Genomic Analysis Reveals the Genetic Determinants Associated With Antibiotic Resistance in the Zoonotic Pathogen Campylobacter spp. Distributed Globally

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The genus Campylobacter groups 32 Gram-negative bacteria species, several being zoonotic pathogens and a major cause of human gastroenteritis worldwide. Antibiotic resistant Campylobacter is considered by the World Health Organization as a high priority pathogen for research and development of new antibiotics. Genetic elements related to antibiotic resistance in the classical C. coli and C. jejuni species, which infect humans and livestock, have been analyzed in numerous studies, mainly focused on local geographical areas. However, the presence of these resistance determinants in other Campylobacter species, as well as in C. jejuni and C. coli strains distributed globally, remains poorly studied. In this work, we analyzed the occurrence and distribution of antibiotic resistance factors in 237 Campylobacter closed genomes available in NCBI, obtained from isolates collected worldwide, in different dates, from distinct hosts and comprising 22 Campylobacter species. Our data revealed 18 distinct genetic determinants, genes or point mutations in housekeeping genes, associated with resistance to antibiotics from aminoglycosides, β-lactams, fluoroquinolones, lincosamides, macrolides, phenicols or tetracyclines classes, which are differentially distributed among the Campylobacter species tested, on chromosomes or plasmids. Three resistance determinants, the blaOXA−493 and blaOXA−576 genes, putatively related to β-lactams resistance, as well as the lnu(AN2) gene, putatively related to lincosamides resistance, had not been reported in Campylobacter; thus, they represent novel determinants for antibiotic resistance in Campylobacter spp., which expands the insight on the Campylobacter resistome. Interestingly, we found that some of the genetic determinants associated with antibiotic resistance are Campylobacter species-specific; e.g., the blaOXA−493 gene and the T86V mutation in gyrA were found only in the C. lari group, whereas genes associated with aminoglycosides resistance were found only in C. jejuni and C. coli. Additional analyses revealed how are distributed the
INTRODUCTION

The discovery and consequent therapeutic use of antibiotics was a remarkable advance in human medicine, which prevented the mortal outcomes of bacterial infections, saving millions of lives during the last century. However, bacteria have evolved through diverse mechanisms, intrinsic and acquired, to withstand the harmful activity of antibiotics. The antibiotic resistance (AR) is mainly generated by the presence of specific resistance genes or point mutations in some housekeeping genes; likewise, the AR can be transferred between bacteria through different mechanisms of DNA exchange, which has greatly increased the occurrence and spread of antibiotic-resistant bacteria worldwide. The development of AR has progressively compromised the effective use of antibiotics, restricting the therapeutic options available to treat the illness produced by antibiotic-resistant pathogenic bacteria. Nowadays, pathogenic bacteria that show resistance to a great diversity of antibiotics represent a serious threat to health worldwide. It has been estimated that infections produced by these bacteria could cause 10 million annual deaths by 2050 (O’Neill, 2014). Faced with this risk to human health, the World Health Organization (WHO) issued a priority global list of antibiotic-resistant pathogenic bacteria for which there is an urgent need to direct research for discovery and development of new antibiotics (WHO, 2017). Importantly, in this WHO report, and also in a published analysis from the United States Centers for Disease Control and Prevention (CDC), antibiotic-resistant Campylobacter spp. were cataloged as a serious health hazard in the world (CDC, 2013; WHO, 2017).

The genus Campylobacter groups biologically diverse species. These are Gram-negative, chemoorganotrophic, non-sporeforming epsilonproteobacteria. Depending on the species, these can be slender, spirally curved-, curved- or straight-rod; with a single polar flagellum, bipolar or multiple flagella, or no flagellum; and microaerobic or anaerobic bacteria (Vandamme et al., 2015). At the time of writing this paper, the genus Campylobacter comprised 32 species and 13 subspecies with validly published names1. Twenty Campylobacter species have been isolated from symptomatic or healthy humans: C. coli, C. concisus, C. curtus, C. fetus, C. gracilis, C. helveticus, C. hominis, C. hyointestinalis, C. insulaeignrae, C. jejuni, C. lari, C. mucosalis, C. peloridis, C. rectus, C. showae, C. sputorum, C. upsaliensis, C. ureolyticus and C. volucris (Man, 2011; Kweon et al., 2015). Some of these Campylobacter species have also been isolated from the gastrointestinal tract of animals, mainly farm animals (poultry, pigs, cattle, and sheep), where Campylobacter spp. reside usually as commensal microorganisms (Silva et al., 2011).

