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ORIGINAL ARTICLE

Multi-step optimization of the filtration method for the isolation of *Campylobacter* species from stool samples

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Abstract

The filtration method (FM) is the most effective isolation technique for *Epsilobacteriaceae* from stool samples. FM's different adaptations make it difficult to compare data between studies. This study was performed in three phases to optimize FM from a routine laboratory perspective. In July–September 2014 (part I), FM was performed on Mueller–Hinton agar containing 5% sheep blood and Columbia agar containing 5% sheep blood. In July 2016 (part II), FM was performed using 0.60-μm pore size polycarbonate filters (0.6-PC filter) and 0.45-μm pore size cellulose acetate filters (0.45-AC filter); in January 2018 (part III), the addition of hydrogen to incubators was studied. On 1146 stools analyzed in part I, the positive samples that showed no growth on the Butzler medium ($n = 22/72$, 30.6%) had improved growth of *Epsilobacteriaceae* when using the Columbia instead of the Mueller–Hinton medium (21/22 strains vs. 11/22, $p < 0.05$). In part II, on 718 stools, 91 strains grew with FM (12.7%), more with 0.6-PC filter (90/91) than with 0.45-AC filter (44/91) ($p < 0.05$). In part III, 578 stools were cultured, 98 *Epsilobacteriaceae* strains grew with FM, and 7% hydrogen finding significantly more *Epsilobacteriaceae* than without hydrogen (90/98, 91.8%, vs. 72/98, 73.5%; $p < 0.05$). The use of a Columbia medium containing 5% sheep blood with 0.6-PC filters incubated at 37 °C in a 7% hydrogen-enriched atmosphere led to an almost fourfold increase in the isolation rate of *Epsilobacteriaceae* among the studied combinations. Reference centers for *Campylobacter* should use standardized protocols to enable the comparison of prevalence in space and time.

Keywords *Campylobacter* · Filtration method · Filter · Hydrogen · Concisus · Gastroenteritis

Introduction

According to the World Health Organization (WHO), *Campylobacter* spp. is the most important bacterial cause of diarrhea in humans [1]. In Europe, since 2005, *Campylobacter* sp. is the most prevalent enteric pathogen reported to the European Centre for Disease Prevention and Control (ECDC), with 71 cases per 100,000 in 2014. Yet, incidence rates vary greatly between countries. The incidence of *Campylobacter* spp. infections is probably underestimated because of a lack of systematic

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approach in the isolation methods and an unsystematic declaration of diagnosed cases [2].

Among the *Epsilobacteriaceae* family, *Campylobacter jejuni* and *Campylobacter coli* are the most isolated species in gastroenteritis in humans [3]. With an optimal growth at 42 °C, *C. jejuni*, *C. coli*, and *Campylobacter lari* are commonly isolated from the intestines of avian species, especially poultry, because of a higher body temperature of those species which represent the main source of infection for humans [4]. They are called "thermophilic *Campylobacter*" in opposition to other *Campylobacter* with a lower optimal growth temperature [5].

During the last few decades, the development of diagnostic techniques has led to the rise of less known and nutritionally fastidious *Campylobacter*, including *Campylobacter rectus*, *Campylobacter upsaliensis*, and *Campylobacter concisus*. Although emergent *Campylobacter* have been associated with gastroenteritis, inflammatory bowel diseases and periodontitis, their pathogenic role remains controversial [3, 6–8].

Molecular tools are convenient to identify bacterial DNA in a sample, especially for fastidious *Campylobacter*. However, they make it impossible to infer if the organism was alive or dead or to obtain an antimicrobial susceptibility profile for potential pathogens; culture methods thus remain essential. Given the lack of systematic approach for the culture of human stools for these emerging *Campylobacter*, the prevalence is difficult to compare between studies.

