Identification and Differentiation of Highly Diverse *Campylobacter concisus* Strains using the MALDI Biotyper

Hans Linde Nielsen[1]*, Mette Mølvadgaard[1], Henrik Nielsen[2,3] and Markus Kostrzewa[4]

[1]Department of Clinical Microbiology, Aalborg University Hospital, Aalborg, Denmark
[2]Department of Infectious Diseases, Aalborg University Hospital, Aalborg, Denmark
[3]Department of Clinical Medicine, Aalborg University, Aalborg Øst, Denmark
[4]Bruker Daltonik GmbH, Bremen, Germany

Abstract

In Danish diarrheic stool samples, the emerging enteric pathogen *Campylobacter concisus* has an incidence at the level of *Campylobacter jejuni*. Nevertheless, *C. concisus* has been neglected in many clinical laboratories due to labour intensive isolation procedures as well as time-consuming PCR-based methods for identification. Fourteen isolates of *C. concisus* were characterised in the MALDI-TOF mass spectrometer and analysed using the MALDI Biotyper and the ClinProTools 3.0 software. The data show that MALDI-TOF MS, as previously described for other *Campylobacter* species, can give a rapid identification of *C. concisus*. Interestingly, the isolates showed a remarkable diversity based upon their mass spectra when compared by visual and computational analysis.

Keywords: *Campylobacter concisus*; MALDI-TOF MS; Identification; Diversity; *Campylobacter jejuni*

Description

Campylobacter jejuni is the most commonly reported bacteriological agent in infectious gastrointestinal disease [1-5]. However, in a population-based study of diarrheic stool samples in North Denmark Region, with a population of 580,000 inhabitants [6-12], the close-related *Campylobacter concisus* had an incidence at the level of *C. jejuni* [13,14]. In a questionnaire study, *C. concisus*-positive patients reported a milder, though more prolonged diarrhoea than did patients with *C. jejuni* [15-18]. Furthermore, recent studies have linked *C. concisus* with inflammatory bowel diseases, since a high prevalence of *C. concisus* DNA has been detected in colonic biopsies of patients with Crohn's disease compared to controls [10,12,19]. Furthermore, *in-vitro* studies have shown that *C. concisus* is capable of invading intestinal epithelial cells, where it may increase apoptosis and the intestinal permeability [7,11,17]. These findings support the understanding of *C. concisus* as an intestinal pathogen. *Campylobacter jejuni* and other *Campylobacter* species can easily be identified to species level with use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [2]. *Campylobacter concisus* does not grow on modified charcoal-cefoperazone-deoxycholate agar (mCCDA) which is routinely used for cultivation mCCDA in western countries. However, *Campylobacter concisus* and other emerging *Campylobacter* species can be isolated in high numbers in stools by use of a filtration technique on 5% yeast-enriched blood agar plates (SSI Diagnostica, Hillerød, Denmark) incubated at 37°C in a microaerobic atmosphere with hydrogen [14]. Identification of *C. concisus* has until recently been based on PCR-based techniques which may seem slow compared to the rapid identification by use of the MALDI-TOF MS. In this study, we evaluated MALDI-TOF MS as a method for the rapid identification of *C. concisus*.

Fourteen isolates of *C. concisus* were tested (Table 1). All isolates were cultured at Department of Clinical Microbiology, Aalborg University Hospital, Denmark. They consisted of seven faecal isolates from seven diarrheic patients. From three of these patients their oral isolate was also tested. Finally, three oral isolates from healthy individuals and the reference strain ATCC 33237 were investigated.

