

AKADÉMIAI KIADÓ

Acta Microbiologica et  
Immunologica Hungarica


69 (2022) 4, 332–344

DOI:  
[10.1556/030.2022.01900](https://doi.org/10.1556/030.2022.01900)  
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## RESEARCH ARTICLE



# Genome analysis of clinical isolate of *Campylobacter fetus* subspecies *fetus* MMM01 from India reveals genetic determinants of pathogenesis and adaptation

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Received: October 9, 2022 • Accepted: October 25, 2022  
Published online: November 7, 2022

## ABSTRACT

In this study we report the whole genome sequencing (WGS) based analysis of blood-borne *Campylobacter fetus* subsp. *fetus* MMM01 isolated from a diabetic patient to obtain deeper insights in to the virulence and host adaptability. The sequenced genome of *C. fetus* subsp. *fetus* MMM01 along with reference genomes retrieved from NCBI was subjected to various *in-silico* analysis including JSpecies, MLST server, PATRIC server, VFAnalyzer, CARD, PHASTER to understand their phylogenetic relation, virulence and antimicrobial resistance profile. The genome had a size of 1,788,790 bp, with a GC content of 33.09%, nearly identical to the reference strain *C. fetus* subsp. *fetus* 82-40. The MLST based phylogenetic tree constructed revealed the polyphyletic branching and MMM01 (ST25) was found to be closely related to ST11, both belong to the sap-A serotype which are more common in human infections. VFAnalyzer identified 88 protein-coding genes coding for several virulence factors including *Campylobacter* adhesion to fibronectin, flagellar apparatus, cytolethal distending toxin operons and *Campylobacter* invasion antigen proteins which enhance the virulence of bacteria along with resistance genes against antibiotics including fluoroquinolone, chloramphenicol, tetracycline, and aminoglycoside in MMM01, which points to enhanced survival and pathogenicity of this zoonotic pathogen. It was interesting to find that MMM01 lacked FGI-II island found in most of the clinical isolates, which encoded CRISPR Cas and prophage II regions. More details about the complexity and evolution of this zoonotic pathogen could be learned from future studies that concentrate on comparative genome analysis using larger genome datasets.

## KEYWORDS

zoonotic pathogens, *Campylobacter fetus*, genome sequencing, comparative genome analysis

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## 1. INTRODUCTION

Campylobacteriosis is a serious bacterial infection caused by *Campylobacter* characterized by diarrhoea (often dysentery), abdominal pain, fever, headache, nausea, and vomiting being the most common clinical symptoms and can last 3–6 days [1]. *Campylobacter fetus*, a pathogen of campylobacterial infections, is gram-negative, motile, spiral-shaped, microaerophilic of the family Campylobacteraceae, and is a widely studied pathogen of livestock such as cattle, sheep, and horses [2]. They are sub-grouped as *C. fetus* subsp. *venerealis* (CFV), *C. fetus*



subsp. *fetus* (CFF) and reptile associated *C. fetus* subsp. *testudium* (CFT) [3–5]. The subspecies within *C. fetus* show a strong affinity towards host preferences compared to other bacterial pathogens [6, 7]. With a relatively small genome size of approximately 1.8 Mbp and a high clonal structure, *C. fetus* subspecies are a well-suited model organism to study pathogen-host adaptation due to their differences in host preference and tissue tropism [2]. CFF infections are severe in ovine groups compared to bovine, where they colonize in the gut and uterus of the animal causing infection following oral transmission [8, 9]. In addition to being pathogenic to hoofed animals, CFF are opportunistic pathogens of humans, transmitted mainly via faeces and contaminated food including processed porcine and cattle meat [10]. Infection can also occur via handling of contaminated equipment or during semen collection in veterinary settings [11, 12]. In humans, it causes systemic infections and acute diarrhoea especially in juveniles, the elderly and immunocompromised individuals resulting in mortality of up to 14% [13]. Though recognized as a leading pathogen causing bacteraemia in humans, its role in mortality is greatly underestimated [14, 15].

The organism is known to colonize the intestine and kidneys, causing acute diarrhoea and systemic infections. CFF has also been isolated from the blood of patients with septicaemia, endocarditis, aneurysms and meningoencephalitis [3]. Complications generally occur among the immunocompromised, HIV positive and aged individuals. In pregnant women, it causes uterine and placental infections; perinatal infections may lead to abortions [16]. It is surmised that the infection can occur at any stage of pregnancy from early to childbirth and has also been reported as the cause of meningitis in the neonates [17, 18]. The first report of *C. fetus* (then known as *Vibrio fetus*) causing abortion in humans was reported in 1947 [19]. The CFF strains have a plethora of virulence factors that assist in their proliferation and infection.

The surface layer (S-layer) is a para-crystalline protein structure made up of S-layer proteins (SLP's) that form a capsule-like cover present on the surface and helps in evading the immune reactions from the host [20, 21]. These SLPs bypass the phagocytosis-mediated host immune system and provide resistance to complement-mediated apoptosis by preventing binding of C3b. This effectively leads to the survival and proliferation of the pathogen within its host [20] while the SLPs also enhance the dissemination of CFF through the bloodstream and spread of infection within the human host [9, 20]. The SLP's are encoded by SLP-encoding genes (*sap* genes), and are evolved to avoid antibody mediated killing through high frequency antigenic variation at DNA level [21, 22]. This helps the pathogen to evade antibodies that specifically target the SLPs by allowing the bacteria to hide from the host immune system and result in repeated infections eventually causing systemic problems [23, 24].

The glycine tolerance test has been used as the gold standard to differentiate between the subspecies in particular, the CFF and CFV. The CFF is tolerant to glycine and

shows luxuriant growth compared to CFV [25]. In recent years, molecular techniques have been developed to differentiate between the subspecies including Multilocus Sequence Typing (MLST) and Pulsed Field Gel Electrophoresis (PFGE) [3]. However, very few studies have focused on whole genome sequencing and comparative genome analysis amongst the CFF strains isolated from diverse sources. Human pathogenic CFF needs to be further explored for their genome composition and evolution as opportunistic pathogens in humans. A comparative study on CFF isolated from clinical settings with its environmental counterparts would give an insight into evolution and acquisition of virulence traits by the bacteria.

In this study, we analyzed the whole genome of a clinical strain to understand the genome composition, distribution of the virulence genes that contribute to the ability of this pathogen to cause infection, and resistance genes that enable the bacterium to persist and proliferate in their ecological niches. The study also focuses on diversity and genetic relatedness among the *Campylobacter* sp. strains isolated from various sources across the globe.

