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RESEARCH ARTICLE

Comparative Genomic Analysis of *Campylobacter jejuni* cj255 Reveals Diverse Genetics, Pathogenicity Determinants and Variation in T6SS

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Campylobacter jejuni mostly associated is a main reason of gastroenteritis. In the current study, we conducted the extensive genome wide comparative analysis of *C. jejuni* strain cj255 recovered from chicken in the area of Islamabad Pakistan. *C. jejuni* strain cj255 genomes comprises of 1,634,595 nucleotide bases, exhibiting about 98% similarity with human pathogenic strain 81116.3, encoding 1711 coding sequences and 39 RNAs. Overall, diverse virulence as well as antibiotic resistance genes such as T6SS were noted among these *C. jejuni* strain cj255 species. Most interesting characteristics of cj255 is the presence of the single locus Type VI secretion system (T6SS) with conserved as well as accessory components similar to strain 001597 while absent in strains 11168, 81116 and RM1221. The derived phylogenetic tree also suggests a possible evolutionary history for these strains and their T6SS cluster. Moreover, ClpB T6SS gene was present among all these strains. Moreover, extensive non-coding RNA prediction studies revealed functionally important non-coding RNAs. The study shows the extensive genetic diversity of *C. jejuni* strain cj255 and other selected strains for T6SS as well as other virulence factors and paves the way for identifying these strains and the pathogenetic co-relation for establishing better epidemiological tools to understand this complex pathogen of strains resourced from Pakistan.

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INTRODUCTION

Campylobacter jejuni is a mobile, microaerophilic gram-negative bacteria, that belongs to epsilon Proteobacteria class. Infections caused by *C. jejuni* are referred to as *C. jejuni* gastroenteritis or empylobacteriosis leads to either watery or bloody diarrhea (Murphy *et al*., 2006). Campylobacteriosis is a leading foodborne illness, which involves livestock origin foods, especially poultry. This pathogen can grow and reproduce in diversified ecological niches even in severe environmental conditions (Stabler *et al.,* 2013; Sheppard and Maiden, 2015).

Previous studies have reported that the virulence genes present in *C. jejuni* genome might be responsible for the ability to survive and contribution of incidence of disease (Suerbaum *et al.,* 2001; Abu-Madi *et al*., 2016). Such as antigenic genes coding for surface exposed proteins, that help in immune system invasion and *flaA* and *flab* flagellin structural genes harboring highly variable regions closely similar to the exposed parts of surface proteins. While other non-immunogenic virulence factors genes appears to be conserved (Meinersmann 2000; Tseng *et al.,* 2009). Moreover, the genome of NCTC 11168 have hardly <1% repeat sequences (Parkhill *et al*., 2000). Consequently, *C. jejuni* would appear to be an ideal organism to study by genome typing.

Among various determinants, secretion systems are reported to have very important role in bacterial interaction with environment. Until now Type I-VII (T1SS-T7SS) secretion systems have been reported in bacteria which are responsible for bacterial invasion in host cell by modifying the bacterial host environment which could lead to bacterial pathogenesis (Green and Mecsas, 2016). These multiple T6SS possibly leads to better survival of different bacteria (Pell *et al.,* 2009).

In the present study the complete genome wide analysis of newly sequenced *Campylobacter jejuni* strain cj255 and representative genomes resourced from chicken and humans were performed to reveal the diversity of toxin like protein secretion systems and their potential effectors that help in bacterial survival in diverse niches and involved in varying virulence patterns.

MATERIALS AND METHODS

Bacterial Isolates Studied: Chicken isolate cj255 was isolated and confirmed according to the method described by Siddiqui *et al*. (2015). Representative *C. jejuni* strains included in this study were cj1 strain resourced from human (Accession no. NZ_AUUL01000000), 11168.3 resourced from human (Accession no. AL111168 AL139074-AL139079), 81116.3 resourced from chicken (Accession no. CP000814), RM1221 resourced from chicken (NC_003912) and 00-1597 resourced from human (Accession no. CP010306).

***Campylobacter jejuni* genomic data:** Genomic data of our *C. jejuni* strain cj255 and representative strains is available at Genomes Online Database (GOLD) (http: //www. genomesonline.org/). Accession numbers of genomes along with information about the reference *C. jejuni* genome sequences utilized in present study are listed in Table 1. Comparison of draft genome of *C. jejuni* cj255 was generated with closely related reference genomes listed in Table 1.

