APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2009, p. 5700–5703 Vol. 75, No. 17
0099-2240/09/$08.00+0 doi:10.1128/AEM.02816-08

Inter- and Intraspecies Plasmid-Mediated Transfer of Florfenicol Resistance in *Enterobacteriaceae* Isolates from Swine▽

Nabin Rayamajhi,[1] Seung Bin Cha,[1] Mi Lan Kang,[1] Su In Lee,[1] Hee Soo Lee,[2] and Han Sang Yoo[1]*

Department of Infectious Disease, College of Veterinary Medicine and KRF Priority Zoonotic Disease Research Institute and Brain Korea 21 for Veterinary Science, Seoul National University, Seoul 151-742,[1] and Division of Bacteriology and Parasitology, National Veterinary Research and Quarantine Service, Anyang 430-757, Kyunggi,[2] South Korea

Received 12 December 2008/Accepted 27 June 2009

Florfenicol resistance was analyzed in 230 enteric pig isolates collected between 1998 and 2006. PCR, plasmid profiling, Southern blot hybridization, and a mixed-broth conjugation assay suggested the intra- and interspecies plasmid-mediated transfer of florfenicol resistance among the isolates that exhibited MICs for florfenicol between 4 to 128 mg/liter.

Florfenicol, a fluorinated chloramphenicol derivative, is a broad-spectrum antimicrobial agent active against a wide range of both gram-positive and gram-negative bacteria. In South Korea, it was initially approved for the treatment of bovine and porcine respiratory disease in 1999 (10, 16). Recent reports have shown increasing use of florfenicol in the treatment of target respiratory pathogens as well as in *Escherichia coli* infection in bovine, porcine, and poultry production (4, 6, 11, 18). This may have lead to the emergence and spread of florfenicol resistance in a wide range of gram-negative and gram-positive bacteria (5, 8, 15, 17, 18). Florfenicol resistance in enteric microbes, such as *E. coli*, *Klebsiella pneumoniae*, and *Salmonella* spp., is of special concern because they share a common gut environment and are liable to transferring the resistance genes through mobile genetic structures and plasmids bearing antibiotic-resistant determinants (1, 2, 17). Florfenicol resistance is mediated by the *floR* gene in gram-negative bacteria, and chromosomal location of this gene, especially in the pentadrug-resistant gene cluster of *Salmonella enterica* serovar Typhimurium phage type (PT) DT104, has attracted wide interest in light of its contribution to multidrug resistance and the development of DT104 detection methods using PCR (1, 7, 9).

Though recent reports have shown increased consumption of florfenicol in farms, no reports are available on the prevalence of florfenicol resistance among microbes of enteric origin from South Korean farms (10, 12, 19). Past work on florfenicol resistance has reported a wide range of MICs for florfenicol for *E. coli* animal isolates, with and without the *floR* gene (12, 14, 17, 18). The *floR* gene, however, has been identified in most *E. coli* isolates with MICs for florfenicol of >8 mg/liter (6, 15, 18). However, some strains with MICs between 8 mg/liter and 16 mg/liter have been reported to have other mechanisms of reduced susceptibility to florfenicol (15, 17). Currently, CLSI breakpoints are approved to indicate florfenicol resistance only for bovine and porcine respiratory disease pathogens (*Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni*); no approved CLSI breakpoint is currently available for enteric bacteria except for *S. enterica* serovar Cholerasuis.

In light of this, our study focused on an analysis of phenotypic and genotypic florfenicol resistance in enterobacteria isolated from the samples of clinically sick animals (pigs) between the years 1998 and 2006. We further tested whether or not this resistance was plasmid mediated. Conjugation experiments were performed to evaluate the ease with which resistance-associated plasmids would move across species or genera of enterobacteria.