Isolation methods for *Campylobacter* vary between laboratories, even in the same country. Most use selective agar plates, developed principally for *C. jejuni-coli*, containing antibiotics to suppress normal fecal flora, incubated at 42 °C in a microaerophilic atmosphere, like the modified charcoal cefoperazone deoxycholate agar (mCCDA), the preferred media for isolating *C. jejuni-coli* [9]. But those conditions inhibit the growth of most of the *Campylobacter* spp. other than *C. jejuni* or *C. coli* (COJC) [6, 10]. Due to their specific growth conditions, slow growth rates, and susceptibility to antibiotics present in the *C. jejuni-coli* selective agar plate, COJC, like *C. concisus*, *C. curvus*, *C. upsaliensis*, and *Campylobacter fetus*, are more difficult to isolate in culture. Specific isolation methods, such as the filtration method (FM) on antibiotic-free agar, are thus warranted, with incubation at a temperature lower (37 °C) than for *C. jejuni* and *C. coli* [11, 12]. An increased hydrogen (H_2) concentration is required to better isolate several *Campylobacter* spp. other than *C. jejuni* or *C. coli* like *C. concisus*, *C. curvus*, *Campylobacter sputorum*, and *Campylobacter hyointestinalis* [11, 13].

An enrichment step is sometimes used by inoculating the sample in a broth before putting the stools on the filter [3, 6]. Most of the times, this enrichment broth is used for samples with low bacteria concentration, like water or food [14, 15]. Brucella broth has been shown to resuscitate injured or dormant *Campylobacter* spp. [16] and has been used for that reason in our laboratory for years instead of saline to dilute stools.

Different agar plates have been used for the FM, most of the times without antibiotics [3]. Mueller–Hinton agar and Columbia blood agar base with defibrinated sheep blood have been used for decades [17], and Columbia media is still suggested as one of the preferred media for *Campylobacter* spp. growth [18].

Material and pore size of the filter used is another possible adaptation of the FM. The 0.65-µm pore size cellulose acetate (CA) filters, used in the first FM [19], are known to have a better sensitivity than 0.45-µm pore size CA filters [20], but they also increase the level of contaminating bacteria. For that reason, our laboratory had chosen the 0.45-CA filter years ago. In 2013, Nielsen et al. compared polycarbonate (PC) 0.6-µm pore size filters and CA 0.65-µm pore size filters and found that *C. concisus* was significantly more isolated using the PC filter with a smaller pass through of commensally fecal flora [21].

In this work, we report the results of a three-step study comparing two types of agar plates, two types of filters, and the presence of H_2 in order to optimize FM from a routine laboratory perspective.

Materials and method

Part I: optimization of the agar medium

From July to September 2014, stool samples referred to our clinical laboratory for *Campylobacter* culture were systematically tested using a selective Butzler's medium [22] (Thermo Fisher Scientific, Erembodegem, Belgium) incubated at 42 °C in a microaerobic atmosphere (CO_2 10%, O_2 5%, H_2 0%) during 48 h according to Vandenberg et al. [12], and FM was performed as described by Lopez [23]. Stool samples were diluted 1:5 in Brucella broth and incubated for 30 min at 37 °C in normal atmosphere. The 0.45-µm pore size CA filters (Porafil, Duren, Germany) (referred as "0.45-CA filter") were placed on two non-selective media: Mueller–Hinton agar containing 5% sheep blood (Thermo Fisher Scientific, Erembodegem, Belgium) and Columbia agar containing 5% sheep blood (Becton–Dickinson, Erembodegem, Belgium). Eight drops of the fecal suspension were placed on top of the membrane and allowed to filter passively for 30 min at 37 °C in normal atmosphere. This procedure was repeated a second time. After filtration, filters were removed. Plates were incubated at 37 °C in a microaerobic atmosphere for 10 days. Colonies suspected to be *Epsilobacteriaceae* were identified by MALDI-TOF MS using the Microflex LT and the IVD 2.2 Biotyper database (Bruker, Bremen, Germany).

Part II: optimization of the pore size filters

In the month of July 2016, stool samples were tested using the Butzler medium and FM on Columbia medium containing 5% sheep blood using 0.45-CA filters and 0.60-μm pore size PC filters (Nuclepore™, Whatman, Kent, UK) (referred as "0.6-PC filter"). Plates were incubated in the same conditions as described in part I. Once a suspected colony was detected, the plate was placed in a jar with an H_2-enriched microaerobic atmosphere (80% N_2—7% CO_2—6% O_2—7% H_2) until the colony was large enough to be identified by MALDI-TOF MS.

