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ORIGINAL RESEARCH

Prevalence Of *mcr-1* Among Cefotaxime-Resistant Commensal *Escherichia coli* In Residents Of Vietnam

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Ryuji Kawahara[1,*]
Diep Thi Khong[2,*]
Ha Viet Le[2]
Quang Ngoc Phan[2]
Thang Nam Nguyen[2]
Takahiro Yamaguchi[1]
Yuko Kumeda[3]
Yoshimasa Yamamoto[4,5]

[1]Department of Microbiology, Osaka Institute of Public Health, Osaka, Japan; [2]Center of Medical, Pharmaceutical Science and Technology Services, Thai Binh University of Medicine and Pharmacy, Thai Binh, Vietnam; [3]Research Center for the 21st Century, Osaka Prefecture University, Osaka, Japan; [4]Life Science Research Center, Gifu University, Gifu, Japan; [5]Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

*These authors contributed equally to this work

Purpose: The dissemination of colistin-resistant bacteria harboring the colistin-resistance gene *mcr-1* in developing countries has recently entered the spotlight as an emerging public health threat, which is attributed to the abuse of colistin use in these countries. However, the prevalence of these bacteria in developing countries has not been extensively investigated. Therefore, in the present study, we examined the prevalence of cefotaxime-resistant commensal *Escherichia coli* harboring *mcr-1* among residents of a representative Vietnamese village and assessed the characteristics of these isolates.

Materials and methods: The stool samples, one stool sample per resident, of 612 residents were cultured on MacConkey agar with cefotaxime. Resulting *E. coli*-like colonies were isolated and examined further for the presence of colistin-resistant extended-spectrum β-lactamase (ESBL)-producing *E. coli* with *mcr-1*. Antibiotic susceptibility tests were performed, and clonal relationship among colistin-resistant isolates was assessed.

Results: Thirty-one of the 451 cefotaxime-resistant *E. coli* isolates were resistant to colistin and the majority possessed *mcr-1*, bla_{CTX-M}, and/or bla_{TEM}, except for two isolates that produced the AmpC β-lactamase. All *mcr-1* ESBL-producing *E. coli* isolates were multidrug-resistant (5–11 antibiotics). The isolates contained various plasmid replicon types, including the most prevalent types IncHI2 (54.8%), IncFIB (48.4%), and IncN (41.9%). In addition, 83.9% of the *mcr-1* ESBL-*E. coli* isolates possessed a transposon IS*Apl1-mcr-1* segment. Furthermore, 77.4% of the *mcr-1* ESBL-*E. coli* isolates belonged to phylogenetic group A. Pulsed-field gel electrophoresis analysis indicated limited clonal expansion of a specific strain.

Conclusion: These results demonstrate the wide dissemination of colistin-resistant ESBL-*E. coli* harboring *mcr-1* among commensal bacteria of rural residents in Vietnam, suggesting possible mobilization of the *mcr-1* gene among ESBL-producing microbiota, which is a great public health concern.

Keywords: *mcr-1*, residents, stool specimens, commensal bacteria, Vietnam

Introduction

Colistin has been recognized as a last-resort antibiotic for the treatment of intractable infections involving multidrug-resistant (MDR) gram-negative bacteria, especially carbapenem-resistant MDR bacteria.[1] However, the increasing usage of colistin in food-producing animals has resulted in a rising prevalence of colistin-resistant bacteria.[2] Moreover, the mobile colistin-resistant gene *mcr-1* was recently discovered in *Escherichia coli* isolates from animals and humans in China.[3] To date, MCR-1-producing *E. coli* strains have been reported in many countries throughout Europe, Asia, and South and North America.[4–6] Therefore, the possible global dissemination of *mcr-1*-positive bacteria has become a serious public health concern.

