Dissemination of the rmtB gene carried on IncF and IncN plasmids among Enterobacteriaceae in a pig farm and its environment

Qiongfen Yao, Zhenling Zeng, Jianxia Hou, Yuting Deng, Liangying He, Wei Tian, Hongqing Zheng, Zhangliu Chen and Jian-Hua Liu

College of Veterinary Medicine, National Reference Laboratory of Veterinary Drug Residues (SCAU), South China Agricultural University, Guangzhou, People’s Republic of China

*Corresponding author. Tel: +86-20-85283824; Fax: +86-20-85283824; E-mail: jhliu@scau.edu.cn

Received 9 May 2011; returned 16 June 2011; revised 12 July 2011; accepted 14 July 2011

Objectives: To investigate the prevalence and characterization of 16S rRNA methylase-producing bacteria in a pig farm and its environment in East China.

Methods: Enterobacteriaceae isolates and metagenomic DNA from 102 pig faecal samples from a pig farm and 97 soil samples taken in or around the farm were screened for the presence of 16S rRNA methylase genes. The clonal relationships of 16S rRNA methylase-positive isolates, plasmid content and other associated resistance genes were also characterized.

Results: Fifty-six rmtB-positive Enterobacteriaceae isolates, including 54 Escherichia coli, 1 Morganella morganii and 1 Proteus mirabilis, were recovered from 55 pig faecal samples. Nineteen rmtB-positive bacteria, including 13 E. coli, 2 M. morganii, 2 Leclercia adecarboxylata, 1 Enterobacter aerogenes and 1 Enterobacter cloacae, were recovered from 16 soil samples. Among the 75 rmtB-positive isolates, 31 and 25 also carried the qepA and blaCTX-M genes, respectively. The qepA gene co-localized with rmtB on the F2:A-:B1 plasmids and the blaCTX-M-65 gene co-localized with rmtB on the F33:A-:B- plasmids. The rmtB gene was also found to be associated with the IncN plasmids. Clonal transmission of rmtB-positive E. coli isolates was observed between different pig groups and soil samples.

Conclusions: Both horizontal gene transfer and clonal spread could be responsible for the dissemination of the rmtB gene in the pig farm and its environment. To our knowledge, this study is the first report of rmtB-positive bacteria from farmland soils and indicates that these antibiotic-resistant bacteria and/or resistance genes could be acquired by humans through the food chain.

Keywords: plasmid replicon type, animal isolates, aminoglycoside resistance

Introduction

Aminoglycosides, such as gentamicin, amikacin, neomycin and apramycin, are widely used in food-producing animals to prevent and control bacterial infections. Resistance to aminoglycosides is mostly due to the acquisition of various modifying enzymes. In recent years, 16S rRNA methylases that confer high-level resistance to all 4,6-disubstituted deoxystreptamine aminoglycosides have been identified in clinical isolates of Gram-negative bacteria.1 To date, seven plasmid-encoded 16S rRNA methylases, comprising armA, rmtA, rmtB, rmtC, rmtD, npmA and rmtE, have been discovered in multiple regions.1–5 16S rRNA methylase genes, especially rmtB and armA, have often been found in association with genes encoding extended-spectrum β-lactamases (ESBLs), such as blaCTX-M.6–8 Since aminoglycosides are often used in combination with β-lactams to treat severe infections caused by ESBL-producing Enterobacteriaceae, the dissemination of 16S rRNA methylase producers has become a serious threat to the clinical use of these important antimicrobials.

Several studies have also identified 16S rRNA methylase genes in bacteria from food animals.5,9,10 In addition, contamination of the natural environment with antimicrobial-resistant bacteria by animal production facilities has been reported; this may eventually impact human health.11–13 Despite the frequent use of aminoglycosides in food animals, little is known about the occurrence of aminoglycoside resistance genes, particularly 16S rRNA methylase genes, in soil environments surrounding animal farms. In the present study, we examined the distribution of 16S rRNA methylase genes in commensal bacteria from...
samples of pig faeces and soil in or around a pig farm in East China and characterized the isolates and plasmids carrying these genes.

