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# High Prevalence and Predominance of the *aph(2″)-If* Gene Conferring Aminoglycoside Resistance in *Campylobacter*

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**ABSTRACT** *Campylobacter* is a major foodborne pathogen, and previous studies revealed that *Campylobacter* isolates from food-producing animals are increasingly resistant to gentamicin in China. The molecular epidemiology and genetic mechanisms responsible for gentamicin resistance in China have not been well understood. In this study, 607 *Campylobacter* isolates of chicken and swine origins collected in 2014 were analyzed, revealing that 15.6% (25/160) of the *Campylobacter jejuni* isolates and 79.9% (357/447) of the *Campylobacter coli* isolates were resistant to gentamicin. PCR detection of the gentamicin resistance genes indicated that *aph(2″)-If* was more prevalent than the previously identified *aacA/aphD* gene and has become the dominant gentamicin resistance determinant in *Campylobacter*. Transformation and whole-genome sequencing as well as long-range PCR discovered that *aph(2″)-If* was located on a chromosomal segment inserted between two conserved genes, Cj0299 and *panB*. Cloning of *aph(2″)-If* into gentamicin-susceptible *C. jejuni* NCTC 11168 confirmed its function in conferring high-level resistance to gentamicin and kanamycin. Molecular typing by pulsed-field gel electrophoresis suggested that both regional expansion of a particular clone and horizontal transmission were involved in the dissemination of the *aph(2″)-If* gene in *Campylobacter*. To our knowledge, this is the first report describing the high prevalence of a chromosomally encoded *aph(2″)-If* gene in *Campylobacter*. The high prevalence and predominance of this gene might be driven by the use of aminoglycoside antibiotics in food animal production in China and potentially compromise the usefulness of gentamicin as a therapeutic agent for *Campylobacter*-associated systemic infection.

**KEYWORDS** gentamicin resistance, *aph(2″)-If*, *Campylobacter*, food safety

*Campylobacter* is a significant cause of foodborne diarrhea in humans, and *Campylobacter jejuni* and *Campylobacter coli* are the main species accounting for the majority of campylobacteriosis (1). Severe cases of the infection require antibiotic therapy, for which fluoroquinolones and macrolides are considered the drugs of choice (2). However, recent studies have indicated that *Campylobacter* isolates are increasingly resistant to these two clinically important classes of antibiotics, posing a serious threat to public health (3, 4). In addition to causing a localized infection in the intestine, *Campylobacter* is also able to induce systemic infections such as bacteremia, for which aminoglycosides (i.e., gentamicin) are the drugs of choice for treatment (2).

In general, gentamicin resistance rates in *Campylobacter* have been reported to be low and stable in most countries (5–8). In the United States, gentamicin resistance in *Campylobacter* was rarely reported before 2007 according to National Antimicrobial Resistance Monitoring System (NARMS) studies; however, an increasing trend of resis-

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tance to aminoglycosides was observed in recent years (9). In 2011, 12.2% of human isolates and 18.1% of retail isolates showed resistance to aminoglycosides (9). In China, the gentamicin resistance rate in *Campylobacter* has been at a much higher level than in other countries (10–12). Several mechanisms of gentamicin resistance have been reported in *Campylobacter*. The gentamicin resistance gene *aacA4* was described in *C. jejuni* isolated from a broiler house (13). A multidrug-resistant plasmid, pCG8245, harboring resistance to gentamicin, was identified in a clinical strain of *C. jejuni*, which was isolated from a U.S. soldier deployed to Thailand (14). The gene conferring gentamicin resistance was initially annotated as *aac(6′)-Ie/aph(2″)-Ia* (also *aacA/aphD*), but it was found to encode the phosphotransferase activity only and thus was renamed *aph(2″)-If* (15). Subsequently, an *aacA/aphD* gene, which was contained in an aminoglycoside resistance genomic island, was reported in *C. coli* in China. The *aacA/aphD* gene encoding a bifunctional enzyme was associated with gentamicin resistance (16). Very recently, several variants of 2″-phosphotransferase accounting for gentamicin resistance were identified in *Campylobacter* in the United States (9, 17).