Genome annotation and analysis: The *C. jejuni* strain cj255 along with representative strains was annotated using RAST annotation server following genome comparative analysis as well as coding sequence prediction analysis. Predictions of genes were performed by Glimmer 2, Gene Mark and GeneMark.hmm. To detect any potentially missing gene, translation of six-frame of the nucleotide sequence slices was performed. The annotation was provided from both sets and supplemented with information from the Clusters of Orthologous Groups and Conserved Domain Database (Tatusov *et al.,* 2000). Prediction of rRNAs was achieved using BLAST against RNA sequence database.

Computational Analysis of T6SS Loci: The genome scale survey was conducted to retrieve the T6SS proteins in *C. jejuni* strains cj255 and representative strains. The homolog of T6SS coding loci identification was carried out by BLASTn and BLASTx analysis using 13 core components of T6SS (Ibrahim *et al.,* 2012). Only T6SS components having hits and homology >50% with their corresponding T6SS genes were retrieved in all *C. jejuni*, and orthologues were identified using reverse-BLAST. Table S1 enlists E-value and bit scores of all the orthologues of the known T6SS components in all selected *C. jejuni* strains. It was considered that all strains having at least 10 known T6SS core components of T6SS were selected and are listed in Table 1.

Comparative analysis of T6SS gene cluster: Nucleotide based comparison of T6SS gene cluster was carried out among the *C. jejuni* strains with WebACT (Abbott *et al.,* 2005; Carver *et al.,* 2005). The parameters were initialized as; E-value having a score of 10, cost to open gap penalty as 5, cost to extend gap penalty as 2, penalty of mismatched nucleotides as -3 and finally the match score as 1.

Protein Family, Function and Domain Search: DNA sequences representing each T6SS locus were further analyzed to identify conserved protein domains in each ORF by cross referencing the available databases such as Protein family and domain annotations were considered for the functional classification of T6SS genes. InterPro and ProDOM databases were used for searching domains (Hunter *et al.,* 2011).

ncRNAs Detection: Noncoding RNAs were predicted using RNAspace. Filtration parameters included alignment size smaller than 50nt identity and possibly E value higher than 0.1. Following the removal of rRNA and tRNA genes, the putative ncRNA genes were filtered which located in the regions with more than average repeats. Moreover, it is also well reported that biologically interesting RNA molecules encode better stable structure than expected by chance (Bonnet *et al*., 2004).

Phylogenetic Study of T6SS Components and 16S rRNA: Phylogenetic studies were conducted using the Molecular Evolutionary Genetic Analysis (MEGA) software version 10 as described by Kumar *et al.,* (2018). T6SS loci amino acids sequences were aligned using ClustalW. Sequences of 16S rRNA corresponding to strains used by Kumar *et al.* (2018) were downloaded from RNA database and 16S phylogenetic tree was constructed.

RESULTS

Retrieval of Genomic Data and Genome features: Chicken isolate cj255 was isolated and confirmed according to the method described by Siddiqui *et al*., 2015 after growing at Campylobacter Blood-Free Selective Agar (mCCDA) Agar supplemented with CCDA Selective Supplement SR0155 after 48h of incubation at 42ºC under microaerophilic condition (Fig. 1). Genomic data of the *C. jejuni* strain cj255 as well as the representative strains was annotated using RAST. The *C. jejuni* strain cj255 draft genome sequence comprises of 1,634,595 bp, representing almost more than 99% of the estimated genome of *C. jejuni* strain cj255 with 25 contigs when compared to the representative strain such as *C. jejuni* strain RM1221, strain 81116.3, strain 00-1597 etc (Table 1). The GC contents of this genome also have small variation, which ranges from 30.3 to 30.7%.Most important elements noted in the *C. jejuni* genomes was lower no. of GC% contents. A circular map of the genome was generated using the CGView comparison tool as shown in Fig. 2. The number of coding sequences(CDS) predicted vary among all strains with substantial no. of differences such as strain *C. jejuni* strain cj12 encodes highest number of CDS 1812 while *C. jejuni* strain 81116.3 and RM1221 lowest 1623. The RNAs varies from 39 to 53 RNAs predicted by RNAmmer and tRNAscan. The variation in coding sequences and GC contents may be due to missing of some genes or may be mutation in the strains.