All isolates were primarily cultivated with use of the filter method

Isolate number and source (Oral or Faecal)	Sex	Age	Clinical characteristic
ATCC 33237 Oral (Human gingival sulcus)	-	-	-
K2 Oral	Female	25	Healthy
K4 Oral	Female	31	Healthy
M2 Oral	Male	26	Healthy
2009-74775 Faecal	Male	29	Crohn's disease
2009-118452 Faecal	Female	19	Crohn's disease
2009-119100 Faecal	Female	63	Collagenous colitis
2010-1718 Faecal	Female	2	Bloody diarrhoea
2010-112100 Oral+Faecal	Female	36	Crohn's disease
2010-113332 Oral+Faecal	Female	57	Diarrhoea
2010-115605 Oral+Faecal	Male	42	Diarrhoea

Table 1: *Campylobacter concisus* isolates used in the study.

as described elsewhere [14] and final reference identification was based on a species specific realtime PCR based on the chaperonin-60 gene and the 23S rRNA gene [1,3]. All isolates were stored at -80°C before being retrieved and sub-cultured on 5% horse blood agar plates, containing 1% yeast extract (SSI Diagnostica, Hillerød, Denmark) for 48 hours in a microaerobic atmosphere with hydrogen at 37°C before analysis.

For MALDI-TOF MS investigation, the extraction method used was according manufacturer's instructions and samples were measured in the MALDI-TOF mass spectrometer (MALDI Biotyper system, Bruker Daltonics). Acquired spectra were analysed using the MALDI Biotyper and the ClinProTools 2.2 software. A dendrogram

*Corresponding author: Hans Linde Nielsen, Department of Clinical Microbiology, Aalborg University Hospital, Hobrovej 18-22, DK-9000 Aalborg, Denmark, Tel: +45-97-66-54-23; Fax: +45-97-66-54-25; E-mail: halin@rn.dk

of the MALDI-TOF profiles was constructed after main spectra (MSP) generation according to the recommendations of the manufacturer, and visual spectra comparison was performed. To assess the stability of the isolates, spectra after three re-cultivation steps, each for 48 hours, were analyzed. Furthermore, daily spectra from all isolates up to one week (after 24 hr, 48 hr, 72 hr, 96 hr, 120 hr, 144 hr and 168 hr) of incubation on the same agar plates were compared.

The fourteen isolates showed very high diversity with no real significant grouping. To investigate if MALDI-TOF MS could be used for a fast and reliable identification of *C. concisus* we evaluated the main spectras (20 per sample) of the 14 isolates and made a comparison with the MALDI Biotyper library containing four main spectras of bacterial strains. In spite of evident differences between the isolates, all strains

were correctly identified as *C. concisus*, with scores over the threshold (log score 2.0) for secure species identification.

To test for the relationship to other closely related *Campylobacter* species, like *C. jejuni* and C. coli, their spectra were compared with the spectra of the different *C. concisus* isolates, and there was a clear separation between *C. concisus* and other *Campylobacter* species, when grouped together in a MSP dendrogram, indicating there is sufficient conserved peaks for species identification (Figure 1). However, two groups could be slightly separated by principle component analysis (not shown) as well as in a MSP dendrogram (Figure 2), although inside these groups variability was also very high. The variability of strain profiles was also confirmed by correlation analysis. The three coupled
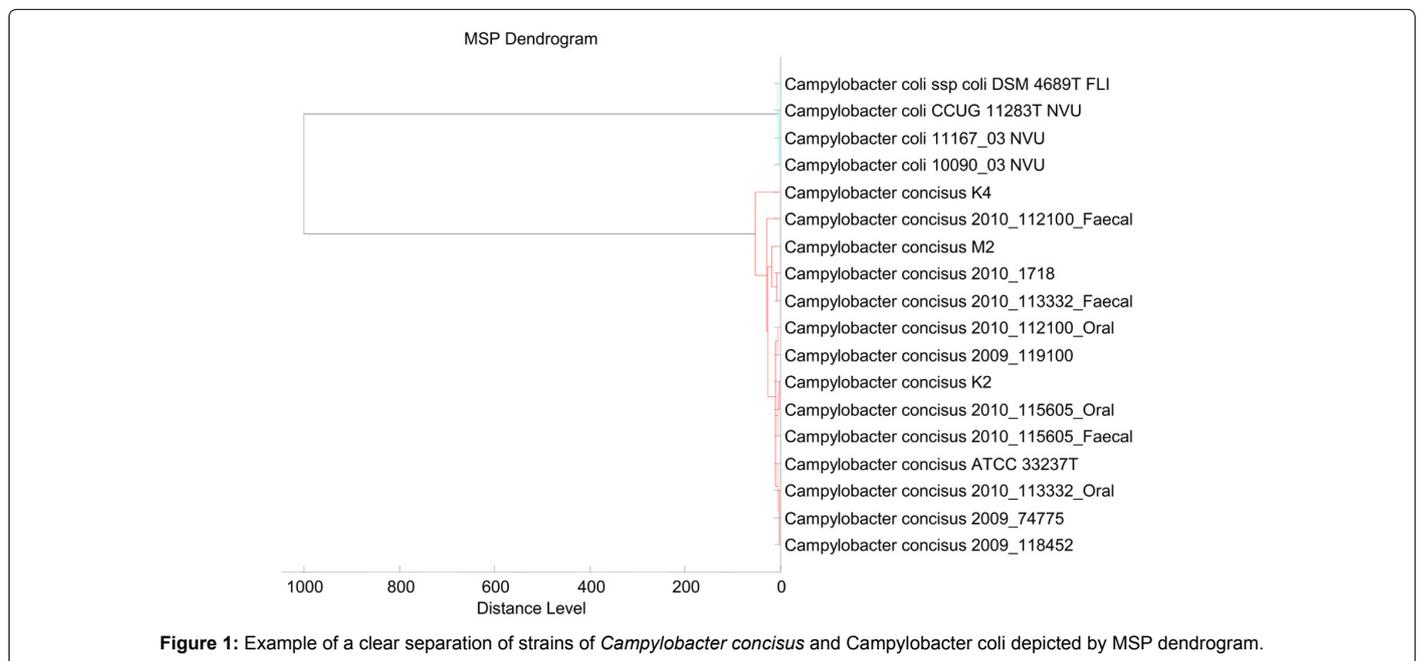