In humans, Campylobacter spp. can cause campylobacteriosis, which is considered the leading food-borne zoonosis and the most common cause of gastroenteritis in the world (EFSA and ECDC, 2018). C. jejuni and C. coli are the Campylobacter species more frequently involved in human gastroenteritis, hence, these two species have been by far the most studied (Man, 2011; Kaakoush et al., 2015). However, other species such as C. concisus, C. lari, C. upsaliensis, and C. ureolyticus, have also begun to be recognized as causative agents of human and animal campylobacteriosis; therefore, they are known as emerging Campylobacter species (Man, 2011).

In 2010, the global burden of Campylobacter infections was 95,613,970 clinical cases; 21,374 deaths and 2,141,926 DALYs (Disability Adjusted Life Years) (Havelaar et al., 2015). Campylobacteriosis may cause mild to severe clinical signs, or even be asymptomatic. The common symptoms of Campylobacter enteric infections include diarrhea (often bloody), fever, abdominal cramps, headache, nausea and/or vomiting. In vulnerable populations, such as very young children, elderly or immunologically compromised patients, this illness can be mortal2. Furthermore, other gastrointestinal manifestations or severe life threatening extragastrointestinal complications may appear (Man, 2011; Kaakoush et al., 2015). Because most Campylobacter enteric infections are self-limiting, antibiotic administration is usually not required. Antibiotic therapy is recommended in patients with severe clinical symptoms, relapses, or a prolonged course of infection (Tang et al., 2017). In these cases, fluoroquinolones such as ciprofloxacin, and macrolides such as erythromycin, are the drugs of choice (Ge et al., 2013).

A rapid and constant increase in the frequency of antibiotic-resistant Campylobacter strains isolated from humans and animals has been recognized worldwide (Luangtongkum et al., 2009; Cody et al., 2010; Tang et al., 2017; Signorini et al., 2018). It has been reported a wide-ranging prevalence of Campylobacter strains resistant to the following antibiotic families: aminoglycosides, β-lactams, cephalosporins, fluoroquinolones, fosfomycins, lincosamides, macrolides, phenicols, quinolones, sulfonamides, and tetracyclines (Ishihara et al., 2004; Karikari et al., 2017; Lee et al., 2017; Premaratne et al., 2017; Agunos et al., 2018; Bailey et al., 2018; Ewers et al., 2018; Iglesias-Torrenes et al., 2018; Khan et al., 2018;)

Keywords: Campylobacter, zoonotic bacteria, antibiotic resistance, resistance genes, genomic analysis, resistome

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1www.bacterio.net/campylobacter.html

2https://www.who.int/news-room/fact-sheets/detail/campylobacter
Signorini et al., 2018; Wei and Kang, 2018; Zhang et al., 2018; Nowaczek et al., 2019; Schiaffino et al., 2019). Moreover, a prevalence of up to 94% of multidrug resistant (MDR; resistant to three or more antibiotic families) Campylobacter isolates, in different parts of the world, has been reported (Zhang et al., 2018). A lot of relevant information about the genetic determinants mediating AR in C. coli and C. jejuni has been reported (Taylor and Courvalin, 1988; Payot et al., 2006; Alfredson and Korolik, 2007; Luangtongkum et al., 2009; Smith and Fratamico, 2010; Ivone, 2013; Tang et al., 2017; Shen et al., 2018); however, the genetic determinants for AR in the rest of the Campylobacter spp., including the emerging species, are greatly unknown.

By its importance for public health and food safety, it is necessary to know the resistome of the genus Campylobacter; the resistome is defined as the collection of AR determinants in a specific bacteria or ecological niche (D’Costa et al., 2006; Wright, 2007; Hu et al., 2017). A very high correlation between the genotype and phenotype for AR has been observed in Campylobacter (Zhao et al., 2016; de Vries et al., 2018; Whitehouse et al., 2018). Therefore, the identification of the Campylobacter genotypes associated with AR could help to choose the best antibiotic treatment against infections by Campylobacter species.

The aim of this study was to gain insight into the genetic determinants that constitute the Campylobacter resistome.

**MATERIALS AND METHODS**

**Bacterial Genomes**

A total of 237 closed genomes (chromosome and plasmid) of Campylobacter spp. were retrieved from the open-access RefSeq: NCBI Reference Sequence Database1 in March 2019. NCBI accession numbers of the 237 genomes, the information about the host, collection date and geographic location of the strains from which the DNA was extracted, sequenced and annotated, as well as the number of plasmids annotated as an assembly unit, are registered in Supplementary File S1.

