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Comparison of Rapid Urease Tests, Staining Techniques, and Growth on Different Solid Media for Detection of *Campylobacter pylori*

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Thirty-nine single antral biopsies (phase 1) and 99 sets of six antral biopsies (phase 2) were collected from 132 patients, and 87 (63%) yielded positive cultures for *Campylobacter pylori*. Of several primary media tested in phase 1, tryptic soy agar and Skirrow agar, each supplemented with 10% whole sheep blood, supported relatively good growth of *C. pylori*. In phase 2, four of the six biopsies in each set were tested with different urease systems. Selective urea agar for rapid identification was the most sensitive (39 of 63 [62%] at 1 h) and specific (100%); however, the difference between this system and the CLOtest was not statistically significant. The remaining two biopsies, one transported in saline and the other transported in a supplemented tryptic soy broth, were ground separately and inoculated onto tryptic soy agar and Skirrow agar, each supplemented with 10% whole sheep blood. In selected instances, 10% horse serum or 10% horse serum and 5 mM urea or 1% cholesterol were also added to the media. Smears stained with a modified Gram stain or acridine orange detected 68% of 63 culture-positive biopsies; no false-positive results were reported. Skirrow agar supported better growth of *C. pylori* than did tryptic soy agar; the nonselective medium was also overgrown with contaminants in 25 to 30% of the positive cultures. Based on colony size, Skirrow agar supplemented with 10% whole sheep blood, 10% horse serum, and 1% cholesterol supported optimal growth of *C. pylori*. Fresh media supported better growth than did prepoured commercial media ($P \leq 0.004$). Saline was a convenient and satisfactory transport medium for antral biopsies.

In 1983 Warren and Marshall reported that they had isolated a spiral bacterium from antral biopsy specimens on moist chocolate agar incubated under microaerophilic conditions (J. R. Warren and B. Marshall, Letter, Lancet i:1273–1275, 1983). Subsequently, several investigators have found an association between this organism, now known as *Campylobacter pylori*, and gastritis (1, 6, 10). As interest in this putative cause-effect relationship between *C. pylori* and gastritis increases, laboratories may be asked to culture biopsy specimens for this organism.

C. pylori generally requires 3 to 7 days to grow on solid primary media. Rapid identification techniques, such as the rapid urease test and various staining procedures, have been described (1, 7, 11). However, results from these rapid tests were compared with culture results obtained by using a limited number and type of media. In another study, aimed at determining the growth requirements of *C. pylori*, a limited number of stock isolates were tested (2). Because different strains of *C. pylori* may show variable growth patterns on solid media, it is important that all media be tested with primary cultures.

We tested a number of different solid media for the support of primary growth and isolation of *C. pylori*. In addition, we tested rapid methods for presumptive identification of *C. pylori*, including urease production and staining techniques, and compared these results with results obtained from cultures.

(Some of these data were presented previously [P. E. Coudron and D. F. Kirby, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, C104, p. 349].)

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MATERIALS AND METHODS

Thirty-nine single antral biopsies and 99 sets of six antral biopsies were collected from 132 patients who, at the time of endoscopy, generally presented with upper abdominal complaints consistent with peptic disease. All patients gave informed consent. Biopsy specimens were collected with an Olympus fiberoptic endoscope. After specimen collection from each patient, the endoscope was thoroughly rinsed with water and with a 2% Staphene solution and then soaked in a 3.2% glutaraldehyde solution (CidexPlus) for 10 min; at the end of each day, the endoscopes were washed in a 2% glutaraldehyde solution (Cidex) for 20 min.

In a preliminary study (phase 1), 39 biopsy specimens were collected, transported to the laboratory in saline (sterile, nonbacteriostatic), and processed in the following manner. Each specimen was thoroughly ground with a mortar and pestle in 1.0 ml of saline. To ensure equal distribution of tissue fragments, the homogenate was flushed up and down in a sterile, disposable plastic pipette and then evenly distributed among 14 plates of solid media. Smears were also prepared for 31 of the biopsies by placing 1 drop of the homogenate on each of four slides.

The smears were air dried and fixed in 100% methanol (except Gram-stained slides, which were heat fixed), and the following stains were applied: (i) Giemsa stain (A.J.P. Scientific, Inc, Clifton, N.J.), (ii) Gram stain, (iii) a modified Gram stain with carbol fuchsin as the counterstain (8), and (iv) a fluorescent acridine orange stain (SpotTest; Difco Laboratories, Detroit, Mich.). The Giemsa and acridine orange stains were applied as recommended by the manufacturers. Slides stained with Giemsa stain, Gram stain, or the modified Gram stain were examined by light microscopy

at a magnification of ×1,000. Slides stained with acridine orange were also examined at ×1,000 (oil), using a Nikon episcopic fluorescence microscope. All slides were examined for up to 10 min for characteristic curved, S-, or U-shaped bacterial cells.