Correspondence: Yoshimasa Yamamoto
Life Science Research Center, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan
Tel/fax +81 58 230 6239
Email yyamamot@sahs.med.osaka-u.ac.jp

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Although the characteristics of animal and human *E. coli* isolates harboring *mcr-1* have been examined in detail,[1,3,4,7,8] there is limited comprehensive information regarding the prevalence and characteristics of colistin-resistant bacteria harboring *mcr-1* among human residents of developing countries. Because the human intestinal flora is an important reservoir of resistance genes,[9,10] the prevalence and characteristics of colistin-resistance in human commensal *E. coli* should be taken into account in prevention and control efforts. Therefore, the aim of this study was to explore the prevalence of *mcr-1*-positive *E. coli* in the feces of residents of a local community in Thai Binh province, Vietnam, which is known to be widely contaminated with extended-spectrum β-lactamase (ESBL)-producing bacteria,[11] and to investigate the molecular characteristics of these strains. These data should provide a useful resource for understanding current threats in the region to help initiate appropriate screening, treatment, and control.

Materials And Methods

Specimen Collection

The bacterial strains used in this study were originally isolated from residents for assessment of the dissemination of ESBL-producing bacteria in Nguyen Xa village, Thai Binh province, Vietnam, from September 2013 to February 2016. The village is a representative rural community in this province, with a reported population of 7730 residents in 2008 households at the time of the study. A total of 612 asymptomatic healthy resident volunteers who had not received any medical treatment for the last 3 months participated in the study. One stool specimen was obtained from each participant using a transport swab with Cary-Blair transport medium (Eiken Chemical, Tokyo, Japan). The study was approved by the ethics committees of Osaka University and Thai Binh University of Medicine and Pharmacy. All participants provided written informed consent and that this was conducted in accordance with the Declaration of Helsinki.

Isolation Of Cefotaxime-Resistant *E. coli*

Stool specimens were plated on MacConkey agar (Nissui, Tokyo, Japan) supplemented with 1 mg/L cefotaxime (CTX-MacConkey) and incubated at 37°C for 18–20 hrs as previously described.[11] Resulting colonies exhibiting *E. coli* characteristics were isolated and confirmed as *E. coli* using biochemical tests with triple sugar iron slants, lysine indole motility medium (BD, New Jersey, USA), cellobiose lactose indole β-glucuronidase medium (Nissui, Tokyo, Japan), and API 20E (bioMerieux, Marcy l'Etoile, France).

Detection Of ESBL- And AmpC-Producing *E. coli*

The presence of ESBLs was confirmed using the double-disc synergy test using CTX and ceftazidime with and without clavulanic acid, as recommended by the Clinical and Laboratory Standards Institute (CLSI 2014, Wayne, PA, USA). Cefoxitin (FOX)-resistant strains were classified as putative AmpC-producing *E. coli*.

Genetic detection and genotyping of β-lactamases were performed by PCR using boiled bacterial suspensions in Tris-EDTA buffer as the template. PCR analysis was performed with universal primers specific to the TEM, SHV, CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M-8/25 groups, as previously described.[12] The PCR products were visualized by 2% agarose gel electrophoresis and stained with GelRed nucleic acid (Biotium, Hayward, CA, USA). The sequence analysis of CTX-M genes was performed as previously described.[13]

Phylogenetic Determination

Phylogenetic groups were determined by triplex PCR using a combination of two genes, *chuA* and *yjaA*, and the DNA fragment TSPE4.C2 as previously described.[14]

mcr-1 Detection And Colistin MIC

The presence of the colistin resistance gene *mcr-1* was detected by PCR with bacterial DNA and sequencing of the resulting products.[3] The colistin MIC of the *mcr-1*-positive isolates was evaluated by ETEST® (bioMerieux) according to the manufacturer's protocol.

Antibiotics Susceptibility

The susceptibility of the ESBL- or AmpC-producing *E. coli* isolates harboring *mcr-1* to 14 antimicrobial agents was tested using the disc diffusion method following the standard procedure of the CLSI, as previously described.[15] The disc diffusion test included the following antibiotics: ampicillin (AMP), FOX, CTX, ceftazidime (CAZ), meropenem (MEM), streptomycin (STR), kanamycin (KAN), gentamicin (GEN), ciprofloxacin (CIP), nalidixic acid (NAC), tetracycline (TET), chloramphenicol (CHL), fosfomycin (FOF), and sulfamethoxazole-trimethoprim (SXT).

