Comparison of Six Media, Including a Semisolid Agar, for the Isolation of Various \textit{Campylobacter} Species from Stool Specimens

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A recently described semisolid blood-free selective motility medium (SSM) (J. Goossens, L. Vlaes, I. Galand, C. van den Borre, and J. P. Butzler, J. Clin. Microbiol. 27:1077-1080, 1989) was compared with two charcoal-based selective media (charcoal-based selective medium [CSM] and modified charcoal cefoperazone deoxycholate agar [CCDA]), two blood-based media (Skirrow medium [SKM] and CampyBAP), and a passive, 0.65-μm-pore-size cellulose acetate membrane filter technique for the recovery of campylobacters from stools of patients with diarrhea. A total of 1,980 specimens were tested, 161 of which were found to be positive for campylobacters. \textit{Campylobacter jejuni} was isolated in 148 specimens (91.9%), \textit{C. coli} was isolated in 12 (7.5%), and \textit{“C. upsaliensis”} was isolated in 1 (0.6%). After 72 h of incubation with a single medium, the cumulative percentages of \textit{Campylobacter}-positive specimens isolated on CSM, CCDA, SKM, and SSM were 97, 83, 80, and 72%, respectively. The filter method alone enabled us to recover 61% of all campylobacters. The \textit{“C. upsaliensis”} strain was isolated by this method only. The highest isolation rates were observed when two media, including CSM, were combined. The combination of CSM and SSM yielded the highest rates (96%), but these were not statistically different from the rates observed with combinations of CSM and SKM (94%) or of CSM and the filter method (91%). Extending the incubation time from 48 to 72 h led to an increase in the isolation rate regardless of the medium used ($P < 0.001$). CSM and CCDA were the most selective media. SKM and CampyBAP appeared to be the most inhibitory media for the isolation of \textit{C. coli}.

\textit{Campylobacter jejuni} and \textit{C. coli} are now recognized as significant causes of diarrhea in humans. Recently, however, several more unusual \textit{Campylobacter} species have been associated with human disease. Several authors have suggested that the clinical significance of \textit{Campylobacter} species must be extended to include \textit{“C. lari,”} \textit{C. hyointestinalis, C. jejuni} subsp. doylei, \textit{C. cryaerophila,} and \textit{“C. upsaliensis”} (6, 16-18, 22, 23).

Different selective media containing antimicrobial agents have been described for culturing campylobacters from feces. Combinations of antibiotics have been used to supplement basal media containing blood as well as blood-free, charcoal-based media (11, 12). The more unusual campylobacters mentioned above, however, may be susceptible to one or more of the antimicrobial agents incorporated in these media. \textit{“C. upsaliensis,”} for instance, is in general highly susceptible to cefoperazone, colistin, vancomycin, rifampin, and trimethoprim (8). Therefore, a filtration technique first described in 1972 by Dekeyser et al. (5) was reintroduced in 1984 by Steele and McDermott (20) and reevaluated by Bolton et al. (4). More recently, a new semisolid blood-free selective motility medium (SSM) was described by Goossens et al. (9). This medium was found to be very selective as well as sensitive.

The present study was undertaken to assess two charcoal-based selective media (11, 12), two blood-based media (3, 19), the filtration method, and the semisolid motility agar of Goossens et al. (9) (SSM). This evaluation was also designed to determine which combination of two media or medium and method would allow the optimal recovery of \textit{Campylobacter} species.

MATERIALS AND METHODS

Media. The charcoal-based selective medium (CSM) (12) was composed of Columbia agar base (M10600; Gibco Ltd., Paisley, Scotland), bacteriological charcoal (L9; Oxoid Ltd., Basingstoke, United Kingdom), hemin (H-2250; Sigma Chemical Co., St. Louis, Mo.), sodium pyruvate (44094 2M; BDH Biochemicals, Poole, United Kingdom), vancomycin (20 mg/liter), cefoperazone (32 mg/liter), and cycloheximide (100 mg/liter). The modified charcoal cefoperazone deoxycholate agar (CCDA) (11) was made of charcoal base (CM 739; Oxoid) and cefoperazone (32 mg/liter; SR 125; Oxoid). The Skirrow medium (SKM) (19) contained blood agar base no. 2 (CM 271; Oxoid) supplemented with 5% lysed horse blood, vancomycin (10 mg/liter), trimethoprim (5 mg/liter), and polymyxin (2,500 IU/liter) (SR 69; Oxoid). CampyBAP (3) was composed of brucella agar supplemented with 10% sheep blood, vancomycin (10 mg/liter), trimethoprim (5 mg/liter), polymyxin (2,500 IU/liter), cephalothin (15 mg/liter), and amphotericin B (2 mg/liter) (no. 21727; BBL Microbiology Systems, Cockeysville, Md.). The semisolid motility agar (SSM) (9) contained Mueller-Hinton broth (no. 12322; BBL) supplemented with 0.32% agar, cefoperazone (32 mg/liter), and trimethoprim lactate (50 mg/liter). For the filter method (20), use was made of 0.65-μm-pore-size cellulose acetate filters with a diameter of 47 mm (SM 11105; Sartorius, Gottingen, Federal Republic of Germany) and blood agar base no. 2 (CM 271; Oxoid) supplemented with 5% sheep blood.
All media, except for CampyBAP, were prepared once weekly at the Leiden University Hospital and stored in the dark at 4°C until use. CampyBAP plates were purchased directly from the manufacturer (BBL) every month.

