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Original Article

Clinical and microbiological characteristics of patients with bacteremia caused by *Campylobacter* species with an emphasis on the subspecies of *C. fetus*

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Abstract *Objectives:* This study was intended to investigate the clinical and microbiological characteristics of patients with bacteremia caused by *Campylobacter* species.
Methods: From April 1998 to May 2014, 56 adults with bacteremia caused by *Campylobacter* species were evaluated. These *Campylobacter* species isolates were confirmed to the species level using 16S rRNA gene sequencing (all isolates) and multiplex PCR analysis (for *C. fetus* only). The performance of identification for *Campylobacter* species by the Bruker Biotyper MALDI-TOF MS was evaluated. The genetic relatedness of *C. fetus* isolates was analyzed by multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE).
Results: The leading underlying medical conditions of these patients were malignancy (46.4%), hypertension (35.7%), and liver cirrhosis (23.2%). The overall 30-day mortality rate was 5.4%. Using 16S rRNA sequencing analysis, 26 isolates of *C. coli*, 11 of *C. jejuni*, and 19 of *C. fetus*, including 15 *C. fetus* subsp. *fetus* and five *C. fetus* subsp. *venerealis*, were identified. Among the five *C. fetus* subsp. *venerealis* isolates recognized by 16S rRNA gene sequencing, only two isolates were *C. fetus* subsp. *venerealis* by multiplex PCR method. The Bruker Biotyper

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MALDI-TOF MS failed to correctly identify *C. fetus* subsp. *venerealis* isolates. MLST analysis of *C. fetus* isolates revealed three STs: ST20 (n = 12), ST11 (n = 5), and ST57 (n = 2), which were compatible with three major PFGE clusters.

Conclusion: Database expansion of MALDI-TOF MS for the correct identification of *C. fetus* to subspecies levels is needed. A novel clone of ST57-PFGE Cluster C of *C. fetus* subsp. *venerealis* was noted.

Introduction

Campylobacter is a genus of microaerophilic Gram-negative, non-spore forming, motile bacteria with a characteristic spiral or corkscrew-like appearance; this bacteria causes puzzling diseases in both humans and animals.[1,2] In addition to *Campylobacter jejuni*, which causes the majority of *Campylobacter* infections, other *Campylobacter* species, such as *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter upsaliensis*, and *Campylobacter lari*, have been reported to be associated with human diseases.[3]

Earlier reports have revealed the incidence, clinical characteristics and outcomes of bacteremia caused by *Campylobacter* species.[4–7] These risk factors include old age, alcoholism, diabetes mellitus, HIV infection, malignancy, and transplantation.[4,5,8] As there are increasing numbers of patients with the abovementioned risk factors, the clinical significance of *Campylobacter* bacteremia has resulted in increased attention, and timely and correct therapeutic intervention is prudent. Thus, it is important to precisely identify these isolates beyond the genus level for both therapeutic options and epidemiological surveillance.

C. fetus can cause intestinal illness and, occasionally, severe extra-intestinal infections.[9] *C. fetus* bacteremia, a rare human infection, occurs almost in the settings of advanced age, immunosuppression, HIV infection, alcoholism, or recent gastrointestinal surgery.[10] Currently, four subspecies—*C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, *C. fetus* subsp. *testudinum* and the variant *C. fetus* subsp. *venerealis* biovar *intermedius* have been identified.[11] These subspecies are very closely related genetically but have different habitats.

Unfortunately, the phenotypic identification of *Campylobacter* species is currently unsatisfactory because of their complex taxonomy, unreliable biochemical tests, special requirements for optimal growth, and the laborious work involved in the process.[12] The hippurate hydrolysis test has been applied to identify *C. jejuni*, and only *C. jejuni* gives a positive reaction with this test. However, hippurate-negative strains of this species are commonly described, and false-positive test results for non-*C. jejuni* species have also been widely recognized.[13,14] Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an accurate, rapid, and inexpensive method for identifying clinically relevant bacteria, including *Campylobacter* species.[15,16] Although the accuracy of identification by MALDI-TOF MS can reach 100% compared with the molecular methods employed for all

Campylobacter species, except *C. jejuni* (99.4%), the use of MALDI-TOF MS for the identification of subspecies of *C. fetus* has not been reported.[15,16]

In this study, we conducted a retrospective analysis of the clinical characteristics and outcomes of all patients with bacteremia caused by different *Campylobacter* species. We also assessed the accuracy of identification using the MALDI-TOF MS system and conventional phenotypic methods for the identification of clinical isolates of genetically confirmed *Campylobacter* species. Genotypes of *C. fetus* isolates were determined by the multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) methods.

Materials and methods

Patients and setting

Patients with *Campylobacter* bacteremia were identified by review of the laboratory records of the National Taiwan University Hospital (NTUH) from April 1998 to May 2014. This hospital, a 2900-bed university-affiliated hospital with all medical and surgical specialties in Taipei, northern Taiwan, provides both primary and tertiary medical care. The medical records of patients with *Campylobacter* bacteremia, including demographics, clinical characteristics, underlying medical conditions, and outcomes, were retrospectively reviewed.