Materials and methods

Sampling and cultivation of bacteria

In April 2010, 102 pigs from a breeding pig farm in East China were randomly selected for rectal swab sampling based on their age and stage of production (sows, boars, fattening pigs or weaned piglets) (Table S1, available as Supplementary data at JAC Online). Ninety-seven soil samples were obtained from inside the pig farm (n=55) and areas within a 500 m radius (n=42) (Table 1). For comparison, 56 soil samples were collected from farmland 50 km away from the pig farm in September 2010. The isolation of bacteria was performed on 200 mg/L amikacin MacConkey agar and tryptic soy agar (TSA) plates and confirmed by API 20E strips. Bacterial isolates that could not be identified by the API 20E system (bioMérieux, France) were subjected to PCR amplification and sequencing of the 16S rRNA locus as described previously.14

PCR amplification

The armA, rmtA, rmtB, rmtC, rmtD, npmA and rmtE genes were detected by PCR as previously described.3,5 The rmtB-positive isolates were also screened for qepA and blaCTX-M genes.15,16 The genotype of blaCTX-M was confirmed by PCR and DNA sequencing.16 The associations of rmtB with Tn3-like elements and qepA with ISCR3 were determined by PCR mapping as described previously.9 Metagenomic DNA of soil samples that contained amikacin-resistant bacteria but not 16S rRNA methylase-positive bacteria were extracted using an UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer’s protocol. The extracted DNA was used for the detection of 16S rRNA methylase genes.

Antimicrobial susceptibility testing

The MICs of ampicillin, cefotaxime, gentamicin, amikacin, tetracycline, chloramphenicol, florfenicol, trimethoprim/sulfamethoxazole, ciprofloxacin and fosfomycin were determined by the agar dilution method. Antimicrobial susceptibility testing was conducted for transconjugants and the other 2 were identified as Enterobacter cloacae (n=1) and Enterobacter aerogenes (n=1). The prevalences of CTX-M type β-lactamases and qepA amongst rmtB-positive isolates are listed in Table 2.

Conjugation experiments and restriction fragment length polymorphism (RFLP) of plasmid DNA

The mobility of 16S rRNA methylase genes was determined by conjugation experiments using a streptomycin-resistant E. coli C600 strain as the recipient. Transconjugants were selected on MacConkey agar plates containing amikacin (200 mg/L) and streptomycin (2000 mg/L). Antimicrobial susceptibility testing was conducted for transconjugants and the transfer of the rmtB gene was confirmed by PCR. Plasmids extracted from transconjugants containing only a single plasmid were digested with EcoRI (TaKaRa Biotechnology, Dalian, China) to analyse the RFLP profile and the sizes of the plasmids.

PCR-based replicon typing

All transconjugants obtained were subjected to replicon typing by a PCR method as described previously.18 Replicon sequence typing of IncF plasmids was performed according to the protocol described by Villa et al.19 and alleles were assigned by submitting the amplicon sequence to the multilocus sequencing database (www.pubmlst.org/plasmid).

Epidemiological typing

The PFGE analysis of XbaI-digested genomic DNA of all rmtB-positive isolates was performed using a CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA, USA) as described by Gautam.20 The PFGE patterns were interpreted based on the criteria published by Tenover et al.21 Enterobacterial repetitive intergenic consensus sequence (ERIC) PCR was performed in isolates that did not yield a satisfactory banding pattern by PFGE.22 After visual inspection, any two isolates that showed two or more different bands were considered to be unrelated.