E. coli ($n = 121$), *S. enterica* serovar Typhimurium ($n = 71$), *S. enterica* serovar Enteritidis ($n = 12$), and *K. pneumoniae* ($n = 26$) strains were obtained from the feces, intestines, lungs, and lymph nodes of pigs with mixed clinical signs of digestive and respiratory disorders after necropsy. Identification of the suspected colonies, isolated from selective media incubated overnight with necropsied samples, was made by Vitek (Vitek system; bioMérieux, Marcy l'Etoile, France). All *Salmonella* spp. were serotyped by slide agglutination and tube agglutination with *Salmonella* O and H group antisera, respectively (Difco Co., Franklin Lakes, NJ), at the National Veterinary Research and Quarantine Service (NVRQS; Anyang, South Korea). Phage typing was performed for the isolates showing ACSSuTF resistance at NVRQS in accordance with the guidelines provided by the Public Health Laboratory Service (PHLS), London, United Kingdom.

MICs of antimicrobial agents were determined by the microbroth dilution method, using microtiter plates that contained florfenicol concentrations of 0.5 to 256 mg/liter in serial twofold dilutions (16). Evaluation of the MIC was performed according to the recommendations of the CLSI (13). The MIC was considered to correspond to the first dilution at which no growth was detectable. *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella* serovar Typhimurium DT104 ATCC 2501 were used for quality control.

PCR was performed using genomic DNA (Wizard genomic DNA purification kit; Promega, Madison, WI) of all the isolates with MICs for florfenicol of ≥4 mg/liter (see Table 2) as a template and a set of *floR*-specific oligonucleotide primers, *Flo-F* (5′-CTGATCGCTCCTTTCGACAT-3′) and *Flo-R* (5′-CCGTGGCGTAACAAATCAC-3′) (GenBank accession no.

* Corresponding author. Mailing address: Department of Infectious Disease, College of Veterinary Medicine, Seoul National University, San 56-1, Shilim 9 dong, Kwanak-Ku, Seoul 151-742, South Korea. Phone: 82-2-880-1263. Fax: 82-2-874-2738. E-mail: yoohs@snu.ac.kr.

▽ Published ahead of print on 10 July 2009.

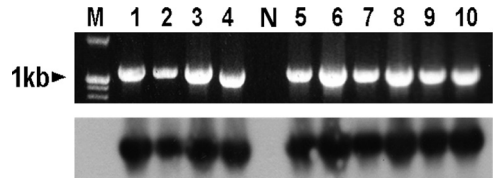

FIG. 1. PCR amplification of the *floR* gene (upper panel). Lane M, 100-bp DNA marker (iNtRON Biotechnology, South Korea); lanes 1 to 4, *E. coli* (03/16; MIC, 16 mg/liter), *E. coli* (04/18; MIC, 32 mg/liter), *Salmonella* serovar Enteritidis (03/22; MIC, 32 mg/liter), and *K. pneumoniae* (04/22; MIC, 32 mg/liter); lane N, negative control; lanes 5 to 10, *E. coli* (04/1; MIC, 16 mg/liter), *Salmonella* serovar Typhimurium (04/13; MIC, 16 mg/liter), *Salmonella* serovar Enteritidis (04/8; MIC, 32 mg/liter), *E. coli* (03/17; MIC, 16 mg/liter), *E. coli* (04/19; MIC, 32 mg/liter), and *K. pneumoniae* (04/21; MIC, 64 mg/liter). Identity confirmation of *floR* gene in these isolates was performed based on the size and Southern blot hybridization of PCR products (lower panel).

DQ647028.2). Amplified PCR product with the expected size of 1,083 bp from one of the isolates was cloned in the pQE-UA 30 vector system (Qiagen) using the manufacturer's protocol. The identity of *floR* gene was confirmed by sequencing. For all other PCR-positive isolates, identity of the *floR* gene was confirmed by the size and Southern blot hybridization result of the PCR products with the biotin-labeled *floR* probe as described below.

Mixed-broth culture mating was performed to observe the transferability of the florfenicol resistance gene, as described by Kang et al. (7). All isolates with the *floR* gene (see Table 2) were included as putative donors in a conjugation experiment. *E. coli* RG488 Rif^r, kindly provided by Je Chul Lee (Kyungpook National University), was used as the recipient to detect the transfer of resistance gene. The transconjugants were selected on MacConkey agar supplemented with florfenicol (2 mg/liter) and rifampin (rifampicin) (100 mg/liter). Transfer frequency was calculated as the number of transconjugants per recipient.