Table 1: Complete Genome wide analysis of *Campylobacter jejuni* strains with cj255

Strains	Size (bp)	G+C content (5)	Number of Contigs	Number of Subsystems	Number of coding sequences	Number of RNAs	Host
Cj255	1,634,595	30.3	25	290	1711	39	Chicken
Cj1	1,752,906	30.3	52	316	1812	42	Human
11168.3	1,641,481	30.6	1	309	1686	53	Human
81116.3	1,628,115	30.5	1	311	1623	53	Chicken
RM1221	1,777,831	30.4	1	311	1623	53	Chicken
00-1597	1,742,047	30.5	1	315	1807	53	Human

Table 2: Complete T6SS analysis of *Campylobacter jejuni* strains

Gene name	Alternate name/function	COG	255	CJ1	11168	81116	RM1221	00-1597
Hcp	Hcp	COG3157	Present	Present	Absent	Absent	Absent	Present
TssM	IcmF	COG3523	Present	Present	Absent	Absent	Absent	Present
TssL	OmpA/MotB	COG3455	Present	Present	Absent	Absent	Absent	Present
TssK	ImpJ/VasE	COG3522	Present	Present	Absent	Absent	Absent	Present
TssJ	lipoprotein/VasD	NO COG	Present	Present	Absent	Absent	Absent	Present
TssA	ImpA	COG3515	Present	Present	Absent	Absent	Absent	Present
TssB	ImpB	COG3516	Present	Present	Absent	Absent	Absent	Present
TssC	ImpC	COG3517	Present	Present	Absent	Absent	Absent	Present
TssE	Uncharacterized	NO COG	Present	Present	Absent	Absent	Absent	Present
TssF	ImpG/VasA	COG3519	Present	Present	Absent	Absent	Absent	Present
TssG	ImpH/VasB	COG3520	Absent	Present	Absent	Absent	Present	Present
TssI	VgrG protein	COG3501	Absent	Present	Absent	Absent	Present	Present
ClpB	ClpB protein	COG0542	Present	Present	Present	Present	Present	Present

Table 3: T6SS gene loci in *Campylobacter jejuni* strain cj255

Contig	Type	Start	Stop	Frame	Strand	Length (bp)	Function/Domain
scf_17371_23_contig_1	CDS	3549	4064	3	+	516	hcp protein
scf_17371_23_contig_1	CDS	5091	4318	-3	-	774	ImpK/VasF, OmpA/MotB
scf_17371_23_contig_1	CDS	6485	5088	-2	-	1398	ImpJ/VasE
scf_17371_23_contig_1	CDS	6941	6495	-2	-	447	lipoprotein/VasD
scf_17371_23_contig_1	CDS	7067	8314	2	+	1248	ImpA
scf_17371_23_contig_1	CDS	8383	8868	1	+	486	ImpB
scf_17371_23_contig_1	CDS	8870	10324	2	+	1455	ImpC
scf_17371_23_contig_1	CDS	10327	10719	1	+	393	Uncharacterized protein
scf_17371_23_contig_1	CDS	10716	12437	3	+	1722	ImpG/VasA

Comparative analysis of *C. jejuni* genomes: Based on site-directed mutagenesis resulting in either loss of colonization or bacterial invasion in host cell, it was identified that several Campylobacter genes are responsible for *in vitro* colonization in chicken or the invasion or adhesion of bacterial into human cells. Many of these genes were also found in *C. jejuni* strain cj255. For example, *cad*F gene *jlp*A and *peb*1A, which encodes a 27-kDa human strains such as cj1, RM1221, 11168.3, 00-1597, and RM1221 respectively or putative adhesion and a 43kDa important outer membrane protein (MOMP) *por*A gene were identified. A putative adhesin recently selected as flpA- encoded fibronectin type-A protein-A was also identified. Not all previously stated *C. jejuni* invasion genes were found in the genome of *C. jejuni* strain cj255 and in cj1 like the absence of an auto transporter *cap*A gene. The presence of readily melting DNA in this region compared to the genome is because this region comprises of more AT bases making it a less thermodynamically stable section, secondly readily melting of DNA also increases mutational rates in this region(Jon *et al.,* 2018).

