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Antimicrobial resistance in *Campylobacter fetus*: emergence and genomic evolution

Linda van der Graaf-van Bloois[1,2], Birgitta Duim[1,2], Torey Looft[3], Kees T. Veldman[4], Aldert L. Zomer[1,2] and Jaap A. Wagenaar[1,2,4,*]

Abstract

Campylobacter fetus is a pathogen, which is primarily associated with fertility problems in sheep and cattle. In humans, it can cause severe infections that require antimicrobial treatment. However, knowledge on the development of antimicrobial resistance in *C. fetus* is limited. Moreover, the lack of epidemiological cut-off values (ECOFFs) and clinical breakpoints for *C. fetus* hinders consistent reporting about wild-type and non-wild-type susceptibility. The aim of this study was to determine the phenotypic susceptibility pattern of *C. fetus* and to determine the *C. fetus* resistome [the collection of all antimicrobial resistance genes (ARGs) and their precursors] to describe the genomic basis of antimicrobial resistance in *C. fetus* isolates over time. Whole-genome sequences of 295 *C. fetus* isolates, including isolates that were isolated in the period 1939 till the mid 1940s, before the usage of non-synthetic antimicrobials, were analysed for the presence of resistance markers, and phenotypic antimicrobial susceptibility was obtained for a selection of 47 isolates. *C. fetus* subspecies *fetus* (*Cff*) isolates showed multiple phenotypic antimicrobial resistances compared to *C. fetus* subspecies *venerealis* (*Cfv*) isolates that were only intrinsic resistant to nalidixic acid and trimethoprim. *Cff* isolates showed elevated minimal inhibitory concentrations for cefotaxime and cefquinome that were observed in isolates from 1943 onwards, and *Cff* isolates contained *gyrA* substitutions, which conferred resistance to ciprofloxacin. Resistances to aminoglycosides, tetracycline and phenicols were linked to acquired ARGs on mobile genetic elements. A plasmid-derived *tet*(O) gene in a bovine *Cff* isolate in 1999 was the first mobile genetic element observed, followed by detection of mobile elements containing *tet*(O)-*aph(3')-III* and *tet*(44)-*ant(6)-Ib* genes, and a plasmid from a single human isolate in 2003, carrying *aph(3')-III-ant(6)-Ib* and a chloramphenicol resistance gene (*cat*). The presence of ARGs in multiple mobile elements distributed among different *Cff* lineages highlights the risk for spread and further emergence of AMR in *C. fetus*. Surveillance for these resistances requires the establishment of ECOFFs for *C. fetus*.

DATA SUMMARY

The short-read data are deposed at the European Nucleotide Archive (ENA) under project PRJEB42468 and accession numbers are listed in Table S1, available with the online version of this article.

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Author affiliations: [1]Department Biomolecular Health Sciences, Division Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands; [2]WHO Collaborating Centre for Reference and Research on Campylobacter and Antimicrobial Resistance from a One Health Perspective / WOAH Reference Laboratory for Campylobacteriosis, Utrecht/Lelystad, Netherlands; [3]Food Safety and Enteric Pathogens Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, IA, USA; [4]Wageningen Bioveterinary Research, Lelystad, Netherlands.

***Correspondence:** Jaap A. Wagenaar, j.wagenaar@uu.nl

Keywords: antimicrobial resistance; *Campylobacter fetus*; ECOFF; plasmid; whole genome sequencing.

Abbreviations: AMR, antimicrobial resistance; ARG, antimicrobial resistance gene; AST, antimicrobial susceptibility testing; C. coli, Campylobacter coli; C. fetus, Campylobacter fetus; Cff, Campylobacter fetus subspecies fetus; Cfv, Campylobacter fetus subspecies venerealis; CIP, ciprofloxacin; CIP-R, ciprofloxacin resistance; C. jejuni, Campylobacter jejuni; ECOFF, epidemiological cut-off values; FQ, fluoroquinolone; gyrA, gyrase A; MIC, minimal inhibitory concentration; ML, maximum likelihood; MLST, multilocus sequence typing; ST, sequence type; T4SS, type-IV secretion system.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. One supplementary figure and one supplementary table are available with the online version of this article.

Impact Statement

The increase in antimicrobial resistance (AMR) in *Campylobacter* is a worldwide public health concern. AMR has been studied in *C. jejuni* and *C. coli* in particular, but very limited in *C. fetus*. Knowledge of AMR in *C. fetus* is important, as *C. fetus* causes bacteraemia with a fatality rate of 14% [65] and may also result in a prolonged treatment with antimicrobials. In our study, we investigated the phenotypic and genomic basis of AMR for a diverse set of 295 *C. fetus* isolates that were isolated from the pre-antimicrobial era (1939) until now.

Non-wild-type AMR for four antimicrobial classes were genotypically explained, but intrinsic resistance to nalidixic acid and trimethoprim, and reduced beta-lactam susceptibility observed from 1943 onwards were not genetically resolved. In early *C. fetus* isolates, only a few antimicrobial resistances were present, which significantly increased by the end of the 1990s. This was mainly due to the acquisition of mobile genetic elements carrying resistance genes conferring resistance to multiple drug classes. Our study demonstrates the need to establish epidemiological cut-off values and clinical breakpoints for *C. fetus*, which will improve monitoring of emerging AMR in *C. fetus*.

INTRODUCTION

Campylobacter fetus (*C. fetus*) is a pathogen for both animals and humans. Currently, *C. fetus* comprises three subspecies: *C. fetus* subspecies *fetus* (*Cff*), *C. fetus* subspecies *venerealis* (*Cfv*) and *C. fetus* subspecies *testudinum* (*Cft*). *Cff* and *Cfv* are primarily associated with mammals, whereas *Cft* is associated with reptiles [1]. *Cfv* is the causative agent of Bovine Genital Campylobacteriosis and is restricted to the genital tract of cattle [2]. In contrast, *Cff* is pathogenic for both animals and humans, and can cause fertility problems in cattle and sheep [2]. *Cff* infections in humans most likely orginate from cattle and sheep, and are acquired by humans after consumption of contaminated meat [3]. *Cff* infections are associated with intestinal illness and severe systemic infections, affecting mostly immunocompromised patients, the elderly and neonates [3]. Systemic *C. fetus* infections require prolonged antimicrobial treatment and infections often relapse. Systemic infections are often treated with carbapenems like imipenem and meropenem, as well as gentamicin and chloramphenicol [4]. *C. fetus* is intrinsically resistant to nalidixic acid [5, 6]. For other antimicrobials, there are no epidemiological cut-off values (ECOFFs) available for *C. fetus* to distinguish between the wild-type and non-wild-type populations, or to determine the clinical breakpoints of *C. fetus* isolates.

The molecular mechanisms of AMR in *C. fetus* have not been extensively studied. Resistance to tetracycline and streptomycin has been linked to *tet*(44) and *ant(6)-Ib* genes on a genomic island in *C. fetus* that also carried a type-IV secretion system (T4SS) [7, 8]. No association between source or genotypes of *C. fetus* isolates and presence of this genomic island has been identified and this island seems to spread in a strain-independent manner through different *C. fetus* lineages [9].

In contrast to the well-described and concerning increase in antimicrobial resistance (AMR) in *Campylobacter coli* (*C. coli*) and *Campylobacter jejuni* (*C. jejuni*) [10], there is little knowledge on the development and spread of AMR in *C. fetus*. The aim of this study was to determine the phenotypic susceptibility patterns, and to correlate them with AMR resistance genes and their precursors in the genomes (resistome) of *C. fetus* isolates, including isolates that were isolated before the usage of non-synthetic antimicrobials (1939 until mid-1940s).

METHODS

Bacterial isolates and whole-genome sequencing

A total of 295 *C. fetus* genomes were included in this study, of which 100 isolates were isolated from humans, 184 from animals (including bovine, ovine, horse and monkey isolates) and 11 isolates from unknown sources. In this study, 100 *C. fetus* isolates obtained between 1939–2021 from humans (n=30), animals (n=59) and unknown sources (n=11) were sequenced with Illumina MiSeq sequencing. Reads were trimmed with TrimGalore v0.4.4 with parameters minimum quality 20 and minimum length 100 [11], and assembled using SPAdes v3.11 with default parameters [12]. Contigs smaller than 200 basepairs and with a coverage lower than 10 were removed from the assemblies. A total of 195 genomes from public available databases were used, including 34 genomes from our previous studies [13, 14] and 161 genomes from ENA study PRJEB8721 [15], which were selected after genome quality check with Checkm v1.1.2 [16] for completeness (>95%) and contamination (<5%). Multi-locus sequence typing (MLST) sequence types (STs) were determined using the PubMLST scheme for *C. fetus* [17]. Surface-layer types were determined by performing *in silico* PCR with *sapA* and *sapB* specific primers [18].

