

Research Article

High Prevalence of *Arcobacter* Carriage in Older Subjects with Type 2 Diabetes

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Received 31 January 2010; Accepted 25 March 2010

Academic Editor: Schwan William

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Arcobacters are potential pathogens related to diarrheic infections and, rarely, septicaemia. This study evaluated the prevalence of *arcobacters* in stool samples of subjects with ($n = 38$) and without ($n = 61$) type 2 diabetes by using cultural and molecular techniques. Three *Arcobacter* positive cultures were found, all among diabetic subjects, whereas molecular analysis showed a carriage rate of 79% and 26.2% in subjects with and without type 2 diabetes ($P < .001$), respectively. The multivariate analysis showed that type 2 diabetes ($\beta = 1.913$; 95%CI: 2.378–19.285; $P < .0001$) and age ($\beta = 1.744$; 95%CI: 2.077–15.766; $P = .001$) were the only factors independently associated with *arcobacters* colonization in this population. Our study demonstrated a high prevalence of *arcobacters* colonization in type 2 diabetic and older subjects. The clinical significance and the potential health risk associated with these emerging species remain to be determined.

1. Introduction

The genus *Arcobacter* currently includes ten species. Pathogenic strains belonging to *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* have been isolated from human specimens, as well as from animal faeces and food of animal origin [1–6]. *Arcobacter nitrofigilis*, *Arcobacter halophilus*, as well as a number of recently published species such as *Arcobacter cibarius*, *Arcobacter marinus*, *Arcobacter thereius* and *Arcobacter mytili*, and the not yet established species *A. sulfidicus* have been isolated from a variety of sources, but not from humans yet [7–13].

Epidemiological data on *arcobacters* colonization in humans are limited [1, 14, 15]; overall, the presence of these potential pathogens seems to be generally low and seldom associated with gastrointestinal disorders. Thus, *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* have been mainly isolated from stool specimens from patients with diarrhoea and in some cases of septicemia [1–3, 16–20].

Most infections are believed to result from the ingestion of contaminated food or water [21, 22], and the ubiquitous environmental distribution of *arcobacters* may be related to the high occurrence of these microorganisms in animal faecal samples [4, 23].

Diabetic subjects seem to be particularly prone to colonization by members of the *Campylobacter* family like *Helicobacter pylori* [24, 25]. However, to date, there are no data on carriage rate in this type of patients by *Arcobacter*, another genus of this family.

In this study we investigated *arcobacters* carriage rates and the clinical characteristics of a group of subjects with and without type 2 diabetes and with no sign or symptom for gastrointestinal disorders.

2. Materials and Methods

2.1. Subjects. A group of nonhospitalized subjects with ($n = 38$) and without ($n = 61$) type 2 diabetes, without any

sign or symptom for gastrointestinal disorders, consecutively attending the outpatient clinic for Metabolic disorders of the Department of Internal Medicine of Messina University, Italy, were recruited into this study. All study subjects gave their informed consent and the study was approved by the local ethical committee.

Lifestyle (socio-economical status, smoking) and clinical data were collected from all participants through a standardized questionnaire. Type 2 diabetes was diagnosed according to ADA 1997 criteria [26], and all diabetic participants were currently treated with diet, oral agents, insulin, or a combination of them. Data on retinopathy, neuropathy, and nephropathy, as diagnosed according to ADA 2008 guidelines [27], as well as data on macrovascular disease, as defined as a composite end-point [28, 29] including documented myocardial infarction, ischaemic heart disease, coronary heart bypass, coronary angioplasty, cerebral thromboembolism and peripheral amputations were also available for diabetic subjects only.

2.2. Clinical and Laboratory Measurements. An accurate physical examination was performed in each participant. Weight, height, body mass index (BMI), waist circumference and blood pressure, were also measured according to standard procedures.

Fasting blood samples were collected from all the participants for measurements of the study parameters. Fasting plasma glucose, lipid concentrations, as well as leukocyte blood count and erythrocyte sedimentation rate (ESR) were measured with standard laboratory methods. Glycated haemoglobin (HbA_{1c}) was measured using an automated high-performance liquid chromatography analyzer (Diamat; Bio-Rad Laboratories, Milan, Italy) in diabetic participants, only; normal range values were 4–6%.

2.3. Specimen Collection. Stool samples from all participants were collected and readily sent to the laboratory for subsequent analyses. On arrival in the laboratory, samples were kept under cooled conditions until culture, which was usually performed within 1 to 3 h of receipt.

