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Whole-Genome Sequencing Analysis Accurately Predicts Antimicrobial Resistance Phenotypes in *Campylobacter* spp.

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The objectives of this study were to identify antimicrobial resistance genotypes for *Campylobacter* and to evaluate the correlation between resistance phenotypes and genotypes using *in vitro* antimicrobial susceptibility testing and whole-genome sequencing (WGS). A total of 114 *Campylobacter* species isolates (82 *C. coli* and 32 *C. jejuni*) obtained from 2000 to 2013 from humans, retail meats, and cecal samples from food production animals in the United States as part of the National Antimicrobial Resistance Monitoring System were selected for study. Resistance phenotypes were determined using broth microdilution of nine antimicrobials. Genomic DNA was sequenced using the Illumina MiSeq platform, and resistance genotypes were identified using assembled WGS sequences through blastx analysis. Eighteen resistance genes, including *tet*(O), bla_{OXA-61}, *catA*, *lnu*(C), *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2′)-If*, *aph(2″)-Ig*, *aph(2″)-Ih*, *aac(6′)-Ie-aph(2″)-Ia*, *aac(6′)-Ie-aph(2″)-If*, *aac(6′)-Im*, *aadE*, *sat4*, *ant(6′)*, *aad9*, *aph(3′)-Ic*, and *aph(3′)-IIIa*, and mutations in two housekeeping genes (*gyrA* and 23S rRNA) were identified. There was a high degree of correlation between phenotypic resistance to a given drug and the presence of one or more corresponding resistance genes. Phenotypic and genotypic correlation was 100% for tetracycline, ciprofloxacin/nalidixic acid, and erythromycin, and correlations ranged from 95.4% to 98.7% for gentamicin, azithromycin, clindamycin, and telithromycin. All isolates were susceptible to florfenicol, and no genes associated with florfenicol resistance were detected. There was a strong correlation (99.2%) between resistance genotypes and phenotypes, suggesting that WGS is a reliable indicator of resistance to the nine antimicrobial agents assayed in this study. WGS has the potential to be a powerful tool for antimicrobial resistance surveillance programs.

Whole-genome sequencing (WGS) technology has become a fast and affordable tool that is revolutionizing research in the fields of genetics, microbiology, and ecology, as well as public health surveillance and response. Recent studies have shown that WGS analysis can potentially be a single, rapid, and cost-effective approach to define resistance genotypes and predict resistance phenotypes of bacteria with great sensitivity and specificity (1–4).

Current laboratory methods, such as broth microdilution, measure antimicrobial susceptibility based on the growth response of bacteria following exposure to the drugs. These methods are advantageous in that they help us understand the concentration of drug necessary to prevent growth of the organism, a measure that is directly related to the clinical success of antimicrobial therapy. However, such phenotypic testing can be costly and time-consuming, especially for bacteria such *Campylobacter* that require special growth conditions. Several antimicrobial susceptibility testing methods have been developed for *Campylobacter*, including agar dilution, disk diffusion, Etest, and broth microdilution (5–7). Each of these methods can test only a limited number of antimicrobials. Reproducibility between laboratories also can be problematic, even when published guidelines are followed, due to variations in medium, incubation conditions, inoculum density, and antimicrobial stability (1, 8). Several studies showed that agreement between susceptibility testing methods for *Campylobacter* varied greatly (6, 7, 9, 10). In addition, clinical resistance breakpoints for many antimicrobials have yet to be established for *Campylobacter*, and for drugs that do have clinical breakpoints, interpretive criteria between the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) differ. Furthermore, phenotypic testing does not provide information on resistance alleles and their genetic context, which can be very valuable in interpreting surveillance data and conducting outbreak investigations. Genetic mechanisms responsible for antimicrobial resistance can help attribute bacteria to a given source, point in time, and/or geographic location. PCR-based methods have been widely used to detect specific resistance genes but are limited in their ability to characterize the resistome of isolates due to the large number of known resistance genes and alleles. Recently, several studies have used WGS-based approaches to accurately predict resistance phenotypes for *Escherichia coli*, *Klebsiella*, *Salmonella*, *Enterococcus*, and *Staphylococcus* (1–3). These studies reported that WGS had >96 to 97% sensitivity and 97 to 100% specificity in predicting resistance phenotypes and concluded that WGS was a realistic alternative to conventional phenotypic antimicrobial susceptibility testing methods.

