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Genomic Profiling of Multidrug Efflux Pumps and Heavy Metal Proteins in Multidrug-resistant *Campylobacter fetus* Isolated from Sheath Wash Samples of Bulls in South Africa

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ABSTRACT

A substantial evolution of resistance mechanisms among zoonotic bacteria has resulted from anthropogenic factors related to the application of antibiotics in human and veterinary medicine, particularly in contemporary agriculture. This issue associated with the presence of heavy metal-laced protein in zoonotic bacteria should be taken seriously with regard to the health of animals and the general people. To address this issue, the present study employed whole genome sequencing to identify the antimicrobial resistance patterns of *Campylobacter fetus* subsp. *fetus* (*Cff*) and *Campylobacter fetus* subsp. *venerealis (Cfv)*, resistance and virulence genes, as well as heavy metal protein. Based on culture method biochemical testing and PCR amplification using particular primer pairs (MG3F-MG4R and VenSF-VenSR), bacteria were isolated and identified as *C. fetus* subsp. *fetus* and *C.fetus* subsp. *venerealis*. Subsequently, antimicrobial disc diffusion tests and whole genome sequencing were performed. Isolated bacteria were resistant to tetracycline at 65%, amoxicillin, and doxycycline at 60%. The resistance was also observed against neomycin at (55%), streptomycin (60%), and gentamycin (55%). Through comprehensive genome sequencing analysis and PCR, multiple efflux pumps linked to multidrug resistance were identified, including the broad-specificity multidrug efflux pump (YkkD), along with *CmeA*, *CmeB*, *CmeC*, and *gryA*.The genome sequence also revealed genes associated with the production of Cytotoxin (*Cdt A*, *B*, and *C*), adhesion and colonization (*VirB10* and *VirB9*), and invasion (*CiaB*). In addition, different genomic features in heavy metal resistance included Cobalt-zinc-cadmium resistance protein (*CzcD*), Tellurite resistance protein (*TehA*), and arsenic efflux pump protein. The findings of the current study revealed that the emergence of bacterial multidrug resistance is increasingly associated with the substantial and growing contribution of Multidrug resistance efflux pumps, as evident in *Cff* and *Cfv*. Therefore, it is crucial to tighten the control of *Cff* and *Cfv* in livestock production to prevent the transfer of genes resistant to humans through the food chain.

Keywords: *Campylobacter fetus*, Heavy metal protein, Multidrug resistance, Multidrug efflux pumps, Virulence factor, Whole genome sequencing

INTRODUCTION

Despite the fact that certain *Campylobacter* spp. coexist as commensals in the digestive systems of ruminants and birds, these zoonotic bacteria cause infections in both humans and animals (Sahin et al., 2017; Babazadeh and Ranjbar, 2022). There are currently 39 species and 16 subsp. of *Campylobacter* (Hlashwayo et al., 2020). The two most well-known and common members of this genus, *Campylobacter jejune* (*C. jejune*) and *Campylobacter coli* (*C. coli*), are mostly blamed for human diarrheal illnesses (Ranjbar et al., 2017; García-Sánchez et al., 2018). *Campylobacter jejune* has been identified as one species that can cause enteritis, which is mostly characterized by diarrhea in various animals, including chickens (Humphrey et al., 2014; Ranjbar and Babazadeh. 2017). Within this group of bacteria, *Campylobacter fetus* subsp. fetus (*Cff*) is identified as the responsible factor for spontaneous abortion in ruminants, whereas *Campylobacter fetus* subsp. *venerealis* (*Cfv*) primarily contributes to bovine genital campylobacteriosis, a well-known sexual transmissible disease (STD) usually characterized by abortion in cattle (Sahin et al., 2017; Hlashwayo et al., 2020). In addition, to being present in cattle, sheep, humans, and other animal species, *Cff* affects a variety of hosts and is thought to be a normal component of the flora in human intestines (Sprenger et al., 2012; Iraola et al., 2017). Random abortions in sheep and cattle are brought on by *Cff* (Iraola et al., 2017). Contrarily, *Cfv* is extremely limited to the genital region of cattle (Silveira et al., 2018). Additionally, several regions worldwide have reported cases of *Campylobacter* species that are antibiotic-resistant (Ibrahim et al., 2018; Neogi et al., 2020). The diversity of encoding genes linked with resistance,

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which can pose major risks to animal and public health, has been identified as a global concern in biology and medicine; hence the mechanisms of resistance bacterial species are a serious worry on a global scale (WHO, 2014; Roca et al., 2015).

Therefore, this study aimed to identify multidrug efflux pumps involved in bacterial resistance, virulent genes implicated in the pathogenicity of bacteria, and heavy metal protein, this study used whole genome sequencing analysis to identify the antimicrobial resistance patterns of *Cff* and *Cfv* to different antibiotic agents.

MATERIALS AND METHODS

Ethical approval

The Department of Agriculture, Forestry and Fisheries (DAFF, Section 20 approval) and the North-West University Animal Production Sciences Research Ethics Committee approved the study under the ethics number NWU-01881-19-A5.

Origin of the isolates

Twenty presumptive isolates of *Campylobacter fetus* were obtained from the Vryburg Veterinary Laboratory in South Africa's North West Province. These isolates originated from Sheath Wash samples collected from bulls in various municipalities within the Dr. Ruth Mopati District, including Naledi (coordinates E24 33' and S26 54'), Mamusa (coordinates E25 27' and S27 24'), Molopo (coordinates E25 32' 42' and S26 00'), and Tswaing (coordinates E25 16' and S26 36').

Sub-culturing and phenotypic identification

To establish an oxygen-deprived setting conducive to the cultivation of *Cf* subsp., these bacteria were propagated on Skirrow's agar. The plates were then placed within a 2.5 L anaerobic jar (Oxoid, England) containing a CampyGen^{TM} sachet CN0025A, and incubated at 37 °C for 72 hours. This method follows the procedures outlined by Acke et al. (2009) and Wieczorek and Osek (2013). The pure colonies of *Campylobacter fetus* isolates were also subjected to various biochemical tests, including gram staining (Fawole and Oso, 2004), an oxidase reaction carried out using oxidase strips paper (Microbact Identification kit, MBO266A, South Africa) following the manufacturer's instructions, and a catalase test carried out using hydrogen peroxide 100 Vol (SAAR3063820LP) following the method used by Tshipamba et al. (2018). The Hippurate test was carried out in adherence to the manufacturer's guidelines, using a Hippurate disk (Remel Inc. 12076 Santa Fe Dr. Lenexa KS 66215, USA). Similarly, the urease test was conducted following the protocol outlined by Bermejo et al. (2002). Additionally, the tolerance to 1% glycine was tested using *brucella* broth supplemented with 1% glycine (Briedis et al., 2002).

Molecular assays

Extraction of genomic DNA

The Zymo extraction kit (Zymo-Research fungal/bacterial soil microbe DNA, D6005 USA), that supplied by Bio lab, South Africa was used to isolate genomic DNA (gDNA) in accordance with the manufacturer's instructions. A Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, USA) was used to quantify the isolated genomic DNA.

PCR amplification for the identification of Cff and Cfv

A Multiplex-polymerase chain reaction was used in this study to confirm *Cff* and *Cfv* using particular primers listed in Table 1. The PCR reactions required a total volume of 50 µl, which was made up of 20 µl of PCR inhibitor-free DNA/DNAse/RNAse-free (Bio-Concept, Switzerland); 4 µl of template DNA; 22 µl of One Taq Quick-Load 2X MM (master mix, Bio-Labs, England); 4 µl of MG3F and MG4R primer (Inqaba Biotec, South Africa). The thermal cycling protocol involved an initial denaturation step at 95 °C for duration of 3 minutes, followed by a series of 35 iterative cycles. Each cycle encompassed sequential phases, including denaturation at 95 °C for 15 seconds, annealing at 54 °C for 15 seconds, and extension at 72 °C for 15 seconds. Subsequent to the cycling regimen, a terminal extension phase was carried out at 72 °C for 5 minutes to complete the process.

Table 1. Different primers used for the identification of *Campylobacter fetus* subspecies

Organism	Target gene	Name of primer	Sequence (5'-3')	Amplicon (bp)	References
Campylobacter fetus subsp. *fetus*	cst	MG3F MG4R	GGTAGCCGCAGCTGCTAAGAT TAG CTACAA TAA CGA CAA CT	750	(Willoughby et al., 2005)
Campylobacter fetus subsp. venerealis	Unknown plasmid	VenSF VenSR	CTTAGCAGTTTGCGATATTGCCATT GCTTTTGAGATAACAATAAGAGCTT	142	(Hum et al., 1997)

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bp: Base pair

Agarose gel electrophoresis of the PCR products

Amplification of PCR product was done through screening for the presence of DNA using 1.5% agarose gel following staining in ethidium bromide (0.5 µg/ml). A molecular ladder of 100bp (Qiagen GelPilot® DNA molecular weight markers (South Africa) was run together with products of PCR amplicons. The gel was then visualized using the Bio-imaging system (Bio-Rad, Molecular imager® Gel Doc™ XR+, Model: Universal Hood II, Serial number: 721BR11002, USA). The existence of DNA bands was also recorded using bio-imaging technology (version 6.00.22). The sample shows a brilliant band (DNA bands), which denotes successful amplification. As shown in Figure 1, the amplicon product at 750 bp was identified as a distinctive feature of *C. fetus* subsp. *fetus* and the amplicon product at 142 bp as a distinctive feature of *C. fetus* subsp. *venerealis* (Hum et al., 1997) as indicated in Figure 2. The negative controls were *C. jejune* (ATCC 33560) and *Escherichia* (*E.*) *coli* (ATCC 25922).

Analysis of DNA sequencing

Amplified PCR products were sent for sequencing to Inquaba, Biotechnology, Pretoria, South Africa. The sequencing was done using the ABI PRISM® 3500XL DNA sequencer (Applied Biosystems, South Africa). The DNA strands were sequenced, and the raw sequence data were cleaned using Finch TV version 1.4.0. Forward and reverse sequences for each isolate were assembled before a Blast search was carried out in the NCBI GenBank. The nucleotide sequence of the PCR amplicons obtained was aligned to sequences in the National Centre for Biotechnology Information (NCBI) Database, using the Blast algorithm search tool, in order to identify sequences with significant similarity and carry out bacterial identification (Altschul et al., 1997). The gene fragments of *Cff* and *Cfv* obtained in this study were then deposited at the NCBI GenBank, and accession numbers were obtained.

Neighbor-Joining phylogenetic tree

The evolutionary lineage was deduced using the Neighbor-Joining technique (Saitou and Nei, 1987), and this was visually represented by a consensus tree produced from 1000 replicated samples (Felsenstein, 1985). Branches that appeared in fewer than 50% of these replications were compressed. The genetic differences, reflecting evolutionary distances, were calculated through the p-distance approach, quantified in terms of variations in genetic bases per generation (Nei and Kumar, 2000). To accommodate variations in rates across genetic sites, a gamma distribution with a shape parameter of 1 was employed. This investigation encompassed a set of 26 nucleotide sequences, as showcased in Figure 3. Specifically, the codon positions considered were the 1st, 2nd, 3rd, and Noncoding positions. Any positions containing gaps or missing data were excluded using the complete deletion option. The final dataset comprised a total of 316 positions. For conducting the evolutionary assessments, the software MEGA X was employed (Kumar et al., 2018).

Antimicrobial susceptibility tests

Based on the disk diffusion method, the antibacterial profile of the *Campylobacter fetus* subsp. was evaluated as described by Bauer et al. (1966). According to several studies, 14 antibiotics were chosen based on their use in veterinary and human medicine (Wieczorek and Osek, 2013; Tafa et al., 2014), as presented in Table 2. The isolated bacteria were subcultured on Columbia blood agar CM0331 (Oxoid, United Kingdom) that had previously been blended with 5% sheep blood and supplemented with *Campylobacter* growth supplement SR023E for the antimicrobial susceptibility test (Oxoid, Thermo Fisher, Basingstoke, United Kingdom). For 72 hours, the sample was incubated under microaerophilic conditions at 37°C. Single colonies from the pure culture were cultured for 24 hours at 37°C in a shaking incubator with five mL of Mueller Hinton broth supplemented with *Campylobacter* selective supplement (Skirrow SR0068E; Oxoid, England). The inoculum was brought to room temperature and allowed to cool after incubation. With a sterile cotton swab, the suspension was streaked over Mueller-Hinton agar's (Merck, Germany) entire surface, supplemented with 5% horse serum (Media Mage product, M60404, South Africa). Antibiotic discs were applied after allowing the inoculated plates to dry for 5 minutes. Subsequently, the plates were placed in an incubator set at 37°C for 24 hours, maintaining microaerophilic conditions. After the incubation period, the zones of inhibition were assessed using a plastic ruler, and the susceptibility, resistance, and intermediate patterns were determined following the guidelines outlined in CLSI (2020). The quality control strains used were *Campylobacter jejuni* (ATCC 33560), *C. coli* (ATCC 33559), *E. coli* (ATCC 25922), and *Campylobacter fetus* subsp. *fetus* (ATCC 27374).

