Applied and Environmental Microbiology, May 2011, p. 3163–3166 Vol. 77, No. 9
0099-2240/11/$12.00 doi:10.1128/AEM.02188-10

Plasmid Typing and Resistance Profiling of *Escherichia fergusonii* and Other *Enterobacteriaceae* Isolates from South Korean Farm Animals▽

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Received 15 September 2010/Accepted 6 March 2011

In this study, we focused on determining the distribution and prevalence of major plasmid replicons in β-lactam-resistant *Escherichia fergusonii* and *Enterobacteriaceae* of animal and human origin. A high degree of plasmid variability and multiple plasmid replicons were observed among the isolates. The IncF and IncI1 replicons were the most prevalent in *E. fergusonii* and *Salmonella enterica* serovar Indiana isolated from swine and poultry in South Korea, respectively. The presence of broad-host-range plasmid replicons such as IncN, IncA/C, IncHI1, and IncHI2 that are associated with important virulence genes and toxins as well as antimicrobial resistance determinants indicates that *E. fergusonii* has the potential to become an important pig pathogen and possible emerging opportunistic zoonotic pathogen.

Several recent studies have reported that *Escherichia fergusonii*, a member of the *Enterobacteriaceae*, is becoming resistant to the available antimicrobial therapy options (2, 17, 23). Among the members of *Enterobacteriaceae*, *E. fergusonii* has a distinct lactose-nonfermenting phenotype closest to that of *E. coli*. There are few reports of clinically significant *E. fergusonii* human and animal infections, suggesting that this organism may cause enteric infections in different hosts (1, 8, 16, 21, 23). Interestingly, we have encountered a high frequency of multidrug-resistant *E. fergusonii* in fecal samples of clinically sick pigs in our laboratory and at the National Veterinary Research and Quarantine Service (NVRQS) of South Korea since 2007. Most of these isolates are resistant to the antibiotics commonly used on farms. In our previous studies of antimicrobial resistance in *Enterobacteriaceae* isolated from farm animals, we identified some important antimicrobial resistance genes associated with transferable plasmids of similar sizes. In addition to antibiotic resistance genes, plasmids may also bear important toxin genes that could be maintained and disseminated to a wide range of microbes, especially members of the *Enterobacteriaceae*, from farm animals that share common environmental niches (9, 10, 13, 15).

In light of the available information on *E. fergusonii* and the role of transferable genetic elements in the acquisition and dissemination of biologic traits affecting success as pathogens, we determined the antimicrobial resistance profiles, plasmid replicons, and plasmid-associated toxins and virulence genes present in β-lactam-resistant *E. fergusonii* to assess the potential risk to animal and public health (3, 5, 7, 11).

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▽ Published ahead of print on 11 March 2011.

We also included β-lactam-resistant isolates of the known opportunistic zoonotic pathogens *E. coli*, *Salmonella*, and *Klebsiella* in this study to determine if certain lineages of transferable genetic factors are involved (15, 19).

Antimicrobial resistance phenotypes, genotypes, and serotypes of the isolates. Forty-six *E. fergusonii* isolates obtained between 2007 and 2009 were tested with common antibiotics used on farms. Ampicillin-resistant isolates were tested for extended-spectrum β-lactamase (ESBL) phenotypes by double disk diffusion tests using three indicator cephalosporins: cefotaxime (CTX), ceftazidime (CAZ), and cefoxitin (FOX), both alone and in combination with amoxicillin-clavulanic acid (AMC) (4).

For all the β-lactam-resistant isolates, we performed PCRs with oligonucleotide primer sets targeting TEM, SHV, CTXM, or AmpC β-lactamases as described previously (19). Because *E. fergusonii* was previously misidentified as a variant biotype of *E. coli* O157:H7 by Vitek, and to determine if some of the isolates carried *E. coli* O157 somatic antigens, 20 randomly selected *E. fergusonii* isolates were checked for cross-reaction with antisera specific for *E. coli* O antigens 157 and 55 at the NVRQS of South Korea (6, 7, 23).

