Chapter

Conventional and Molecular Detection Methods of the Opportunistic Bacterial Pathogen *Campylobacter concisus*

Mohsina Huq and Taghrid Istivan

Abstract

Campylobacter concisus is an emerging pathogen that causes gastroenteritis and is a suspected cause of inflammatory bowel diseases. Its importance is enhanced by the chronic sequela that results from acute infection. This bacterium has been under-diagnosed in intestinal infectious diseases, and its clinical importance has not been determined yet. In order to establish the implication of this emerging bacterial species in human gastroenteritis and other infections, different approaches and procedure have been performed, where molecular typing methods have played a central role. The chapter provides a comprehensive past and recent updates on the detection of *C. concisus* by biochemical and molecular methods.

Keywords: *Campylobacter concisus*, hydrogen-requiring microaerophilic, opportunistic pathogen, PCR, PCR-DGGE, MALDI-TOF

1. Introduction

Campylobacter concisus is a fastidious, microaerophilic and hydrogen-requiring mesophile. It is a Gram-negative curved rod bacterium that is normally found in the human oral cavity and is actively motile with a single polar flagellum [1] with a cell size of (0.5–1) × (2–6) μm [2]. This small, non-pigmenting, asaccharolytic bacterium [3] usually grows slowly and requires enriched media. Such characteristics could be linked to its small genome that has a low G + C content (34% -38%) [3]. Unlike other *Campylobacter* spp., *C. concisus* does not have any known primary animal reservoir yet. Probably the human gastrointestinal tract is its only habitat where potential infections may spread via the inter-personal route. However it is worth to note that *C. concisus* was reported to be isolated from slaughtered porcine samples but not from live animals [4], while its DNA has been detected in few animal sources, such as the saliva of domestic pets using PCR-DGGE method [5], and in diarrheic faecal samples from domestic dogs by quantitative PCR [6].

Historically, in 1981 Tanner et al. [3] first recognised *C. concisus* as a member of the microflora of the oral cavity. Oral cavity parts are lips, buccal mucosa, teeth, gums, tongue, the floor of the mouth below the tongue, and the hard bony roof and soft palate. Twenty years later, in addition to its status as a coloniser of the oral cavity in humans, *C. concisus* (with other *Campylobacter* spp.) was considered as an

opportunistic pathogen under certain medical conditions by Macuch, Tanner [7]. Since the recognition, there have been many approaches to detect and isolate this bacterium, but it remains unclear whether *C. concisus* is an opportunistic pathogen of inflamed tissues, an oral pathogen, or is simply a commensal of the oral cavity. Here we focus on past and current isolation and detection techniques used to detect this bacterium in clinical samples and other sites of the human's gastrointestinal tract.

2. *C. concisus* in the oral cavity

Several studies examined the composition of the subgingival microbiota of children and found that the detection rate of *C. concisus* in permanent teeth is significantly higher than that of indeciduous teeth ($p < 0.001$) [8, 9]. The prevalence of *C. concisus* in the human oral cavity was detected by a PCR targeting the 16S rRNA gene and it was found in 100% (11/11) of saliva samples collected from healthy individuals [5]. Similar outcomes have been reported by Dewhirst et al. [10], when both cultivation and molecular methods were used to identify the human's oral microbiota. Another study conducted by Zhang et al. [11] also suggested that *C. concisus* was commonly present in the human oral cavity. The study reported the isolation of *C. concisus* from saliva of healthy controls, where 75% (44/59) were culture positive and 97% (57/59) were PCR positive. It is worth to mention here that our research team has successfully isolated this bacterium from 100% healthy human adults, using conventional culture and molecular techniques (unpublished data). These collective data indicate that the human oral cavity is the primary colonisation site of this bacterium.

The association of *C. concisus* with human periodontal diseases is also well reported [3, 9, 12], for example this bacterium was found attached to the teeth in higher numbers than other sites of the oral cavity in patients with periodontitis [13]. Immune responses against *C. concisus* in persons with periodontal diseases were also investigated, with higher antibody levels detected in periodontally diseased subjects compared to healthy controls [14]. Yet in other studies the bacterium was reported to be associated with gingivitis, periodontal sites in addition to healthy sites [15]. *C. concisus* was also reported to be detected in bleeding sites more than non-bleeding sites in periodontitis [8, 9, 16], and in enlarged lesions of gingivitis [17]. The same group reported in a later study that it was more associated with periodontitis in smokers than non-smokers participants [18]. The association of *C. concisus* with periodontitis was also supported by significantly higher isolation rates when gingival crevicular fluids (GCF) of patients were positive for aspartate aminotransferase (AST) compared to patients with negative result for AST [19]. Later, *C. concisus* was included into one of the six successional complexes that are believed to be involved in periodontal diseases [20].