Here, we report the prevalence of gentamicin resistance and the associated resistance mechanisms in *Campylobacter* isolates of food animal origin in China. For the first time, we detected the *aph(2″)-If* gene in China. In addition, we found that this gene was located on the chromosome instead of plasmids. Molecular typing of the *Campylobacter* isolates indicated that the *aph(2″)-If* gene was disseminated by both clonal expansion and horizontal transmission. Additionally, the *aph(2″)-If* gene is more prevalent than *aacA/aphD* and has become the predominant determinant conferring gentamicin resistance in *Campylobacter* in China. These findings provide new insights into the epidemiology and spread of aminoglycoside resistance in *Campylobacter*.

## RESULTS AND DISCUSSION

**Gentamicin resistance in *Campylobacter* isolates.** In total, 62.9% (382/607) of *Campylobacter* isolates were resistant to gentamicin. Specifically, 15.6% (25/160) of *C. jejuni* isolates and 79.9% (357/447) of *C. coli* isolates were resistant to gentamicin. The proportions of gentamicin-resistant *C. jejuni* strains ranged from 0 to 37.5% among Guangdong, Ningxia, Shandong, Henan, and Shanghai, while the proportions of gentamicin-resistant *C. coli* strains ranged from 35.7% to 100% among the five regions (Table 1). The distributions of gentamicin MICs of *C. jejuni* and *C. coli* are shown in Fig. 1, which revealed that gentamicin resistance was much more prevalent in *C. coli* than in *C. jejuni* isolates ($P < 0.0001$, Fisher's exact test). Generally, the rate of resistance of *Campylobacter* to gentamicin is low (<2%) in other countries (8, 18, 19). However, recent studies performed in China suggested that the frequency of gentamicin resistance is high in *Campylobacter*, especially in *C. coli* isolated from swine and broiler chickens (23.2% to 95.4%) (10–12). The high prevalence of gentamicin resistance in *Campylobacter* found in this study is consistent with previous findings in China. According to the antimicrobial usage data collected in 2013, the total consumption of antibiotics was 162,000 tons in China, of which 83,200 tons was used in animals (20, 21). Additionally, antimicrobial usage records suggested that aminoglycoside agents, such as amikacin and neomycin, were commonly used to prevent and control bacterial diseases in food-producing animals in China (3). The exact reason for the high prevalence of gentamicin resistance in China is unknown, but it might be driven by the extensive use of aminoglycoside antibiotics in animal production.

**Prevalence of gentamicin resistance genes in *Campylobacter* isolates.** All 607 *Campylobacter* isolates (160 *C. jejuni* and 447 *C. coli* isolates; see Table S1 in the supplemental material) were examined by PCR for detecting various aminoglycoside resistance determinants. The *aph(2″)-If* gene was identified in 10.0% (16/160) of *C. jejuni* and 57.7% (258/447) of *C. coli* isolates (Table 1), indicating its common presence and higher prevalence in *C. coli* ($P < 0.0001$, Fisher's exact test). Notably, all the gentamicin-resistant *C. coli* isolates from Shandong province harbored the *aph(2″)-If* gene (Table 1), indicating that *aph(2″)-If* is responsible for gentamicin resistance in the *C. coli* isolates derived from this province. This finding is in contrast to a previous study conducted by

**TABLE 1** Prevalence of gentamicin resistance and the associated resistance determinants *aph(2″)-If* and *aacA/aphD*

| Location of isolation | Host | No. of gentamicin-resistant isolates/total no. of isolates (%) | | No. of *aph(2″)-If*-positive isolates/total no. of isolates (%) | | No. of *aacA/aphD*-positive isolates/total no. of isolates (%) | |
|---|---|---|---|---|---|---|---|
| | | *C. jejuni* | *C. coli* | *C. jejuni* | *C. coli* | *C. jejuni* | *C. coli* |
| Guangdong | Chicken | 6/16 (37.5) | 103/119 (86.6) | 3/16 (18.8) | 51/119 (42.9) | 1/16 (6.3) | 38/119 (31.9) |
| Ningxia | Chicken | 6/37 (16.2) | 23/23 (100) | 5/37 (13.5) | 11/23 (47.8) | 1/37 (2.7) | 8/23 (34.8) |
| | Swine | 2/6 (33.3) | 11/20 (55.0) | 1/6 (16.7) | 9/20 (45.0) | 0/6 (0) | 0/20 (0) |
| Shandong | Chicken | 0/1 (0) | 160/163 (98.2) | 0/1 (0) | 160/163 (98.2) | 0/1 (0) | 0/163 (0) |
| Shanghai | Chicken | 9/25 (36.0) | 25/52 (48.1) | 7/25 (28.0) | 2/52 (3.8) | 2/25 (8.0) | 17/52 (32.7) |
| | Swine | | 26/52 (50.0) | | 20/52 (38.5) | | 5/52 (9.6) |
| Henan | Chicken | 2/75 (2.7) | 4/4 (100) | 0/75 (0) | 0/4 (0) | 0/75 (0) | 0/4 (0) |
| | Swine | | 5/14 (35.7) | | 5/14 (35.7) | | 0/14 (0) |
| Total | | 25/160 (15.6) | 357/447 (79.9) | 16/160 (10.0) | 258/447 (57.7) | 4/160 (2.5) | 68/447 (15.2) |