Pulsed-Field Gel Electrophoresis (PFGE) Analysis

*Xba*I-digested genomic DNA samples from the *E. coli* isolates were analyzed on a CHEF-DR III System (Bio-Rad, Hercules, CA, USA) following the detailed methods reported previously.[16]

Plasmid Replicon Typing

Plasmid replicon typing of the *mcr-1*-harboring *E. coli* isolates was assessed by multiplex PCR as previously described.[17–20]

Transposon ISApl1 Detection

The presence of IS*Apl1-mcr-1* was assessed by PCR with TaKara Gflex DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) and the primers ISApl1-mcr1-F (5′-GCGCAAAAT CGCAGTCG-3′) and ISApl1-mcr1-R (5′-TGTAGGGCAT TTTGGAGCATG-3′) according to the manufacturer's instructions. The cycle conditions were as follows: initial denaturation at 95°C for 5 mins; 30 cycles of denaturation for 10 s at 98°C, annealing for 15 s at 55°C, and extension for 1 min at 68°C; and a final extension at 68°C for 7 mins. The approximate 1000-bp PCR products were verified by direct sequencing.

Results

Prevalence Of Colistin-Resistant *E. coli* Harboring *mcr-1* In Vietnamese Residents

The characteristics of the 612 participants are listed in Table 1. Bacterial growth on CTX-MacConkey was assessed for all participant stool specimens. One colony exhibiting *E. coli* characteristics from each participant specimen was obtained and subjected to further microbiological analysis. ESBL production was observed in 375 of the 451 *E. coli* isolates (83.1%). Moreover, 21 of the 451 isolates (4.7%) were AmpC producers.

PCR analysis revealed that 31 of the 451 isolates (6.9%) possessed the *mcr-1* gene.

Table 1 Prevalence Of Colistin-Resistant *E. Coli* Harboring *Mcr-1* In Residents In Vietnam

No. of participants		612	
Age	Median Range	40.5 2–83	
Sex	Male (%)	54.8	
No. of *E. coli* isolates on CTX-MacConkey		451/612[a]	73.7%
ESBL producers		375/451[b]	83.1%
AmpC producers		21/451	4.7%
mcr-1-Positive isolates		31/451	6.9%

Notes: [a]No. of positive *E. coli* isolates/no. of specimens tested. One representative *E. coli* isolate was obtained from each specimen. [b]No. of positive isolates/no. of isolates tested.

Characteristics Of Colistin-Resistant *E. coli* Isolates Harboring *mcr-1*

As shown in Table 2, the colistin MIC values of the *mcr-1*-positive *E. coli* isolates ranged from 3 to 6 μg/mL, as determined using ETEST. Phylogenetic typing of the isolates showed that group A was the most prevalent phenotype of the *mcr-1*-positive *E. coli* isolates (77.4%, 24/31), with other less common groups identified (B1, 6.5%; B2, 6.5%; D, 9.7%).

Assessment of antibiotic susceptibility showed nearly 100% resistance to AMP and CTX, while resistance to FOX, a second-generation cephamycin, and CAZ, a third-generation cephalosporin, was only 6.5% and 9.7%, respectively. Resistance levels to aminoglycosides, tetracycline, phenicols, and folic acid inhibitors were quite high (51.6–90.3%). Moreover, over half of the *mcr-1*-harboring *E. coli* isolates exhibited resistance (54.8%) to quinolones. However, no carbapenem-resistant isolates were identified.