## 2. MATERIALS AND METHODS

### 2.1. Genome sequencing, assembly and validation

Sequencing the whole genome of CFF MMM01 was performed earlier using the Ion Torrent PGM platform with 200-bp fragmentation chemistry (Bioserve Biotechnologies, India) [26]. Before proceeding to assembly, the quality of single ended raw reads was validated using the FASTQC tool [27] and trimmed to remove sequencing adapters and low-quality reads using the Trimmomatic software v.0.38.0 [28]. Geneious Prime R9 (<https://www.geneious.com>) was used for reference-based assembly of the trimmed reads against the genome of clinical strain CFF 82-40. To assess the quality of assembled sequences, the Quality Assessment Tool for Genome Assemblies (QUAST) was used [29]. The taxonomic identity of the genome sequence was confirmed using JSpecies v1.2.1 to measure the pairwise average nucleotide identity (ANI) with the reference sequence CFF 82-40 [30]. Further, the GGDC 2.0 server was used to perform digital DNA-DNA hybridization [31]. For comparative analysis, 19 CFF strains and 5 *C. jejuni* genome sequences were retrieved from the NCBI GenBank (Table A1). By calculating the Average Nucleotide Identity (ANI) using JSpecies, the sequence similarity of the sequenced strain was compared to genome sequences retrieved from NCBI GenBank [30]. The calculated ANI values were tabulated, and Heatmapper ([www.heatmapper.ca](http://www.heatmapper.ca)) was used to visualise them.

### 2.2. Multilocus sequence typing and phylogenetic analysis

The sequence type (ST) of the CFF MMM01 was investigated based on allelic profiles of seven housekeeping genes using the MLST 2.0 server [32]. A Neighbour-Joining phylogenetic tree was constructed using the concatenated



sequence alignments of the seven housekeeping genes of CFF MMM01 and global sequences with default parameters, and visualised using iTOL v3 [33].

2.3. Genome annotation and characterization

On the PATRIC server, structural gene prediction and functional annotation was performed using the Rapid Annotations Subsystems Technology tool kit (RASTtk) [34]. VFanalyzer was used to detect the presence of virulence factors in the genome of CFF MMM01 [35]. PATRIC and the CARD web portal was used to predict the presence of antibiotic resistance genes.

2.4. Fetus genomic island (FGI) analysis

CFF MMM01 genome was visualised in the BLAST atlas, using the Blast Ring Image Generator (BRIG) v 0.95 [36] with CFF 82-40 genome as reference sequence. Additionally, the whole genome data was subjected to reference-based alignment using the MAUVE alignment tool [37] in Geneious Prime 9 to compare the genome composition of CFF MMM01 and reference strain CFF 82-40. The aligned sequences were analysed for genomic islands that conferred virulence. The EasyFig v2.2.2 software was used to create a linear genomic comparison map [38]. PHASTER [39], a phage search tool, was employed to search prophage sequences in the assembled genome of CFF MMM01.

3. RESULTS

3.1. Genome properties and characterization

The sequence quality of the 209,580 raw reads generated by the Ion Torrent PGM platform, with 33% GC content, was evaluated and qualified for downstream analysis. The trimmed reads, when subjected to reference-based genome assembly, resulted in a genome of 1,788,790 bp in length and GC content of 33.09% with 17.5× coverage. Table 1 shows a summary of the assembly and basic genome statistics.

Table 1. Summary of the genome assembly QC and annotation details of CFF-MMM01 sequenced genome

Raw reads	209,580
Raw reads post trim	208,802
Total length	1,788,790 bp
GC%	33.09
Contig N50	169,872
Coverage	17.5x
No. of CDS	2,105
No. of tRNA	41
No. of rRNA	3
Proteins with functional assignments	1,728
Hypothetical proteins	377
% G + C difference	0.22
ANIb (%) to reference CFF 82-40	99.58%
dDDH (%) identity to CFF 82-40	97.40%
Prophage region identified	1
Multilocus sequence type (MLST)	ST-25

Analysis from RAST identified 2,105 coding DNA sequences, 41 tRNA sequences, and 3 rRNA sequences. Among the CDS identified, 1,728 genes were assigned functional categories and 377 predicted as hypothetical proteins. JSpecies was used to calculate a genome-to-genome comparative analysis of average nucleotide identity (ANI), which was visualised in a heatmap (Figure A1). Clinical isolates were found to have a higher genetic similarity than environmental isolates. The highest ANI was shared by CFF MMM01, CFF 82-40, and CFF H1-UY.

3.2. MLST and phylogenetic analysis

CFF MMM01 was identified as sequence type (ST) 25 by MLST profiling as seen in Table A2. This sequence type was found to be closely related to Cff06569, a bovine pathogen identified as ST-11 (pubMLST id: 299), which was isolated in Belgium in 1985 from a calf with systemic infection. The phylogenetic tree constructed using concatenated housekeeping genes (Fig. 1) revealed polyphyletic branching between the *C. fetus* strains of clinical and environmental (including veterinary) origin. The CFF MMM01 was also found to be closely related to ST 11.

3.3. Virulence profiling

VFanalyzer identified 88 protein-coding genes in the genome of CFF MMM01, coding for 10 different virulence factors from 7 different VF classes (Table A3). The genome of CFF MMM01 also revealed three sets of Cdt toxin operons. In CFF MMM01, the *sap* genes can be seen mapped alongside 82-40 found within FGI1. A set of type II toxin-antitoxin systems (*hipA* and *yafQ*), were found nestled in this region as seen in Fig. 2. The genome of CFF MMM01 contained a majority of the structural and functional components of the flagellar apparatus. as well as three sets of Cdt toxin operons. It did not harbor genes encoding the Type III Secretion System (T3SS) or Type VI Secretion System (T6SS). A *Cia* gene, *ciaB*, was also detected in the MMM01 genome and the S layer protein, which consists of *sapA* and *sapA*-like genes, was observed to be present only in CFF strains. Only CFF strains 82-40 and 493 lacked genes for capsular biosynthesis. Genes encoding secretion systems were found in four CFF strains (CFF - 006A 0059, 98/v445, NW ME1, and 13/344) but not in our genome sequence. CFF MMM01 and CFF 82-40 were also compared with five *C. jejuni* strains in the interspecific comparison (Figure A2). It was also noted that CFF MMM01 had a partial gene cluster that is involved in capsular biosynthesis and transport. The Cytolethal Distending Toxin gene, was predominantly present in CFF strains (8 in CFF 82-40 and 9 in CFF MMM01) (3 each in other CFF strains) as compared to *C. jejuni* strains, there was no interspecific variation in the genes coding for motility and export apparatus.