us, in which the *aacA/aphD*-containing genomic island was found to account for gentamicin resistance in *C. coli* isolates derived during 2008 to 2009 from Shandong province (16). These results strongly suggest that *aph(2″)-If* recently emerged and became the predominant gentamicin resistance determinant in *Campylobacter* in Shandong province. The shift in the gentamicin resistance determinants during 2008 and 2009 to 2014 may be due to the rapid expansion of a *C. coli* clone containing *aph(2″)-If* (see the genotyping result).

The prevalence of the *aacA/aphD* gene was 2.5% (4/160) and 15.2% (68/447) in the *C. jejuni* isolates and *C. coli* isolates, respectively (Table 1). Similarly to the *aph(2″)-If* gene described above, *aacA/aphD* is more prevalent in *C. coli* than in *C. jejuni* ($P < 0.0001$, Fisher's exact test). In addition, we analyzed the gentamicin resistance genes reported in other countries, such as *aacA4* and the variants of 2″-phosphotransferase, none of which were identified in the *Campylobacter* isolates examined in this study. However, neither *aph(2″)-If* nor *aacA/aphD* was found in 20.0% (5/25) of *C. jejuni* and 8.7% (31/357) of *C. coli* isolates that were resistant to gentamicin, suggesting that these isolates may harbor unknown gentamicin resistance mechanisms. These results indicate that *aph(2″)-If* and *aacA/aphD* genes are responsible for gentamicin resistance in most of the *Campylobacter* isolates in China, with *aph(2″)-If* as the predominant resistance determinant. Notably, *aph(2″)-If* and *aacA/aphD* coexisted in some *Campylobacter* isolates, but they were inserted in different sites of the chromosome (data not shown), which could facilitate the transmission of gentamicin resistance along with other antimicrobial resistance genes.

**Transferability and the gene environment of *aph(2″)-If*.** The genomic DNAs of two *aph(2″)-If*-positive strains with high-level resistance to gentamicin, *C. coli* HS11B and *C. jejuni* CN9, were used as donors to transform *C. jejuni* NCTC 11168 by natural

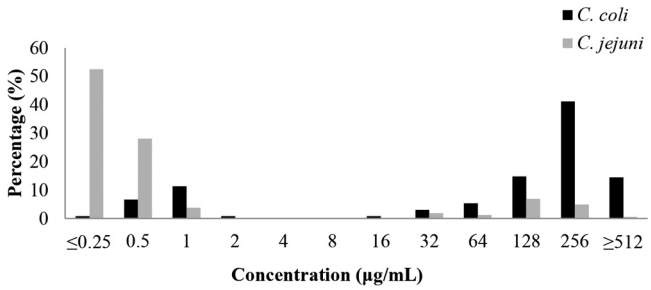


**FIG 1** Distributions of gentamicin MICs for *C. coli* ($n = 447$) and *C. jejuni* ($n = 160$) isolates. The breakpoint for gentamicin resistance is 8 $\mu$g/ml.