**In silico Identification of Genes Associated With Antibiotic Resistance**

Datasets from publicly available resistance gene databases Comprehensive Antibiotic Resistance Database (CARD)2 (Jia et al., 2017) and the National Database of Antibiotic Resistant Organisms (NDARO3), were downloaded (March 2019) and used to identify the presence of genes associated with AR in the 237 Campylobacter genomes, by following two different approaches. In the first one, the BPGA software (Chaudhari et al., 2016) was applied to clustering the resistance genes from CARD and NDARO with those of the Campylobacter genomes, using the USEARCH clustering tool with default parameters (a cutoff set of 50% amino acid identity and 20 random permutations). In the second approach, a BLASTp search was performed4 with all predicted ORFs from the Campylobacter genomes, against the products of resistance genes from CARD and NDARO, using an E value cutoff of 10E-5, a selected threshold of 50% amino acid identity and minimum coverage of 60% of the query sequence length. Both approaches were compared to ensure the identification of the respective gene associated with AR, on the base of the best hit.

**RESULTS**

**Identification of Genetic Determinants Associated With AR in Campylobacter**

Genetic elements associated with AR, i.e., specific genes and point mutations in housekeeping genes, were sought by in silico analysis in a total of 237 closed publicly available Campylobacter genomes, as described in the “Materials and Methods” section. The 237 genomes assessed spanned 22 species of Campylobacter: C. avium (1), C. coli (22), C. concisus (3), C. cuniculorum (1), C. curvis (1), C. fetus (11), C. gracilis (1), C. helveticus (1), C. hepticus (1), C. hominis (1), C. hyointestinalis (2), C. iguanorum (3), C. insulaenigrae (3), C. jejuni (163), C. laniae (1), C. lari (8), C. peloridis (1), C. pinnipediorum (5), C. sputorum (4), C. subantarcticus (2), C. ureolyticus (1) and C. volucris (1); the number of genomes tested for each species is indicated between parenthesis. Most genomes analyzed were from C. jejuni (68.8%) and C. coli (9.3%); genomes from the 20 remaining Campylobacter species denoted 21.9% of the total. Important to note, in our analysis, we considered a genome as that including the sequence of both the chromosome and plasmids, when present, from the respective strain.

As depicted in Figure 1, 15 acquired genes associated with resistance to 5 distinct antibiotic classes were identified. Those more frequently found were blaOXA–61 and blaOXA–184, showing a prevalence of 32.5% (77/237 genomes) and 27.8% (66/237 genomes), respectively, both coding class D oxacillinase (OXA)-type β-lactamas (Figure 1). Acquired resistance to some β-lactams antibiotics has been associated with β-lactamases production in many organisms, including Campylobacter

1https://www.ncbi.nlm.nih.gov/refseq/
2https://card.mcmaster.ca/
3https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/
4https://blast.ncbi.nlm.nih.gov/Blast.cgi
5https://cge.cbs.dtu.dk/services/ResFinder/
6https://www.megasoftware.net
Resistance Genotypes of Campylobacter spp.

FIGURE 1 | Frequency of genetic determinants associated with antibiotic resistance on Campylobacter genomes. Genetic determinants for antibiotic resistance (resistance genes and mutations in housekeeping genes targets of antibiotics) present in 237 closed publicly available genomes belonging to 22 Campylobacter species, were identified by in silico analysis as described in “Materials and Methods” section. The colors indicate the antibiotic class to which the respective genetic determinant putatively confers resistance. *T86I, T86K, T86V, P104T or T86A/D90Y substitutions; **A2074G or A2074G/A2075G point mutations; ***K43R, K88R or K43R/K88R substitutions.

(Taylor and Courvalin, 1988; Alfredson and Korolik, 2005; Griggs et al., 2009). Notably, our analysis revealed 2 additional putative OXA-type β-lactamase genes, *bla*<sub>OXA−493</sub> (prevalence of 5.49%; 13/237 genomes) and *bla*<sub>OXA−576</sub> (prevalence of 0.42%; 1/237 genomes) (Figure 1), which are annotated in the respective genomes, but had not been previously reported. All these genes for β-lactamases are located on chromosome (Table 1), which is consistent with the fact that plasmids coding β-lactamases have not been described in Campylobacter.

In addition to genes for β-lactamases, we found that 16.5% of analyzed genomes (39/237 genomes) harbor the *tet*(O) gene (Figure 1), which codes for the ribosomal protection protein involved in resistance to tetracyclines (Sougakoff et al., 1987). Consistent with previous reports indicating the presence of *tet*(O) either on plasmid or chromosome (Pratt and Korolik, 2005; Dasti et al., 2007), we found that 30 and 9 of the analyzed genomes carry this resistance gene on plasmid and chromosome, respectively (Table 1). It should be noted that Campylobacter plasmids are usually classified according to the genes they carry, those harboring the *tet*(O) gene have been called pTet plasmids (Marasini et al., 2018). From 87 plasmids annotated as an assembly unit separated from chromosome in the total of Campylobacter genomes tested, 30 were pTet and only one plasmid containing an AR gene other than *tet*(O) was found; the 31 plasmids harboring AR genes show sizes ranging from 29,115 to 180,543 bp (Figure 2). These pTet plasmids carry the *tet*(O) gene alone or together with other AR genes; interestingly, all of them, including that no pTet, are present only on genomes from *C. coli* and *C. jejuni* (Figure 2).