Genome analysis and phylogeny

Genomes were annotated using Prokka v1.13 [19] and *gyrA* sequence alignments were extracted using the query_pan_genome option of Roary v3.12.0 with default parameters [20]. A core-genome alignment was generated using Parsnp v1.2 with default

parameters [21] and recombination regions were masked using Gubbins v2.3.4 with default parameters [22]. A maximum-likelihood (ML) tree was constructed from the masked whole-genome alignment using IQ-Tree v2.2.0 with default settings and 1000 bootstraps [23]. The tree was visualized using iTol v6.5.4 [24].

Analysis of antimicrobial resistance genes

ARGs and chromosomal mutations associated with antimicrobial resistance were determined in the *C. fetus* genomes using Abricate v0.8.2 [25] with the databases of Resfinder v2.1 (database date 1 September 2021) [26], CARD (database date 20 April 2018) [27], AMRFinderPlus (database date 20 April 2018) [28] and Pointfinder v3.0 [29].

To identify ARGs that are known to be present in *C. coli* and *C. jejuni* genomes, but were not annotated in the *C. fetus* genomes, a local BLASTp search with no cut-off parameters was performed. The following protein sequences of *C. coli* and *C. jejuni* were used as reference: for multi-drug resistance gene *cfr*(C) gene AQM75611.1 of *C. coli* plasmid KX686749 was used [30]; *C. jejuni* protein Y18300.1 was used for DNA topoisomerase *parC* [31]*; C. jejuni* protein WP_063844288.1 for trimethoprim-resistant repeat-containing dihydrofolate reductase *dfrA* [32]; and *C. jejuni* protein AF466820 for multi-drug efflux system genes *cmeABC* [33]. The resistance-nodulation-division (RND)-type efflux pump region was aligned using MAFFT v7.450 [34] and an approximate maximum-likelihood tree of this alignment was built with FastTree v2.1.11 with a generalized time-reversible (GTR+CAT) model [35].

Location of contigs (plasmid or chromosomal) were predicted using RFPlasmid v0.0.15 with default parameters [36] using the *Campylobacter* model, and contigs with votes between 0.4–0.6 were considered as 'unknown'. Analysis of the chromosomal insertion sites of mobile elements was performed with a local BLASTp search (using cut-off values of 98% identity and 98% coverage) with the following proteins as reference for the flanking core genes; for mobile element 1 CFV97608_1279 encoding a MCP-domain signal transduction proteinand CFV97608_1381 encoding sodium/proline symporter *putP*; and for mobile element 2 CFV97608_1018 encoding chaperone protein *htpG* and CFV97608_1023 encoding fumarate hydratase class II *fumC*. Sequences between the flanking core genes were extracted manually, and only sequences that contained both flanking core reference genes on the same contig were included. Sequences were annotated using Prokka v1.13 [19] and gene cluster comparisons were visualized using Clinker v0.0.37 [37].

Antimicrobial susceptibility testing

C. fetus genomes were arranged in groups with unique genomic resistance patterns when considering resistance genes, gyrase A (*gyrA*) substitutions, and MLST STs, which resulted in 25 different unique groups. Of each group, one isolate was randomly selected for phenotypic antimicrobial susceptibility testing. Additionally, a selection of 28 isolates without resistance markers from different subspecies, years and hosts were included for phenotypic antimicrobial susceptibility testing (Table 1). These 28 isolates without resistance markers were selected from different time periods as follows; period before 1946, three isolates per subspecies from different isolation years; period from 1950 to 1959, the only two available *Cff* isolates and six *Cfv* isolates from different years; period from 1960 to 1985, where possible we selected an isolate every 5 years from both subspecies resulting in two *Cff* and four *Cfv* isolates; and in the period from 1986 to 2021, we selected seven *Cfv* and two *Cff* isolates without antimicrobial resistance markers. The selected isolates are highlighted in blue in Table S1.

Antimicrobial susceptibility testing (AST) was performed on 53 selected *C. fetus* isolates by microbroth dilution according to CLSI document VET06, first edition, January 2017 for 23 different antimicrobials or combinations of antimicrobials (Table 1), but for *C. fetus*, no specific ECOFFs are available. Therefore, EUCAST *C. coli*/*C. jejuni* ECOFFs [38] were used for interpretation of the results and the tested *C. fetus* isolates were considered wild-type (susceptible) or non-wild-type (resistant) for the following antimicrobials with available EUCAST *C. coli*/*C. jejuni* ECOFFs: ampicillin, azithromycin, chloramphenicol, ciprofloxacin, doxycycline, erythromycin, florfenicol, gentamicin, nalidixic acid, streptomycin, tetracycline and trimethoprim/sulfamethoxazole. For the tested antimicrobials lacking EUCAST *C. coli*/*C. jejuni* ECOFFs (colistin, cefotaxime, cefquinome, ceftazidime, enrofloxacin, kanamycin, meropenem, neomycin, sulfamethoxazole, trimethoprim, tigecycline), isolates were considered susceptible (wild-type) or potentially reduced susceptible (non-wild-type) based on MIC distributions.

RESULTS

Antimicrobial susceptibility testing

A total of 53 isolates (*Cff n*=33, *Cfv n*=20) were selected for phenotypic antimicrobial susceptibility testing, however six *Cfv* isolates repeatedly failed to grow in the AST-broth and were excluded for AST (orange in Table 1), resulting in a selection of 47 isolates for AST (blue in Table 1).

In this study, susceptibility testing of *C. fetus* isolates was performed to monitor for the presence of antimicrobial resistance and not for clinical purposes. Overall, *Cff* isolates was more resistant to antimicrobials compared to *Cfv* isolates, as *Cfv* isolates were only intrinsically resistances to nalidixic acid and trimethoprim. The oldest *Cff* isolate, which was isolated in 1942, was resistant

Table 1. Selected *C. fetus* genomes with antimicrobial susceptibility testing results. AST results are presented as MIC in μg ml^{-1}, with susceptible (S) and resistant (R) (highlighted in yellow) classification based on EUCAST *C. coli/C. jejuni* ECOFFs. For the tested antimicrobials that do not have *C. coli/C. jejuni* ECOFFs, non-wild-type isolates with increased MIC-values are marked with an asterisk (*) and are highlighted in orange