Multidrug resistance pattern

This study investigated the profiles of multidrug resistance in Gram-negative bacteria exhibiting resistance to three or more antibiotic classes (Table 2), as outlined in prior research (Falagas et al., 2006; Paterson and Doi, 2007; Kallen et al., 2010). Consequently, any instances of *Cff* and *Cfv* demonstrating in vitro resistance to three or more antibiotic classes were categorized as bacteria displaying multidrug resistance.

Detection of antibiotic resistance genes using a polymerase chain reaction

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All multidrug-resistant *Campylobacter fetus* subsp. *fetus* and *venerealis* strains were tested for antimicrobial resistance genes, as shown in Table 3. None of the multidrug-resistant *Cff/Cfv* was PCR-positive for those specific genes, according to the PCR results. As a result, two isolated bacteria with multidrug resistance profiles, *Cff* (MT138645.1) and *Cfv* (MT138649.1), were chosen for further investigation and subjected to whole genome sequencing.

Table 2. Guideline of antibiotic resistance of Enterobacteriaceae according to the CLSI (2020)

Class	ATB	Concentration of antibiotics (Disc/ mg)	Zone diameter breakpoints (mm)		
			Susceptible S	Intermediate I	Resistant R
Beta-lactam	Ampicillin	10	≥ 17	14-16	≤ 13
Beta-lactam	Amoxicillin	10	≥18	14-17	≤ 13
Macrolide	Erythromycin	15	≥ 23	14-22	≤ 13
Macrolide	Azithromycin	15	≥ 13	-	≤ 12
Aminoglycoside	Neomycin	30	≥ 15	13-14	≤ 12
Aminoglycoside	Streptomycin	10	≥15	12-14	≤ 11
Aminoglycoside	Gentamicin	10	≥15	13-14	≤12
Quinolone	Ciprofloxacin	5	≥ 26	22-25	≤ 21
Quinolone	Nalidixic acid	30	≥ 19	14-18	≤ 13
Quinolone	Norfloxacin	5	≥ 17	13- 16	≤ 12
Quinolone	Enrofloxacin	10	≥ 17	13 – 16	≤ 12
Tetracycline	Tetracycline	30	≥ 15	12-14	≤ 11
Tetracycline	Doxycycline	30	≥ 14	11-13	≤ 10
Amphenicol	Chloramphenicol	30	≥ 18	13-17	≤ 12

CLSI: Clinical Laboratory Standard Institute

Table 3. Primers used for the detection of resistance genes in *Campylobacter fetus*

Target gene	Primer sequence	Annealing temperature (°C)	Amplicon size (bp)	Target antibiotic	Reference
tetO	F-GCGTTTTGTTTATGTGCG R-ATGGACAACCCGACAGAAG	54	559	tetracycline	(Pratt and Korolik, 2005)
bla$_{OXA-61}$	F- AGAGTATAATACAAGCG R- TAGTGAGTTGTCAAGCC	54	372	B-lactams	(Obeng et al., 2012)
erm(B)	F: GGG CAT TTA ACG ACG AAA CTG G R: CTG TGG TAT GGC GGG TAA GT	52	421	Macrolide	(Wang et al., 2014)
aadE	F: GCTGCCGCTGGAACT R: TCTTTTGCCGAATCACA	55	527	Aminoglycoside	(Wang et al., 2014)
qnrA	**F:** ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTTAGGTCA	55	516	Quinolone	(Wang et al., 2008)
aphA-3-1	F: TGCGTAAAAGATACGGAAG **R:** CAATCAGGCTTGATCCCC	54	701	erythromycin	(Obeng et al., 2012)

bp: Base pair

Genome sequencing

Whole-genome sequencing was performed on the gDNA of multidrug resistant *Cff* and *Cfv* (MT138645; MT138649). Enzymatic fragmentation of extracted gDNA samples was used (NEB Ultra II FS Kit). AMPure XP beads were used to size and select the resulting DNA fragments (200-500bp). End-repaired fragments were ligated to each fragment with Illumina-specific adapter sequences. The samples were individually indexed before undergoing a second size selection step. Each sample was separately labeled with an index before undergoing a subsequent size selection phase. Following this, quantification was performed using a fluorometric technique, and the samples were diluted to achieve a consistent concentration of 4nM. Subsequently, the sequencing of these samples was carried out using Illumina's NextSeq platform along with a NextSeq 300 cycle kit (Illumina, USA), following the manufacturer's instructions.

Genome assembly and annotation

The Kbase bioinformatics platform was used to analyze genome sequences with the default parameters (Arkin et al., 2018). FastQC-V0.11.5 was used to assess the raw reads for quality control, and Trimmomatic-v0.36 was used to trim the low-quality reads (Bolger et al., 2014). SPAdes-V3.13.0 (Bankevich et al., 2012) was utilized for the genome assembly. To achieve functional annotation of the genome, the NCBI Prokaryotic Genome Annotation Pipeline (Haft et al., 2018) and Rast_SDK V0.11 (Aziz et al., 2008) were employed. Patrick's annotation was also used to create a circular

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view of the genome (Wattam et al., 2017). The Pathogen Finder tool version 1.1 was used to predict the pathogenic potential of the isolates (Cosentino et al., 2013). Furthermore, genome sequences were submitted to an IS finder to identify the insertion sequences, the type of insertion sequences possessing an open reading frame (ORF), and the type of insertion sequence family to which they belong (Zhang et al., 2000).

Prediction of antimicrobial resistance genes and virulence factors

Antimicrobial resistance and virulence genes were identified utilizing Rapid Annotation Subsystem Technology (Rast) version 1.8.1 on the Kbase platform (The United States Department of Energy Systems Biology Knowledgebase) (Arkin et al., 2018), in addition to the Pathosystems Resource Integration Center (PATRIC, Brettin et al., 2015; Wattam et al., 2017). The functional annotation of genome features was carried out using kmers-v2, kmers-v1, and protein similarity. The final genome included coding DNA sequences (CDS), coding genes, and proteins, all of which possessed functional annotations.

PCR-based functional analysis of predicted antibiotic resistance genes

The predicted antibiotics resistance genes identified by whole-genome analysis, such as *CmeA*, *CmeB, CmeC,* and *gryA,* which are susceptible to inducing a multidrug resistance profile, were confirmed for their functional characteristics in isolated bacteria using PCR with specific primers listed in Table 4. The PCR amplification conditions were carried out per the methods used by the different studies listed in Table 4.

Prediction of Campylobacter virulence factors

With whole genome sequencing, the predicted virulence genes involved in cytotoxin production (*CdtA*, *CdtB*, and *CdtC*), adhesion, and colonization (*virB11* and *CiaB*) were further screened for functional characteristics in isolated bacteria using specific reported primer pairs shown in Table 5.

Table 4. Primers used to amplify predicted resistance genes in *Campylobacter fetus*

Target gene	Primer sequences	Annealing Temp (˚C)	Amplicon size (bp)	Reference
CmeA	F:TAGCGGCGTAATAGTAAATAAAC R:ATAAAGAAATCTGCGTAAATAGGA	49.8	435	(Olah et al., 2006)
CmeB	F: TCCTAGCAGCACAATATG R: AGCTTCGATAGCTGCATC	54	241	(Obeng et al., 2012)
CmeC	F: CAAGTTGGCGCTGTAGGTGAA R: CCCCAATGAAAAATAGGCAGAGTA	52	431	(Olah et al., 2006)
gryA	F: TTT TTA GCA AAG ATT CTG AT R: CAA AGC ATC ATA AAC TGC AA	50	265	(Zirnstein et al., 1999)

bp: Base pair

Table 5. Primer pair used for the detection of selected virulence genes in *Campylobacter fetus*

Target gene	Primer sequences	Annealing Temp (˚C)	Amplicon size (bp)	Reference
virB11	Fw: TCTTGTGAGTTGCCTTACCCCTTTT Rv: CCTGCGTGTCCTGTGTTATTTACCC	53	494	(Datta et al., 2003)
CdtA	FW: CTATTACTCCTATTACCCCACC RV: AATTTGAACCGCTGTATTGCTC	57	422	(Martínez et al., 2006)
CdtC	FW: ACTCCTACTGGAGATTTGAAAG Rv: CACAGCTGAAGTTGTTGTTGTTGGC	57	339	(Martínez et al., 2006)
CdtB	Fw: AGGAACTTTACCAAGAACAGCC Rv: GGTGGAGTATAGGTTTGTTGTC	57	531	(Martínez et al., 2006)
CiaB	Fw: TTTTATCAGTCCTTA Rv: TTTCGGTATCATTAGC	42	986	(Datta et al., 2003)

bp: Base pair

Phylogenetic genome analysis

The phylogenetic genome tree was created using the Kbase platform's Insert Genome into Species Tree version 2.2.0 application. Users can create a species tree using a collection of 49 cores, universal genes specified by clusters of orthologous groups (COG) gene families. The process mixes the genome(s) supplied by the user with a set of closely similar genomes retrieved from the public Kbase genomes database. The degree of relatedness was determined by alignment similarity with a subset of 49 COG domains. The user's genomes were put into precisely selected multiple sequence alignments (MSA) for each COG family. These curated alignments were adjusted with GBLOCKS to remove poorly aligned MSA segments. The MSAs were then concatenated, and a phylogenetic tree was built using Fast Tree

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(version 2.1.10), a fast method for estimating maximum likelihood phylogeny between the user's genome(s) and the set of genomes identified as analogous in the previous step (Price et al., 2010; R Project, 2013). In addition, a comparison of genome sequences based on proteins was done using online seed Viewer version 2.0; the complete genome of *Cff* 82-40 was used as a reference strain.

Statistical analysis

SPSS Statistics software (version 23.0) was employed for the analysis of descriptive statistics, encompassing frequencies and percentages. This software was also utilized to assess the presence of *Cf/Cfv* isolates along with their patterns of antimicrobial resistance. To establish connections between the geographical region and the occurrence of isolates, as well as their antimicrobial resistance profiles, Pearson's chi-square test of association was adopted ($p \leq 0.05$).

Furthermore, the Kruskal-Wallis test and Mann-Whitney's U were applied to investigate whether there were substantial differences in resistance levels across various antibiotics and between *Cff* and *Cfv*. In scenarios where statistically significant results were observed, cross-tabulations were employed to elucidate the relationships between the variations in resistance and other variables, such as the geographical region and the antibiotic profiles of *Cff* and *Cfv*.

RESULTS

Agarose gel electrophoresis of the DNA samples obtained from sheath wash samples revealed the presence of distinct amplification products. Specifically, a prominent 750 bp band was observed, which is characteristic of *Cff*, and a separate 142 bp band was detected, indicative of *Cfv* (Figures 1, 2, and 3). The statistical results showed that 70% of the isolated bacteria were identified as *C. fetus* subsp. *fetus,* and 30% were identified as *C. fetus* subsp. *venerealis* (Figure 4). The statistical analysis of results obtained from molecular identification, using the likelihood ratio of the Chi-square test, revealed no significant association between the areas ($p > 0.05$).

