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Genome Analysis of Antimicrobial Resistance Genes and Virulence Factors in Multidrug-Resistant *Campylobacter fetus* Subspecies Isolated from Sheath Wash

Mpinda Edoaurd Tshipamba*[1], Ngoma Lubanza[1] and Mulunda Mwanza[1]

[1]*Department of Animal Health, School of Agriculture, Faculty of Agriculture, Science and Technology, Mafikeng Campus, North-West University, Private Bag X2046, Mmabatho, 2735, South Africa*

*Corresponding author's Email: edotshipamba@gmail.com; ORCID: 0000-0003-4469-3200

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ABSTRACT

Campylobacter fetus subspecies are mostly characterized by reproductions problems in cattle and sheep. This study aimed to study the genetic profile and assess the genes mechanism of resistance and their virulence factors using genome sequence analysis. A total of 59 confirmed *Campylobacter fetus* subspecies based on molecular assays and DNA sequencing were subjected to antimicrobial susceptibility test against 14 antibiotic agents representing the five classes of antibiotics using the disc diffusion method. In addition, sequencing the genome of all strains induced complete resistance against all tested antibiotics. The results of the antimicrobial test indicated that 54.4% had a resistance profile, 26.3% were intermediate, while 19.3% were observed to be susceptible. The Whole Genome Sequencing (WGS) result revealed the presence of different genes, such as Broad-specificity multidrug efflux pump and 16S rRNA (guanine^{527}-N^{7})-methyltransferase (gidB), efflux pump conferring antibiotic resistance (MacA and MacB), protein-altering cell wall charge conferring antibiotic resistance (PgsA), which have never been reported in *Campylobacter fetus* subspecies. The WGS also revealed the presence of genes that involved in colonization, adhesion, motility, and invasion, such as type IV secretion system protein (VirD4), S-Layer, cytolethal distending toxin (A, B, and C), *Campylobacter* invasion antigen (CiaB), and fic domain protein (fic) were among important CDS. The presence of these uncommon genes explains the resistance of *Campylobacter fetus* subspecies against different tested antibiotics. The results of this study can be used to implement molecular surveillance of *Campylobacter fetus* subspecies and conduct further studies on the resistance mechanism in these subspecies.

Keywords: Broad-specificity multidrug efflux pump, *Campylobacter fetus* subspecies, Genome analysis, Methyltransferase gidB, Multidrug resistance.

INTRODUCTION

Embryonic deaths, temporary infertility with mild endometritis, and abortions can be caused by *Campylobacter fetus* subspecies in the herd, which resulted in substantial production losses and economic hardship for producers worldwide (Bellows et al., 2002; Morrell et al., 2011). Venereal diseases such as bovine genital campylobacterosis are considered as the primary cause of reproductive loss (McCool et al., 1988). Experimental studies revealed that treating carrier bulls has been done by victimization, either vaccination with a monovalent oil-based immunizing agent or recurrent antimicrobial agent. Furthermore, several of the revealed antimicrobial treatment protocols that used antimicrobial agents are not commercially obtainable. What is more, it had been confirmed that none of the revealed studies had provided robust proof supporting a treatment of choice that eliminates shedding in infected bulls (Vasquez et al., 1983; Truyers et al., 2014).

On the other hand, researchers discovered that vaccination itself is not ample to manage a pestilence. However, habitually testing and culling of infected bulls is suggested. Testing and culling procedures have a considerable price related to the premature loss of high price animals; particularly, considering the value of breeding bulls (Guerra et al., 2014; Erickson et al., 2017). A study conducted on the analysis of long action oxytetracycline and a monovalent immunizing agent for the management of *Campylobacter fetus* subsp. *venerealis* disease in bulls ended that there have been no vital variations between treated and untreated bulls. No matter the diagnostic applied, treatment with two label doses of this regime failed to stop the shedding of *Campylobacter fetus* subsp. *venerealis* in treated bulls, therefore, is not suggested as an efficient management strategy (Erickson et al., 2017). The resistance against long action oxytetracycline observed by Erickson et al. (2017) may be correlated with the previous findings obtained by Mak et al. (2014), that researchers indicated that bacteria species contain a variable number of resistance mechanisms against

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different antimicrobial agents for their self-defense to ensure their complete protection. This resistance mechanism is, for the most part, co-regulated through the expression of resistance genes (Mak et al., 2014).

Thus considering the importance of *Campylobacter fetus* subspecies relatedness to food, public, and animal health and their resistance profile to different antibiotics, this study aimed to study the genetic profile and assess genes mechanism of resistance and their virulence factors by using genome sequence analysis.

MATERIALS AND METHODS

Ethical approval

Ethical consideration for this study was submitted and approved by the Animal Production Sciences Research Ethics Committee of the North-West University (NWU-AnimProdRec), South Africa. The study procedures were performed in accordance with the requisite ethical standards.

Study design and area

This was a cross-sectional descriptive study conducted in Dr. Ruth Mompati District Municipality in North-West Province, South-Africa, from January 2018 to December 2019, focusing on positive *Campylobacter fetus* subspecies collected from the state veterinary services and processed at the North-West University Laboratory at Mafikenf Campus for further analysis.

Bacterial identification

Positive *Campylobacter fetus* subspecies isolated from sheath wash and obtained from bulls that were from in the North-West province in South Africa were transported in Steve medium was spread plated on the surface of Skirrow's agar (Tryptose blood Agar Base, Oxoid, UK) mixed with 7% sheep blood and supplemented with *Campylobacter* selective supplement (Skirrow, SR0069E, Oxoid, England). Plates were then incubated anaerobically at 37˚C for 72 hours using an anaerobic Jar 2.5 L (Oxoid, England) containing CampyGenTM sachet CN0025A (Oxoid, England) to produce a microaerophilic atmospheric condition for the growth of *Campylobacter fetus*. All the plates were firstly examined for growth after the 72 hours incubation period, and if growth was not observed, the plates were re-incubated for a further 48 hours. After the incubation period, all the plates were subjected to morphological identification (a small colony, mucoid, greyish, flat colony with irregular edges). The suspected plates of *Campylobacter fetus* were considered for further analysis (Acke et al., 2009).

Molecular assays for the identification of *Campylobacter fetus* subspecies

Genomic DNA extraction

The extraction of genomic DNA was carried out according to the standard molecular methods (Di Giannatale et al., 2014) using Zymo-Research Kit (Bio-Lab, South Africa) following the manufacturer's instructions. The extracted genomic DNA was then quantified spectrophotometrically using a Nanodrop® ND-1000. The genomic DNA was kept in fresh DNA tubes to serve as a DNA template for subsequent PCR.

Polymerase chain reaction

The confirmation of members of the genus *Campylobacters fetus* subsp in this study was performed using Polymerase Chain Reaction (PCR). A total volume of 50 µL containing 20 µL PCR 2xMaster Mix (Bio-Labs, Iso 9001, England), 4 µL template DNA, 22 µL nuclease DNA free water (Bio-concept ltd, ISO 9001, paradiesrain 14, Switzerland) and 4µL of oligonucleotide primer (Forward and Reverse) were prepared and mixed in PCR tubes. The DNA was amplified using a Thermal cycler (Bio-Rad, T100^{TM} Thermal cycler, Model: T100^{TM} Thermal cycler, serial number: 621BR19028, Singapore) as it has been presented in table 1 (Schulze et al., 2006; Wangroongsarb et al., 2011).

The DNA sequencing analysis

The amplified PCR products of isolated bacteria were sent to Inqaba Biotechnology (Pretoria, South Africa) for sequencing. The sequences and chromatograms were observed with Bio-systems. Forward and reverse sequences were compared and corrected for conformity. Blast program tools were applied to search for the sequences (Altschul et al., 1997) to find the closest match for each one in the Gen-Bank. The closest sequences were then downloaded and aligned with the original sequences using clustal and edited using Finch TV version 1.4.0.