**TABLE 2** MICs of aminoglycoside antibiotics for various isolates and constructs

| Antimicrobial agent | MIC (μg/ml) for isolate or construct[a]: | | | | | | |
|---|---|---|---|---|---|---|---|
| | ATCC 33560 | NCTC 11168 | HS11B | NT-HS11B | CN9 | NT-CN9 | 11168-*aph(2″)-If* |
| Gentamicin | 1 | 1 | >512 | >512 | 512 | 512 | 512 |
| Kanamycin | 8 | 8 | >512 | >512 | >512 | >512 | >512 |
| Neomycin | 4 | 4 | >512 | 256 | 256 | 32 | 4 |
| Streptomycin | 4 | 4 | 128 | 4 | 128 | 4 | 4 |
| Amikacin | 2 | 4 | 256 | 64 | 128 | 32 | 4 |
| Apramycin | 2 | 4 | 4 | 4 | 4 | 4 | 4 |

[a]NT-HS11B and NT-CN9 are transformants of NCTC 11168 with donor DNA from *C. coli* HS11B and *C. jejuni* CN9, respectively, while 11168-*aph(2″)-If* is the NCTC 11168 construct containing a cloned *aph(2″)-If*.

transformation. The transformants, NT-HS11B and NT-CN9, showed >512- and 512-fold increases in the MICs of gentamicin, respectively, compared with NCTC 11168 (Table 2). These findings indicated that the resistance to gentamicin in HS11B and CN9 can be transferred by natural transformation. PCR and DNA sequencing of the products revealed the existence of the *aph(2″)-If* gene in NT-HS11B and NT-CN9. Furthermore, the transformants NT-HS11B and NT-CN9 showed decreased susceptibility to kanamycin, neomycin, and amikacin (Table 2), which are commonly used for disease control in broiler chickens in China.

Subsequently, one of the NT-HS11B transformants was investigated by whole-genome sequencing. The draft genome of NT-HS11B was compared with NCTC 11168, revealing that the backbone of the transformant was NCTC 11168, but a 10,550-bp segment was inserted between Cj0299 and *panB*, which are highly conserved on *C. jejuni* and *C. coli* genomes. This inserted segment contained 11 open reading frames (ORFs), including 8 antimicrobial resistance genes, among which 7 [*aph(2″)-If*, *aac*, *aadA*, *aph2*, *aad9*, *aphA3*, and *aphA7*] encode aminoglycoside-modifying enzymes and one (*cat*) mediates chloramphenicol resistance. Thus, this genomic island can mediate resistance to multiple aminoglycoside antimicrobials, which was shown by the MICs of the transformants that had increased MICs of gentamicin, kanamycin, neomycin, and amikacin (Table 2). It is conceivable that use of either of the aminoglycosides should provide selection pressure for this genomic island. Comparative analysis suggested that most ORFs in this segment were highly similar to the ORFs located on plasmid pCG8245 (GenBank accession number AY701528) in *C. jejuni* but with additional genes, such as the chloramphenicol resistance gene *cat* (Fig. 2) (14). The *aph(2″)-If* gene was located immediately upstream of Cj0299 in NT-HS11B and showed 100% nucleotide identity to the "*aph(2″)-If*" in pCG8245 and 78% nucleotide identify to the APH(2″)-Ia domain with only phosphotransferase activity of the bifunctional enzyme AAC(6′)-Ie/APH(2″)-Ia (14, 16). In addition, the comparison with the previously reported aminoglycoside resistance genomic island (16) was also exhibited in the results shown in Fig. 2. In general, the novel multidrug resistance island identified in this study showed a very low similarity with the segments of pN29701 (CP004067) and SX81 (JQ655275) (16, 17) but was highly similar to the segment of pCG8245 (AY701528) (14).

S1 pulsed-field gel electrophoresis (S1-PFGE) and Southern hybridization verified that the *aph(2″)-If* gene was located on the chromosome instead of a plasmid (data not shown). Furthermore, long-range PCR was performed to determine the insertion of the segment in the field isolates using primers located in the conserved regions of the Cj0299 and *panB* genes, respectively. An amplicon of ~10 kb was obtained from HS11B, CN9, and several other representative gentamicin-resistant *Campylobacter* isolates derived from different provinces (data not shown). The DNA sequencing results of the long-range PCR products revealed that the DNA segment in these isolates showed >99% nucleotide identity to that of NT-HS11B. These results indicate that the *aph(2″)-If*-containing segment is inserted in a conserved location on the chromosome and is widely spread in the gentamicin-resistant *Campylobacter* isolates in China.