The disk diffusion test results revealed that all *E. fergusonii* isolates were resistant to more than three antibiotics commonly used on farms. Of the isolates, eight exhibited resistance to ampicillin while one *E. fergusonii* isolate showed reduced sensitivity to the indicator cephalosporins. PCR and sequencing results showed that all eight ampicillin-resistant isolates carried the TEM-1 gene, while the single *E. fergusonii* isolate with reduced sensitivity to the indicator cephalosporins carried an additional CTXM-15 β-lactamase gene. The antimicrobial phenotypes and genotypes of these strains are listed below (see Table 3). None of the *E. fergusonii* isolates identified in this study were positive for *E. coli* O antigens 157 and 55.

Transfer of antimicrobial resistance, toxins, and virulence factors. Mixed broth culture mating was performed with the azide-resistant *E. coli* J53AzR strain as a recipient for all ampicillin-resistant *E. fergusonii* isolates. Similar conjugation ex-

TABLE 1. Description of oligonucleotide primers used for detection of toxins and virulence genes in this study

Target gene	Oligonucleotide primer sequence (5′–3′)		Location	Amplicon size (bp)
	Forward	Reverse		
F4	GCCTGGATGACTGGTGATTT	TCTGACCGTTTGCAATACCC	Plasmid, *fae* locus	715
F5	TTGGGCAGGCTGCTATTAGT	TAGCACCACCAGACCCATTT	Plasmid, *fan* locus	222
F6	GCGTGCATCGAAATGAGTT	GGTGGTTCCGATGTATGCTT	Chromosome, *fas* locus, or plasmid	589
F18	CTTTCACATTGCGTGTGGAG	ATTCGACGCCTTAACCTCCT	Plasmid, *fed* locus	441
F41	GGAGCGGGTCATATTGGTAA	CTGCAGAAACACCAGATCCA	Chromosome	941
STa	GAAACAACATGACGGGAGGT	GCACAGGCAGGATTACAACA	Plasmid, *estA* gene	229
STb	CCTACAACGGGTGATTGACA	CCGTCTTGCGTTAGGACATT	Plasmid, *estB* gene	480
LT	GGTTTCTGCGTTAGGTGGAA	GGGACTTCGACCTGAAATGT	Plasmid	605
Stx2e	TGGTGTCAGAGTGGGGAGAA	TACCTTTAGCACAATCCGCC	Plasmid/chromosome	351
EAST1	CCATCAACACAGTATATCCGA	GGTCGCGAGTGACGGCTTTGT	Plasmid	111
AIDA-I	TGGTGGGAAAACCACTGCTA	TAGCCGCCATCACTAACCAG	Plasmid	771
pAA	CCATAAAGACAGCTTCAGTGAAAA	GTATTACTGGTACCACCACCATCA	Plasmid	162
eae	CCCGAATTCGGCACAAGCATAAGC	CCCGGATCCGTCTCGCCAGTATTCG	Chromosome/plasmid	881
stx	GAGCGAAATAATTTATATGTG	TGATGATGGCAATTCAGTAT	Plasmid/chromosome	518
est	TTAATAGCACCCGGTACAAGCAGG	CCTGACTCTTCAAAAGAGAAAATTAC	Plasmid/chromosome	147
elt	TCTCTATGTGCATACGGAGC	CCATACTGATTGCCGCAAT	Plasmid/chromosome	322
ipaH	GTTCCTTGACCGCCTTTCCGATAC CGTC	GCCGGTCAGCCACCCTCTGAGAGTAC	Plasmid	619
aggR	GTATACACAAAGAAGGAAGC	ACAGAATCGTCAGCATCAGC	Plasmid	254
CVD432	AGACTCTGCCGAAAGACTGTATC	ATGGCTGTCTGTAATAGATGAGAAC	Plasmid	194
aspU	GCCTTTGCGGGTGGTAGCGG	AACCCATTCGGTTAGAGCAC	Plasmid	282

periments were also performed for four *E. coli* isolates from cattle and pigs kindly provided by S. K. Lim from NVRQS (15), single isolates of *E. coli* and *K. pneumoniae* from swine from our previous research work (20), a *Salmonella enterica* serovar Montevideo clinical isolate kindly provided by *lebsiella* J. Y. Kim (12), three *Salmonella enterica* serovar Indiana poultry isolates from recent research work (19), and two *K. pneumoniae* clinical isolates (10252 and 10255) obtained from the Korean Type Culture Collection (KTCC) (18) to determine if the *E. fergusonii* isolates contained similar transferable genetic factors to these other pathogens.