Bacterial isolates

Blood cultures were processed using the BACTEC blood culture system (Becton Dickson, Sparks, MD, USA), and Gram-negative bacteria demonstrating a curved or spiral-shaped appearance that reacted positively to oxidase and catalase were preliminarily identified as *Campylobacter* species through the use of conventional biochemical methods. In brief, isolates were also grown on Campy-BAP agar plates (BBL Microbiology System, Cockeysville, MD, USA) after they were incubated for 48 h in a microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2) at 25 °C, 37 °C, or 42 °C. Isolates that grew well at 42 °C but not at 25 °C and that were resistant to cephalothin (30-μg disk diffusion method) were identified as either *C. jejuni* or *C. coli*. These thermophilic *Campylobacter* isolates were considered to be *C. jejuni* if they were positive for hippurate hydrolysis and were considered *C. coli* if they were negative for hippurate

hydrolysis. *C. fetus* was identified if the isolate grew at 25 °C but not at 42 °C and was susceptible to cephalothin (30-μg disk). All these isolates were preserved for further phenotypical and genotypic characterization in this study.

Molecular identification

All preserved *Campylobacter* isolates were confirmed to the species level by 16S rRNA gene sequencing. Multiplex PCR (*cad*F, *hip*O and *asp* genes) was carried out to confirm the subspecies level for all *C. fetus* isolates.[17,18] The following primer combinations were used: Ps5/1 (5′-TATG-GAGAGTTTGATCCTGG-3′) and Ps3/1 (5′-GTTAAGCTGTTA-GATTTCAC-3′), Ps5/2 (5′-AGCGTTACTCGGAATCACTG-3′) and Ps3/2 (5′-ACAGCCGTGCAGCACCTGTC-3′), and Ps5/3 (5′-AACCTTACCTGGGCTTGATA-3′) and Ps3/3 (5′-AAG-GAGGTGATCCAGCCGCA-3′). The 16S rRNA gene sequencing data of these isolates were deposited into GenBank, and the accession numbers of the submitted nucleotide sequences were reported. Identification of *C. fetus* isolates to the subspecies level was in accordance with the previous description by Hum et al.[19] *C. fetus* subsp. *venerealis* JCM2529[T] (http://www.jcm.riken.jp/cgi-bin/jcm/jcm_number?JCM=2528) obtained from Japan Collection of Microorganisms (JCM, Saitama, Japan) was used as controls for 16S rRNA sequencing and multiplex PCR analysis.

Identification by API Campy system

All isolates were analyzed using a commercial identification system (API Campy system, bioMérieux, France), which is a standardized system for the identification of *Campylobacter* species using 20 biochemical tests. The identification of each isolate was shown using a 7-digit profile number and API Campy analytical profile index software.[20] Identification using the API system was categorized as excellent if the percentage of identification was ≥99% and the T-index was ≥0.75, very good if the percentage of identification was ≥99% and the T-index was ≥0.5, good if the percentage of identification was ≥90% and the T-index was ≥0.25, and acceptable if the percentage of identification was ≥80% and the T-index was ≥0.0.[21]

Bruker Biotyper MALDI-TOF MS

For the analysis of *Campylobacter* isolates using the Bruker Biotyper MALDI-TOF MS system, samples were prepared as previously described.[22] A single colony from a blood agar plate (BD Microbiology System) was subjected to an ethanol—formic acid extraction procedure for microorganism profiling. Briefly, colonies were transferred into 300 μl of distilled water and 900 μl of ethanol using a 5-μl inoculating loop. The suspension was pelleted after centrifugation at 12,000 rpm for 2 min, dried, and then reconstituted in 50 μl of 70% formic acid. After incubation for 30 s, 50 μl of acetonitrile (Sigma—Aldrich) was added. The suspension was then centrifuged at 12,000 rpm for 2 min. A volume of 1.0 μl of the supernatant was applied to a 96-spot polished steel target (Bruker Daltonik GmbH, Bremen, Germany) plate and dried. A saturated solution of 1.0 ml of MALDI

matrix (HCCA; Bruker Daltonik) was applied to each sample and dried. Measurements were performed with a Bruker MicroFlex LT MALDI-TOF MS (Bruker Daltonik GmbH) using FlexControl software with Compass Flex Series version 1.3 software and a 60 Hz nitrogen laser (337 nm wavelength). Spectra were collected in the linear positive mode in a mass range covering 1960 to 20,132 *m/z*. Spectra ranging from the mass-to-charge ratio (*m/z*) 2000 to 20,000 were analyzed using a Bruker Biotyper MALDI-TOF MS automation control and the Bruker Biotyper 3.1 software and library (version 3.1.66, with 4613 entries; Bruker Daltonics). The interpretation criteria used in our study, suggested by the manufacturer, were as followings: a score of ≥2.000 indicated species level identification, a score of 1.700—1.999 indicated genus level identification, and a score of <1.700 was interpreted as no identification. All isolates with discrepant identification results between the molecular and Bruker Biotyper MALDI-TOF MS methods were retested twice.

MLST for *C. fetus* isolates

Seven loci used in a previously described *C. fetus* MLST scheme were required to design the primers for MLST: *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*.[23] Distinct allele sequences were assigned arbitrary allele numbers for each locus in a *C. fetus* MLST database (http://pubmlst.org/campylobacter/), and the seven numbers constituted an allelic profile or sequence type (ST). Allele numbers for new sequences and ST numbers for new allelic profiles are available by submission to the database.