Campylobacter is a leading cause of foodborne illness in the United States. The National Antimicrobial Resistance Monitoring System (NARMS [http://www.fda.gov/AnimalVeterinary/Safety

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Health/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/default.htm]) tracks resistance in *Campylobacter* from human clinical specimens, retail meats, and food production animals. The methods of antimicrobial susceptibility testing and the number of antimicrobials tested for *Campylobacter* have changed over the years since the NARMS program was created in 1996. The U.S. National Strategy for Combating Antibiotic Resistant Bacteria emphasizes the need for improved surveillance and data sharing, highlighting the importance of universal, rapid, reproducible, and cost-effective methods that can predict resistance phenotypes and can also define resistance mechanisms for surveillance purposes and clinical diagnostics. Before adopting a sequence-based approach for determining antimicrobial resistance, we must first evaluate performance, reproducibility, practicality, and agreement with results of traditional phenotypic methods. The objectives of this study were to use WGS to identify antimicrobial resistance genotypes of *Campylobacter* spp. and to correlate these results with resistance phenotypes identified by *in vitro* antimicrobial susceptibility testing (AST) to determine the efficacy of WGS as an alternative susceptibility testing method.

MATERIALS AND METHODS

Bacterial isolates. A total of 114 *Campylobacter* species isolates (82 *C. coli* and 32 *C. jejuni*) obtained from 2000 to 2013 as part of the NARMS surveillance program were selected based on resistance to multiple antimicrobials with different resistance profiles and representing a variety of sources. Isolates included 40 from human clinical specimens, 59 from retail meats, and 15 from cecal samples of food production animals (young chicken, turkey, heifer, and steer) (see Table S1 in the supplemental material). *Campylobacter* species isolates were grown on tryptic soy agar supplemented with 5% sheep blood (Remel, Lenexa, KS) under microaerobic conditions (85% nitrogen, 10% carbon dioxide, and 5% oxygen). All isolates were stored at −80°C in brucella broth with 20% glycerol until use.

Antimicrobial susceptibility testing (AST). The MIC of each drug tested was determined by broth microdilution using a Sensititre automated antimicrobial susceptibility system in accordance with the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Nine antimicrobial agents with the potential for treating *Campylobacter* (11) were tested in different concentration ranges: azithromycin (AZI; 0.15 to 64 μg/ml), ciprofloxacin (CIP; 0.015 to 64 μg/ml), clindamycin (CLI; 0.03 to 16 μg/ml), erythromycin (ERY; 0.03 to 64 μg/ml), florfenicol (FFN; 0.03 to 64 μg/ml), gentamicin (GEN; 0.12 to 32 μg/ml), nalidixic acid (NAL; 4 to 64 μg/ml), telithromycin (TEL; 0.015 to 8 μg/ml), and tetracycline (TET; 0.06 to 64 μg/ml). *Campylobacter jejuni* ATCC 33560 was used as a quality control according to the Clinical Laboratory and Standards Institute (CLSI) guidelines. In this report, we used epidemiological cutoff values (ECOFFs) as defined by the EUCAST (www.eucast.org) to interpret susceptibility. We refer to isolates with a non-wild-type interpretation as "resistant" and those with a wild-type interpretation as "susceptible." ECOFFs define resistance as any acquired decrease in susceptibility (which is sometimes described as being "microbiologically resistant" as opposed to being "clinically resistant") based on breakpoints used to guide therapy. ECOFF breakpoint values for *C. jejuni* and *C. coli* were different for some drugs. The lowest MICs of the nonsusceptible population are as follows: AZI, ≥0.5 μg/ml for *C. jejuni* and ≥1 μg/ml for *C. coli*; CLI, ≥1 μg/ml for *C. jejuni* and ≥2 μg/ml for *C. coli*; ERY, ≥8 μg/ml for *C. jejuni* and ≥16 μg/ml for *C. coli*); and TET, ≥2 μg/ml for *C. jejuni* and ≥4 μg/ml for *C. coli*. For other drugs, *C. jejuni* and *C. coli* have the same lowest MICs of the nonsusceptible population: CIP, ≥1 μg/ml; FFN, ≥8 μg/ml; GEN, ≥4 μg/ml; NAL, ≥32 μg/ml; and TEL, ≥8 μg/ml.