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Analysis of the antimicrobial profile of *Campylobacter fetus* subspecies

The evaluation of the antimicrobial profile of *Campylobacter fetus* in this study was carried out using the Kirby-Bauer disc diffusion method (BAKWS and Turck, 1966; Washington and Wood, 1995). The results were interpreted based on the guidelines of the Clinical and Laboratory Standards Institute guidelines (institute, 2013). Multiple drug resistance was defined as resistance to two or more classes of antimicrobials tested. The antibiotics panel selected for screening *Campylobacter fetus* subspecies were the one mostly used in the case of campylobacteriosis in both veterinary and human medicine. The following 14 antibiotics were used: Erythromycin (15 mg), Azithromycin (15 mg), Streptomycin (10mg), Neomycin (30 mg), Gentamicin (10 mg), Ampicillin (10 mg), Amoxicillin (10 mg), Ciprofloxacin (5 mg), Enrofloxacin (5 mg), Norfloxacin (5 mg), Doxycycline (5 mg), Tetracycline (30 mg), Chloramphenicol (30 mg) and Nalidixic Acid (30 mg).

Genome sequencing, assembly, and annotation

The extracted genome DNA samples were fragmented using an enzymatic approach (NEB Ultra II FS Kit, BioLabs, England). The DNA fragmented results were sized selected (200-500 bp), using AMPure XP beads, the fragments were end-repaired, and Illumina specific adapter sequences were ligated to each fragment. Each sample was individually indexed, and a second size selection step was performed. Samples were then quantified, using a fluorometric method, diluted to a standard concentration (4 nM), and then sequenced on Illumina's NextSeq platform, using a NextSeq 300 cycle kit, following a standard protocol per manufacturer's instruction to generate a total number of 43,826,295 reads and 41,141,918 reads with 2X 300bp paired-end read length and the coverage was 40X respectively for *Campylobacter fetus* subsp *venerealis* NW_ME2 and *Campylobacter fetus* subsp. *fetus* NW_ME1.

The sequenced data generated were analyzed using appropriate bioinformatics tools, with default parameters, through the Kbase platform (Arkin et al., 2018). The sequenced data was filtered for low-quality reads and adapter regions using Trimmomatic- v0.36 (Bolger et al., 2014), and Fast QC-version 0.11.5 was employed to assess the quality of data. The genome assembly was performed using SPAdes- version 3.13.0 (Bankevich et al., 2012). The functional annotation of the entire draft assembly was carried out with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Haft et al., 2018) and Rapid Annotations using Subsystems Technology (RAST, version 11) (Aziz et al., 2008).

Statistical analysis

Data were analyzed using SPSS (version 23.0). Descriptive statistics such as frequencies and percentages were applied to determine the occurrence of bacteria and the antimicrobial profile of the isolated bacteria. Pearson's chi-square test of association was used to ascertain the association between the area and occurrence of bacteria and antimicrobial profile. Furthermore, the study used the Kruskal Wallis test and the Mann-Whitney's U to ascertain whether the resistance differed significantly across the antibiotics and whether the resistance differed across the bacterial species. For significant tests, cross-tabulations (Tables 2 and 3) were used to explain the association of the difference in resistance against different antibiotic profile of the isolated bacteria. Based on Kruskal Wallis test results, the p-value <0.05 was considered significant, and they have been presented in tables 4,5, and 6.

Table 1. Primers and PCR conditions

Primers	Conditions	References
MG3F (5'-GGTAGCCGCAGCTGCTAAGAT-3') MG4R (5'-TAG CTACAA TAA CGA CAA CT-3').	-Initial denaturation: 95°C for 15 seconds -Second denaturation: 96°C for 15 seconds - Annealing: 60°C for 60 seconds - Extension: 72°C for 90 seconds The processes of the second denaturation, annealing, and extension were repeated for 35 cycles and a final extension done at 180°C for 10 min	(Schulze et al., 2006)
CFCH57F (GCAAGTCGAACGGAGTATTA) CF1054R (GCAGCACCTGTCTCAACT)	-Initial denaturation: 94°C for 5 minutes - Second denaturation: 94°C for 60 seconds - Annealing at 50°C for 60 sec - Extension: 72°C for 60 seconds - The processes of the second denaturation, annealing, and extension were repeated for 30 cycles and a final extension done at 720C for 10 min	(Wangroongsarb et al., 2011).

DNAse free water, *Staphylococcus aureus* ATCC 25923 and *Campylobacter Jejune* ATCC® BAA-1153™ were used in this study as negative controls for PCR.

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Table 2. The occurrence of antimicrobial profile of *Campylobacter fetus* subsp. *fetus* isolated from sheath wash

Cross-tabulation of the antimicrobial profile of isolated bacteria				
Bacteria	**Antibiotics**	**Resistance**		
		Resistant	**Intermediate**	**Susceptible**
Campylobacter fetus subsp. *fetus*	Tetracycline	59.3%	29.6%	11.1%
	Doxycycline	44.4%	44.4%	11.1%
	Ampicillin	63.0%	29.6%	7.4%
	Amoxicillin	59.3%	29.6%	11.1%
	Erythromycin	25.9%	48.1%	25.9%
	Azithromycin	38.5%	34.6%	26.9%
	Neomycin	57.7%	19.2%	23.1%
	Streptomycin	48.1%	33.3%	18.5%
	Gentamicin	44.4%	25.9%	29.6%
	Ciprofloxacin	63.0%	22.2%	14.8%
	Nalidixic acid	85.2%	11.1%	3.7%
	Norfloxacin	33.3%	29.6%	37.0%
	Enrofloxacin	37.0%	33.3%	29.6%
	Chloramphenicol	51.9%	37.0%	11.1%
	Total	50.8%	30.6%	18.6%

Table 3. Phenotypic antimicrobial profile of *Campylobacter fetus* subsp. *venerealis* isolated from sheat wash

Bacteria	**Antibiotics**	**Cross-tabulation of the antimicrobial profile**		
		Resistant	**Intermediate**	**Susceptible**
Campylobacter fetus subsp. *venerealis*	Tetracycline	71.0%	25.8%	3.2%
	Doxycycline	54.8%	32.3%	12.9%
	Ampicillin	67.7%	16.1%	16.1%
	Amoxicillin	58.1%	22.6%	19.4%
	Erythromycin	48.4%	16.1%	35.5%
	Azithromycin	51.6%	22.6%	25.8%
	Neomycin	64.5%	32.3%	3.2%
	Streptomycin	71.0%	16.1%	12.9%
	Gentamicin	48.4%	29.0%	22.6%
	Ciprofloxacin	51.6%	19.4%	29.0%
	Nalidixic acid	61.3%	22.6%	16.1%
	Norfloxacin	45.2%	29.0%	25.8%
	Enrofloxacin	45.2%	19.4%	35.5%
	Chloramphenicol	67.7%	12.9%	19.4%
	Total	57.6%	22.6%	19.8%

RESULTS

Antimicrobial resistance profile of *Campylobacter fetus* subspecies

The overall results of the antimicrobial resistance profile revealed that 54.4% of the isolated bacteria indicated a resistance profile against the antimicrobial tested, 26.3% presented intermediate profile, while a small portion of isolated bacteria 19. 3% were observed to susceptible as they have been demonstrated in figure 1.

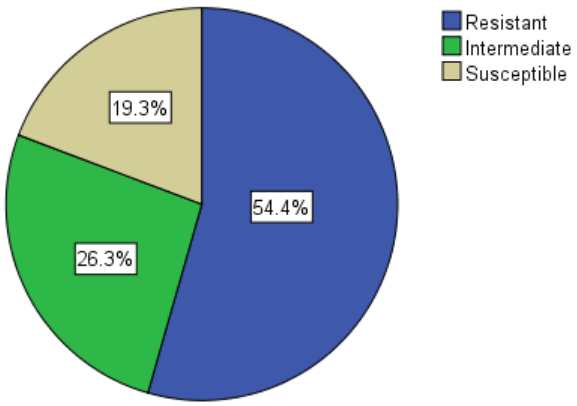

Figure 1. Overall profiles of *Campylobacter fetus* subspecies subjected to the antimicrobial test

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The occurrence of antimicrobial resistance profile of *Campylobacter fetus* subspecies

The analysis revealed that the antibiotic resistance of the *Campylobacter fetus* subsp *fetus* differed across the different antibiotics. For instance, it was observed that more strains of this subspecies were resistant to nalidixic acid (85.2%) than those that were resistant to ampicillin or ciprofloxacin (63%), as presented in table 2. Some strains of these subspecies were found to be more susceptible to norfloxacin (37%) than those that were susceptible to gentamicin (29.6%). It was also observed that 71.0% of *Campylobacter fetus* subsp.*venerealis* were resistant to tetracycline, 71.0% streptomycin, while 61.3% were resistant to nalidixic acid, 67.7% were resistant to chloramphenicol, and 35.5% of the isolates were found to be susceptible to erythromycin (Table 3).