Briefly, single colonies of the donor and recipient strains grown in tryptic soy broth (TSB) (Difco) were mixed and incubated at 37°C for 20 h. MacConkey agar supplemented with sodium azide (200 μg/ml) and ampicillin (100 μg/ml) was used to select for transconjugants. Single colonies of all the donors and transconjugants were picked from MacConkey agar plates and cultured overnight in TSB (19). PCR was performed with DNA extracted from both the donor and transconjugant strains, targeting 20 different plasmid-associated toxins and virulence factors using the primers listed in Table 1 (14, 22).

Lateral transfer of ampicillin resistance and the TEM-1 gene was observed in six of eight *E. fergusonii* isolates. Transconjugants of one *E. fergusonii* strain contained both TEM-1 and CTXM-15 genes and an AM-C-Te-SXT resistance phenotype. Similarly, we confirmed the transferability of β-lactam resistance of the 5 *E. coli*, 2 *K. pneumoniae*, 2 *S.* Indiana, and 1 *S.* Montevideo β-lactam-resistant isolates included in this study.

Plasmid profiling of the donors and transconjugants of all isolates (*E. coli*, *K. pneumoniae*, and *Salmonella* spp.) revealed transfer of plasmids larger than 90 kbp. Among the isolates tested for toxin and virulence factors, one *E. fergusonii* isolate positive for TEM and CTXM-15 carried STa, LT, F4, and F18.

TABLE 2. Oligonucleotide primers used for plasmid replicon typing

Name	Target site	Oligonucleotide primer sequence (5′–3′)		Amplicon size (bp)
		Forward	Reverse	
HI1	*parA-parB*	GGAGCGATGGATTACTTCAGTAC	TGCCGTTTCACCTCGTGAGTA	471
HI2	Iterons	TTTCTCCTGAGTCACCTGTTAACAC	GGCTCACTACCGTTGTCATCCT	644
I1	RNAI	CGAAAGCCGGACGGCAGAA	TCGTCGTTCCGCCAAGTTCGT	139
X	*oriγ*	AACCTTAGAGGCTATTTAAGTTGCTGAT	TGAGAGTCAATTTTTATCTCATGTTTTAGC	376
L/M	RepA,B,C	GGATGAAAACTATCAGCATCTGAAG	CTGCAGGGGCGATTCTTTAGG	785
N	*repA*	GTCTAACGAGCTTACCGAAG	GTTTCAACTCTGCCAAGTTC	559
FIA	Iterons	CCATGCTGGTTCTAGAGAAGGTG	GTATATCCTTACTGGCTTCCGCAG	462
FIB	*repA*	GGAGTTCTGACACACGATTTTCTG	CTCCCGTCGCTTCAGGGCATT	702
W	*repA*	CCTAAGAACAACAAAGCCCCCG	GGTGCGCGGCATAGAACCGT	242
Y	*repA*	AATTCAAACAACACTGTGCAGCCTG	GCGAGAATGGACGATTACAAAACTTT	765
P	Iterons	CTATGGCCCTGCAAACGCGCCAGAAA	TCACGCGCCAGGGCGCAGCC	534
FIC	*repA2*	GTGAACTGGCAGATGAGGAAGG	TTCTCCTCGTCGCCAAACTAGAT	262
A/C	*repA*	GAGAACCAAAGACAAAGACCTGGA	ACGACAAACCTGAATTGCCTCCTT	465
T	*repA*	TTGGCCTGTTTGTGCCTAAACCAT	CGTTGATTACACTTAGCTTTGGAC	750
FII_S	*repA*	CTGTCGTAAGCTGATGGC	CTCTGCCACAAACTTCAGC	270
F_{repB}	RNAI/*repA*	TGATCGTTTAAGGAATTTTG	GAAGATCAGTCACACCATCC	270
K	RNAI	GCGGTCCGGAAAGCCAGAAAAC	TCTTTCACGAGCCCGCCAAA	160
B/O	RNAI	GCGGTCCGGAAAGCCAGAAAAC	TCTGCGTTCCGCCAAGTTCGA	159