PFGE for *C. fetus* isolates

DNA from the 19 *C. fetus* isolates was digested by the restriction enzyme *Sma*I at 25 °C, and the restriction fragments were separated in a CHEF-DR III unit (Bio-Rad Laboratories, Hercules, CA, USA) at 6 V for 18 h with initial switch of 6.8 s and final switch of 35.4 s according to the instruction manual. The PFGE results and dendrogram were analyzed with BioNumerics Version 5.10 (settings: Dice, UPGMA with 1% optimization and 1% band tolerance). Isolates exhibiting PFGE profiles with greater than 80% similarity were considered to be closely related strains.[24]

Results

16S rRNA gene sequencing for all *Campylobacter* isolates

During the study period, fifty-six patients with *Campylobacter* bacteremia were identified. Tables 1 and 2 show the identification results according to 16S rRNA gene sequencing for the 56 *Campylobacter* species isolate to the species level, with >99% sequence similarity at the level of the 16S rRNA sequence. Three different *Campylobacter* species, including *C. jejuni* (11 isolates), *C. coli* (26 isolates), and *C. fetus* (19 isolates), were identified. Among the 19 *C. fetus* isolates, fourteen isolates were reported as

Table 1 The identification of 11 *Campylobacter jejuni* isolates and 26 *Campylobacter coli* isolates recovered from patients with bacteremia who were treated at the National Taiwan University Hospital.

No.	Identification (ID) results						
	16S rRNA sequencing		API Camp			Bruker Biotyper MALDI-TOF MS	
	Species	ID %	Species	ID %	T-index value	Species	Score value
1.	*C. jejuni*	99.9	*C. jejuni* subsp. *jejuni* 2	99.90	0.39	*C. jejuni*	2.226
2.	*C. jejuni*	100	*C. jejuni* subsp. *jejuni* 2	99.90	0.74	*C. jejuni*	2.156
3.	*C. jejuni*	99.2	*C. jejuni* subsp. *jejuni* 2	97.20	0.23	*C. jejuni*	2.261
4.	*C. jejuni*	100	*C. jejuni* subsp. *jejuni* 2	99.90	0.33	*C. jejuni*	2.257
5.	*C. jejuni*	99.2	*C. jejuni* subsp. *jejuni* 2	99.90	0.25	*C. jejuni*	2.395
6.	*C. jejuni*	100	*C. jejuni* subsp. *jejuni* 2	99.90	0.37	*C. jejuni*	2.423
7.	*C. jejuni*	100	*C. jejuni* subsp. *jejuni* 2	99.90	0.35	*C. jejuni*	2.036
8.	*C. jejuni*	100	*C. jejuni* subsp. *jejuni* 2	99.90	0.48	*C. jejuni*	2.282
9.	*C. jejuni*	100	*C. jejuni* subsp. *jejuni* 2	99.90	0.48	*C. jejuni*	2.326
10.	*C. jejuni*	100	*C. jejuni* subsp. *jejuni* 2	99.90	0.37	*C. jejuni*	2.153
11.	*C. jejuni*	100	*C. jejuni* subsp. *jejuni* 2	99.90	0.45	*C. jejuni*	2.158
12.	*C. coli*	99.9	*C. coli*	99.70	0.36	*C. coli*	2.22
13.	*C. coli*	100	*C. coli*	99.40	0.32	*C. coli*	2.176
14.	*C. coli*	100	*C. coli*	96.90	0.43	*C. coli*	1.957
15.	*C. coli*	100	*C. coli*	99.50	0.77	*C. coli*	2.146
16.	*C. coli*	100	*C. coli*	99.90	0.82	*C. coli*	2.076
17.	*C. coli*	100	*C. coli*	99.30	0.52	*C. coli*	2.089
18.	*C. coli*	100	*C. coli*	95.80	0.42	*C. coli*	2.186
19.	*C. coli*	100	*C. coli*	99.90	0.63	*C. coli*	2.106
20.	*C. coli*	99.9	*C. coli*	92.20	0.75	*C. coli*	1.898
21.	*C. coli*	100	*C. coli*	99.30	0.66	*C. coli*	2.212
22.	*C. coli*	99.9	*C. coli*	90.70	0.46	*C. coli*	1.814
23.	*C. coli*	99.4	*C. coli*	99.80	0.78	*C. coli*	1.897
24.	*C. coli*	99.9	*C. coli*	99.90	0.42	*C. coli*	2.16
25.	*C. coli*	99.9	*C. coli*	99.90	0.42	*C. coli*	2.214
26.	*C. coli*	99.9	*C. coli*	99.00	0.63	*C. coli*	2.212
27.	*C. coli*	99.1	*C. coli*	99.70	0.36	*C. coli*	2.233
28.	*C. coli*	99.9	*C. coli*	99.90	0.6	*C. coli*	2.117
29.	*C. coli*	99.9	*C. coli*	97.90	0.34	*C. coli*	2.259
30.	*C. coli*	99.9	*C. coli*	97.90	0.34	*C. coli*	2.206
31.	*C. coli*	100	*C. coli*	99.90	0.82	*C. coli*	2.032
32.	*C. coli*	99.9	*C. coli*	99.40	0.32	*C. coli*	1.872
33.	*C. coli*	100	*C. coli*	99.70	0.25	*C. coli*	1.961
34.	*C. coli*	99.9	*C. coli*	96.20	0.5	*C. coli*	2.028
35.	*C. coli*	99.9	*C. coli*	95.80	0.42	*C. coli*	2.066
36.	*C. coli*	100	*C. coli*	90.70	0.46	*C. coli*	1.978
37.	*C. coli*	100	*C. coli*	99.90	0.42	*C. coli*	1.804