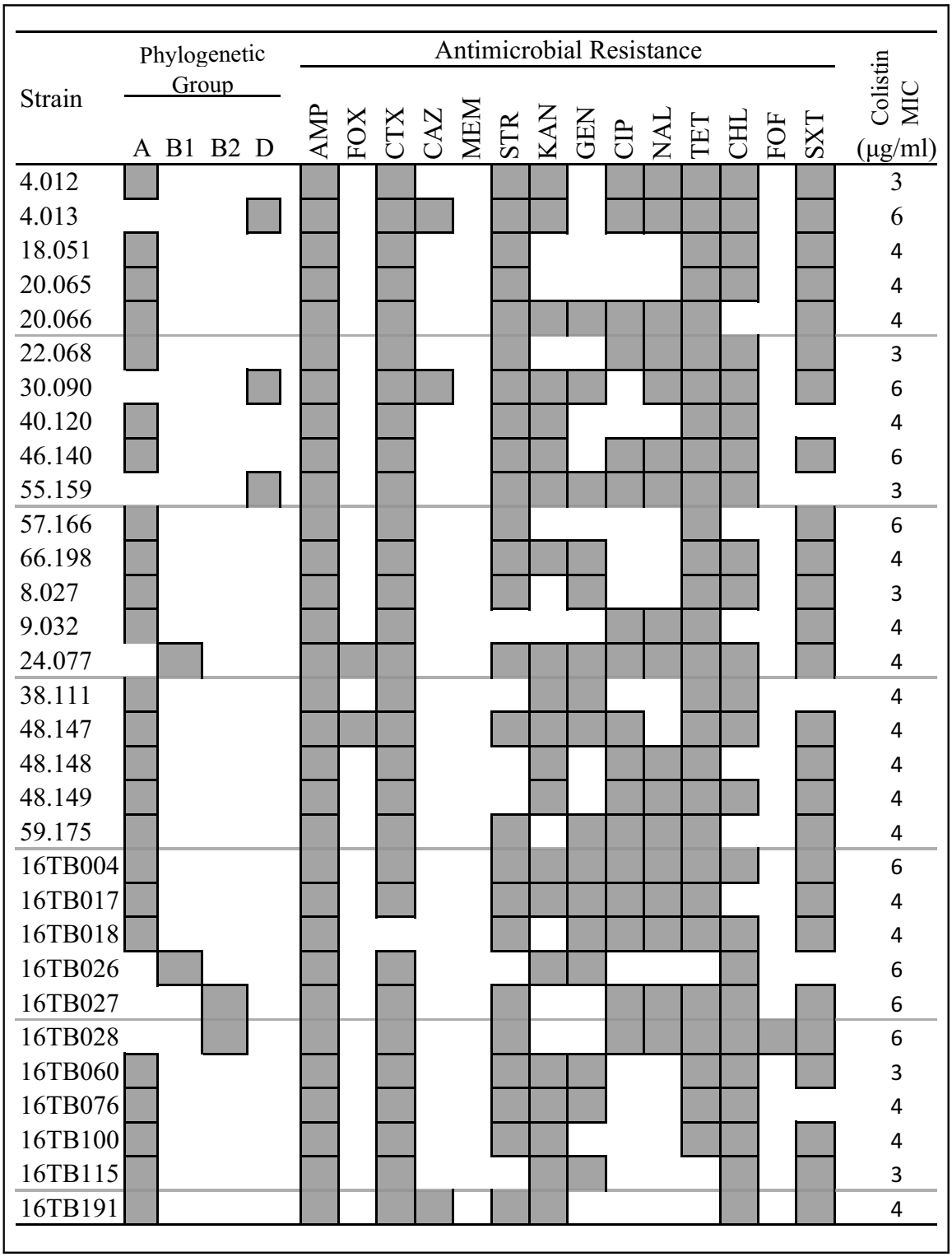
Genetic Features Of Colistin-Resistant *E. coli* Isolates Harboring *mcr-1*

Genetic analysis showed that 74.2% (23/31) of the *mcr-1*-harboring *E. coli* isolates belonged to the CTX-M-9 group (Table 3). The $bla_{\text{CTX-M-9}}$ gene was identified in 11 of 23 CTX-M-9 group isolates. Twelve (40%, 12/30) ESBL-producing isolates possessed both the CTX-M and TEM genes.

A wide variety of plasmid replicon types were present in the *mcr-1*-harboring *E. coli* isolates. The most frequently detected replicon type was HI2 (54.8%), followed by FIB (48.4%) and N (41.9%), whereas B/C, FIC, HI1, U, X2, and X3 were not detected. Replicon types A/C, FIB, and P were detected in the *E. coli* isolates harboring *mcr-1* as a single replicon type.

