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Presence and Antimicrobial Susceptibility of RE-*cmeABC*-Positive *Campylobacter* Isolated from Food-Producing Animals, 2014–2016

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ABSTRACT

Campylobacter spp. are the leading cause of human gastroenteritis worldwide. RE-CmeABC is a newly identified resistance-enhancing multidrug efflux pump of *Campylobacter* spp. (*C.* spp.) that confers high-level resistance to fluoroquinolones, phenicols, macrolides, and tetracyclines (TETs), all of which are critical drugs in both human and veterinary medicine. In this study, we analyzed the presence and antimicrobial susceptibility of RE-*cmeABC*-positive *Campylobacter* isolates of food-animal origin from three representative regions (Shandong, Shanghai, and Guangdong) in China over three successive years, from 2014 to 2016. A total of 1088 *Campylobacter* isolates (931 *C. coli* and 157 *C. jejuni*) were recovered from the RE-*cmeABC* screening. We detected 122 (11.2%) RE-*cmeABC*-positive isolates of chicken origin, including 111 (70.7%) *C. jejuni* and 11 (1.2%) *C. coli*. This multidrug efflux pump is more prevalent among *C. jejuni* than *C. coli*. The level of resistance was significantly different in 111 RE-*cmeABC*-positive *C. jejuni* versus 46 RE-*cmeABC*-negative *C. jejuni* for florfenicol, clindamycin, and erythromycin ($P < 0.05$), but not for ciprofloxacin (CIP), TET, and gentamicin (GEN). However, the isolates harboring RE-*cmeABC* could shift the minimum inhibitory concentration distribution to the higher range for CIP and TET. Pulsed-field gel electrophoresis (PFGE) analysis suggested that horizontal transmission might be involved in the dissemination of RE-*cmeABC* in Shanghai and Guangdong, while clonal expansion was predominant in Shandong. Three isolates shared the indiscriminate PFGE types of RE-*cmeABC*-positive *C. jejuni* isolates in Shanghai and Guangdong, and four isolates in Shanghai and Shandong. Our study suggests the possibility of a wide dissemination of RE-*cmeABC* in *Campylobacter* of food-animal origin, which would pose a significant threat to public health.

1. Introduction

Campylobacter spp. are Gram-negative thermophilic bacteria that are major etiologic agents for human foodborne illness (campylobacteriosis) worldwide [1,2]. Among all *Campylobacter* species, *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) are the leading causes of human gastroenteritis [3]. Moreover, *C. jejuni* infection may lead to autoimmune conditions known as Guillain–Barré syndrome (GBS) and Miller Fisher syndrome [2]. Poultry, contaminated water, and raw milk are the most frequent vehicles for illness [4]. Chickens are the major host of *Campylobacter* due to their suitable intestinal environment, and thus play a critical role as the typical source of *Campylobacter* infections in industrialized nations [5].

Campylobacter infections are generally self-limiting and treated with supportive therapy, such as maintenance of hydration and electrolyte balance [6]. However, antimicrobial chemotherapy is recommended for patients with severe symptoms, extraintestinal infections, or acquired immune deficiency syndrome (AIDS) [6]. Fluoroquinolones and macrolides are the drugs of choice to treat campylobacteriosis [7], while tetracycline (TET) and gentamicin (GEN) are used to treat the systemic infection caused by *Campylobacter* [7]. However, widely used antimicrobial agents, such as ciprofloxacin (CIP) and azithromycin, have been challenged by drug resistance in both human and animal isolates. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria in 2017 showed that 57.7% of *C. jejuni* and 63.5% of *C. coli* of human origin were resistant to CIP [8]. Data from the National Antimicrobial Resistance Monitoring System (NARMS) report in the United States indicated that CIP resistance increased in *C. coli* from humans (34% in 2012 to 40% in 2015) and continued

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to increase or remained high in *C. jejuni* isolates from chickens (ranging between 22% and 28% during 2012–2015) [9]. Hence, the US Centers for Disease Control and Prevention (CDC) listed drug-resistant *Campylobacter* as a serious antibiotic resistance threat to public health in 2013 [10]. Similarly, the World Health Organization (WHO) listed fluoroquinolone-resistant *Campylobacter* species as high-priority pathogens in 2017 [11].