C. fetus subsp. *fetus,* and five isolates were identified as *C. fetus* subsp. *venerealis.*

Identification of *C. fetus* subspecies by multiplex PCR

All 14 *C. fetus* subsp. *fetus* isolates identified by 16S rRNA sequencing methods were concordantly identified by multiplex PCR. Among the five *C. fetus* subsp. *venerealis* isolates recognized by 16S rRNA gene sequencing analysis, two isolates were identified as *C. fetus* subsp. *venerealis* and the other three were identified as *C. fetus* subsp. *fetus.*

Clinical characteristics and outcomes of patients with *Campylobacter* bacteremia

The clinical characteristics of the 56 patients with *Campylobacter* bacteremia are summarized in Table 3. Ten patients (17.9%) presented with fever, and 1 patient developed the metastatic complication infectious spondylodiscitis. The leading underlying diseases were malignancy (26 patients, 46.4%), hypertension (20 patients, 35.7%), and cirrhosis (13 patients, 23.2%). Thirteen (68.4%) *C. fetus* bacteremia patients presented with cirrhosis or renal insufficiency, whereas only 4 (15.4%) *C. coli* bacteremia patients and no (0%) *C. jejuni* bacteremia patients

Table 2 Identification of 19 *Campylobacter fetus* isolates recovered from patients with bacteremia who were treated at the National Taiwan University Hospital.

No.	Multiplex PCR	Identification (ID) results							
		Growth in 1% glycine	16S rRNA sequencing		API Camp			Bruker Biotyper MALDI-TOF MS	
			Species	ID %	Species	ID %	T-index value	Species	Score value
1.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	99.6	*C. fetus* subsp. *fetus*	71.30	0.86	*C. fetus* subsp. *fetus*	2.134
2.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	99.6	*C. fetus* subsp. *fetus*	97.50	0.96	*C. fetus* subsp. *fetus*	2.292
3.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	99.6	*C. fetus* subsp. *fetus*	92.60	0.81	*C. fetus* subsp. *venerealis*	2.171
4.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	99.6	*C. fetus* subsp. *fetus*	68.40	0.84	*C. fetus* subsp. *fetus*	2.338
5.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	99.6	*C. fetus* subsp. *fetus*	71.30	0.86	*C. fetus* subsp. *fetus*	2.262
6.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	99.9	*C. fetus* subsp. *fetus*	50.60	0.97	*C. fetus* subsp. *fetus*	2.12
7.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	99.6	*C. fetus* subsp. *fetus*	68.40	0.84	*C. fetus* subsp. *fetus*	2.119
8.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	100	*C. fetus* subsp. *fetus*	68.40	0.84	*C. fetus* subsp. *venerealis*	2.276
9.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	100	*C. fetus* subsp. *fetus*	68.40	0.84	*C. fetus* subsp. *venerealis*	2.348
10.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	99.9	*C. fetus* subsp. *fetus*	44.80	0.75	*C. fetus* subsp. *fetus*	2.401
11.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	100	*C. fetus* subsp. *fetus*	95.10	0.54	*C. fetus* subsp. *fetus*	2.348
12.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	99.5	*C. fetus* subsp. *fetus*	95.10	0.54	*C. fetus* subsp. *fetus*	2.353
13.	*C. fetus* subsp. *venerealis*	−	*C. fetus* subsp. *venerealis*	100	*C. fetus* subsp. *fetus*	64.80	0.86	*C. fetus* subsp. *fetus*	1.762
14.	*C. fetus* subsp. *venerealis*	−	*C. fetus* subsp. *venerealis*	99.3	*C. fetus* subsp. *fetus*	68.10	0.87	*C. fetus* subsp. *fetus*	1.814
15.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *venerealis*	99.9	*C. fetus* subsp. *fetus*	92.60	0.81	*C. fetus* subsp. *fetus*	2.367
16.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *venerealis*	99.3	*C. fetus* subsp. *fetus*	68.10	0.87	*C. fetus* subsp. *fetus*	2.262
17.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	98.9	*C. fetus* subsp. *fetus*	64.80	0.86	*C. fetus* subsp. *fetus*	2.407
18.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *fetus*	97,7	*C. fetus* subsp. *fetus*	68.10	0.87	*C. fetus* subsp. *fetus*	2.383
19.	*C. fetus* subsp. *fetus*	+	*C. fetus* subsp. *venerealis*	99.9	*C. fetus* subsp. *fetus*	92.60	0.81	*C. fetus* subsp. *fetus*	2.356

Table 3 Clinical characteristics of 56 patients with *Campylobacter* bacteremia treated at the National Taiwan University Hospital.