Strainnumber in analysis	Original strain number	Host	Year	Country	Subspecies	MLST ST	54layer serotype	Resistance genes: tetracycline	aminoglycoside	chloramphenicol	fluoroquinolone (gyrA mutation)	23S rRNA	Location	Antimicrobial Susceptibility Testing Results: Aminoglycoside	Aminoglycoside	Aminoglycoside	Aminoglycoside	β-lactams	β-lactams	β-lactams	β-lactams	β-lactams	phenicol	phenicol	Fluoroquinolones	Fluoroquinolones	Fluoroquinolones	macrolide	macrolide	polymyxin	Tetracycline	Tetracycline	Tetracycline	folate pathway antagonist	folate pathway antagonist	folate pathway antagonist
														Gentamicin	Kanamycin	Neomycin	Streptomycin	Ampicillin	Meropenem	Cefotaxime	Ceftazidime	Cefquinome	Chloramfenicol	Florfenicol	Ciprofloxacin	Enrofloxacin	Nalidixic acid	Azithromycin	Erythromycin	Colistin	Doxycycline	Tetracycline	Tigecycline	Trimethoprim	Sulfamethoxazole	trimethoprim / sulfamethoxazole
18S02849-1	18S02849-1	Bovine	1939	USA	Cfv	4	A							0.5 (S)	4 (S)	2 (S)	2 (S)	<=1 (S)	<=0.03 (S)	<=0.25	2	0.25	<=8 (S)	0.12 (S)	<=0.12 (S)	0.06	32 (R)	<=2 (S)	<=1 (S)	<=1	<=0.06 (S)	0.12 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.25/4.75 (S)
18S03803-1	18S03803-1	unknown	1942	USA	Cfv	4	A							<=0.12 (S)	2 (S)	1 (S)	0.5 (S)	<=1 (S)	<=0.03 (S)	<=0.25	1	0.12	<=8 (S)	0.12 (S)	<=0.12 (S)	0.06	16 (S)	<=2 (S)	<=1 (S)	<=1	<=0.06 (S)	<=0.06 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.25/4.75 (S)
18S03802-1	18S03802-1	unknown	1942	USA	Cfi	3	B							<=0.5 (S)	1 (S)	0.12 (S)	4 (S)	4 (S)	<=0.03 (S)	2 (*)	8 (*)	>0.5 (*)	<=8 (S)	0.5 (S)	0.25 (S)	0.12	64 (R)	<=2 (S)	2 (S)	8 (*)	0.25 (S)	<=0.5 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.25/4.75 (S)
18S03806-1	18S03806-1	unknown	1943	USA	Cfi	3	B							0.25 (S)	2 (S)	2 (S)	1 (S)	2 (S)	0.06 (S)	4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	1 (S)	<=0.12 (S)	0.06	>64 (R)	<=2 (S)	<=1 (S)	<=1	0.25 (S)	1 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.12/2.38 (S)
18S02845-1	18S02845-1	unknown	1945	USA	Cfv	4	A							<=0.12 (S)	0.25 (S)	0.25 (S)	<=0.25 (S)	<=1 (S)	<=0.03 (S)	<=0.25	<=0.5	0.06	<=8 (S)	0.12 (S)	<=0.12 (S)	0.06	16 (S)	<=2 (S)	<=1 (S)	<=1	0.12 (S)	<=0.06 (S)	<=0.25 (S)	32 (*)	<=8 (S)	0.5/9.5 (S)
18S03808-1	18S03808-1	unknown	1945	USA	Cfi	3	B							<=0.12 (S)	*n.g.*	*n.g.*	0.5 (S)	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	<=0.12 (S)	*n.g.*	>64 (R)	*n.g.*	<=1 (S)	*n.g.*	*n.g.*	1 (S)	*n.g.*	*n.g.*	*n.g.*	*n.g.*
18S03233-1	18S03233-1	Bovine	1952	USA	Cfv	4	A							0.5 (S)	2 (S)	0.5 (S)	2 (S)	<=1 (S)	<=0.03 (S)	0.5	4	0.5	<=8 (S)	0.5 (S)	<=0.12 (S)	0.06	>64 (R)	<=2 (S)	<=1 (S)	<=1	0.25 (S)	0.25 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.06/1.19 (S)
19S00906-1	19S00906-1	Bovine	1955	USA	Cfv	4	A					T-C (2074)		<=0.12 (S)	1 (S)	0.5 (S)	<=0.25 (S)	<=1 (S)	<=0.03 (S)	<=0.25	<=0.5	0.06	<=8 (S)	0.12 (S)	<=0.12 (S)	0.06	16 (S)	<=2 (S)	2 (S)	2	<=0.06 (S)	<=0.06 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.25/4.75 (S)
18S02857-1	18S02857-1	Bovine	1956	USA	Cfi	3	B							<=0.12 (S)	2 (S)	1 (S)	1 (S)	4 (S)	0.12 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	<=0.12 (S)	0.12	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.25 (S)	0.5 (S)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
18S03241	18S03241	Bovine	1958	USA	Cfv	4	A							<=0.12 (S)	0.25 (S)	<=0.06 (S)	<=0.25 (S)	<=1 (S)	<=0.03 (S)	<=0.25	<=0.5	0.03	<=8 (S)	0.12 (S)	<=0.12 (S)	0.03	16 (S)	<=2 (S)	<=1 (S)	2	<=0.06 (S)	<=0.5 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.12/2.38 (S)
18S02859-1	18S02859-1	Bovine	1959	USA	Cfv	4	A							<=0.12 (S)	1 (S)	0.25 (S)	<=0.25 (S)	<=1 (S)	<=0.03 (S)	<=0.25	<=0.5	0.06	<=8 (S)	0.25 (S)	<=0.12 (S)	0.03	32 (R)	<=2 (S)	<=1 (S)	8	0.12 (S)	<=0.5 (S)	<=0.25 (S)	>32 (*)	16 (S)	0.5/9.5 (S)
18S03823-1	18S03823-1	Ovine	1959	USA	Cfi	11	A							<=0.5 (S)	2 (S)	0.25 (S)	1 (S)	4 (S)	0.25 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.25 (S)	0.12	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.5 (S)	1 (S)	<=0.25 (S)	>32 (*)	32 (S)	2/38 (S)
18S02863	18S02863	Ovine	1962	USA	Cfi	2	A					D91E		<=0.12 (S)	1 (S)	1 (S)	0.5 (S)	<=1 (S)	<=0.03 (S)	2 (*)	8 (*)	>0.5 (*)	<=8 (S)	1 (S)	0.5 (S)	0.5	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.25 (S)	1 (S)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
18S03831-1	18S03831-1	Bovine	1964	USA	Cfv	4	A							<=0.12 (S)	2 (S)	0.25 (S)	0.5 (S)	<=1 (S)	<=0.03 (S)	<=0.25	<=0.5	0.06	<=8 (S)	0.5 (S)	<=0.12 (S)	0.06	32 (R)	<=2 (S)	<=1 (S)	4	0.12 (S)	<=0.5 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.5/9.5 (S)
18S03841-1	18S03841-1	Bovine	1966	USA	Cfi	6	A							<=0.12 (S)	2 (S)	2 (S)	1 (S)	2 (S)	0.25 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	<=0.12 (S)	0.12	64	<=2 (S)	<=1 (S)	16 (*)	0.25 (S)	0.5 (S)	<=0.25 (S)	>32 (*)	32 (S)	1/19 (S)
18S03824-1	18S03824-1	Ovine	1969	USA	Cfi	2	A					D91E		0.25 (S)	2 (S)	1 (S)	1 (S)	2 (S)	0.12 (S)	4 (*)	8 (*)	>0.5 (*)	<=8 (S)	1 (S)	0.5 (S)	0.5	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.5 (S)	1 (S)	<=0.25 (S)	>32 (*)	16 (S)	0.5/9.5 (S)
78-1	78-1	Human	1978	USA	Cfi	3	B							0.25 (S)	4 (S)	2 (S)	1 (S)	4 (S)	0.25 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.25 (S)	0.12	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.25 (S)	0.5 (S)	<=0.25 (S)	>32 (*)	16 (S)	0.5/9.5 (S)
92203	92203	Bovine	1992	Argentina	Cfv	4	A							<=0.12 (S)	1 (S)	0.25 (S)	0.5 (S)	<=1 (S)	<=0.03 (S)	<=0.25	<=0.5	0.12	<=8 (S)	0.25 (S)	<=0.12 (S)	0.03	32 (R)	<=2 (S)	<=1 (S)	4	0.12 (S)	<=0.5 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.5/9.5 (S)
CCUG33900	CCUG33900	Bovine	1995	France	Cfv	4	A							<=0.12 (S)	1 (S)	<=0.06 (S)	<=0.25 (S)	<=1 (S)	<=0.03 (S)	<=0.25	<=0.5	0.06	<=8 (S)	0.5 (S)	<=0.12 (S)	0.015	32 (R)	8 (S)	<=1 (S)	<=1	<=0.06 (S)	<=0.5 (S)	<=0.25 (S)	>32 (*)	32 (S)	1/19 (S)
ERR1069024	BS 458/99	Bovine	1999	Germany	Cfi	3	B	*tet(O)*	*aph(3')-III*				Mobile element 2	<=0.12 (S)	1 (S)	2 (S)	0.5 (S)	2 (S)	0.06 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	1 (S)	0.5 (S)	0.5	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	8 (R)	16 (R)	<=0.25 (S)	>32 (*)	<=8 (S)	0.25/4.75 (S)
ERR1046006	2975	Human	2002	Taiwan	Cfi	11	A	*tet(O)*					Mobile element 3	<=0.12 (S)	2 (S)	2 (S)	0.5 (S)	8 (S)	0.25 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	4 (S)	1 (S)	0.5	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	8 (R)	>32 (R)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
02298	02298	Bovine	2002	Argentina	Cfv	4	A							<=0.12 (S)	0.25 (S)	<=0.06 (S)	<=0.25 (S)	<=1 (S)	0.06 (S)	<=0.25	<=0.5	0.25	<=8 (S)	2 (S)	<=0.12 (S)	0.03	32 (R)	<=2 (S)	<=1 (S)	2 (S)	0.12 (S)	<=0.5 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.25/4.75 (S)
ERR1046007	923	Human	2003	Taiwan	Cfi	11	A	*tet(O)*	*aph(3")-III, ant(6)-Ia*	*cat*			*Tet(O)* on mobile element 3 / *aph, ant, cat* on mobile element 4	<=0.12 (S)	>32 (*)	>32 (*)	16 (R)	16 (R)	1 (S)	>4 (*)	>8 (*)	>0.5 (*)	32 (R)	4 (S)	1 (S)	0.5	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	16 (R)	>32 (R)	<=0.25 (S)	>32 (*)	32 (S)	1/19 (S)
03293	03293	Bovine	2003	Argentina	Cfv	4	A							<=0.12 (S)	1 (S)	0.25 (S)	0.5 (S)	<=1 (S)	<=0.03 (S)	<=0.25	<=0.5	0.12	<=8 (S)	0.25 (S)	<=0.12 (S)	0.03	32 (R)	<=2 (S)	<=1 (S)	4 (S)	<=0.06 (S)	<=0.5 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.5/9.5 (S)