3. *C. concisus* in acute gastroenteritis and chronic gastrointestinal diseases

The correlation between *C. concisus* and gastroenteritis was first reported in 1989 by Vandamme et al. [21]. *C. concisus* along with other *Campylobacter* spp. such as *C. upsaliensis, C. hyointestinalis,* and *C. fetus* have been reported as causative agents of gastroenteritis, but the bacterium remained unidentified when conventional culture techniques with antibiotics in the culture medium were used [22–24]. The introduction of hydrogen to the microaerophilic incubation conditions

significantly improved the isolation rates of *C. concisus* from patients with diarrhoea [25]. In 1995 a study in Sweden reported that 6% of the total cases of children with diarrhoea were found to be related to *C. concisus* [26]. Another study conducted in the same year, in Melbourne, reported that 56% of the "*Campylobacter* like organisms" isolated from children with diarrhoea, were identified to be *C. concisus* by conventional culture techniques [27]. The identity of these isolates was also confirmed by molecular techniques in a following study which concluded that *C. concisus* was associated with diarrhoea particularly in infants between 0–35 months of age [28]. Van Etterijck et al. [29] did not report a significant difference in *C. concisus* isolation rate from children with and without diarrhoea (9% in control and 13.2% in patients). However, other studies reported that this bacterium is associated with gastroenteritis cases in children [30–32]. Furthermore, *C. concisus* DNA has been detected in stool samples of patients with gastroenteritis in several studies [24, 33–36], with Nielsen et al. [37] reporting the incidence of *C. concisus* in patients with gastroenteritis, almost as high as the common *C. jejuni* or *C. coli* in a population-based study in Denmark. A more recent study associated *C. concisus* with travellers' diarrhoea in Nepal using 16S *rRNA* PCR of *Campylobacter* [38].

C. concisus was linked to inflammatory bowel diseases (IBD) since 2009 [11] when it was first isolated from stool samples of IBD patients [39]. Furthermore, Zhang et al. [11] found a significantly higher prevalence of *C. concisus* in children with CD than in controls ($p < 0.001$). In 2010, *C. concisus* DNA was detected in faecal samples of CD patients, in a significantly higher ratio (65%) than that of healthy and non-IBD controls (33%) [40]. Hence, in 2011, *C. concisus* was considered to be associated with UC cases [41]. Furthermore, the prevalence of *C. concisus* DNA was significantly higher in biopsy specimens ($p = 0.0019$) of adult UC patients (33.3%) as compared with controls (10.8%), which was supported by another study in the same year [42]. Ismail et al. [43] compared enteric and oral *C. concisus* isolates from eight patients with IBD (four UC and four CD) and six controls by multi-locus sequence typing (MLST), invasion assays, protein analysis, and scanning electron microscopy. Interestingly, the MLST results showed that the majority (87.5%) of *C. concisus* isolates from IBD patients were in one cluster compared to those from the control group (28.6%) ($p < 0.05$). This study provided the first evidence that patients with IBD are colonised with specific oral *C. concisus* strains and these strains may undergo natural recombination. Exotoxin 9, a putative virulence factor which may be associated with increased survival in the cell [44], and the zonula tight junction occludens toxin (Zot) [45] have been associated with virulence properties of *C. concisus* isolates from IBD cases.

4. Identification

4.1 Laboratory diagnosis, isolation, and detection of *C. concisus* in clinical specimens

Since *C. concisus* is usually present with other commensal microorganisms, the filtration culture techniques and/or molecular identification methods are more reliable than standard culture methods.

4.2 Culture and incubation conditions

C. concisus is routinely cultured on Columbia agar base or blood agar base supplemented with 5–6% defibrinated horse blood (HBA) in a special gas mixture containing 7% H_2, 7% CO_2, 5–7% O_2 and ~ 79% N_2 in an anaerobic jar incubated

for 48–72 hours at 37 °C [28, 46, 47]. The microaerophilic growth conditions can also be generated by evacuating an anaerobic jar to −7 bar and then gassing with a mixture of 10% H_2, 10% CO_2 and ~ 80% N_2 [46, 47]. *C. concisus* appears on HBA as colonies measuring 1–2 mm in diameter, round, entire, semi translucent and grey in colour [28].