Results

Incidence of 16S rRNA methylases

High-level amikacin-resistant bacteria were present in 55 out of the 102 (53.9%) pig faecal samples. Fifty-six isolates from all 55 faecal samples carrying amikacin-resistant bacteria were positive for rmtB. In contrast, among the 87 soil samples harbouring amikacin-resistant bacteria, only 19 isolates from 16 soil samples were positive for rmtB (Table 1). The other six 16S rRNA methylase-negative bacteria were not identified in any sample. Metagenomic DNA of the other 71 soil samples where 16S rDNA methylase-positive bacteria were not identified was further screened for 16S rRNA methylase genes. Again, no such sample was positive for any 16S rRNA methylase gene.

Characterization of RmtB producers

Of the 56 rmtB-positive isolates recovered from pig faeces, 54 were identified as E. coli and the other 2 were identified as Morganella morganii and Proteus mirabilis (Table 2). The 19 RmtB producers recovered from soil samples were identified as E. coli (n=13), M. morganii (n=2), Leclercia adecarboxylata (n=2), Enterobacter aerogenes (n=1) and Enterobacter cloacae (n=1).

PFGE was successfully performed in 54 E. coli isolates, which were grouped into 32 clonal patterns, designated A to FA (Table 2). Four E. coli isolates carrying both rmtB and blaCTX-M-65, including three from soil samples inside the pig farm and one from the farmland soil outside the farm, belonged to the same PFGE pattern (Table 2). Three M. morganii strains (one from pig faeces and two from soil) and two L. adecarboxylata strains had indistinguishable ERIC patterns.

PFGE-relatedness

Plasmid analysis

Plasmids carrying rmtB from 58 isolates were successfully transferred to recipients by conjugation. Cefotaxime resistance and reduced susceptibility to ciprofloxacin were co-transferred, which was due to the co-transfer of blaCTX-M-65 and qepA genes. IncFII, IncFIB and IncN replicons were detected in 42, 30 and 13 transconjugants, respectively. Two plasmid replicons (IncFII in...
F2:A-:B1 plasmids carrying both M. morganii role of animal farms in spreading resistance genes. Future studies are required to explore more animal farms and one farm for the prevalence of 16S rRNA methylase genes, animal origin in farmland may contaminate plants and vegetables from the pig farm into the farmland. Resistant bacteria of rmtB might have contributed to the spread of to the pig feedlot had not been treated with animal manures from similar plasmid restriction patterns, with a size of ≏ plasmid restriction patterns, with a size of rmtB four IncN plasmids carrying only we report for the first time the occurrence of prevalent in pig faecal samples in a pig farm in China. In addition, the results obtained in this study showed that was highly considered part of the tribe Proteae within the family Enterobacteriaceae, is ubiquitous in the environment and may cause opportunistic infections in humans. L. adecarboxylata is distributed widely in nature and has been isolated from food, water and other environmental sources, as well as various clinical specimens. It has been described as a novel opportunistic human pathogen since 1997. The clinical significance of rmtB-producing L. adecarboxylata and M. morganii is not clear. However, the spread of the rmtB gene to indigenous soil bacteria, such as L. adecarboxylata and M. morganii, is worth special attention because these organisms are better adapted for survival in the soil and thus may serve as a long-term reservoir of antimicrobial resistance.

The rmtB gene is commonly found to be associated with qepA, and rmtB-qepA has been reported to be co-localized on IncF plasmids. In the present study, rmtB and qepA were found located on very similar F2:A-:B- plasmids, which have been disseminated in pigs as well as soils inside and outside the pig farm. In addition, rmtB is also associated with blaCTX-M-65 on the F33:A-:B- plasmids, which have been disseminated in pigs as well as soils inside and outside the pig farm. Interestingly, the RFLP plasmid patterns of the F33:A-:B- plasmids after EcoRI digestion in these studies were very similar to the patterns of F33:A-:B- plasmids carrying ' rmtB blaCTX-M-65 genes in Enterobacteriaceae from pets in Southern China. It seems that the F33:A-:B- plasmids have been efficiently and widely diffused in Enterobacteriaceae of different animal species and may be responsible for disseminating both rmtB and blaCTX-M-65 in China. Coexistence of rmtB and qepA or blaCTX-M-65 in the same plasmid may contribute to the dissemination of ESBL producers or plasmid-mediated quinolone resistance genes through co-selection.