**Specimens.** A total of 1,980 stool specimens submitted between 10 July and 1 December 1989 for culturing of enteric pathogens were tested. Specimens collected by swabs were not analyzed. No transport medium was used. A total of 1,094 stool specimens (55.3%) were tested in the microbiology department of the Leiden University Hospital, a 1,000-bed hospital serving Leiden and surrounding areas. The other 886 stool specimens (44.7%) were tested in the Regional Public Health Laboratory at the Leyenburg Hospital in The Hague, an 800-bed hospital serving The Hague and located about 15 mi (ca. 24 km) from the Leiden University Hospital.

Approximately 70% of the samples were tested on the day of collection; almost 88% were tested within 24 h, and 96% were tested within 48 h.

**Inoculation.** Approximately 0.5 g (or 0.5 ml) of feces was suspended in 3 ml of brain heart infusion broth. Selective agars were inoculated with 2 drops of the fecal suspension delivered with a Pasteur pipette. The semisolid agar was inoculated directly with a loopful of feces placed in the medium at the periphery of the plate (diameter, 50 mm). For the filter technique, a cellulose membrane was applied to a blood agar plate. Six drops were delivered with a Pasteur pipette to the top of the membrane and allowed to filter at 37°C. The filters were removed after 1 h, and the plates were incubated.

**Incubation.** All culture plates were incubated microaerobically by including H2- and CO2-generating envelopes without catalysts (GasPak 70304; BBL) in anaerobic jars (BBL). The manufacturer advises against this practice because of the explosiveness of accumulated H2. However, the low explosive level of H2 is probably never reached, so this practice has therefore been used on a fairly large scale throughout Europe. SKM, CampyBAP, and SSM were incubated at 42°C. These media were originally designed for use at 42°C. SKM has already been shown to lack selectivity when used at 37°C (4). CCDA and CSM are, however, selective at 37°C. Therefore, we decided to incubate CSM, CCDA, and the medium used with the filter method at 37°C. All plates were incubated for 3 days and inspected daily.

**Identification.** Presumptive identification was based on colony morphology, Gram staining, and oxidase and catalase tests. In addition, the strains were tested for hippurate hydrolysis, growth at 25 and 42°C, and susceptibility to nalidixic acid and cephalothin. Nalidixic acid-resistant strains were further screened for H2S and DNase production (12-14), anaerobic growth in the presence of 1 g of trimethy1amine-N-oxide hydrochloride per liter, and growth on Mueller-Hinton agar supplemented with 1.5% NaCl (2). These reagents enabled us to differentiate between nalidixic acid-resistant C. coli and "C. lari."

**Semi-quantitative scoring of growth.** The growth of Campylobacter colonies as well as of fecal contaminants was scored as follows: −, no growth; +, 1 to 10 colonies; ++, 11 to 100 colonies; ++++, >100 colonies. These estimations were performed on all solid media on days 1, 2, and 3.

**Statistical analysis.** The McNemar test, the Cochran Q test, and the chi-square test were performed (1), and $P < 0.05$ was regarded as statistically significant.

### RESULTS

In total, 161 campylobacter strains were detected in 1,980 stool specimens by all media. C. jejuni was isolated in 148 specimens (91.9%), C. coli was isolated in 12 (7.5%), and "C. upsaliensis" was isolated in 1 (0.6%). The differences in isolation rates at the two locations were not statistically significant. The isolation rate for samples plated on the day of collection was not different from that for those plated on the other day. With the exception of the "C. upsaliensis" strain, which was isolated only by the filter method, all strains were isolated by at least two different media.

**Isolation of campylobacters by one medium or method.** The cumulative numbers and percentages of specimens found positive for campylobacters by one medium or method are shown in Table 1.