The presence of the transposon, IS*Apl1*, a key component contributing to the mobilization of *mcr-1*,[21] in the *mcr-1 E. coli* isolates was also investigated. Because the PCR used in this study targeted only the IS*Apl1-mcr-1* segment, only the upstream portion of IS*Apl1* that is directly attached to *mcr-1* was detected. The majority of *mcr-1*-harboring *E. coli* isolates (83.9%, 26/31) had the IS*Apl1-mcr-1* segment.

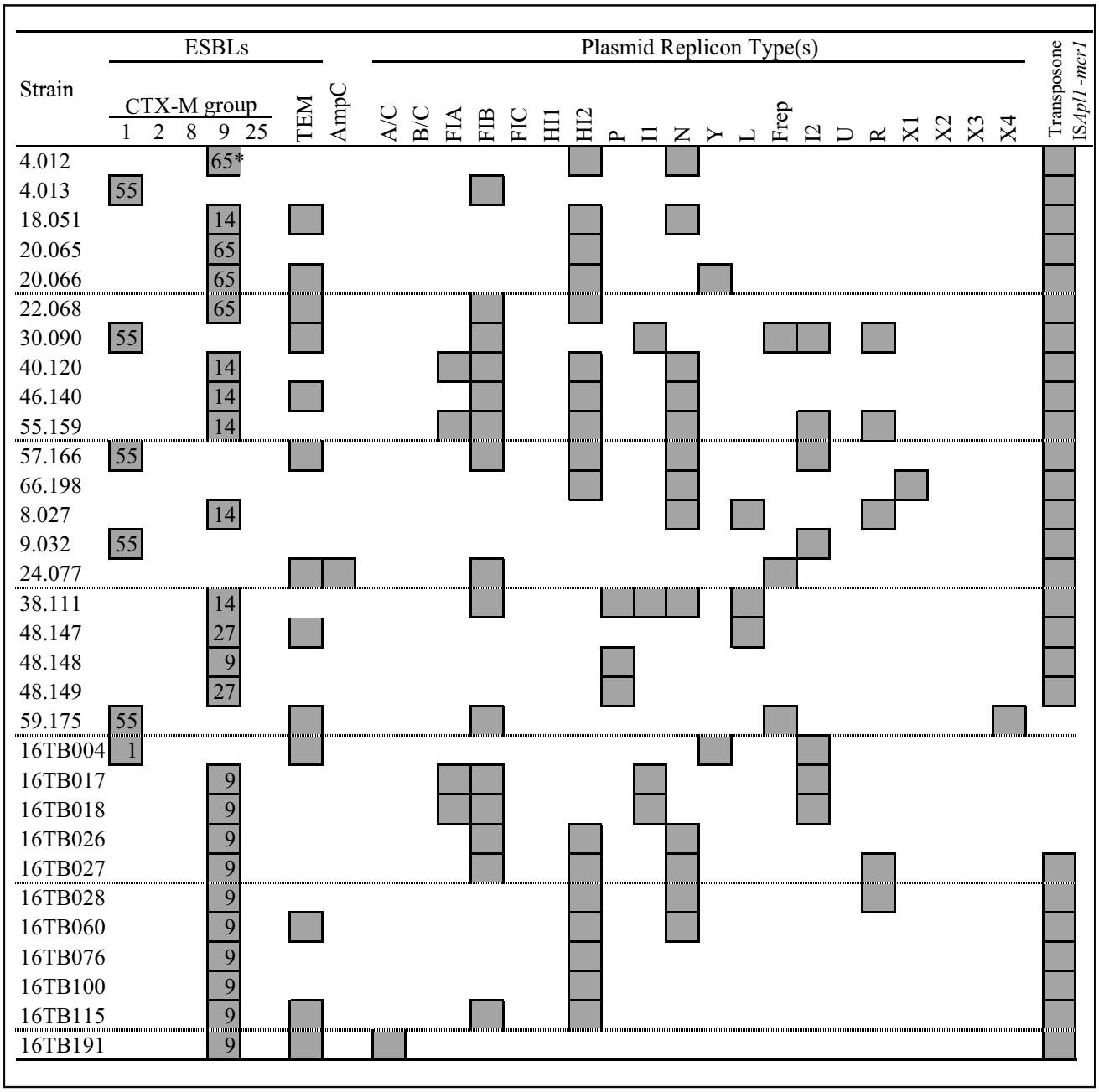
Table 2 Characteristics Of Colistin-Resistant *E. Coli* Isolates Harboring *mcr-1*



Notes: Gray squares indicate the detected phylogenetic group or antibiotic resistance. White squares in phylogenetic group and antimicrobial resistance indicate not detected and susceptibility, respectively.