2.4. Cultural Methods. For the isolation of *Arcobacter* spp., direct plating and enrichment procedures [30] were applied to faecal samples emulsified in approximately 1 g/mL of sterile saline.

2.5. Molecular Methods. PCR assay was performed to confirm presumptive isolates and was directly on DNA extracted from stool samples, according to the manufacturer's instructions (QIAamp DNA Stool Mini Kit (QIAGEN)).

Three pairs of primers targeting the 16S and 23S genes were used in PCR assays for the detection and identification of arcobacters [31]. The CRY1-CRY2 primers amplified a 257-bp fragment from *A. cryaerophilus*, the ARCO-BUTZ a 401-bp fragment from *A. butzleri*, and the ARCO-SKIR a 641-bp fragment from *A. skirrowii* [31]. The primers were synthesized by MWG-Biotech (Mannheim, Germany).

The amplification was performed on a PCR Sprint Thermal Cycler (Hybaid, UK), as previously described [32].

Briefly, PCR conditions provided an initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C (1 min), annealing at 50°C (1 min), extension at 72°C (1 min), and a final extension at 72°C for 10 min. A negative control lacking the DNA template was included in each experiment. The *Arcobacter* strains used as positive controls in the PCR tests included *A. butzleri* ATCC 49616^T, *A. cryaerophilus* ATCC 43157, and *A. skirrowii* ATCC 51132^T. PCR products were then separated by electrophoresis on an 1.5% agarose gel, and DNA fragments were visualized by UV illumination, after staining with ethidium bromide.

A random sample of positive PCR products from both isolates ($n = 3$) and faecal DNA ($n = 26$) was confirmed by direct sequencing using primers described by Houf et al. [31] at the Genelab, ENEA, Rome (Italy).

2.6. Statistical Analysis. Statistical analysis was performed using the SPSS program, version 11.0 for Windows (SPSS Inc. Chicago, IL). Data are given as mean \pm SD. We used χ^2 test to compare categorical measures and the analysis of variance (ANOVA) for continuous measures. Linear regression models were determined using a stepwise selection procedure for all the factors, entering the continuous variables with the use of appropriate transformations. All statistical comparisons are two-tailed, and they were considered significant at the $P < .05$ level.

3. Results

3.1. Clinical Characteristics of Study Population. Clinical characteristics of the study population according to the presence of type 2 diabetes are summarized in Table 1. Overall, diabetic patients were older, with an acceptable metabolic control despite the relatively long duration of diabetes. Diabetic subjects also showed significantly higher values of BMI, waist circumference, fasting blood glucose, and triglycerides and lower concentrations of total, LDL- and HDL-cholesterol as compared to nondiabetic subjects.

Infection laboratory indexes (ESR, leukocyte blood count, and percentage of neutrophils) were within the normal range, although with some differences between the two groups (Table 1).

Also the percentage of smokers was higher in diabetic versus controls ($P = .05$), whereas no other significant differences were noted between the two groups.

3.2. Prevalence of *Arcobacter* Colonization in the Study Population. Data on the prevalence of *Arcobacter* in the examined stool samples, as determined both by cultural and molecular techniques, are shown in Table 1.

Only 3 of the 99 cultures were positive (3%), while *Arcobacter* DNA was detected by PCR analysis in 46 (46.5%) stool samples including the 3 samples positive by culture that belonged to diabetic subjects. In particular, molecular evidence of *Arcobacter* was noted in 30 (79%) subjects with and 16 (26.2%) without type 2 diabetes ($P < .001$).

TABLE 1: Clinical characteristics and *Arcobacter* carriage rate in subjects with and without diabetes.