Unlike the United States and Europe, China lacks a complete antimicrobial resistance surveillance network for *Campylobacter*. Surveillance on *Campylobacter* in parts of China has shown that CIP resistance has reached a high level, with the most noticeable rates being observed in Jiangsu (85.2%) [12], Beijing (87.5%) [13], Shenzhen (89.7%) [14], and Shanghai (97.4%) [15]. Furthermore, the *Campylobacter* isolated from broiler chickens and swine in five provinces during 2008–2014 exhibited 100% resistance to CIP [16], suggesting that this drug may no longer be suitable for the treatment of *Campylobacter* infection in these areas.

The main mechanism of *Campylobacter* resistance to fluoroquinolones is mediated by point mutations in the quinolone resistance-determining region of GyrA and the tripartite multidrug efflux pump CmeABC [17,18]. CmeABC belongs to the resistance-nodulation-division (RND) protein family, and consists of a membrane fusion protein (CmeA), an inner membrane transporter (CmeB), and an outer membrane protein (CmeC) [19]. As a predominant efflux pump for *Campylobacter*, CmeABC extrudes toxic compounds as well as structurally diverse antimicrobials including fluoroquinolones, phenicols, macrolides, and TETs [20]. It is notable that a potent variant of CmeABC, named RE-CmeABC, has recently been found in *Campylobacter* [21]. Using basic local alignment search tool (BLAST), the unique CmeB shares 80.5%–81.2% amino acid sequence identity with wide-type CmeB in RE-*cmeABC*-negative strains; and its differences mostly contribute to the enhanced resistance level to various antibiotics. Furthermore, RE-CmeABC not only promotes the emergence of fluoroquinolone-resistant mutants under selective pressure, but also confers exceedingly high-level resistance (minimum inhibitory concentration (MIC) of CIP $\geq$ 128 mg·L^{-1}) to fluoroquinolone in the presence of GyrA mutations [21].

Since the RE-*cmeABC* genes were first identified in 2014, no surveillance of RE-*cmeABC* genes in *Campylobacter* collected from the same regions has been conducted. We therefore investigated the presence and antimicrobial susceptibility of RE-*cmeABC*-positive *Campylobacter* isolates of food-animal origin from slaughterhouses and farms in three representative regions of China during 2014–2016.

2. Materials and methods

2.1. Isolation, screening, and mutation detection of *RE*-cmeABC-*positive* Campylobacter *isolates*

We analyzed the presence and epidemic trend of RE-*cmeABC* in *Campylobacter* over three successive years (2014–2016) in three regions of China (Guangdong, Shandong, and Shanghai). A total of 1088 *Campylobacter* isolates (931 *C. coli* and 157 *C. jejuni*) were recovered, of which the isolates from 2014 have been published previously [21]. These *Campylobacter* strains were isolated from the cecal contents, carcasses, feces, and retail meat of swine and chickens under our laboratory's annual antimicrobial resistance surveillance program during 2014–2016. All of the *Campylobacter* strains were grown on Mueller–Hinton (MH) agar (Sigma–Aldrich, Inc., USA) at 42 °C under microaerobic conditions (5% oxygen, 10% carbon dioxide, 85% nitrogen) for 18–24 h. All isolates were screened for the presence of the RE-*cmeB* gene using the primers RE-*cmeB*F (5′-CGTATTGCACGATTATTTGGAC-3′) and RE-*cmeB*R (5′-ATCGTTATCAAACCCTCTATGTGCC-3′). To investigate whether the high-level resistance to CIP was relevant to the coharboring of the DNA gyrase *gyrA* mutation and RE-*cmeABC*, multiplex-mismatch amplification mutation assay-polymerase chain reaction (MAMA-PCR) was used to detect the single nucleotide mutation (C-257 to T) that is frequently observed in the *gyrA* gene of all RE-*cmeABC*-positive strains [22].