On another hand, genes conferring resistance to phenicols or lincosamides were detected in a very small proportion of the analyzed genomes. Only 2 genomes harbor the *cat* gene (prevalence of 0.84%), which codes for a chloramphenicol acetyltransferase that is associated with resistance to
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found. The different AR genotypes and their prevalence on genomes from *C. coli*, *C. jejuni*, and other *Campylobacter* species, are shown in Supplementary Figures S1–S3. *bla*<sub>OXA</sub>−<sub>−61</sub> and *tet*<sub>(O)</sub> were the most common AR genotypes present on genomes from *C. coli*, with a prevalence of 23.8% (5/21 genomes) and 14.3% (3/21 genomes), respectively; whereas *bla*<sub>OXA</sub>−<sub>184</sub> (prevalence of 41.1%; 58/141 genomes) and *bla*<sub>OXA</sub>−<sub>61</sub> (prevalence of 28.4%; 40/141 genomes) were the predominant AR genotypes on genomes from *C. jejuni*; and *bla*<sub>OXA</sub>−<sub>493</sub>/gyrA T86V and *bla*<sub>OXA</sub>−<sub>493</sub> were the AR genotypes more common on genomes from other *Campylobacter* species, with a prevalence of 31.8% (7/22 genomes) and 27.3% (6/22 genomes), respectively (Figure 4). Location (chromosome or plasmid) of the AR determinants for some of the AR genotypes is shown in Figure 3B. All AR genotypes present in the *Campylobacter* plasmids are displayed in Figure 2.

Genetic Determinants Associated With AR That Are *Campylobacter* Species-Specific

Interestingly, our analysis revealed that some genetic determinants for AR are present only in particular *Campylobacter* species. For instance, despite the great representativeness of genomes from *C. jejuni* (163 genomes), and with less abundance from *C. coli* (22 genomes), the *bla*<sub>OXA</sub>−<sub>493</sub> gene was only found on genomes from 4 of the 6 species that integrate the *C. lari* group.

| Campylobacter spp. | ID_Plasmid       | Antibiotic resistance gene(s)            | Size (bp) |
|------------------|------------------|------------------------------------------|-----------|
| *C. coli*        | NZ_CP017879.1    | *tet*(O)                                  | 46,186    |
| *C. coli*        | NZ_CP017866.1    | *tet*(O)                                  | 46,193    |
| *C. coli*        | NZ_CP011017.1    | *tet*(O)                                  | 29,115    |
| *C. jejuni*      | NZ_CP014745.1    | *tet*(O)                                  | 116,883   |
| *C. jejuni*      | NZ_CP017857.1    | *tet*(O)                                  | 119,543   |
| *C. jejuni*      | NZ_CP022078.1    | *tet*(O)                                  | 46,448    |
| *C. jejuni*      | NZ_CP022471.1    | *tet*(O)                                  | 46,746    |
| *C. jejuni*      | NZ_CP017861.1    | *tet*(O)                                  | 45,025    |
| *C. coli*        | NZ_CP017877.1    | *tet*(O)/aph(3′)-Illa                    | 55,234    |
| *C. coli*        | NZ_CP013035.1    | *tet*(O)/aph(3′)-Illa                    | 44,064    |
| *C. jejuni*      | NZ_CP007752.1    | *tet*(O)/aph(3′)-Illa                    | 46,761    |
| *C. jejuni*      | NZ_CP020775.1    | *tet*(O)/aph(3′)-Illa                    | 45,904    |
| *C. jejuni*      | NZ_CP017030.1    | *tet*(O)/aph(3′)-Illa                    | 50,689    |
| *C. jejuni*      | NC_022354.1      | *tet*(O)/aph(3′)-Illa                    | 49,002    |
| *C. coli*        | NZ_CP017872.1    | *tet*(O)/aph(2′)-Ig/aph(3′)-Illa/sat-4   | 55,122    |
| *C. coli*        | NZ_CP023546.1    | *tet*(O)/aph(2′)-Ig/aph(3′)-Illa/sat-4   | 55,122    |
| *C. coli*        | NC_022355.1      | *tet*(O)/aph(2′)-Ig/aph(3′)-Illa/sat-4   | 55,127    |
| *C. jejuni*      | NZ_CP023544.1    | *tet*(O)/aph(2′)-Ig/aph(3′)-Illa/sat-4   | 55,132    |
| *C. coli*        | NZ_CP017026.1    | *tet*(O)/aph(3′)-Iila                    | 180,543   |
| *C. coli*        | NZ_CP007180.1    | *tet*(O)/nu(AN2)                          | 48,422    |
| *C. coli*        | NZ_CP007182.1    | *tet*(O)/aph(3′)-Illa                    | 47,962    |
| *C. jejuni*      | NZ_CP028186.1    | *tet*(O)/aph(2′)-Igain(9)/ant(6)-Iap(3′)-Illa/sat-4 | 66,602 |
| *C. coli*        | NZ_CP018901.1    | *aph(3′)-Iila                             | 125,964   |