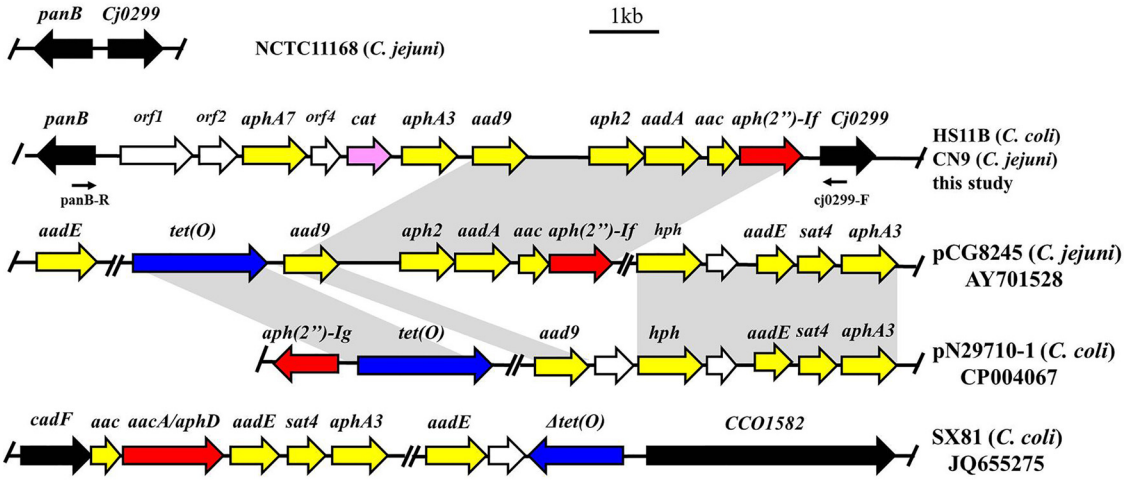


**FIG 2** Chromosomal organization of the *aph(2″)-If*-carrying segment in *C. coli* HS11B and *C. jejuni* CN9 in comparison with plasmid pCG8245, pN29710-1, the multidrug resistance genomic island of *C. coli* SX81, and *C. jejuni* NCTC 11168. Arrows indicate the positions and directions of transcription of the genes. The locations of primers panB-F and cj0299-R used to detect the unique genomic island are indicated. The gray-shaded areas represent regions sharing 99% DNA identity. Gentamicin resistance genes are colored red, while other aminoglycoside resistance genes are colored yellow. Chloramphenicol and tetracycline genes are colored pink and blue, respectively.

Previously, *aph(2″)-If* together with other aminoglycoside resistance genes was detected on a plasmid in *C. jejuni* (14), and the genomic islands harboring aminoglycoside resistance and multidrug resistance were identified between *cadF* and *CCO1582* on the chromosome in *C. coli* (16, 22). In the present study, multiple aminoglycoside resistance genes and a *cat* gene are found to be located in a novel spot between Cj0299 and *panB*, which has never been reported previously. This novel integration site raises the possibility of homologous recombination through natural transformation. In addition, the multidrug resistance island is not associated with any known mobile element, such as insertion elements (ISs) and transposons, suggesting that homologous recombination mediated by natural transformation is likely involved in the dissemination of the genomic islands described in this study.

**Functional confirmation of the *aph(2″)-If* gene.** Previously, the *aph(2″)-If* gene on plasmid pCG8245 was interrupted by insertional mutagenesis to demonstrate its function in conferring gentamicin resistance (14). To formally demonstrate that *aph(2″)-If* alone was responsible for the high-level gentamicin resistance, this gene was cloned from HS11B into *C. jejuni* NCTC 11168 and inserted between Cj0299 and *panB* on the chromosome. This construct, 11168-*aph(2″)-If*, showed 512-fold and >64-fold increases in the MICs of gentamicin and kanamycin, respectively, compared with NCTC 11168 (Table 2). This result indicated that *aph(2″)-If* alone confers high-level resistance to both gentamicin and kanamycin in *Campylobacter*. The *aph2* gene was also contained in the segment between Cj0299 and *panB*. Although it was predicted to be active, it did not confer gentamicin resistance in either *Escherichia coli* or *C. jejuni* as determined in a previous study (14). Therefore, we did not further analyze the *aph2* gene by functional cloning in this study. Interestingly, the cloned *aph(2″)-If* gene alone did not affect the MICs of neomycin and amikacin (Table 2), indicating that the elevated MICs of neomycin and amikacin in NT-HS11B and NT-CN9 were due to other aminoglycoside resistance genes in the genomic island.