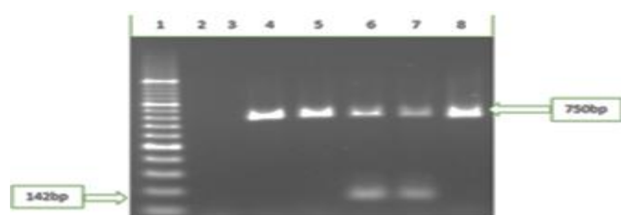

Figure 1. Agarose gel electrophoresis of DNA samples of *Campylobacter fetus* showing amplification at 750bp characteristic of *C. fetus* subsp. *fetus* and 142bp characteristic of *Campylobacter fetus* subsp. *venerealis*

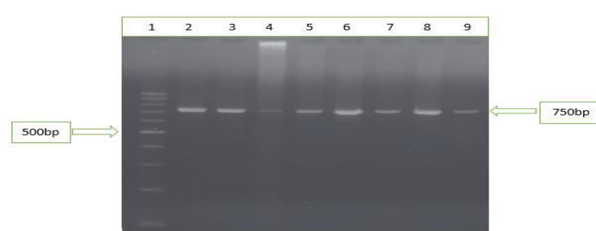

Figure 3. Amplification of DNA samples of *Campylobacter fetus* subsp. *fetus* (750bp)

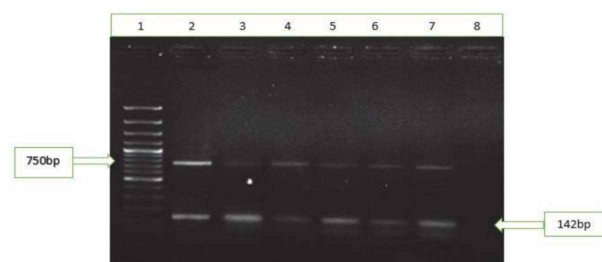

Figure 2. Amplification of DNA samples of *Campylobacter fetus* subsp. *venerealis* (142bp)

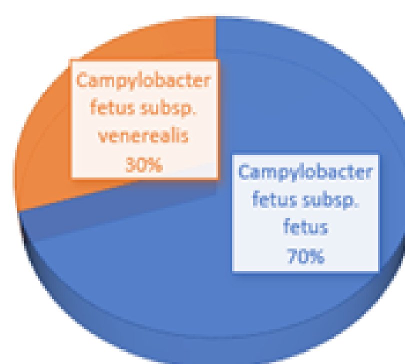

Figure 4. Incidence of *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* isolated from sheath wash of bulls

Neighbor-Joining phylogenetic tree results

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Using the 16S rRNA gene sequences, a neighbor-joining phylogenetic tree was built to assess the resemblances among the isolates from this experiment. It revealed that the *Cff* and *Cfv* isolates recovered in this study were closely related, as shown in Figure 5.

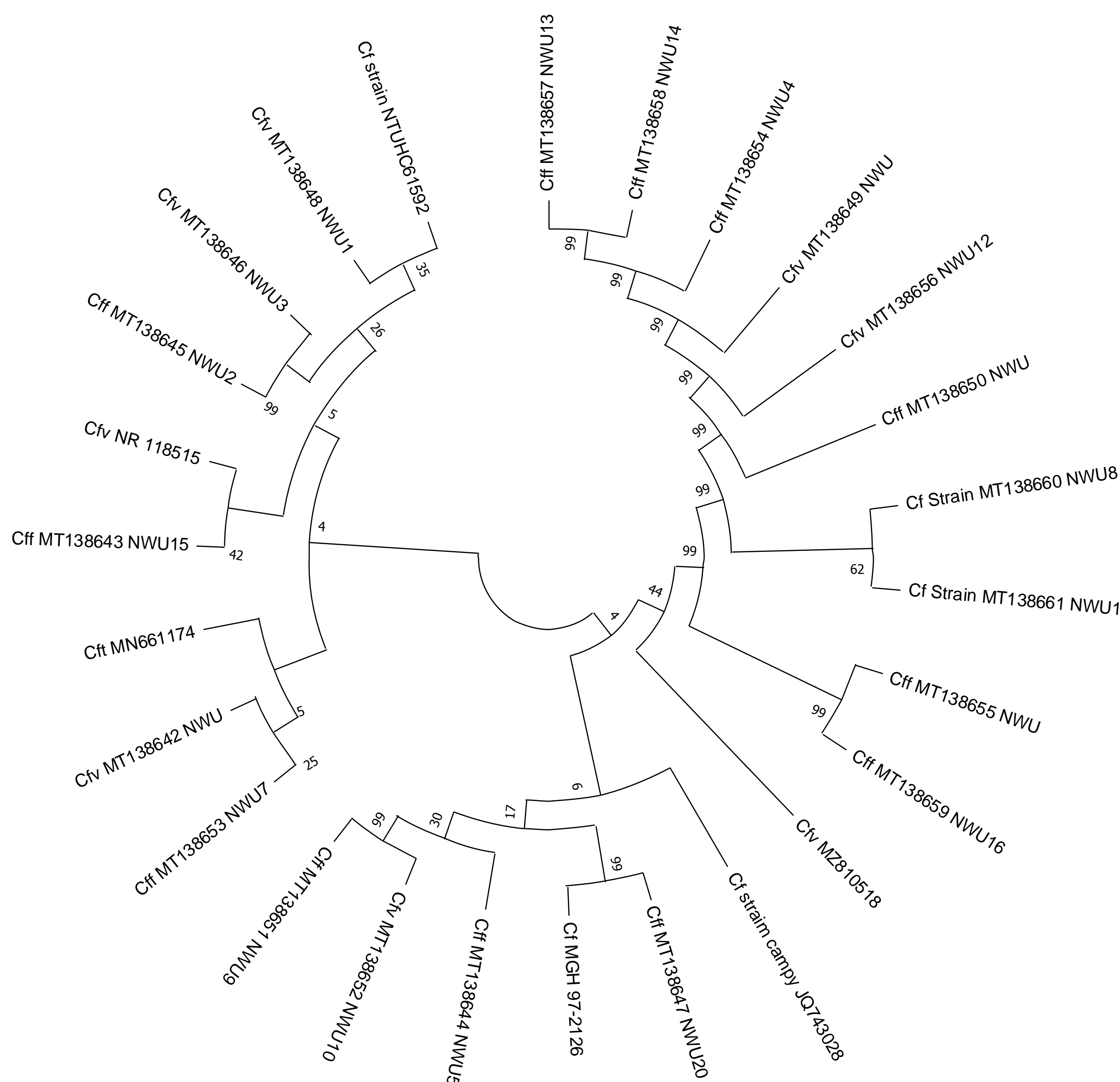

Figure 5. Neighbour-joining phylogenetic tree showing the relatedness among *Campylobacter* spp.

Antimicrobial susceptibility profiles of isolates

The antimicrobial test results reveal that an overall 52% of isolated bacteria were observed to be resistant to the antibiotics tested against, and 18% of these isolates exhibited a susceptibility profile to the antibiotics, as shown in Figure 6. When assessed individually by antibiotics, it was observed that 65% of the isolated bacteria displayed resistance against tetracycline and amoxicillin. Moreover, 60% of the isolated bacteria exhibited resistance to doxycycline, ampicillin, streptomycin, and nalidixic acid as shown in Figure 7. In addition, resistance to neomycin and gentamycin was also observed in 55% of isolated bacteria. As can be seen in Table 6, the obtained results of the Chi-square test revealed that antibiotic resistance did not depend on the collection area ($p > 0.05$).

Table 6. Independent T-test of statistical significance

Independent sample T-test / Antibiotic	Sum of squares	df	Mean square	F	Sig
Between groups	0.600	3	0.200	1.000	0.418
Within groups	3.200	16	0.200		

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Total	3.800	19

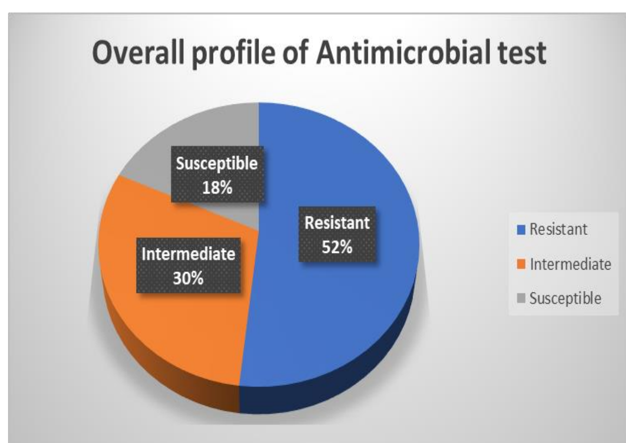

Figure 6. Overall profile of antimicrobial test *against Campylobacter fetus*

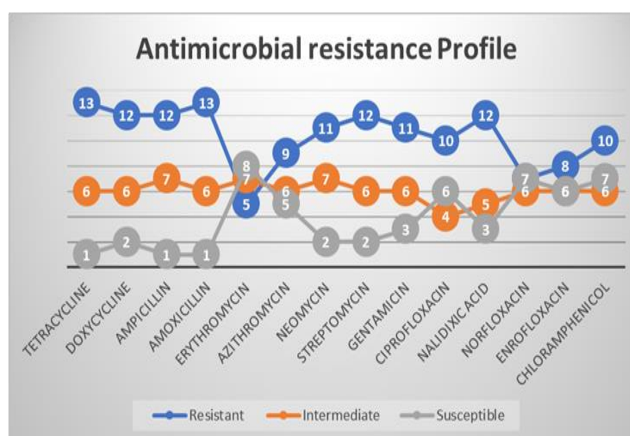

Figure 7. Frequency of antimicrobial resistance profile of *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* (n = 20)

Multidrug resistance profile of the isolated bacteria

Of 20 isolated bacteria subjected to an antimicrobial test, 45% exhibited a multidrug resistance profile to different antibiotic patterns (Table 7).

Whole-genome sequencing results

The confirmed *Campylobacter fetus* subsp. *fetus* (MT138645) and *Campylobacter fetus* subsp. *venerealis* (MT138649) displaying multidrug resistance profile were further selected and characterized by whole genome sequencing to predict genes involved in the mechanism of resistance, virulence factors as well as heavy metal protein. Results of whole genome sequencing revealed numerous genes (Figures 8 and 9), some linked to the mechanism of resistance (Table 8), Insertion sequences (Table 9), virulence factors (Table 10), as well as heavy metal protein (Table 11).

Table 7. Multidrug resistance pattern of *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*

Antibiotics pattern	Acc. Nb	Isolates name	Antimicrobial profiles
TE-DOX-AMP-AMO-ERY-AZT-NEO-STR-NAL-NOR-CHL	MT138645.1	*Cff*	MDR
TE-DOX-AMP-AMO-AZT-NEO-STR-GEN	MT138653.1	*Cff*	MDR
TE-DOX-AMP-AMO-NEO-STR-GEN-CIP	MT138660.1	*Cfstrain*	MDR
TE-DOX-AMO-ERY-AZT-NEO-NAL-NOR-ENR	MT138661.1	*Cfstrain*	MDR
TE-DOX-AMP-ERY-NEO-STR-GEN-CIP-NAL-NOR-ENR-CHL	MT138655.1	*Cff*	MDR
DOX-AMP-AMO-AZT-NEO-STR-GEN-CIP-NAL-ENR-CHL	MT138650.1	*Cff*	MDR
TE-DOX-AMP-AMO-STR-GEN-CIP-NAL-NOR-ENR-CHL	MT138656.1	*Cfv*	MDR
TE-DOX-AMP-AMO-STR-GEN-CIP-NAL-NOR-ENR-CHL	MT138648.1	*Cfv*	MDR
TE-DOX-AMP-AMO-AZT-STR-GEN-CIP-NAL-NOR-CHL	MT138649.1	*Cfv*	MDR

Acc.Nb: Accession number; MDR: Multidrug resistant; *Campylobacter fetus* subsp. *fetus: (Cff); Campylobacter fetus subsp. venerealis: (Cfv).*

Virulence factor analysis using genomic sequencing

Virulence gene analysis was carried out, and several genes were identified with established associations related to bacterial motility and chemotaxis. These genes include FliP, FliL, FliM, and FliN. In contrast, others are linked to cytotoxin production (*Cdt A*, *B*, and *C*), adhesion, colonization (*VirB10* and *VirB9*), and *Campylobacter* invasion antigen B (*CiaB*), as outlined in Table 10.