04554	04554	Bovine	2004	Argentina	Cfi	5	B					D91E, A114V		<=0.12 (S)	2 (S)	1 (S)	1 (S)	2 (S)	0.06 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.25 (S)	0.12	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.5 (S)	1 (S)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
ERR976358	2006/367h	Human	2006	France	Cfi	6	A	*tet(44)*	*ant(6)-Ib*				Mobile element 1	<=0.12 (S)	2 (S)	2 (S)	>16 (R)	2 (S)	0.12 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.5 (S)	0.25	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	16 (R)	32 (R)	<=0.25 (S)	>32 (*)	32 (S)	0.5/9.5 (S)
ERR1046010	1592	Human	2006	Taiwan	Cfi	20	A	*tet(O)*	*aph(3')-III*				AMR genes on short contigs, locations unknown	<=0.12 (S)	>32 (*)	8 (*)	0.5 (S)	4 (S)	0.12 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.5 (S)	0.25	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	4 (R)	16 (R)	<=0.25 (S)	>32 (*)	64 (S)	2/38 (S)
ERR976364	2008/568h	Human	2008	France	Cfi	6	A	*tet(44)*	*ant(6)-Ib*			D91Y	Mobile element 1	<=0.12 (S)	2 (S)	1 (S)	>16 (R)	4 (S)	0.12 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	16 (R)	>2 (*)	>64 (R)	<=2 (S)	<=1 (S)	<=1	32 (R)	>32 (R)	<=0.25 (S)	>32 (*)	16 (S)	0.5/9.5 (S)
ERR1069017	08CS0024	Bovine	2008	Germany	Cfi	6	A	*tet(44)*	*ant(6)-Ib*			T87I	Mobile element 1	<=0.12 (S)	2 (S)	1 (S)	>16 (R)	16 (R)	0.25 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	4 (S)	>16 (R)	>2 (*)	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	16 (R)	>32 (R)	<=0.25 (S)	>32 (*)	16 (S)	0.5/9.5 (S)
ERR1046013	0003304-2	Human	2009	Taiwan	Cfi	11	A	*tet(O)*	*aph(3')-III*				*Tet(O)* on mobile element 3 / *aph(3')* location unknown	<=0.12 (S)	>32 (*)	>32 (*)	1 (S)	16 (R)	0.5 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	4 (S)	1 (S)	0.5	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	8 (R)	>32 (R)	<=0.25 (S)	>32 (*)	32 (S)	1/19 (S)
ERR1069029	11CS0098	Ovine	2011	Germany	Cfi	3	B					D91Y		0.25 (S)	2 (S)	2 (S)	1 (S)	4 (S)	0.12 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	16 (R)	>2 (*)	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.5 (S)	1 (S)	<=0.25 (S)	>32 (*)	32 (S)	2/38 (S)
WBT01109	WBT01109	Bovine	2011	United Kingdom	Cfv	4	A							<=0.12 (S)	*n.g.*	*n.g.*	<=0.25 (S)	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	<=0.12 (S)	*n.g.*	16 (S)	*n.g.*	<=1 (S)	*n.g.*	*n.g.*	<=0.5 (S)	*n.g.*	*n.g.*	*n.g.*	*n.g.*
15S04243-1	15S04243-1	Human	2013	NL	Cfi	22	B					A14T		0.25 (S)	2 (S)	1 (S)	1 (S)	4 (S)	0.06 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.5 (S)	0.12	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.5 (S)	1 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.5/9.5 (S)
15S04042-1	15S04042-1	Human	2014	NL	Cfi	3	B	*tet(44)*	*ant(6)-Ib*				Mobile element 1	0.25 (S)	2 (S)	1 (S)	>16 (R)	4 (S)	0.12 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.25 (S)	0.25	>64 (R)	<=2 (S)	2 (S)	16 (*)	16 (R)	>32 (R)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
15S04041-1	15S04041-1	Human	2014	NL	Cfi	2	B					D91E		<=0.12 (S)	2 (S)	2 (S)	1 (S)	2 (S)	0.06 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.5 (S)	0.25	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.25 (S)	0.5 (S)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
15S04240-1	15S04240-1	Human	2015	NL	Cfi	2	A					D91E		0.25 (S)	2 (S)	1 (S)	1 (S)	2 (S)	0.06 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.5 (S)	0.5	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.5 (S)	0.5 (S)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
16S05798	16S05798	Human	2015	NL	Cfi	20	A					D91N		<=0.12 (S)	2 (S)	2 (S)	1 (S)	2 (S)	0.06 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	8 (R)	>2 (*)	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.25 (S)	0.5 (S)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
17S03759-1	17S03759-1	Human	2016	Germany	Cfi	3	B	*tet(44)*	*ant(6)-Ib*				Mobile element 1	<=0.12 (S)	2 (S)	2 (S)	>16 (R)	4 (S)	0.5 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.5 (S)	0.25	>64 (R)	<=2 (S)	2 (S)	16 (*)	8 (R)	>32 (R)	<=0.25 (S)	>32 (*)	32 (S)	1/19 (S)
2160129049	2160129049	Equine	2016	NL	Cfi	5	B					T87I		<=0.12 (S)	2 (S)	2 (S)	0.5 (S)	2 (S)	0.06 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	1 (S)	16 (R)	>2 (*)	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.25 (S)	2 (S)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
16S00675-10	16S00675-10	Equine	2016	NL	Cfi	6	A					D91Y		0.25 (S)	2 (S)	2 (S)	1 (S)	8 (S)	0.12 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	16 (R)	>2 (*)	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	1 (S)	0.5 (S)	<=0.25 (S)	>32 (*)	32 (S)	1/19 (S)
17S01351-1	17S01351-1	Ovine	2017	NL	Cfi	2	A	*tet(44)*	*ant(6)-Ib*			D91E	Mobile element 1	<=0.12 (S)	1 (S)	1 (S)	>16 (R)	2 (S)	0.06 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	1 (S)	0.5 (S)	0.5	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	8 (R)	>32 (R)	<=0.25 (S)	>32 (*)	16 (S)	0.5/9.5 (S)
17S01135-1	17S01135-1	Bovine	2017	Brazil	Cfv	4	A							<=0.12 (S)	1 (S)	0.12 (S)	0.5 (S)	<=1 (S)	0.12 (S)	<=0.25	<=0.5	0.12	<=8 (S)	0.25 (S)	<=0.12 (S)	0.03	32 (R)	<=2 (S)	<=1 (S)	4	0.12 (S)	<=0.5 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.5/9.5 (S)
17S01353-1	17S01353-1	Ovine	2017	NL	Cfi	3	B					T87I		0.25 (S)	2 (S)	2 (S)	1 (S)	4 (S)	0.5 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	16 (R)	>2 (*)	>64 (R)	<=2 (S)	<=1 (S)	16 (*)	0.25 (S)	1 (S)	<=0.25 (S)	>32 (*)	32 (S)	1/19 (S)
18S04140-1	18S04140-1	Human	2018	Belgium	Cfi	6	A					T87I		<=0.12 (S)	0.25 (S)	0.25 (S)	0.5 (S)	<=1 (S)	<=0.03 (S)	2 (*)	>8 (*)	>0.5 (*)	<=8 (S)	1 (S)	16 (R)	>2 (*)	>64 (R)	<=2 (S)	<=1 (S)	<=1	0.25 (S)	1 (S)	<=0.25 (S)	>32 (*)	<=8 (S)	0.5/9.5 (S)
19S00791-1	19S00791-1	Human	2019	India	Cfi	25	A					T87I		0.25 (S)	2 (S)	2 (S)	1 (S)	2 (S)	0.06 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	16 (R)	>2 (*)	>64 (R)	<=2 (S)	<=1 (S)	>16 (*)	0.25 (S)	0.5 (S)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
19S01514-1	19S01514-1	Human	2019	Belgium	Cfi	5	B					A14V		<=0.12 (S)	1 (S)	2 (S)	2 (S)	2 (S)	0.12 (S)	>4 (*)	>8 (*)	>0.5 (*)	<=8 (S)	2 (S)	0.25 (S)	0.25	>64 (R)	<=2 (S)	<=1 (S)	>16 (*)	0.5 (S)	1 (S)	<=0.25 (S)	>32 (*)	16 (S)	0.5/9.5 (S)
21S00955-1	21S00955-1	Bovine	2021	Netherlands	Cfi	6	A							<=0.12 (S)	0.5 (S)	0.12 (S)	0.5 (S)	<=1 (S)	<=0.03 (S)	0.5	1	>0.5 (*)	<=8 (S)	2 (S)	<=0.12 (S)	0.12	>64 (R)	<=2 (S)	<=1 (S)	4	0.5 (S)	1 (S)	<=0.25 (S)	>32 (*)	16 (S)	1/19 (S)
No growth in AST medium																																				
18S02853-1		Bovine	1954	USA	Cfv	4	A							*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*
18S02858		Bovine	1957	USA	Cfv	4	A							*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*
18S03801-1		Bovine	1972	USA	Cfv	4	A							*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*
18S03851-1		Bovine	1983	USA	Cfv	4	A							*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*
18S03852-1		Bovine	1983	USA	Cfv	4	A							*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*
17S01138-1		Bovine	2017	Brazil	Cfv	4	A							*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*	*n.g.*