4.3 Identification by cultural and biochemical properties

As *C. concisus* is a fastidious slow growing bacterium that is biochemically inert or inactive, it has been under-reported due to difficulties in isolation and improper identification. Sensitivity to cephalothin and nalidixic acid, growth temperature and colony colour have been used to identify *C. concisus* [48]. Arylsulfatase activity test is another important test, used to differentiate it from *C. mucosalis* and *C. upsaliensis* [49]. *C. concisus* was misidentified as *C. mucosalis* when initially isolated from samples other than the oral cavity [21]. Now, *C. concisus* is reported more often from patients with diarrhoea and other sites because of improvement of the culture system and the use of the stool filtration technique named the "Cape Town Protocol" [50]. In Cape Town, South Africa, the identification rate was reported to be increased by 31% when this technique was used with incubation in a hydrogen-enriched environment [31, 51]. However, as mentioned earlier, recently Nielsen et al. [52] demonstrated the polycarbonate filter is superior to the cellulose acetate filter for detection of *C. concisus*.

The phenotypic characteristics used to identify *C. concisus* in several studies are listed in **Table 1**.

Test	[3]	[2]	[53]	[54]
Active motility	+	+	+	+
Oxidase	+	+ (60–93%)	+	V
Catalase	—	—	—	—
Urease	—	—	—	NA
Hippurate hydrolysis	NA	—	—	—
Benzidine reaction	+			
Indoxyl acetate hydrolysis	NA	—	—	—
Nitrate reduction	+	+ (14–50%)	+	(−)
Selenite reduction		+ (14–50%)		NA
H_2S/TSI	+	—[b]	−/+	NA
Benzyl viologen reduction	+		NA	NA
Neutral red reduction	+		NA	NA
Growth at 25 °C	NA	—	—	
Growth at 42 °C	NA	+ (60–93%)	—	(+)
Growth stimulated by formate and fumarate	+		NA	NA
Alpha-hemolysis	NA		NA	—
MacConkey agar	NA		NA	—
Nutrient agar	—		NA	—
Growth on minimal media	—	—	NA	
NaCl (2.0%)	NA	+ (14–50%)	NA	—

Test	[3]	[2]	[53]	[54]
Glycine (1.0%)	NA		NA	—
Safranin (0.02%)	+	+ (14–50%)	NA	v
Sodium deoxycholate (0.1%)	+	+ (14–50%)	NA	—
Nalidixic acid (32 mg/ml)	+	+ (60–93%)	R	+
Cephalothin (32 mg/ml)	NA	NA	S	—
Metronidazole (4 mg/ml)	MIC: 0.5–2	+ (14–50%)	NA	—

[b]*Trace quantities, (−) most strains are negative, (+) most strains are positive, v variable, R Resistant, S Sensitive, NA not available.*

Table 1.
Biochemical characteristics of C. concisus.

4.4 Isolation from clinical samples

There is no standard technique for the isolation of *C. concisus* from faeces, saliva or tissue. However, the most common technique used to isolate this bacterium from faeces is the 'Cape Town protocol', which involves filtration of samples onto enriched media such as HBA containing antibiotics or onto antibiotic free HBA [31, 32]. Initially the faecal sample is suspended in liquid medium or phosphate buffered saline (PBS) at 1:2 to 1:10, then, 4–5 drops are placed on a cellulose acetate filter (pores size 0.65 μm) positioned on HBA. The soaked filter should be kept on the medium for approximately 10 min to allow the small sized bacterial cells to pass through its pores. Once the filter is discarded, a streak dilution of the primary inoculum is performed then the plate is incubated for 3–5 days in the gas mixture conditions as previously explained [46].