Transfers of resistance to florfenicol, tetracycline, streptomycin, kanamycin, and sulfamethoxazole in transconjugants were confirmed by MIC, following the procedures described above. Presence of the florfenicol resistance gene, *floR*, in the transconjugants was confirmed by PCR and DNA hybridizations.

Single colonies of the transconjugants (*E. coli* RG488 Rif^r) of *E. coli* 03/16, *E. coli* 04/18, *Salmonella* serovar Enteritidis 03/22, and *K. pneumoniae* 04/22 and single colonies of isolates that failed to transfer the florfenicol resistance (*E. coli* 04/1, *Salmonella* serovar Typhimurium 04/13, and *Salmonella* serovar Enteritidis 04/8) in broth conjugation assay were picked from the MacConkey agar plates with and without antibiotics. Plasmid DNA was extracted using the midi extraction kit (Qiagen, Valencia, CA) following the manufacturer's protocol and was digested by the EcoRI restriction enzyme. The digested plasmids and PCR-amplified products for all the *floR*-positive strains (see Table 2) were electrophoresed through agarose

TABLE 1. Susceptibilities, amplification, and hybridization patterns of *floR* in *E. coli*, *Salmonella* serovar Typhimurium, *Salmonella* serovar Enteritidis, and *K. pneumoniae* isolates

Strains[c]	Yr/no.	FFC MIC (mg/liter)	PCR result[a]	TF	FCC/RIF MIC (TCs) (mg/liter)	Southern blot hybridization[b]
E. coli	04/1	16	+			−
	04/3	32	+			−
	05/9	32	+			−
	05/13	4	−			−
	03/14	8	−			−
	03/16	16	+	1.3×10^{-5}	16/>256	+
	03/17	16	+			−
	04/18	32	+	1.4×10^{-5}	16/>256	+
	04/19	32	+			−
ST (PT193)	04/13	16	−			−
	04/26	16	−			−
	04/15	16	−			−
ST (U302)	05/24	128	+			−
ST (PT120)	05/21	128	+			−
ST (DT104)	03/41	32	+			−
SE	04/8	32	+			−
	03/22	32	+	1.3×10^{-4}	16/128	+
KP	04/21	64	+			−
	04/22	32	+	1.2×10^{-4}	16/128	+
E. coli (RG488 Rif^r)		<0.5	−			−

[a] PCR amplification of *floR* gene, using whole-cell genomic DNA.
[b] Hybridization signal was in the plasmid DNA of the transconjugants (*E. coli* RG4888 Rif) of *E. coli* 03/16 (MIC of 16 mg/liter), *E. coli* 04/18 (MIC of 16 mg/liter), *Salmonella* serovar Enteritidis 03/22 (MIC of 16 mg/liter), and *K. pneumoniae* 04/22 (MIC of 16 mg/liter).
[c] ST, *Salmonella* serovar Typhimurium; SE, *Salmonella* serovar Enteritidis; KP, *Klebsiella pneumoniae*; FCC, florfenicol; RIF, rifampin; TF, transfer frequency; TCs, transconjugant.

TABLE 2. Susceptibilities of antimicrobial agents for the donor and the transconjugant strains

Strain	MIC (mg/liter)[a]					
	KAN	TE	SuM	STR	FCC	RIF
E. coli 03/16	>256	>256	>256	128	16	2
Transconjugant	128	128	128	128	16	>256
E. coli 04/18	>256	>256	>256	>256	32	4
Transconjugant	>256	>256	>256	64	16	>256
Salmonella serovar Enteritidis 03/22	128	128	>256	>256	32	<0.5
Transconjugant	128	64	>256	>256	16	128
K. pneumoniae 04/22	>256	64	>256	>256	32	<0.5
Transconjugant	128	32	128	>256	16	128

[a] KAN, kanamycin; TE, tetracycline; SuM, sulfamethoxazole; STR, streptomycin; FCC, florfenicol; RIF, rifampin.

gels and transferred to a positively charged nylon membrane (GE Healthcare, Little Chalfont, England). Hybridization experiment was performed by using a psoralen-biotin (BrightStar psoralen-biotin nonisotopic labeling kit; Ambion Inc, Austin, TX)-labeled PCR product of the *floR* gene. Detection was performed using a BrightStar BioDetect nonisotopic detection kit (Ambion Inc., Austin, TX) following the manufacturer's protocol.

