F33:A−:B− and F2:A−:B− Plasmids Mediate Dissemination of \textit{rmtB-bla\textsubscript{CTX-M-9}} Group Genes and \textit{rmtB-qepA} in Enterobacteriaceae Isolates from Pets in China

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Recently, the production of 16S rRNA methylases by Gram-negative bacilli has emerged as a novel mechanism for their high-level resistance to aminoglycosides (9, 31). Seven plasmid-mediated 16S rRNA methylase genes, \textit{rmtA}, \textit{rmtB}, \textit{rmtC}, \textit{rmtD}, \textit{rmtE}, \textit{armA}, and \textit{npmA}, have been identified so far in multiple species of Enterobacteriaceae (3, 6, 9, 13, 31). These resistance determinants are globally disseminated, with \textit{rmtB} and \textit{armA} being the most frequently reported types worldwide (3, 5, 9, 10, 11, 13, 19, 27, 29, 32). However, even though aminoglycosides are widely used in pets to treat Gram-negative bacterial infections, the prevalence of 16S rRNA methylases in bacteria from pets is not known. In addition, the 16S rRNA methylase genes, especially \textit{rmtB}, are commonly associated with \textit{bla\textsubscript{CTX-M}} genes (1–3, 8, 13, 19, 27, 29, 32). In our recent study on the distribution of extended-spectrum \textit{b lactamase} genes in Enterobacteriaceae isolates (119), some CTX-M-producing isolates showed significantly reduced activity of aminoglycosides, especially \textit{gentamicin}, which is the most often used aminoglycoside in pets (3, 5, 6, 9). Of the 135 CTX-M producers, 60 (44.9%) were positive for \textit{rmtB}, and 5 (3.7%) were positive for \textit{armA} (Table 1). The two dominant types of IncF plasmids, F2:A−:B−, carrying \textit{rmtB-qepA}, and F33:A−:B−, carrying the \textit{rmtB-bla\textsubscript{CTX-M-9}} group genes (and especially \textit{bla\textsubscript{CTX-M-9G}}), shared restriction patterns within each incompatibility group.

The present study included 135 CTX-M-producing \textit{Enterobacteriaceae} isolates (119 \textit{E. coli}, 11 \textit{Klebsiella pneumoniae}, 3 \textit{Enterobacter cloacae}, and 2 \textit{C. freundii} isolates) recovered from healthy or diseased pets (dogs and cats) in Guangdong, China, during 2006 and 2008. The ESBL genes in most of these strains have been characterized previously (18, 25).

The gene type of \textit{bla\textsubscript{CTX-M}} was confirmed by PCR and DNA sequencing (25). In addition, this study also included 132 \textit{Enterobacteriaceae} isolates previously confirmed to be CTX-M negative that were collected from pets in Guangdong province of China during 2006 and 2008 (18, 25). The prevalence of 16S rRNA methylase genes was identified by PCR using previously designed primers (5, 6, 9). Of the 135 CTX-M producers, 60 (44.9%) were positive for \textit{rmtB} and 5 (3.7%) were positive for \textit{armA} (Table 1). No isolate was positive for the \textit{rmtA}, \textit{rmtC}, \textit{rmtD}, \textit{rmtE}, or \textit{npmA} genes. The \textit{rmtB} gene was also detected in 9 (7.0%) of the 132 CTX-M-negative isolates. Therefore, of the 69 \textit{rmtB}-positive isolates, 60 were CTX-M producers, and most of the enzymes produced belonged to the CTX-M group. Since the plasmid-mediated fluoroquinolone efflux pump gene \textit{qepA} is frequently associated with \textit{mtb} (1, 2, 17, 22, 23, 28, 32), RmtB-producing isolates were screened for the \textit{qepA} gene (17). Our results showed that 31 (44.9%) RmtB-producing isolates were positive for \textit{qepA}. The pulsed-field gel electrophoresis analysis revealed that most of the \textit{rmtB}-positive isolates were clonally unrelated (Table 1).

The transferability of the \textit{rmtB} genes was studied by conjugation experiments as previously described (7). When plasmid cotransfer occurred, the transformation experiment was carried out. The presence of \textit{rmtB}, \textit{qepA}, and \textit{bla\textsubscript{CTX-M}} in the transconjugants and transformants was confirmed by PCR as previously described (5, 17). Of the 69 \textit{rmtB}-positive isolates, 73 transconjugants/transformants containing \textit{rmtB} were obtained from 66 isolates, with 6 donors generating two or three transconjugants carrying different plasmids and resistance genes. \textit{rmtB} was cotransferred with \textit{bla\textsubscript{CTX-M}} or \textit{qepA} genes in 33 and 25 transconjugants/transformants, respectively (Table 2). In addition, 8 transconjugants carried \textit{rmtB}, \textit{qepA}, and \textit{bla\textsubscript{CTX-M-9G}} simultaneously.