To isolate *C. concisus* from tissue samples such as intestinal biopsies, the homogenised sample is spread on HBA plates containing 10 mg/ml of each trimethoprim and vancomycin prior to incubation under the suitable growth conditions [11, 42]. Alternatively, a two-step enrichment-filtration method can be used [55] as follows: Step 1, the biopsy is enriched by initial incubation for 48 h in microaerophilic conditions in a tube containing 3 ml of Ham's F-12 medium with foetal bovine serum (5% FBS) and 10 μg/ml of vancomycin; step 2, filtration of 200 μl of the enrichment broth from the growth mixture obtained from step 1 onto HBA medium containing 10 μg/ml of vancomycin; followed by incubation in similar growth conditions for 2–4 days [55].

The isolation of *C. concisus* from saliva samples can be achieved by streaking 6 μl of saliva on a HBA medium containing 10 μg/ml vancomycin and incubation under the above mentioned growth condition for 3 days. The mixed bacterial culture is then collected as a suspension in BHI broth and filtered using cellulose filter (pores size 0.65 μm) on a fresh HBA plate and incubated for 2 days [56]. However, this method might not reflect the original load of *C. concisus* in saliva due to potential further growth during both incubation periods. Furthermore, commensals that are resistant to vancomycin could also compete and reduce the growth of *C. concisus*.

5. Detection and confirmation by molecular methods

Historically, *C. concisus* has been identified based on conventional methods such as culturing. This technique poses many challenges and can provide false negative results due to several external factors. Molecular biology allows more reliability as

well as a higher sensitivity when detecting the presence or absence of the pathogens. Therefore, a variety of molecular methods have been developed to detect *C. concisus*. For a bacterium of a fastidious nature, like *C. concisus*, molecular techniques can improve detection and identification in clinical samples. However, genetic variations should be considered in these detection methods otherwise some strains might be missed.

5.1 Direct detection of DNA in clinical samples

The presence of *C. concisus* DNA was investigated directly in faeces, intestinal biopsy, and saliva samples [11, 35, 56]. Initially a primer set (C412F and C1288R) designed by Linton et al. [22] to amplify the 16S rDNA gene (816 bp), was used as one step PCR to detect *C. concisus* from colonic biopsies [11]. Then, to identify *C. concisus*, the PCR product was sequenced and aligned to published sequences [11]. Soon after, a specific nested PCR was developed by Man et al. [40] to detect *C. concisus* in faecal specimens targeting the 16S rDNA gene. In the first PCR step, the primer set (C412F and C1288R) designed by Linton et al. [22], was used, while in the second step a new primer set (ConcisusF and ConcisusR) was developed to amplify a specific 560 bp region from the first PCR product of the 16S rDNA gene. Man et al. [40] applied this PCR to detect *C. concisus* from children's stool samples with CD, non-IBD patients and healthy controls. Later, this nested PCR method was also applied to detect *C. concisus* from saliva samples collected from IBD patients and healthy controls [56]. This nested PCR has been used for *C. concisus* DNA detection in other human clinical specimens including intestinal biopsies and saliva [40–42, 56].

Later on, Huq et al. [35] developed a multiplex PCR (m-PCR) to detect *C. concisus* and other *campylobacter* spp.. in faecal samples, based on the size of PCR product. When this m-PCR was applied on spiked faecal samples, *C. concisus, C. jejuni*, and *C. coli* were specifically identified at 10^5 cells/gm of faeces. However, as *C. concisus* is present in very low numbers in intestinal samples, using the nested PCR could be more sensitive than the m-PCR method.

5.2 Molecular confirmation and typing

The first specific PCR used for *C. concisus* identification was developed in 1995 by Bastyns et al. [57] using the forward primer MUC1 and a mix of two reverse primers CON1/CON2 to amplify the 23S rDNA region of *C. concisus* isolates. In 2004, we modified the this PCR method used by Bastyns et al. [57] to identify and group 19 clinical isolates from children with diarrhoea into two genomospecies using primers MUC1/CON1 (genomospecies A) and MUC1/CON2 (genomospecies B) [28]. However, there were some reports on that the primers designed for this PCR constantly cross reacted with *C. showae* and *Wolinella succinogenes* and produced a similar size PCR product [31].

Other techniques which successfully identified *C. concisus* were later developed including a two-step identification scheme for *Campylobacter, Arcobacter* and *Helicobacter* based on analysis of the 16S rRNA gene by PCR-RFLP (PCR-restriction fragment length polymorphism) by Marshall et al. [58]. Another PCR assay was developed, in 2001, from a 1.6 kb DNA fragment isolated from *C. concisus* genomic library for molecular identification, where a single PCR product was obtained without any cross reaction from other *Campylobacter* spp. [51]. Another primer set (Pcisus5-F and Pcisus6-R) developed by Matsheka et al. [51] was initially used to amplify DNA fragments (344 bp) obtained from a *C. concisus* genomic library and later showed to specifically amplify *C. concisus* [28, 35, 51]. This primer set amplifies *gyrB* [35].