Biopsy homogenates were inoculated onto several primary media. Except for chocolate agar (BBL Microbiology Systems, Cockeysville, Md.), media were obtained from Difco, prepared in the laboratory, and inoculated within 2 weeks. Prepoured modified Thayer-Martin medium (Remel, Lenexa, Kans.) was also inoculated. Specimens were inoculated onto each type of medium in duplicate and in some instances, in triplicate. Inoculation of a total of 14 plates of solid media with each specimen ensured a comparable volume of inoculum per plate per specimen. Inoculated media were incubated at 35°C under microaerophilic conditions in an anaerobic jar containing a catalyst and a campylobacter gas-generating envelope (Gas Generating Kit; Oxoid U.S.A., Inc., Columbia, Md.).

After 5 to 7 days of incubation, isolates that were urease (Christensen urea agar), catalase (3% hydrogen peroxide solution), oxidase (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride), and phosphatase (*p*-nitrophenyl phosphate in Tris buffer [3]) positive and showed characteristic cell morphology when stained with the modified Gram stain were identified as *C. pylori*. Eight isolates were confirmed as *C. pylori* by C. W. Moss, Centers for Disease Control, Atlanta, Ga., on the basis of cellular fatty acid analysis. Growth of *C. pylori* was assessed semiquantitatively as follows: no growth in any quadrant was considered 0, growth in quadrant 1 was considered 1+, growth in quadrant 2 was considered 2+, and growth in quadrant 3 was considered 3+.

In the second part of the study (phase 2), biopsies from each of 99 sets were tested for *C. pylori* by two rapid techniques, urease production and staining procedures, and results from these tests were compared with culture results obtained with selected primary media. A set consisted of six biopsy specimens that were collected from each patient per visit. One biopsy specimen was placed in a transport medium consisting of tryptic soy broth (no glucose) supplemented with 10% horse serum and 5 mM urea, and the remaining specimens were placed in saline. The specimens were held at room temperature and transported to the laboratory, and most were processed within 2 to 4 h after collection (see below).

Upon arrival in the laboratory, four biopsy specimens that were transported in saline were immediately tested for urease production by placing one tissue specimen in each of the following media: (i) 0.5 ml of a commercially available (lyophilized), urea broth medium for rapid identification (Remel); (ii) a custom-made medium with a compound base, the selective urea agar for rapid identification (Remel); (iii) a medium in the CLOtest strip (Delta West Ltd., Western Australia, Australia); and (iv) the conventional Christensen urea agar medium (Difco). The media were preheated to 35°C prior to inoculation and maintained at 35°C after inoculation. The test was read as positive when a red or pink color developed around the biopsy specimen. Tests were read at 15 min and 1, 3, and 20 h.

The remaining two biopsy specimens, one transported in supplemented tryptic soy broth and the other in saline, were ground with a separate mortar and pestle in 1.0 ml of the respective transport medium and processed as described for phase 1. Smears from each biopsy were stained with the modified Gram stain and acridine orange and examined as described above by a technologist who was unaware of the urease test results.

TABLE 1. Relative growth of *C. pylori* on different primary solid media for culture-positive specimens

Medium[a]	*n*[b]	No. (%) of times *C. pylori* was isolated with indicated relative growth[c]			
		0	1+	2+	3+
ChocA	7	3 (42)	2 (29)	2 (29)	
ChocA (no IsoVitaleX)	6	4 (66)	1 (17)	1 (17)	
MTM	6	2 (33)	2 (33)	2 (33)	
BHIA + 7% LHB	18	6 (33)	8 (45)	4 (22)	
TSA + 5% WSB	24	5 (21)	11 (46)	8 (33)	
TSA + 10% WSB	24	1 (4)	12 (50)	9 (38)	2 (8)
TSA + 20% WSB	8		2 (25)	6 (75)	
TSA + 10% WRB	8	1 (12)	3 (38)	4 (50)	
TSA + 10% WHuB	6		3 (50)	3 (50)	
SkirA + 5% WSB	9		4 (44)	4 (44)	1 (12)
SkirA + 10% WSB	15		2 (13)	11 (74)	2 (13)
SkirA + 10% WSB + 10% HS	8		1 (12)	7 (88)	

[a] Abbreviations: ChocA, chocolate agar; MTM, modified Thayer-Martin medium; BHIA, brain heart infusion agar; LHB, laked horse blood; TSA, tryptic soy agar; WSB, whole sheep blood; WRB, whole rabbit blood; WHuB, whole human blood; SkirA, Skirrow agar; HS, horse serum.