Abbreviations: AMP, ampicillin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; MEM, meropenem; STR, streptomycin; KAN, kanamycin; GEN, gentamicin; CIP, ciprofloxacin; NAC, nalidixic acid; TET, tetracycline; CHL, chloramphenicol; FOF, fosfomycin; SXT, sulfamethoxazole-trimethoprim.

Table 3 Genetic Features Of Colistin-Resistant *E. Coli* Isolates Harboring *mcr-1*



Notes: [a]Number indicates the CTX-M gene identified. Gray squares indicate the identified gene group, replicon type, or transposon. White squares indicate not detected.

Clonal Relationship Among Colistin-Resistant *E. coli* Isolates Harboring *mcr-1*

PFGE was conducted to assess the clonal relationship among the 31 *mcr-1 E. coli* isolates, which included 24 phylogenetic group A isolates. Representative dendrograms of PFGE of the isolates are shown in Figure 1. A common PFGE pattern was only observed in strains 16TB017/16TB018 and 16TB027/16TB028. Strains 16TB017 and 16TB018 were isolated from residents belonging to the same household, and strains 16TB027 and 16TB028 were also isolated from the same, albeit different, households. The remaining isolates did not demonstrate any similarity.

Discussion

This study revealed that the prevalence of colistin/CTX-resistant commensal *E. coli* harboring *mcr-1* among residents of a rural community in northern Vietnam is as high as 5.1% (31 positives/612 residents tested), although the