**Molecular typing and phylogenetic analysis of *aph(2″)-If*-carrying *C. jejuni* and *C. coli* isolates.** To understand if the *aph(2″)-If*-carrying *Campylobacter* isolates were genetically related, 40 isolates (28 of *C. coli* and 12 of *C. jejuni*), representing different regions and antimicrobial susceptibility patterns, were selected to perform pulsed-field gel electrophoresis (PFGE) analysis with SmaI digestion (Fig. 3). Using 80% genetic similarity as a cutoff, the 28 *C. coli* isolates were grouped into 4 clusters (PFGE patterns represented by multiple strains) and 16 unique PFGE patterns (PFGE patterns repre-

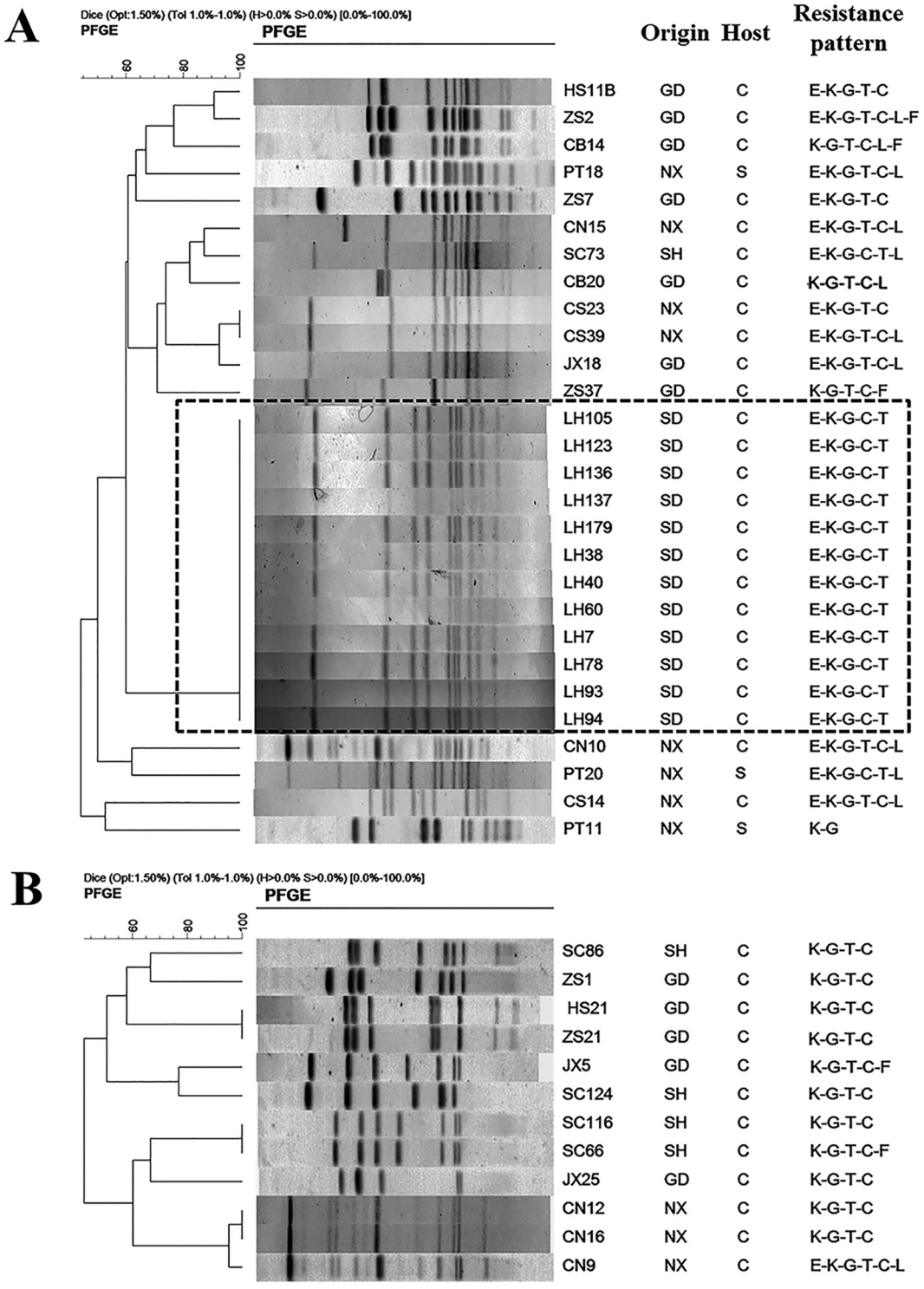


**FIG 3** PFGE analysis of *aph(2″)-If*-positive *C. coli* (A) and *C. jejuni* (B) isolates. SmaI was used for digestion of the genomic DNA. Isolation sources include Guangdong (GD), Shandong (SD), Ningxia (NX), and Shanghai (SH). Hosts include chicken (C) and swine (S). Abbreviations of antimicrobial agents: C, ciprofloxacin; T, tetracycline; E, erythromycin; F, florfenicol; L, clindamycin; K, kanamycin; G, gentamicin.

sented by a single strain) (Fig. 3A). The 12 *C. jejuni* isolates were grouped into 3 clusters and 9 unique PFGE patterns (Fig. 3B). In general, the *C. coli* and *C. jejuni* isolates from different regions were genetically diverse; however, the 12 *C. coli* isolates originating from Shandong (LH strains) showed identical PFGE patterns, suggesting that these

isolates are clonally related. This finding supports the notion that the predominance of *aph(2″)-If* in gentamicin-resistant *C. coli* isolates in Shandong province is related to regional expansion of a particular clone. Overall, findings in this study revealed both genetic diversity and regional clonality of *aph(2″)-If*-carrying isolates, suggesting that both horizontal transmission and clonal expansion were involved in dissemination of *aph(2″)-If* in China.

In summary, this study reports the high prevalence of the *aph(2″)-If* gene conferring high-level gentamicin resistance in *C. coli* in China. This gene is widely disseminated among *Campylobacter* isolates derived from chickens and swine. Horizontal transmission and clonal expansion are likely involved in the dissemination of *aph(2″)-If*. These findings provide new