Reports on the uses of antimicrobial agents have shown that the use of florfenicol gradually increased from 387 kg/year in 2001 to 17,159 kg in 2005 (10). More than half of this increase was used in the pig industry alone, followed by poultry and bovine farms (10). This figure could increase for subsequent years because the in vitro antimicrobial activity of florfenicol against respiratory pathogens is very effective, and no report of resistance in target pathogens is available from South Korea (16). In this study, 7.43% (9/121) of *E. coli*, 8.45% (6/71) of *Salmonella* serovar Typhimurium, 16.6% (2/12) of *Salmonella* serovar Enteritidis, and 7.69% (2/26) of *K. pneumoniae* strains exhibited MICs for florfenicol that ranged from 4 to 128 mg/liter. The *floR* gene was amplified by 14 out of 19 isolates that exhibited MIC for florfenicol of ≥4 mg/liter (Fig. 1). Two *E. coli* (MIC, 4 and 8 mg/liter) and three *Salmonella* serovar Typhimurium (MIC, 16 mg/liter each) isolates (Table 1) did not amplify the *floR* gene. The reduced susceptibility of these isolates to florfenicol might be due to the involvement of other mechanisms or resistance genes (15, 17).

Past work on florfenicol resistance in *E. coli* animal isolates has reported various ranges of MICs. North American and European *E. coli* strains carrying the *floR* gene from animal origin have been reported to have MICs for florfenicol of 16 to ≥256 mg/liter and of ≥128 mg/liter, respectively (15). However, MIC values for florfenicol of isolates from South Korea (this study) and *E. coli* isolates from China were substantially less than those described earlier (6, 11, 15). This indicates that the MIC for florfenicol could vary geographically, so a comparative study of the florfenicol resistance gene from different geographical regions might help elucidate its mechanism.

Conjugation assay showed the transfer of reduced florfenicol susceptibility by 4 out of 14 strains. These transconjugants that harbored the plasmid of 23 kb were also resistant to tetracycline, streptomycin, kanamycin, and sulfamethoxazole. Results of the MIC determination of these antibiotics for donor strains (*E. coli* 03/16, *E. coli* 04/18, *Salmonella* serovar Enteritidis 03/22, and *K. pneumoniae* 04/22) and their transconjugants are listed in Table 2. Out of those 14 strains, 10 isolates amplified the *floR* gene in PCR and failed to transfer *floR* gene in conjugation experiments. From those 10 isolates, plasmid extraction was carried out for three randomly selected *E. coli* (04/1; MIC, 16 mg/liter), *Salmonella* serovar Typhimurium (04/13; MIC, 16 mg/liter), and *Salmonella* serovar Enteritidis (04/8; MIC, 32 mg/liter) strains. None of these three strains showed *floR*-specific probe hybridization in the plasmid profile and Southern blot (Fig. 2, lanes 1 to 3) indicating the chromosomal location of the *floR* gene in these isolates. The *floR*-specific probe hybridization of plasmid DNA of the transconjugants (*E. coli* RG488 Rif^r) of *E. coli* 03/16, *E. coli* 04/18, *Salmonella* serovar Enteritidis 03/22, and *K. pneumoniae* 04/22 indicated the presence of both single (Fig. 2, left panel, lanes 4, 6, and 7) and multiple copies (Fig. 2, left panel, lane 5) of this gene. Likewise, the hybridization signal in the different digested fragments of plasmid DNA indicated the different orientations of the *floR* gene in the conjugative plasmid of these strains. The