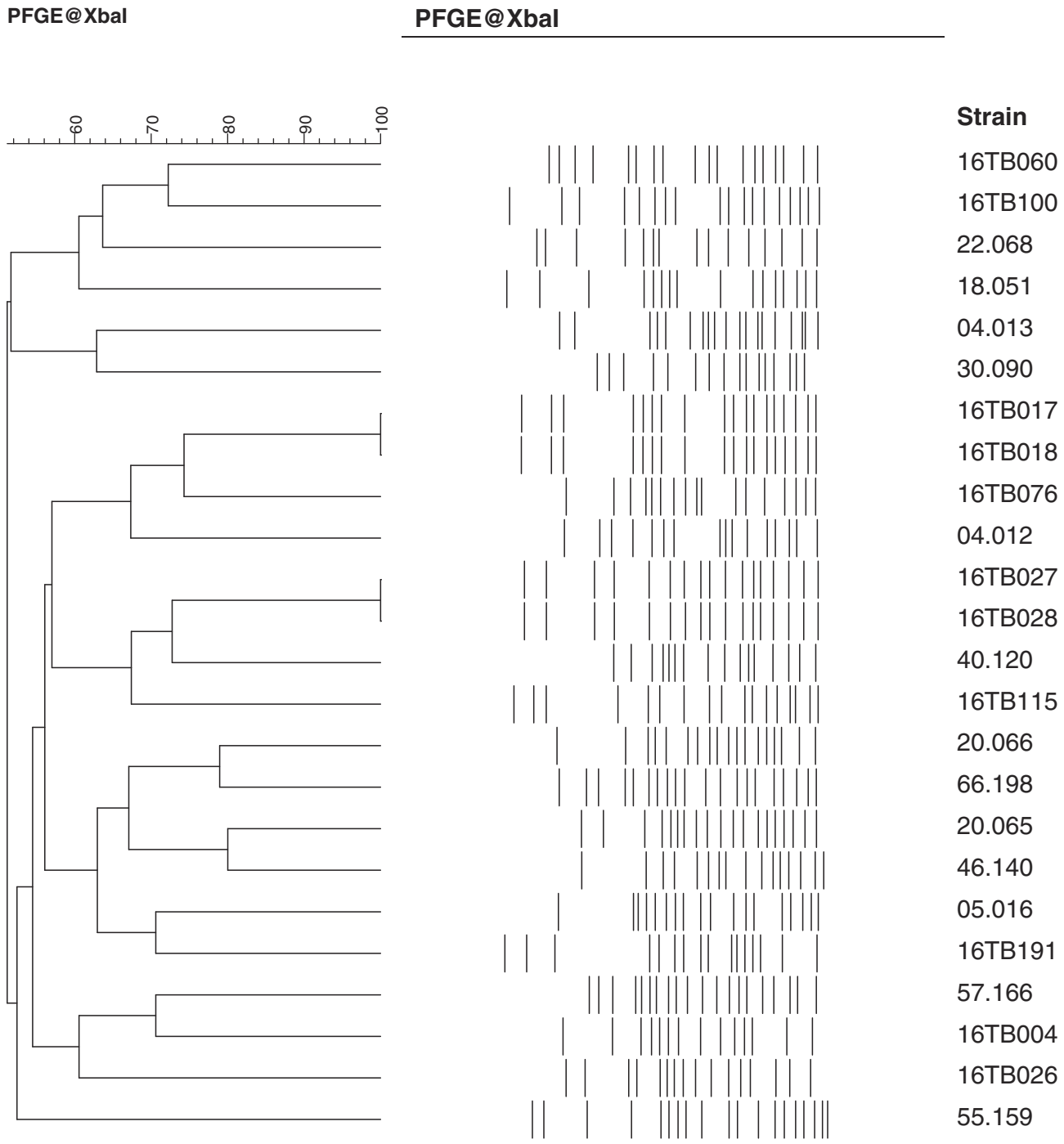


Figure 1 Representative dendrogram of PFGE patterns of colistin-resistant *E. coli* isolates harboring *mcr-1*.

isolates assessed were selected based only on CTX resistance. Trung et al[7] also reported a relatively high prevalence (17.9%) of *mcr-1*-carrying bacteria among residents of a rural area in South Vietnam. We have also detected a very high prevalence (69.4%) of colistin-resistant *E. coli* with *mcr* in stool specimens of healthy residents in Vietnam using selective medium for colistin-resistant bacteria, such as CHROMagar™ COL-APSE (CHROMagar, Paris, France), as primary selection for isolation of bacteria.[22] Therefore, the prevalence of colistin-resistant bacteria seems to be dependent on the medium used for isolation of bacteria. Nevertheless, the results of these recent studies, including the present study, indicate a wide dissemination of *mcr-1*-positive commensal *E. coli* in Vietnam. In addition, the finding of such non-negligible numbers of isolates possessing both ESBL and colistin-

resistance genes highlights a high risk of extensively drug-resistant bacteria circulating in the area.

Dissemination studies conducted in other countries demonstrated the absence of *mcr-1* isolates in healthy people of Switzerland[23] and the Netherlands.[24] In contrast, 4.9% of the Chinese resident samples[25] were found to be carriers of *mcr-1*-positive bacteria. Therefore, at the moment, the degree of the spread of *E. coli* harboring *mcr-1* in healthy individuals has only been demonstrated in Asian countries, although there have been several reports of bacteria harboring *mcr-1* in food and food-producing animals worldwide.[26]

Phylogenetic analysis revealed that the majority of the *mcr-1*-harboring *E. coli* isolates belonged to phylogenetic group A, with a few group B1, B2, and D isolates detected. These results are similar to the prevalence of phylogenetic groups in the normal human intestinal microbiota; A is the predominant group, followed by B2, whereas B1 and D are less common.[27] Thus, the frequency of *mcr-1*-harboring *E. coli* clone phylogenetic groups in the residents of this Vietnamese village may reflect the normal intestinal microbiota in humans.