PCR-based plasmid replicon typing was performed to characterize the conjugative plasmids carrying \textit{rmtB} (4). The IncFII, IncFIB, and IncN replicon types were detected in 58 (79.5%), 5 (6.8%), and 4 (5.5%) of the plasmids from the 73
TABLE 1. Distribution of 16S rRNA methylase genes among Enterobacteriaceae isolates and diversity of these isolates

| CTX-M type(s) of β-lactamase (no. of isolates) | No. (%) of isolates positive for: | No. of PFGE subtypesa |
|-----------------------------------------------|---------------------------------|----------------------|
|                                               | mrtB   | armA   | qepAb           |                                       |
| CTX-M-9 type (88)                             | 46 (52.3) | 4 | 17 (37.0)       |                                       |
| CTX-M-14 (57)                                 | 22     | 2      | 10 (45)         | (4)                                  |
| CTX-M-24 (13)                                 | 9      | 6      | 8 (1)           |                                       |
| CTX-M-65 (9)                                  | 9      | 1      | 6 (1)           |                                       |
| CTX-M-27 (8)                                  | 5      | 2      | 8              |                                       |
| CTX-M-9 (1)                                   | 1      | 1      |                |                                       |
| CTX-M-9 type and CTX-M-1 type (17)             | 10 (58.8) | 7 |                |                                       |
| CTX-M-14, CTX-M-82 (1)                        | 1      | 1      |                |                                       |
| CTX-M-14, CTX-M-55 (9)                        | 5      | 5      | 7 (1)           |                                       |
| CTX-M-65, CTX-M-55 (1)                        | 1      | 1      |                |                                       |
| CTX-M-14, CTX-M-64, CTX-M-55 (1)              | 1      | 1      |                |                                       |
| CTX-M-14, CTX-M-64, CTX-M-61 (1)              | 1      | 1      |                |                                       |
| CTX-M-27, CTX-M-64 (1)                        | 1      |        |                |                                       |
| CTX-M-14, CTX-M-3 (1)                         | 1      | 1      |                |                                       |
| CTX-M-9, CTX-M-55 (1)                         | 1      |        |                |                                       |
| Any CTX-M-1 type (30)                         | 4 (13.3) | 1 | 3              |                                       |
| CTX-M-55 (17)                                 | 2      | 1      | 14 (2)         |                                       |
| CTX-M-64 (1)                                  |        |        | 1              |                                       |
| CTX-M-3 (6)                                   | 2      | 2      | 4 (2)          |                                       |
| CTX-M-15 (6)                                  |        |        | 5              |                                       |
| CTX-M-positive isolates (135)                 | 60 (44.4) | 5 | 27 (45.0)      |                                       |
| CTX-M-negative isolates (132)                 | 9 (6.8) | 4      |                |                                       |
| All isolates (267)                            | 69 (25.8) | 5 | 31 (44.9)      |                                       |

a Only mrtB-positive isolates were screened for the qepA gene.
b The number of nontypeable isolates is indicated in parentheses. PFGE, pulsed-field gel electrophoresis.

transconjugants, respectively, with 7 other transconjugants carrying two replicons (FII in combination with FIB or N) (Table 2). To better clarify the IncF plasmids, a replicon sequence typing scheme discriminating IncF plasmid variants, described by Villa et al. (26), was used to characterize the IncFII and IncFIB replicons. Among these transconjugants, 10 and 3 different alleles were identified for the FII and FIB replicons, respectively (Table 2). The F2 allele was detected in 24 out of the 34 transconjugants that carried both mrtB and qepA, and the F33 allele, a new F allele identified in this study, was detected in 12 out of the 22 transconjugants that carried both mrtB and blaCTX-M-9G. Three transconjugants obtained from one E. coli donor (0113DDF) carried different IncF plasmids (p0113J, p0113-1, and p0113-2T) encoded by different resistance genes (Table 3), indicating the coexistence of three unrelated plasmids in one bacterium. It has been demonstrated that the IncF plasmids possess great versatility in intracellular adaptation due to the rapid evolution of the regulatory sequences of the replicons (21, 26). The coexistence and maintenance of three IncF plasmids that belong to different subgroups, F2:A—B—, F35:A—B—, and F33:A—B—, in the same bacterial strain indicated preliminarily that these differences in the F alleles could result in the emergence of compatible plasmids.