WGS and assembly. Genomic DNA was extracted using a Qiagen DNeasy blood and tissue kit (Qiagen, Gaithersburg, MD) per the manufacturer's instructions. Sequencing libraries were prepared with a Nextera XT DNA sample preparation kit (Illumina, Inc., San Diego, CA) and sequenced on an Illumina MiSeq instrument (Illumina) with a 250-bp paired-end protocol according to the manufacturer's instructions. The sequencing reads were demultiplexed by MiSeq reporter software (Illumina). Genome sequences were assembled using CLC Genomics Workbench, version 6.0.2 (CLC bio, Germantown, MD), and the assembled sequences were exported as fasta files for further analysis. Depth of coverage of ≥30 (30×) was required to ensure sequence quality. Isolates that had less than 30× sequence coverage were resequenced.

Antibiotic resistance gene identifications. Previously reported antibiotic resistance genes were downloaded from GenBank to a local database that contained 2,546 resistance genes from a number of bacterial sources, with gene variants representing resistance to all major antimicrobial classes (12). Additional databases published previously were used as references (13, 14). Assembled draft genomic sequences were generated to detect resistance genes using the blastx program, version 2.2.27 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (15), where putative resistance genes were compared with the reference database described above. The blastx results were processed using in-house PERL scripts to identify the most related resistance proteins, with a cutoff set at 50% sequence length and 85% amino acid identity. To examine the specific genomic mutations associated with resistance to quinolones and to macrolides/lincosamides/ketolides (MLKs), the *gyrA* gene and 23S rRNA gene were identified from the assembled sequences using blastx and extracted using in-house PERL scripts. The genes were then aligned using the MEGA program, version 5.0 (www.megasoftware.net) (16). The amino acid in GyrA at position 86, which is associated with resistance to quinolones (17), and nucleotides in the 23S rRNA at positions 2074 and 2075, which are associated with resistance to macrolides/lincosamides/ketolides (18), were examined. Five isolates showing discrepancies between phenotypic and genotypic resistance for AZI, CLI, and TEL were further analyzed for mutations in the three copies of the 23S rRNA using raw sequence reads. Mutations in L4 and L22 proteins were also checked for each of these five isolates.

Correlation of susceptibility phenotypes and genotypes. A total of 1,026 phenotypic data points were generated among the 114 isolates by AST. The correlation of resistance phenotypes obtained by AST and genotypes by WGS analysis was determined for each of nine antimicrobials tested. Specifically, each interpretation (resistance or susceptible) for a given AST result was manually compared with the presence or absence of a known corresponding resistance gene(s) and/or specific mutations. The percentage of correlation between resistance phenotypes and genotypes was calculated. Susceptible phenotypes were also compared with genotypes for the nine antimicrobials tested. Identification of colocated resistance genes was performed by identifying multiple resistance genes present on individual contigs. Elements were identified as carried on plasmids based on homology to well-characterized plasmids, whereas those identified as chromosomal were based on the presence of known chromosomal genes.

Nucleotide sequence accession numbers. WGS results of 114 *Campylobacter* species isolates used in this study were submitted to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The GenBank accession numbers of individual isolates are listed in Table 1.

RESULTS

Quality of WGS. WGS data of the 114 isolates were assembled to construct draft genomes with a median N_{50} value of 106,874 bp and a mean of 107,559 ± 47,616 bp. The median number of contigs was 87, with a mean of 105 (range, 23 to 670).

AST profiles. Each of the 114 isolates was tested for susceptibility to nine antimicrobials using the standard broth microdilu-