6. Detection by MALDI-TOF

Matrix-assisted laser desorption/ionisation (MALDI) with time-of-flight mass spectrometry (TOFMS) is a technique developed more than three decades ago, which can be used to detect and characterise pathogens on the basis of larger biomolecules. Few studies proved the feasibility to identify *C. concisus* using MALDI-TOF-MS analysis of protein biomarkers from protein extracts of cell lysates or from whole cells [59–61]. The first attempt was in 2005 [62] using MALDI-TOF-MS to identify a number of *Campylobacter* species from their protein biomarkers, where they have identified a 10.5-kDa protein as the DNA-binding protein HU, and the potential species-identifying biomarker ions (SIBI) for *C. concisus* strains. Later this DNA-binding protein HU (10.5-kDa) was suggested to be used as a strain-specific biomarker for analysis by 'top-down' proteomics techniques [59]. However, a confirmed *C. concisus* isolate, by sequencing part of the 16S rRNA gene, could not be identified by MALDI-TOF by using database 3995 main spectra (June 2011) [63]. While a score ≥ 2.0 is considered reliable species identification, and between 1.7 and 2.0 represent reliable identification at the genus level, the isolate had only a score of 1.62, as *C. concisus* was not included in the database. The first successful identification and characterisation of *C. concisus* by MALDI-TOF and ClinProTools 2.2 software was in 2016 [60]. The study correctly identified all 14 *C. concisus* strains, despite evident differences between the isolates, with a scores ≥2.0 for secure species identification. There was a clear separation between other *Campylobacter* species and *C. concisus* by grouping of MSP dendrogram, with sufficient conserved peaks found for species identification. However, no distinguished biomarker has been identified to differentiate between the two genomospecies which can be easily differentiated by the 23S rDNA PCR [28]. Recently the lipo-oligosaccharide (LOS) structure (an important virulence factor which activates TLR4) of *C. concisus* clinical isolates correlated the inflammatory potential of each isolate with bacterial virulence by MALDI-TOF MS [61]. The presence of multiple bands in the SDS-PAGE profiles of *C. concisus* and *C. jejuni* LOS indicated their heterogeneity. The mass spectrometric analyses of lipid A indicated a novel hexa-acylated diglucosamine moiety, which cloud be an indicator of a potential virulence property.

7. Diversity of strains

As *C. concisus* is a genetically diverse organism, there is no standard molecular method yet to fully address this diversity. The standard typing of *C. concisus* could determine whether isolates obtained from diarrhoeic or IBD patients differ from those colonising healthy individuals [7, 64–66]. Applying such standard typing methods would help researchers to have a better understanding of *C. concisus* transmission, natural habitat, virulence and the host's immunological responses [67].

7.1 Typing by protein profiling techniques

It has been suggested that sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunotyping were excellent tools for *C. concisus* identification. SDS-PAGE has been applied successfully to distinguish *C. concisus* from other small bacteria with very similar characteristics, such as *C. mucosalis*, other species of Gram-negative rods, as well as non-pigmenting and asaccharolytic bacteria [68, 69]. Other studies have used SDS-PAGE to identify clusters within *C. concisus;* the number of clusters identified varied from two to five [28, 65]. Therefore, the protein profiling technique could be discriminative for *C. concisus*

isolates, but the discriminative power might be improved if combined with another typing technique such as genotyping. It should also be noted that protein profiling failed to separate *C. concisus* isolates from IBD patients and healthy controls [43].