2.2. Antimicrobial susceptibility testing

The standard agar dilution method recommended by the Clinical & Laboratory Standards Institute (CLSI) M45 (2016) was used to determine the MICs of various antimicrobial agents for all *Campylobacter* isolates [23]. The tested antimicrobial agents included: GEN, erythromycin (ERY), CIP, TET, and clindamycin (CLI). Standardized MIC breakpoints for phenicols were not available for *Campylobacter* from the CLSI. Therefore, we utilized the breakpoint as recommended by the NARMS [24]. *C. jejuni* ATCC 33560 was used as the quality-control strain.

2.3. Molecular typing

A total of 97 representative *Campylobacter* isolates containing RE-*cmeABC* were genotyped by pulsed-field gel electrophoresis (PFGE), conducted with a CHEF-DR III apparatus (Bio-Rad Laboratories, Inc., USA) in accordance with the protocol for *Campylobacter* [25]. The DNA of *Campylobacter* was digested with SmaI, while *Salmonella* H9812 was digested with XbaI used as the reference marker. The results were analyzed with InfoQuest FP software version 4.5 (Bio-Rad Laboratories, Inc., USA).

2.4. Data collection and statistical analysis

A descriptive analysis of the percentage and prevalence (together with 95% confidence intervals (CIs)) was performed using the functions provided in Excel 2016 (Microsoft, USA). Univariable analysis among RE-*cmeABC*, *Campylobacter* species, and MIC values of antimicrobial agents was conducted using Prism 7.0 (GraphPad Software, USA), which was adopted to select variables with $P \leq 0.05$. The association between RE-*cmeABC* positivity and the variables was examined by odds ratio (OR). The differences between the rates were tested by X^2 or Fisher's exact test, if appropriate.

3. Results and discussion

3.1. Presence of *RE*-cmeABC *in* Campylobacter

Among the 1088 *Campylobacter* isolates tested, 122 (11.2%, 95% CI: 9.4–13.2) RE-*cmeABC*-positive isolates, all from chicken, were detected. These included 111 (111/157, 70.7%, 95% CI: 62.9–77.7) *C. jejuni* and 11 (11/931, 1.2%, 95% CI: 0.6–2.1) *C. coli*. RE-*cmeABC*-positive *C. jejuni* were significantly more prevalent compared with RE-*cmeABC*-positive *C. coli* (70.7% versus 1.2%, $P < 0.0001$) over three years (Fig. 1(a)). The prevalence difference between *C. jejuni* and *C. coli* was similar to a previous study that examined *Campylobacter* isolates from 2012 to 2014 [21]. Furthermore, the prevalence of RE-*cmeABC* in *C. jejuni* isolates from the two studies increased from 34.7% (189/544) to 70.7% (111/157) ($P < 0.0001$), while RE-*cmeABC*-positive *C. coli* isolates decreased from 3.2% (47/1458) to 1.2% (11/931) ($P < 0.0001$). This result suggested that the transformation of RE-*cmeABC* in *C. jejuni* is more frequent than in *C. coli*. This finding indicated that *C. jejuni* has become the dominant reservoir for RE-*cmeABC*. Moreover, all RE-*cmeABC*-positive *Campylobacter* were isolated from chicken samples, possibly