3.4. Antimicrobial resistance profiling

Potential antimicrobial-resistant (AMR) traits in CFF MMM01 were identified using the Comprehensive Antibiotic Resistance Database (CARD). Also, the annotated genome was analysed



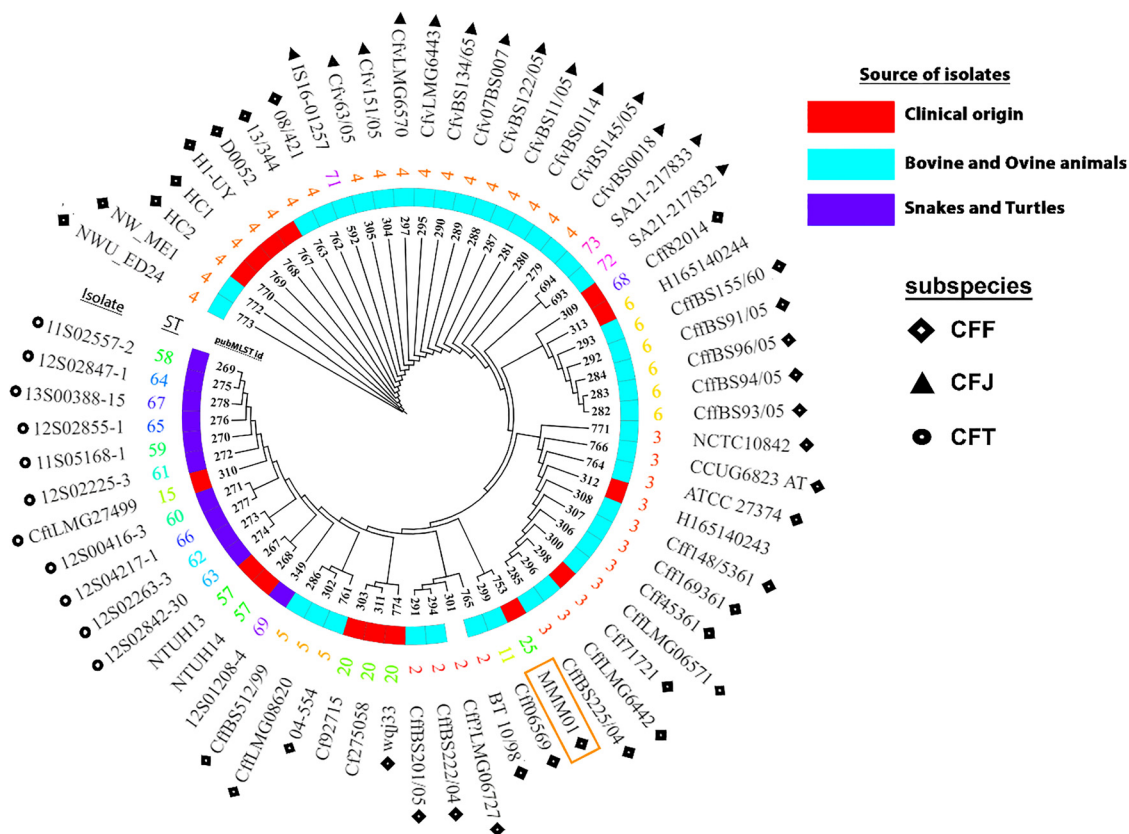


Fig. 1. MLST based phylogenetic analysis of *Campylobacter fetus* strains available in pubMLST database. The query strain is marked with ‘a star’

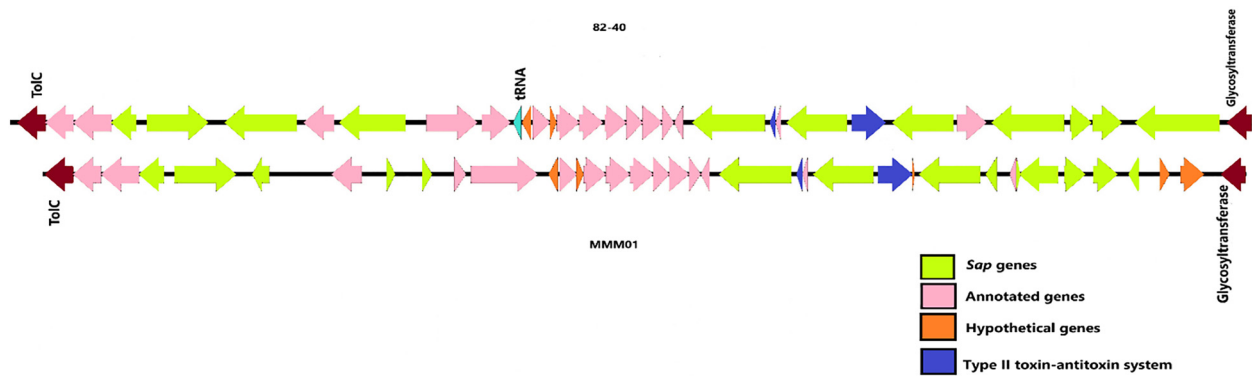


Fig. 2. Linear genomic representation of the *sap* genes aligned between the reference strain 82-40 and the MMM01 found inside the FGI-I genomic island

for the presence of antimicrobial resistance genes using PAT-RIC server (Table A3).

Antibiotic resistance found in CFF MMM01 was encoded by the genes, *cmeABC*, *macA*, *macB*, and *ykkCD*, and also multidrug resistance efflux pumps. The isolate had the most gene clusters identified by RAST annotation for the Multi-drug Resistance Efflux Pump subsystem, as shown in Figure A3. Fluoroquinolone, chloramphenicol, tetracycline, and aminoglycoside resistance genes were present in the sequenced genome.

3.5. Genomic islands comparison

The genome of CFF MMM01 mapped against the genome of CFF 82-40 using BRIG, showed most of the regions as identical (Fig. 3). CFF MMM01 contained the Fetus Subspecies Definition Region (FSDR), the conserved region, and the subspecies confirmation marker. The reference strain CFF 82-40 harbors two Fetus Genomic Islands (FGIs), FGI-I and FGI-II. FGI-I (size - 65,092 bp) is located between genomic regions 431,396 bp and 496,461 bp while FGI-II (size - 23,758 bp) is located between regions 654,904 bp and