Part III: optimization of the atmosphere

In the month of January 2018, stool samples were tested with 0.6-PC filter, as described for July 2016, but incubation was done in two distinct ways. For each stool sample, one plate was incubated as described for July 2016 and another one was directly placed in a jar with an H_2enriched microaerobic atmosphere (80% N_2—7% CO_2—6% O_2—7% H_2) to compare the number of positive specimen and rapidity of growth with and without H_2.

Statistics were calculated using computer software EpiInfo7 version 7.2.1.0, p values were calculated using McNemar test, and χ^2 to compare the numbers of *Epsilobactericeae* obtained in each group of the three parts of the study.

Results

Among the 1146 stool samples tested during part I, 72 samples were positive by culture (6.3%) on at least one of the three media. Table 1 shows *Epsilobacteriaceae* identified in 1146 stool cultures according to culture method used: *C. jejuni* was the most frequently isolated species ($n = 58$, 80.5%) followed by *C. concisus* ($n = 5$, 6.9%).

Table 1 *Epsilobacteriaceae* identified in 1146 stool cultures according to culture method used, with comparison of two agar types for the filtration method. Numbers between brackets are strains that did not grow on Butzler's media. (Part I of the study)

	All agar	Butzler	MH[a]	Col[b]
Campylobacter jejuni	58 (10)	48	37 (6)	39 (9)
Campylobacter concisus	5 (5)	0	1 (1)	5 (5)
Helicobacter pullorum	4 (4)	0	2 (2)	4 (4)
Campylobacter coli	2 (0)	2	1 (0)	1 (0)
Campylobacter upsaliensis	2 (2)	0	1 (1)	2 (2)
Campylobacter curvus	1 (1)	0	1 (1)	1 (1)
Total	72 (22)	50	43 (11)	52 (21)

Incubation at 37 °C in a microaerobic atmosphere

[a] filtration method performed on Mueller Hinton agar containing 5% sheep blood

[b] filtration method performed on Columbia agar containing 5% sheep blood

Of the positive samples that showed no growth on the Butzler medium ($n = 22$), *Epsilobacteriaceae* growth was improved when using the Columbia (21 strains) instead of the Mueller–Hinton medium (11 strains) (McNemar, $p < 0.05$).

During the second part of the study, 718 stool samples were tested. Ninety-three stool cultures were positive (12.9%) with one ($n = 90$) or two ($n = 3$) different species of *Epsilobacteriaceae* isolated. Table 2 shows *Epsilobacteriaceae* identified in 718 stool cultures according to culture methods used: *C. concisus* was the most frequently isolated *Epsilobacteriaceae* (44/96, 45.8%), followed by *C. jejuni* (37/96, 38.5%).

Ninety-one strains grew with the FM, of which 57 were only recovered using FM. All but one grew with the 0.6-PC filter (90/91), only 44 grew with the 0.45-CA filter (McNemar, $p < 0.05$). Species-wise, only *C. concisus* and *C. jejuni* were statistically significantly more isolated with the 0.6-PC compared to the 0.45-CA filter. When both were positive, the mean time-to-detection with the 0.6-PC filter was 3.41 days (median 3 days) and 3.67 days (median 3 days) with the 0.45-CA filter. Strain identification resulted in 158 MALDI-TOF analysis for the 0.6-PC filters and 44 for the 0.45-CA filters. The number of plates contaminated with commensal flora after 10 days of culture was 18.4% (132/718) for the 0.6-PC filters and 10.2% (73/718) for the 0.45-CA filters (McNemar, $p < 0.05$).

Thirty-four strains grew on both Butzler and 0.6-PC filter; the mean time-to-growth and time-to-identification on Butzler's medium was 1 day (median = 1), compared to 2.65 days (median = 2) for the 0.6-PC filter. Five strains grew on the Butzler medium only.