Relationship between resistance profile and antibiotics

Pearson Chi-square test indicated that they were no association between the area from where samples were collected and the occurrences of isolated bacteria ($p>0.05$) as presented in table 5. The Kruskal Wallis test results indicated that the antibiotic resistance of *Campylobacter fetus* subsp. *fetus* differed significantly in accordance with the different tested antibiotics ($p<0.05$). However, there was no significant difference in the antibiotic resistance across the different antibiotics for the *Campylobacter fetus* subsp. *venerealis* ($p>0.05$), as presented in table 4. The Mann-Whitney's U test presented that the antibiotic resistance to nalidixic acid differed significantly across the two bacterial species ($p<0.05$) as presented in table 6.

Encoding of resistance genes in *Campylobacter fetus* subspecies using genome sequencing

The analysis revealed numerous genes involved in the resistance and virulence, as presented in tables 7 and 8. Additionally, it was observed that the present genome *Campylobacter fetus* subsp. *fetus* NW_ME1 contained six mobile element proteins, *Campylobacter fetus* subsp. *venerealis* _NW_ME2 was observed to have nine mobile element proteins. It was also observed that genes MurA (UDP-N-acetylglucosamine 1-carboxyvinyltransferase), Dxr (1-deoxy-D-xylulose 5-phosphate reductoisomerase) were exclusively found in *Campylobacter fetus* subsp. *fetus* NW_ME1. Furthermore, the encoded genes were located at different positions of the DNA strand and had a different DNA length, as is demonstrated in figures 2 and 7.

General genome feature and data availability

Data from our genome shotgun project of *Campylobacter fetus* subsp. *venerealis* NW_ME2 and *Campylobacter fetus* subsp. *fetus* NW_ME1 have been deposited at DDBJ/ENA/Gene Bank under the accession numbers JAATTN000000000 and JAAVIZ000000000. The version published in this paper was JAATTN010000000 and JAAVIZ010000000. The Raw reads were also submitted in NCBI/ SRA and are available in NCBI under Bio project PRJNA614613 and PRJNA614609. The genome of *Campylobacter fetus* subsp. *venerealis* NW_ME2 has a total length of 1,891,894 bp with an average G + C content of 33.2%, while *Campylobacter fetus* subsp. *fetus* NW_ME1 has a total length of 1,845,025 nucleotides with a GC content of 33.4%. Both isolates harbor resistance genes, and virulence factors were demonstrated in figures 8 and 9. The nucleotide sequences with high similarities (≥ 95%) were also submitted in the NCBI Gene bank and they are available under these accession numbers: MT138642; MT138643; MT138644; MT138645; MT138646; MT138647; MT138648; MT138649; MT138650; MT138651; MT138652; MT138653; MT138654; MT138655; MT138656; MT138657; MT138658; MT138659; MT138660; MT138661

Pathogenomics and phylogenetic analysis

The Pathogenomics analysis using Pathogen Finder version 1.1 revealed that our input organism *Campylobacter fetus* subsp. *fetus* NW_ME1 was predicted to be a human pathogen. The probability of being a human pathogen was 0.914, whose input proteome coverage (%) was % 0.16 and matched with pathogenic tree families (Table 9).

Phylogenetic analysis

Phylogenetic analysis constructed by inserting genome into species tree version 2.2.0 through Kbase platform. The phylogenetic analysis revealed that the multidrug-resistant *Campylobacter fetus* subspecies, which were isolated from sheath wash and obtained from bulls that were from the North-West province in South Africa, were highly similar and clustered tightly into one specific phylogenetic subgroups in the phylogenetic tree as it has been presented in figure 10.

Table 4. Kruskal Wallis test for association between the area in which the samples were collected and the occurrence of *Campylobacter fetus* subspecies

Bacteria		Resistance
Campylobacter fetus subsp. *fetus*	Chi-Square	34.652
	df	13
	p-value	0.001
Campylobacter fetus subsp. *venerealis*	Chi-Square	19.705
	df	13
	p-value	0.103

a. Kruskal Wallis Test; b. Grouping Variable: Antibiotic; df: the degree of freedom

Table 5 Association between the area in which samples were collected and the occurrence of *Campylobacter fetus* subspecies.

Chi-Square Tests	Value	df	p-value
Pearson Chi-Square	0.728	3	0.867

df: degree of freedom

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Table 6. Relationship between *Campylobacter fetus* subspecies resistance and different antibiotics tested against

Antibiotic	Statistical test	Resistance
Tetracycline	Mann-Whitney U	361.500
	P-value	0.287
Doxycycline	Mann-Whitney U	384.000
	P-value	0.552
Ampicillin	Mann-Whitney U	413.500
	P-value	0.926
Amoxicillin	Mann-Whitney U	400.000
	P-value	0.744
Erythromycin	Mann-Whitney U	378.500
	P-value	0.508
Azithromycin	Mann-Whitney U	361.500
	P-value	0.475
Neomycin	Mann-Whitney U	348.000
	P-value	0.308
Streptomycin	Mann-Whitney U	328.500
	P-value	0.108
Gentamicin	Mann-Whitney U	390.500
	P-value	0.638
Ciprofloxacin	Mann-Whitney U	356.000
	P-value	0.275
Nalidixic acid	Mann-Whitney U	314.500*
	P-value	0.039
Norfloxacin	Mann-Whitney U	356.000
	P-value	0.300
Enrofloxacin	Mann-Whitney U	410.000
	P-value	.887
Chloramphenicol	Mann-Whitney U	376.000
	P-value	0.448

a. Grouping Variable: Bacteria

Table 7. Genes resistant encoded in *Campylobacter fetus* subsp. *venerealis* NW_ME2 and *Campylobacter fetus* subsp *fetus* NW_ME1

Genes	Name	Function
YkkCD	Broad-specificity multidrug efflux pump *YkkC*	EFP-CAR
YKKCD	Broad-specificity multidrug efflux pump *YkkCD*	EFP-CAR
CmeABC	Multidrug efflux system, membrane fusion component => *CmeA*	EFP-CAR
CmeABC	Transcriptional repressor of CmeABC operon, *CmeR*	RME–ARGe
CmeABC	Multidrug efflux system, outer membrane factor lipoprotein => *CmeC*	EFP-CAR
CmeABC	Multidrug efflux system, inner membrane proton/drug antiporter (RND type) => *CmeB*	EFP-CAR
gidB	16S rRNA (guanine(527)-N(7))-methyltransferase	Ge-CR-Ab
rpoB	DNA-directed RNA polymerase beta subunit	AT-TSsp
rpoC	DNA-directed RNA polymerase beta' subunit	AT-TSsp
rho	Transcription termination factor Rho	AT-TSsp
Ddl	D-alanine ligase	AT-TSsp
MacB	Macrolide export ATP-binding/permease protein MacB	EFP-CAR
MacA	Macrolide-specific efflux protein MacA	EFP-CAR
7a-HSDH-like	Enoyl-[acyl-carrier-protein] reductase, 7-alpha-HSDH-like => refractory to triclosan	AT-REPLp
MurA	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	AT-SUSCsp
dxr	1-deoxy-D-xylulose 5-phosphate reductoisomerase	AT-SUSCsp
EF-G	Translation elongation factor G	AT-SUSCsp
EF-Tu	Translation elongation factor Tu	AT-SUSCsp
NimB	Nitroimidazole resistance protein NimB	A-Inc ENZ
Iso-tRNA	Isoleucyl-tRNA synthetase	AT-SUSCsp
S10p	SSU ribosomal protein S10p (S20e)	AT-SUSCsp
inhA, fabI	Enoyl-[acyl-carrier-protein] reductase [NADH]	AT-SUSCsp
Alr	Alanine racemase	AT-SUSCsp
PgsA	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	Prot alt CWCh-CAR
gyrB	DNA gyrase subunit B	AT-SUSCsp
gyrA	DNA gyrase subunit A	AT-SUSCsp
S12p	SSU ribosomal protein S12p	AT-SUSCsp
TolC	Outer membrane protein TolC	Out Mem EF Prot-CAR

EFP-CAR (Efflux pump conferring antibiotic resistance), RME–ARGe (regulator modulating the expression of antibiotic resistance genes), Ge-CR-Ab(Gene conferring resistance via absence), AT-TSsp (antibiotic target in susceptible species), AT-REPLp (antibiotic target replacement protein), AT-SUSCsp (antibiotic target in susceptible species), A-Inc ENZ (antibiotic inactivation enzyme), Prot alt CWCh-CAR (protein-altering cell wall charge conferring antibiotic resistance), Out Mem EF Prot-CAR (Out membrane efflux protein conferring antibiotic resistance).