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Table 8. Resistant genes encoded in multidrug-resistant *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*

Genes	Classification of genes	Size of the gene		Predicted using		Targeted ATB	Reference
		Start (bp)	End (bp)	Patrick	Rast/Kbase		
Na+ driven multidrug efflux pump	Efflux pump conferring antibiotic resistance	26.905	28.228		+	*Ampicillin *Penicillin * Streptomycin *Erythromycin	(Huda et al., 2001) (Morita et al., 2000)
Broad-specificity multidrug efflux pump YkkCD	Efflux pump conferring antibiotic resistance	16.617	16.938	+	+	* Streptomycin, *Chloramphenicol * Tetracycline	(Jack et al., 2000)
ABC transporter multidrug efflux pump, fused ATP-binding domains	Multidrug efflux transporter	57.833	59.489		+	*Induce resistance against several antibiotics such as: *Macrolides *Tetracyclines *Chloramphenicol	(Orelle et al., 2019)
RND efflux system, outer membrane lipoprotein CmeC	Efflux pump conferring antibiotic resistance	28.672	30.088	+	+	*Fluoroquinolones *Macrolides *Quinolones	(Lin et al. 2002b)
RND efflux system, inner membrane transporter CmeB	Efflux pump conferring antibiotic resistance	30.080	33.227	+	+	*Cephalosporins, *Fusidic acid, *Fluoroquinolones *Macrolide	(Lin et al., 2002 a)
RND efflux system, membrane fusion protein CmeA	Efflux pump conferring antibiotic resistance	33.226	34.348	+	+	*Cephalosporins, *Fusidic acid, *Fluoroquinolones *Quinolones *Macrolides	(Lin et al., 2002 a)
Transcriptional repressor of CmeABC operon, CmeR	Efflux pump conferring antibiotic resistance	34,477	35,119	+	+	*Cephalosporins, *Fusidic acid, *Fluoroquinolones *Quinolones *Macrolides	(Lin et al., 2002 a)
Outer membrane protein TolC	Efflux pump conferring antibiotic resistance	244	1,546		+	* Fluoroquinolone	(Zgurskaya et al., 2009)
Transcriptional regulator, MarR (Multiple antibiotic resistance repressor) family	Drug efflux pump	1	427	+		*Fluoroquinolones *Beta-lactam	(Beggs et al., 2020)
Macrolide export ATP-binding/permease protein MacB	Efflux pump conferring antibiotic resistance	8,114	10,040	+	+	*Macrolides Erythromycin	(Kobayashi et al., 2001)
Macrolide-specific efflux protein MacA	Efflux pump conferring antibiotic resistance	10,036	11,221	+	+	*Macrolides Erythromycin	(Kobayashi et al., 2001)
GidB (16SrRNA (guanine(527)-N(7))-methyltransferase	Gene conferring antibiotic resistance	702	1,251	+	+	*Aminoglycosides *Streptomycin	(Okamoto et al., 2007)

+: Detected, -: not detected; RND (Resistance – Nodulation –Cell Division)

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Table 9. Important insertion sequencing family encoded in the genome of *Campylobacter fetus* subspecies

	Family IS 1182/ IS Length1536bp		
Accession number	Transposition	Origin of	Host
NZ_GG692850	ND	*E. faecalis*	*E. faecalis* T2
Left flank	Direct repeat	Right flank	DR length
CATATATAAA	AAAGTAGCTGCTAAAGATAGCAGCTACT TT	TTAGCGTTAA	30
ORF number		ORF function	
1		Transposase	
	Family IS1182/ IS Length 1935		
Accession number	Transposition	Origin	Host
U35635	ND	*Staphylococcus haemolyticus*	*Staphylococcus haemolyticus* Y176
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
ORF number		ORF function	
3		Transposase	
	Family Tn3/ IS Length 4948		
Accession number	Transposition	Origin	Host
HM769901	ND	*Salmonella enterica*	*Salmonella enterica* subsp. enterica serovar Wien plasmid pZM-3
Left flank	Direct repeat	Right flank	DR Length
TGTGGTATGG	GAAAA	CAAACAGCGC	5
ORF number		ORF function	
3		Passenger gene	
	Family IS 607/ IS Length 2030bp		
Accession number	Transposition	Origin	Host
AM260752	ND	*Cf*	*Cfv*
Left flank	Direct Repeat	Right flank	DR Length
-	-	-	-
ORF number		ORF function	
2		Transposase	
	FamilyIS4/ IS Length 1653bp		
Accession number	Transposition	Origin	Host
AY566173	ND	*Bacillus thuringiensis*	*Bacillus thuringiensis* subsp. pakistani
Left flank	Direct Repeat	Right flank	DR Length
-	-	-	-
ORF number		ORF function	
1		Transposase	
	Family IS1634 / IS Length 1910bp		
Accession number	Transposition	Origin	Host
AF272977	ND	*Mycoplasma hyopneumoniae*	*Mycoplasma hyopneumoniae*
Left flank	Direct repeat	Right flank	DR Length
TAAACAATCT	AGAAATTTTTAAAAAAACCTAGGT TTTTTTTAAAAATTTCTTTGAAAAC TGAAATTTAGATTAGAACGGCCAT ATTTTTT	TATATTGTAT	80
ORF number		ORF function	
1		Transposase	
	Family IS4/ IS Length 1653bp		
Accession number	Transposition	Origin	Host
AY566173	ND	*Bacillus thuringiensis*	*Bacillus thuringiensis* subsp. *pakistani*
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
ORF number		ORF function	
1		Tansposase	
	Family IS4/ IS Length26		
Access number	Transposition	Origin	Host
AJ605334	ND	*Bacillus cereus*	*Bacillus cereus* As4-12
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
ORF number		ORF function	
1		Passenger gene	
	Family IS Kra/ IS Length 2802bp		
Accession number	Transposition	Origin	Host
NZ_AEWF01000011	ND	*Candidatus Odyssella*	*Candidatus Odyssella* thessalonicensis L13 HMO
Left flank	Direct repeat	Right flank	DR Length
GGTAGATAG	-	AAATATATT	0
ORF number		ORF function	
3		Passenger gene (2) Transposase	
	Family IS3/ IS Length 1226		
Accession number	Transposition	Origin	Host

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-	ND	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* *Klebsiella pneumoniae* KP1276 plasmid pIA/C-KLUC
Left flank	Direct Repeat	Right flank	DR Length
TACACAA	CCT	TTTACTG	3
GGTTTCGCATCAGTAAAA	GG	TTGTGTATTCAGAACAAA	2
ORF number		ORF function	
3		Transposase	
	Family IS4/ IS Length 5396bp		
Accession number	Transposition	Origin	Host
NC_002146	ND	*Bacillus anthracis*	*Bacillus anthracis*
Left flank	Direct Repeat	Right flank	DR Length
GTTAAAATGT	AGATGGGACCC	CTTCTTATTT	11
ORF number		ORF function	
5		Passenger gene (3) Transposase (2)	
	Family IS NCY/ IS Length 1619		
Accession number	Transposition	Origin	Host
NC_003901	ND	*Methanosarcina mazei*	*Methanosarcina mazei*
Left flank	Direct repeat	Right flank	DR length
TTTATATTTACA	GGATTTTTT	ACGTGTTTAAT	9
ORF number		ORF function	
1		Transposase	
	Family IS 1595/ IS Length 1665bp		
Accession number	Transposition	Origin	Host
ABCF01000016	ND	*Bacillus sp.*	*Bacillus* sp. SG-1
Left flank	Direct repeat	Right flank	DR Length
ATCCAAAGTCCCCCGTAT	AAAAAGAA	AGACCTTCCGCGAAAC	8
ORF number		ORF function	
2		Transposase (1) Passenger gene (1)	
	Family ISAs1/ IS Length1241		
Accession number	Transposition	Origin	Host
NZ_ABIZ00000000.1	ND	*Verrucomicrobium spinosum*	Verrucomicrobium spinosum DSM 4136
Left flank	Direct repeat	Right flank	DR Length
CCCGGATCTG CAGAGACCGA GAATCCCTTC CTTTTGGCAG CGCATAAAGC	TCCAGGTATC GTTGTGGGA AGAAGGGACC GGGCACTCGC GTCCGTTCAG	GTTGTCACCA CTACAAGCCA CGGCATCAGG TGACAGGAGA TTTACGCCAT	10 9 10 10 10
ORF number		ORF function	
1		Transposase	
	Family ISAs1/ IS Length 1326bp		
Accession number	Transposition	Origin	Host
U24571	Y	*Vibrio cholerae*	*Vibrio cholerae* O22 and *Vibrio cholerae* O155 *Vibrio cholerae* O139 Bengal *Vibrio cholerae* M045 *Vibrio cholerae* O2
Left flank	Direct repeat	Right flank	DR length
GCTAA TAGTA ATGAC	ACGAGCAATG ATCCACCTTA ACGAAGTGCA	AGCCC TAACA TCACT	10 10 10
ORF number		ORF function	
1		Transposase	
	Family IS30/ IS Length 1521bp		
Accession number	Transposition	Origin	Host
AJ564386	ND	*Mycoplasma bovis*	*Mycoplasma bovis* isolate 2610
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
ORF number		ORF function	
1		Transposase	
	Family IS5/ IS Length 930bp		
Accession number	Transposition	Origin	Host
-	ND	*Methylobacterium dichloromethanicum*	*Methylobacterium dichloromethanicum* DM4
Left flank	Direct repeat	Right flank	DR length
GCCTCAACT	TCTAAGT	GTCTGTCCG	7
ORF number		ORF function	
3		Transposase	
	Family ISL3/ IS length 1245bp		

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Accession number	Transposition	Origin	Host
NC_010296	ND	*Microcystic aeruginosa*	*Microcystic aeruginosa* NIES 843
Left flank	Direct repeat	Right flank	DR length
TTCGGCAGAA CTACTGACTC CGCTATCGTC	AAGGGTC CTAACCC GATTTAG	TATATTCTTC TAAGAACAAT ACTTACAAAA	7 7 7
ORF number		ORF function	
1		Transposase	
	Family IS6/ IS length 790bp		
Accession number	Transposition	Origin	Host
X53951	ND	*Staphylococcus aureus*	*Staphylococcus aureus* plasmid pUW3626 *Staphylococcus aureus* plasmid pSH6 *Staphylococcus aureus* plasmid pSK41
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
ORF number		ORF function	
1		Transposase	
	Family IS 256/ IS Length 1313bp		
Accession number	Transposition	Origin	Host
X71808	ND	*Streptococcus thermophilus*	*Streptococcus thermophilus* AO54 *Streptococcus thermophilus* CNRZ368
Left flank	Direct repeat	Right flank	DR length
TTAC ATTA TTTTTTTTGA	ACCTAATC TATTCTAG AAAAAATG	AATT TTAT ACAATTGAAA	8 8 8
ORF number		ORF function	
1		Transposase	
	Family IS 481/ IS Length 1023bp		
Accession number	Transposition	Origin	Host
AF034434	ND	*Vibrio cholerae*	*Vibrio cholerae* N16961
Left flank	Direct repeat	Right flank	DR length
-	-	-	-
1		Transposase	
	Family IS 66/ IS length 2709bp		
Accession number	Transposition	Origin	Host
-	ND	*Escherichia coli*	*Escherichia coli* *Escherichia coli* O121:H19 51104
Left flank	Direct repeat	Right flank	DR length
GGTTCAGACC TTTACCTGTA AGACAGTGAC GGACATGCCA CGGCAACTGA CTGTTGTTCA TAGAGTGCGA	CTTTTTTT TGACCTAC GGATGTTG TTGTTTTC CAGAAATC AATGGCGA TTGCTGTG	AATGATGATG GCCGCATGGA TCAAGATATT TGACTGTTGG TCAGCAATGA CAACAATGGC AACAACAGAC	8 8 8 8 8 8 8
ORF number		ORF function	
3		Accessory gene (2) Transposase (1)	

Orf: open reading frame; DR: Direct Repeat, ND: Not defined

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Table 10. Whole-genome sequence for the prediction of virulence factors in multidrug resistance *Cff* and *Cfv*