to colistin, nalidixic acid and trimethoprim, and showed increased MICs to cefotaxime, cefquinome and ceftazidime. In the *Cff* isolates, resistances to tetracyclines and aminoglycosides were observed for the first time in isolates from 1999 and 2003, respectively.

Aminoglycosides

Aminoglycoside-mediated resistance genes were present in nine *Cff* genomes with *ant(6)–Ib* as the most abundant gene (*n*=6), followed by *aph(3′)-III* (*n*=3) which co-occurred with *ant(6)*–Ia in one isolate. The three isolates that contained the *aph(3′)-III* gene were resistant to both kanamycin (MIC >32 µg ml^{-1}), and neomycin (MIC ≥8 µg ml^{-1}), whereas the isolates containing *ant(6)* genes were resistant to streptomycin (MIC ≥16 µg ml^{-1}). All *C. fetus* isolates were susceptible for gentamicin (MIC ≤0.5 µg ml^{-1}), showing that the identified aminoglycoside-mediated resistance genes did not confer resistance to gentamicin.

Beta-lactams

Three *Cff* isolates were considered resistant to ampicillin with a MIC of 16 µg ml^{-1}. A gene conferring resistance to ampicillin in *C. coli* and *C. jejuni* is the chromosomal located $bla_{\text{OXA-61}}$ gene [39]. However, this gene was not present in any of the *C. fetus* genomes. For cefotaxime, ceftazidime and cefquinome, no ECOFFs for *C. coli/C. jejuni* were available. However, we noticed increased MICs for all *Cff* isolates, except the most recent *Cff* isolate 21S00955-1 isolated in 2021, which showed the same level MICs for third-generation cephalosporin's cefotaxime and ceftazidime as wild-type *Cfv* isolates.

Fluoroquinolones

Six *gyrA* substitutions were present in the *C. fetus* genomes; A14V, A14T, D91E, D91N, D91Y and T87I (Table 1), of which D91Y and T87I were previously identified to confer ciprofloxacin resistance in *C. fetus* and other bacterial *gyrA* genes including *C. jejuni* [40]. However, the positions of the *gyrA* substitutions were different than described by Bénéjat *et al.* [41], who identified the *gyrA* substitutions at amino acid positions 86 and 90 (substitutions T86I, D90E, D90G, D90N, D90Y) using PCR amplicons, which are most likely the same *gyrA* substitutions as found in the genome sequences analysed in this study.

The authors of a study with 123 *C. fetus* isolates from patients in France proposed to use an ECOFF with a MIC>=0.5 µg ml^{-1} for ciprofloxacin resistance in *C. fetus* isolates [41] equal to the EUCAST ECOFF for *C. coli/C. jejuni* (in accordance with EFSA guidelines implemented in European legislation [Commission Implementation Decision (EU) 2020/1729, Table 3] [42]. Nine *C. fetus* isolates were resistant to ciprofloxacin with a MIC ≥8 µg ml^{-1}, which contained the *gyrA* substitutions D91N, D91Y or T87I. The other detected *gyrA* substitutions A14V, A14T and D91E did not confer phenotypic ciprofloxacin resistance (MIC ≤0.5 µg ml^{-1}). Ciprofloxacin resistance correlated with reduced susceptibility for enrofloxacin since all ciprofloxacin-resistant isolates expressed an increased MIC for enrofloxacin and vice versa (Table 1).

C. fetus is known as intrinsically resistant to nalidixic acid [5], and all *C. fetus* isolates showed MICs of ≥16 µg ml^{-1} for nalidixic acid.

Trimethoprim/sulfamethoxazole

For trimethoprim and sulfamethoxazole, no separate ECOFFs were available, and the ECOFF for *Campylobacter* spp. for the combination trimethoprim/sulfamethoxazole is >16/304 µg ml^{-1}. *C. fetus* is intrinsically resistant to trimethoprim [5], and all *C. fetus* isolates showed MICs>32 µg ml^{-1}. For sulfamethoxazole, all *C. fetus* isolates showed MICs≤64 µg ml^{-1} and were presumably susceptible for sulfamethoxazole. This correlated with the expected susceptibility of all *C. fetus* isolates for the combination trimethoprim/sulfamethoxazole (MIC ≤1/19 µg ml^{-1}) since isolates can only be resistant for this combination if they are resistant to both individual antimicrobials.

Macrolides

Nucleotide transitions A2075G and A2074C/G in the 23S rRNA gene is the most common mechanism for high-level resistance to macrolides in *C. coli* [43]. Only *Cfv* isolate 19S00906-1, isolated in 1955 in the USA carried a single nucleotide mutation in the 23S rRNA gene, resulting in a T→C mutation at base 2074. However, none of the tested *C. fetus* isolates were resistant to the two tested macrolides (azithromycin and erythromycin) showing that this T2074C mutation in the 23S rRNA gene did confer phenotypic macrolide resistance in *C. fetus*.

Phenicols

Of the 295 *C. fetus* genomes, one single genome (ERR1046007) contained a *cat* gene encoding chloramphenicol resistance, which was located in a gene cluster with *ant(6)-Ib* and *aph(3′)-III* resistance genes. The isolate containing the *cat* gene cluster was isolated from a human infection in Taiwan with a MIC of 64 µg ml^{-1} for chloramphenicol, whereas all *cat*-negative isolates expressed MIC-values of ≤8 µg ml^{-1}, showing that this *cat* gene was conferring chloramphenicol resistance in *C. fetus* isolates.

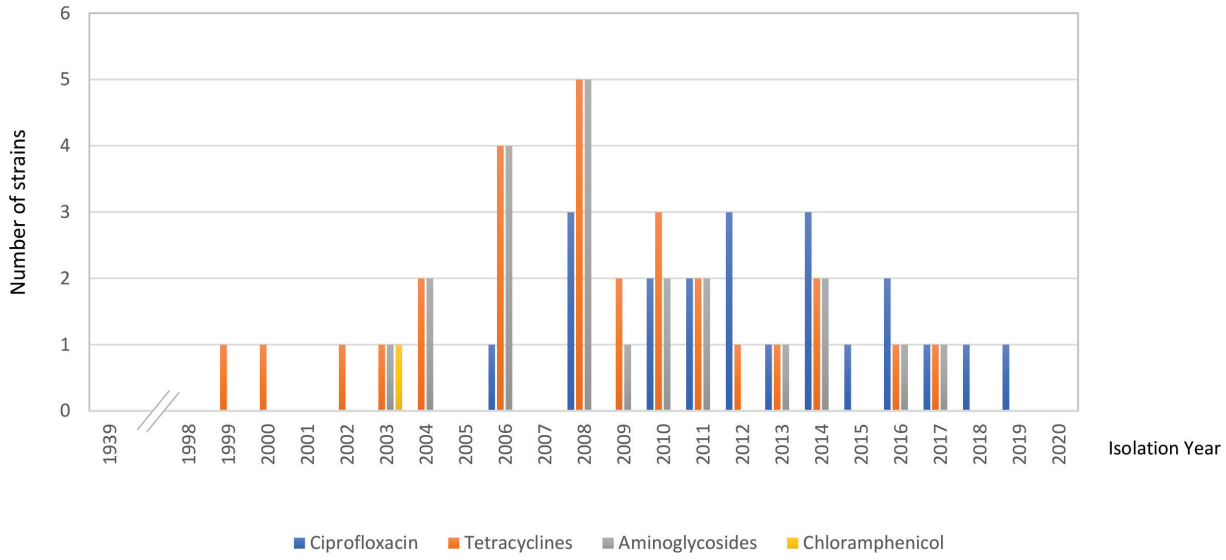

Fig. 1. Number of *C. fetus* isolates harbouring resistance genes and *gyrA* substitutions causing ciprofloxacin resistance, presented per isolation year. The *x*-axis is disrupted from 1939 to 1998 since isolates in that period did not contain any resistance markers.