<table>
<thead>
<tr>
<th>Medium or method</th>
<th>No. (%) of specimens found positive for campylobacters after incubation:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Day</td>
</tr>
<tr>
<td>SKM</td>
<td>43 (27)</td>
</tr>
<tr>
<td>CampyBAP</td>
<td>47 (29)</td>
</tr>
<tr>
<td>CCDA</td>
<td>19 (12)</td>
</tr>
<tr>
<td>CSM</td>
<td>22 (14)</td>
</tr>
<tr>
<td>SSM</td>
<td>37 (23)*</td>
</tr>
<tr>
<td>Filter</td>
<td>9 (6)</td>
</tr>
</tbody>
</table>

* A total of 1,980 stool specimens were tested, and 161 were found positive for campylobacters by all methods.

**Isolation of campylobacters by two or more media.** Table 2 shows the numbers and percentages of specimens found positive for campylo-

### TABLE 1. Specimens found positive for campylobacters by one medium or method

<table>
<thead>
<tr>
<th>Medium or method</th>
<th>No. (%) of specimens found positive for campylobacters after incubation:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Day</td>
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<tr>
<td>SKM</td>
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</tr>
<tr>
<td>CCDA</td>
<td>19 (12)</td>
</tr>
<tr>
<td>CSM</td>
<td>22 (14)</td>
</tr>
<tr>
<td>SSM</td>
<td>37 (23)*</td>
</tr>
<tr>
<td>Filter</td>
<td>9 (6)</td>
</tr>
</tbody>
</table>

a A total of 1,980 stool specimens were tested, and 161 were found positive for campylobacters by all methods.

b $P < 0.005$, as compared with CCDA and SSM.

c $P < 0.05$, as compared with SSM and CCDA.

d $P < 0.005$, as compared with CampyBAP and SSM.

e $P < 0.05$, as compared with CCDA and CSM.

f $P < 0.05$, as compared with all media.

**Isolation of campylobacters by two or more media.** After 72 h of incubation, CSM, CCDA, and SKM enabled us to recover the highest numbers of campylobacters. The differences in isolation rates among these three media were not statistically significant. A total of 128 strains were isolated on both CCDA and CSM, 6 strains grew only on CCDA, and 12 strains grew only on CSM; none of these were C. coli.

Extending the incubation time to 72 h instead of 48 h led to significant increases in isolation rates with all separate media ($P < 0.001$).

**Isolation of campylobacters by two media or by a medium combined with the filter method.** Table 2 shows the numbers and percentages of specimens found positive for campylo-

### TABLE 2. Specimens found positive for campylobacters by a combination of two media or medium and method after 72 h of incubation

<table>
<thead>
<tr>
<th>Medium or method</th>
<th>No. (%) of specimens found positive for campylobacters with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SKM</td>
</tr>
<tr>
<td>CampyBAP</td>
<td>135 (84)</td>
</tr>
<tr>
<td>CCDA</td>
<td>149 (93)</td>
</tr>
<tr>
<td>CSM</td>
<td>151 (94)</td>
</tr>
<tr>
<td>SSM</td>
<td>145 (90)</td>
</tr>
<tr>
<td>Filter</td>
<td>139 (86)</td>
</tr>
</tbody>
</table>

a A total of 1,980 stool specimens were tested, and 161 were found positive for campylobacters by all methods.

b Not significant, as compared with CSM plus the filter method.
bacters by a combination of two media or medium and method. The highest isolation rates were found when combinations including CSM or CCDA were used. No statistical significance was achieved in combinations of CSM or CCDA with SSM, SKM, CampyBAP, or the filter method.

**Semi-quantitative scoring of growth of campylobacters.** The growth of campylobacters after 72 h of incubation did not reveal major differences among the four selective agars. The filter method, however, yielded less abundant growth of campylobacters (P < 0.05).

**Semi-quantitative scoring of growth of contaminants.** After 72 h of incubation, SKM was the least selective medium (Table 3). A total of 1,820 of the 1,980 inoculated SKM plates showed growth of contaminants. More than half of these were heavily contaminated. CSM and CCDA appeared to be the most selective agars. Almost 40% of the 1,980 inoculated plates showed no growth of contaminants after 72 h of incubation. The predominant contaminants on these two media were *Candida* spp., which could always be easily differentiated from *Campylobacter* spp. by colony morphology.

**Isolation of *C. coli*.** The rate of isolation of *C. coli* was significantly lower on CampyBAP than was that of *C. jejuni* on this medium (42 versus 72%; P < 0.01). The rate of isolation of *C. coli* on SKM was lower as well (58 versus 82%; P = 0.05). The highest rates of isolation of *C. coli* were found on CSM and CCDA (83 and 75%, respectively).