Genes	Classification of genes	Size		Predicted using		Reference
		Start (bp)	End (bp)	Patrick	Rast	
Flip (Flagellar biosynthesis protein FliP)	Motility, Chemotaxis, Invasion, Phase variation	2	477	+	+	(Ohnishi et al., 1997)
FlgC (Flagellar basal-body rod protein FlgC)	Motility, Chemotaxis, Invasion, Phase variation	1.990	2.485	+	+	(Shippy et al., 2014)
FliM (Flagellar motor switch protein FliM)	Motility, Chemotaxis, Invasion, Phase variation	5,476	6.568	+	+	(Park et al., 2006)
FliN (Flagellar motor switch protein FliN)	Motility, Chemotaxis, Invasion, Phase variation	6.560	7.391	+	+	(Brown et al., 2005)
FliI (Flagellum-specific ATP synthase FliI)	Motility, Chemotaxis, Invasion, Phase variation	2.952	3.570	+	+	(Imada et al., 2007)
FliQ (Flagellar biosynthesis protein FliQ)	Motility, Chemotaxis, Invasion, Phase variation	1.102	1.369	+	+	(Chaban et al., 2018)
CdtA (cytolethal distending toxin subunit A)	Cytotoxin production	6.041	6.863		+	(Moolhuijzen et al., 2009)
CdtB (Cytolethal distending toxin subunit B)	Cytotoxin production	5.232	6.033		+	(Moolhuijzen et al., 2009)
CdtC (Cytolethal distending toxin subunit C)	Cytotoxin production	4.684	5.233		+	(Moolhuijzen et al., 2009)
VirB3 (Inner membrane protein forms channel for type IV secretion of T-DNA complex)	Adhesion and colonisation	1.187	3.053		+	(Silva et al., 2021)
VirB4 (ATPase required for both assembly of type IV secretion complex and secretion of TDNA complex)	Adhesion and colonisation	820	3.628		+	(Silva et al., 2021)
VirB10 (Inner membrane protein of type IV secretion of T-DNA complex, TonB-like)	Adhesion and colonisation	241	1.474		+	(Silva et al., 2021)
VirB8 (Inner membrane protein forms channel for type IV secretion of T-DNA complex)	Adhesion and colonisation	505	1.174		+	(Silva et al., 2021)
VirB9 (Forms the bulk of type IV secretion complex that spans outer membrane and periplasm	Adhesion and colonisation	55	706		+	(Silva et al., 2021)
VirB5 (Minor pilin of type IV secretion complex)	Adhesion and colonisation	45	1,075		+	(Silva et al., 2021)
VirB1(Bores hole in peptidoglycan layer allowing type IV secretion complex assembly)	Adhesion and colonisation	3.889	4.681		+	(Silva et al., 2021)
CiaB (*Campylobacter* invasion antigen B)	Invasion and colonisation	1.755	3.495		+	(Scallan et al., 2011)
SLP (Surface-Layer protein)	Colonisation, Adherence, and evasion	112	1,063			(Blaser, 1993)
VirB11 (ATPase required for both assembly of type IV secretion complex and secretion of TDNA complex	Adhesion and colonisation	1.882	2.802			(Silva et al., 2021)
VirD4 (Like coupling protein)	Adhesion and colonisation	748	2.584			(Silva et al., 2021)
Fic (Fic domain protein, BT_4222 type)	Adhesion and colonisation	1.739	2.660			(Sprenger et al., 2012)

+: Detected, -: not detected

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Table 11. Using the entire genome to predict the presence of heavy metal proteins in drug resistance *Cfv* and *Cff*

Genes	Classification of genes	Size		Predicted using		Reference
		Start (bp)	End (bp)	Patrick	Rast	
Cobalt-zinc-cadmium resistance protein (CzcD)	Ion efflux system involved in bacterial metal resistance	1,941	2,925	+	+	(Intorne et al., 2012)
Tellurite resistance protein (tehA)	Ion efflux system involved in bacterial metal resistance	11,595	12,669	+	+	(Nguyen et al., 2021)
Mercuric ion reductase (Mer)	Ion efflux system involved in bacterial metal resistance	45,527	46,844	+	+	(Christakis et al., 2021)
Arsenic efflux pump protein	Ion efflux system involved in bacterial metal resistance	13,456	14,722	+	+	(Shen et al., 2013)
Molybdopterin-guanine dinucleotide biosynthesis protein (MobB)	Ion efflux system involved in bacterial metal resistance	122	647	+	+	(Leimkühler and Iobbi-Nivol, 2016)
Molybdenum transport ATP-binding protein ModC	Ion efflux system involved in bacterial metal resistance	2,434	3,163	+	+	(Leimkühler and Iobbi-Nivol, 2016)
Molybdopterin-guanine dinucleotide biosynthesis protein (MobA)	Ion efflux system involved in bacterial metal resistance	4,098	4,641	+	+	(Leimkühler and Iobbi-Nivol, 2016)
Magnesium and cobalt transport protein (CorA)	Ion efflux system involved in bacterial metal resistance	10,648	11,380	+	+	(Kersey et al., 2012)
Nickel responsive regulator (NikR)	Ion efflux system involved in bacterial metal resistance	8,988	9,411	+	+	(Budnick et al., 2018)
hydrogenase nickel incorporation-associated protein (HypB)	Ion efflux system involved in bacterial metal resistance	9,434	10,223	+	+	(Maier and Benoit, 2019)
Ferric receptor CfrA	Ion efflux system involved in bacterial metal resistance	21,131	21,464	+	+	(Zeng et al., 2009)
Ferric siderophore transport system, periplasmic binding protein (TonB)	Ion efflux system involved in bacterial metal resistance	19,381	20,107	+	+	(Naikare et al., 2013)
Ferric iron ABC transporter, ATP-binding protein	Ion efflux system involved in bacterial metal resistance	6,914	7,916	+	+	(Zeng et al., 2009)
Ferrous iron transport protein B	Ion efflux system involved in bacterial metal resistance	7,498	9,421	+	+	(Zeng et al., 2009)

+: Detected, -: not detected

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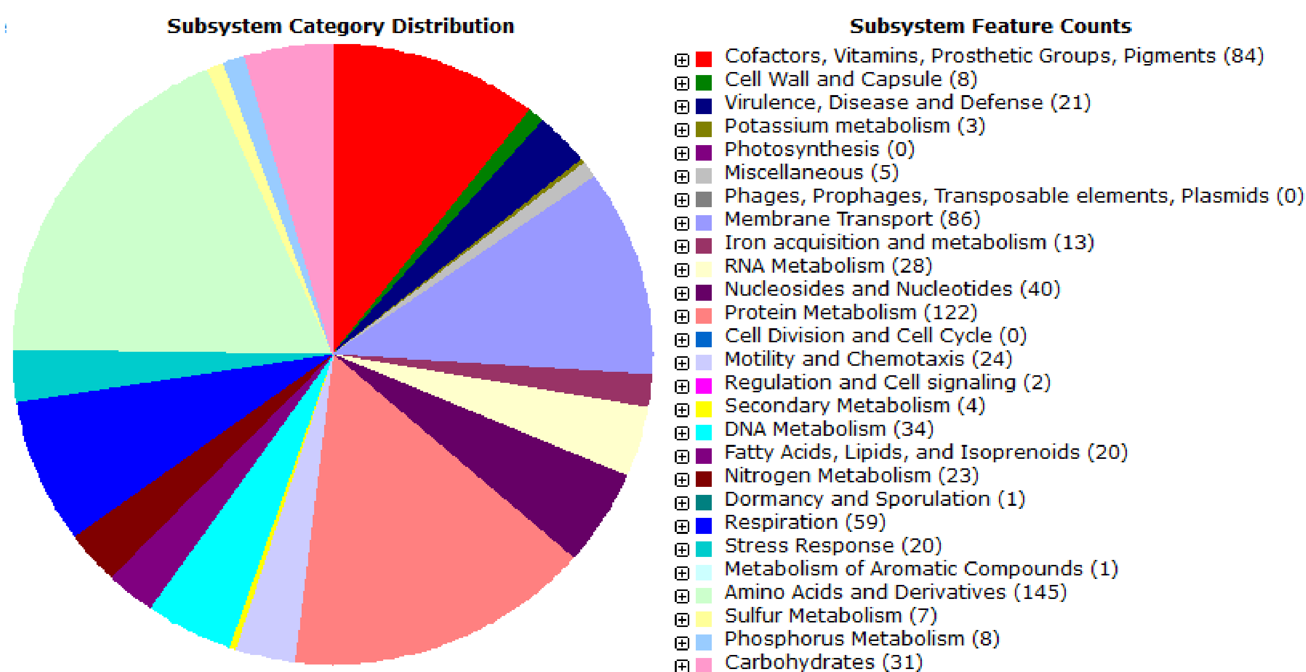

Figure 8. System distribution of *Campylobacter fetus subsp.fetus_* NWU_ED24 generated using seed viewer. Distribution of the subsystem in different colors and its corresponding number of gene features in numerical numbers

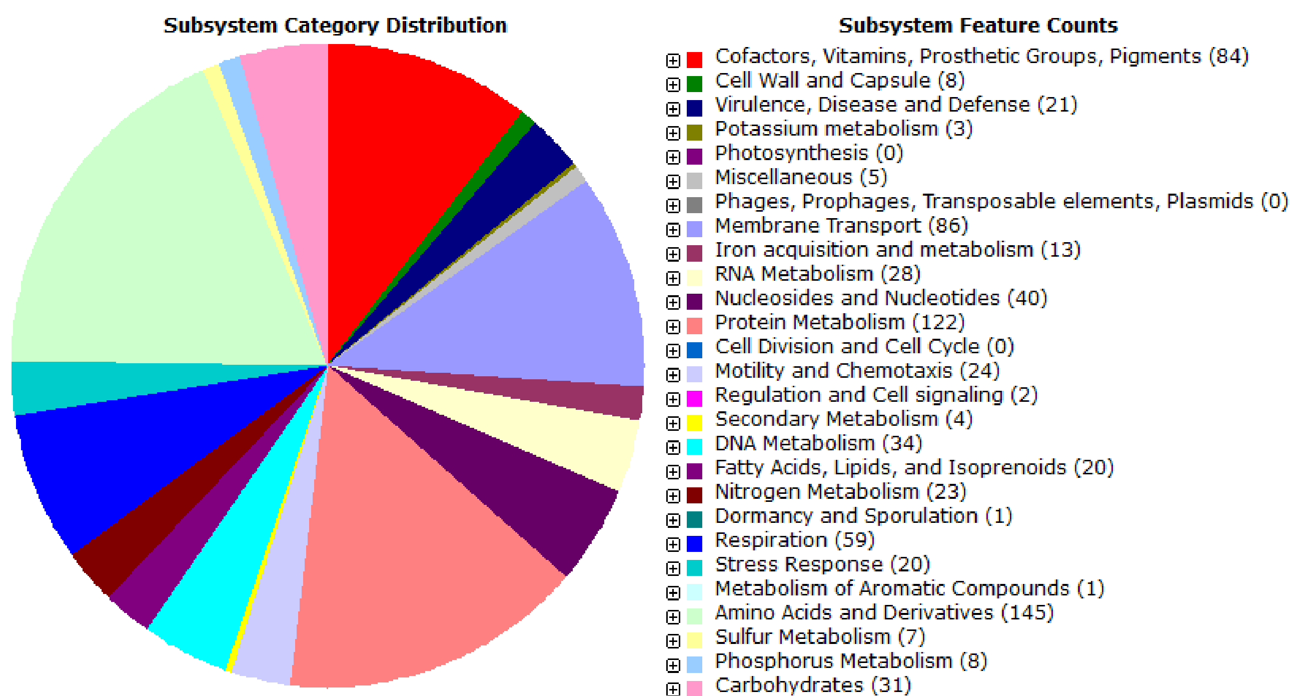

Figure 9. System distribution of *Campylobacter fetus subsp.venereals_* NWU_ED23 generated using seed viewer. Distribution of the subsystem in different colors and its corresponding number of gene features in numerical numbers

Functional characteristic of the predicted resistance and virulence genes using PCR

The results of the PCR analysis of the predicted resistance and virulence genes in *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* are summarized in Tables 12, 13, as well as Figures 10, 11, and 12. The overall PCR results showed a prevalence of *CmeA* 8(61.5%), *CmeB* 10(76.9%), *CmeC* 12(92.3%), and *gyrA* 13(100%) in *Campylobacter fetus* subsp. *fetus*. Moreover, the prevalence rates of *CmeA* (76.9%), *CmeB* (92.3%), *CmeC* (92.3%), and *gyrA* (100%) were observed in multidrug-resistant *Campylobacter fetus* subsp. *venerealis*. Furthermore, in *Cff* the statistical analysis of the virulence genes revealed the occurrence of *Cdt*A (76.9%), *CdtB* (100%), *CdtC* (61.5%), *CiaB*

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(61.5%), and *vir*B11 (46%). Meanwhile, in *Cfv* the PCR results also showed the presence of C*dt*A (69%), *CdtB* (84.6%), *CdtC* (69%), *CiaB* (76.9%), and *virB*11 (38%). The results indicated no significant relationship between the resistance genes detected in *Cff* and *Cfv* (p > 0.05).