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Table 8. Virulence factors encoded in *Campylobacter fetus* subspecies Isolated from sheath wash

Property	Genes	Product	Classification
Virulence factor	fliI	Flagellum-specific ATP synthase FliI	Motility, Chemotaxis, Invasion, Phase variation
Virulence factor	CheY	Chemotaxis regulator - transmits chemoreceptor signals to flagellar motor components CheY	Chemotaxis, motility
Virulence factor	GidA	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	Invasion
Virulence factor	flgC	Flagellar basal-body rod protein FlgC	Motility, Chemotaxis, Invasion, Phase variation
Virulence factor	pseB	UDP-N-acetylglucosamine 4,6-dehydratase (inverting)	Biosynthesis, synthesis of both functional flagella and lipopolysaccharides
Virulence factor	fliP	Flagellar biosynthesis protein FliP	Motility, Chemotaxis, Invasion, Phase variation
Virulence factor	flhA	Flagellar biosynthesis protein FlhA	Motility, Chemotaxis, Invasion, Phase variation
Virulence factor	fliM	Flagellar motor switch protein FliM	Motility
Virulence factor	fliQ	Flagellar biosynthesis protein FliQ	Motility, Chemotaxis, Invasion, Phase variation
Virulence factor	fliN	Flagellar motor switch protein FliN	Motility, Chemotaxis, Invasion, Phase variation
Virulence factor	CdtA	Cytolethal distending toxin subunit A	Cytotoxin production
Virulence factor	CdtB	Cytolethal distending toxin subunit B	Cytotoxin production
Virulence factor	CdtC	Cytolethal distending toxin subunit C	Cytotoxin production
Virulence factor	MotA	Flagellar motor rotation A	Motility
Virulence factor	MotB	Flagellar motor rotation B	Motility
Virulence factor	VirB3	Inner membrane protein forms channel for type IV secretion of T-DNA complex	Adhesion and colonization
Virulence factor	VirB4	ATPase required for both assembly of type IV secretion complex and secretion of T-DNA complex	Adhesion and colonization
Virulence factor	VirB10	Inner membrane protein of type IV secretion of T-DNA complex, TonB-like	Adhesion and colonization
Virulence factor	VirB8	Inner membrane protein forms channel for type IV secretion of T-DNA complex	Adhesion and colonization
Virulence factor	VirB9	Outer membrane and periplasm component of type IV secretion of T-DNA complex has secretin-like domain	Adhesion and colonization
Virulence factor	VirB9	Forms the bulk of type IV secretion complex that spans outer membrane and periplasm	Adhesion and colonization
Virulence factor	VirB5	Minor pilin of type IV secretion complex	Adhesion and colonization
Virulence factor	VirB1	Bores hole in peptidoglycan layer allowing type IV secretion complex assembly	Adhesion and colonization
Virulence factor	CiaB	*Campylobacter* invasion antigen B	Invasion, colonization
Virulence factor	SLP	S-Layer protein	Adherence, evasion
Virulence factor	VirB11	ATPase required for both assembly of type IV secretion complex and secretion of T-DNA complex	Adhesion and Colonization
Virulence factor	VirD4	Type IV secretion system protein	Adhesion and colonization
Virulence factor	VirD4	Like coupling protein	Adhesion and colonization
Virulence factor	fic	Fic domain protein, BT_4222 type	Adhesion and colonization
Virulence factor	TrbE	Conjugative transfer protein TrbE	Adhesion and colonization
Virulence factor	TrbD	Conjugative transfer protein TrbD	Adhesion and colinization
Virulence factor	TrbC	Conjugative transfer protein TrbC	Adhesion and colonization
Virulence factor	TrbI	Conjugative transfer protein TrbI	Adhesion and colonization
Virulence factor	TrbG	Conjugative transfer protein TrbG	Adhesion and colonization
Virulence factor	TrbF	Conjugative transfer protein TrbF	Adhesion and colonization
Virulence factor	TrbL	Conjugative transfer protein TrbL	Adhesion and colonization
Virulence factor	TrbJ	Conjugative transfer protein TrbJ	Adhesion and colonization
Virulence factor	TraT	IncF plasmid conjugative transfer surface exclusion protein TraT	Adhesion and colonization
Virulence factor	TrbB	Conjugative transfer protein TrbB	Adhesion and colonization

Table 9. Matched pathogenic families with *Campylobacter fetus* subsp. *fetus* NW_ME1

Accession No	Organism	Class	Protein function	Protein number	Identity (%)
CP000792	*Campylobacter. concisus*13826 complete genome	Epsilonproteo bacteria	Thymidylate synthase, flavin dependent	EAT99108	80.1
CP000767	*Campylobacter. curvus* 525.92 Complete genome	Epsilonproteo bacteria	Ribosomal protein S2	EAU00943	81.25
CP000538	*Campylobacter. Jejune* subsp. *jejune* 81 – 176 Complete genome	Epsilonproteo bacteria	Ribosomal protein L22	EAQ72756	85.45

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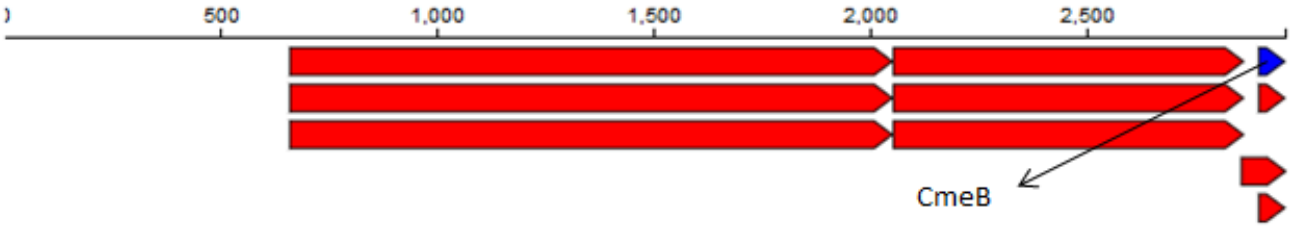

Figure 2. *Campylobacter fetus* subsp. *venerealis*: RND efflux system inner membrane transporter CmeB, Location: 2,897-2,960 (+ Strand). DNA Lengh 63

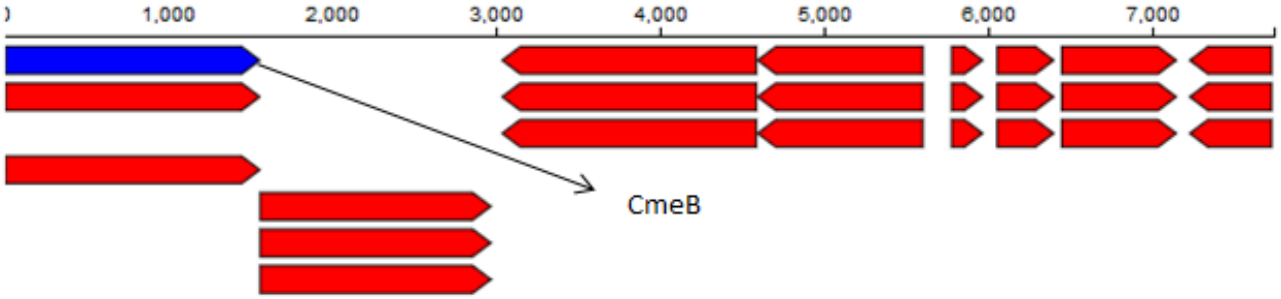

Figure 3. *Campylobacter fetus* subsp. *fetus*: RND efflux inner membrane transporter CmeB, Location: 2-1,562 (+ Strand). DNA Length 1560