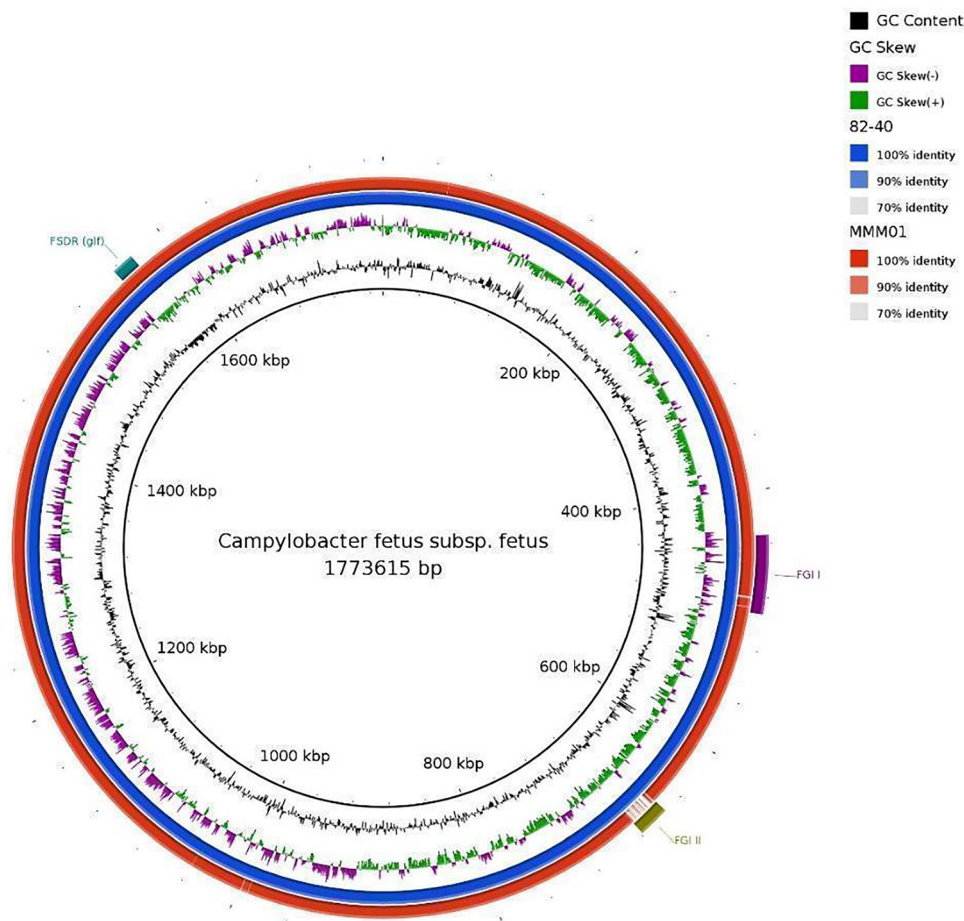


Fig. 3. The genome atlas of *Campylobacter fetus* subsp. *fetus* MMM01 generated using BLAST Ring Image Generator (BRIG) against reference genome *C. fetus* subsp. *fetus* 82-40

678,662 bp. Except for a few incomplete genes, FGI-II was missing from its corresponding genomic region in CFF MMM01. MAUVE alignment was used to confirm the missing region of FGI-II, and visualised using Easyfig (Fig. 4). Between ORFs tRNA and *gp27*, the genome of CFF MMM01 showed complete absence of regions harboring 30 ORF, including CRISPR Cas and Prophage II regions

earlier identified in the genome of reference strain CFF 82-40. PHASTER identified an incomplete prophage region that shares similarity to PHAGE\_Altero\_vB\_AmaP\_AD45\_P1\_NC\_021532 (28.8 Kb) located at 1,148,064 bp -1,176,879 bp region on the sequenced genome of this study. This prophage sequence comprised 36 ORFs and a GC content of 29.97%.

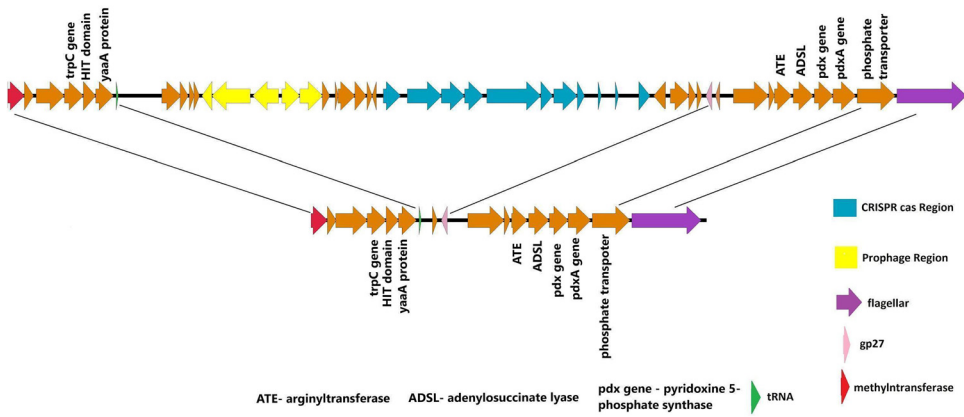


Fig. 4. Linear genomic representation of the FGI 2 region in the 82-40 strain (top) and the MMM01 strain (bottom). The FGI 2 region including a CRISPR-Cas's region and a Prophage region is completely absent in the MMM01 strain



## 4. DISCUSSION

*C. fetus* is a zoonotic pathogen that has been linked to systemic illness in people with immunosuppression, infancy, liver disease, malignancy, and diabetes mellitus [18], with infections that could be fatal. Using MALDI TOF MS, *C. fetus* was isolated and identified from the blood culture of a 60-year-old patient with type II diabetes and chronic kidney disease in 2013 [26], which following confirmation as CFF strain, was sequenced, genome data submitted to NCBI GenBank and assigned accession numbers. The study presents genome wide analysis of CFF MMM01 and comparison to other global sequences available.

The query genome MMM01, used in this study, was found to be nearly identical to the genome of the reference strain CFF 82-40 (1.77 Mbp) [2]. The reduced genome size is a common characteristic of pathogens that exhibit host tropism [6]. However, the genome size of isolates among members of *C. fetus* subspecies varies greatly, ranging from 1.2 to 1.9 Mb [14]. As the ANI and dDDH results have confirmed, the sequenced strain in this study belongs to the same subspecies, CFF, since the dDDH % value exceeds the threshold limit (dDDH >79%) [40]. And as the heatmap clearly shows the clinical strains sharing more genetic similarities which could be due to the strains being isolated from patients within the same hospital. The highest ANI was shared by CFF MMM01, CFF 82-40, and CFF H1-UY, a human pathogen isolated from rural workers and belonging to the bovine-associated sequence type. Similarly, MMM01 is a clinical isolate with a sequence type that was found to be closer to that of bovine species.

Due to the difficulty in distinguishing between isolates, more reliable molecular techniques such as MLST need to be developed for subspecies and isolate discrimination. The MMM01 sequence type (ST 25) was found to be closely related to the strain Cff06569, which is a bovine pathogen identified as ST-11 (pubMLST id: 299), which was isolated in Belgium in 1985 from a calf with systemic infection. A study by Iraola et al (2017) [41] has suggested that isolates identified with a well-known bovine pathogen sequence type ST-4 have been observed to infect humans implying its zoonotic potential. Looking at the phylogenetic tree it was evident that the sequence type and source of the isolates were independent of each other. However, when compared to other human and veterinary isolates, the majority of *C. fetus* isolates of reptile origin were genetically distant [4]. The majority of *C. fetus* isolates belonged to ST – 4, 3, and 6, which included both clinical and veterinary isolates [42, 43]. The congruence of ST in *C. fetus* has been found to have a direct correlation with their *sap* types and ST 11, as identified to be related to the CFF MMM01 strain, both are known to belong to the *sap*-A serotype. Based on 16S rRNA, MLST phylogeny, and DNA hybridization analysis, the proposed evolutionary history of three CFF serotypes suggests that all of them are capable of infecting humans. Human infections were most common in the *sap*-A serotype, followed by *sap*-B, and both evolved from a mammalian

ancestor [14]. The evolutionary stability of the *C. fetus* isolates could explain the diversity in MLST and inconsistency in differential branching of isolates belonging to different subspecies [42].