	Type 2 diabetic subjects	Non diabetic subjects	P
<i>n</i>	38	61	
Male sex <i>n</i> (%)	21 (55.3)	33 (54)	—
Age (years)	63.1 ± 9.3*	54.3 ± 7.2	<.0001
BMI (kg/m ²)	29.5 ± 4.3	27.1 ± 2.9	.001
Waist circumference (cm)	98.3 ± 9.2	91.6 ± 9.8	.001
Low instruction level <i>n</i> (%)	30 (78.9)	40 (65.6)	—
Hypertension <i>n</i> (%)	20 (52.6)	25 (41.0)	—
Current smokers <i>n</i> (%)	15 (39.5)	13 (21.3)	.05
Diabetes duration (years)	10.9 ± 8.8	—	—
Fasting blood glucose (mg/dL)	146.7 ± 49.3	90.8 ± 6.3	<.0001
HbA _{1c} (%)	7.06 ± 1.34	—	—
Total Cholesterol (mg/dL)	178.5 ± 37.9	199.4 ± 27.0	.001
LDL-Cholesterol (mg/dL)	104.1 ± 41.2	123.6 ± 28.0	.006
HDL-Cholesterol (mg/dL)	48.7 ± 9.4	54.4 ± 10.4	.008
Triglycerides (mg/dL)	132.6 ± 50.2	110.7 ± 50.6	.04
ESR (mm/hr)	10.3 ± 6.1	12.8 ± 6.2	.02
Leukocyte Blood Count/mm ³	7317 ± 1552	6651 ± 1506	.04
Neutrophils (%)	58.6 ± 7.6	61.5 ± 5.6	.04
Positive cultures for <i>Arcobacter n</i> (%)	3 (7.9)	0	—
<i>A. butzleri n</i> (%)	1 (33.3)	—	
<i>A. cryaerophilus n</i> (%)	1 (33.3)	—	
<i>A. butzleri</i> and <i>A. cryaerophilus n</i> (%)	1 (33.3)	—	
Positive PCR for <i>Arcobacter n</i> (%)	30 (78.9)	16 (26.2)	.001
<i>A. butzleri n</i> (%)	3 (10)	2 (12.25)	
<i>A. cryaerophilus n</i> (%)	12 (40)	9 (56.25)	
<i>A. butzleri</i> and <i>A. cryaerophilus n</i> (%)	15 (50)	5 (31.25)	

*Mean ± SD. Only significant *P*-values are presented.

BMI: Body mass index.

HbA_{1c}: Glycated haemoglobin, available for diabetic subjects only.

ESR: Erythrocyte Sedimentation Rate.

Of the 3 positive cultures, the PCR analysis revealed that one was positive for *A. butzleri*, another for *A. cryaerophilus*, and the third for both *A. butzleri* and *A. cryaerophilus*.

The sequences obtained from three of our *Arcobacter* isolates (MTF1, MTF2, MTF3) confirmed the PCR data, showing a 100% sequence similarity with *A. butzleri* (GenBank accession no.: AY621116) for MTF1 and a 98% sequence similarity to *A. cryaerophilus* (GenBank accession no.: X80383; AB081300) for both MTF2 and MTF3.

Of the 46 *Arcobacter* PCR positive samples, 21 (45.6%) were positive for *A. cryaerophilus*, 20 (43.5%) for both *A. butzleri* and *A. cryaerophilus*, and 5 (10.9%) for *A. butzleri*.

Comparing the sequences of 26 PCR products, randomly selected among the 46 positive faecal DNA samples, with previously published sequences in the GenBank database, a very high degree of similarity of our sequences with *A. butzleri* (accession no.: CP000361; AY621116; EU252504) and *A. cryaerophilus* (accession no.: X80383; AB081300) was found.

Neither *A. skirrowii* isolates nor *A. skirrowii* PCR products were found in our study. Figure 1 show a representative

example of PCR analysis of DNA extracted from faecal samples.

The GenBank accession numbers for our nucleotide sequences are as follows: GU116587, GU116588 and GU116589 for the isolates MTF1, MTF2, MTF3, respectively, and from GU108194 to GU108219 for the faecal DNA sequences.

3.3. Clinical Differences in Diabetic and Nondiabetic Subjects, According to *Arcobacter* Colonization. When clinical characteristics of subjects with or without *Arcobacter* carriage were compared in the diabetic and non diabetic groups separately (Table 2), diabetic subjects with evidence of arcobacters were significantly older as compared to those without it (*P* < .001), whereas no differences were noted in the other clinical characteristics between the two groups (Table 2). Similarly, no differences were noted in diabetes-related variables, such as diabetes duration, current therapy with oral hypoglycaemic agents and/or insulin, and the rate of micro- and/or macrovascular complications between diabetic subjects with or without *Arcobacter* colonization (data not shown).

