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Semisolid Blood-Free Selective-Motility Medium for the Isolation of Campylobacters from Stool Specimens

HERMAN GOOSSENS,* LINDA VLAES, INGRID GALAND, CHANTAL VAN DEN BORRE, AND JEAN-PAUL BUTZLER

World Health Organization Collaborating Centre for Enteric Campylobacter, St. Pieters University Hospital, 322 Hoogstraat, B-1000 Brussels, Belgium

Received 26 October 1988/Accepted 23 January 1989

Isolation of *Campylobacter jejuni* and *C. coli* from stool specimens is done by growing campylobacter colonies on solid selective media with or without blood. However, recognition of these colonies can be difficult. Therefore, we decided to evaluate an isolation procedure based on the swarming of campylobacters through a semisolid medium. We developed a semisolid blood-free selective motility (SSM) medium which is composed of Mueller-Hinton broth with 0.4% agar and supplemented with cefoperazone (30 μg/ml) and trimethoprim (50 μg/ml). The SSM medium was compared with our previously described Butzler Medium Virion (Goossens et al., J. Clin. Microbiol. 24:840–843, 1986) and blood-free medium (Bolton and Coates, J. Appl. Bacteriol. 54:115–125, 1983) with cefoperazone (32 μg/ml) (Bolton et al., J. Clin. Pathol. 37:956–957, 1986). Of 1,890 routine stool specimens tested, 100 were found to be positive for campylobacters: 95 were recovered with the SSM medium, 94 with the Virion medium, and 90 with the blood-free medium. The SSM medium performed equally well whether it was incubated in the special incubator or the candle jar. Only 4.4 and 7.3% of the plates grew contaminating fecal flora when incubated in the special incubator and the candle jar, respectively. Clearly the SSM medium is easy, quick, cheap, sensitive, and more selective than any other medium which has been developed so far and does not require the addition of blood. We believe that this medium has a future in the routine microbiology laboratory in developed as well as in developing countries.

Campylobacter jejuni and *C. coli* are now considered a common cause of diarrhea throughout the world. Isolation of these organisms has evolved from a filtration technique in 1972 (4) to the simpler procedure of adding selective antibiotic supplements to solid media in 1977 (12). Subsequently, other antibiotic combinations were proposed as selective supplements for basal media with blood (1, 3, 5–7, 9). Even a blood-free, charcoal-based medium has been developed successfully by Bolton and Coates (2).

However, recognition of campylobacter colonies on these media can be difficult for several reasons: first, contaminating fecal flora may overgrow campylobacters; second, fecal flora may mimic campylobacter colonies; and third, campylobacter colonies may not always appear as swarming flat colonies. Thus, recovery of campylobacters from stool specimens can be time-consuming; very often staining of suspected colonies will have to be done to confirm the typical campylobacter morphology. Finally, developing countries are having trouble in isolating campylobacters because supplies of sterile blood are not readily available and because the blood-free basal medium is expensive.

For these reasons, we have been looking for other isolation systems for several years. In this paper, we report on a new isolation procedure for campylobacters based on their swarming through a semisolid blood-free selective motility (SSM) medium.

MATERIALS AND METHODS

Development of SSM medium. (i) Effect of antibiotics. To be certain that the antibiotics present in the semisolid medium were not inhibiting swarming of campylobacters, we decided to test *C. jejuni* and *C. coli* strains with cefoperazone MICs of ≤100 μg/ml (MICs of trimethoprim are always ≥100 μg/ml). For this purpose, we selected strains from a MIC study performed in early 1988 on 200 *C. jejuni* and *C. coli* strains isolated at our hospital. In this way we were hoping to select the most cefoperazone susceptible strains. Finally, we were able to find 10 strains with MICs of ≤100 μg/ml. Four media were tested: a control medium without antibiotics, the final SSM medium, and SSM medium without trimethoprim or without cefoperazone. The strains tested were suspended in phosphate-buffered saline (PBS) to a McFarland no. 1 standard and 40 μl was inoculated into the medium at the periphery of the plate. After incubation for 48 h under the appropriate gas atmosphere (6% oxygen, 6% carbon dioxide, and 88% nitrogen), we measured the distance from the site of inoculation to the migration line.