**FIGURE 2** | Antibiotic resistance patterns from *Campylobacter* plasmids. *Campylobacter* species harboring the respective plasmid, NCBI accession number for each plasmid, genes for antibiotic resistance, and size (bp) for each plasmid, are indicated. Distinct antibiotic resistance patterns are displayed in different color.
FIGURE 3 | Resistance or multidrug resistance genotypes harbored on genomes of *Campylobacter* spp. (A) Multidrug resistance genotype containing the *inhA* (AN2) gene on a 48,422 bp plasmid from the *C. coli* RM5611 isolate. (B) Resistance or multidrug resistance genotypes containing different combinations of genetic determinants associated with antibiotic resistance on chromosome and/or plasmid. The name of the *Campylobacter* species from which the respective genome was obtained, as well as the NCBI accession number and size (bp) of genomes are shown. The colors indicate the antibiotic class to which the respective genetic determinant putatively confers resistance. Brown, β-lactams; pink, tetracyclines; blue, phenicols; green, lincosamides; and red, aminoglycosides. The genes shown with white color are not related to antibiotic resistance. The asterisks indicate truncated genes. The number (n) of genomes harboring each antibiotic resistance genotype is specified on the right. Arrows denote the direction of transcription for each gene.

(Miller et al., 2014; Costa and Iraola, 2019): *C. insulaenigrae* (3/3 genomes), *C. lari* (7/8 genomes), *C. subantarcticus* (2/2 genomes) and *C. volucris* (1/1 genomes) (Supplementary File S1); the number of genomes carrying the *bla*<sub>OXA</sub>−493 gene with respect to the total of genomes tested for each species, is indicated between parenthesis. Hence, *bla*<sub>OXA</sub>−493 was the predominant gene for β-lactamases that was identified in the *C. lari* group (prevalence of 92.9%, 13/14 genomes); only one genome from this group presents the *bla*<sub>OXA</sub>−184 gene and none carries the *bla*<sub>OXA</sub>−61 or *bla*<sub>OXA</sub>−576 genes. In contrast, the *bla*<sub>OXA</sub>−61 gene was confined to *C. jejuni* (63/163 genomes) and *C. coli* (14/22 genomes) species, as well as the *bla*<sub>OXA</sub>−184 gene (66 genomes) was mostly present in *C. jejuni* (65/163 genomes); only one genome of *C. helveticus* also carries the *bla*<sub>OXA</sub>−184 gene (1/1 genome) (Supplementary File S1). Furthermore, aminoglycoside resistance genes were only detected in *C. coli* (12/22 genomes) and *C. jejuni* (11/163 genomes) (Supplementary File S1).

On another hand, the T86V substitution in GyrA (with a prevalence of 24.2% with respect to the total of 87 GyrA substitutions, 8/33 genomes) was detected only in species of the *C. lari* group: *C. insulaenigrae* (3/3 genomes), *C. lari* (1/1 genomes), *C. peloridis* (1/1 genomes), *C. subantarcticus* (2/2 genomes) and *C. volucris* (1/1 genomes) (Supplementary File S1). In contrast, the T86I mutation in GyrA (with a prevalence of 66.7% with respect to the total of 33 GyrA substitutions, 22/33 genomes) was identified mostly in genomes of *C. jejuni* (18/22), but not in those from the *C. lari* group (Supplementary File S1).

Prevalence of AR Genotypes in *Campylobacter* and Its Association With Collection Date, Host and Geographic Location