TABLE 1 Accession numbers of *Campylobacter* isolates

Isolate no.	GenBank accession no.
N20344	JAJU00000000
N20402	JAJV00000000
41922	JAKL00000000
41932	JAJW00000000
41939	JAJX00000000
41946	JAJE00000000
41955	JAJY00000000
41957	JAJZ00000000
41958	JAKA00000000
41965	JAJI00000000
41974	JAKS00000000
41976	JAKB00000000
41986	JAJJ00000000
N39665	LBEV00000000
N39671	LBEU00000000
N39677	LBET00000000
N40944	LBES00000000
N40946	LBER00000000
N41652	LBEQ00000000
N41661	LBEP00000000
N42547	LBEN00000000
N43850	LBEM00000000
N44396F	LBDZ00000000
N44721	LBEL00000000
N44984F	LBDW00000000
N45963	LBEK00000000
N46876	LBEJ00000000
N47608F	LBDS00000000
N47960	LBEI00000000
N48647F	LBDR00000000
N49243	LBEH00000000
N49244	LBEG00000000
N49249	LBEF00000000
N49369F	LBDQ00000000
N51183F	LBDN00000000
N51226F	LBDL00000000
N51619	LBEE00000000
N51691	LBEC00000000
N51712	LBEB00000000
N51987	LBEA00000000
41923	JAKM00000000
N1630	JOUH00000000
N6388	JOUY00000000
N7454	JOUZ00000000
N13165	JAJT00000000
41898	JAJK00000000
41900	JAKC00000000
41902	JAKD00000000
41912	JAKH00000000
41918	JAKJ00000000
41921	JAKK00000000
41943	JAKQ00000000
41953	JAJQ00000000
41963	JAJR00000000
41970	JAJH00000000
41971	JAJS00000000
N44406F	LBDY00000000
N44505F	LBDX00000000
N51201F	LBDM00000000
N51684	LBED00000000
N534	JOUW00000000
N26070	JOUN00000000
N9077	JOVF00000000
N14840	JOVV00000000
N18323	JOUJ00000000
N18725	JOUK00000000
N23392	JOUM00000000
N26699	JOUP00000000
N279	JOUQ00000000
N287	JOVY00000000
N3506	JOUR00000000
N3508	JOUS00000000
N4677	JOUV00000000
N6401	JOVZ00000000
N7464	JOVA00000000
N8957	JOVC00000000
N9016	JOVD00000000
N9095	JOVH00000000
41936	JAKP00000000
N14784	JOVU00000000
N15870	JOVW00000000
N1636	JOUI00000000
N20320	JOVX00000000
N23169	JOUL00000000
N26697	JOUO00000000
N4517	JOUT00000000
N8133	JOVB00000000
41904	JAJL00000000
41905	JAKE00000000
41908	JAKF00000000
41910	JAKG00000000
41914	JAKI00000000
41915	JAJM00000000
41917	JAJN00000000
41927	JAJG00000000
41933	JAKN00000000
41934	JAKO00000000
41944	JAJO00000000
41945	JAJP00000000
41964	JAKR00000000
41975	JAKT00000000
41985	JAKU00000000
N42205	LBEO00000000
N45714F	LBDV00000000
N46573F	LBDU00000000
N51090F	LBDO00000000
N5441	JOUX00000000
N15262	JOUG00000000
N462	JOUU00000000
N9036	JOVE00000000
N9093	JOVG00000000
N46596F	LBDT00000000
N50039F	LBDP00000000
41973	JAJF00000000

tion method. Resistance was most commonly observed to TET (94.7%, $n = 108$), followed by GEN (68.4%, $n = 78$), CIP/NAL (46.6%, $n = 53$), AZI (46.5%, $n = 53$), ERY/CLI (45.6%, $n = 52$), and TEL (43%, $n = 49$). All isolates were susceptible to FFN. Thirteen resistance phenotypes were identified, and the most common resistance phenotype was TET-GEN ($n = 40$), followed by AZI-CLI-ERY-TEL-CIP-NAL-GEN-TET ($n = 19$), CIP-NAL-GEN-TET ($n = 16$), AZI-CLI-ERY-TEL-TET ($n = 15$), and AZI-CLI-ERY-TEL-CIP-NAL-TET ($n = 9$) (Table 2).

TABLE 2 Number of *Campylobacter* isolates with different resistance patterns

Resistance profile	No. of isolates by species and sample source					
	C. jejuni			*C. coli*		
	Animal	Meat	Human	Animal	Meat	Human
AZI CLI ERY GEN CIP NAL TET	0	0	1	0	0	0
AZI CLI ERY GEN TET	0	0	0	2	0	0
AZI CLI ERY TEL	0	1	0	0	3	0
AZI CLI ERY TEL CIP NAL	0	0	0	0	1	0
AZI CLI ERY TEL CIP NAL GEN TET	0	0	10	3	1	5
AZI CLI ERY TEL CIP NAL TET	0	1	1	0	7	0
AZI CLI ERY TEL TET	0	3	0	0	12	0
AZI CLI TEL CIP NAL TET	0	0	0	0	1	0
AZI ERY TEL CIP NAL TET	0	0	0	0	1	0
CIP NAL	0	1	0	0	0	0
CIP NAL TET	0	1	1	0	2	0
CIP NAL GEN TET	0	1	6	3	1	5
GEN TET	0	2	3	7	20	8

Detection of resistance genes, mutations, and resistance gene cassettes. Eighteen resistance genes were identified in our 114 isolates, including *tet*(O), bla_{OXA-61}, *catA*, *lnu*(C), *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2′)-If*, *aph(2″)-Ig*, *aph(2″)-Ih*, *aac(6′)-Ie-aph(2″)-Ia*, *aac(6′)-Ie-aph(2″)-If*, *aac(6′)-Im*, *aadE*, *sat4*, *ant(6′)*, *aad9*, *aph(3′)-Ic*, and *aph(3′)-IIIa*. The *tet*(O), bla_{OXA-61}, *catA*, and *lnu*(C) genes encode resistance to tetracycline, beta-lactams, chloramphenicol, and lincomycin, respectively (19–22). The remaining 14 genes encoded various aminoglycoside-modifying enzymes; only *aac(6′)-Ie-aph(2″)-Ia*, *aac(6′)-Ie-aph(2″)-If*, *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2′)-If*, *aph(2″)-Ig*, and *aph(2″)-Ih* are known to encode resistance to GEN (23).