(ii) Sensitivity testing. To test the sensitivity of the SSM medium, we made McFarland no. 1 suspensions in PBS of 11 *C. jejuni* and *C. coli* strains isolated from routine stool specimens and continued to make 10-fold dilutions in PBS. From these dilutions, we simultaneously inoculated 125 μl into SSM medium for measuring the distance in swarming and 125 μl onto solid agar plates for colony counts. In order to work under "ideal" circumstances (i.e., mimicking a clinical sample with highly motile strains), we used only fresh isolates.

(iii) Swarming in SSM medium incubated in candle jars. We compared with swarming of *C. jejuni* and *C. coli* through SSM medium incubated at 42°C in the special incubator (with 6% oxygen, 6% carbon dioxide, and 88% nitrogen) and in a candle jar. We tested five different *Campylobacter* strains in duplicate experiments. The strains were suspended in PBS to McFarland no. 1, and 40 μl was inoculated into the medium at the periphery of the plate. Swarming was again expressed as the distance in millimeters between the site of inoculation and the migration line.

Evaluation of SSM medium with clinical stool specimens. (i)

* Corresponding author.

TABLE 1. Effect of trimethoprim and cefoperazone on the swarming of *C. jejuni* and *C. coli*[a]

Strain	Biotype	MIC (μg/ml)		Swarming distance (mm)			
		CEF	TMP	No antibiotics	CEF (30 μg/ml)	TMP (50 μg/ml)	CEF (30 μg/ml) plus TMP (50 μg/ml)
D368	*C. coli* I	50	>100	44.0	42.0	45.5	37.5
H166	*C. jejuni* II	12.50	>100	36.5	33.5	36.0	33.5
J92	*C. jejuni* I	25	>100	50.0	50.0	50.0	50.0
M322	*C. jejuni* II	50	>100	41.5	50.0	39.5	39.5
N114	*C. coli* II	50	>100	50.0	50.0	50.0	50.0
R194	*C. coli* I	100	100	50.0	50.0	50.0	50.0
S216	*C. coli* I	100	>100	46.5	50.0	40.5	50.0
U25	*C. coli* I	100	100	41.5	50.0	42.5	36.0
U27	*C. coli* I	100	100	46.0	40.5	40.5	42.5
V258	*C. coli* I	50	>100	47.5	50.0	48.0	46.0
Range				36.5–50.0	33.5–50.0	36.0–50.0	33.5–50.0
Mean				45.4	46.6	44.3	43.5

[a] The effects of trimethoprim (TMP) and cefoperazone (CEF) on swarming were determined in SSM medium incubated at 42°C for 48 h. Results are the mean of two experiments.

Media. The SSM medium was composed as follows: Mueller-Hinton broth (GIBCO Laboratories) with 0.4% agar (Difco Laboratories) and cefoperazone (30 μg/ml) and trimethoprim (50 μg/ml) as the selective agents. The SSM medium was compared with two solid media: our previously described selective medium, Butzler medium Virion (Institut Virion, Zurich, Switzerland) (6) composed of cefoperazone (30 μg/ml), rifampin (10 μg/ml), and amphotericin B (2 μg/ml); and blood-free medium (2) (Oxoid CM 739) supplemented with cefoperazone (32 μg/ml) (Oxoid SR 125). The media were prepared twice weekly and stored in the dark at 4°C until use.

(ii) Specimens. A total of 1,890 stool specimens submitted to our laboratory for routine culturing of enteric pathogens between 4 May and 1 October 1988 were tested.

(iii) Inoculation. Within 24 h of collection, feces samples were inoculated directly onto the media; emulsification in saline to obtain a smooth heavy suspension was carried out of solid stools inoculated on the solid media. This procedure was not necessary for the semisolid medium. For this SSM medium, a loopful of feces was placed into the medium at the periphery of the agar plate (diameter of the plate, 50 mm).

(iv) Incubation. All media were incubated at 42°C in a special incubator from which two-thirds of the air had been evacuated and replaced, leading to a final gas mixture of approximately 6% oxygen, 6% carbon dioxide, and 88% nitrogen. The SSM medium was also incubated in a candle jar of the last 1,113 stool specimens tested, between 22 July and 1 October 1988. The plates were incubated at 5 p.m. Results were recorded the next day, after 18 h, and the second day, after 42 h. Plates containing suspected campylobacter growth, either as colonies on solid media or as cells swarming through the semisolid media, were stained with crystal violet.

(v) Identification and biotyping. Oxidase and catalase tests were done. All isolates were biotyped by the Lior biotyping scheme (10).

Statistical analysis. The chi-square test was performed, and $P < 0.05$ was taken as statistically significant.