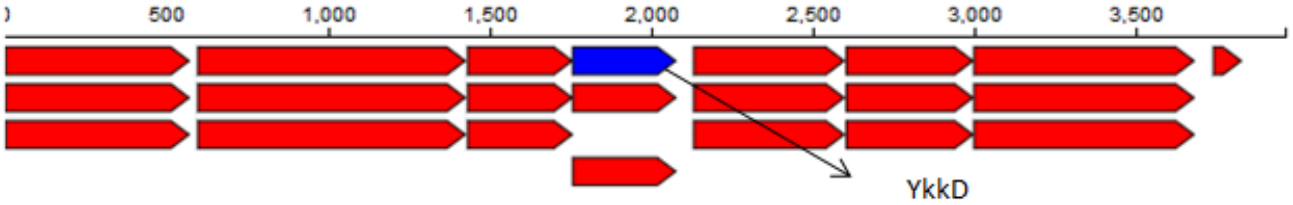

Figure 4. *Campylobacter fetus* subsp. *fetus*: Broad-specificity multidrug efflux pumps YkkD. Location 1, 757 – 2, 078 (+ Strand), DNA Length: 321

Figure 5. *Campylobacter fetus* subsp. *venerealis*: Broad-specificity multidrug efflux pumps YkkD. Location: 2, 471 – 2, 792 (- Strand), DNA Length: 321

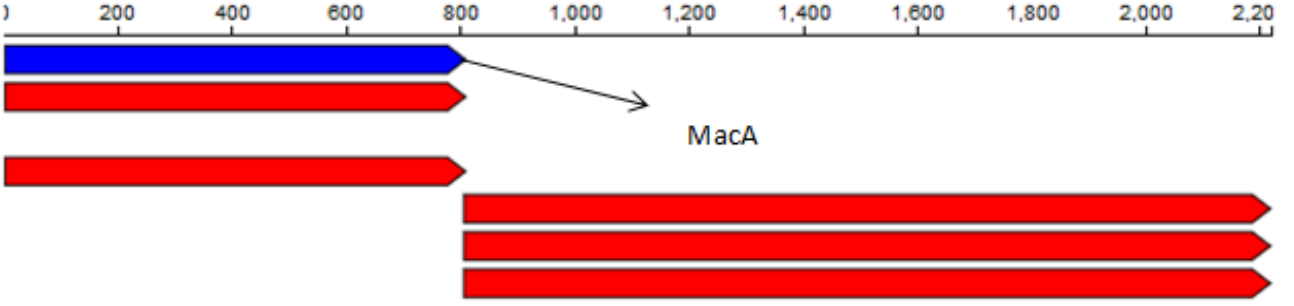

Figure 6. *Campylobacter fetus* subsp. *fetus*: Macrolide efflux protein MacA. Location: 2 – 809 (+ Strand), DNA Length: 807

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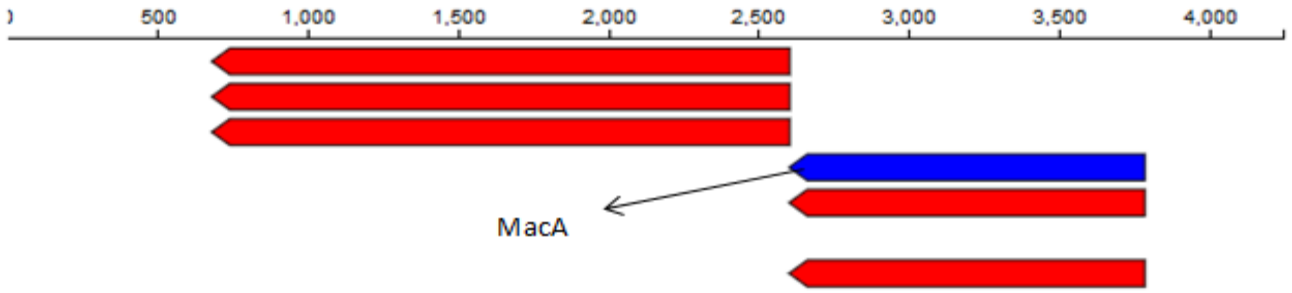

Figure 7. *Campylobacter fetus* subsp. *venerealis*: Macrolide specific efflux protein MacA. Location: 2, 599 – 3, 784 (-Strand), DNA Length: 1, 185

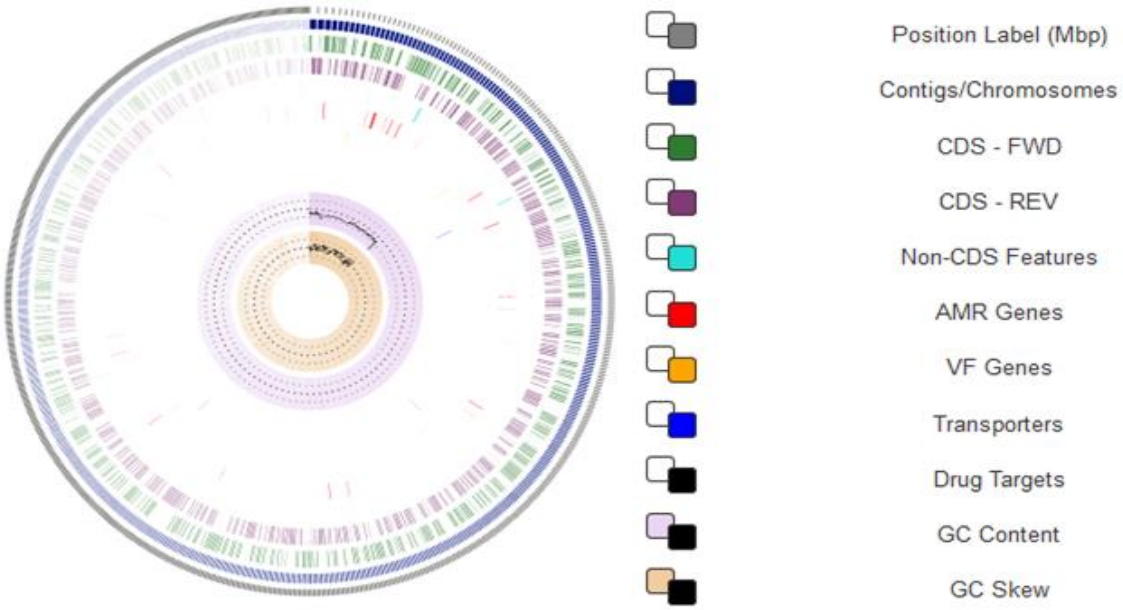

Figure 8. Circular genome map of *Campylobacter*. *fetus* subsp. *fetus* NW_ME1. The overview of the genome map of *Campylobacter fetus* subsp. *fetus* NW_ME1 generated via Patrick annotation version 3.6.3. The tracks on the viewer are displayed as concentric rings, from outermost to innermost: Position, Contigs, CDS-Forwards, CDS- Reverse, Non-CDS features, GC content, and GC skew