In addition, mutations in the two housekeeping genes, *gyrA* and 23S rRNA, were identified. A mutation of T86I in GyrA was detected in all CIP^r/NAL^r isolates, and mutations in the 23S rRNA at position A2074T and/or A2075G were detected in all ERY^r isolates (17, 18). These same 23S rRNA mutations were detected in all AZI^r, CLI^r, and TEL^r isolates except one (N9077). Overall, resistance genotypes were diverse, and most isolates carried multiple resistance genes in additional to one or two housekeeping gene mutations that conferred resistance (see Table S1 in the supplemental material). Many isolates with the same resistance phenotype had different resistance genes; further details on the genotype-phenotype correlations and discrepancies follow later in the Results section. A total of 55 resistance genotypes were identified, with the following being the most common types: *aadE aad9 aph(2″)-Ig aph(3′)-IIIa sat4 tet*(O) ($n = 19$); *aadE aad9 aph(2″)-Ig aph(3′)IIIa* bla_{OXA-61} *sat4 tet*(O) ($n = 8$); and *aad9 aph(2″)-Ih aph(3′)-IIIa* bla_{OXA-6} *sat4 tet*(O)GyrA T86I 23S rRNA A2075G ($n = 9$). The details of resistance phenotypes and genotypes of the 114 isolates are listed in Table S1.

Several resistance gene cassettes were identified in distinct genetic locations (Fig. 1). A pTet plasmid was found in many TET^r isolates and often carried multiple resistance genes, including *aadE*, *aad9*, *aph(2″)-Ig*, *aph(3′)-IIIa*, *sat4*, and *tet*(O) (Fig. 1). In addition, *tet*(O)was chromosomally encoded in one isolate (41898), which has also been found previously (24, 25). Since shotgun sequencing on the MiSeq platform provides fragmentary genomes assembled as contigs, not all resistance gene locations were determined.

Correlation of susceptibility phenotypes and genotypes. All 108 TET^r isolates carried *tet*(O), which encodes a ribosomal protection protein (24), but none of the 6 TET^s isolates carried this gene. Therefore, the correlations between both resistance and susceptible phenotypes and genotypes for TET were 100% (Table 3). Fifty-three CIP^r/NAL^r isolates had a GyrA T86I mutation, whereas no *gyrA* mutations were detected in any of the 61 CIP^s/NAL^s isolates. Thus, the correlations between resistance/susceptible phenotypes and genotypes for quinolones were also 100% (Table 3).

Among 78 GEN^r isolates, 76 carried one of the following seven GEN^r genes: *aac(6′)-Ie-aph(2″)-Ia*, *aac(6′)-Ie-aph(2″)-If*, *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2″)-Ig*, or *aph(2′)-If or aph(2″)-Ih*. One GEN^r isolate (41945) carried two GEN^r genes, *aph(2″)-If* and *aac(6′)-Ie-aph(2″)-Ia*. Another GEN^r isolate (N13165) carried three aminoglycoside resistance genes [*ant*(6), *aph*(3′)-*IIIa*, and *sat4*], but each of these is predicted to cause resistance to aminoglycosides other than gentamicin. The correlation between resistance phenotypes and genotypes was 98.7% (77/78) for GEN. Thirty-six GEN^s isolates carried one or more aminoglycoside resistance genes, but each was predicted to be responsible for resistance to aminoglycosides other than gentamicin. The correlation between susceptible phenotypes and genotypes for GEN was also 100%.

Previous studies indicated that 23S rRNA mutations at positions 2074 and 2075 were major contributors for resistance to macrolides (M), lincosamides (L), and ketolides (K) in *Campylobacter* spp. (26–28). Among 52 ERY^r isolates, 51 of them contained 23S rRNA mutations at A2075G, and 1 carried a mutation at A2074T, from analysis of the assembled genomes. These mutations were not detected in the 62 ERY^r isolates.