insights into the epidemiology of aminoglycoside resistance in *Campylobacter* and highlight the tremendous adaptability of this major foodborne pathogen to antibiotic selection pressure. Considering the usefulness of gentamicin in treating systemic infections caused by *Campylobacter*, the rising resistance to this class of antibiotics should be concerning, and measures should be taken to reduce the prevalence of antibiotic-resistant *Campylobacter*.

## MATERIALS AND METHODS

***Campylobacter* isolates and susceptibility testing.** In total, 607 *Campylobacter* isolates (160 *C. jejuni* and 447 *C. coli* isolates) were investigated in this study. These strains were isolated from cecal contents of chickens and feces of swine from Guangdong, Ningxia, Henan, and Shandong provinces and Shanghai (see Table S1 in the supplemental material) in 2014. All the *Campylobacter* strains were grown on Mueller-Hinton (MH) agar (Sigma-Aldrich, MO, USA) at 42°C under microaerobic conditions (5% $O_2$, 10% $CO_2$, and 85% $N_2$). The standard agar dilution method was used to determine the MICs of gentamicin for *Campylobacter* according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (23). Those isolates that showed resistance to gentamicin were further examined for their susceptibility to kanamycin, neomycin, streptomycin, amikacin, and apramycin. The reference strain *C. jejuni* ATCC 33560 was used as a quality control strain. The resistance of antimicrobial agents was interpreted according to the criteria of CLSI (23). Antimicrobial agents were obtained from the China Institute of Veterinary Drug Control (Beijing, China).

**PCR detection of gentamicin resistance genes in *Campylobacter* isolates.** The known gentamicin resistance genes, including *aph(2″)-If*, *aacA/aphD*, *aph(2″)-Ig*, and *aacA4*, were detected in *Campylobacter* isolates by PCR and sequencing. The primers used in this study are listed in Table S2. The PCR mixture was composed of 12.5 $\mu$l of Ex-Taq (TaKaRa, Dalian, China), 0.5 $\mu$l of each primer, 0.5 $\mu$l of chromosomal DNA template prepared by boiling as described previously (24), and 11 $\mu$l of sterile distilled water. The PCR was conducted in a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the conditions described in Table S2.