Analysis of the antibiotic susceptibility of the *mcr-1*-harboring *E. coli* isolates revealed that all isolates were MDR, ie, resistant to at least one antibiotic drug from three or more antibiotic classes.[28] However, resistance to FOX and CAZ was less common among these isolates. In addition, the overall antibiotic resistance profile of the *mcr-1*-harboring *E. coli* isolates was similar to that reported from ESBL-producing *E. coli* isolated from food in Vietnam.[12] Therefore, it is possible that the *mcr-1* gene may have been transmitted to ESBL-producing *E. coli*.

Indeed, nearly all of the *mcr-1*-harboring *E. coli* isolates were ESBL producers except for two isolates, which were class C β-lactamase producers, as determined by both phenotypic and genetic assays. The majority of these ESBL-*mcr-1* isolates possessed CTX-M-9 group genes; the $bla_{\text{CTX-M-14}}$ gene was identified in nearly one-third of the CTX-M-9 group and *mcr-1* isolates tested. In contrast, a recent paper on MDR *E. coli* harboring *mcr-1* reported that most isolates from pig were co-harboring CTX-M-1 group genes.[29] The difference between this previous study and our study may be due to the difference in specimen source.

Although it remains unclear whether the *mcr-1* and CTX-M genes are located on the same plasmid, the present results demonstrate that ESBL-producing *E. coli* isolates with CTX-M-9 group genes are dominant among the colistin-resistant commensal *E. coli* in residents of the community. A previous study showed that *mcr-1* and CTX-M-9 group genes are co-transferred by one or more plasmid types.[30] Moreover, the *mcr-1*-carrying plasmid identified in the first-ever discovered *mcr-1* isolate is known to harbor the CTX-M-9 group, $bla_{\text{CTX-M-14}}$.[31]

Plasmid type plays an important role in the spread of *mcr-1*, not only in the human intestinal microbiota but also in community settings. Therefore, characterization of the plasmids in *mcr-1*-harboring *E. coli* is important for understanding their dissemination. Replicon typing of the present isolates revealed a high diversity of plasmid backbones. The major replicon types of the isolates were HI2 (54.8%), FIB (48.4%), and N (41.9%), which have been reported as *mcr-1*-associated replicon types.[3,32,33] Replicon type I2, which is known as an *mcr-1* disseminator,[34] was detected in 22.6% (7/31) of the isolates. Moreover, plasmid replicon type X4 was identified in one of our isolates, which was recently identified as a common *mcr-1*-carrying plasmid replicon type in Enterobacteriaceae from food, animal, and human samples recovered in different countries.[5] However, since we assessed the replicon type of all plasmids in the isolates, the replicon types of the plasmid(s) harboring the *mcr-1* gene remain unclear. In spite of this limitation, these results indicate that the diversity of plasmid types among *mcr-1* isolates may contribute to the successful spread of the *mcr-1* gene among different *E. coli* clones.

Besides class 1 integrons,[35] the transposon IS*Apl1*, is also known to be involved in horizontal gene transfer and maybe a key factor contributing to the widespread dissemination of the *mcr-1* gene.[36] In this study, transposon IS*Apl1* with *mcr-1* was detected in the majority of the isolates, except for six. As IS*Apl1* can be lost through improper recombination,[36] the IS*Apl1-mcr-1* segment may not have been detected in all of the *mcr-1* harboring isolates. Zurfluh et al[33] reported that the *mcr-1* gene may be mobilized independently. The possible occurrence of the *mcr-1* gene or gene module mobilization among plasmids in the *E. coli* clones is also supported by the PFGE analysis, showing the limited clonal expansion of *mcr-1*-harboring *E. coli*.

Overall, this study suggests that the wide dissemination of colistin-resistant ESBL-producing *E. coli* harboring *mcr-1* in commensal bacteria in Vietnamese residents involves diverse clones with a variety of plasmid replicon types. Mobilization of the *mcr-1* gene module among microbiota under certain circumstances such as exposure to colistin including suboptimal treatment during therapy is speculated as one of the likely causes of this dissemination.[37] Such wide dissemination of the *mcr-1* gene in the commensal bacteria of healthy people

constitutes a great public health concern in regard to the prevention, monitoring, and treatment of colistin-resistant bacteria.

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Disclosure

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