Five discrepancies between phenotypes and genotypes were observed for AZI, CLI, and TEL. Isolate N9077 showed low-level resistance to AZI (MIC of 1 μg/ml), CLI (MIC of 2 μg/ml), and TEL (MIC of 8 μg/ml) but was susceptible to ERY based on EUCAST resistance breakpoints. Further analysis of the mutations in all three copies of 23S rRNA and in the ribosomal L4 and L22 proteins revealed that N9077 displayed no mutations in any of three copies of 23S rRNA but an amino acid change in the L22 protein, A86E. Mutations in the 23S rRNA gene were reported to be associated with high-level resistance to ERY, whereas mutations in the L4 and L22 proteins appeared to mediate a low-to-intermediate level of resistance to ERY in *Campylobacter* (28). Thus, this mutation may account for the resistance pattern observed for N9077. Four other isolates were highly resistant to AZI and ERY but susceptible to either CLI or TEL and shared a mutation in three copies of 23S rRNA at A2075G and no mutations in L4 and L22 proteins. Isolate N26070 had high MICs to AZI (>64 μg/ml), ERY (>64 μg/ml), and TEL (8 μg/ml) but was susceptible to CLI, with an MIC of 0.25 μg/ml. Three other isolates (N46596F, N50039F, and 41973) showed high levels of resistance to AZI (>64 μg/ml) and ERY (>64 μg/ml) and low levels of resistance to CLI (2 to 4 μg/ml) but were susceptible to TEL (4 μg/ml). The AST was repeated for each isolate, and the

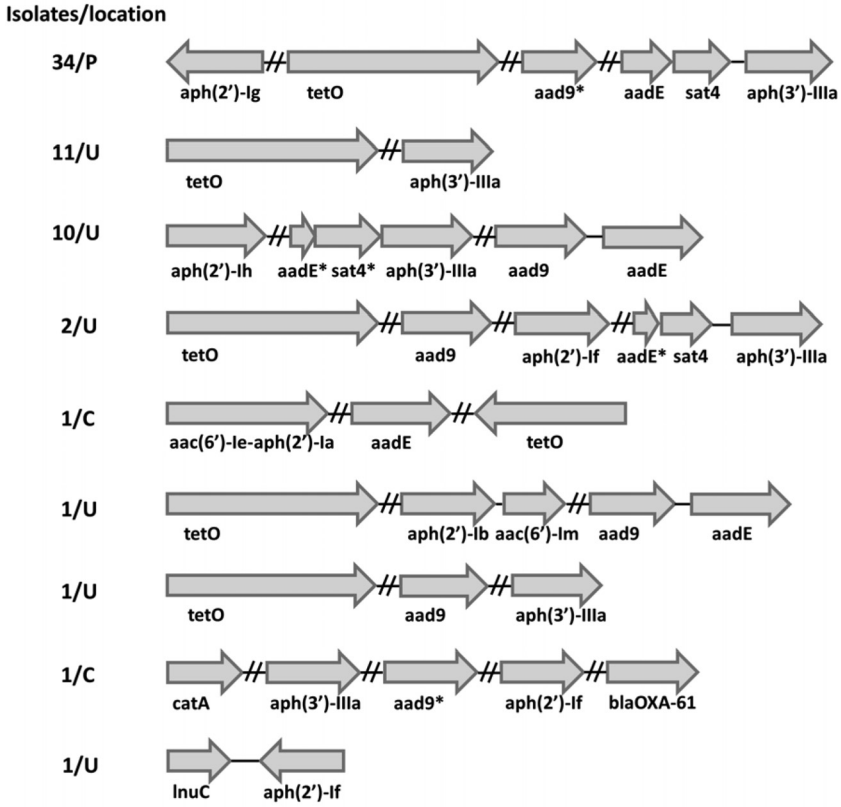

FIG 1 Depiction of selected resistance gene cassettes. Several genetic elements are shown, with resistance genes labeled. The number of isolates with each element, as well as the likely genetic location of each, is also depicted (U, unknown; C, chromosome; P, plasmid). Asterisks indicate genes that are truncated or disrupted and may be nonfunctional.

MIC values remained unchanged. Despite these discrepancies, the correlations between resistance phenotypes and genotypes for AZI, CLI, and TEL were 98.1% (52/53), 98.1% (51/52), and 98% (48/49), respectively. The correlations between susceptible phenotypes and genotypes for AZI, CLI, and TEL were 100% (61/61), 98.4% (61/62), and 95.4% (62/65), respectively (Table 3). All isolates were susceptible to FFN, and no known genes associated with FFN resistance were detected. Overall, among 1,026 antimicrobial phenotypic tests, there were only eight discrepancies, and a high correlation between genotype and phenotype was observed (1018/1026, 99.2%) (Table 3).