RESULTS

Development of SSM medium. Initially, we had carried out a study on 150 stool specimens, inoculated in the semisolid medium, with various concentrations and combinations of trimethoprim and cefoperazone. This study showed that trimethoprim at 50 μg/ml with cefoperazone at 30 μg/ml gave optimal selectivity (results not shown). This antibiotic combination did not inhibit swarming of *C. jejuni* and *C. coli* through the semisolid medium (Table 1).

SSM medium showed high sensitivity, taking into account the lowest number of colony-forming units per plate which was still able to produce swarming through the SSM medium (Table 2).

Finally, we found that swarming of *C. jejuni* and *C. coli* occurred equally well in the special incubator and in the candle jar (results not shown).

Evaluation of SSM medium for routine isolation of campylobacters from stool specimens. The two different types of swarming of campylobacters in the SSM medium are illustrated in Fig. 1A and B. Most campylobacters showed continuous swarming through the medium (Fig. 1A); however, a minority showed a cloudy type of swarming (Fig. 1B). In any case, swarming of campylobacters could be easily distinguished from the swarming of contaminating fecal flora (Fig. 1C). The latter showed a dark white and often discontinuous type of swarming, with an odor which was totally different from the soft and sweet-smelling odor of campylobacters. However, staining was mandatory to confirm the typical spiral morphology of campylobacters.

TABLE 2. Sensitivity of SSM medium[a]

Strain	Biotype	No. of CFU/plate	Swarming distance (mm)
B643	*C. jejuni* I	10	19
D459	*C. jejuni* I	3	41
E326	*C. jejuni* II	15	23
E328	*C. jejuni* I	5	32
E329	*C. jejuni* I	5	22
G231	*C. jejuni* II	6	26
G234	*C. jejuni* I	13	33
L238	*C. jejuni* II	9	28
M432	*C. coli* I	3	35
N147	*C. jejuni* I	1	23
S281	*C. jejuni* IV	4	36
Range		1–15	19–41
Mean		6.7	28.9

[a] The smallest inoculum that produced swarming was determined.

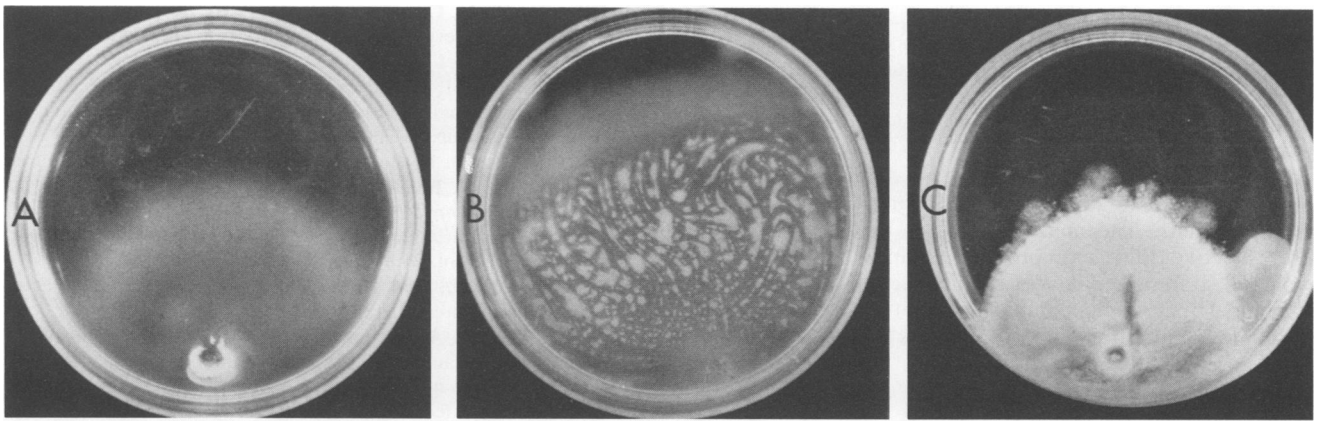

FIG. 1. Swarming in SSM medium. Feces samples were inoculated with a loop (bottom). A campylobacter migration line can be seen (top) at a distance of 24 mm in panel A and 47 mm in panel B. A migration line of contaminating fecal flora can be seen (top) at a distance of 26 mm in panel C. Swarming of campylobacters is totally different from swarming of contaminating fecal flora.