Characteristics	No. (%) of patients with bacteremia caused by indicated Campylobacter species			
	C. fetus (n = 19)	C. coli (n = 26)	C. jejuni (n = 11)	Overall (n = 56)
Age (yr), mean (range)	59 (37—89)	55 (24—82)	45 (8—72)	54 (8—89)
Male	13 (68.4)	15 (57.7)	7 (63.6)	35 (62.5)
Underlying diseases				
Diabetes mellitus	4 (21.1)	2 (7.7)	1 (9.1)	7 (12.5)
Hypertension	8 (42.1)	9 (34.6)	3 (27.3)	20 (35.7)
Coronary artery disease	2 (10.5)	1 (3.8)	1 (9.1)	4 (7.1)
Heart failure	0 (0)	3 (11.5)	0 (0)	3 (5.4)
Cirrhosis	9 (47.4)	4 (15.4)	0 (0)	13 (23.2)
Lung disease	0 (0)	2 (7.7)	0 (0)	2 (3.6)
Renal insufficiency	9 (47.4)	1 (3.8)	0 (0)	10 (17.9)
Autoimmune diseases	0 (0)	2 (7.7)	0 (0)	2 (3.6)
Malignancy	8 (42.1)	11 (42.3)	7 (63.6)	26 (46.4)
Hematologic malignancy	3 (15.8)	3 (11.5)	3 (27.3)	9 (16.1)
Leukemia	0 (0)	0 (0)	2 (18.2)	2 (3.6)
Lymphoma	2 (10.5)	1 (3.8)	1 (9.1)	4 (7.1)
Multiple myeloma	1 (5.3)	1 (3.8)	0 (0)	2 (3.6)
Myelodysplastic syndrome	0 (0)	1 (3.8)	0 (0)	1 (1.8)
Solid tumor	5 (26.3)	8 (30.8)	4 (36.4)	17 (30.4)
Breast	0 (0)	0 (0)	0 (0)	0 (0)
Lung	0 (0)	1 (3.8)	1 (9.1)	2 (3.6)
Liver	3 (15.8)	3 (11.5)	2 (18.2)	8 (14.3)
Gastric	0 (0)	1 (3.8)	0 (0)	1 (1.8)
Colonic	1 (5.3)	0 (0)	0 (0)	1 (1.8)
Pelvic	1 (5.3)	2 (7.7)	0 (0)	3 (5.4)
Others	0 (0)	1 (3.8)[a]	1 (9.1)[b]	2 (3.6)
Transplantation	1 (5.3)	2 (7.7)	1 (9.1)	4 (7.1)
Liver	0 (0)	1 (3.8)	0 (0)	1 (1.8)
Kidney	1 (5.3)	1 (3.8)	0 (0)	2 (3.6)
Peripheral blood stem cell	0 (0)	0 (0)	1 (9.1)	1 (1.8)
30-day mortality	1 (5.3)	2 (7.7)	0 (0)	3 (5.4)

[a] Nasopharyngeal carcinoma.
[b] Giloblastoma multiforme.

presented with cirrhosis or renal insufficiency. The proportions of *C. fetus*, *C. coli*, and *C jejuni* bacteremia patients with a history of malignancy were 42.1%, 42.3%, and 63.6%, respectively. Among the 19 patients with *C. fetus* bacteremia, ten patients (52.6%) had chronic liver diseases, either hepatitis B virus or hepatitis C virus infection, or alcohol-related, and nine patients (47.4%) presented with gastrointestinal mucosal insults, including recent gastrointestinal bleeding, non-steroidal anti-inflammatory drug use, or leukopenia. As for the two patients with *C. fetus* subsp. *venerealis* bacteremia, one each presented with multiple myeloma and HBV-related cirrhosis with hepatocellular carcinoma and both had leukopenia. Among the 56 patients, three (5.4%) died within 30 days of hospitalization, including one (5.3%) with *C. fetus* subsp. *fetus* bacteremia and two (7.7%) with *C. coli* bacteremia.