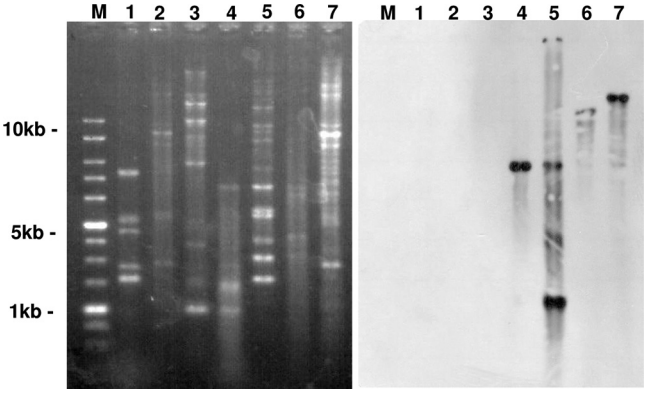

FIG. 2. Southern blot hybridization (left) and EcoRI restriction (right) profiles of plasmids extracted from *E. coli*, *Salmonella* serovar Typhimurium, *Salmonella* serovar Enteritidis, and *K. pneumoniae* isolates with different MICs. Lanes M, 1-kb DNA marker; lanes 1 to 3, *E. coli* (04/1; MIC, 16 mg/liter), *Salmonella* serovar Typhimurium (04/13; MIC, 16 mg/liter), and *Salmonella* serovar Enteritidis (04/8; MIC, 32 mg/liter) that showed no transfer of florfenicol resistance in broth conjugation experiments; lanes 4 to 7, transconjugants (*E. coli* RG4888 Rif) of *E. coli* (03/16; MIC, 16 mg/liter), *E. coli* (04/18; MIC, 16 mg/liter), *Salmonella* serovar Enteritidis (03/22; MIC, 16 mg/liter), and *K. pneumoniae* (04/22; MIC, 16 mg/liter). The hybridization signal in the different digested fragments of plasmid DNA indicated the different orientations of the *floR* gene in the conjugative plasmid of these strains. Likewise, the hybridization signal in the plasmid DNA of the transconjugant (*E. coli* RG4888 Rif) of *E. coli* (04/18; MIC, 16 mg/liter) (lane 4) indicated the multiple copies of this gene.

floR gene has also been extensively described in *Salmonella* serovar Typhimurium epidemic strain DT104. However, only one such PT was identified among the *Salmonella* serovar Typhimurium (MIC, 32 mg/liter) isolates in our study. Two other *floR*-positive *Salmonella* serovar Typhimurium isolates were identified as PT 302 and 120. Three *Salmonella* serovar Typhimurium isolates, identified as PT 193 (MIC, 16 mg/liter), did not amplify the *floR* gene (Table 1). None of these *Salmonella* serovar Typhimurium PTs transferred florfenicol resistance in the broth conjugation experiments.

Our findings are in agreement with those of previous reports on florfenicol resistance in *E. coli* and support the clinical relevance of an MIC breakpoint of 32 mg/liter in *E. coli* and other enteric bacteria as proposed by Singer et al. (17). Likewise, plasmid profiling, Southern blotting, phage typing, and conjugation experiment results indicated that emergence and dissemination of *floR* genes among the *Enterobacteriaceae* are not due to the prevalence of DT104 or other closely related PTs (4). Indeed, it could be inferred that *floR*-bearing promiscuous plasmids in these groups of enteric microbes are in circulation due to the local selection pressure imposed by the use of antimicrobial agents on farms (7, 10, 12).

This work was supported by Korea Research Foundation grants (KRF-2006-21-E00011 and KRF-2006-005-J502901), a BK-21 grant for Veterinary Science, and a Bio-Green 21 grant (20070401-034-009-007-01-00), RDA, South Korea.