Genome wide analysis and genetic architecture of T6SS in *C. jejuni:* The genomes of investigated *C. jejuni* and its related species range from 1,628,115 bp to 1,752,906 bp. A comparison of genomes of cj255 (first T6SS +ve chicken isolate from Pakistan) with cj1 (T6SS +ve clinical isolate from Thailand), strain 11168.3 (clinical isolate from UK), 81116.3 (chicken isolate from UK), RM1221 (chicken isolate from USA), 108 (clinical isolate from Netherlands) and 00-1597 (clinical isolate from Canada) showed that strain cj255 is more closely related to strain 11168.3. Annotation and genomic comparison was performed by RAST, seed viewer. Interestingly, all the selected genomes were negative for the presence of T6SS in plasmids, phages, prophages and transposable elements. All the genomes showed absence of siderophores. Arsenic resistance encoding genes were seen in all except strain 81116.3. While the three reference genomes i.e. 11168.3, 81116.3 and RM 1221 with which Type VI harboring strains were compared, lacked any of the secretion system i.e. Type I – Type VIII secretion systems.

The most distinctive feature of *C. jejuni* cj255 is the presence of T6SS and absence of Type I-V, VII and VIII secretion systems. The cj1 strain has Type IV secretion system in addition to T6SS (Table 2). Moreover, absence of genes for iron transport is unique to this strain. Other features that were exclusively present in this strain were; Heat shock *dna*K gene cluster of MiaB family protein, possibly involved in tRNA or rRNA modification; NADPH-dependent 7-cyano-7-deazaguanine reductase of RNA metabolism; GMP synthase for purine biosynthesis; Periplasmic nitrate reductase component NapD of Nitrogen metabolism; Ferrous iron transport permease Efeu; 3R- hydroxymyristoyl dehydratase of fatty acids synthesis; Thiazole biosynthesis protein ThiH of Thiamine biosynthesis; Lipoprotein releasing system transmembrane protein LolC; Beta, 1,3-glucosyl-transferase LOS core oligosaccharide biosynthesis; Pyruvate flavodoxin oxidoreductase and pyruvate kinase of central carbohydrate metabolism; Dihydrodipicolinate reductase and Dihydrodipicolinate synthase of Amino acid biosynthesis and L-serine dehydratase of Amino acid utilization.

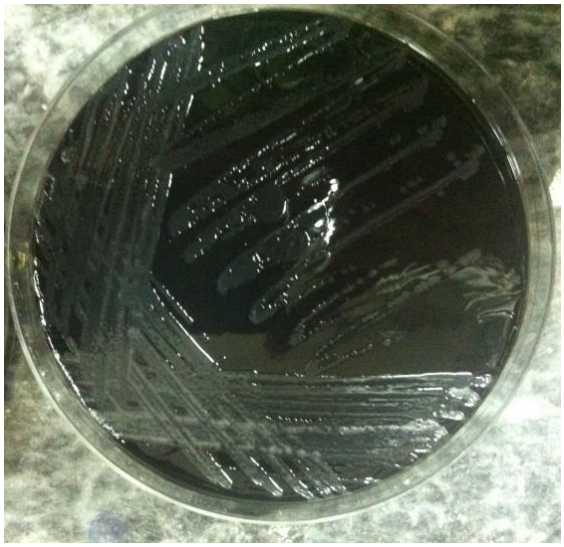

Fig. 1: Characteristics grey, moist flat spreading colonies of *C. jejuni* on Campylobacter Blood-Free Selective Agar (mCCDA) Agar supplemented with CCDA Selective Supplement SR0155 after 48h of incubation at 42°C under microaerophilic condition.