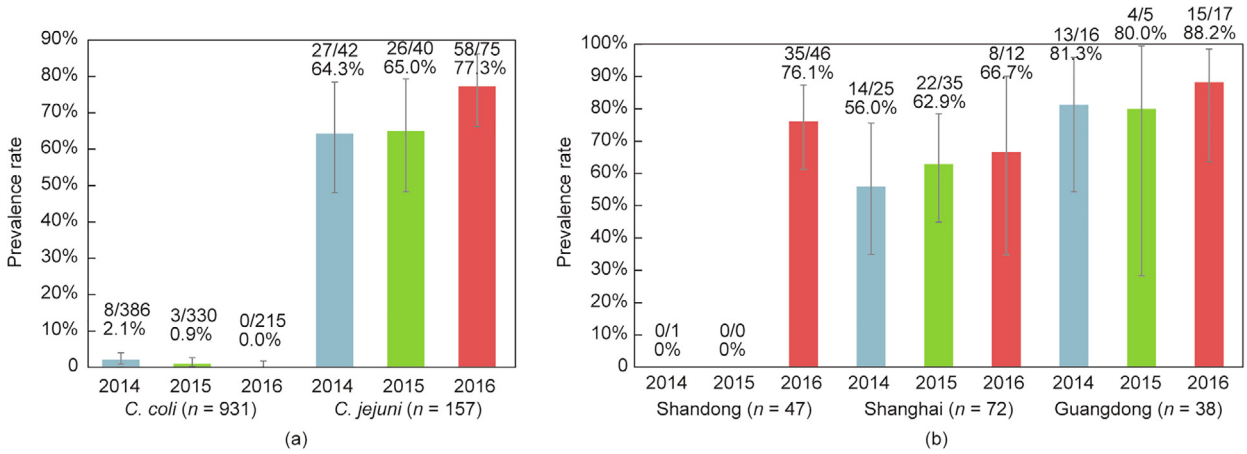

Fig. 1. Prevalence rate of RE-*cmeABC*-positive isolates from three regions by year. (a) Comparison of RE-*cmeABC*-positive isolates in *C. coli* and *C. jejuni* from the three regions by year; (b) percentage of RE-*cmeABC*-positive *C. jejuni* isolates from three regions by year. The error bar represents 95% CI.

because *C. jejuni* is the dominant species in poultry [26]. Due to the low isolation rate and limited number of RE-*cmeABC*-positive *C. coli*, we only compared RE-*cmeABC*-positive *C. jejuni* from various regions and years.

The percentages of RE-*cmeABC*-positive *C. jejuni* isolates for each year are shown in Fig. 1(a). The positive isolates maintained a high level and did not significantly increase during the three years of the study ($P > 0.05$). Similarly, the prevalence of RE-*cmeABC*-positive *C. jejuni* from Guangdong and Shanghai slightly increased over the three years of sampling (Fig. 1(b)). However, the rate from Shandong remained at a low level during 2014 and 2015, while a rapid emergence of high prevalence (76.1%, 35/46, 95% CI: 61.2–87.4) occurred in 2016 (Fig. 1(b)). These data are of particular concern with regards to public health, as RE-*cmeABC*-positive *C. jejuni* now presents at a high rate in these major livestock and poultry production areas of China.

3.2. Antimicrobial susceptibility profiles

Antimicrobial susceptibility testing (AST) showed that all RE-*cmeABC*-positive *C. jejuni* were fully resistant to CIP, followed by their resistance to TET, florfenicol (FFC), GEN, CLI, and ERY, with the resistance rates of 99.1%, 74.8%, 45.9%, 13.5%, and 11.7%, respectively (Table 1). To assess the contribution of RE-*cmeABC* toward antimicrobial susceptibility to *C. jejuni*, we supplemented the AST of RE-*cmeABC*-negative *C. jejuni* for analysis. The resistance rates were significantly different in the RE-*cmeABC*-negative versus RE-*cmeABC*-positive *C. jejuni* isolates for FFC, CLI, and ERY ($P < 0.05$), but were not significantly different for CIP, TET, and GEN ($P > 0.05$) (Table 1). The resistance of RE-*cmeABC*-positive isolates to FFC was significantly higher than that of RE-*cmeABC*-negative isolates (74.8% vs. 39.1%, $P < 0.0001$). The OR of FFC for RE-*cmeABC*-positive isolates (OR = 4.61) were significantly higher

Table 1
MIC profiles of *C. jejuni* with or without RE-*cmeABC*.