Polymyxins

The majority of *Cff* isolates showed relatively high MIC-values for colistin (MIC ≥16 µg ml^{-1}), whereas all *Cfv* isolates showed lower MIC-values for colistin (MIC ≤8 µg ml^{-1}). The mechanism by which the MIC-values of *Cff* were elevated could not be resolved. Colistin resistance can be associated with lipopolysaccharide modification via different routes [44], and changes in the electrostatic networks of the outer membrane can protect bacteria against the action of polymyxins [45], but it is unknown which mechanism causes colistin resistance in *Campylobacter* spp.

Tetracyclines

Two different variants of tetracycline-resistant genes were present in the *C. fetus* genomes, the *tet*(O) and *tet*(44) genes. Susceptibility for tetracycline was tested on 11 isolates carrying *tet*(O) and *tet*(44) genes and all 11 isolates expressed MIC-values of ≥16 µg ml^{-1} for tetracycline. The other 36 isolates did not carry tetracycline resistance genes and were all susceptible to tetracycline. These results indicated that both *tet* gene variants can confer tetracycline resistance in *C. fetus*. In *C. coli/C. jejuni,* isolates with MIC-values of >2 µg ml^{-1} are considered tetracycline resistant. Applying this EUCAST ECOFF for interpretation of the *C. fetus* MIC-values can correctly distinguish the resistant population from the susceptible population based on the presence or absence of *tet* genes. The tetracycline resistance correlated with an increased MIC ≥4 µg ml^{-1} for doxycycline for all tetracycline resistant isolates. None of the isolates showed an increased MIC for tigecycline.

Presence of resistance markers in public *C. fetus* genomes

A total of 295 *C. fetus* genomes (*Cff n*=166; *Cfv n*=129) were analysed for the presence of ARGs and chromosomal point mutations, using three different databases with resistance genes; Resfinder [26], CARD [27], AMRFinderPlus [28] and Pointfinder [29]. Remarkably, resistance genes and *gyrA* substitutions were only detected in *Cff* genomes, and none in the 129 *Cfv* genomes. In our set, 16% of the *Cff* genomes contained resistance genes (27 out of 166) of which 85% were isolates from human cases (*n*=23) mostly obtained from Taiwan (15 out of 23) (Table S1). The human isolates carrying resistance genes were mainly from Taiwan and belonged to MLST ST11 and ST20 (Fig. 2 and Table S1), showing that these isolates belonged to at least two different *C. fetus* lineages.

Substitutions in the *gyrA* gene, known to be associated with resistance to fluoroquinolones (FQs), was first detected in *Cff* in 1962 where the substitution D91E was found in an ovine isolate from the USA, however without conferring phenotypic resistance to ciprofloxacin. In *Cff* isolates from later dates, D91E and other *gyrA* substitutions appeared, such as substitution T87I conferring ciprofloxacin and enrofloxacin resistance, which was detected in 2006 (Fig. 1). The first detected mobile resistance gene was the tetracycline gene *tet*(O) in a bovine isolate that was isolated in 1999 in Germany. After 1999, multiple genes conferring resistance to aminoglycoside (*aph(3')-III, ant(6)-Ia*), tetracycline (*tet*(O), *tet*(44)) and phenicol (*cat*) were increasingly detected in *C. fetus* genomes and were geographically dispersed (Fig. 1 and Table 1).

The resistance genes *tet*(O)-*aph(3′)* and *tet*(44)-*ant(6)-I* were distributed across the different lineages of the *C. fetus* genomes, showing that there has been more than one introduction of these genes (Fig. 2). Four *gyrA* substitutions (A14T, D91N, D91Y and T87I) were randomly distributed over the genomes, whereas *gyrA* substitutions A14V and D91E, without conferring ciprofloxacin resistance, showed to be associated with specific *C. fetus* lineages (Fig. 2).

The resistance gene *cfr*(C) was detected using a Resfinder search in five *Cff* genomes with an extremely low coverage of 7.46% protein identity. A local BLAST search using the *C. coli cfr*(C) gene sequence (AQM75611.1) [46] resulted in a BLAST-hit of 83 bp with a short (252 bp) hypothetical protein in the five *Cff* genomes, suggesting that this sequence is in *C. fetus* most likely not associated with a *cfr*(C) resistance gene.

The *parC* gene that confers quinolone resistance in other *Campylobacter* species has not been detected in *C. fetus* before. Using a local BLASTp search with the *C. jejuni parC* sequence (Y18300.1) [31] resulted in only insignificant hits (<40% coverage, <75% identity), confirming that no homologs of the *C. jejuni parC* gene are present in the analysed *C. fetus* genomes.

In all analysed *C. fetus* genomes, no functional genomic markers for beta-lactam and macrolide resistance were found. Cephalosporin resistance, as well as resistances to other antimicrobials in *Campylobacter* spp. can be associated with mutations in the *cmeABCR* region encoding a RND multi-drug efflux pump [47, 48]. All *C. fetus* genomes contained a *cmeABCR* region and we identified multiple point mutations in the *cmeABCR* genes, which were associated with different MLST STs of isolates (Fig. S1), but the specific point mutations could not be associated with the phenotypic resistance patterns of the isolates.

Mobile genetic elements containing ARGs

In-depth analysis of the contigs carrying resistance genes was performed to study if the ARGs were located on the chromosome or on a plasmid. Two chromosomal insertion sites of ARGs carrying mobile elements were identified. One mobile element (named mobile element 1) was located between CFV97608_1279 encoding a MCP-domain signal transduction protein and CFV97608_1381 encoding the sodium/proline symporter *putP* (Fig. 3). This insertion side was assembled in a single contig in 247 genomes and of these, 24 genomes contained an inserted mobile element in this location. The gene organization of the mobile elements at this insertion side was diverse and a respresentive of each element is shown in Fig. 3. Mobile element 1 carrying ARG cluster *tet*(44) and *ant(6)-Ib* was found in 11 genomes and was homologous to the previously described mobile island (accession number FN594949). In 13 genomes, the inserted mobile element 1 carried only T4SS encoding genes without AMR encoding genes, including the previously described pathogenicity island carrying a T4SS and *fic* genes that was present in the same insertion site in isolate 97/608 and partly in isolate 03/293 [49]. All isolates carrying mobile element 1 with the ARG cluster *tet*(44) and *ant(6)-Ib* were from Europe (the Netherlands, Germany and France) and originated from different sources (human, bovine, ovine), and comprised different MLST *C. fetus* lineages. This shows that mobile element 1 is not restricted to a single *C. fetus* lineage, and most likely has been acquired multiple times in the *C. fetus* population (Fig. 2). Of the 11 isolates that harboured gene cluster *tet*(44) and *ant(6)-Ib*, six isolates were included in the phenotypic antimicrobial susceptibility testing, and all six isolates were confirmed to be resistant to tetracycline and streptomycin.

A second mobile element (named mobile element 2) that was flanked by CFV97608_1018 encoding chaperone protein *htpG* and CFV97608_1023 encoding fumarate hydratase class II *fumC* contained another mobile element (named 'mobile element 2' in Fig. 3), harbouring the ARG cluster *tet*(O)-*aph(3′)-III* and T4SS encoding genes. Insertion site 2 could be studied on single contigs in 285 genomes. The full mobile element containing the ARG cluster and T4SS encoding genes was detected in two genomes, and mobile elements without ARGs were detected in three genomes (Fig. 3).