Since most of the mrtB-qepA and mrtB-blaCTX-M-9G gene combinations were associated with the F2:A—B— and F33:A—B— plasmids, respectively (Table 3), these plasmids were subjected to restriction enzyme digestion analysis to clarify whether a specific plasmid had been disseminated among the isolates. Plasmids extracted from the transconjugants or transformants containing only a single plasmid were digested with the endonucleases EcoRI and BamHI (TaKaRa Biotechnology, Dalian, China). Twenty-one F2:A—B— plasmids carrying both mrtB and qepA were obtained, 18 of which, including one plasmid bearing mrtB, qepA, and blaCTX-M-9G showed identical plasmid restriction patterns. Interestingly, these patterns are the same as or highly similar to the patterns obtained from F2:A—B— plasmids carrying both mrtB and qepA in E. coli isolates obtained from pigs, the environment, and farmers in 2002 (Table 3) (7). It is suggested that this F2:A—B— plasmid is a rather stable plasmid circulating in different members of Enterobacteriaceae and is present in different animal and human reservoirs in China. The F2:A—B— plasmids are also associated with blaCTX-M genes in Enterobacteriaceae isolates from China, Hong Kong, South Korea, Vietnam, Italy, Canada, the United Kingdom, and Belgium (12, 14, 20, 24, 26, 30, 34). Further studies are needed to address the mechanisms underlying the worldwide dissemination of these plasmid types.

Twelve F33:A—B— plasmids carrying both mrtB and blaCTX-M-9G (including seven carrying blaCTX-M-65) and one F33:A—B— plasmid carrying only mrtB showed the same BamHI digestion profiles and highly similar EcoRI digestion

TABLE 2. Replicon sequence typing of plasmids in transconjugants carrying mrtB

| Resistance gene(s) in transconjugant (n)b | No. of plasmids with indicated replicon type(s)a | F1 | F2 | F18 | F33 | B1 | F34 | F35 | B20 | F42 | F22 | F36 | F36 | Unknown |
|-------------------------------------------|-----------------------------------------------|----|----|-----|-----|----|-----|-----|-----|-----|-----|-----|-----|---------|
| mrtB (15)                                 | 1                                             | 1  | 3  | 1   | 2   |    |     |     |     |     |     |     |     | 7       |
| mrtB, blaCTX-M-9G (22)                    |                                               | 4  | 12 | 2   |     |    |     |     |     |     |     |     |     | 3       |
| mrtB, blaCTX-M-65 (2)                     |                                               |    |    |     |     |    |     |     |     |     |     |     |     | 2       |
| mrtB, qepA (25)                            |                                               | 1  | 1  | 22  |     |    |     |     |     |     |     |     |     |         |
| mrtB, qepA, blaCTX-M-9G (8)               |                                               | 1  | 1  | 2   | 1   | 1  | 1   |     |     |     |     |     |     | 1       |
| mrtB, qepA, blaCTX-M-15G (1)              |                                               | 1  | 1  |     | 1   | 1  | 1   |     |     |     |     |     |     |         |
|                                           | Total (73)                                     | 2  | 1  | 1  | 31  | 14 | 2   | 1   | 1   | 1   | 1   |     | 13      |

a n, number of transconjugants carrying the indicated resistance gene(s).
b N, IncN; F, IncFII allele; B, IncFIB allele.
profiles (Table 3). \( \text{bla}_{\text{CTX-M-65}} \) is one of the dominant CTX-M types in animal isolates obtained in China after 2005 (15, 16, 33) and has also been identified as colocalized with \( \text{mttB} \) in pRB1 in an \( E. \text{coli} \) isolate from a patient in the United States (8). This suggests that the increasing prevalence of CTX-M-65 in \( E. \text{coli} \) isolates may be due to the dissemination of plasmids carrying both \( \text{mttB} \) and \( \text{bla}_{\text{CTX-M-65}} \).

In conclusion, the dissemination of \( \text{mttB} \), \( \text{qepA} \), and \( \text{bla}_{\text{CTX-M}} \) genes in \( \text{Enterobacteriaceae} \) isolates from pets is mediated mainly by the F2A::A1 and F33:A1 plasmids. The coexistence of these resistance determinants in a single plasmid increases the selection by one or more of the antimicrobials used in clinical practice. Therefore, prudent use of antimicrobial agents in pets is urgently needed.