Antimicrobial agents	Number of resistance isolate (resistance rates, 95% CI)		OR	*P* value
	RE-*cmeABC*-positive ($n = 111$)	RE-*cmeABC*-negative ($n = 46$)		
CIP	111 (100.0%)	46 (100.0%)	—	—
TET	110 (99.1%, 95.1%–99.97%)	44 (95.7%, 85.2%–99.5%)	5.00	0.206
FFC	83 (74.8%, 65.6%–82.5%)	18 (39.1%, 25.1%–54.6%)	4.61	< 0.0001
GEN	51 (45.9%, 36.4%–55.7%)	21 (45.7%, 30.1%–61.0%)	1.01	0.973
CLI	15 (13.5%, 7.8%–21.3%)	15 (32.6%, 19.5%–48.0%)	0.32	0.006
ERY	13 (11.7%, 6.4%–19.2%)	13 (28.3%, 16.0%–43.5%)	0.34	0.011

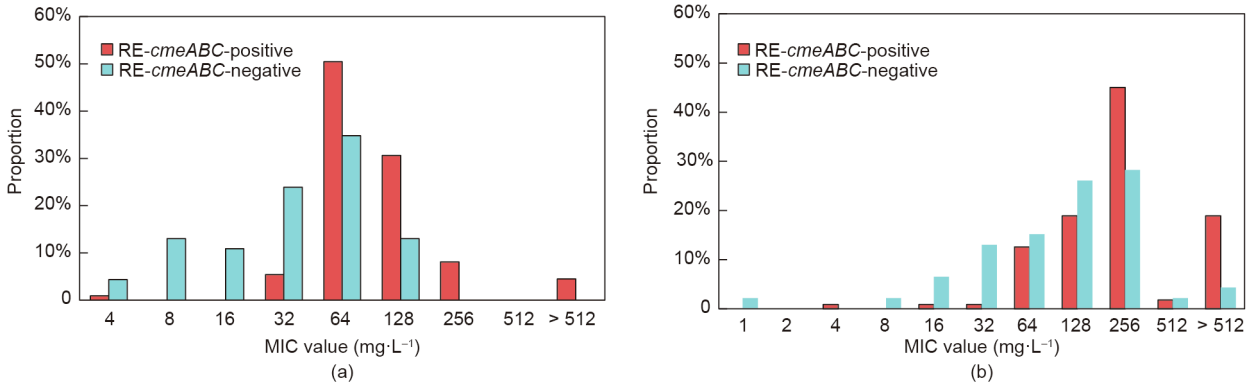

Fig. 2. Distribution of CIP and TET MICs for RE-*cmeABC*-positive isolates and -negative isolates. (a) Distribution of CIP MICs for *C. jejuni*; (b) distribution of TET MICs for *C. jejuni*.

than those for RE-*cmeABC*-negative isolates (Table 1). FFC, which belongs to the phenicols, is unapproved for clinical use, but has been used extensively for the treatment of respiratory diseases in food-producing animals [27]. Therefore, this antimicrobial agent may play an important role in the selection of RE-*cmeABC*-positive *Campylobacter* in animal production. Unlike FFC resistance,