DISCUSSION

In this study, comprehensive resistance genotypes of 114 *Campylobacter* isolates along with their correlations with resistance phenotypes were determined. To accomplish this, resistance mechanisms for each of nine antimicrobials tested were defined. Eighteen resistance genes along with a number of resistance-associated mutations were identified. There was a high correlation between phenotypic resistance to a given drug tested and the presence of one or more resistance genes expected to confer resistance to that drug. For four antimicrobial agents, tetracycline, ciprofloxacin, nalidixic acid, and erythromycin, there was a 100% correlation of the presence of putative resistance genes or mutations and observed resistance phenotypes. A few discrepancies were observed for gentamicin, azithromycin, clindamycin, and telithromycin. All isolates were susceptible to florfenicol, and no genes associated with florfenicol resistance were found. The results suggest that WGS has the potential to accurately predict resistance phenotypes for which genetic mechanisms have been defined.

The aminoglycoside class of antimicrobials is of particular interest because a total of 14 aminoglycoside resistance genes were found in our study. We identified seven GEN^r genes, but only *aph(2″)-If* and *aph(2″)-Ig* were shared in isolates recovered from humans, poultry, and poultry meats. Our recent study showed that the genetically diverse APH(2″) family was a major contributor to GEN^r in *Campylobacter*, with amino acid identity between the subfamilies ranging from 26% to 52% (23). Using this new knowledge, we were able to successfully correlate resistance genotypes and phenotypes for the vast majority of isolates. Among the six isolates with discrepancies between resistance/susceptible phenotypes and genotypes, only one was due to a discrepancy with gentamicin. This discrepancy may be due to the presence of resistance mechanism(s) that have not yet been described or to sequence gaps from the draft genome that prevented detection of one of the known gentamicin resistance genes. This indicates a significant limitation of the WGS approach and necessitates further analysis of the mechanism of gentamicin resistance in this isolate.

The other five isolates displayed discrepancies between resistance phenotypes and genotypes for AZI, CLI, and TEL. AZI, CLI, and TEL belong to the macrolide (M), lincosamide (L), and ketolide (K) antimicrobial classes, respectively. These classes of antimicrobials are structurally diverse but share overlapping binding

TABLE 3 Correlations of resistance phenotype and genotype in *Campylobacter*

Drug class	Drug(s) tested by AST	No. of isolates with R or S phenotype ($n = 114$)[a]	Presence of resistance gene(s) or mutation(s) corresponding to resistance phenotype (no. of isolates)	Correlation between genotype and phenotype (%)[b]
Tetracycline	TET	R, 108	*tet*(O) ($n = 108$)	100 (108/108)
		S, 6	None	100 (6/6)
Quinolones	CIP, NAL	R, 53	GyrA T86I ($n = 53$)	100 (53/53)
		S, 61	None	100 (61/61)
Aminoglycosides	GEN	R, 78	*aph(2″)-If* ($n = 18$), *aph(2″)-Ig* ($n = 37$) *aph(2″)-Ih* ($n = 13$), *aph(2″)-Ib* ($n = 1$), *aph(2″)-Ic* ($n = 4$), *aac(6′)-Ie-aph(2″)-Ia* ($n = 2$), *aac(6′)-Ie-aph(2″)-If* ($n = 1$), *aac(6′)-Ie-aph(2″)-Ia-aph(2″)-If* ($n = 1$)	98.7 (77/78)[c]
		S, 36	None	100 (36/36)
Macrolides	AZI	R, 53	23S rRNA A2075G($n = 51$), 23S rRNA A2074T ($n = 1$)	98.1 (52/53)[d]
		S, 61	None	100 (61/61)
	ERY	R, 52	23S rRNA A2075G ($n = 51$), 23S rRNA A2074T ($n = 1$)	100 (52/52)
		S, 62	None	100 (62/62)
Lincosamides	CLI	R, 52	23S rRNA A2075G ($n = 50$), 23S rRNA A2074T ($n = 1$)	98.1 (51/52)[d]
		S, 62	23S rRNA A2075G ($n = 1$)	98.4 (61/62)[e]
Ketolides	TEL	R, 49	23S rRNA A2075G ($n = 47$), 23S rRNA A2074T ($n = 1$)	98 (48/49)[d]
		S, 65	23S rRNA A2075G ($n = 3$)	95.4 (62/65)[f]
Phenicols	FFN	S, 114	None	100 (114/114)