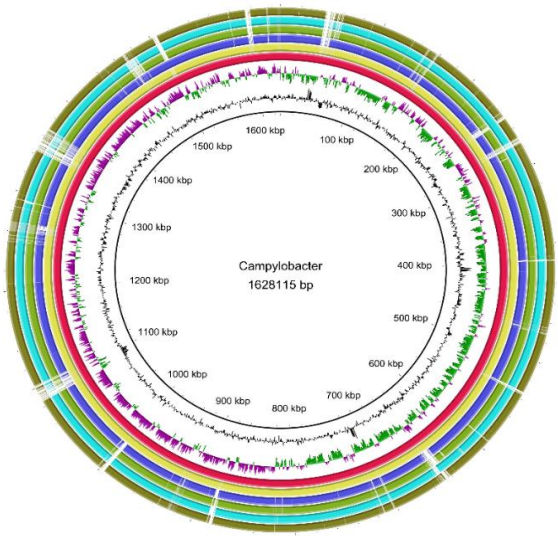

Fig. 2: Circular map of *Campylobacter genomes* features generated with the CGview tool. From inner to outer : Ring 1: GC Skew, Ring 2: GC Skew, Ring 3: 81116, Ring 4: CJ1, Ring 5: 255, Ring 6: 1597, Ring 7: NTC11168, Ring 8: RM1221.

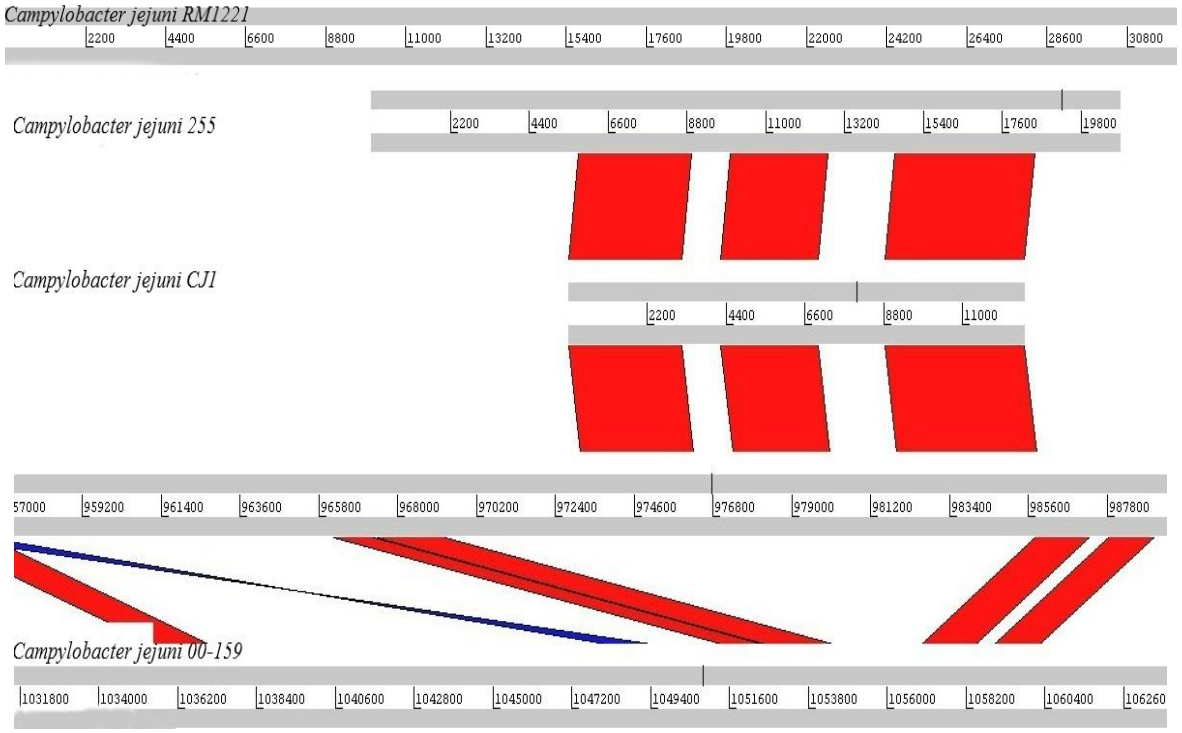

Fig. 3: Comparison of the T6SS locus of *Campylobacter jejuni strain 255* and representative strains by TBLASTN analysis using WebACT and visualized with ACT software.

Analysis of the *C. jejuni* genome sequences revealed the presence of the previously described T6SS (Fig. 3). Blast analysis revealed that the T6SS locus is located within 3 contigs of the cj1 genomic sequence, specifically in contigs. To further analyze this T6SS gene cluster, the contigs were annotated using the RAST pipeline (Table 3).