Genetic factors associated with AR (genes or point mutations) were identified in 77.6% of the *Campylobacter* genomes analyzed (184/237 genomes; 1 to 8 factors per genome). No genetic determinants associated with AR were found on genomes from
C. concisus, C. curvus, C. gracilis, C. hepaticus, C. hominis, and C. pinnipediorum. Genomes carrying factors that putatively confer resistance to 1 or 2 classes of antibiotics were considered to represent a resistance genotype, those carrying factors related with resistance to 3 or more antibiotic classes were considered to be a multidrug resistance (MDR) genotype, and those lacking factors for AR were taken as susceptible genotypes. Most studies on AR in Campylobacter have been performed on the C. coli and C. jejuni species, whereas this phenomenon remains poorly explored for the other Campylobacter species. Thus, to gain more insight about the AR in C. coli and C. jejuni, as well as in the Campylobacter species less studied, we decided to compare the prevalence of resistance or MDR genotypes between genomes from C. coli (22 genomes), C. jejuni (163 genomes) and the other Campylobacter species analyzed (52 genomes). For the genomes from C. coli, the resistance genotype is the most represented (prevalence of 63.6%; 14/22 genomes), followed by the MDR genotype (prevalence of 31.8%; 7/22 genomes) and finally the susceptible genotype (prevalence of 4.6%; 1/22 genomes) (Figure 5). For genomes from C. jejuni, the resistance genotype is also the predominant (prevalence of 78.5%; 128/163 genomes), then the susceptible genotype (prevalence of 13.5%; 22/163 genomes) and at last the MDR genotype (prevalence of 8%; 13/163 genomes) (Figure 5). In contrast, for the genomes from the other Campylobacter species, the susceptible genotype is predominant (prevalence of 57.7%; 30/52 genomes), followed by the resistance genotype (prevalence of 40.4%; 21/52 genomes) and only one MDR genotype was found (prevalence of 1.9%; 1/52 genomes) (Figure 5).

Then, we analyzed the prevalence of the Campylobacter AR genotypes according to the host, collection date and geographical location from where the respective bacteria were isolated (Supplementary File S1). The C. coli isolates from farm animals carry resistance or MDR genotypes, with prevalence of 61.1% (11/18 genomes) and 33.3% (6/18 genomes), respectively, whereas the only 2 C. coli isolates from humans present the resistance genotype (Figure 6A). For C. jejuni, the MDR genotype has a higher prevalence in isolates from farm animals (25.8%; 8/31 genomes) compared with those from humans (3.7%; 4/109 genomes); the opposite was observed for the resistance genotype (Figure 6B). For the other Campylobacter species, the resistance genotype is predominant in bacteria isolated from marine animals (58.3%; 7/12 genomes), whereas the only MDR genotype found in this group is present in the C. laniænae NCTC 13004 strain isolated from a human (6.7%; 1/15 genomes) (Figure 6C). Notably, our analyses show a considerable increase in the presence of MDR genotypes from C. coli (Figure 7A) and C. jejuni (Figure 7B), as well as in the presence of resistance genotypes from the other Campylobacter species group (Figure 7C), in bacteria collected in the last decade (2010–2018), compared with previous decades. Additionally, our results indicate a very high prevalence of AR genotypes in C. coli and C. jejuni isolates from both Europe and North America (88–100%); being the MDR genotype from both C. coli and C. jejuni more predominant in North America (Figures 8A,B). Likewise, our analyses indicate a higher prevalence of AR genotypes from the other Campylobacter species group in Europe (56.3%) with respect to North America (35.7%) (Figure 8C).

**DISCUSSION**

Previous studies have analyzed the prevalence of genetic determinants for AR in genomes from C. coli and C. jejuni...
strains isolated in specific geographic regions (Weis et al., 2016; Zhao et al., 2016; Cantero et al., 2018; de Vries et al., 2018; Fabre et al., 2018; Whitehouse et al., 2018). In the present study, we identified genetic determinants associated with AR in 237 genomes from 22 different Campylobacter species with global distribution.

We found 15 distinct AR genes and point mutations related to AR in 3 housekeeping genes in the Campylobacter genomes tested (Figure 1). Notably, 3 of the AR genes had not been previously reported in Campylobacter: Insu(AN2), putatively associated with lincosamides resistance, and 2 putative β-lactams resistance genes, blaOXA−493 and blaOXA−576. Two β-lactamases coding genes, blaOXA−61 and blaOXA−184, were the most abundant AR genes in the analyzed genomes from C. coli and C. jejuni, which is consistent with the results from previous studies (Griggs et al., 2009; Weis et al., 2016; Zhao et al., 2016; de Vries et al., 2018; Fabre et al., 2018; Whitehouse et al., 2018). In contrast, in the analyzed genomes from species other than C. coli and C. jejuni, the blaOXA−493 β-lactamase coding gene was the predominant (Supplementary File S1). On another hand, even though our study indicates a low occurrence of determinants associated with aminoglycoside resistance in the Campylobacter genomes tested, these determinants displayed a great diversity, since 9 different aminoglycoside resistance genes, as well as point mutations in rpsL, were found (Figure 1). These findings are in agreement with those reported previously (Qin et al., 2012; Zhao et al., 2015, 2016; Cantero et al., 2018; Fabre et al., 2018; Hormeno et al., 2018; Whitehouse et al., 2018).