[b] *n*, Number of biopsies that were inoculated onto each medium.

[c] Relative growth was scored as follows: no growth in any quadrant was considered 0, growth in quadrant 1 was considered 1+, growth in quadrant 2 was considered 2+, and growth in quadrant 3 was considered 3+.

Biopsy homogenates were inoculated onto selected fresh primary media. These media included tryptic soy agar and Skirrow agar, each of which was prepared from base medium with the same lot number. Both media were supplemented with 10% whole sheep blood, and in selected instances, 10% horse serum or 10% horse serum and 5 mM urea or 1% cholesterol were also added. Because many laboratories use commercial media, 37 of the biopsies tested in phase 2 were also inoculated onto prepoured Skirrow media (Remel and BBL). All media were inoculated and incubated as described for phase 1. Growth was assessed semiquantitatively and, in addition, the relative colony size on each media was recorded as <0.5, 0.5 to 1.0, or >1.0 mm.

Data comparing detection of *C. pylori* with different urease systems and by growth on various media were analyzed by the Fisher exact test.

RESULTS

Of the 39 single biopsies (phase 1) and 99 sets of biopsies (phase 2) that were collected, 24 (62%) and 63 (64%), respectively, yielded positive cultures for *C. pylori*. In phase 1, 16 of the 24 culture-positive biopsies were stained, and characteristic curved organisms were seen in the following number of smears: Giemsa stain, 5 (31%); Gram stain (conventional), 9 (56%); modified Gram stain, 11 (69%); and acridine orange stain, 12 (75%). For the 15 culture-negative biopsies, no false-positive results were recorded with any stain; i.e., no streptococci or enteric bacilli were misread as curved, S-shaped organisms.

The relative growth of *C. pylori* on different primary media for the 24 culture-positive biopsies taken in phase 1 is given in Table 1. Because of previous reports, some media were inoculated more often than other media. Tryptic soy agar and Skirrow agar, each supplemented with 10% whole sheep blood, supported better growth of *C. pylori* than did chocolate agar, modified Thayer-Martin medium, or supplemented

TABLE 2. Comparison of rapid urease tests for detection of *C. pylori* in 63 culture-positive specimens

Urease test[a]	No. (%) of biopsies positive at indicated time			
	15 min	1 h	3 h	20 h
UB	16 (25)	27 (43)	37 (59)	49 (78)
SUA	29 (46)	39 (62)	45 (71)	52 (83)
CLOtest	21 (33)	34 (54)	42 (67)	49 (78)
CUA	12 (19)	23 (37)	29 (46)	34 (54)

[a] Abbreviations: UB, urea broth for rapid identification (Remel); SUA, selective urea agar for rapid identification (Remel); CLOtest (Delta West Ltd.); CUA, Christensen urea agar (Difco).

brain heart infusion agar. Growth on all types of blood supplement appeared to be about equal. Medium supplemented with horse serum also supported good growth. On the basis of staining and culture results for biopsies collected in phase 1, additional tests were performed with a larger number of biopsies.

Results obtained by using four rapid urease systems to detect *C. pylori* in 63 phase 2 culture-positive biopsies are given in Table 2. Selective urea agar for rapid identification was the most sensitive urease medium tested. The CLOtest detected fewer culture-positive biopsies than did the selective medium; however, the difference was not statistically significant ($P = 0.101$ at 15 min). Thirty-six culture-negative biopsies were also tested with the same systems and at the same time periods. At 20 h, 4 (11%) and 5 (14%) false-positive results were seen with the urea broth for rapid identification and the Christensen urea agar, respectively.

Results of two direct staining methods, the modified Gram stain and the acridine orange stain, used in phase 2 to detect *C. pylori* essentially agreed with the preliminary findings in phase 1; both staining methods detected 43 (68%) of the 63 culture-positive biopsies. Of 36 culture-negative biopsies stained, no false-positive results were seen. We also found that saline was a better transport medium than the supplemented broth for direct staining purposes.