More extensive computational analysis showed that the hcp protein which is hemolysin co-regulated protein and VgrG protein which is known as valine glycine repeat G play role to translocate into the target cell via the dynamic activity of T6SS by penetrating the membrane we not found in the any genomes of *C. jejuni* selected in our study (Spear *et al.*, 2015). Moreover, our bioinformatics analysis further revealed no identification of more than one T6SS loci as there was no any information regarding the presence of T6SS components encoded in different regions of the *C. jejuni* genome. We performed a (basic local alignment search tool) BLAST analysis in order to identify T6SS orphan components and we didn't identify any single vgrG gene either in the cluster or as orphan components. vgr*G* proteins are essential for T6SS function so their presence along with the previously identified T6SS locus suggest that *C. jejuni* may lead to the prediction that T6SS may functionally be associated with other genes instead of vgrG in *C. jejuni* (Fig. 4).

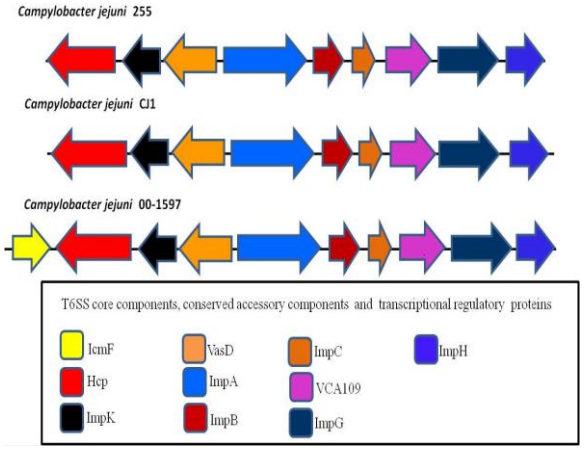

Fig. 4: Locus of T6SS core and conserved accessory components in *Campylobacter jejuni* strain cj255 which also exist in *Campylobacter jejuni* strain cj1 and other strains are represented with a different color.

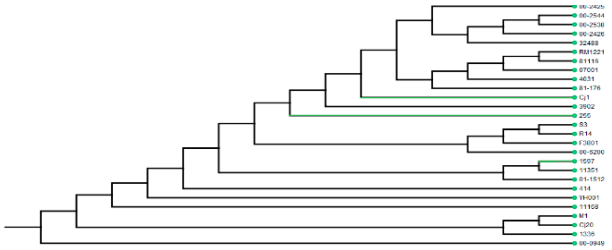

Fig. 5: Phylogenetic relationship of 16s rRNA sequences was performed using CLC sequence viewer version 7.5. The strain cj255 is closely associated in the cluster of cj1, RM1221 and 3902.

Non-coding RNA analysis: Using RNAspace and QRNA in the *C. jejuni* strain cj255 and after removing tRNA and rRNA genes, the putative ncRNA genes with stable secondary structure and located in the intergenic regions were selected. It is interested to note that only *C. jejuni* strain 00-1597 encodes 54 putative ncRNA, *C. jejuni* strain 11168 encodes 55 ncRNA and *C. jejuni* strain rm1221 encodes 54 putative ncRNA while ncRNA were absent in *C. jejuni* strain cj255, *C. jejuni* strain cj1 and *C. jejuni* strain 81116. The list of these non-coding RNAs will be proven to be valuable tools for studying the roles of specific proteins in the cell by gene expression studies.

Phylogenetic analysis: Phylogenetic analysis was performed to highlight the association between the *C. jejuni* species using T6SS genes. Multiple sequence alignment leading to tree construction with Mega 6 was achieved. Various clusters of T6SS obtained could be evaluated as of bearing different gene organization and regulation patterns designated specifically for the specialized secretion machinery. This study could probably indicate that *C. jejuni* strains making a separate cluster may play different functional roles, providing a competitive benefit in specific environmental conditions. It has been noted (Fig. 5) that *C. jejuni* strain cj255 is in an individual cluster and showing an out group species from rest of the strains. It may be the possibility that *C. jejuni* strain cj255 has been evolved by horizontal gene transfer.