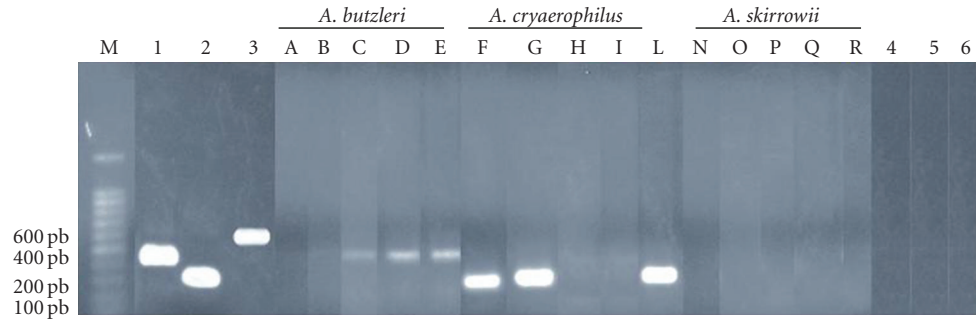


FIGURE 1: Example of the PCR analysis of DNA extracted from the 3 reference strains and of five representative faecal samples using the species specific primers. Lane M, 100-bp ladder; lanes 1–3 PCR products of the control strains: *A. butzleri* ATCC 49616^T (401-bp), *A. cryaerophilus* ATCC 43157 (257-bp), and *A. skirrowii* ATCC 51132^T (641-bp), respectively. Lanes A, F, N, and B, G, O results obtained for samples 1 and 2, respectively, showing only amplicons of the expected size for *A. cryaerophilus*; lanes C, H, P and D, I, Q, results obtained from samples 3 and 4, respectively, showing only amplicons of the expected size for *A. butzleri*; lanes E, L, R, results obtained from sample 5 showing amplicons of the expected size for *A. butzleri* and *A. cryaerophilus*. None of the samples showed amplicons with the *A. skirrowii*-specific primers; lanes 4 to 6, negative controls.

TABLE 2: Clinical characteristics in diabetic and nondiabetic subjects with or without evidence of *Arcobacter* colonization.

	Type 2 diabetic subjects		Non diabetic subjects	
	Without <i>Arcobacter</i> colonization	With <i>Arcobacter</i> colonization	Without <i>Arcobacter</i> colonization	With <i>Arcobacter</i> colonization
<i>n</i>	8 (21.1)	30 (78.9)	45 (73.8)	16 (26.2)
Male sex <i>n</i> (%)	3 (37.5)	18 (60.0)	24 (53.3)	9 (56.3)
Age (years)	54.1 ± 8.9*	65.8 ± 7.8 [†]	54.0 ± 7.0	55.9 ± 8.2
BMI (kg/m ²)	31.9 ± 6.4	28.8 ± 3.4	27.0 ± 2.8	27.7 ± 3.2
Waist circumference (cm)	99.4 ± 11.8	98.0 ± 8.7	90.2 ± 9.2	95.9 ± 10.6 ^{††}
Low instruction level <i>n</i> (%)	5 (62.5)	25 (83.3)	30 (66.6)	10 (62.5)
Current smokers <i>n</i> (%)	3 (37.5)	12 (40.0)	10 (22.2)	3 (18.8)
Dyslipidemia <i>n</i> (%)	5 (62.5)	22 (73.3)	16 (35.5)	5(31.2)
Hypertension <i>n</i> (%)	3 (37.5)	17 (56.6)	14 (31.1)	11 (68.8) [†]
Fasting blood glucose (mg/dL)	136.5 ± 48.0	149.4 ± 50.1	90.0 ± 6.3	93.1 ± 5.9
HbA _{1c} (%)	7.2 ± 1.2	7.1 ± 1.3	—	—
Total Cholesterol (mg/dL)	165.2 ± 42.0	182.0 ± 36.6	198.8 ± 28.2	201.2 ± 23.5
LDL-Cholesterol (mg/dL)	91.7 ± 35.8	107.4 ± 42.5	124.5 ± 29.6	119.6 ± 22.1
HDL-Cholesterol (mg/dL)	46.6 ± 8.8	49.3 ± 9.7	54.1 ± 9.6	55.1 ± 12.6
Triglycerides (mg/dL)	141.7 ± 42.8	130.2 ± 52.3	111.0 ± 53.6	109.9 ± 42.7

*Mean ± SD. Only significant *P*-values are presented.

[†] *P* < .001 versus subjects without *Arcobacter* colonization.

^{††} *P*:.05 versus subjects without *Arcobacter* colonization.

BMI: Body mass index.

HbA_{1c}: Glycated haemoglobin, available for diabetic subjects only.

In the non diabetic group (Table 2), carriers of arcobacters had a significantly higher waist circumference (*P* = .05) and a higher rate of hypertension (*P* < .001) as compared to noncarriers. No other significant differences in clinical characteristics were noted between the two groups.