A high global incidence of Campylobacter isolates resistant to ciprofloxacin (fluoroquinolones) and erythromycin (macrolides) has been reported before (Liangtongkum et al., 2009; Signorini et al., 2018; Sproston et al., 2018). However, our analysis showed a low occurrence of mutations in the gyrA and 23S rRNA genes (Figure 1), which are associated with resistance to fluoroquinolones and macrolides, respectively. Nevertheless, multidrug efflux pumps can also mediate resistance to antibiotics of different classes, such as aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, macrolides and tetracyclines, among others (Blair et al., 2014). The efflux pumps are ancient elements that play important physiological roles in bacteria; but notably, they can also export antibiotics out of the cell, thus reducing their intracellular concentration and, as consequence, confer AR (Blair et al., 2014; Blanco et al., 2016). CmeABC is the only multidrug efflux pump characterized in Campylobacter; it was shown to be involved in resistance to structurally different antibiotics including β-lactams, fluoroquinolones, macrolides, and tetracyclines (Lin et al., 2002; Liangtongkum et al., 2009; Guo et al., 2010; Shen et al., 2018). In addition to specific genes and point mutations for AR, we also detected genes that code for multiple efflux pumps in Campylobacter (Supplementary File S2). Genes for the CmeABC system, MacB family, macrolide ABC transporter and TolC family efflux pumps, were found in the bulk of analyzed genomes. Besides, genes for other efflux pumps were found only in some genomes, such as those for the AcrB/AcrD/AcrF family, hydrophobe/amphiphile efflux-1 family and multidrug efflux SMR transport efflux pumps. Important to note, given that the efflux pumps can confer resistance to multiple antibiotics, to facilitate interpretation, they were not considered in our analysis as determinants for AR.

A major finding of our work was the Campylobacter species-specific distribution of some genetic determinants for AR. Campylobacter species cluster in 5 discrete phylogenic clades (Costa and Iraola, 2019). One is the C. lari group, which is composed of six species: C. insulaenigræ, C. lari, C. ornithocola, C. peloridis, C. submarcticus, and C. vulnificus (Miller et al., 2014; Costa and Iraola, 2019). These species are highly related at the genome level and have been isolated from similar hosts and environments (Miller et al., 2014). We found the blaOXA−493 gene and the T86V substitution in GyrA, exclusively in species from the C. lari group (Supplementary File S1). The T86V mutation in GyrA had also been previously reported in the C. lari species (Piddock et al., 2003; Weis et al., 2016). In contrast, aminoglycoside resistance genes were only detected in the classical C. coli and C. jejuni species (Supplementary File S1). Previous studies also have indicated the presence of aminoglycoside resistance genes in
FIGURE 6 | Frequency of susceptible, resistance or multidrug resistance (MDR) genotypes on Campylobacter genomes from isolates collected from different hosts. Genotypes from (A) C. coli, (B) C. jejuni and (C) other 20 Campylobacter species: C. avium, C. concisus, C. curvus, C. fetus, C. gracilis, C. helveticus, C. hepaticus, C. hominis, C. hyointestinalis, C. iguaniorum, C. insulaenigrae, C. lari, C. lari, C. peloridis, C. pinnipediorum, C. sputorum, C. subantarcticus, C. ureolyticus, and C. volucris. Farm animals include chicken, bovine, pig, sheep, turkey, and rabbit; marine mammals comprise seal, sea lion, penguin, albatross, gull, and shellfish; other animals encompass iguana, lizard, snake, cat, and alpaca. Only those hosts with more available information are displayed. The number (n) of genomes corresponding to each host is shown above the columns.

FIGURE 7 | Frequency of susceptible, resistance or multidrug resistance (MDR) genotypes on Campylobacter genomes from isolates collected in different decades. Genotypes from (A) C. coli, (B) C. jejuni and (C) other 9 Campylobacter species: C. avium, C. concisus, C. curvus, C. fetus, C. hepaticus, C. iguaniorum, C. lari, C. pinnipediorum, and C. sputorum. Only those decades with more available information are displayed. The number (n) of genomes corresponding to each decade is shown above the columns.
C. jejuni and C. coli (Qin et al., 2012; Zhao et al., 2015, 2016; Cantero et al., 2018; Fabre et al., 2018; Whitehouse et al., 2018); however, the presence of these genes in species other than C. jejuni and C. coli had not been analyzed. Thus, our data show that the genetic variability between the different clades of Campylobacter also involves the determinants for AR. Due to the limited number of genomes from non-C. coli/C. jejuni species (52 genomes), we cannot discard the possibility that aminoglycoside resistance genes are present in these species, but in very low prevalence.