**Natural transformation and whole-genome sequencing.** The natural transformation assay was performed in accordance with the method described previously with minor modifications (25). The whole-genomic DNA of high-level-gentamicin-resistant *Campylobacter* isolates was used as the donor, while the reference strain of *C. jejuni* NCTC 11168 served as the recipient. Briefly, the recipient cells were spread on MH agar, and then, 1 $\mu$g of genome DNA of the donor was added onto the cells followed by culture for 7 h at 42°C under microaerobic conditions. The cells were collected and plated on the selective plate containing gentamicin (10 $\mu$g/ml) and then incubated for 72 h at 42°C under microaerobic conditions. Transformation without donor DNA was used as a negative control. Single colonies of transformants were picked and subcultured on gentamicin-containing plates for purity. The antimicrobial susceptibility of the transformants was determined to confirm the phenotype of gentamicin resistance. Subsequently, the transformants were subjected to whole-genome sequencing using an Illumina HiSeq 2500 sequencer (Berry Genomics Company, Beijing, China).

**S1-PFGE and Southern blotting.** S1 nuclease pulsed-field gel electrophoresis (PFGE) and Southern blotting were performed to determine the location of gentamicin resistance gene *aph(2″)-If* on plasmids or on the chromosome. S1 nuclease (TaKaRa, Dalian, China) was used to digest the agarose gel plugs containing the cells of gentamicin-resistant isolates and then separated by PFGE under the following conditions: 0.5× Tris-borate-EDTA, 1% agarose solution for 11 h at 6 V/cm and 14°C, with a pulse angle of 120° and the initial and final switch of 2 s and 55 s, respectively, as described previously with minor modifications (26). Southern blotting was performed to detect the location of *aph(2″)-If*. The probe was amplified using specific primers (If-F, 5′-TTGGTGAGGGTTATGACAGC, and If-R, 5′-CACTTCCTTAATTTTTC ATCTTTGC) for the *aph(2″)-If* gene and then labeled with a digoxigenin (DIG) High Prime I DNA labeling and detection starter kit for hybridization. Hybridization was conducted at 42°C for 16 h. Membranes were washed twice at room temperature (22 to 25°C) in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS for 5 min and twice at 68°C in 0.1× SSC-0.1% SDS for 15 min. The signals from the bands were visualized using a nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) color detection kit (Roche Diagnostics, Mannheim, Germany) according to the recommendations of the supplier.

**Functional cloning of the *aph(2″)-If* gene.** In order to demonstrate the function of the *aph(2″)-If* gene, we cloned it into aminoglycoside-susceptible *Campylobacter* NCTC 11168. Briefly, several pairs of

primers were designed to amplify the entire *aph(2″)-If* gene together with its upstream Cj0299 gene (primers P1 and P2), the *cat* gene (primers P3 and P4) in HS11B, and the *panB* (primers P5 and P6) gene in NCTC 11168 using the online assembly tool NEBuilder (New England Biolabs, Ipswich, MA). These primers are listed in Table S2. Then, the 3 fragments were assembled with NEBuilder HiFi DNA assembly master mix (New England Biolabs, Beverly, MA, USA) according to the protocol provided by the manufacturer. Subsequently, the assembled 3 fragments were used as the template, the primers P1 and P6 were used to amplify the donor DNA, and *C. jejuni* NCTC 11168 was used as the recipient strain for natural transformation according to the method mentioned above. The transformants were selected with 8 $\mu$g/ml chloramphenicol.

**PFGE.** PFGE analysis of *Campylobacter* strains was performed using SmaI as the restriction endonuclease according to the protocol for *Campylobacter* (27). *Salmonella* strain H9812 digested by XbaI was used as the reference marker. PFGE results were analyzed by using the InfoQuest software, version 4.5 (Bio-Rad Laboratories).

**Accession number(s).** The sequence described in this paper has been deposited at GenBank under accession number KX272768.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00112-17.

**SUPPLEMENTAL FILE 1,** PDF file, 0.1 MB.

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