At multivariate analysis, performed in the whole population and including in the model all the variables that showed significant differences between subjects with and without arcobacters (age, waist circumference, hypertension, and diabetes), type 2 diabetes (β = 1.913; *P* < .0001) and age (β = 1.744; *P* = .001) were confirmed as the only factors

independently associated with *Arcobacter* colonization in this population, with a OR of 6.8 (95%CI: 2.378–19.285) in diabetic versus non diabetic subjects, and an OR of 5.7 (95%CI: 2.077–15.766) in older versus younger subjects.

4. Discussion

Although data on healthy carriers are available worldwide [14, 15, 33], our study is the first investigation on the prevalence of *Arcobacter* carriage in diabetic subjects without specific gastrointestinal symptoms in Italy.

We found that type 2 diabetes and older age were associated with an impressively high rate of *Arcobacter* carriage. Overall, we found a high prevalence of *Arcobacter* colonization with 46 positive cases by molecular analysis and 3 positive cases by cultural methods, as evaluated by both direct plating and enrichment procedures. Compared to the cultural method, the PCR analysis identified 43 more cases, namely 41.4% cases positive for *A. cryaerophilus*, and 25.2% for *A. butzleri*; thus confirming, as previously reported [32], the superiority of the molecular method over the cultural one in arcobacters detection. However, several other reasons may explain the very low detection rate for *Arcobacter* with cultural methods in our study, including the differences on isolation methods [17, 23, 34]. First of all, it has been indicated that CAT agar supplemented media that have been used in this study are not able to adequately inhibit other faecal microorganisms [23, 35–37], that might mask arcobacters, thus resulting in a lack of detection by cultural methods. This was clearly improved in other studies using the membrane filtration method that produced a reduction of the competitive microbiota [14, 23, 35–37]. Other authors found almost equal results between the m-PCR detection methods [31] from the enrichment *Arcobacter* broth supplemented with CAT, by culturing the broth after passive filtration (0.45 mm membrane filters) on blood agar [23, 36].

Consistent with another study [15], we found that *A. cryaerophilus* was the most frequently occurring species in all the examined samples, whereas other authors have identified *A. butzleri* as the dominant species [1, 17].

Furthermore, our study reported, for the first time in humans, a concurrent colonization with two different *Arcobacter* species, namely *A. butzleri* and *A. cryaerophilus*, an occurrence that has been reported for animals [5, 38], for water and food products [23, 35]. Thus, the coexistence of two species in the same stool samples was found in a single patient with cultural method, but it was shown at higher rates by PCR (20 patients, 43.5%). The latter findings suggest the possibility of multiple sources of contaminations or the consumption of water or food products simultaneously contaminated with both species.

Epidemiological data on the occurrence of arcobacters in humans show different prevalence rates, both in asymptomatic and symptomatic subjects [1, 14, 15], especially those obtained by molecular methods [1, 33, 39]. Although our detection rate is considerably higher when compared to these studies, the prevalence obtained in our diabetic patients with no gastrointestinal disease is hardly comparable with the variable data obtained from healthy subjects, of different ages [1, 14, 15]. Furthermore, the generally low socio-economical status of subjects participating to our study may be another issue in favouring arcobacters transmission [40], although no differences were noted in the instruction level between carriers and non carriers of these microorganisms, either in diabetic or in control subjects.

Several metabolic and nonmetabolic factors, such as age, hypertension, waist circumference, and diabetes, were all associated with arcobacters carriage in our study participants; however, in the whole study group, only type 2

diabetes and an older age were significant and independent risk factors for *Arcobacter* colonization in this population, with a risk that was 7- and 6-fold higher in diabetic and older subjects as compared to nondiabetic and younger ones, respectively.

The very high arcobacters carriage rate found in our diabetic patients largely confirms their generally high susceptibility to infections, related to the hyperglycaemic *milieu* that favours bacterial growth, as well as to an altered motility of gastroenteric tract, as a consequence of autonomic neuropathy and/or microangiopathy, or to the impaired humoral and cellular host defence [20, 24, 25].

Notably, the very high prevalence of arcobacters detected in type 2 diabetic and older patients, together with the potential decline of their immune system competency [41], may pose these subjects at higher risk of infection.

Although conducted in a small sample, our results clearly indicate that older subjects and those with type 2 diabetes are at greater risk for arcobacters carriage; large prospective studies are needed to evaluate the potential health risk associated with arcobacter colonization in these high risk groups.

Acknowledgment

This study was supported by research grants (2005 financing) from the University of Messina, Italy.

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