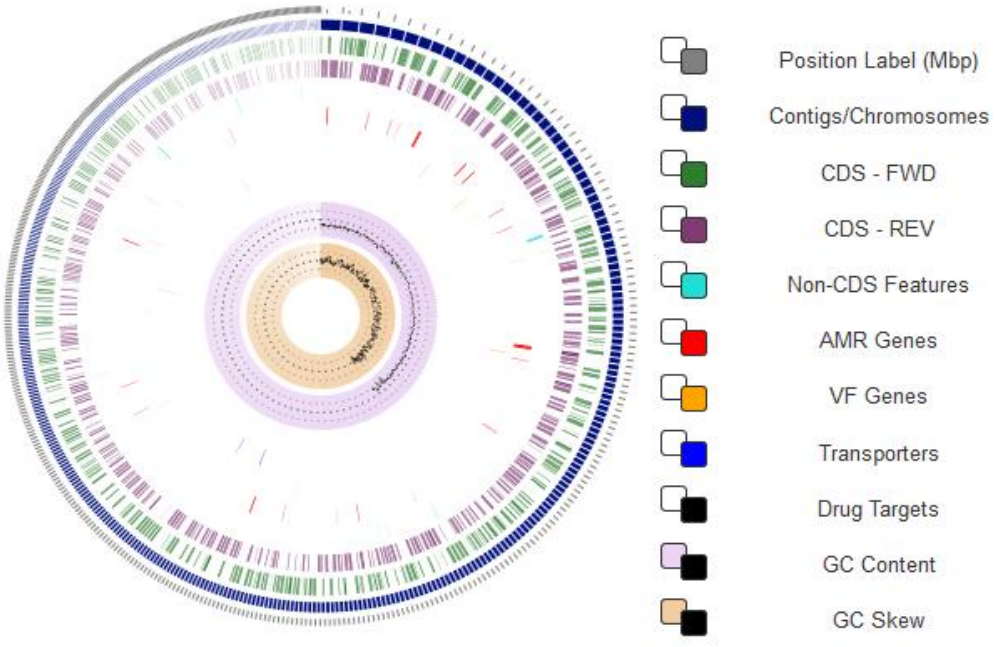

Figure 9. Circular genome map of *Campylobacter fetus* subsp. *venerealis* NW_ME2 generated using Patrick annotation-circular, from outside to inner ring contigs, CDS on the forward, CDS on the reverse, non CDS features, CDS with homology to known antimicrobial resistance genes, virulence genes, transports, drug targets GC content and GC skew.

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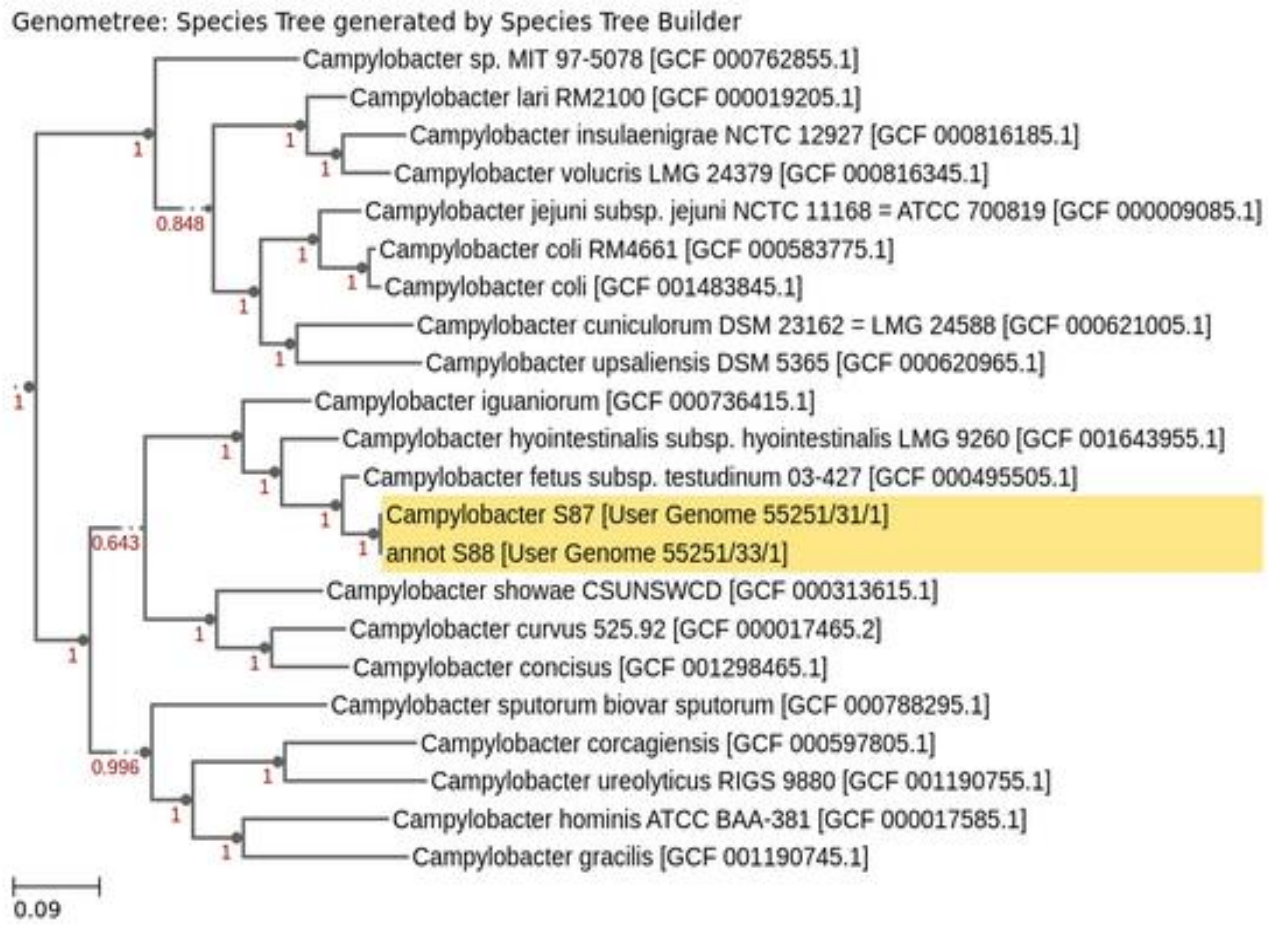

Figure 10. Whole-genome Neighbor joining phylogenetic tree. Phylogenetic tree of the complete genome of *Campylobacter fetus* subsp. *fetus* NW_ME1 and *venerealis* NW_ME2 align with 20 complete *Campylobacter* genomes; both *Campylobacter fetus* subspecies isolated in this study expressedmore significant homology. The digits indicated the distance or divergence between species (genome) used in the tree. The scale bar indicates 0.09 nucleotide substitutions per nucleotide position

DISCUSSION

The current study revealed that most of the *Campylobacter fetus* subspecies were multidrug-resistant to almost all the antibiotics tested against, as demonstrated in tables 2 and 3. The resistance of *Campylobacter fetus* subsp. *fetus* to different antibiotics was also reported in a study on the temporal prevalence of antimicrobial resistance in *Campylobacter* spp. from beef cattle in Alberta Feedlots in Canada. The researchers indicated that the *Campylobacter fetus* (39%) was resistant to doxycycline and tetracycline. The same study presented that isolated *Campylobacter fetus* (97%) were highly resistant to nalidixic acid (Tremblay et al., 2003). This corroborates with the findings of this study, that 85.2% of *Campylobacter fetus* subsp. *fetus* and 61.3% of *Campylobacter fetus* subsp. *venerealis* were observed to be highly resistant against nalidixic acid. The resistance of *Campylobacter fetus* subsp. *fetus* was also reported by Kenar and Gökçe (2019). The bacteria were isolated from the livers of slaughtered ruminants and aborted ovine fetuses. Their results, based on the disk diffusion method, revealed that the highest resistance was manifested against ciprofloxacin and tetracycline (45.45%) while the highest sensitivity was against gentamicin (90.91%) and chloramphenicol (100%) (Kenar and Gökçe, 2019).