REFERENCES

1. **Briggs, C. E., and P. Fratamico.** 1999. Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. Antimicrob. Agents Chemother. **43:**846–849.
2. **Carattoli, A.** 2001. Importance of integrons in the diffusion of resistance. Vet. Res. **32:**243–259.
3. Reference deleted.
4. **Cloeckaert, A., S. Baucheron, G. Flaujac, S. Schwarz, C. Kehrenberg, J. L. Martel, and E. Chaslus-Dancla.** 2000. Plasmid-mediated florfenicol resistance encoded by the *floR* gene in *Escherichia coli* isolated from cattle. Antimicrob. Agents Chemother. **44:**2858–2860.
5. **Doublet, B., S. Schwarz, C. Kehrenberg, and A. Cloeckaert.** 2005. Florfenicol resistance gene *floR* is a part of a novel transposon. Antimicrob. Agents Chemother. **49:**2106–2108.
6. **Du, X., C. Xia, J. Shen, B. Wu, and Z. Shen.** 2004. Characterization of florfenicol resistance among calf pathogenic *Escherichia coli*. FEMS Microbiol. Lett. **236:**183–189.
7. **Kang, H. Y., Y. S. Jeong, J. Y. Oh, S. H. Tae, C. H. Choi, D. C. Moon, W. K. Lee, Y. C. Lee, S. Y. Seol, D. T. Cho, and J. C. Lee.** 2005. Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. J. Antimicrob. Chemother. **55:**639–644.
8. **Kehrenberg, C., and S. Schwarz.** 2005. Plasmid-borne florfenicol resistance in *Pasteurella multocida*. J. Antimicrob. Chemother. **55:**773–775.
9. **Khan, A. A., M. S. Nawaz, S. A. Khan, and C. E. Cerniglia.** 2000. Detection of multidrug-resistance *Salmonella typhimurium* DT104 by multiplex polymerase chain reaction. FEMS Microbiol. Lett. **182:**355–360.
10. **Korea Food and Drug Administration (KFDA).** 2005. Establishment of control system of antibiotics for livestocks. Korea Food and Drug Administration, Seoul, South Korea.
11. **Li, X. S., G. Q. Wang, X. D. Du, B. A. Cui, S. M. Zhang, and J. Z. Shen.** 2007. Antimicrobial susceptibility and molecular detection of chloramphenicol and florfenicol resistance among *Escherichia coli* isolates from diseased chickens. J. Vet. Sci. **8:**243–247.
12. **Lim, S. K., H. S. Lee, H. M. Nam, Y. S. Cho, J. M. Kim, S. W. Song, Y. H. Park, and S. C. Jung.** 2007. Antimicrobial resistance observed in *Escherichia coli* strains isolated from fecal samples of cattle and pigs in Korea during 2003–2004. Int. J. Food Microbiol. **116:**283–286.
13. **National Committee for Clinical Laboratory Standards.** 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, 2nd ed. Approved standard M31-A2. National Committee for Clinical Laboratory Standards, Wayne, PA.
14. **Pritchett, L. C., M. E. Konkel, J. M. Gay, and T. E. Besser.** 2000. Identification of DT104 and U320 phage type among *Salmonella enterica* serotype Typhimurium isolates by PCR. J. Clin. Microbiol. **38:**3484–3488.
15. **Schwarz, S., C. Khenberg, B. Doublet, and A. Cloeckaert.** 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol. Lett. **28:**519–542.
16. **Shin, S. J., S. G. Kang, N. Rayamajhi, M. L. Kang, and H. S. Yoo.** 2005. Evaluation of the antimicrobial activity of florfenicol against bacteria isolated from bovine and porcine respiratory disease. Vet. Microbiol. **106:**73–77.
17. **Singer, R. S., T. E. Patterson, A. E. Meier, J. K. Gibson, H. L. Lee, and C. W. Maddox.** 2004. Relationship between phenotypic and genotypic florfenicol resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **48:**4047–4049.
18. **White, D. G., C. Hudson, J. J. Maurer, S. Ayers, S. Zhao, M. D. Lee, L. Bolton, T. Foley, and J. Sherwood.** 2000. Characterization of chloramphenicol and florfenicol resistance in *Escherichia coli* associated with bovine diarrhea. J. Clin. Microbiol. **38:**4593–4598.
19. **Yang, S. J., K. Y. Park, S. H. Kim, K. M. No, B. K. Lee, and Y. H. Park.** 2002. Antimicrobial resistance in *Salmonella enterica* serovars Enteritidis and Typhimurium isolated from animals from Korea: comparison of phenotypic and genotypic resistance characterization. Vet. Microbiol. **86:**295–301.