DISCUSSION

The advancement in the Next generation sequencing (NGS) which determines the complete bacterial genomics DNA in a single sequence run and reveal information on virulence, resistance and typing. These informations could be used molecular diagnostics techniques. In our study, the confirmed isolate based on previous studies Siddiqui *et al*., 2015 were processed for whole genome sequence as well as comparative genomics analysis. The genomic data of the *C. jejuni* strain cj255 as well as the representative strains revealed significant differences between *C. jejuni* strains cj255 with representative strains. Most important elements noted in the *C. jejuni* genomes was lower no. of GC% contents. Numerous ecological factors are known to distress the genomic GC-content in bacteria such as availability of nitrogen or oxygen in the environment or the diversity of environments encountered by an organism and growth temperature (Hershberg and Petrov, 2010; Bohlin *et al.*, 2018). The base composition of Genomic DNA is known to have significant effect on genome functioning as well as species ecology (Foerstner *et al.*, 2005; Almpanis *et al.*, 2018). The RNAs varies from 39 to 53 RNAs among all strains. The variation in coding sequences may be due to missing of some genes or may be mutation in the strains (Monteville *et al.*, 2003; De Varies *et al*., 2017). Many of these genes were also found in *C. jejuni* strain cj255. For example, *cad*F, *jlp*A and *peb*1A genes required for Campylobacter host adhesion (Monteville *et al.*, 2003), and *por*A gene: a potential adhesion and a porin (Moser *et al.*, 1997), were identified. A putative adhesin recently selected as flpA- encoded fibronectin type-A protein-A, which has been shown to be necessary for efficient cell adherence and chicken colonization (Flanagan *et al.*, 2009), was identified. Not all previously stated *C. jejuni* invasion genes were found in the genome of *C. jejuni* strain cj255 and in cj1 like the absence of an auto transporter *cap*A gene, stated to be linked with chickens colonization and human epithelial cells adherence. Though, several *C. jejuni* isolates have been found having no capA gene (Flanagan *et al.*, 2009), but still the phenotypic and genotypic relationship of such strain with missing capA gene is yet to be identified.

T6SSs are secretion machineries and have the capability of directly injecting toxins into eukaryotic cells (Cianfanelli *et al.*, 2016). The presence of T6SS in bacteria is known as the virulent weapon which kills directly their competitors by secreting effectors and successful colonization in mammalian gut (Cianfanelli *et al.*, 2016; Sana *et al.*, 2017). The comparison of genomes of *C. jejuni* strain cj255 revealed that it encodes T6SS in chicken isolate from Pakistan which is the first report of T6SS with strain *cj*1, 11168.3, 81116.3, RM1221, 108 and 00-1597showed that these are more closely related to strain 11168.3. The most distinctive feature of *C. jejuni* cj255 is the presence of Type VI secretion system and absence of Type I-V, VII and VIII secretion systems. Moreover, the absence of VgrG and Hcp protein (the core orphan components of T6SS) which contribute in antimicrobial resistance and virulence in bacteria (Carruthers *et al.*, 2013) further anticipate that mechanism of virulence of T6SSin *C. jejuni* may be via only T6SS accessory components identified in our study or via RND efflux proteins. Various types of T6SS are known to play role in DNA or protein transport via membrane in response to particular environmental signal (Green and Mecsas, 2016). The IcmF family protein TssM is a

conserved T6SS component and putative NTPase in which the importance of its Walker; A nucleotide binding site for Hcp secretion remains controversial (Sana *et al.,* 2017).

The prediction of ncRNA shows that these non-coding RNAs will prove to be valuable tools for studying the roles of specific proteins in the cell by gene expression studies using RNA sequence, microarray and RT-PCR technologies and can help to target specific genes, thus shutting off expression of the protein product, the effects of these can be observed. Phylogenetic study of T6SS shows that *C. jejuni* strains making a separate cluster may play different functional roles, providing a competitive benefit in specific environmental conditions. It may be the possibility that *C. jejuni* strain cj255 has been evolved by horizontal gene transfer.

Conclusions: Whole genome-wide analysis of *C. jejuni* leads to the identification of many genes responsible for bacterial survival in host belonging to divergent ecological niches and for exhibiting varying disease outcomes. These genes could also play a significant role in designing primer to identify *C. jejuni* resourced from Pakistan. Moreover, this observation could possibly indicate different functional roles being played by each gene cluster, helping the bacteria in its adaptation and in competing with other organisms present in certain environmental niches.

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Authors contribution: ZN conducted, FMS, GZ, NN, AH, contributed in computational analysis with ZN, MI, HB wrote the paper, guide the students in experimentation.

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