Figure 1: Example of a clear separation of strains of *Campylobacter concisus* and Campylobacter coli depicted by MSP dendrogram.
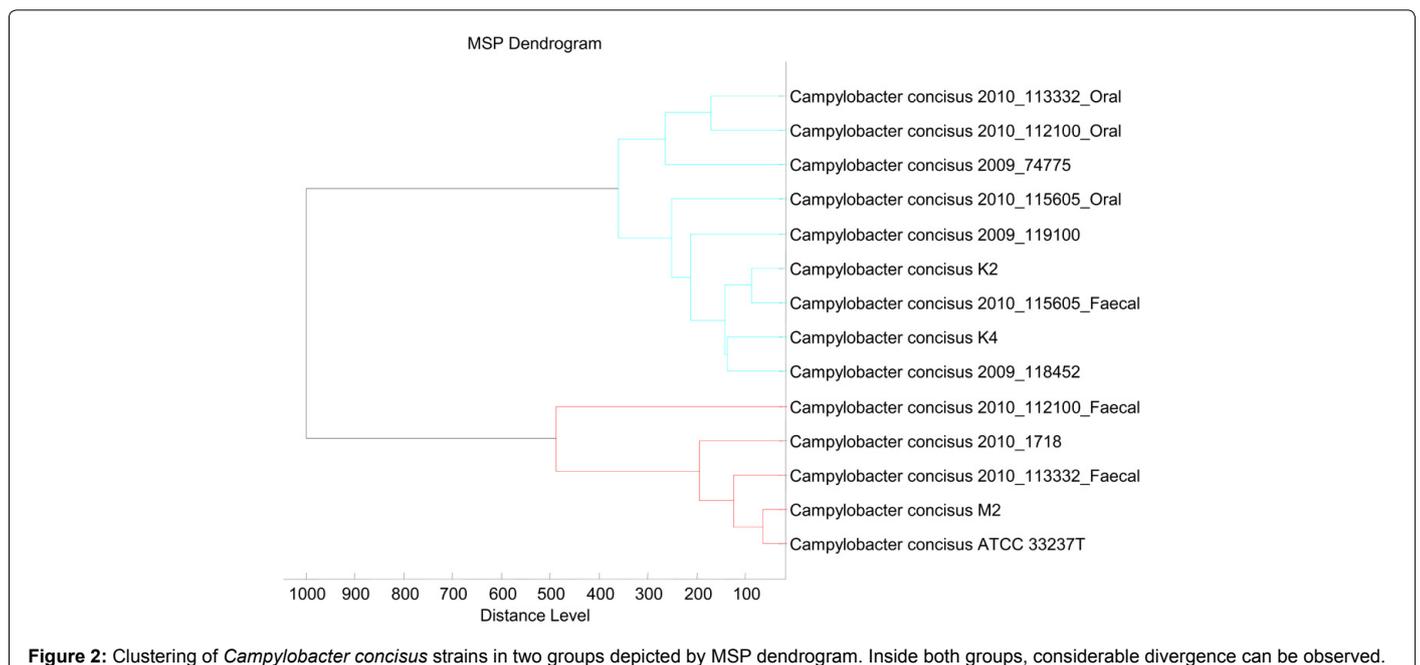