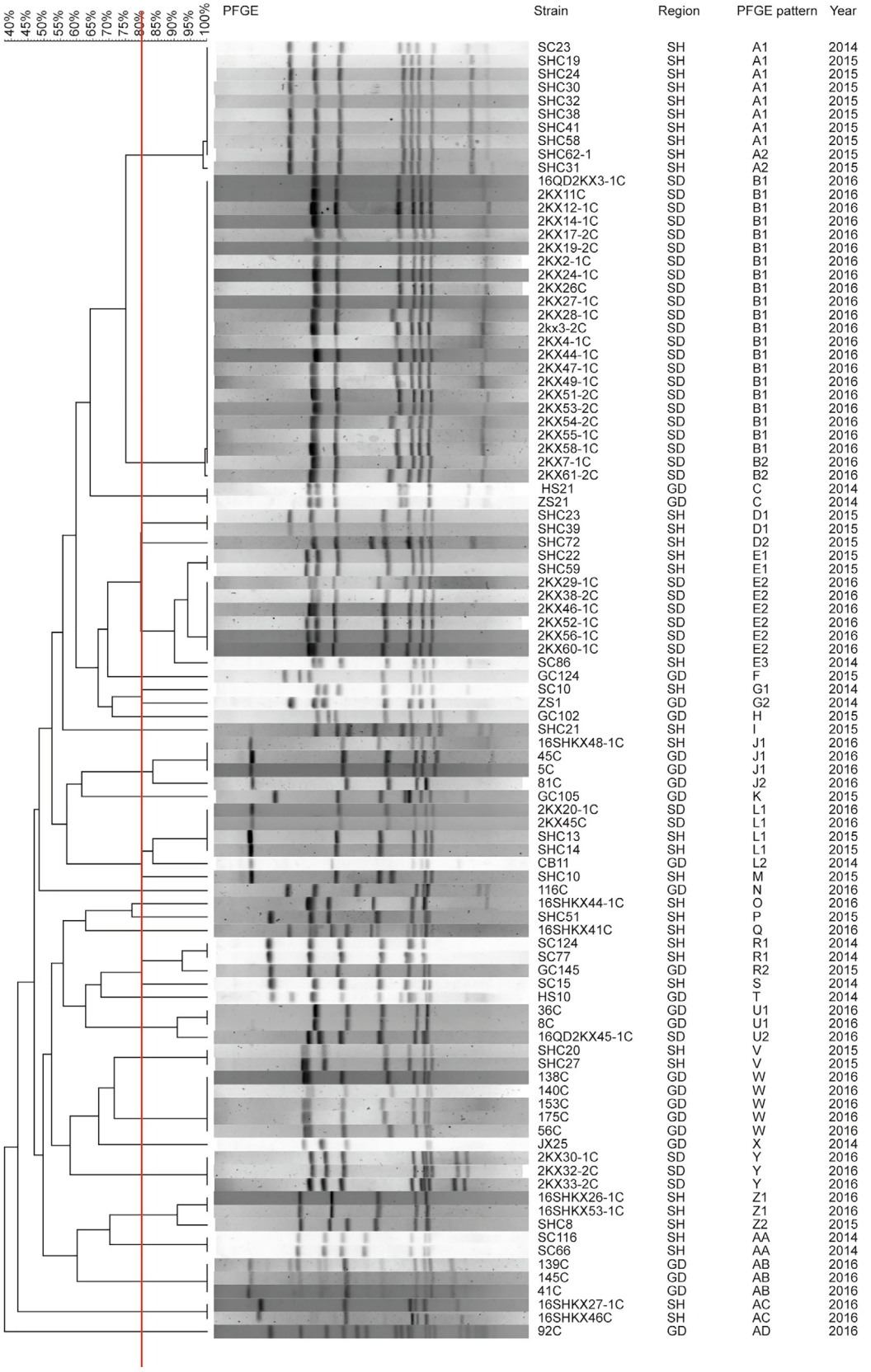

Fig. 3. PFGE typing of 97 representative RE-*cmeABC*-positive *C. jejuni* collected from 2014 to 2016. SmaI was used for PFGE analysis. The regions included Shandong (SD), Shanghai (SH), and Guangdong (GD).

the resistance levels to CLI and ERY in RE-*cmeABC*-positive were significantly lower than those of RE-*cmeABC*-*negative* (OR = 0.32 and 0.34, respectively) (Table 1). Although RE-*cmeABC* could contribute a four-fold increase in the MIC of ERY, it was insufficient to cross the breakpoint on its own, unless the target-gene mutations of 23S rRNA were present as well [28]. These data suggest that RE-*cmeABC* may not be the major resistance mechanism of CLI and ERY in *C. jejuni*.