7.2 Genomic typing by PCR and other techniques

There is no documented standard technique for genotyping *C. concisus* isolates. The first genetic method to type *C. concisus* was DNA–DNA hybridization [21]. The authors used electrophoretic protein profiles, immunotyping and DNA: DNA hybridization to identify 22 strains named as EF (E Falsen) group 22 which were identified as *C. concisus*. The 22 strains showed a considerable heterogeneity (42%) with the *C. concisus* type strain. The genetic diversity was later confirmed by analysis of 100 *C. concisus* isolates using randomly amplified polymorphic DNA (RAPD) [29, 70]. Another approach, which is a modification of the 23S rDNA PCR amplification method of Bastyns et al. [57], was used successfully used to type *C. concisus* by Istivan et al. using either the specific primer set (MUC1 and CON1) or (MUC1 and CON2) [28, 46]. In this system, isolates amplified by MUC1 and CON1 were assigned to genomospecies A while those amplified by MUC1 and CON2 were designated as genomospecies B [28, 66, 71].

The multi-locus sequence typing (MLST) was another technique applied to type 70 oral and intestinal *C. concisus* isolates from 8 patients with IBD and 6 healthy controls [43]. Subsequently, the neighbour-joining tree divided these isolates into 26 types and two major groups. Most isolates (87.5%) in cluster 1 were from IBD patients compared with only 28.6% in cluster 2 ($P < 0.05$). It was also reported that all of the invasive *C. concisus* isolates were localised in cluster 1 [43]. Two major groups were also demonstrated by MLST using a different set of housekeeping genes (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *ilvD*, and *pgm*), applied to 60 *C. concisus* faecal isolates [72]. However, in both of these studies, it was not clear whether the two major groups were correlated with genomospecies A and B. A more recent study in 2016, Nielsen et al. [60] determined the genetic diversity of 67 *C. concisus* isolates from Danish diarrheic patients using MLST and specific differences in the 23S rRNA, and reported the high diversity of *C. concisus* with 53 sequence types (STs). However, dendrogram profiles of each allele showed a division into two groups, which was more or less correlated with genomospecies A and genomospecies B but had no association to the clinical severity of disease.

In addition to the previous techniques, the pulsed field gel electrophoresis (PFGE) indicated the diversity of *C. concisus* isolates and assigned them into two groups according to the source of the isolates (faeces and oral cavity) and into several subgroups [73]. Furthermore, *C. concisus* isolates have been allocated into four groups using the amplified fragment length polymorphism analysis technique (AFLP) [66, 71]. AFLP groups 1 and 2 aligned with genomospecies A and B (based on 23S rDNA PCR); while groups 3 and 4 could not be amplified by 23S rDNA PCR [66].

7.3 Typing by denaturing gradient gel electrophoresis (DGGE)

The PCR-DGGE technique was initially used to evaluate the microbial diversity in complex environments [5]. In environmental microbiology applications, universal primers are designed to target the 16S rDNA gene for the detection of mixed bacterial communities and differentiation of *Campylobacter, Helicobacter* and *Arcobacter* from clinical samples and *C. concisus* [5]. The PCR product is separated by polyacrylamide gel electrophoresis based on the use of different melting temperatures and its mobility in gradient denaturation of formamide and urea [74]. Previously, the DGGE technique was applied on 21 *C. jejuni* and one *C. coli* isolates using primer sets targeting the flagellin gene (fla-DGGE) [75]. A study conducted on DNA extracted from human saliva using PCR-DGGE to detect *Epsilobacteria*

(Campylobacters, Helicobacters and Arcobacters and related bacteria) reported that three reference *C. concisus* strains fell into two different DGGE profile groups [5]. However, Cornelius et al. [76] assigned *C. concisus* isolates from healthy volunteers and diarrhoea specimens into only one DGGE profile group by a semi-nested PCR-DGGE from 16S rRNA gene. The authors suggested that PCR-DGGE can be a useful tool for a direct detection of Epsiloproteobacteria.

Elshagmani [77] used Muyzer primer sets (518R: R-ATTACCGCGGCTGCTGG; 341F-GC: F-CCTACGGGAGGCAGCAG and 907R: R-CCGTCAATTCMT TTGAGTTT) [78, 79] to amplify the 16S DNA of *C. concisus*. Those primers were originally designed to detect and analyse the genetic diversity of mixed bacterial populations in environmental samples. As the DNA of all *C. concisus* tested isolates could be amplified using both Muyzer primer sets (1 and 2) to amplify 16S rDNA, it was suggested these sets could be used in clinical samples to detect *C. concisus* in mixed extracted DNA samples. Moreover, the analysis showed that all *Campylobacter* spp. isolates can be divided into four distinct groups that were defined as group I, II, III and IV. All *C. concisus* genomospecies B isolates fall into group II consistently, however, most genomospecies A isolates were allocated to group I but some were allocated in group II [77].