In conclusion, both the horizontal gene transfer of IncF or IncN plasmids and clonal spread are responsible for the dissemination of the rmtB gene in the pig farm and its environment. The spread of 16S rRNA methylases and ESBL and PMQR genes to environmental bacteria is alarming. More attention should be paid to the combination with IncFIB) were present simultaneously in 30 transconjugants. Within FII replicons, F2 and F33 alleles were identified in 18 and 24 transconjugants, respectively. Within FIB replicons, only B1 was identified (Table 2).

Twenty transconjugants carrying only one plasmid were randomly selected for plasmid restriction enzyme digestion analysis according to plasmid replicon type or F allele (six F33:A-:B-, two F33:A-:B1, seven F2:A-:B- and five IncN) (Table 2). F33:A-:B- plasmids carrying both rmtB and blaCTX-M-65 shared the same or very similar plasmid restriction patterns, with a size of ≏ 70 kb. F2:A-:B1 plasmids carrying both rmtB and qepA showed the same plasmid restriction patterns, with a size of ≏ 100 kb. The four IncN plasmids carrying only rmtB also shared the same plasmid restriction patterns, with a size of ≏ 35 kb (Table 2).

### Discussion

The results obtained in this study showed that rmtB was highly prevalent in pig faecal samples in a pig farm in China. In addition, we report for the first time the occurrence of M. morganii and environmental origin M. morganii and environmental origin L. adecarboxylata producing RmtB, M. morganii, which is considered part of the tribe Proteae within the family Enterobacteriaceae, is ubiquitous in the environment and may cause opportunistic infections in humans. L. adecarboxylata is distributed widely in nature and has been isolated from food, water and other environmental sources, as well as various clinical specimens. It has been described as a novel opportunistic human pathogen since 1997. The clinical significance of rmtB-producing L. adecarboxylata and M. morganii is not clear. However, the spread of the rmtB gene to indigenous soil bacteria, such as L. adecarboxylata and M. morganii, is worth special attention because these organisms are better adapted for survival in the soil and thus may serve as a long-term reservoir of antimicrobial resistance.

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### Table 1. Prevalence of 16S rRNA methylase-producing isolates and distribution of qepA and blaCTX-M genes among these isolates

<table>
<thead>
<tr>
<th>Sample source</th>
<th>No. of samples containing amikacin-resistant bacteria (%)</th>
<th>No. of rmtB-positive samples (%)</th>
<th>No. of rmtB-positive isolates</th>
<th>No. of PFGE subtypes of rmtB-positive E. coli isolates</th>
<th>No. of qepA-positive isolates among RmtB producers (%)</th>
<th>No. of CTX-M-positive isolates among RmtB producers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sow faeces</td>
<td>20</td>
<td>20 (100.0)</td>
<td>20 (100.0)</td>
<td>12 (2)</td>
<td>2 (10.0)</td>
<td>10 (50.0)</td>
</tr>
<tr>
<td>Boar faeces</td>
<td>20</td>
<td>12 (60.0)</td>
<td>12 (60.0)</td>
<td>8 (4)</td>
<td>6 (50.0)</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td>Fattening pig faeces</td>
<td>42</td>
<td>17 (40.5)</td>
<td>17 (40.5)</td>
<td>8 (1)</td>
<td>16 (88.9)</td>
<td>3 (16.7)</td>
</tr>
<tr>
<td>Weaned piglet faeces</td>
<td>20</td>
<td>6 (30.0)</td>
<td>6 (30.0)</td>
<td>6 (6)</td>
<td>2 (33.3)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>Soil inside pig feedlot</td>
<td>102</td>
<td>55 (53.9)</td>
<td>55 (53.9)</td>
<td>56 (54)</td>
<td>28 (7)</td>
<td>26 (42.9)</td>
</tr>
<tr>
<td>Soil from farmland adjacent to pig farm</td>
<td>55</td>
<td>49 (89.1)</td>
<td>12 (21.8)</td>
<td>15 (9)</td>
<td>7 (6)</td>
<td>3 (23.1)</td>
</tr>
<tr>
<td>Soil from farmland far away</td>
<td>42</td>
<td>19 (45.2)</td>
<td>3 (7.1)</td>
<td>3 (3)</td>
<td>3 (66.7)</td>
<td>1 (33.3)</td>
</tr>
</tbody>
</table>

aThe number of E. coli isolates is indicated in parentheses.