Consistent with previous reports (NARMS, 2012; de Vries et al., 2018; Whitehouse et al., 2018), we found that the C. coli genomes harbor the highest number of AR determinants, compared with the other Campylobacter species analyzed (Supplementary File S1). Furthermore, in agreement with previous studies (Thakur et al., 2010; Wieczorek et al., 2015), our results show a higher prevalence of MDR genotypes in C. coli (31.8%; 7/22 genomes) with respect to C. jejuni (8%; 13/163 genomes). Notably, 86.4% (19/22 genomes) of the C. coli genomes possess 1–4 plasmids; in contrast, only 14.1% (23/163 genomes) of the C. jejuni genomes carry 1 or 2 plasmids, and 25% (13/52 genomes) of the genomes from the remaining 20 Campylobacter species tested contain 1–5 plasmids (Supplementary File S1). Interestingly, most genes associated with AR in C. coli are located on plasmids, whereas in the other Campylobacter species are placed on chromosome (Supplementary File S1), which could explain, at least in part, the higher number of AR determinants and MDR genotypes in C. coli compared with the other Campylobacter species. Remarkably, in non-C. coli/C. jejuni species, AR genes located on plasmids were not identified (Figure 2 and Supplementary File S1), Campylobacter spp. have mechanisms for conjugation and natural transformation, and transferrable AR has been documented in this genus (Taylor and Courvalin, 1988). Moreover, it has been proposed that Campylobacter gained some AR genes from Gram-positive bacteria (Taylor and Courvalin, 1988; Zilhao et al., 1988; Alfredson and Korolik, 2007). Thus, the AR genes located on plasmids, or on other mobile elements, imply a threat for the appearance of new Campylobacter strains resistant to antibiotics.

We found a reduced number of genetic determinants for AR in Campylobacter, in comparison with the huge number of genetic determinants for AR present in other bacteria such as Enterococcus spp. (Torres et al., 2018), Escherichia coli (Poirel et al., 2018) or Salmonella enterica (McDermott et al., 2018), which share hosts and niches with Campylobacter spp. The reason why Campylobacter spp. maintain in general a low number of genetic determinants for AR remains to be determined.

Our results, together with previous studies, reveal a high prevalence of Campylobacter AR genotypes worldwide, not only from the classical C. jejuni and C. coli species, but also, although still with less extent, from emerging Campylobacter species; these AR genotypes can be present in bacteria residing in different hosts such as humans and different animals (Figures 5, 6, 8 and Supplementary File S1). Even more worringly, our data support
that the prevalence of these bacteria carrying AR genotypes has been increasing with time (Figure 7).

Although the phenotype conferred by all the resistance genetic determinants identified in this study needs to be tested, a very high correlation between the presence of genetic determinants for AR and the respective phenotype has been reported in Campylobacter spp., which reaches up to 100% of correspondence for some specific antibiotics (Nirdnoy et al., 2005; Chen et al., 2013; Zhao et al., 2015, 2016; Fabre et al., 2018; Whitehouse et al., 2018). Hence, it has been suggested that analysis of genomic data, for the identification of genetic determinants associated with AR, has the potential to reliably predict resistance phenotypes (Zhao et al., 2016; Whitehouse et al., 2018; Feldgarden et al., 2019).

Thus, our study, together with other reports, provide genetic determinants that can be used to predict AR in Campylobacter spp., which could greatly help to select the best antibiotic therapy against infections caused by these bacteria. Additionally, our study further expands the knowledge on the genetic elements that shape the resistome of the genus Campylobacter and on the scattering of these AR genetic determinants between the Campylobacter species.

DATA AVAILABILITY STATEMENT
All datasets generated for this study are included in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS
DP-M and VB contributed to the conception and design of the study, as well as the discussion of the results. DR-M, IM-F, RS, and LL carried out the bioinformatics work. DP-M analyzed the bioinformatics results, created the figures, and wrote the manuscript. VB edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.513070/full#supplementary-material

FIGURE S1 | Frequency of resistance or multidrug resistance (MDR) genotypes on C. coli genomes. Thirteen antibiotic resistance genotypes were detected in 21 genomes from C. coli isolates. For each genotype, the antibiotic class to which they putatively confer resistance is indicated.

FIGURE S2 | Frequency of resistance or multidrug resistance (MDR) genotypes on C. jejuni genomes. Twenty antibiotic resistance genotypes were detected in 141 genomes from C. jejuni isolates. For each genotype, the antibiotic class to which they putatively confer resistance is indicated.

FIGURE S3 | Frequency of resistance or multidrug resistance (MDR) genotypes on genomes from other Campylobacter species. Eleven antibiotic resistance genotypes were detected in 22 genomes from other Campylobacter species isolates: C. avium, C. fetus, C. helveticus, C. hyointestinalis, C. ignaviornum, C. insulaeaeae, C. lanienae, C. lari, C. peloridis, C. sputorum, C. subantarcticus, C. ureolyticus, and C. volucris. For each genotype, the antibiotic class to which they putatively confer resistance is indicated.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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