**Nucleotide sequence accession numbers.** New replication sequences described in this work were deposited in the GenBank database with assigned accession numbers GU477621, HQ706665, HQ706666, HQ706667, HQ882837, HQ882838, and HQ882839.

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**REFERENCES**

1. Bercot, B., et al. 2010. Low prevalence of 16S methylases among extended-spectrum-beta-lactamase-producing \( \text{Enterobacteriaceae} \) from a Turkish hospital. J. Antimicrob. Chemother. 65:797–798.
2. Bercot, B., L. Poirel, and P. Nordmann. 2008. Plasmid-mediated 16S rRNA methylases among extended-spectrum \( \beta \)-lactamase-producing \( \text{Enterobacteriaceae} \) isolates. Antimicrob. Agents Chemother. 52:4526–4527.
3. Bogaerts, P., et al. 2007. Emergence of \( \text{ArmA} \) and \( \text{RmtB} \) aminoglycoside resistance 16S rRNA methylases in \( \text{Escherichia coli} \) isolates from pigs. J. Antimicrob. Chemother. 59:159–164.
4. Carattoli, A., et al. 2005. Identification of plasmids by PCR-based replicon typing. J. Microbiol. Methods 63:219–228.
5. Chen, L., et al. 2007. Emergence of \( \text{RmtB} \) methylase-producing \( \text{Escherichia coli} \) and \( \text{Enterobacter cloacae} \) isolates from pigs in China. J. Antimicrob. Chemother. 59:880–885.
6. Davis, M. A., et al. 2010. Discovery of a gene conferring multiple-aminoglycoside resistance in \( \text{Escherichia coli} \). Antimicrob. Agents Chemother. 54:2666–2669.
7. Deng, Y., et al. 2014 January, posting date. Dissemination of IncFI plasmids carrying \( \text{mttB} \) and \( \text{qepA} \) in \( \text{Escherichia coli} \) from pigs, farm workers, and the environment. Clin. Microbiol. Infect. doi:10.1111/1469-0891.2011.03472.x.
8. Doi, Y., J. M. Adams-Haduch, and D. L. Paterson. 2008. \( \text{Escherichia coli} \) isolate coproducing 16S rRNA methylase and CTX-M-type extended-spectrum \( \beta \)-lactamase isolated from an outpatient in the United States. Antimicrob. Agents Chemother. 52:1204–1205.
9. Doi, Y., and Y. Arakawa. 2007. 16S rRNA methylation: emerging resistance mechanism against aminoglycosides. Clin. Infect. Dis. 45:88–94.
10. Du, X. D., et al. 2009. Plasmid-mediated \( \text{ArmA} \) and \( \text{RmtB} \) 16S rRNA methylases in \( \text{Escherichia coli} \) isolated from chickens. J. Antimicrob. Chemother. 64:1328–1330.
11. González-Zorn, B., et al. 2005. \( \text{ArmA} \) and aminoglycoside resistance in \( \text{Escherichia coli} \). Emerg. Infect. Dis. 11:954–956.
12. Ho, P. L., et al. 2011. Complete sequencing of the FII plasmid pHK01, encoding CTX-M-14, and molecular analysis of its variants among \( \text{Escherichia coli} \) from Hong Kong. J. Antimicrob. Chemother. 66:752–756.
13. Kang, H. Y., et al. 2009. Characterization of conjugal plasmids carrying antibiotic resistance genes encoding 16S rRNA methylase, extended-spectrum \( \beta \)-lactamase, and/or plasmid-mediated AmpC beta-lactamase. J. Microbiol. 47:88–75.
14. Kim, J., et al. 2011. Characterization of IncF plasmids carrying the \( \text{bla}_{\text{CTX-M-14}} \) gene in clinical isolates of \( \text{Escherichia coli} \) from Korea. J. Antimicrob. Chemother. 66:1263–1268.
15. Li, J., et al. 2010. Dissemination of cefotaxime-M-producing \( \text{Escherichia coli} \)
isolates in poultry farms, but not swine farms, in China. Foodborne Pathog. Dis. 7:1387–1392.
16. Li, L., et al. 2010. Characterization of antimicrobial resistance and molecular determinants of beta-lactamase in Escherichia coli isolated from chickens in China during 1970–2007. Vet. Microbiol. 144:505–510.