Table 3 shows the results of a comparison between our previously described selective medium (6), the blood-free medium (2), and the newly developed SSM medium. A total of 100 patients were found to be positive for campylobacters. No statistically significant differences in isolation rates were found between the different media. The SSM medium performed equally well whether it was incubated in the special incubator or the candle jar: of 1,113 stool specimens, 53 were found positive for campylobacters whether the SSM medium was incubated in the special incubator or the candle jar. We noticed that the degree of swarming of campylobacters through the SSM medium was the same (40.4 mm) whether the medium was incubated in the special incubator or the candle jar.

However, incubation in the candle jar was found to allow greater growth of contaminating fecal flora that was statistically significant: 4.4% with the special incubator versus 7.3% with the candle jar ($P < 0.01$).

DISCUSSION

We have developed a semisolid selective motility (SSM) medium, which is composed of Mueller-Hinton broth with 0.4% agar and to which cefoperazone (30 μg/ml) and trimethoprim (50 μg/ml) were added.

Mueller-Hinton is among the best basal broths for the growth of campylobacters, provided that a good batch is selected, because there is significant batch-to-batch variation. The agar concentration also necessitates careful selection, because we noted that a change in agar lot number markedly affected the degree of swarming (the optimal agar concentration ranges from 0.28 to 0.40%, depending on the batch and commercial source).

TABLE 3. Comparison of SSM, Butzler medium Virion, and blood-free charcoal medium

Medium[a]	No. of patients positive for campylobacters
SSM only	4
BMV only	1
CCDA only	0
SSM + BMV	5
SSM + CCDA	2
BMV + CCDA	4
SSM + BMV + CCDA	84

[a] SSM, Butzler medium Virion (BMV), and blood-free charcoal medium (CCDA) were incubated at 42°C for 42 h. A total of 1,890 stool specimens were tested, identifying campylobacter infections in 100 patients.

There are many reasons why we would strongly recommend the use of this SSM medium by microbiologists. First of all, the medium is very cheap and easy to prepare; Mueller-Hinton broth and agar are the only basal ingredients. The use of small petri dishes (diameter, 5 cm) further reduces the cost of the medium and are very easily placed into the candle jar (two plates can be put next to each other in a 2.5-liter jar). Only two antibiotics have to be incorporated (inhibition of gram-positive organisms and yeasts is no longer necessary). The kit, consisting of the basal medium as well as the new supplements of trimethoprim and cefoperazone, is now available commercially from Institut Virion AG, Zurich, Switzerland. Second, the medium is easy to interpret; plates are read only for swarming, not for organisms. Third, the typical spiral forms of campylobacters were observed exclusively with the SSM medium. Indeed, coccoid transformation, as can be seen with campylobacters on solid media, was never seen in the semisolid medium. Fourth, contamination with fecal flora in the SSM medium is much lower than with any other selective medium which has been developed so far. Indeed, only 4.4% of the SSM plate were contaminated with fecal flora versus 29.3% for Butzler medium Virion (6), 42.6% for Karmali medium (10), 65.3% for Skirrow medium (8), 59.7% for Preston medium (6), and 37.6% for the blood-free medium (6). As a consequence, the time saved with the SSM medium is considerable. Fifth, SSM medium is highly sensitive. Sixth, the antibiotic supplement in the SSM medium did not inhibit the growth of *C. coli*, as has been shown for other supplements (6, 11). Indeed, inhibition of *C. coli* is mainly due to the presence of polymyxins, which were omitted from the SSM medium. Seventh, the SSM medium does not require the addition of blood. This has several advantages; commercially available blood is expensive, sterile blood might be difficult to obtain, especially in developing countries, and finally, the addition of blood can cause variable and misleading results (11).

A disadvantage of the SSM medium is that nonmotile campylobacters might not be identified; however, all five *C. jejuni* which were only isolated on solid media were found to be highly motile, and subculture in the SSM medium showed a swarming of ≥50 mm after 48 h. We have no explanation

for why these five *C. jejuni* were missed with the SSM medium, especially because the plates did not show any contamination with fecal flora. Nor do we have any explanation for why six *C. jejuni* were missed with Butzler medium Virion and 10 were missed with the blood-free medium.

In conclusion, we have developed a semisolid selective motility medium for the recovery of campylobacters from stool specimens. The medium is easy, quick, cheap, and more selective than any other medium which has been developed so far and does not require the addition of blood. We believe that this medium has a future in the routine microbiology laboratory in developed as well as in developing countries.

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