The relative growth of *C. pylori* on selected primary solid media for 63 phase 2 culture-positive biopsies was scored as described for phase 1 (data not shown). Colony sizes of *C. pylori* on the same media are shown in Table 3. In 25 to 30% of the cultures, the nonselective media were overgrown with contaminating organisms. In general, little difference in the relative growth or colony size (Table 3, >1.0 mm column, $P \geq 0.151$) of *C. pylori* was observed between cultures of biopsies that were transported in saline and cultures of biopsies that were transported in supplemented tryptic soy broth. On the basis of colony size, Skirrow agar supported better growth of *C. pylori* than did tryptic soy agar (Table 3, base medium plus blood only, >1.0 mm column, $P = 0.017$). The numbers of colonies of *C. pylori* on Skirrow agar supplemented with blood and the same medium supplemented with blood, horse serum, and urea or cholesterol were nearly equal. However, the sizes of the colonies were larger on fresh Skirrow medium supplemented with both horse serum and cholesterol than on fresh Skirrow medium without these ingredients (Table 3, >1.0 mm column, $P = 0.010$).

TABLE 3. Colony size of *C. pylori* on selected primary solid media

Medium[a]	TM[b]	*n*[c]	No. (%) of times colonies were of indicated size (mm)		
			<0.5	0.5–1.0	>1.0
TSA + WSB	S	38	27 (71)	9 (24)	2 (5)
	T	33	24 (73)	7 (21)	2 (6)
TSA + WSB + HS	S	41	18 (44)	19 (46)	4 (10)
	T	39	12 (31)	19 (49)	8 (20)
SkirA + WSB	S	56	18 (32)	25 (45)	13 (23)
	T	57	25 (44)	14 (25)	18 (31)
SkirA + WSB + HS	S	58	11 (19)	25 (43)	22 (38)
	T	58	13 (22)	22 (38)	23 (40)
SkirA + WSB + HS + 5 mM Urea	S	58	13 (23)	28 (48)	17 (29)
	T	59	14 (24)	25 (42)	20 (34)
SkirA + WSB + HS + 1% Chol	S	57	9 (16)	22 (38)	26 (46)
	T	58	11 (19)	24 (41)	23 (40)

[a] See Table 1, footnote *a*; concentration of WSB and HS was 10%; Chol, cholesterol.
[b] Abbreviations: TM, transport medium; S, saline; T, tryptic soy broth supplemented with 10% horse serum and 5 mM urea.
[c] Sixty-three culture-positive biopsies were inoculated onto all media. However, *C. pylori* failed to grow on some media or media were overgrown with contaminants (TSA media only).

TABLE 4. Relative growth of *C. pylori* on primary Skirrow media

Medium	TM[a]	No. (%) of times *C. pylori* was isolated ($n = 26$[b]) with indicated relative growth[c]			
		0	1+	2+	3+
SkirA + 10% WSB[d] (Difco; fresh)	S	1 (4)	11 (42)	13 (50)	1 (4)
	T	0	11 (42)	13 (50)	2 (8)
Skirrow (Remel; prepoured)	S	4 (15)	14 (54)	8 (31)	0
	T	2 (8)	16 (61)	7 (27)	1 (4)
Skirrow (BBL; prepoured)	S	8 (31)	16 (61)	2 (8)	0
	T	11 (42)	10 (39)	5 (19)	0

[a] See Table 3, footnote *b*.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.
[d] See Table 1, footnote *a*.

The relative growth and colony sizes of *C. pylori* on three different primary Skirrow media are shown in Tables 4 and 5. On the basis of the relative number of colonies, fresh Skirrow medium (Difco) supported better growth of *C. pylori* than did BBL prepoured medium (Table 4, 2+ column, $P < 0.001$), and on the basis of colony size, fresh medium supported better growth than did either BBL or Remel prepoured medium (Table 5, >1.0 mm column, $P \leq 0.004$).

DISCUSSION

Several investigators have used the Giemsa or Gram stain to detect *C. pylori* in histological sections of antral biopsies

TABLE 5. Colony size of *C. pylori* on primary Skirrow media

Medium	TM[a]	*n*[b]	No. (%) of times colonies were of indicated size (mm)		
			<0.5	0.5–1.0	>1.0
SkirA + 10% WSB[c] (Difco; fresh)	S	25	10 (40)	5 (20)	10 (40)
	T	26	10 (38)	6 (24)	10 (38)
Skirrow (Remel; prepoured)	S	22	19 (86)	2 (9)	1 (5)
	T	24	19 (79)	4 (17)	1 (4)
Skirrow (BBL; prepoured)	S	18	18 (100)	0	0
	T	15	15 (100)	0	0

[a] See Table 3, footnote *b*.
[b] See Table 1, footnote *b*. *C. pylori* failed to grow on some media (see Table 4).
[c] See Table 1, footnote *a*.