Table 12. Resistance genes present by PCR in *Campylobacter fetus*

Isolated bacteria \ Antimicrobial resistance genes detected in isolated Bacteria using PCR	*CmeA*	*CmeB*	*CmeC*	*gyrA*
Campylobacter fetus subsp. *fetus*	8/13 61.5%	10/13 76.9%	12/13 92.3%	13/13 100%
Campylobacter fetus subsp. *venerealis*	10/13 76.9%	12/13 92.3%	12/13 92.3%	13/13 100%

Table 13. Virulence genes detected in *Campylobacter fetus*

	Virulence genes detected in the isolated bacteria using PCR				
Isolated bacteria	***CdtA***	***CdtB***	***CdtC***	***CiaB***	***virB11***
Campylobacter fetus subsp. *fetus*	10/13 76.9%	13/13 100%	8/13 61.5%	8/13 61.5%	6/13 46%
Campylobacter fetus subsp. *venerealis*	9/13 69%	11/13 84.6%	9/13 69%	10/13 76.9%	5/13 38%

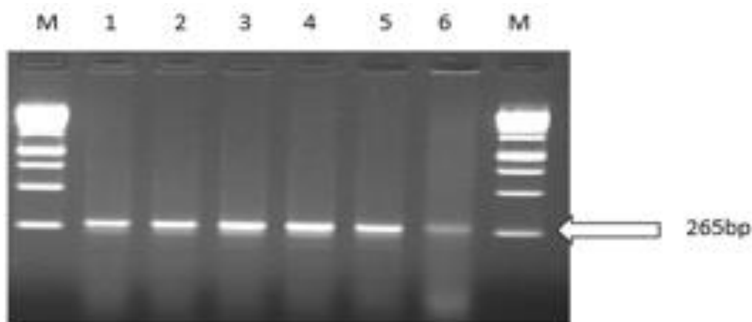

Figure 10. Amplification of *gyrA* gene at 256bp of *Campylobacter fetus* subsp.: Lane A (Molecular marker 100bp), Lane 1 to 3 (*Campylobacter fetus* subsp. *venerealis*) and Lane 4 to 6 (*Campylobacter fetus* subsp. *fetus*)

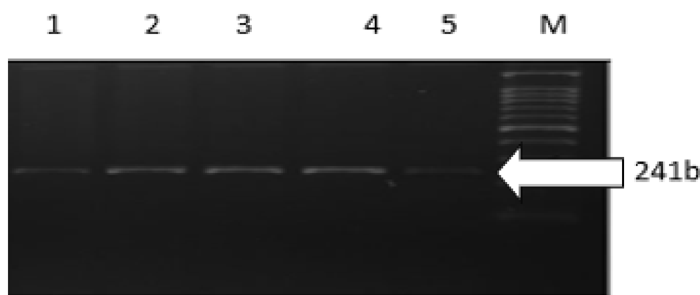

Figure 11. Electrophoresis of *CmeB*, Lane M (Molecular maker 100bp), Lane (1 to 3) *Campylobacter fetus* subsp. *fetus* and Lane (4 and 5) *Campylobacter fetus* subsp. *venerealis*

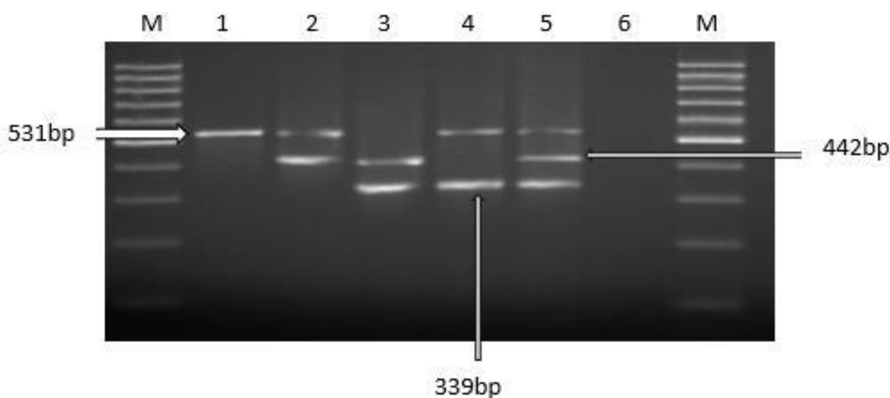

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Figure 12. Electrophoresis m-PCR for the detection of *CdtB* (531bp), *CdtA* (442bp), and *CdtC* (339bp) in *Cff* and *Cfv*. Lane M (Molecular Marker 100bp). In lanes 1 to 3 *Cff*, Lanes 4 and 5 *Cfv*, and Lane 6, no amplification was observed in one *Campylobacter fetus* subsp. *venerealis*

Comparison of genome sequencing based on proteins

The comparison results for annotated proteins across genomes of *Cf* subsp. revealed that most protein sequence identities ranged between 95 and 99.9% (Figure 13). The hit with the highest values (100%) was observed for DNA gyrase subunit B (Figure 14), hypothetical protein (Figure 15), flagellar hook protein FlgE (Figure 16), Flagellar hook-length control protein Flik (Figure 17), while Figure 18 shows Bcr/Cfla (multidrug resistance transporter) family, Czcd (cobalt-zinc-cadmium resistance protein) indicate in Figure 19, cytolethal distending toxin subunit C (Figure 20), possible abortive infection phage resistant protein (Figure 21), LSU rRNA pseudouridine (2605) synthase (Figure 22) and ribosomal RNA large subunit methyltransferase N (Figure 23). Additionally, standard protein identity similarities ranging between 10 and 80% were observed for certain proteins, such as Flavodoxin (Figure 24), Nitroimidazole resistant protein (NimB, Figure 25), Cytolethal distending toxin subunit A (Figure 26), zinc ABC transporter, substrate-binding protein ZnuA (Figure 27), thioredoxin reductase (Figure 28), SAM-dependent methyltransferase (Figure 29), Type IV fimbrial assembly ATPase, PilB (Figure 30), inner membrane protein creates a channel for the type IV secretion of the T-DNA complex. The protein VirB3 acts as an ATPase, necessary for both the assembly of the type IV secretion complex and the secretion of the T-DNA complex. This is depicted in Figure 31. The chromosomal regions containing the gene of interest were compared with the corresponding regions in four similar organisms. The graphical representations were centered around the focal gene, which was highlighted in red and labeled as "1." Genes exhibiting comparable sequences were assigned the same number and marked in red. Genes showing consistent positioning in at least four other species were connected functionally and enclosed in gray background boxes (Figures 14-31).

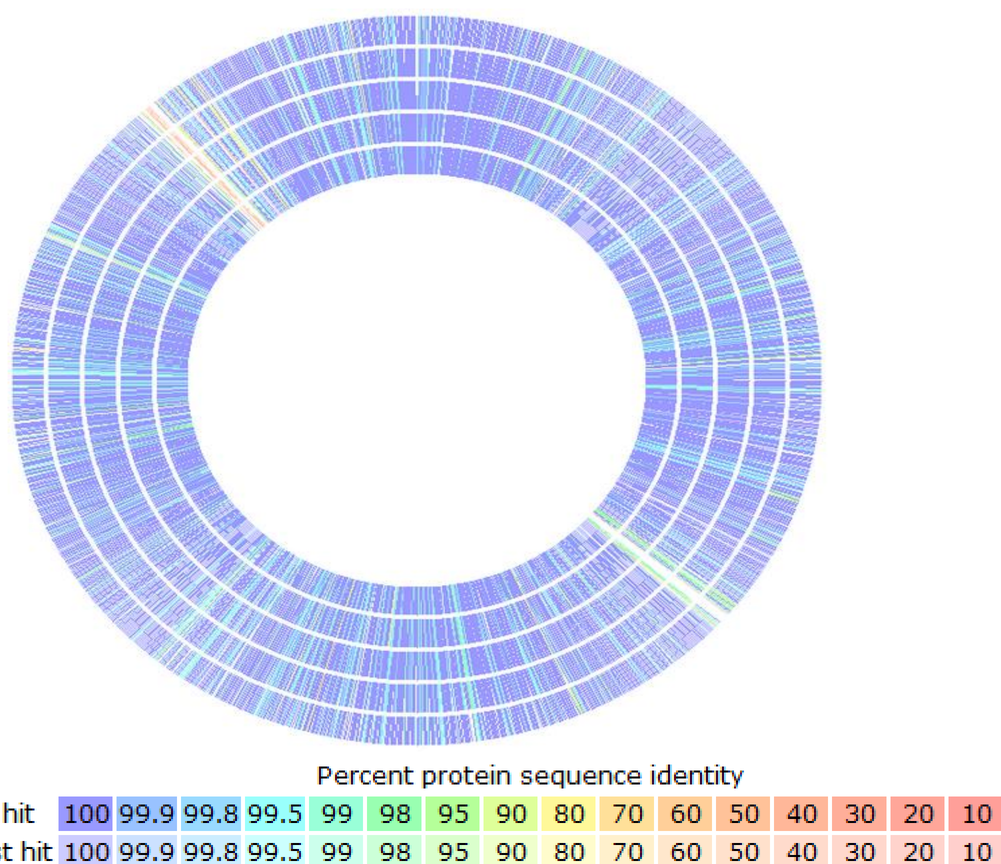

Figure 13. Comparison of the whole genome of *Cf* subspecies based on proteomic

Comparison of genome sequencing based on proteins

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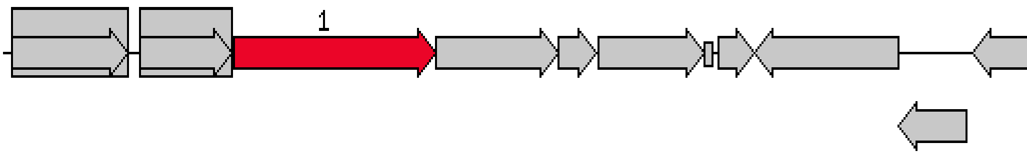

Figure 14. DNA gyrase subunit B of *Campylobacter fetus*, size 2322bp, 774aa

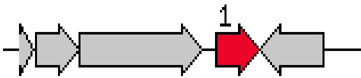

Figure 15. The hypothetical protein of *Campylobacter fetus*, size 423bp, 141aa

Figure 16. Flagellar hook protein FlgE of *Campylobacter fetus,* size 636bp, 212aa

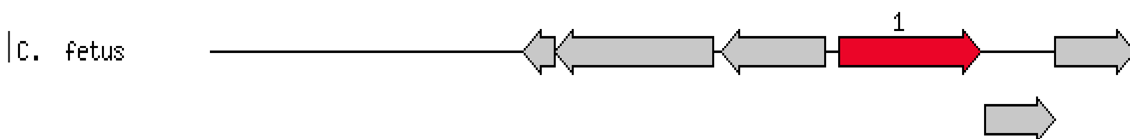

Figure 17. Flagellar hook-length control protein Flik of *Campylobacter fetus,* size 1602bp, 534aa

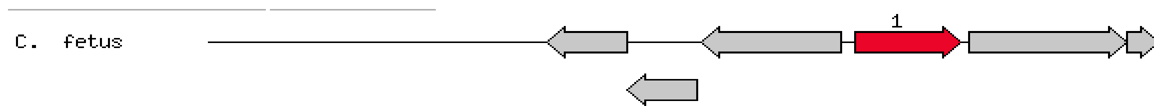

Figure 18. Multidrug-resistant transporter Bcr/CflA family of *Campylobacter fetus*, size 1203bp, 401aa

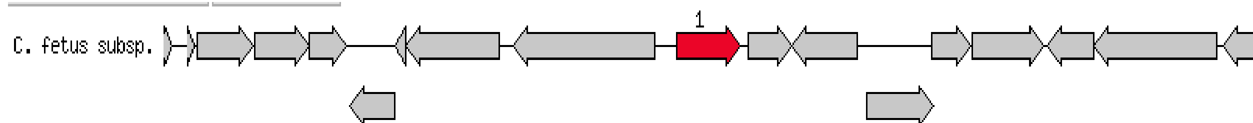

Figure 19. Cobalt-zinc-cadmium resistant protein Czcd of *Campylobacter fetus*, size 945bp, 315aa