7.4 Typing by *rrn* analysis

The diversity of the ribosomal RNA (*rrn*) operon (5S rRNA, 16S rRNA, 23S rRNA genes, and the ITS regions) is considered a useful tool for differentiation of the heterogeneous *C. concisus* species [80]. The sequences of *C. concisus rrn* operons were used in a recent study for the purpose of strain typing and delineation of phylogenetic relationships within these operons. A total of 38 indels were identified in the *rrn* operon within *C. concisus* genome. Five indels found in the 23S rRNA gene were significantly associated with either genomospecies A or B ($p \leq 0.05$). The phylogenetic trees generated from 15 *rrn* operons and 23S rRNA genes also demonstrated sequence differences between strains within the *rrn*. Hence, the study confirmed that *C. concisus* can be classified into two genomospecies (A& B) based on the presence of the indels in the *rrn* operon and the 23S rRNA gene is a more reliable target for *C. concisus* typing than the 16S rRNA gene [80].

7.5 Whole genome sequencing

Whole-genome sequencing (WGS) is becoming increasingly available and affordable technique. Until 2011, there was only one *C. concisus* fully sequenced genome available for a strain (id. 13826) isolated from faeces of acute gastroenteritis patient and sequenced in 2007. The second *C. concisus* strain (UNSWCD) isolated from an intestinal biopsy of a patient with Crohn's disease was sequenced in 2011 [81]. Only 76% of genes were homologues between *C. concisus* 13826 and UNSWCD [82]. More *C. concisus* strains isolated from various clinical sources were sequenced and their genomes showed evidence of gene shuffling in *C. concisus* [83]. Few years later, another study defined the *C. concisus* core-genome and identified genomospecies-specific genes [84]. It concluded that the *C. concisus* core-genome, housekeeping genes, and the 23S rRNA gene consistently divided the 36 strains used in the study into two genomospecies. The study also reported novel genomic islands that contain type IV secretion system and putative effector proteins, in addition to other new genomic features. A study by our team investigated the *rrn* operon (5S rRNA, 16S rRNA, 23S rRNA genes, and the ITS regions) for four newly sequenced whole genomes extracted from intestinal and oral *C. concisus* strains, along with eight available WGSs online and established a clear correlation between the *rrn* operons and genomospecies [80].

More recently, the complete genome sequence of the *C. concisus* type strain ATCC 33237 and the draft genome sequences of eight additional well-characterised *C. concisus* strains were added to the database [85]. This was followed by a study in 2018 which analysed the genomes of 63 oral *C. concisus* strains isolated from patients with IBD and healthy controls, of which 38 genomes were newly sequenced. The genomes were examined to identify pathogenic molecular markers and the researchers reported a *C. concisus* molecular marker, which is a novel secreted enterotoxin B homologue (csep1-6bpi) potentially associated with active CD [86]. Moreover, in 2018 [87], a study to identify *C. concisus* virulence properties and adaptations capability to reside in the GI tract, produced robust genome sequencing data and comprehensive pangenome assessment from 53 new *C. concisus* strains. The researchers identified few genetic differences between oral and gut isolates from the same patient and suggested that the variability in bacterial secretion system content may play an important role in their virulence potential [87].

8. Conclusion

This chapter discussed the various approaches used to identify and differentiate *C. concisus*, since it was identified and named almost 40 years ago. This bacterium has been associated with periodontal diseases, acute enteritis, and IBDs, with the strongest evidence relating to acute and chronic intestinal diseases. However, its identification has always been challenging due to its inert biochemical characteristics and to the extremely high degree of genetic heterogeneity. The studies presented and explored in this chapter show that *C. concisus* is a genetically diverse species, but the extent of the difference between strains remains largely unknown. However, with the limitation of biochemical tests to identify *C. concisus*, molecular detection approaches including the PCR, of 23S rDNA, DGGE, m-PCR, MALDI-TOF and whole genome sequencing, have all made the identification and differentiation of this bacterium much easier than before.

Conflict of interest

There is no conflict of interest between the authors.

Author details

Mohsina Huq and Taghrid Istivan*
Biosciences and Food Technology, School of Science, RMIT University, Melbourne, Australia

*Address all correspondence to: Taghrid.istivan@rmit.edu.au

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