Performance of the API Campy system

Tables 1 and 2 present the identification results from the API Campy system. Among the 11 *C. jejuni* isolates confirmed by 16S rRNA sequencing, ten isolates were identified as *C. jejuni* subsp. *jejuni* with a percentage of identification of $\geq$99.90% and a T-index $\geq$0.25. One isolate was identified as *C. jejuni* subsp. *jejuni* with a low T-index value (percentage of identification: 97.2%; T-index value: 0.23). All 26 *C. coli* isolates were interpreted as *C. coli*, with the percentage of identification ranging from 90.7% to 99.9% and the T-index value ranging from 0.25 to 0.82. Of the 19 *C. fetus* isolates, fourteen isolates were correctly reported as *C. fetus* subsp. *fetus* by API Campy system, whereas five *C. fetus* subsp. *venerealis* isolates identified by the 16S rRNA sequencing method were all identified as *C.*
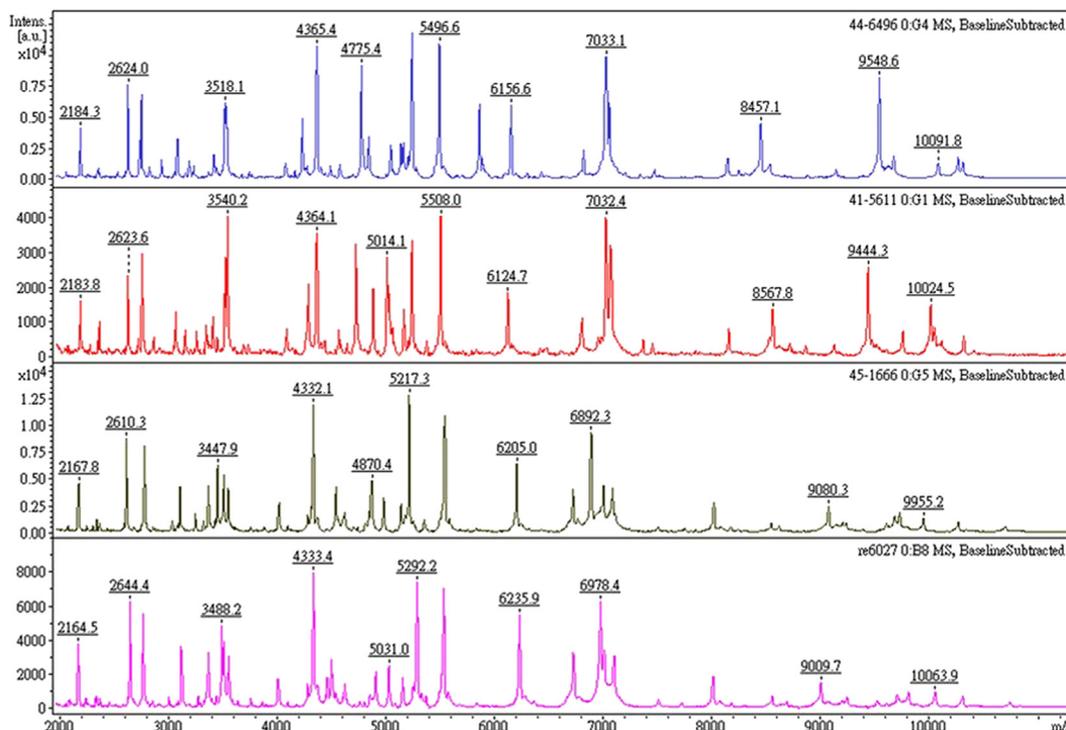
Figure 1. MALDI-TOF MS spectra of the isolates. Spectra of (A) *C. jejuni*, (B) *C. coli*, (C) *C. fetus* subsp. *fetus*, and (D) *C. fetus* subsp. *venerealis* generated by Bruker Biotyper MALDI-TOF MS. The absolute intensities of the ions are shown on the *y*-axis, and the masses (*m/z*) of the ions are shown on the *x*-axis. The *m/z* value represents the mass-to-charge ratio.
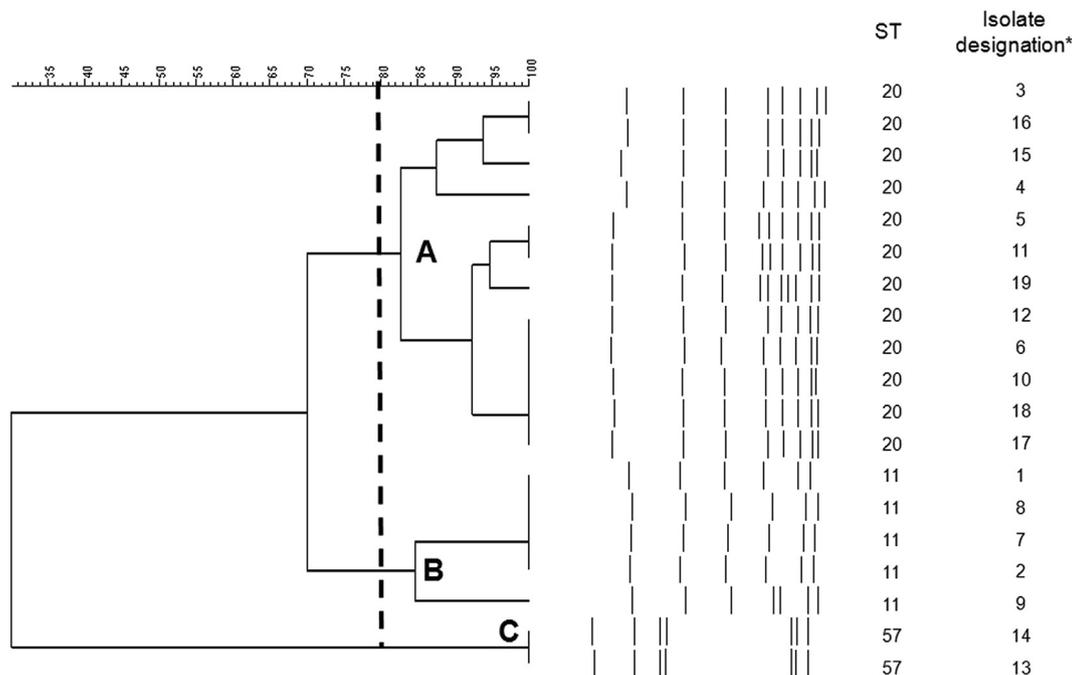

Figure 2. Pulsed-field gel electrophoresis profiles using the restriction enzyme *Sma*I and a dendrogram of the 19 *C. fetus* isolates. With a cut-off at 80% of similarity, three major clusters (A, B, and C) were identified.

fetus subsp. *fetus*. Among the 14 isolates that were correctly reported as *C. fetus* subsp. *fetus* by the API Campy system, only 4 *C. fetus* subsp. *fetus* isolates were reported comfortably, whereas the other 10 isolates were identified as *C. fetus* subsp. *fetus* with a low percentage of identification, ranging from 44.8% to 71.3%.