[a] R, resistant; S, susceptible.
[b] The values in parentheses represent the number of isolates with a resistance element(s)/number of isolates with the phenotype.
[c] One isolate (N13165) had a GEN^r phenotype, but no known GEN^r gene was detected.
[d] One isolate (N9077) had an AZI^r CLI^r TEL^r phenotype, but it did not have a mutation in the 23S rRNA at A2075G.
[e] One isolate (26070) was CLI^s, but it had a mutation in 23S rRNA at A2075G.
[f] Three isolates (N46596F, N50039F, and 41973) were TEL^s, but they each had a mutation in 23S rRNA at A2075G.

sites in the peptidyl transferase region of 23S rRNA. Some resistance determinants alter part of the common binding site, simultaneously reducing susceptibility to more than one of the MLK antimicrobials as well as to streptogramins and oxazolidinones (29, 30). There are several resistance mechanisms to MLKs, including methylation of 23S rRNA by an acquired group of rRNA methylase genes (*erm*), point mutations in 23S rRNA genes, and mutations in ribosomal proteins L4 and L22 and efflux pumps (30, 31). The major contribution to MLK resistance was due to point mutations in the 23S rRNA at position 2074 or 2075 in our current study, and no *erm* genes were detected. Our findings indicated that although the A2075G point mutation in the 23S rRNA is a major mechanism of resistance to MLKs, not all isolates with this mutation showed resistance to lincosamides and ketolides. Additional genetic factors influencing susceptibility to these antibiotics may be responsible for this discrepancy; thus, further studies of lincosamide and ketolide resistance mechanisms in *Campylobacter* are warranted.

All 53 CIP^r/NAL^r isolates had a T86I mutation in the GyrA, and this mutation was not seen in any of the 61 susceptible isolates. Our previous study showed that the single mutation at T86I in GyrA was associated with decreased susceptibility (4-fold to 128-fold) to all eight fluoroquinolones tested (unpublished data). Other reports also indicated that mutation at T86I in GyrA was one of the most common mechanisms for fluoroquinolone resistance in *Campylobacter* (26, 27, 32).

In addition to aminoglycoside resistance genes and the *tet*(O) gene, one isolate (41975) carried the *catA* gene and two isolates (41904 and 41994) had *lnu*(C) genes, which are associated with resistance to chloramphenicol and lincomycin, respectively. *lnu*(C) was first detected in *Streptococcus agalactiae*, which was resistant to lincomycin but susceptible to clindamycin and erythromycin (22). We believe this is the first report of *lnu*(C) in *Campylobacter*. Chloramphenicol and lincomycin were not included in our tested panel, so the correlation of resistance phenotypes and genotypes for these two genes could not be evaluated. More than half of the isolates ($n = 66$) carried $bla_{\text{OXA-61}}$, a finding that has been reported by other investigators (33). *Campylobacter* has intrinsic resistance to penicillins and most cephalosporins; therefore, beta-lactams are not recommended to treat *Campylobacter* infections (34–36).

We recognized several limitations of this study despite the high degree of correlation between resistance phenotypes and genotypes. For instance, genotypic prediction of resistance relies on highly curated databases of known resistance determinants and cannot identify mechanisms that have yet to be defined. Therefore, phenotypic susceptibility testing is still necessary to fully account for resistance and to detect new mechanisms of resistance. Furthermore, the use of whole-genome shotgun sequencing results in fragmentary genomes with sequences assembled into contigs. This makes it possible for some genes to go undetected and often makes it difficult to determine whether resistance genes are located on plasmids or the chromosome. Also gene expression levels and/or synergistic effects among different resistance mechanisms can impact the MIC levels, which would not be determined by WGS. More work is also necessary to understand how the presence of one or more resistance genes for a given drug correlates with the likelihood of successful treatment of patient infections with that drug. Despite these drawbacks, our study clearly showed that WGS can accurately predict resistance phenotypes for which genetic mechanisms have been defined. Furthermore, with bacterial genome sequencing costs now approximately $50 per genome (37), reagent costs associated with WGS have become

similar to those for AST. In addition, while traditional AST panels can test only a limited number of drugs, WGS data can reveal all resistance genetic elements, including resistance to drugs not tested by AST. We believe that knowing which antimicrobial resistance genes are present in bacterial populations is critical to developing new strategies to combat antimicrobial resistance, including improving antimicrobial resistance and foodborne disease surveillance programs.

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