectrum β-lactamase.

from animals, such as cattle, worldwide[14,39–43] (Tables 4 and 5)[14,36,39,40,43–50]. The ESBL-producing isolates were more prevalent in diseased cattle than healthy cattle and were obtained from different sources. The bla_{CTX} is the most important ESBL-related gene for ESBL-producing *E. coli* from cattle and its genotypes are associated with the geographic area (Tables 3 and 5). The prevalent genotypes of the bla_{CTX} are $bla_{CTX-M14}$ and $bla_{CTX-M15}$ worldwide. However, in this study, $bla_{CTX-M55}$ in the isolates from Yunlin and Tainan was the most prevalent genotype and has been reported from cattle in China. The $bla_{CTX-M55}$ isolates were genetically identical from the same farm in Yunlin, implying clonal dissemination of $bla_{CTX-M55}$ *E. coli* in Yunlin.

In the present study, we determined $bla_{CTM-M14}$, $bla_{CTM-M15}$, and $bla_{CTM-M55}$ responsible for ESBL in *E. coli*. However, $bla_{CTM-M14}$ could not present simultaneously with $bla_{CTX-M15}$ or $bla_{CTM-M55}$ in the same isolate, indicating that $bla_{CTM-M14}$ and $bla_{CTX-M15}$ or $bla_{CTM-M55}$ may exist mutually exclusively, possibly on different incompatibility plasmids, and therefore, the transferability of $bla_{CTM-M14}$ and $bla_{CTX-M15}$ or $bla_{CTM-M55}$ associated elements need to be confirmed. Additionally, some MDR ESBL-producing isolates carried AmpA and AmpC *bla* genes simultaneously, such as bla_{CTX-M2} and bla_{CMY-2}, in hospitalized animals (Table 5).[35] In the present study, 28.1% isolates carried AmpA and AmpC genes simultaneously (Table 3). Carbapenem-resistant *E. coli* can occur in ESBL- and AmpC-producing *E. coli* isolates with mutations in *ompF* and *ompC* genes,[51,52] or be through introduction of the chromosomal- and plasmid-encoded carbapenamase, to become carbapenem-resistant.[53]

MDR fluoroquinolone-resistant ESBL isolates 31 and 58 with both AmpA and AmpC genes, differed in bla_{CTX-M} genotype and pulsotypes and were located in Chiayi and Tainan, respectively. These results demonstrate different origins of these two isolates. The presence of these isolates in current cow farms and the mechanism of reduced susceptibility to carbapenem need to be further investigated. To avoid the emergence of such resistant isolates and to prevent the spreading of these isolates, the use of cephalosporins and fluoroquinolones should be limited in treating cow infection isolates, and enhanced sanitation of milk processing and transportation is needed.

In conclusion, genetically divergent *E. coli* isolates were less resistant to traditional antimicrobials and not related to human pathogens. Emergence of MDR fluoroquinolone-resistant ESBL producers with reduced susceptibility to carbapenem may threaten the public health.

Conflicts of interest

All authors declare that they have no conflicts of interest relevant to this article.

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