Delving into the physical structure of this pathogen we have the S layer, which is a capsule-like assembly of paracrystalline SLPs with molecular weight ranging from 97 to 149 kDa and forms the outermost surface of the CFF cell [2]. The S layer proteins differ between CFF strains of different types. Antigenic variation causes phenotypic switching of SLPs, resulting in a high frequency of DNA rearrangements at the *sap* locus in the genome, thus allowing CFF strains to successfully evade the host immune system [2, 24]. CFF MMM01 is of *sap*-A type and hence its genome revealed six *sapA*-like genes and one *sapA* gene. *sapA* homologs have previously been found clustered together and expressed by unique *sapA* promoters [24]. This presentation is crucial to the colonization and immune evasion from the host demonstrated by the bacteria. According to studies, *sap*-A type CFF strains are more common in human infections as compared to *sap*-B type strains found more commonly in non-human hosts [44]. The type II toxin-antitoxin systems (*hipA* and *yafQ*) found in the FGI1 region in the query strain are known to be small genetic elements made up of toxic proteins and their corresponding antitoxin protein [45]. The cytolethal distending toxin (Cdt) is a membrane-associated tripartite protein toxin found in many bacteria, but notably in *Campylobacter* species, where it is a major virulence factor. The three subunits of this multi-subunit protein, CdtA, CdtB, and CdtC are encoded by an operon containing three closely related corresponding genes, *cdtA*, *cdtB*, and *cdtC* [46]. As seen, the genome of CFF MMM01 identified three sets of Cdt toxin operons and the Cdt mechanism requires the expression of all the three genes in its operon. The CdtB subunit's DNase-I-like activity, prevents cell division by arresting the cell cycle by causing double-stranded DNA breaks; the CdtA and CdtC subunits mediate as binding and internalisation factors to the host cell membrane [47]. It is apparent that the Cdt toxin may act as one of the major virulence attributes found in the genome of CFF MMM01, enhancing virulence by boosting persistence of infection in humans. The immunopathological interaction of *Campylobacter* spp. has been well documented as it activates a series of signalling pathways in the host, resulting in an inflammatory response and upregulation of cytokine production [48]. The ability of the pathogen to adhere to host cells is primarily performed by adhesion factors, and determines the success of infection in response to the host immune response [49]. The *Campylobacter* adhesion to fibronectin (*cadF*) gene which is a highly conserved, helps the pathogen adhere to and enter epithelial cells by binding to the extracellular matrix protein, fibronectin and interacting with integrin receptors [50]. The motility genes *flaA* and *flaB*, coding for flagellar filaments, assist *cadF* to reach target cells [49]. As also noted both the T3SS and T6SS, considered key virulence attributes of most pathogens including some *Campylobacter* spp., were absent in the CFF MMM01 genome [47]. It is likely that the invasion proteins



in CFF MMM01 are secreted via the flagellar apparatus. Campylobacter invasion antigen (Cia), is a protein secreted by *Campylobacter* members via the T3SS or flagellar apparatus that not only enhances invasion and colonization but also aids the intracellular survival of the pathogen in the host. The flagellar hook complex, whose subunits were also identified in the genome, is required for the delivery of these Cia proteins. The Cia gene, *ciaB* that was found in the query genome also stimulates bacterial uptake and aids pathogen internalization by altering the host signaling cascade [51]. CFF strains each, of clinical and environmental origin, were chosen for intraspecific comparison and also included our sequenced genome as seen in Fig. 5. Interestingly, whilst CFF 82-40 lacked the capsular biosynthesis genes, MMM01 as noted had a partial gene cluster that is involved in capsular biosynthesis and transport which was also identified in *C. jejuni* strains. Based on the observations, it could be concluded that CFF strains have a range of virulence attributes and share virulence determinants with other *Campylobacter* species such as *C. jejuni*.

The various resistances towards antimicrobials are as vital if not more, in the infection process of this pathogen. Quinolones and fluoroquinolones (FQ) are increasingly being used to treat diarrhoea and severe infection in immunocompromised patients caused by Gram-negative pathogens including *Campylobacter* species. The mechanism involving chromosomal mutations modifying DNA gyrase or DNA topoisomerase IV, which are targets for quinolones in both gram-negative and gram-positive pathogens, has been attributed to FQ resistance [52]. Quinolone/FQ is being

increasingly used in patients and as a consequence there is an increase in quinolone-resistance in several bacterial species including *Campylobacter*. The resultant antimicrobial resistance has been found in patients recovering from antibiotic therapy, both in hospitals and also in the veterinary and fisheries sector, where farm animals have been exposed to antibiotics. FQs in general are known to inhibit the activity of DNA gyrase or topoisomerase IV [53]. In *Campylobacter* sp., the FQ resistance is due to a point mutation in the quinolone resistance-determining region of *gyrA* (QRDR) [52, 54]. Mutations within *gyrA* at Thr-86, Asp-90, and Ala-70 were involved in resistance seen in *Campylobacter* species [53]. This, in tandem, with the multidrug efflux pump *cmeABC* found in *Campylobacter* sp. like *C. jejuni*, contributes to quinolone and FQ resistance [55].

As previously noted, CFF MMM01 is very similar to CFF 82-40 and comparing the genomes with each other shows a lot of interesting details. The BRIG map detected the Fetus Subspecies Definition Region (FSDR), the conserved region, which is specific to CFF strains with a lower GC content of 29.4%, encodes genes that are thought to be involved in surface carbohydrate metabolism [2]. The *sap* genes in *C. fetus* are found in FGI-I and are involved in conferring virulence to the bacterium [56]. The *sap* locus, along with tRNA and a putative ABC-transporter, is located within genomic island FGI-I in CFF. A similar genomic island region, called Venerealis Genomic Island (VGI) III, was discovered in CFV strain 84-112, and showed homology to FGI-I. In the genome of CFF MMM01, FGI-I was present and intact, similar to the reference genome CFF 82-40.