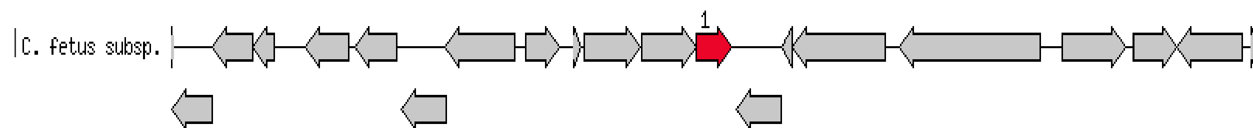

Figure 20. Cytolethal distending toxin subunit C of *Campylobacter fetus*, size 549bp, 183aa

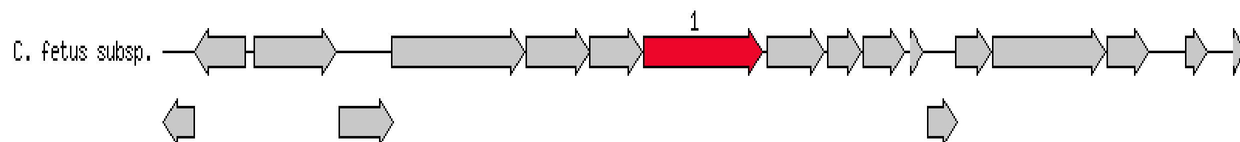

Figure 21. Possible abortive infection phage-resistant protein of *Campylobacter fetus*, size 1746bp, 588aa

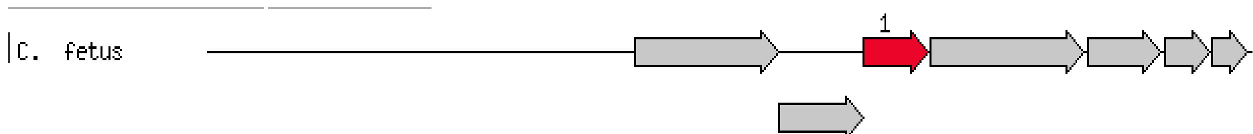

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Figure 22. LSU rRNA pseudouridine (2605) synthase of *Campylobacter fetus*, size 765bp, 255aa

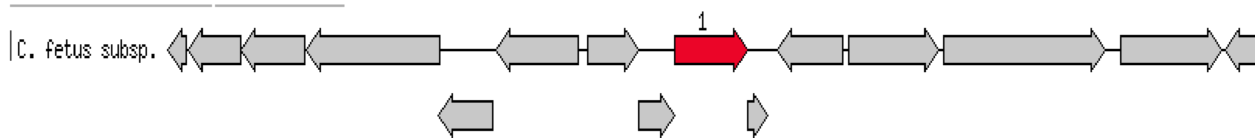

Figure 23. Ribosomal RNA large subunit methyltransferase of *Campylobacter fetus*, size 1065bp, 355aa

Figure 24. The flavodoxin of *Campylobacter fetus*, size 273bp, 91aa

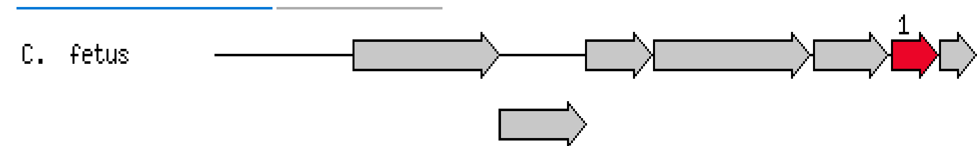

Figure 25. The nitroimidazole resistant protein NimB of *Campylobacter fetus*, size 516bp, 172aa

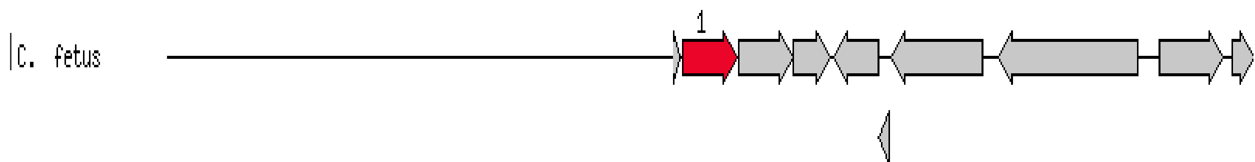

Figure 26: Cytolethal distending toxin subunit A of *Campylobacter fetus*, size 822bp, 274aa

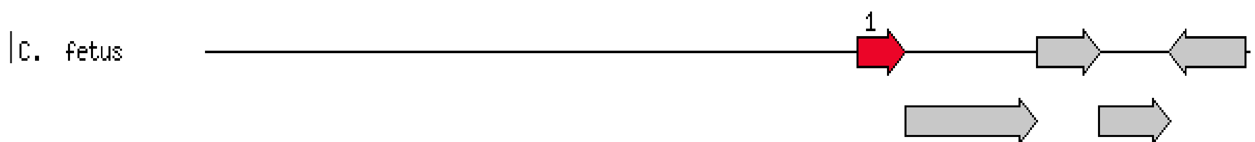

Figure 27. Zinc ABC transporter, substrate-binding protein ZnuA of *Campylobacter fetus*, size 588bp, 196aa

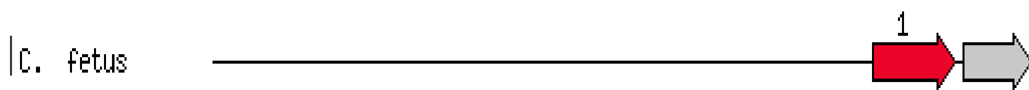

Figure 28. Thioredoxin reductase of *Campylobacter fetus*, size 945bp, 315aa

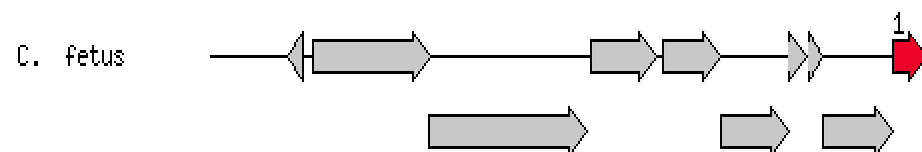

Figure 29. SAM –dependent methyltransferase of *Campylobacter fetus*, size 387bp, 129aa

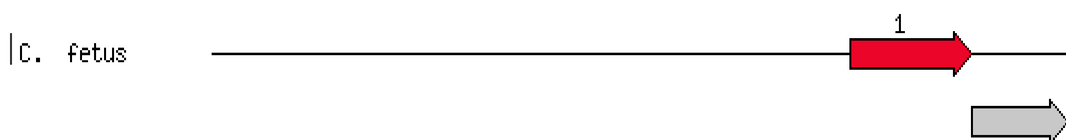

Figure 30. Type IV fimbrial assembly ATPase, PilB of *Campylobacter fetus*, size 1389bp, 463aa

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Figure 31. Vir-like type4 secretion system of *Campylobacter fetus*

Analysis of the phylogenetic genome

Analysis of the phylogenetic genome of bacterial genomes of *Cff* and *Cfv* revealed similar bacterial genomes clustered tightly together into different phylogenetic subgroups in the phylogenetic tree (Figure 32). The phylogenetic tree was constructed using the complete genomes of NWU_ED24 of *Campylobacter fetus* subsp. *fetus* and NWU_ED23 of *Campylobacter fetus* subsp. *venerealis*, both aligned with 20 other complete *Campylobacter* genomes. Notably, the two *Campylobacter fetus* subsp. isolated in this research demonstrated a pronounced level of similarity and clustered together. The numerical values represented the extent of distance or divergence between the various species (genomes) incorporated in the tree. A scale bar was included to illustrate a rate of 0.09 nucleotide substitutions per nucleotide position.

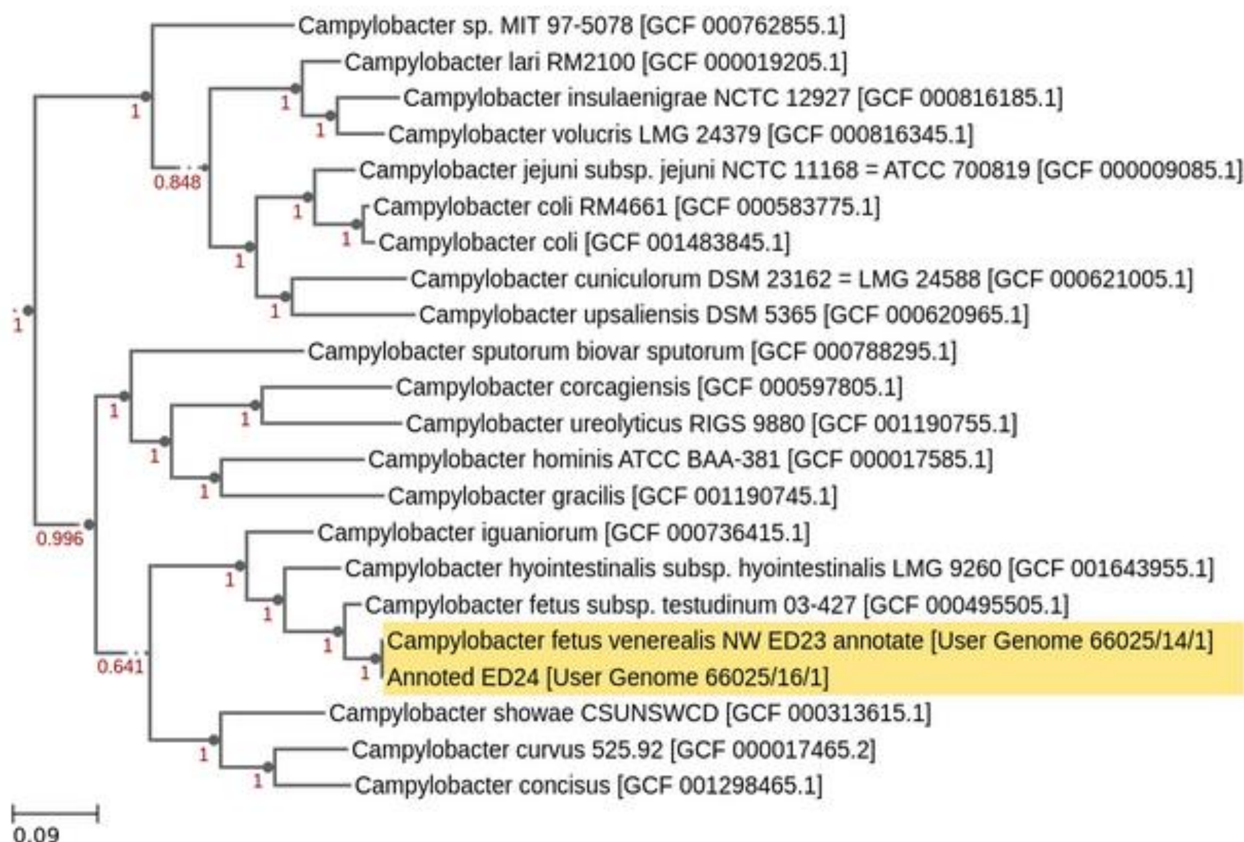

Figure 32. Neighbour-joining complete genome phylogenetic tree of *Campylobacter* spp.

DISCUSSION

Most studies on *Campylobacter fetus* recovered from livestock preputial wash focused on the diagnostic, epidemiology, and economic impact of the infection (Mshelia et al., 2010; Michi et al., 2016; Sahin et al., 2017). This research focused on the *in-silico* prediction of genes involved in the mechanism of resistance of these zoonotic bacteria to various antibiotic agents and virulence factors that can cause disease. The antimicrobial test results revealed that the bacteria isolated in this study were multidrug-resistant, with most *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* exhibiting resistance profiles against tetracycline, doxycycline, and chloramphenicol, as shown in Table 7.

Additionally, results indicated that the initial stage of drug resistance often involved the expression of several efflux pumps, even when they provided only minimal resistance. This preliminary resistance step subsequently paves the

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way for a more substantial resistance level, achieved through the acquisition of chromosomal mutations targeting antibiotics (Schmalstieg et al., 2012; El Meouche and Dunlop, 2018; Frimodt-Møller et al., 2018).