Performance of the Bruker Biotyper MALDI-TOF MS system

Thirty peaks in the spectra of the *Campylobacter* species isolates were generated by ClinProtools 3.0 using the Quick

Classifier algorithm (Bruker Daltonics). The results of the identification by Bruker Biotyper MALDI-TOF MS to the species level (11 *C. jejuni* isolates, 26 *C. coli* isolates, and 19 *C. fetus* isolates) were identical to those by 16S rRNA sequencing analysis. All 11 *C. jejuni* isolates had high identification score values, ranging from 2.036 to 2.423, whereas the identification score values for the 26 *C. coli* isolates ranged from 1.804 to 2.259, including 18 isolates with score values of 2.028−2.259 and eight isolates with score values of 1.804−1.978 (genus level identification). Two subspecies of *C. fetus* were identified, namely 17 isolates of *C. fetus* subsp. *fetus* and two isolates of *C. fetus* subsp. *venerealis*. Among the 14 *C. fetus* subsp. *fetus* isolates confirmed by 16S rRNA gene sequencing, twelve isolates were properly reported with high identification score values (from 2.119 to 2.407). Despite high identification score values (2.171 and 2.276, respectively), the remaining 2 isolates were misidentified as *C. fetus* subsp. *venerealis*. All of the five *C. fetus* subsp. *venerealis* isolates confirmed by 16S rRNA gene sequencing were misidentified as *C. fetus* subsp. *fetus* by Bruker Biotyper MALDI-TOF MS, with the identification scores ranging from 1.762 to 2.367. Bruker Biotyper MALDI-TOF MS profiles of the four *Campylobacter* species/subspecies are illustrated in Fig. 1.

MLST of *C. fetus*

Among the 19 *C. fetus* isolates, 12 belonged to ST20 (including three identified as *C. fetus* subsp. *venerealis* by 16S rRNA sequencing analysis only), five were ST11 and two were ST57 (both were concordantly identified as *C. fetus* subsp. *venerealis* by both 16S rRNA gene sequencing and multiplex PCR methods).

PFGE of *C. fetus*

Cluster analysis and a dendrogram of the PFGE profiles of the 19 *C. fetus* isolates revealed three major clusters (Clusters A, B, and C) based on an 80% similarity. All 12 *C. fetus* subsp. *fetus* ST 20 isolates belonged to Cluster A, the five *C. fetus* subsp. *fetus* ST 11 isolates belonged to Cluster B, and the two *C. fetus* subsp. *venerealis* ST 57 isolates belonged to Cluster C. No geographically epidemiological relationship within the three clusters was noted during the observed period (Fig. 2).

Discussion

Campylobacter bacteremia primarily occurs in immunocompromised hosts or those with debilitating conditions, including liver cirrhosis, malignancy, and renal insufficiency,[1,7,8] which is compatible with our results. The overall 30-day mortality rate associated with *Campylobacter* bacteremia was 5.4% in our cohort, whereas the reported data on the mortality of *Campylobacter* bacteremia are contradictory, ranging from 2.5% to 28%.[1,5,6,8] Many factors have been attributed to the wide-ranging mortality rate associated with *Campylobacter* bacteremia, including age, underlying diseases, inadequate antibiotic treatment,[4] transient *C. fetus* or *C. jejuni* bacteremia,[25] and HIV infection.[7] In addition, the

misidentification of *Campylobacter* species by phenotypic methods in previous studies may also account for the inconsistent results observed. Further studies considering the above factors to explore the fetal outcome of *Campylobacter* bacteremia are needed.

In view of differing virulence, clinical presentation, high mortality, and creeping antibiotic resistance, it is prudent to properly and rapidly identify these pathogens.[1,6,7] Unfortunately, the identification of *Campylobacter* species has been ambiguous in the past due to several of their characteristics, including slow growth, special requirements for their growth environment, atypical strain existence with unusual phenotypic profiles, and close genetic associations between some species. Here, we presented a comparison of accuracy in *Campylobacter* identification between conventional and molecular methods.

The multiplex PCR assay to detect the target genes *cstA* and *parA* described by Hum et al. has been widely used for subspecies identification of *C. fetus*. However, this method is less successful for the genotypic identification of *C. fetus* subsp. *venerealis* isolates.[26] In Hum's research, all *C. fetus* subsp. *fetus* were correctly identified by the multiplex PCR assay, whereas 2 (2.1%) of 97 isolates, identified as *C. fetus* subsp. *venerealis*, were discordant with those made by macro-restriction profile analysis and/or probabilistic phenotypic methods. Willoughby et al.[26] reported that the *parA* gene was obtained for only 14 (43.8%) of 32 phenotypical *C. fetus* subsp. *venerealis* isolates. They suggested that the presence of an unusual clone of this subspecies in the United Kingdom may account for the failure of multiplex PCR genotyping. In our study, among the five *C. fetus* subsp. *venerealis* isolates identified by the 16S rRNA sequencing analysis, three were identified as *C. fetus* subsp. *fetus* and two were *C. fetus* subsp. *venerealis* by the multiplex PCR method. Interestingly, results from MLST and PFGE analysis suggest that identification results by multiplex PCR method is more accurate than 16S rRNA sequencing analysis because the three *C. fetus* subsp. *venerealis* identified by the 16S rRNA sequencing analysis belonged to main clone of *C. fetus* subsp. *fetus* ST (ST20-PFGE Cluster A). However, the possibility of presence of two clones (ST20-PFGE Cluster A and ST57-PFGE Cluster C) of *C. fetus* subsp. *venerealis* revealed by the 16S rRNA sequencing analysis could not be excluded. Further surveys are warranted to determine whether there was a novel *C. fetus* subsp. *venerealis* clone (ST57-PFGE Cluster C) circulating in Taiwan.