Figure 2: Clustering of *Campylobacter concisus* strains in two groups depicted by MSP dendrogram. Inside both groups, considerable divergence can be observed.

sets of oral and faecal strains were also clearly different strains, and detailed visual spectra analysis revealed a unique profile for each strain. Investigation of spectra which previously have been used to construct the MSP in the bruker database also confirmed high intraspecies diversity. Finally, all isolates were very stable as they showed no change in the analysis after the three re-cultivation steeps, or after one week of incubation (not shown).

The introduction of MALDI-TOF mass spectrometry into the routine laboratory has significantly improved the possibilities of a highly accurate identification of *Campylobacter* species, saving time and cost compared to conventional methods [2]. Nevertheless, *C. concisus*, a highly prevalent species in routine diarrheic stools, has been neglected in many clinical laboratories due to isolation procedures that use a labour intensive filter technique. Our data show that MALDI-TOF MS, as previously described for other *Campylobacter* species, can give a rapid identification of *C. concisus*. Interestingly, the isolates of *C. concisus* showed a remarkable diversity based upon their mass spectras when compared against each other by visual and computational analysis. In agreement with earlier reports [4,8,9], the intra-species diversity of *C. concisus* seems to be very high. This may be due to the oral nature of this species. Therefore, regarding genetic diversity the bacterium can be compared to the closely related *Helicobacter pylori* [6], but in contrast to *H. pylori*, the mass spectra of *C. concisus* strains still contain a significantly higher number of conserved peaks. This enables identification of the species with only a few database entries which cover the spectral diversity while a much higher number is necessary for coverage of *H. pylori* diversity. On the other hand, the very high intra-species diversity might facilitate epidemiological studies and strain tracking of *C. concisus*. Quite recently, Zautner et al., showed that MALDI-TOF MS could be used to discriminate specific subtypes of *C. jejuni* with different clinical relevance [18]. Similarly, we suggest that MALDI-TOF might play a useful role in subtyping of *C. concisus*. This information may prove essential for clinicians since *C. concisus* has been linked to different clinical manifestations like Crohn's disease, chronic and acute gastroenteritis, and from healthy individuals. The clear difference in spectra between oral and faecal isolates from the same individuals suggests that intestinal disease is not necessarily caused by endogenous oral strains but possibly by transmission of intestinal-type pathogenic strains. Consequently, further studies are needed to investigate a larger cohort of different isolates with differences in the clinical severity. In conclusion, we advocate for culture and MALDI-TOF MS identification of *C. concisus* in diarrheic stool samples. Culture of *C. concisus* has the advantage of antibiotic susceptibility surveillance as well as epidemiological investigation in which MALDI-TOF may be used. Future studies are needed to evaluate the exact role of MALDI-TOF MS including more isolates to fully clarify the diversity and strains tracking within *C. concisus*.

Acknowledgment

References

1. Bastyns K, Chapelle S, Vandamme P, Goossens H, De Wachter R (1995) Specific detection of Campylobacter concisus by PCR amplification of 23S rDNA areas. Mol Cell Probes 9: 247-250.

2. Bessède E, Solecki O, Sifré E, Labadi L, Mégraud F (2011) Identification of Campylobacter species and related organisms by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Clin Microbiol Infect 17: 1735-1739.