This study also used the whole genome to identify the broad-specific multidrug efflux pump. This gene is a genetic determinant of resistance to chloramphenicol and tetracycline. For example, in *E. coli* and *Bacillus subtilis*, this multidrug efflux pump was found to be responsible for resistance to tetracycline, chloramphenicol, and streptomycin (Jack et al., 2000). This efflux pump was reported recently in *Campylobacter jejuni* (Aksomaitiene et al., 2021). The results indicated the presence of multiple efflux pumps within the bacterial genomes of both *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*. A study by Nikaido and Pagès (2012) demonstrated that overexpression of these efflux pumps reduced susceptibility by lowering intracellular antibiotic concentrations.

The present study has identified various efflux pumps, including Na+-driven multidrug efflux pumps, ABC transporter multidrug efflux pumps, fused ATP-binding domains, TolC, and the ATP-binding/permease protein MacB involved in macrolide export. These pumps can induce resistance to a wide range of antibiotics. Multidrug efflux transporters pose a significant challenge in the context of antibiotic resistance mechanisms, as they enable bacteria to evade most existing treatment methods. One category of multidrug efflux pumps employs ATP hydrolysis to expel drugs and falls within the extensive ATP-binding cassette (ABC) transporter superfamily, as Lubelski et al. (2007) noted. Conversely, another study has confirmed that ATP-binding cassette-type drug efflux transporters contribute to resistance against either single drugs or multiple drugs. These transporters are particularly prevalent in Gram-positive bacteria, where they frequently provide protection against internally generated antibiotics and harmful peptides, as highlighted by Zgurskaya (2009). On the contrary, efflux pumps are recognized as significant factors contributing to inherent antibiotic resistance in Gram-negative pathogens, as outlined by Zgurskaya (2009).

Moreover, the CmeABC pump was identified across all bacterial genomes utilized in this investigation. The efflux system, CmeABC, falls under the resistance-nodulation-division (RND) category of efflux pumps and plays a significant role in conferring both intrinsic and acquired resistance to a variety of antimicrobials in *Campylobacter jejuni*, as documented by Su et al. (2017). The study also revealed the presence of a Na+-driven multidrug efflux pump. This specific pump has been previously detected in Gram-negative bacteria such as *Vibrio cholera* and *Vibrio* parahaemolyticus, as reported by Morita et al. (2000) and Huda et al. (2001). Based on an experimental investigation, it was deduced that the Na+-driven multidrug efflux pump could potentially impact the antibiotic resistance levels for substances like ampicillin, penicillin, streptomycin, and erythromycin, as indicated by Huda et al. (2001). Furthermore, these multidrug efflux pumps, identified within bacterial genomes, have been recognized for their significant role in expelling harmful substances from the cell's interior to the external environment prior to these substances reaching their intended targets. This process represents a paramount mechanism of drug resistance, as highlighted in the findings by Holmes et al. (2016).

In current study, important families of insertion sequences, such as IS3, IS5, IS4, IS6, IS1182, ISL3, Tn3, ISAs1, ISNCY, IS1595, IS1634, IS6, IS1634, IS110, IS30, IS607, ISKra, IS982, IS21, IS607, ISLre2, IS256, IS30, IS481, ISH3, IS91, IS256 and IS 1380 were identified (Table 9). Some insertion sequences detected in this study have been linked to the spread of antimicrobial-resistant genes. Among the identified insertion sequences, IS1, IS2, and IS5 have been shown to activate the expression of neighboring genes (Mahillon and Chandler, 1998). The Tn3 family was found in both bacterial genomes in this study. According to reports, the Tn3 family plays an important role in transposing bacterial plasmids, influencing both the structure and properties of these replicons (Szuplewska et al., 2014). Furthermore, insertion sequences from the IS6, Tn3, IS4, and IS1 families have been strongly associated with several antimicrobial-resistant genes (Razavi et al., 2020). As a result of the findings of this study, the multidrug-resistant profile observed in *Cff* and *Cfv* might result from the interaction of different efflux pumps and insertion sequences found in both bacterial genomes.

The genome sequences of *Cf* subsp. were examined for virulence factors associated with bacterial pathogenicity, and the results revealed the presence of genes associated with motility, adherence, invasion, and toxin production, as shown in Table 10, 13, and Figure 12. According to research, the presence of the three subunits of cytolethal-distending toxin requires full toxin activity in *Campylobacter* species (No et al., 2002; Lapierre et al., 2016). The findings of the current study are consistent with the previous study's findings, as the three subunits of the cytolethal distending toxin *CdtABC* were encoded in all bacterial genomes (Lapierre et al., 2016).

Moreover, the outcomes of the present study align with those of Asakura et al. (2007), who documented the presence of cytolethal-distending toxin subunits A, B, and C within *Cf*. These genetic components are recognized for their role in enhancing the pathogenic potential of the associated bacteria, ultimately fostering persistent infections, as noted by Pons et al. (2019). The current study also identified different heavy metal proteins in all bacterial genomes of *Cff*_NWU ED24 and *Cfv*_NWU ED23, as indicated in Table 11. Among them, the ferric receptor gene has been reported to be present in *Campylobacter jejuni* isolated from chicken feces (Zeng et al., 2009). This gene was responsible for facilitating high-affinity iron acquisition within *Campylobacter jejuni* and was essential for successful colonization

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within animal intestines, as explained by Zeng et al. (2009). Additionally, researchers unveiled that iron-regulated Outer Membrane Proteins (OMPs) stand as pivotal virulence determinants in bacteria. They play a crucial role in bacterial adaptation to various host environments, chiefly by facilitating the uptake of iron, as highlighted by Lin et al. (2002). Studies underscore the universal importance of iron for the survival and growth of all Gram-negative bacteria, as Stintzi et al. (2008) noted.

This result aligns harmoniously with the outcomes of the current investigation, where the presence of this protein was predicted in both the *Cff*_NWU ED24 and *Cfv*_NWU ED23 bacterial genomes, as evidenced in Table 11. An empirical study focusing on *Campylobacter jejuni* unveiled that CfrA is a promising target for developing a subunit vaccine against *Campylobacter* infections. This is because antibodies specific to CfrA can effectively hinder its functionality, posing a threat to the bacterium's survival and growth, as demonstrated by Zeng et al. (2009).

The findings of the present study can be considered for the development of a new vaccine against these two subspecies' infections. Furthermore, the heavy metal proteins found in the bacterial genomes of *Cff*_NWU ED24 and *Cfv*_NWU ED23 (Table 11) have been linked to ion binding, transport, and catabolism, as well as the Efflux of inorganic and organic compounds (Aminur et al., 2017). The study's findings highlight the significance of these zoonotic bacteria, which can play an important role in accumulating heavy metals from polluted environments and their potential transfer to humans through the food chain, posing a serious public health concern.

The comparative genomic analysis further demonstrated the presence of the cobalt-zinc-cadmium resistance protein within the bacterial genomes. This specific protein has also been identified in other bacteria, such as Gluconacetobacter diazotrophicus PAl 5 and *E. coli*, as documented by Nies (1995) and Intorne et al. (2012). Additionally, the outcomes unveiled the existence of the multidrug-resistant transporter belonging to the Bcr/CflA family in both bacterial genomes. The Bcr/CflA, drug resistance transporter, encompasses 12 membrane-spanning segments. Known members with functional activity include Bcr (associated with bicyclomycin resistance) in *E. coli*, as reported by Bentley et al. (1993), flor (linked to chloramphenicol and florfenicol resistance) in *Salmonella typhimurium*, as indicated by Braibant et al. (2005), and CmlA (associated with chloramphenicol resistance) discovered in Pseudomonas plasmid R1033, as highlighted by Bissonnette et al. (1991). The comparative genomic assessment also highlighted consistent retention of gene content and arrangement within the genomes of *Cff*_NWUED24 and *Cfv*_NWU_ED23. ED23 (Figure 13 and Figures 14 to 31).

Cytolethal distending toxins CdtABC were found conserved among bacterial genomes. These findings concur with previous studies stating that these genes are well-conserved in both *Cff* and *Cfv* (Ali et al., 2012). Type IV secretion system-related genes, such as *virB* genes, were also conserved in both subspecies. This has also been reported by van der Graaf–van Bloois et al. (2016). Based on the protein comparison (Figure 13), the genomic comparison showed high similarities among bacterial genomes of *Cff*_NWU_ED24 and *Cfv*_NWU_ED23. This finding aligns with previous studies, which revealed that these two subspecies are very similar, closely related, and have identical 16S rRNAs. This finding was also confirmed by van der Graaf–van Bloois et al. (2016), who reported that *Cf* subsp. could be considered genetically identical species with low genetic diversity compared to other *Campylobacter* species.

The whole genome neighbor-joining phylogenetic tree, which was based on 16S rRNA gene sequences extracted from 22 complete genomes of *Campylobacter* species (Figure 32), revealed that both bacterial genomes in the current study, colored in yellow, were tightly clustered together. Due to their 16S rRNA gene sequence homology and a higher degree of similarity in their genomes (Figure 32), these results align with earlier observations concerning the *Cf* subsp. established by Moolhuijzen et al. (2009).

The bacterial genomes of *Cff* _NWU ED24 and *Cfv* _NWU ED23 were found to be distantly related to other *Campylobacter* spp. genomes *Campylobacter* spp. MT97-5078, *Campylobacter lari* RM2100, *Campylobacter insulaenigrae* NCT12927, *Campylobacter hominis* ATCC BAA-381, and *Campylobacter gracilis*, for example, had higher genetic diversity.

On the other hand, it was discovered that the bacterial genome of *Cfv*_NWU ED23 was closely related to the bacterial genome of *Campylobacter fetus* subsp. *testudinum 03-427* was located on a separate clade near *Campylobacter hyointestinalis* subsp. *hyointestinalis LMG 9260*, while the bacterial genome of *Cff*_NWU ED24, was distantly located to *Campylobacter hominis*, identified as a non-human pathogen among all *Campylobacter* genomes used in the neighbor-joining phylogenetic tree (Lawson et al., 2001).

CONCLUSION

This study has unearthed compelling evidence underscoring the heightened and pivotal role of multidrug-resistant (MDR) efflux pumps in the emergence of bacteria resistant to multiple drugs, exemplified by *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*. The revelations underscore the pervasive presence of these MDR efflux

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pumps encoded across all bacterial genomes and firmly link them to the intrinsic or acquired resistance profiles of these zoonotic bacteria.

These findings signify a clarion call for further exploration into the implications and roles of these MDR efflux pumps within the context of *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*. This avenue of inquiry holds promise for developing innovative pharmaceutical interventions designed to effectively counteract their influence. Moreover, the emergence of heavy metal proteins within the realm of multidrug-resistant zoonotic bacteria warrants a grave concern for human health. It is imperative to magnify the focus on these repercussions in order to curtail their rapid dissemination.

The urgency of this matter necessitates an intensified spotlight on these findings, drawing the attention of researchers and stakeholders alike to collectively combat the escalating threat posed by these multidrug-resistant bacteria.

DECLARATION

Acknowledgments

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Availability of data and materials

The genome data project of *Campylobacter fetus* subsp. *venerealis* and *Campylobacter fetus* subsp. *fetus* were deposited in DDBJ/ENA/GenBank and are accessible under the accession number JACASH000000000 and JACASG000000000, respectively. The version described in this manuscript is JACASH010000000 and JACASG010000000. The raw reads were also submitted to the NCBI SRA under accession number SRX8607292, BioSample number SAMN15356083, and Bio Project number PRJNA641553 for *Campylobacter fetus* subsp. *venerealis* and SRX8607532 and Bio Sample number SAMN15356666 for *Campylobacter fetus* subsp. *fetus.*

Ethical consideration

The authors confirm that all authors have reviewed and submitted the manuscript for the first time in this journal.

Authors' contributions

Prof. Mwanza conceived the project and secured funding. Dr. Lubanza provided technical assistance. K. Molefe helped with data analysis. M.E Tshipamba participated in the project's conception, sample collection, laboratory analysis, and document writing; Prof. Mwanza edited the manuscript. The final manuscript has been read and approved by all of the authors.

Competing interests

The authors state that they do not have any competing interests.

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