TABLE 3. Description of the β-lactam-resistant isolates, plasmid replicon types, antibiogram, toxins, and virulence factors of donor and transconjugants of *Enterobacteriaceae* isolated from human and animals in South Korea[b]

Species	Donors					Transconjugants		
	Source[a]	Enzyme(s)	T/VF	Replicons (Inc)[d]	Resistance[c]	Enzymes	T/VF	Replicons (Inc)
E. fergusonii	Pig/Fe	TEM-1	*eae*	I1, F	Am-C-Te-SXT	TEM-1	—	F
E. fergusonii	Pig/Fe	TEM-1	—	I1, F, FIB	Am-C-Te-SXT	TEM-1	—	I1, F
E. fergusonii	Pig/Fe	TEM-1	*eae*	I1, F	Am-C-Te-SXT	TEM-1	—	I1, F
E. fergusonii	Pig/Fe	TEM-1	—	I1, F, FIB, Y, N	Am-C-Te-SXT	TEM-1	—	I1
E. fergusonii	Pig/Lu	TEM-1	*eae*	I1, HI2	Am-C-Te-SXT	TEM-1	—	F
E. fergusonii	Pig/Fe	TEM-1	—	F, Y, A/C	—	—	—	—
E. fergusonii	Pig/Fe	TEM-1, CTXM-15	Sta, LT, F4, F18	I1, Frep, FIB, X, N	Am-C-Te-SXT	TEM-1, CTXM-15	F18	I1, F, FIB
E. coli	Cattle/Fe	CTXM-14	*eae*	F, FIB, HI1	Am-C-Te-SXT	CTXM-14	—	F, FIB
E. coli	Dog/Fe	TEM-1, CTXM-14	—	F	AM-SXT	CTXM-14	—	F
E. coli	Cattle/Fe	TEM-1, CTXM-14	—	F, FIA, FIB, B/O, A/C	Am-C-Te-SXT-GM	TEM-1, CTXM-14	—	B/O, A/C
E. coli	Pig/Fe	TEM-1, CTX-15	—	I1, F, FIB, B/O	Am-C-Te-SXT	TEM-1, CTXM-15	—	I1
E. coli	Pig/Fe	TEM-1, DHA-1	EAST1	I1, F, FIB, FIA, Y, HI1	Am-C-Te-SXT	DHA-1	—	F
S. Montevideo	Human/Fe	TEM-1, DHA-1	—	HI2	Am-C-Te-GM	DHA-1	—	HI2
K. pneumoniae	KTCC	TEM-1, DHA-1	—	F, FIB, A/C, —	Am-C-Te-SXT	DHA-1	—	A/C
K. pneumoniae	KTCC	TEM-1, DHA-1	—	—	—	—	—	—
K. pneumoniae	Pig/Fe	SHV-28, DHA-1	—	F	Am-C	DHA-1	—	F
S. Indiana	Poultry/Fe	TEM-1, DHA-1	—	I1, F, FIB, HI2	Am-Te-SXT	TEM-1, DHA-1	—	I1
S. Indiana	Poultry/Fe	TEM-1, DHA-1	—	I1, F, FIB, N,HI2	Am-Te-SXT-GM	TEM-1, DHA-1	—	I1
S. Indiana	Poultry/Fe	TEM-1, DHA-1	—	N, HI2	—	—	—	—

[a] Fe, fecal specimen; Lu, lung specimen.
[b] T, toxins; VF, virulence factors; —, not identified.
[c] Am, ampicillin; C, chloramphenicol; Te, tetracycline; SXT, sulfa plus trimethoprim; GM, gentamicin.
[d] Inc, incompatibility group.

A transconjugant of this isolate was positive for TEM, CTXM-15, and F18. Four *E. fergusonii* isolates carrying TEM-1 and an *E. coli* pig isolate with DHA-1 were positive for the *eae* and EAST1 genes, respectively.