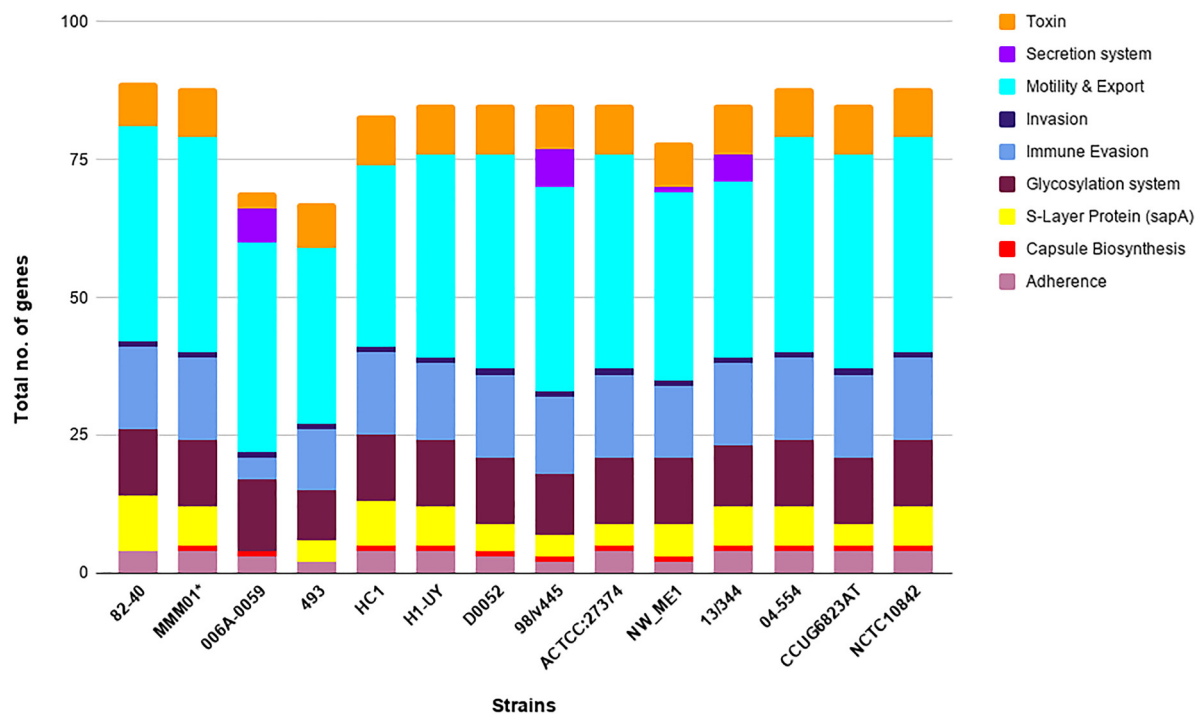


Fig. 5. The comparative analysis of number of gene/gene clusters encoding virulence factors identified by VFDB database present in different strains of CFF from clinical and environmental origin analyzed in this study



According to an earlier report, the 82-40 strain FGI-II contained two CRISPR arrays [2]. Both CFF and CFV strains contain CRISPR-repeats. These repeats were discovered in CFV within VGI-IV, in a single locus known as *Cfv\_CRISPR*. Likewise, two CRISPR-arrays were discovered in FGI-II in CFF 82-40, each labelled *Cff\_CRISPR\_1* and *Cff\_CRISPR\_2*. While *Cfv\_CRISPR* and *Cff\_CRISPR\_1* appear identical, including direct repeats (DR), leader sequences, and spacers, *Cff\_CRISPR\_2* appears to be distinct and lacks homology. The discovery of CRISPR regions suggests that this pathogen has a natural competence for acquiring DNA fragments from its environment and incorporating them into its genome [57]. The ORFs included some of the phage associated genes such as capsid maturation protease, head morphogenesis protein, crossover junction endodeoxyribonuclease, DNA helicase, and HNH endonucleases which are highly conserved among phages. It suggests a possible biological role in the stimulation of homologous recombination by nicking DNA [58]. Due to the robust CRISPR mediated defence that prevents horizontal gene acquisition, the genomes of *Campylobacter* spp. exhibit limited prophage diversity [59].

The genome of CFF, which was isolated from the blood culture of a patient with type 2 diabetes and chronic kidney disease, revealed information concerning virulence and phylogenetic relationships. The MLST based phylogenetic analysis showed interspecies diversity among the three *Campylobacter fetus* subspecies. The identified virulence genes showed inter-species sharing of some virulence genes among members of *Campylobacter* spp. The findings of this study will be useful in determining the various virulence attributes as well as mechanisms responsible for the pathogenesis, survival and dissemination in the environment. The results presented in this study are limited due to the small number of isolates studied; a comparative genome analysis based on a larger genome dataset would provide more information about the complexity and evolution of this zoonotic pathogen.

The genome of CFF MMM01 was earlier deposited in DDBJ/ENA/GenBank and given the accession JRX000000000. The new MLST profile of CFF MMM01 (pubMLST id:753) has also been submitted to the pubMLST database (<https://pubmlst.org>).

**Conflict of interest:** The authors declare that they have no conflict of interest.

## ACKNOWLEDGEMENTS

The authors thank Nitte (Deemed to be University) for providing computational infrastructural facilities for the execution of this research and the Department of Science and Technology for funding a fellowship to Deepak Sebastian Pinto. The authors would like to acknowledge the public databases from where the genomic data and tools were retrieved for analysis.

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## Appendix

Table A1. Summary of strains used in the study

Isolate	Source	Country and	Year	Accession No.
<i>CFF isolates</i>				
CCUG 6823 AT	Sheep brain	United Kingdom	2019	VXKL00000000
HC2	Human cerebrospinal fluid	Uruguay	2016	QJTS00000000
HC1	Human blood	Uruguay	2016	QJTR00000000
D0052	Human abscess	USA	2019	VOWW00000000
08/421	Fetal abomasum from <i>Bos taurus</i>	Argentina	2008	SOOT00000000
006A-0059	Human feces	Canada	2006	FAVB00000000
H1-UY	Human blood	Uruguay	2013	JYCP00000000
NWU_ED24	<i>Bos taurus</i>	South Africa	2019	JACASG00000000
13/344	Fetal abomasum from <i>Bos taurus</i>	Argentina	2013	SOYX00000000
98/v445	Bovine preputial scraping	United Kingdom	2015	LMBH00000000
ATCC27374	Sheep fetus brain	Brazil	2016	MKEI00000000
NW_ME1	<i>Bos taurus</i>	South Africa	2018	JAAVIZ00000000
493	Human blood	China	2016	JAAOXK00000000
BT 10/98	sheep	United Kingdom	1999	LRAL00000000
Cff110800-21-2	Bull preputial scraping	Netherlands	2000	LSZN00000000
wqj33	Human blood	China	2018	WLYG00000000
NCTC10842	Sheep brain	–	1972	LS483431
04/554	Bovine	–	–	CP008808
82-40	Human Blood	–	–	CP000487
MMM01	Human blood	India	2013	JRKX00000000
				(Updated in this study)
<i>CJ isolates</i>				
RM1221	Chicken	USA	2019	CP066242
269.97	Human blood	South Africa	–	CP000768
81116 (NCTC11828)	Human	–	1982	NC_009839
81-176	Human	USA	2006	NC_008787
NCTC 11168	Human	–	1977	NC_002163