The *tet*(O) gene was present in three different *C. fetus* lineages belonging to MLST ST3, ST20 and ST11 (Fig. 2), and co-occured with the resistance gene *aph(3′)-III* in two ST11 and all ST20 genomes. In ST3 and ST11 isolates that only carried the *tet*(O) gene, the gene was located on a plasmid-derived mobile element (named 'mobile element 3'), which harboured T4SS genes and multiple hypothetical proteins (Fig. 3). Mobile element 3 (~32 kbp) carrying the *tet*(O) gene shared high similarity (99%) with a 17 kb region of the *C. coli* RM5611 plasmid pRM5611_48 kb (CP007180.1) and a 16 kb region of the *C. jejuni* S3 plasmid pTet (CP001961.1) [50]. Two MLST ST11 genomes (ERR1046016 and ERR1046007) contained the *tet*(O) gene as well as *aph(3′)-III* and *aph(3′)-III-ant*(6)-*Ib-cat* genes, respectively. However, the gene neighbourhood of the *aph(3′)-III* gene in ERR1046016 was not clear as the gene was located on a small 8 kb contig.

Another mobile element (named 'mobile element 4') was identified in genome ERR1046007, which consisted of a 16 kb contig containing *cat, ant*(6)-*Ib,* and *aph(3′)-III* genes (Fig. 3). This resistance gene cluster was uniquely present in this *C. fetus* isolate from Taiwan, and not in any of the other analysed 295 *C. fetus* genomes. A region of 8 kb of this contig including gene *aph(3′)-III* showed high similarity (99% identity and 83% query coverage) with *C. jejuni* plasmid pAR-0413–1 of *C. jejuni* strain AR-0413. This *C. jejuni* strain is a multi-drug-resistant strain, carrying multiple resistance genes (GenBank accession number CP044170.1).

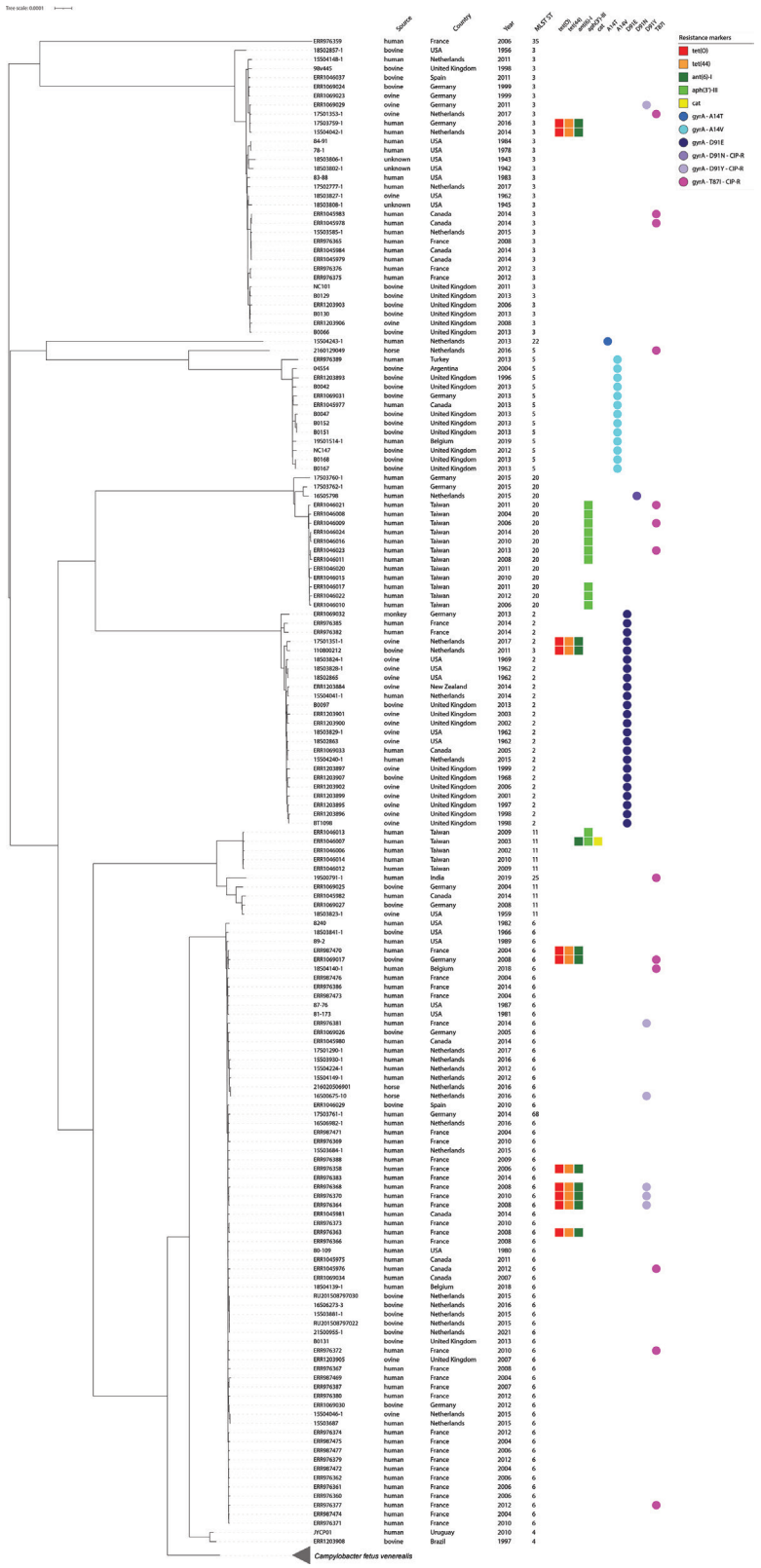

Fig. 2. ML phylogenetic genome tree with isolation source, country and genomic resistance markers. The cluster with *C. fetus* subspecies *venerealis* genomes is collapsed, the *Cfv* genomes were negative for all genomic markers. The tree scale at the top represents year ago. USA: United States of America.

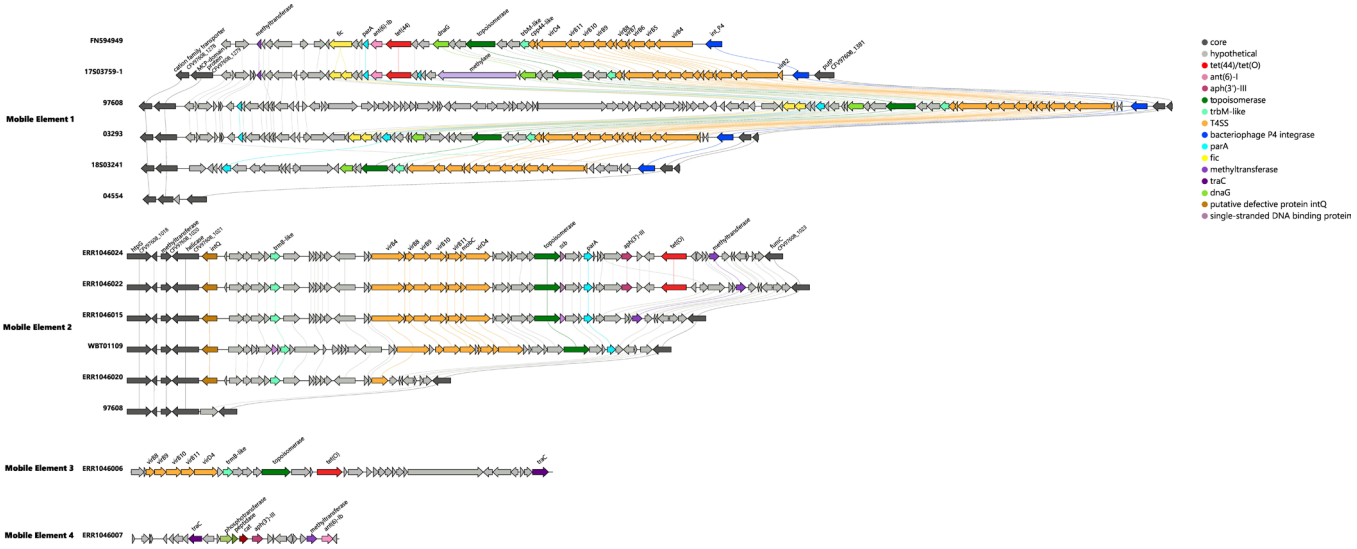

Fig. 3. Schematic representation of mobile elements in *C. fetus* genomes containing antimicrobial resistance genes. Shown are the flanking core genes (grey) of the insertion sites of mobile element 1 and 2, as well as the mobile elements without ARGs inserted at the same sides for mobile element 1 and 2.