## DISCUSSION

Our findings confirm the results of previous studies (4, 12, 15) that the charcoal-based media CSM, CCDA, and SKM are effective for isolating *Campylobacter* spp. from clinical specimens (Table 1). CSM yielded the highest number of isolates in this study (Table 1), but no statistical significance was achieved when CSM was compared with CCDA and SKM. However, CCDA and CSM were markedly more selective than was SKM (Table 3). SSM, CampyBAP, and the filter method were the least successful after 72 h of incubation.

Only 72% of the campylobacters cultured by all media were isolated on SSM (Table 1). This rate is lower than that reported in a study by Goossens et al. (9). They obtained isolation rates of 95 and 90% on SSM and CCDA, respectively. There are several reasons which could at least partially explain this discrepancy. First, our "gold standard" consisted of a combination of six different media, as compared with three in the study of Goossens et al. Second, we incubated our plates for a total of 72 h, as compared with 48 h in the study of Goossens et al. This extension of the incubation time to 72 h, which led to a significant number of additional isolates (Table 1), could well have been a crucial factor. In our study, the rate of isolation on SSM after 48 h of incubation was very similar to that on CCDA, in contrast to the differences after 72 h of incubation (Table 1). A total of 144 strains were isolated after 48 h of incubation on all media. Therefore, the sensitivities on SSM and CCDA were 70% (101 of 144) and 75% (106 of 144), respectively. If we had used SSM, CCDA, and SKM medium as the gold standard, as did Goossens et al., we would have obtained higher sensitivities, approaching those reported by Goossens et al.

The difference in isolation rates between SKM (80%) and CampyBAP (75%) was not statistically significant.

The filter method was the least sensitive method (61%). This isolation rate is lower than that reported by Bolton et al. (4) with the same 0.65-μm-pore-size cellulose acetate filter purchased from the same manufacturer. These authors extended the incubation time to 7 days. However, only one additional strain was isolated between days 3 and 7. Thirteen percent of the plates were heavily contaminated in our study (Table 3). The failure to isolate campylobacters by this method may be explained partially by the heavy growth of contaminants.

We found CampyBAP and SKM to be less suitable media for the isolation of *C. coli*. CSM isolated the highest numbers of *C. coli*, in accordance with the findings of Gun-Munro et al. (10). They isolated considerably more *C. coli* on CCDA than on SKM. Ng et al. (15) also found CSM to be less inhibitory for the recovery of *C. coli*. However, in their study *C. coli* generally grew well on SKM.

Extending the incubation time to 72 h led to a significant increase in isolation rate, regardless of the medium used, stressing the importance of the recommendation made by Bolton et al. (4) to incubate selective *Campylobacter* media for 72 h. Many clinical microbiology laboratories still incubate selective *Campylobacter* media for only 48 h.

Maximal recovery of *Campylobacter* spp. requires more than one medium or method. The use of more than two media is probably not cost-effective and is certainly very laborious for a busy diagnostic laboratory. Using a combination of two media, we achieved isolation rates of about 95%. The highest rates were obtained with CSM or CCDA in combination with SSM. These rates were slightly, although not significantly, higher than those obtained when one of the two charcoal-based media was used with SKM, CampyBAP, or the filter method.

The more unusual campylobacters, such as *"C. upsaliensis,"* are in general highly susceptible to drugs, such as cefoperazone, vancomycin, and trimethoprim, that are present in selective media (8). In a recent study (8), only 4 of 99 *"C. upsaliensis"* strains were isolated on selective media. All strains were recovered by a filter method. However, Taylor et al. (21) and Walmsley and Karmali (24) isolated *"C. upsaliensis"* strains on CCDA and CSM, respectively. Overall, it is likely that none of the presently available selective media are entirely satisfactory for the isolation of these catalase-negative or weakly positive (CNW) strains (7). Consequently, CNW campylobacters may be a more common cause of gastroenteritis than has been recognized, since they often remain undetected (15). Maximal recovery of *"C. upsaliensis"* will probably require a combination of a selective agar and a more labor-intensive isolation method.

On the basis of the present study, we conclude that when the maximal recovery of *Campylobacter* spp. is sought, a combination of two media, including CSM, will lead to high isolation rates. Since CSM and CCDA were more selective than were the other media, the greater ease of detection of...
suspected colonies on these media may be advantageous. Although CSM in combination with SSM isolated the highest numbers of campylobacters, no statistical significance was achieved in comparisons with CSM and SKM or CSM and the filter method. Since we isolated only one "C. upsaliensis" strain, no recommendation with respect to the isolation of CNW campylobacters can be made on the basis of the presented data. We have not been able to confirm the high sensitivity of the semisolid medium reported by Goossens et al. (9). Additional studies should be performed to evaluate this medium.

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REFERENCES