Table A2. Details of multilocus sequence typing for sequenced strain

Locus	Allele Length	Allele
Cfe_aspA	477	Cfe_aspA_1
Cfe_glnA	477	Cfe_glnA_5
Cfe_gltA	402	Cfe_gltA_2
Cfe_glyA	507	Cfe_glyA_2
Cfe_pgm	501	Cfe_pgm_1
Cfe_tkt	459	Cfe_tkt_8
Cfe_uncA	489	Cfe_uncA_1

Table A3. Details of the Virulence factors and AMR genes identified from genome of CFF MMM01 by VFAnalyzer

VFclass	Virulence factors	Related genes identified in CFF MMM01 genome
Adherence	CadF MOMP PEB1/CBF1	<i>cadF</i> (peg.522) <i>porA</i> (peg.529,530) <i>pebA</i> (peg.1040)
Colonization and Immune evasion	Capsule biosynthesis and transport S-layer proteins	Undetermined (peg.1520) <i>sapA</i> -like (peg. 1382, 1703, 1704, 1720, 1723, 1731) <i>sapA</i> (peg. 1726)
Glycosylation system	N-linked protein glycosylation	<i>pglA</i> (peg.1243); <i>pglB</i> (peg. 1244); <i>pglC</i> (peg.1242); <i>pglD</i> (peg. 1241); <i>pglE</i> (peg.1217); <i>pglF</i> (peg.1216); <i>pglG</i> (peg.1215); <i>pglH</i> (peg.1246); <i>pglI</i> (peg.1245); <i>pseB</i> (peg.1525); <i>pseE</i> (peg.1530); <i>pseH</i> (peg.1526)
Immune evasion	Lipooligosaccharides (LOS)	Undetermined (peg. 1260-1265; 1268-1277)
Invasion	Campylobacter invasion antigen	<i>ciaB</i> (peg.1155)

(continued)



Table A3. Continued

VFclass	Virulence factors	Related genes identified in CFF MMM01 genome
Motility and export apparatus	Flagella	Undetermined (peg.11); Undetermined (peg.972); <i>flaA</i> (peg.1528); <i>flab</i> (peg.1527); <i>flaC</i> (peg.57); <i>flaD</i> (peg.568); <i>flag</i> (peg.234); <i>flgB</i> (peg.590); <i>flgC</i> (peg.589); <i>flgD</i> (peg.10); <i>flgE2</i> (peg.1653); <i>flgE</i> (peg.9); <i>flgG2</i> (peg.59); <i>flgG</i> (peg.60); <i>flgH</i> (peg.697); <i>flgI</i> (peg.237); <i>flgK</i> (peg.241); <i>flgR</i> (peg.1316); <i>flhA</i> (peg.971); <i>flhB</i> (peg.475); <i>flhF</i> (peg.1400); <i>flhG</i> (peg.1401); <i>fliA</i> (peg.1403); <i>fliD</i> (peg.233); <i>fliE</i> (peg.588); <i>fliF</i> (peg.401); <i>fliG</i> (peg.400); <i>fliH</i> (peg.399); <i>fliI</i> (peg.1582); <i>fliL</i> (peg.803); <i>fliM</i> (peg.1404); <i>fliN</i> (peg. 489); <i>fliP</i> (peg.637); <i>fliQ</i> (peg.339); <i>fliR</i> (peg.1299); <i>fliS</i> (peg.232); <i>fliY</i> (peg.1405); <i>motA</i> (peg.364); <i>motB</i> (peg.365)
Toxin	Cytolethal distending toxin	<i>cdtA</i> (peg.29, 833, 836); <i>cdtB</i> (peg.28, 832, 835); <i>cdtC</i> (peg.27, 831, 834)
Antimicrobial Genes	AMR Mechanism	Genes
	Antibiotic inactivation enzyme	<i>Mph(C)</i> family, <i>NimB</i>
	Antibiotic target in susceptible species	<i>Alr</i> , <i>Ddl</i> , <i>dxr</i> , <i>EF-G</i> , <i>EF-Tu</i> , <i>gyrA</i> , <i>gyrB</i> , <i>inhA</i> , <i>fabI</i> , <i>Iso-tRNA</i> , <i>kasA</i> , <i>MurA</i> , <i>rho</i> , <i>rpoB</i> , <i>rpoC</i> , <i>S10p</i> , <i>S12p</i>
	Antibiotic target replacement protein	<i>7a-HSDH-like</i>
	Efflux pump conferring antibiotic resistance	<i>CmeABC</i> , <i>MacA</i> , <i>MacB</i> , <i>YkkCD</i>
	Gene conferring resistance via absence	<i>gidB</i>
	Protein altering cell wall charge conferring antibiotic resistance	<i>PgsA</i>
	Regulator modulating expression of antibiotic resistance genes	<i>CmeABC</i>

\*Note: peg - protein encoding gene.

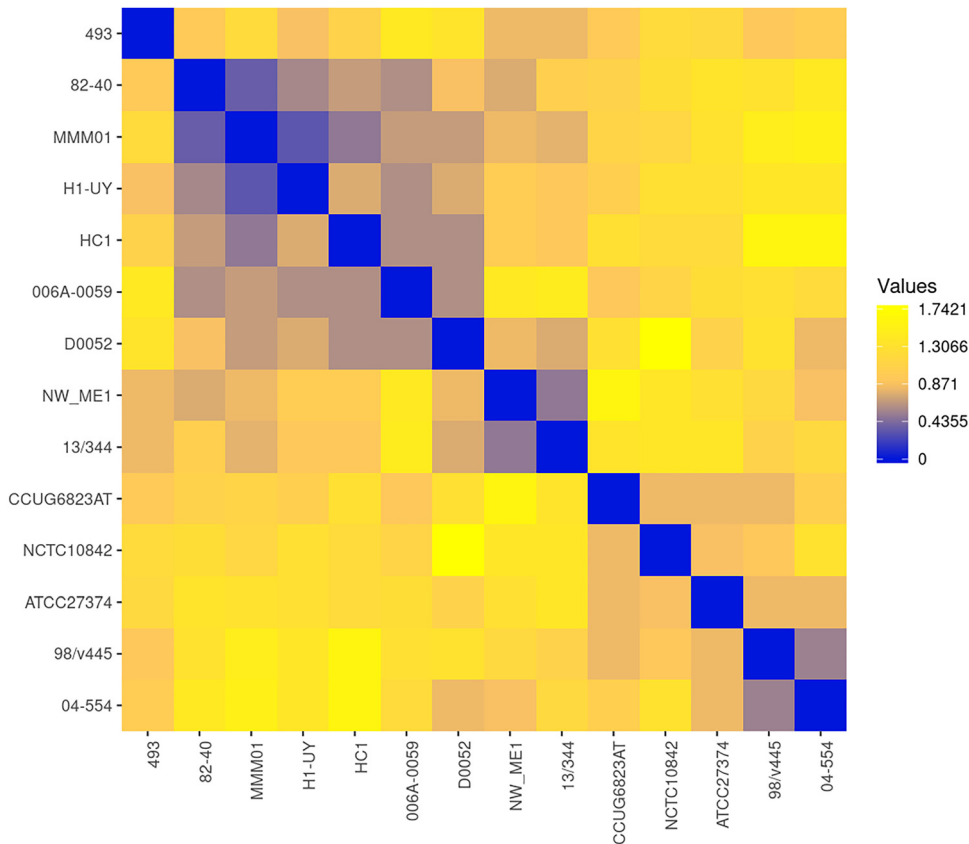


Fig. A1. Heatmap based on ANI% values calculated based on Spearman Rank Correlation method. A distinction between clinical and environmental isolates observed in terms of nucleotide identity percentage