DISCUSSION

In this study, whole-genome sequencing and antimicrobial susceptibility testing was applied to retrospectively investigate the emergence of antimicrobial resistance in *C. fetus*. We were fortunate to have access to a very extensive collection of *C. fetus* genomes from different subspecies and genomic lineages, with the inclusion of *C. fetus* isolates isolated before the usage of non-synthetic antimicrobials.

One limitation of the study is that the isolates are isolated with different methods, and from cases with different backgrounds. Most human *Cff* isolates were from clinical cases possibly treated with antimicrobials, which could have been selected for more resistant isolates. On the other hand, isolates from human blood samples are often isolated without use of selective media [3]. This is in contrast with animal-derived isolates that were isolated during screening procedures from healthy animals using selective media or isolates are isolated with the non-selective filter method described by the World Organisation for Animal Health (WOAH) [51]. The differences in diagnostic isolation methods and the background of samples make it difficult to perform a reliable comparison in this study on the prevalence of AMR markers between *C. fetus* isolates from human and animal origin, or their geographical location.

The lack of knowledge about AMR in *C. fetus* is reflected by the fact that for *C. fetus* no epidemiological cut-off values or clinical breakpoints have been determined for AMR in *C. fetus*. In this study, we used EUCAST ECOFFs available for *C. coli*/*C. jejuni* in accordance with EFSA guidelines [42] implemented in European legislation [Commission Implementation Decision (EU) 2020/1729, Table 3] to determine if a *C. fetus* isolate was resistant or susceptible. In cases where ECOFFs for *C. coli*/*C. jejuni* were lacking, *C. fetus* isolates were considered susceptible (wild-type) or potentially reduced susceptible (non-wild-type) based on MIC distributions. In a study with 100 *C. fetus* isolates that were isolated from humans [52], the use of EUCAST *C. coli*/*C. jejuni* ECOFFs for *C. fetus* were evaluated, and showed that the ECOFFs for ampicillin, amoxicillin-clavulanate, erythromycin and tetracycline can be applied for *C. fetus*. In our study, ampicillin, erythromycin and tetracycline were tested, however, all isolates were susceptible for erythromycin and no genomic markers were found for ampicillin, and therefore, the use of *C. coli*/*C. jejuni* ECOFFs could not be validated. For tetracycline, a perfect correlation between the presence of resistance genes *tet*(O)/*tet*(44) and tetracycline resistance was shown, confirming that the tetracycline EUCAST *C. coli*/*C. jejuni* ECOFF can be applied for *C. fetus* isolates. Sifré *et al.* [52] concluded that AST of ciprofloxacin for *C. fetus* should not be performed with a disc diffusion method, as all *C. fetus* diameters were distributed around the ECOFF of *C. coli*/*C. jejuni*. In our study we tested MIC distributions, and a considerable difference for ciprofloxacin resistance was shown, whereby susceptible wild-type isolates had a MIC ≤1 µg ml^{-1} and presumably resistant non-wild-type isolates had a MIC ≥8 mg l^{-1}, which could be linked to *gyrA* mutations D91N, D91Y and T87I.

Multiple studies on antimicrobial resistance of *C. fetus* describe the MIC of isolates [53, 54], but since no susceptibility criteria are available for *C. fetus*, different cut-off values or breakpoints determined for other *bacterial species have been used to determine if an isolate is resistant or susceptible,* e.g. CLSI guidelines for *Enterobacteriaceae* [55] and CLSI guidelines for bacteria that grow

aerobically [56, 57]. This emphasizes the need to determine ECOFFs and clinical breakpoints specific for *C. fetus* as a lack of standardized cut-offs may lead to treatment failure or overtreatment.

The genetic resistance markers in the *C. fetus* genomes could be associated with the phenotype of the isolates, e.g. the *gyrA* substitutions with ciprofloxacin resistance, tetracycline and aminoglycoside resistance encoding genes with their resistance phenotype, and the *cat* gene with chloramphenicol resistance. However, not all phenotypical resistances or increased MIC-values, could be genetically explained, comprising increased MICs for cefotaxime, ceftazidime, cefquinome for all but one *Cff* isolate, and resistance to ampicillin, colistin, nalidixic acid and trimethoprim. Besides the intrinsic resistances to nalidixic acid and trimethoprim, the genetically unexplainable *C. fetus* resistances comprised mainly beta-lactam antimicrobials. This suggested that *Cff* is most likely intrinsically resistant to third- and fourth-generation cephalosporins, just like *C. coli* and *C. jejuni*, but it remains suprising that *Cfv* seems to be susceptible for these antimicrobials. In addition, in *C. jejuni*, resistance to ampicillin and other beta-lactams is observed in strains that do not produce the beta-lactamases, suggesting other mechanisms can confer beta-lactam resistance [58]. Resistance to cephalosporins may in part be due to the size and charge restrictions that outer membrane porins impose on molecules trying to enter the cell [59], and beta-lactams could be substrates of multi-drug efflux pumps [33, 60]. These alternative resistance mechanisms are very complex and can hamper the detection and characterization of genetic determinants involved in these resistances in *Campylobacter*. Therefore, with the shift towards sequence-based methods for AMR surveillance, it is important to include phenotypic assays for at least a subset of the isolates.

The first *gyrA* substitution associated with ciprofloxacin resistance was observed in a human *Cff* isolate that was isolated in 2006 and since then occurred in other *Cff* genomes from sources as bovine, ovine and horses. The first-generation quinolones (oxolinic acid and flumequine) were licensed for use in food animals at the beginning of the 1980s in some European countries, and FQs were licensed for animal use during the late 1980s and early 1990s in the EU [60]. For human use, (fluoro) quinolones were released in the mid-1980s [61]. The rising trend of FQ resistance has been observed in ruminant-derived *C. coli* and *C. jejuni* strains from the USA in the late 2000s [61]. It has been suggested that the rising trend in FQ resistance is caused by the increased use of fluoroquinolones in the control of respiratory diseases in cattle production [62]. Furthermore, development of FQ resistance can increase the fitness of bacteria, resulting in persistence of FQ resistance in *C. jejuni* strains, even in the absence of antimicrobial selection pressure [63].

Antimicrobial resistance genes are usually acquired from other bacteria via mobile genetic elements, such as plasmids, transposons and integrons. *Cfv* genomes in general contained more mobile elements compared to *Cff* genomes [13, 14, 49], but remarkably, all 129 analysed *Cfv* genomes did not carry resistance genes and all identified resistance genes detected, were present in *Cff* genomes. Human *C. fetus* infections are considered to be foodborne, and the main reservoirs are cattle and sheep [3, 64]. The resistance genes found in the *C. fetus* genomes were presumably transmitted from other *Campylobacter s*pp., like *C. jejuni* and *C. coli* since the *Cff* mobile elements containing the aminoglycoside and tetracycline resistance genes shared parts of the gene content with *C. jejuni* and *C. coli* plasmids. But since we did not find plasmids in *C. fetus,* which share a high coverage and homology with entire plasmids of other *Campylobacter* spp., we presume that acquisition of plasmids in *C. fetus* genomes from other *Campylobacter* spp. does not occur frequently. However, the occurrence of mobile elements containing ARGs and chromosomal antimicrobial resistance markers in *C. fetus* genomes increased from 1999 onwards, showing that AMR in *C. fetus* is emerging.

Conclusion

In *C. fetus* an increase in antimicrobial resistance is described with clear differences between two subspecies. Genomic markers for AMR were only detected in *Cff* isolates and increased significantly from the end of the 1990s. In addition to the known intrinsic nalidixic acid resistance, intrinsic resistance to trimethoprim was found phenotypically, without identification of the genetic basis. Resistance for aminoglycosides, tetracycline and phenicols could be linked to the presence of resistance genes, and *gyrA* substitutions were defined, which were conferring resistance to fluoroquinolones. ARGs were widely distributed on mobile elements containing resistance genes, and were mainly present in human *Cff* genomes but distributed in distinct *Cff* lineages, which highlight the risk for spread and further emergence of antimicrobial resistance in *C. fetus*. Surveillance for these resistances requires the establishment of epidemiological cut-off values and clinical breakpoints, which will improve monitoring the emerging AMR in *C. fetus* supporting the development of treatment guidelines for *C. fetus* infections in humans.

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Author contributions
Designed the study: L.G., B.D., J.W. Performed the experiments: L.G., B.D., T.L., K.V., A.Z. Analysed the data: L.G., B.D., K.V., A.Z., J.W. Writing the paper: all authors.

Conflicts of interest
The authors declare that they have no competing interests.

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