3. Chaban B, Musil KM, Himsworth CG, Hill JE (2009) Development of cpn60-based real-time quantitative PCR assays for the detection of 14 Campylobacter species and application to screening of canine fecal samples. Appl Environ Microbiol 75: 3055-3061.

4. Engberg J, Bang DD, Aabenhus R, Aarestrup FM, Fussing V, et al. (2005) Campylobacter concisus: an evaluation of certain phenotypic and genotypic characteristics. Clin Microbiol Infect 11: 288-295.

5. European Centre for Disease Prevention and Control (2014) Annual epidemiological report 2014-food and waterborne diseases and zoonoses. Stockholm. ECDC.

6. Ilina EN, Borovskaya AD, Serebryakova MV, Chelysheva VV, Momynaliev KT, et al. (2010) Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the study of Helicobacter pylori. Rapid Commun Mass Spectrom 24: 328-334.

7. Kaakoush NO, Deshpande NP, Wilkins MR, Tan CG, Burgos-Portugal JA, et al. (2011) The pathogenic potential of Campylobacter concisus strains associated with chronic intestinal diseases. PLoS One 6: e29045.

8. Kalischuk LD, Inglis GD (2011) Comparative genotypic and pathogenic examination of Campylobacter concisus isolates from diarrheic and non-diarrheic humans. BMC Microbiol 11: 53.

9. Mahendran V, Octavia S, Faruk Demirbas O, Sabrina S, Ma R, et al. (2015) Delineation of genetic relatedness and population structure of oral and enteric Campylobacter concisus strains by analysis of housekeeping genes. Microbiology 161: 1600-1612.

10. Mahendran V, Riordan SM, Grimm MC, Tran TA, Major J, et al. (2011) Prevalence of Campylobacter species in adult Crohn's disease and the preferential colonization sites of Campylobacter species in the human intestine. PLoS One 6: e25417.

11. Man SM, Kaakoush NO, Leach ST, Nahidi L, Lu HK, et al. (2010) Host attachment, invasion, and stimulation of proinflammatory cytokines by Campylobacter concisus and other non-Campylobacter jejuni Campylobacter species. J Infect Dis 202: 1855-1865.

12. Mukhopadhya I, Thomson JM, Hansen R, Berry SH, El-Omar EM, et al. (2011) Detection of Campylobacter concisus and other Campylobacter species in colonic biopsies from adults with ulcerative colitis. PLoS One 6: e21490.

13. Nielsen HL, Ejlertsen T, Engberg J, Nielsen H (2013) High incidence of Campylobacter concisus in gastroenteritis in North Jutland, Denmark: a population-based study. Clin Microbiol Infect 19: 445-450.

14. Nielsen HL, Ejlertsen T, Nielsen H (2015) Polycarbonate filtration technique is noninferior to mCCDA for isolation of Campylobacter species from stool samples. Diagn Microbiol Infect Dis 83: 11-12.

15. Nielsen HL, Engberg J, Ejlertsen T, Bücker R, Nielsen H (2012) Short-term and medium-term clinical outcomes of Campylobacter concisus infection. Clin Microbiol Infect 18: E459-465.

16. Nielsen HL, Engberg J, Ejlertsen T, Nielsen H (2013) Clinical manifestations of Campylobacter concisus infection in children. Pediatr Infect Dis J 32: 1194-1198.

17. Nielsen HL, Nielsen H, Ejlertsen T, Engberg J, Günzel D, et al. (2011) Oral and fecal Campylobacter concisus strains perturb barrier function by apoptosis induction in HT-29/B6 intestinal epithelial cells. PLoS One 6: e23858.

18. Zautner AE, Masanta WO, Tareen AM, Weig M, Lugert R, et al. (2013) Discrimination of multilocus sequence typing-based Campylobacter jejuni subgroups by MALDI-TOF mass spectrometry. BMC Microbiol 13: 247.

19. Zhang L, Man SM, Day AS, Leach ST, Lemberg DA, et al. (2009) Detection and isolation of Campylobacter species other than C. jejuni from children with Crohn's disease. J Clin Microbiol 47: 453-455.