In the third part of the study, 578 stools were cultured for *Epsilobacteriaceae* using Butzler's medium and by two FM using two different atmospheres (with or without 7% H_2). In total, 99 *Epsilobacteriaceae* grew on one of the three media (98/578 stool sample positive, 17.0%). Among the positive stool samples, *C. concisus* was the most frequently isolated *Epsilobacteriaceae* (60/98, 61.2%), followed by *C. jejuni* (19/98, 19.4%). Table 3 shows the distribution of *Epsilobacteriaceae* identified in the 578 stool cultures according to culture method used. Enriched H_2 atmosphere significantly increased the recovery of *Epsilobacteriaceae* compared to atmosphere without H_2 (90/99, 90.9%, and 72/99, 72.7%, respectively: McNemar $p < 0.05$), notably for *C. concisus* (45/60 vs. 55/60, $p < 0.05$), *C. ureolyticus* (3/9 vs. 8/9, $p < 0.05$), and *C. curvus* (4/7 vs. 6/7, $p < 0.5$). The median time-to-growth and time-to-identification was 4 days (min 2, max 12) and 6 days (min 2, max 14), respectively, for cultures incubated without H_2. The presence of H_2 allowed to remove the time interval between growth and identification, with an identical median time-to-event of 3 days (min 2, max 7) for both growth and identification.

Table 2 *Epsilobacteriaceae* identified in 718 stool cultures according to culture method used, with comparison of two filter types for the filtration method. Numbers between brackets are strains that did not grow on Butzler's media. (Part II of the study)

	All techniques	Butzler	Filter 0.6-PC[a]	Filter 0.45-CA[b]
Campylobacter concisus	44 (44)	0	44 (44)	10 (10)
Campylobacter jejuni	37 (3)	34	35 (3)	28 (1)
Campylobacter coli	7 (2)	5	4 (2)	3 (1)
Campylobacter curvus	6 (6)	0	5 (5)	2 (2)
Campylobacter upsaliensis	1 (1)	0	1 (1)	1 (1)
Arcobacter butzleri	1 (1)	0	1 (1)	0 (0)
Total	96 (57)	39	90 (56)	44 (15)

Incubation at 37 °C in a microaerobic atmosphere, if suspected colony detected: plate placed in a jar with an H_2-enriched microaerobic atmosphere (80% N_2—7% CO_2—6% O_2—7% H_2)

[a] Columbia agar containing 5% sheep blood and 0.60-µm pore size polycarbonate filters

[b] Columbia agar containing 5% sheep blood and 0.45-µm pore size cellulose acetate filters

Discussion

Our work shows that COJC are missed when culturing stool samples onto a selective medium only (from 30.6 to 82.8% according to our results). The three-step improvement increased *Epsilobacteriaceae* positivity rates from 5.2% (61/1146) to 17.0% (98/578). Columbia agar containing 5% sheep blood with 0.6-PC filters incubated in H_2-containing atmosphere was the best combination of all the tested FM variations in terms of optimization of COJC culture from human stools, confirming results from previous studies using the same 0.6-PC filter [7, 21] and H_2 atmosphere [7, 11, 24]. Columbia media was confirmed as a good medium for *Campylobacter* sp. growth [18].

Although CA filters have been used in most studies about FM, PC filters are better for the isolation of *C. concisus*, as shown in this study and by others [7, 21]. Our study is nevertheless limited by the fact that filter modifications were done on two parameters at the same time, pore size and filter type. Nielsen et al. suggested that the difference between PC and CA could be linked to the surface of the filters: the smoother surface of the PC and its consistent pore size being more efficient than the rough surface of the CA that could entrap particles leading to an inferior penetration of the motile *Epsilobacteriaceae* [21].

For historical reason [22] and ease of use according to the laboratory technologists, the selective medium used in our laboratory is the Butzler medium, which is infrequently used in other microbiology laboratories where more common media, like CCDA [25], are preferred.

Another limitation is that we did not test different H_2 concentrations in our incubator; the only test was without H_2 and with 7% H_2. Lastovica et al. reported higher *Campylobacter* sp. positivity rate (21%) with increased (40%) H_2 concentration, but our positivity rate (17%) is quite similar, using our three-step improvement [26]. The high H_2 percentage is one of the key elements of the "Cape Town protocol", with generated H_2 concentration greater than 40% [26], which is quite elevated compared to the 3–7% H_2 used in other studies [3, 27]. We, probably like other investigators, were concerned about safety issues associated to such a high proportion of flammable gas in the growth atmosphere of the laboratory.

