2020, Scienceline Publication
World's Veterinary Journal
World Vet J, 10(4): 465-480, December 25, 2020

ISSN 2322-4568

DOI: https://dx.doi.org/10.29252/scil.2020.wvj57-1

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

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ORGINAL ARTICLE
pii: S232245682000057-1-10
Received: 27 Sept 2020
Accepted: 10 Nov 2020

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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