RE-*cmeABC* has been shown to have an enhanced exporter function compared with wide-type *cmeABC*, the latter of which can pump drugs out of cells; this is true for fluoroquinolones, phenicols, macrolides, and TETs [21]. Although there is no significant difference between RE-*cmeABC*-positive and -negative isolates in their resistance to CIP and TET, the isolates harboring RE-*cmeABC* shifted the MIC distribution to a higher range for both CIP and TET (Fig. 2), which was consistent with previous results [21]. Interestingly, the high-level resistant ($\geq$ 16-fold of MIC breakpoint) isolates of CIP and TET were significantly higher in RE-*cmeABC*-positive isolates than in RE-*cmeABC*-negative isolates ($P < 0.0001$ and $P = 0.0004$, respectively). These results indicate that RE-CmeABC increases the antibiotic MICs at the population level, which is consistent with previous results [21]. As a verification of the mechanism of high-level resistance to CIP, we detected the single nucleotide mutation (C-257 to T) that is frequently observed in the *gyrA* gene. However, all RE-*cmeABC*-positive isolates harbored the C257T mutation, which further confirmed that the combination of the GyrA mutation and RE-CmeABC contributes to the high-level resistance to CIP. It is unknown whether the MIC values are related to the expression level of CmeABC. CmeABC is primarily regulated by CmeR, which binds to the promoter region of the cmeABC operon and functions as a transcriptional repressor [29].

3.3. Genotyping

The 97 representative RE-*cmeABC*-positive *C. jejuni* (37 from Shanghai, 25 from Guangdong, and 35 from Shandong) were analyzed by PFGE. Using a cutoff of 80% pattern similarity, the RE-*cmeABC C. jejuni* isolates were clustered into 30 PFGE patterns, including 13 unique patterns and 17 clusters (Fig. 3). The isolates from Shanghai showed the most patterns (eight clusters and seven unique patterns), followed by the Guangdong isolates (11 clusters and six unique patterns), and the Shandong isolates (five clusters). No predominant clones existed in the Shanghai and Guangdong isolates, except for pattern A (27.0%, 10/37) and pattern W (20%, 5/25). These findings suggested that horizontal transmission may be involved in the dissemination of RE-*cmeABC*-positive *C. jejuni* in Shanghai and Guangdong. In contrast, pattern B (65.7%, 23/35) was the predominant clone in Shandong, which indicates that regional expansion of a particular clone accounts for a larger proportion of the dissemination. Pattern R was shared by three isolates from Shanghai and Guangdong, while pattern U was shared by three isolates from Guangdong and Shandong. Moreover, strain 16SHKX48-1C, which was isolated from Shanghai, showed pattern J and had 100% homology to the isolates collected from Guangdong in 2016. Meanwhile, four isolates of pattern L, which were collected from Shanghai and Shandong, also shared 100% homology. These findings suggest that some RE-*cmeABC*-positive *C. jejuni* with the same PFGE patterns have spread into various regions, and that these isolates are stably transferrable and appear to be highly adaptable.

4. Conclusion

This study investigated the presence and antimicrobial susceptibility of RE-*cmeABC*-positive *Campylobacter* from food-producing animals in three regions of China during the three successive years of 2014–2016. By comparing our results with previously collected RE-*cmeABC*-positive *Campylobacter* from 2012 to 2014, we observed a shift of this multidrug efflux pump from *C. coli* to *C. jejuni* in chickens. The FFC resistance rate of RE-*cmeABC*-positive isolates was significantly higher than that of RE-*cmeABC*-negative isolates, while the CIP and TET resistance levels were not significantly different between these isolates. However, RE-*cmeABC* was found to increase the MIC distribution of the antibiotics. Moreover, several PFGE-indiscriminate RE-*cmeABC*-positive *C. jejuni* isolates were identified in both Shanghai and Guangdong, as well as in both Guangdong and Shandong. This finding suggests the possibility of a wide dissemination of RE-*cmeABC* in *Campylobacter*, which would poses a significant threat to human health.

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Compliance with ethics guidelines

Dejun Liu, Weiwen Liu, Xing Li, Hong Yao, Zhangqi Shen, Yang Wang, and Jianzhong Shen declare that they have no conflict of interest or financial conflicts to disclose.

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