Our study showed discrepant results in the API Campy system for the identification of some *Campylobacter* species. In our study, the accuracy of the API Campy system reached up to 90.9% for *C. jejuni* and 100% for *C. coli*. However, the API Campy system failed to correctly identify *C. fetus* to the subspecies level in our study. All 5 *C. fetus* subsp. *venerealis* isolates were erroneously reported as *C. fetus* subsp. *fetus* by the API Campy system. Although all 14 *C. fetus* subsp. *fetus* isolates were correctly recognized by the API Campy system, only 4 (28.6%) isolates were reported comfortably with a percentage of identification $\geq$ 90% and a T-index $\geq$ 0.25. Previous studies revealed similar results. Martiny et al. showed that the API Campy system allowed for the correct identification of

92.8%, 55.4%, 28.6%, 37.5%, 40%, and 100% of *C. jejuni* subsp. *jejuni*, *C. coli*, *C. fetus*, *C. upsaliensis*, *C. lari*, and *C. jejuni* subsp. *doylei* isolates, respectively.[20] Bessède et al. reported outstanding results by the API Campy system for the identification of *C. sputorum* and *C. upsaliensis*, except *C. lari*, which showed a correct identification rate of only 50%.[15]

We noted excellent agreement between molecular methods and Bruker Biotyper MALDI-TOF MS for the identification of common *Campylobacter* species, except for *C. fetus* subspecies. The similar performance of MALDI-TOF MS has also been previously reported. Martiny et al. demonstrated good consistency between MALDI-TOF MS and several PCR-based identification methods, except that MALDI-TOF MS correctly identified only 83.3% of *C. curvus*.[20] Bessède et al. demonstrated that the accuracy of MALDI-TOF MS reached up to 100% compared with the gold standard for all *Campylobacter* species, except for *C. jejuni* (99.4%).[15] For the identification of *C. fetus* subspecies, which were not evaluated in either Martiny's or Bessède's studies, significant discordance between 16S RNA sequencing and MALDI-TOF MS was noted in our study. Even with high score values (2.171 and 2.276, respectively), 14.3% (2/14) of the *C. fetus* subsp. *fetus* isolates, were misidentified as *C. fetus* subsp. *venerealis*, whereas all *C. fetus* subsp. *venerealis* isolates were misidentified as *C. fetus* subsp. *fetus*.

Although both the API Campy system and MALDI-TOF MS have a similar capacity to identify the common clinical *Campylobacter* pathogens to the species level, the latter method provides greater advantages. In terms of time consumption, it requires less than 5 min to perform identification via MALDI-TOF MS after a colony is applied to the steel target plate, whereas the API Campy system method necessitates that the colony is incubated on strips for 24 h ± 2 h to obtain a 7-digit profile number. In terms of cost, the expendables used for each isolate identification is less expensive with MALDI-TOF MS than with the API Campy system ($1 and $16—18, respectively). In term of negligence, the API Campy system requires experienced staff to correctly read the reaction of each strip in order to obtain the correct 7-digit profile number, whereas MALDI-TOF MS does not.

The MLST and PFGE methods have been widely applied for epidemiological studies, as both have effective discriminatory power in evaluating the genetic relatedness among *Campylobacter* strains. Thakur et al. demonstrated that PFGE using restriction enzyme *Sma*I or *Kpn*I was more discriminatory than MLST for *C. coli* genotyping.[27] Duim et al. and Oh et al. observed that MLST and PFGE provided the same value for epidemiological typing of *C. jejuni*.[28,29] Escher et al. showed a 100% correlation between the results of the PFGE and MLST genotypic analyses of *C. fetus* subsp. *fetus*.[30] In agreement with previous studies, MLST and PFGE showed similar discriminatory power for *C. fetus* in the present study. Typing by MLST resulted in *C. fetus* isolates being assigned to three STs: ST20 (12/19, 63.2%), ST11 (5/19, 26.3%) and the newly designated ST57 (2/19, 10.5%). PFGE also grouped 19 *C. fetus* isolates into 3 major clusters (Clusters A, B, and C). The genetic homogeneity of *C. fetus* in our study indicated possible epidemiological links and underestimated circulation of these strains in northern

Taiwan. Further survey epidemiological analysis of human- and poultry-derived *C. fetus* is needed as a common source cannot be excluded.

There are some limitations to our study. First, only 19 isolates of *C. fetus* within only one medical institution were uncovered in our study. This population cannot provide a comprehensive understanding of the epidemiology of this disease. Second, other *Campylobacter* species, such as *C. lari*, *C. upsaliensis*, and *C. insulaenigrae*, have also been documented in humans; however, our study did not assess the accuracy of the API Campy system and MALDI-TOF in the identification of these relatively rare pathogens. Further investigation with a greater number of different *Campylobacter* species is needed to highlight the reliability of these tools. Third, although 16S rRNA sequencing has been viewed as the gold standard for bacterial identification, this approach still has some weaknesses, such as the quality of sequences in the databases and the high similarity between sequences of some species.[7] Thus, for extremely genetically related species, such as *C. fetus* subspecies, additional molecular techniques are required to confirm reliable identification.

In conclusion, it is of clinical and epidemiological importance to identify *Campylobacter* beyond the genus level, and MALDI-TOF MS offered great advantages over the API Campy system for the identification of *Campylobacter* species, including *C. jejuni*, *C. coli* and *C. fetus*, as it allows for rapid and reliable identification. Database expansion of Bruker Biotyper MALDI-TOF MS for the correct identification of *C. fetus* to subspecies levels (e.g. *C. fetus* subsp. *venerealis*) is needed.

Conflicts of interest

All authors declare no conflict of interest.

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