Our combination added-value was mainly related to non-thermophilic campylobacters, such as *C. concisus* (1/1146 with 0.45-CA filter, MH agar and no H_2 vs. 55/578 with 0.60-PC filter, Columbia agar and 7% H_2, $p < 0.05$), which role as

Table 3 *Epsilobacteriaceae* identified in 578 stool cultures according to culture method used, with comparison of two incubator atmosphere (with or without 7% H2). (Part III of the study)

	All	Butzler	FM[a] with H_2	FM[a] without H_2
Campylobacter concisus	60	0	55	45
Campylobacter jejuni	19	17	17	16
Campylobacter ureolyticus	9	0	8	3
Campylobacter curvus	7	0	6	4
Campylobacter fetus	2	0	2	2
Campylobacter sp.	1	0	1	1
Campylobacter upsaliensis	1	0	1	1
Total	99	17	90	72

Incubation at 37 °C in a microaerobic atmosphere or plate directly placed in a jar with an H_2-enriched microaerobic atmosphere (80% N_2—7% CO_2—6% O_2—7% H_2)

[a] *FM* filtration method

an emergent pathogen is discussed and actively studied [3, 7]. The debate on this topic is not closed, but we need reliable tools to obtain a clear and satisfactory answer in the years to come.

C. jejuni is considered the most frequently encountered *Campylobacter* spp. but was outdriven by *C. concisus* in our study, given the performance of the 0.60-PC filters and the H_2 atmosphere, as previously reported [7, 11, 21, 24]. However, the use of both a selective culture and FM was shown to increase the recovery of *C. jejuni* and *C. coli* too [12]. But the choice of the selective medium, Butzler vs. mCCDA, could influence that result too [9].

The accuracy of MALDI-TOF MS and its short turnaround time compared to other identification techniques was previously shown [28]. The routine use of MALDI-TOF MS in our laboratory was of great support in analyzing the multiple colonies isolated, considering that the contamination of the agar plate by commensal flora was more important with the 0.60-PC filter than with the 0.45-CA filter, as expected. Beside its speed, the small quantity of bacteria required for the MALDI-TOF MS analysis was another advantage of this technology, especially for *Campylobacter* spp. that grow slowly to form small colonies. Still, in the study phases before H_2 use, a time laps remained between growth and identification. H_2 allowed colonies to grow better and be immediately identified by MALDI-TOF MS with a median time of only 3 days (min 2, max 7 days) between plating and identification.

The improved FM significantly overloaded the laboratory, due to the increased number of positive stool cultures for COJC. Given the above, we decided to review our reading procedure to only look at the incubated plates on days 2 and 5 postplating, rather than every day from days 2 to 10. We also elected to discard the culture after day 5 instead of day 10 (in part III of the study, only 1 of 97 positive took 7 days to grow; the 96 others took 5 days or less). This led to a significant reduction of the workload, despite a frank increase in the number of *Epsilobacteriaceae* isolated.

This work highlights the importance of the diagnostic method used when comparing *Campylobacter* sp. rate found in different studies across the world; geography is not the only variable, considering that a change in filter could almost double the *Campylobacter* sp. positivity rate [26]. In the context of studies of the pathogenic role of *Campylobacter* spp., National Reference Centers should use the most accurate tools to isolate them, using a standardized protocol, allowing to compare prevalence between studies, places, and over time. Currently, variations in prevalence are probably due more to variants in diagnostic methods than to differences in epidemiology [26].

Conclusion

Standardization and optimization of COJC growth conditions are needed to warrant improvement in the diagnosis and epidemiological understanding of campylobacteriosis. This work confirms the importance of the FM, preferably onto a Columbia containing 5% sheep blood medium with 0.60-PC filters in an atmosphere enriched with H_2 (7%), in addition to the conventional culture onto selective medium.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed were in accordance with the ethical standards of our institutional research committee.

Informed consent Data were totally anonymized before analysis, no consent had to be obtained considering the methodology of the present work.

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