bThe number of non-typeable isolates is indicated in parentheses.
Table 2. Details of rmtB-positive isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Bacterial species</th>
<th>PFGE or ERIC-PCR type</th>
<th>bla&lt;sub&gt;CTX-M&lt;/sub&gt; gene and qepA&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Plasmid replicon type</th>
<th>Plasmid RFLP pattern&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Resistance phenotype&lt;sup&gt;g&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>IS75, IS89a, IS90a</td>
<td>EC</td>
<td>G1, G2, G3, G4</td>
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<td>A1</td>
<td>CIP, CTX, (CHL, FFC, SXT, TET), FOS</td>
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<tr>
<td>BP11, SP38</td>
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<td>N</td>
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<td>SP28, SP32</td>
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<td>O, R, P2, NT</td>
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<td>(CIP), CTX, CHL, FFC, SXT, TET, FOS</td>
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<td>A3</td>
<td>CTX, CHL, SXT, TET, FOS</td>
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<tr>
<td>FP85, FP91, FP93, FP96, FP69</td>
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<td>C1, C2</td>
<td>qepA</td>
<td>F2:A-</td>
<td>B</td>
<td>CIP, CHL, FFC, SXT, TET</td>
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<td>U, T, AB, Y, Z, AA</td>
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<td>B</td>
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<tr>
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<td>N</td>
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<td>I</td>
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<td>ND</td>
<td>SXT, TET, FOS</td>
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</table>

<sup>a</sup>Isolates sharing the same number were obtained from the same sample. Isolates with transconjugants are underlined. SP, sow faeces; BP, boar faeces; FP, fattening pig faeces; WP, weaned piglet faeces; IS, soil inside the farm; OS, soil of farmland adjacent to the pig farm; FLS, soil of other farmland. Plasmids of isolates in bold were digested with EcoRI.

<sup>b</sup>EC, Escherichia coli; MM, Morganella morganii; LA, Leclercia adecarboxylata; PM, Proteus mirabilis; ENC, Enterobacter cloacae; ENA, Enterobacter aerogenes.

<sup>c</sup>PFGE types were assigned as A, B, C, … by visual inspection of the macrorestriction profile. Patterns that differed by fewer than six bands were considered to represent subtypes within the main group (e.g. G1, G2,…). ERIC-PCR types were assigned as I and II. NT, non-typeable isolates. ND, not determined.

<sup>d</sup>Genes that were transferred by conjugation as determined by PCR are underlined.

<sup>e</sup>ND, not determined; N, IncN-type plasmid.

<sup>f</sup>RFLP patterns differing by only a few bands (n = 1–3) were assigned to the same RFLP profile. ND, not determined.

<sup>g</sup>All isolates and transconjugants were resistant to gentamicin, amikacin and ampicillin. CIP, ciprofloxacin; CTX, cefotaxime; CHL, chloramphenicol; FFC, florfenicol; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; FOS, fosfomycin. Patterns transferred by conjugation are underlined. The criterion for florfenical was ≥16 mg/L. Resistance to antibiotics appearing in parentheses was not present in all isolates.

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contamination of the natural environment by clinically important resistant bacteria and resistance genes from animal farms.

Acknowledgements
We thank Sheng Chen (The Hong Kong Polytechnic University) for revision of the manuscript.

Funding
This work was supported by grants from the National Natural Science Foundation of China (no. 30972218 and no. U1031004).

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

None to declare.

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