Replicon typing of the isolates. Replicon typing was performed with the oligonucleotide primers listed in Table 2 as described by Johnson et al. (10). The donors and transconjugants of all the β-lactam-resistant isolates (*E. fergusonii*, *E. coli*, *K. pneumoniae*, and *Salmonella* spp.) carried different replicons either alone or in combination. IncF and IncI1 were the most common replicons among the isolates and were identified in combination with repFIA or/and FIB. Likewise, IncY, IncX, IncN, IncA/C, IncHI1, and IncHI2 were also detected in the isolates. The distributions of replicon types in all the donors and transconjugants are presented in Table 3.

Interestingly, replicon typing of the transconjugant of only the *E. fergusonii* isolate with transferable CTXM-15 and F18 plasmids revealed the presence of two plasmids of 120 and 180 kb with three replicon types: IncF, IB, and I1. Because it was not clear which replicon type carried the CTXM-15 gene, it is unlikely that the IncI1 plasmid was selected at a later stage in *E. fergusonii*, as all ampicillin-resistant isolates except for one carried IncI1 replicons. This indicates that IncI1 in *E. fergusonii* could have other essential roles, such as encoding type IV pili and contributing to adhesion and invasion, as seen in Shiga-toxigenic *E. coli* (3). Replicon typing of two CTXM-14-positive *E. coli* isolates showed the presence of the IncF plasmid in both the donors and transconjugants, indicating that the IncF plasmid might have carried the CTXM-14 gene along with the resistance determinants for ampicillin and sulfa plus trimethoprim (9). Similarly, the transconjugant of *E. coli* that carried CTXM-15, IncF, I1, and B/O contained only a single replicon (I1) and CTXM-15, indicating involvement of this plasmid and CTXM-15. This result suggests that the IncF plasmid is a more primitive plasmid in the *E. coli* isolates, as it carries antibiotic resistance determinants to more classical (i.e., older) antibiotics used on farms (9). In contrast, IncI1 appears to have been selected later in time, corresponding to the use of expanded-spectrum cephalosporins on farms.

A single isolate that was negative for IncI1 had two other replicons: IncF and IncY. We found that most *E. fergusonii* isolates that carried the additional replicons of IncFIB and IncFIA also contained IncF (Table 3). IncF plasmids are commonly found in the fecal flora of humans and animals, indicating that they may have other functions related to the host, apart from antibiotic resistance (5). Although this plasmid is also known to contribute to host virulence by carrying toxin and serum resistance genes that are also present in *E. coli* O157:H7 and *Salmonella*, none of the *E. fergusonii* isolates showed any reaction to *E. coli* O antigens 157 and 55 (3). To the best of our knowledge, this is the first study to investigate plasmid replicons among different *Enterobacteriaceae* isolates of farm origin in South Korea.

Conclusions. Although *E. fergusonii* infections have been reported in both humans and animals, there are very few, if any, reports of this microbe in South Korea. The first *E. fergusonii* isolate in South Korea was first identified in our laboratory in 2001 from the fecal sample of a clinically sick pig. This was followed by the isolation of eight *E. fergusonii* strains from fecal samples and one from a lung specimen in 2007 and an

additional 34 fecal isolates between 2008 and 2009. Despite the low percentage of ampicillin-resistant *E. fergusonii* isolates detected in this study compared to our previous study of *E. coli*, the presence of clinically important plasmid replicons carrying CTXM-15, toxins, and virulence factors, such as *eae*, STa, LT, F4, and F18, which have known roles in the pathogenesis of enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *E. coli*, indicates that *E. fergusonii* could establish itself as a potential swine pathogen and emerging opportunistic zoonotic agent of public health importance (1, 6, 16). Furthermore, because *E. fergusonii* has already been found to cause disease suggestive of salmonellosis in animals with clinical manifestations including abortion, scour, and mastitis, more frequent isolation of *E. fergusonii* from clinical specimens of farm origin is expected as this pathogen is further characterized (1, 8, 16, 21, 23).

We are thankful to Bernard Beall and Bob Gertz for reading over earlier versions of the manuscript.

This work was supported by a grant from a BK-21 grant and a Bio-Green 21 grant (20070401-034-009-007-01-00), ipet 110032-3, and the Research Institute of Veterinary Science, Seoul National University, South Korea.

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