These findings slightly differ from the results of the present study; though both studies used disc diffusion methods, it was observed that the resistance to ciprofloxacin and tetracycline was higher when compared with the previous study, respectively 63.0% and 59.3%. On the other hand, the susceptibility of gentamicin and chloramphenicol was very low, 29.6%, and 11.1%, respectively. Tremblay and Gaudreau (1998) evaluated the susceptibility profile for a total number of 59 *Campylobacter fetus* subsp. *fetus* against eight antimicrobial agents using the disc diffusion method, agar dilution, and E-test. The findings of their study concluded that *Campylobacter fetus* subsp. *fetus* was observed to be susceptible to meropenem, gentamicin, ampicillin, and imipenem. Further, the findings revealed that *Campylobacter fetus* subsp. *fetus* (27%) exhibited resistance to profile to tetracycline for both methods used disk diffusion and agar dilution (Tremblay and Gaudreau, 1998).

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The results of the current study were different from previous ones as isolated *Campylobacter fetus* were not all susceptible to ampicillin and gentamicin; furthermore, imipenem and meropenem were not used in this study; this difference might be related to the source of isolated bacteria. In the present study, the isolated bacteria were obtained from sheath wash, while the previous study was obtained from clinical samples. In this study, it was also observed the resistance of *Campylobacter fetus* subsp. *venerealis* to different antibiotics such as tetracycline, ampicillin, neomycin, streptomycin and other antimicrobial agents as presented in table 3.

The phenotypic antimicrobial resistance profile of *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* have never yet been investigated and reported in South Africa; this was one of the primary reasons for this study to investigate the genes involved in the mechanism of resistance in these two subspecies. These results aligned with observation revealed by researchers in the past few decades. An increasing number of isolated *Campylobacter* spp. have developed resistance to fluoroquinolones and other antimicrobials such as macrolides, aminoglycosides, and beta-lactams. In the current study, resistance against these classes of antibiotics was observed among the isolated bacteria. There is strong evidence indicating that the spread and dissemination of antibiotic resistance observed in many organisms are linked to the use of the antimicrobial agent in animal production and veterinary medicine for growth promoter and diseases prevention, which is also observed in *Campylobacter* spp. in humans (Wieczorek and Osek, 2013). In this study, both *Campylobacter fetus* subsp. *fetus* and *venerealis* expressed resistance to nalidixic acid, which confirmed that resistance to nalidixic acid had been proved to be an important marker to distinguish between *C. jejuni* and *C. fetus* (Taylor et al., 1985).

The WGS analysis revealed that isolated *Campylobacter fetus* subsp. *fetus* and *venerealis* harbored 28 resistance genes and 39 virulence factors. Among the encoded resistance genes, some have never been reported in *Campylobacter fetus* subsp. *fetus* and *venerealis*, such as gidB, ykkc, ykkcd, rho, MacB, and MacA, suggesting a new upcoming mechanism of resistance in these subspecies. Among the genes encoded, some are genetic determinants for fluoroquinolone (gyrA and gryB), for chloramphenicol, and tetracycline (ykkc and ykkcd), for aminoglycoside (gidB). Additionally, multidrug resistance efflux pumps (CmeABC), Broad-specificity multidrug efflux pump (ykkc and ykkcd), efflux pump conferring antibiotic resistance (MacA and MacB), and protein-altering cell wall charge conferring antibiotic resistance (PgsA) were among important CDS encoded. In *Escherichia coli* the expression of ykkc or ykkd was observing to induce phenotypic multidrug-resistant profile in bacteria, including resistance to neutral drugs, cationic and anionic, as it is shown in figure 4 (Jack et al., 2000). Further, the overexpression of these efflux pumps contributes to reducing susceptibility by decreasing the intracellular concentration of the antimicrobial agents (Nikaido and Pagès, 2012). This finding corroborates with the result of the current study, which observed the expression of different efflux pumps as they have been presented in table 7. The study revealed that collaborating MacB, in concert with the adaptor MacA and the outer membrane exit duct, TolC, could underpin the efflux of various drugs and export of virulence factors from multiple Gram-negative bacterial species. Additionally, a study revealed that in Gram-negative bacteria such as *Stenotrophomonas maltophilia*, *Escherichia coli*, and *Salmonella* spp MacA, MacB together with Tolc conferred resistance to numerous classes of antibiotics respectively aminoglycosides, macrolides as well as polymyxins (Nishi et al., 2003; Lin et al., 2014). These findings correlated with the results of the current study because MacA, MacB, and Tolc were encoded in both *Campylobacter fetus* subsp. *fetus* and *venerealis*, and this clarified why our isolated bacteria presented a resistance profile to macrolides and aminoglycosides antibiotics. On the other hand, in this study, the multidrug resistance efflux pumps (CmeABC) were encoded in both isolated bacteria. The efflux pump (CmeABC) has been pointed out to have an important role in the emergence of fluoroquinolone-resistant *Campylobacter* (Yan et al., 2006). It has also been reported that CmeABC is an important multidrug efflux pump in *Campylobacter* species and contributes to its intrinsic and acquired resistance to a broad spectrum of antibiotics, including fluoroquinolone resistance (Lin et al., 2002; Pumbwe and Piddock, 2002; Lin et al., 2003). The CmeABC is distributed in tripartite multidrug efflux pumps, which consist of CmeC (outer membrane protein), *CmeA* (periplasmic fusion protein) and CmeB (inner membrane efflux transporter) figure 3 (Lin et al., 2002).

This finding aligned with the results of the present study, that different operon of the efflux pump were detected in *Campylobacter fetus* subsp. *venerealis* and *Campylobacter fetus* subsp. *fetus*.. It has also been reported that these three proteins (CmeA), (CmeB) and (CmeC) are encoded by a three-gene operon (cmeABC) and function together to form a membrane channel that extruded toxic substrates directly out of *Campylobacter* cells (Lin et al., 2002). The CmeABC contributed actively to the intrinsic and acquired resistance of *Campylobacter* species to structurally diverse antimicrobials (Lin et al., 2002; Pumbwe and Piddock, 2002; Luo et al., 2003).

Additionally, this multidrug efflux pump (CmeABC) is well known to confer resistance against bile. It is required for the growth of *Campylobacter* spp. in media containing bile that serves to colonize the intestinal tract in animals (Lin et al., 2003). Furthermore, In this study, the transcriptional repressor of CmeABC, operon *CmeR* was also detected in isolated bacteria subjected to the whole genome analysis. It has been revealed that *CmeR* interacts with the cmeABC promoter and modulated the expression of cmeABC. On the other hand, the CmeR is known to improve the production and expression of a multidrug efflux pump (Lin et al., 2005). In the present study, the isolated bacteria were

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phenotypically resistant to macrolide tested against erythromycin and azithromycin. The genome sequencing reveals the presence of MacB (macrolide export ATP binding permease protein) as well as MacA gene as it has been shown in figures 6 and 7.

It has been reported that MacB, along with its periplasmic adaptor protein MacA, was first identified in *Escherichia coli* transporter genes as providing resistance to macrolide drugs in a strain lacking the major RND efflux pump AcrAB (Kobayashi et al., 2001). It is also known that these genes MacA and MacB have never been reported in *Campylobacter fetus* subspecies around Sound Africa, and this might be the first report. Another study investigated the role of MacAB in Gram-negative species such as *Stenotrophomonas maltophilia* and found that MacA and B together with Tolc conferred resistance to a variety of macrolides, aminoglycosides, and polymyxins in Gram-negative species (Nishi et al., 2003; Lin et al., 2014).

These findings corroborated with the results of this study, in which MacA and B were detected in isolated bacteria, and phenotypic resistance to macrolide and aminoglycoside was also observed. The auxiliary studies of the MacAB-Tolc framework examined by the Zgurskaya research facility illustrated that the ATPase movement of reconstituted MacB is subordinate to intaglio MacA. Assist, the same consider affirmed that MacAB interceded antimicrobial resistance in vivo requires the nearness of the external layer efflux channel TolC (Tikhonova et al., 2007). Tikhonova and his co-authors corroborated with the findings of this study that the presence of outer membrane efflux channel Tolc was detected in the isolated bacteria and observed in this study that the presence of gene gidB encoded with methyltransferase. This gene has been pointed out as conferring high-level antimicrobial resistance in Gram-negative such as *salmonella* (Mikheil et al., 2012). The research found out that the cancellation of gidB conferred resistance to the aminoglycoside antimicrobials, such as neomycin and streptomycin, whereas gidB protein had a noteworthy part within the modification of antimicrobial susceptibility (Mikheil et al., 2012). This might be the only explanation related to the aminoglycoside observed in this study. Moreover, alteration inside gidB has been linked to a low level of streptomycin resistance in some bacterial species and tall recurrence of the development of streptomycin-resistant mutants, which was too detailed in *Campylobacter upsaliensis* (Nishimura et al., 2007; Okamoto et al., 2007; Olkkola et al., 2015).