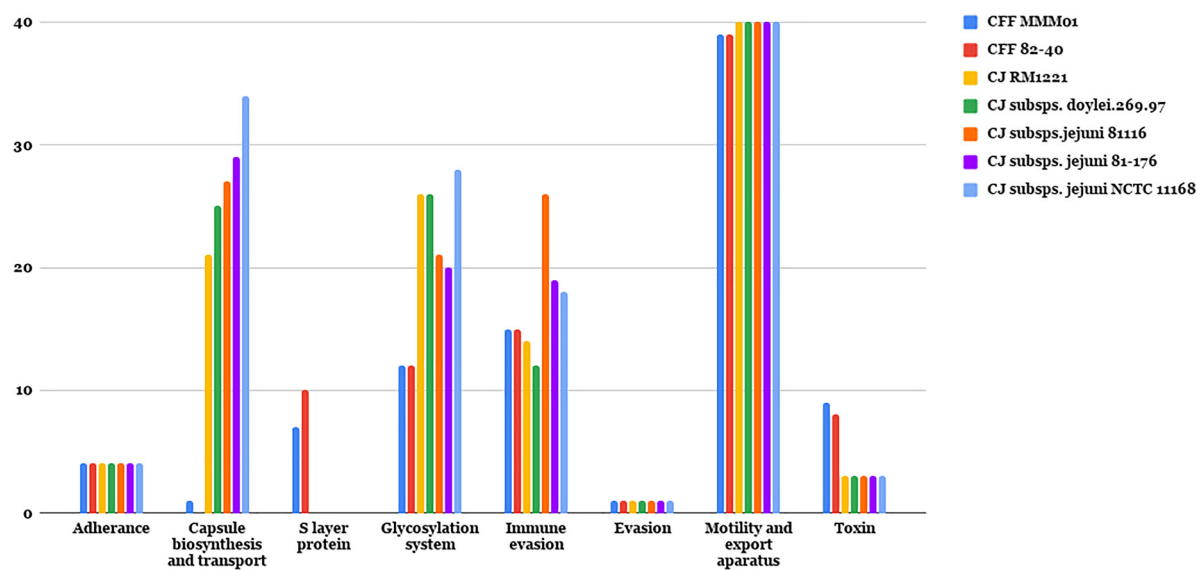


Fig. A2. The comparative analysis of the number of gene/gene clusters encoding virulence factors identified by the VFDB database present in different strains of CFF and CFJ

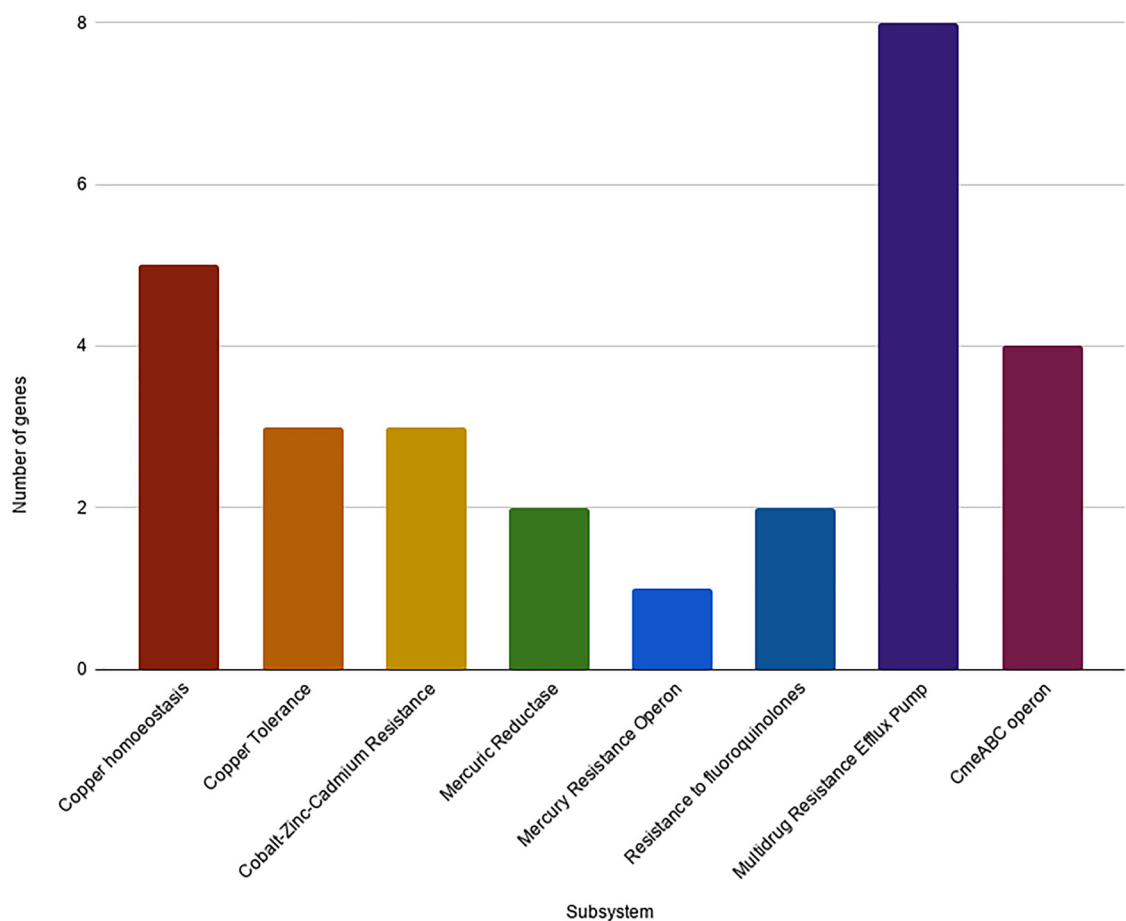


Fig. A3. Antibiotic and heavy metal resistance genes identified using comprehensive analysis with RAST server for genome of CFF MMM01