17. Liu, J.-H., et al. 2008. Prevalence of plasmid-mediated quinolone resistance determinants QepA, Qnr, and AAC(6’)-Ib-cr among 16S rRNA methylase RmtB-producing Escherichia coli isolates from pigs. Antimicrob. Agents Chemother. 52:2992–2993.
18. Ma, J., et al. 2009. High prevalence of plasmid-mediated quinolone resistance determinants qnr, aac(6’)-Ib-cr, and qepA among cefotaxime-resistant Enterobacteriaceae isolates from companion and food-producing animals. Antimicrob. Agents Chemother. 53:519–524.
19. Ma, L., et al. 2009. Widespread dissemination of aminoglycoside resistance genes armA and rmtB in Klebsiella pneumoniae isolates in Taiwan producing CTX-M-type extended-spectrum beta-lactamases. Antimicrob. Agents Chemother. 53:104–111.
20. Nguyen, N. T., et al. 2010. The sudden dominance of bla_{CTX-M} harbouring plasmids in Shigella spp. circulating in Southern Vietnam. PLoS Negl. Trop. Dis. 4:e702.
21. Osborn, A. M., F. M. da Silva Tatley, L. M. Steyn, R. W. Pickup, and J. R. Saunders. 2000. Mosaic plasmids and mosaic replicons: evolutionary lessons from the analysis of genetic diversity in IncFII-related replicons. Microbiology 146:2267–2275.
22. Park, Y. J., J. K. Yu, S. I. Kim, K. Lee, and Y. Arakawa. 2009. Accumulation of plasmid-mediated fluoroquinolone resistance genes, qepA and qnrS1, in Enterobacter aerogenes coproducing RmtB and class A beta-lactamase LAP-1. Ann. Clin. Lab. Sci. 39:55–59.
23. Pe´richon, B., et al. 2008. Sequence of conjugative plasmid pIP1206 mediating resistance to aminoglycosides by 16S rRNA methylation and to hydrophilic fluoroquinolones by efflux. Antimicrob. Agents Chemother. 52:2581–2592.
24. Smet, A., et al. 2010. Complete nucleotide sequence of CTX-M-15-plasmids from clinical Escherichia coli isolates: insertional events of transposons and insertion sequences. PLoS One 5:e11202.
25. Sun, Y., et al. 2010. High prevalence of bla_{CTX-M} extended-spectrum beta-lactamase genes in Escherichia coli isolates from pets and emergence of CTX-M-64 in China. Clin. Microbiol. Infect. 16:1475–1481.
26. Villa, L., A. García-Fernández, D. Fortini, and A. Carattoli. 2010. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. J. Antimicrob. Chemother. 65:2518–2529.
27. Wu, Q., Y. Zhang, L. Han, J. Sun, and Y. Ni. 2009. Plasmid-mediated 16S rRNA methylases in aminoglycoside-resistant Enterobacteriaceae isolates in Shanghai, China. Antimicrob. Agents Chemother. 53:271–272.
28. Yamane, K., et al. 2007. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an Escherichia coli clinical isolate. Antimicrob. Agents Chemother. 51:3354–3360.
29. Yan, J. J., et al. 2004. Plasmid-mediated 16S rRNA methylases conferring high-level aminoglycoside resistance in Escherichia coli and Klebsiella pneumoniae isolates from two Taiwanese hospitals. J. Antimicrob. Chemother. 54:1007–1012.
30. Yi, H., et al. 2010. Sequence analysis of pKF3-70 in Klebsiella pneumoniae: probable origin from R100-like plasmid of Escherichia coli. PLoS One 5:e10141.
31. Yokoyama, K., et al. 2003. Acquisition of 16S rRNA methylase gene in Pseudomonas aeruginosa. Lancet 362:1888–1893.
32. Yu, F. Y., et al. 2010. High prevalence of plasmid-mediated 16S rRNA methylase gene rmtB among Escherichia coli clinical isolates from a Chinese teaching hospital. BMC Infect. Dis. 10:184.
33. Yuan, L., et al. 2009. Molecular characterization of extended-spectrum beta-lactamase-producing Escherichia coli isolates from chickens in Henan Province, China. J. Med. Microbiol. 58:1449–1453.
34. Zhao, F., et al. 2010. Sequencing and genetic variation of multidrug resistance plasmids in Klebsiella pneumoniae. PLoS One 5:e10141.