It has been moreover detailed that in microorganisms, gidB influenced antimicrobial sensitivity by a process that includes a post-transcriptional alteration (Mikheil et al., 2012). In this study, Broad-specificity multidrug efflux pump *YkkCD* was encoded in isolated bacteria; the gene was reported in Gram-positive *Bacillus subtilis* and *Escherichia coli.* It has been reported that in *Escherichia coli* strain DH5α when the gene ykkc and ykkd were expressed, a broad-spectrum multidrug resistance phenotype was observed to different antimicrobial agents, such as chloramphenicol, streptomycin, and tetracycline. Further resistance was also observed to a broader range of toxic compounds (Jack et al., 2000). Considering different genes encoded in our isolated bacteria subjected to the WGS, it is possible to conclude that the multidrug resistance observed in this study might be linked to these genes.

Moreover, some genes found in this study, such as gibB, ykkc, ykkcd, MacA, and MacB, have never been reported in *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* isolated from the sheath wash, their presence in this species might be considered as a new upcoming mechanism of resistance in *Campylobacter fetus* subspecies.

The WGS analysis also revealed presence of numerous genes (table 8) associated with bacterial motility and chemotaxis (fliI, flip, flhA, fliM, cheY, fliQ and fliN). Among them, FliM, FliG, and FliN are important proteins which form switch complex C ring. This complex interacts with the CheY and CheZ chemotaxis proteins, in addition to contact components of the motor that determine the direction of flagellar rotation. In the current study, flagellar motor rotation (MotA and B) were also detected. It has been reported that these flagella conferred swimming motility and distinctive darting motility, which was necessary for infection of humans to cause diarrheal disease and in animals to promote commensalism (Balaban and Hendrixson, 2011). The FliM and FliN proteins have also been reported in *Escherichia coli* and *Salmonella* spp, where they form complexes beneath FliG (Paul et al., 2011; McDowell et al., 2016). The middle domain of FliM contains a CheC-like domain that was not detected in the present study, which forms a continuous belt in the middle of the C ring (Park et al., 2006; Sircar et al., 2015).

In contrast, CheY, together with FliM impact the clockwise or counter-wise motor rotation for chemotaxis (Mathews et al., 1998; Szurmant et al., 2003; Ahn et al., 2013). It has also been reported that in bacterial FliG, FliM, and FliN proteins act as a molecular switch, enabling the motor to spin in both counterclockwise and clockwise directions (Morimoto and Minamino, 2014) while MotA and MotB act as a proton pathway to couple proton flow through the channel with torque generation (Blair and Berg, 1990). Another study reported that MotA/B complex of alkaliphilic *Bacillus clausii* could conduct both H^+ and Na^+ at different pH ranges (Terahara et al., 2008). Additionally study conducted on utilitarian bi-directional flagellar motor uncovered that the two rotor proteins, FliM and FliN, illustrated significantly enthusiastic turnovers between the motor and their cytoplasmic pools (Morimoto and Minamino, 2014). The FliM turnover played an important role in switching the direction of flagellar motor rotation (Delalez et al., 2010; Yuan et al., 2012). In the present study, the responsible gene for adhesion and colonization was identified as virB11. This gene has been detected in retail chicken breast and fecal samples obtained from chicken, suggesting that chicken is a

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potential factor that could invade human intestinal cells with the gene (Thakur et al., 2010; Lapierre et al., 2016). However, in this study, virB11 was detected from sheath wash obtained from the bull, which also suggested that cattle, in general, might constitute a risk factor in the transmission of these genes to humans. Present findings revealed that flagellar and adhesion genes were exceptionally preserved in *Campylobacter* species as suggested in other studies (Thakur et al., 2010; Koolman et al., 2015; Lapierre et al., 2016). In the current study, other virulence marker determinants, including Cytolethal Distending Toxin (A, B, and C), were present in the isolated bacteria. Other studies revealed that three subunits are required for full toxin activity (No et al., 2002; Lapierre et al., 2016). It has also been indicated that these genes played an important role in the host mucosal inflammatory response for Interleukin-8 (IL-8) released by intestinal cells (Perera et al., 2007). Moreover, these genes have been pointed to cause diarrhea by interfering with the division and differentiation of the intestinal crypt cells (Wieczorek et al., 2018). The ciaB marker, which plays a significant role both in the invasiveness and in the colonization of the epithelial cells, was also identified in *Campylobacter fetus* in this study. Moreover, this gene has been detailed to easier the conquering of epithelial cells of the gastrointestinal tract, where it delivered expanding harm to the columnar epithelial cells, which led to swelling and adjusting of attacked cells as a result of the cytotoxin and enterotoxin activities (Sopwith et al., 2006; Perera et al., 2007).

In this study, the type IV secretion system VirD4 was detected only in *Campylobacter fetus* subsp. *fetus* NW_ME1. The type IV translocation pilus is encoded by the gene VirB operon that consists of eleven genes (virB1-virB11); translocation is also dependent on an additional gene virD4, which encodes the type IV coupling protein T4CP (van der Graaf–van Bloois et al., 2016). These findings align with the results of the present study that virB5, virB1, virB9, virB8, virB4, virB3, and virD4 (like coupling protein) were detected in both isolated bacteria. In this study, fic domain protein was found in the isolated bacteria, and it has been reported that this gene had significant roles in multiple cellular processes, including disrupting the host cell processes that were important to pathogen survival and replication after transmission into eukaryotic cells (Worby et al., 2009). Furthermore, the surface layer protein was encoded in this study in both isolated bacteria, and it is well known that surface layer proteins are responsible for the pathogenicity of *Campylobacter fetus* subspecies (Blaser et al., 1987; Pei and Blaser, 1990). On the other hands, the study revealed that both the surface layer proteins and T4SS regions are suggested to have a role in the pathogenicity of *Campylobacter fetus* subspecies, and it might be possible that these features have a synergistic role in immune escape (van der Graaf–van Bloois et al., 2016). Among the important genes encoded in this study, it was also observed the presence of GidA tRNA uridine 5-carboxymethyl aminomethyl modification enzyme, which is presented in table 8. This gene has been considered to be part of the major virulence mechanism in Gram-positive and negative bacteria such as *Escherichia coli*, *Salmonella* spp and *staphylococcus* but never been reported in *Campylobacter fetus* subspecies (Shippy and Fadl, 2014).

CONCLUSION

The findings of this study indicated that *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* subjected to antimicrobial tests were observed to be multidrug resistance to almost antibiotics tested against. The WGS analysis of *Campylobacter fetus* subsp. *fetus* NW_ME1 and *Campylobacter fetus* subsp. *venerealis* NW_ME2 revealed the presence of different genes involved in the mechanism of resistance of the isolated bacteria and virulence factors that are susceptible to initiate the disease. Furthermore, the study encoded genes that have never been reported in *Campylobacter fetus* subsp. *fetus* and *venerealis* such as Broad-specificity multidrug efflux pump (ykkc and ykkd), 16S rRNA (guanine^{527}-N^{7}) methyltransferase (gidB), efflux pump conferring antibiotic resistance (MacA and MacB) suggesting a new resistance mechanism among these subspecies.

DECLARATIONS

Acknowledgments

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Authors' contributions

Mulunda Mwanza provided the funds for the project, participated in the drafting of the research proposal, and participated in the revisions of the final draft of the manuscript. Lubanza Ngoma provided technical support in the laboratory. Mpinda Edoaurd Tshipamba, designed the project, elaborated its plan, collected the samples, conducted the laboratory work, analyzed the results, and wrote the final manuscript for the publication.

Competing interests

The authors of this study declared that there is no financial conflict related to this work, which can negatively impact its publication.

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