(1, 7, 11). To facilitate ease and speed of detection, we prepared smears directly from ground specimens. Smears stained with Giemsa or Gram stain showed poor contrast between bacterial cells and background material. Our results agree with those of Barrett et al. (L. J. Barrett, B. J. Marshall, K. Dye, J. Sarazin, R. McCallum, and R. L. Guerrant, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, C101, p. 348), who recently reported that the sensitivity of the direct Gram stain is generally poor and that the procedure is tedious, time consuming, and distinctly inferior to culture. In our study, carbol fuchsin (modified Gram stain) stained the organisms a deep pink and provided good contrast between organisms and background. With the fluorescent acridine orange stain, bacterial cells appeared orange, in contrast to background material, which appeared yellow-green.

Parsonnet et al. (9) recently described a simple rinse-imprint technique for rapid detection of *C. pylori*. Gastric biopsies were rinsed in glucose phosphate broth and blotted, and an impression smear was made, Gram stained, and examined under oil for 5 min. The biopsy specimens were then ground and inoculated onto brucella agar with 5% horse blood and modified Thayer-Martin medium. Of 32 culture-positive biopsies, typical curved organisms were detected in smears from 30. We performed the imprint technique with 12 culture-positive biopsies (phase 2) and detected characteristic organisms in nine smears which were stained with the modified Gram stain and examined for 10 min. In contrast, typical organisms were seen in 11 of the 12 smears prepared from ground biopsy material.

A second rapid technique for detecting *C. pylori* in gastric biopsies is the urease test (7). Our results with the CLOtest differ from results reported by Marshall et al. (7), who detected up to 75 and 92% of 79 patients infected with *C. pylori*, at 20 min and 3 h, respectively. These investigators defined an infected patient as one who had typical organisms on Gram stain or in histologic sections or was culture positive. A possible explanation for the difference in results may be the extensive number and type of media used in our study. Patients harboring few organisms may not be detected by using a limited number of culture media.

Investigators have used a wide variety of media to grow *C. pylori* (2, 4, 5, 9, 10). However, it is important that there have been few studies that compared recovery and growth of *C. pylori* on different primary media. We found that the addition of blood to media not only enhanced the growth of *C. pylori* but also helped to distinguish *C. pylori* colonies from those of other bacteria on primary media: colonies of *C. pylori* appeared distinctly clear. During phase 1, it became evident in many instances that, although the numbers of colonies of *C. pylori* on different media were nearly equal, the sizes of the colonies differed greatly. On the basis of colony size, we found that the addition of horse serum to fresh Skirrow medium improved the growth of *C. pylori* slightly ($P = 0.066$) and that the addition of horse serum and cholesterol improved growth more significantly ($P = 0.010$).

Biopsy specimens were held in transport media at room temperature prior to culture. The times for holding biopsies ranged from 1 to 8.5 h, with a mean time of 2.9 ± 1.2 h. Holding biopsy material in this manner does not appear to affect the recovery of *C. pylori*. Barrett et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1988) demonstrated that *C. pylori* could be recovered from biopsy specimens that were held in saline for 0.5 to 2 h as readily as from biopsy specimens that were cultured immediately in the endoscopy suite. Goodwin et al. (4) found that biopsies could be kept at 4°C for 5 h without loss of viability of *C. pylori*. We tested recovery of *C. pylori* from biopsies stored for longer times, including two biopsies that were held for 24 h at 4°C in saline, a third biopsy that was stored for 72 h at 4°C, and a fourth biopsy that was frozen for 72 h at −70°C in tryptic soy broth plus 23% glycerol. In all instances, the numbers and sizes of colonies were slightly decreased relative to growth from specimens that were cultured within 2.5 h after collection. The question of holding specimens for more than 4 h should be tested with a larger number of biopsy specimens.

Goodwin et al. (4) also reported that grinding biopsy specimens in a ground-glass grinder in 0.3 ml of 20% glucose consistently gave much heavier growth of *C. pylori* than merely mincing the specimen. In our study, biopsy material was prepared by grinding each specimen in 1 ml of the respective transport medium (supplemented tryptic soy broth or saline). In 12 instances, an extra biopsy specimen (culture positive) was transported in saline and ground in 1 ml of 20% glucose. No difference in the numbers or sizes of colonies of *C. pylori* was observed between biopsies ground in saline and biopsies ground in glucose. We recommend that a biopsy specimen be prepared for culture by thoroughly grinding the specimen in saline.

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