

Optimal Combination of Media for Primary Isolation of *Helicobacter pylori* from Gastric Biopsy Specimens

RAFFAELE PICCOLOMINI,^{1*} GIOVANNI DI BONAVENTURA,¹ DAVIDE FESTI,²
GIOVANNI CATAMO,¹ FRANCESCO LATERZA,² AND MATTEO NERI²

Laboratory of Clinical Microbiology, Department of Biomedical Sciences,¹ and Department of Medicine and Aging Sciences,² "G. D'Annunzio" University, I-66100 Chieti, Italy

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The aim of the present study was to compare eight media, four nonselective and four selective media, to determine the best combination of media for the primary isolation of *Helicobacter pylori*. Over a period of 5 months, mucosal antral biopsy specimens were obtained from 222 consecutive dyspeptic patients undergoing endoscopy. Biopsy samples were plated in parallel on all eight media. Egg yolk emulsion agar (EYE), Skirrow's medium, Dent's medium, and modified Thayer-Martin medium were used as selective media; modified chocolate agar (MCHOC), Triptycase soy agar (TSA), brucella agar, and brain heart infusion agar were used as nonselective media. Overall, by using these eight media, *H. pylori* was recovered from biopsy specimens from 114 of 222 patients, yielding an isolation rate of 51%. Comparison of all possible combinations of the eight media showed that the highest rate of isolation of *H. pylori* was 100% (114 of 114) with EYE-MCHOC, followed by 96.5% (110 of 114) when EYE-TSA was used. Conversely, it was found that none of the media used alone yielded a 100% rate of recovery (the maximum recovery rate was 95%, which was achieved with EYE). These results indicate that the association of EYE and MCHOC yielded the maximum recovery of *H. pylori* from gastric biopsy specimens. Therefore, the use of selective and nonselective media in parallel offers optimal recovery rates with only a slight increase in costs.

Since the first description by Warren and Marshall (30) in 1983, *Helicobacter pylori* has been recognized as an important gastroduodenal pathogen. *H. pylori* infection is one of the most frequent bacterial infections in the world, and this organism has been acknowledged to be a crucial factor in several different diseases ranging from gastritis to gastric malignancies (1, 3, 6, 9, 12-14, 25). These observations explain the interest of investigators in developing accurate diagnostic methods.

At present, culture of this bacterium from gastric antral biopsy specimens is a reference technique in bacteriology and is essential for drug susceptibility testing and analysis of putative virulence factors. Although it is usually considered a tedious, time-consuming, and expensive procedure, culturing on solid medium is the standard technique used in most laboratories for the isolation of *H. pylori* from gastric biopsy specimens. Over the years, there have been a number of reports on the media that can be used for the successful culture of *H. pylori*. Originally, Marshall et al. (19) used brain heart infusion chocolate agar supplemented with 7% horse blood. Subsequently, a variety of media, selective and nonselective, or a combination of both have been proposed for use in the primary isolation of *H. pylori* (2, 7, 11, 16, 21, 28, 31), but the optimal method of recovery still remains to be established.

Resistance of *H. pylori* to metronidazole and macrolides has emerged worldwide and now constitutes a major problem in therapy (4, 22, 29). This justifies the increasing use of culture in testing for *H. pylori* infection because it is the only diagnostic method that allows us to assess the susceptibility of this organism to antimicrobial agents.

The objective of this study was therefore to compare and evaluate classic and new selective and nonselective media in

order to determine the combination of media yielding the optimal recovery of *H. pylori* from antral mucosal biopsy specimens.

(Part of this study was presented at the IX International Workshop on Gastroduodenal Pathology and *Helicobacter pylori* in Copenhagen, Denmark, 16 to 19 October 1996 [26].)

MATERIALS AND METHODS

Biopsy specimens. Three gastric mucosal antral biopsy specimens were collected from each of 222 consecutive dyspeptic patients undergoing endoscopy. Gastric biopsy specimens were placed in sterile tubes containing 1 ml of transport medium consisting of modified Cary-Blair medium (24) and were stored at 4°C. The delay between the removal of the specimens and the inoculation onto culture media did not exceed 3 h. One biopsy specimen was used for histologic examination (Giemsa stain). The other two biopsy specimens for culture were finely minced in a tissue grinder (Wheaton, Millville, N.J.) in 1 ml of sterile saline solution and were vortexed at a high speed. Serial 10-fold dilutions of the homogenate were made up to 10⁻³ of the original concentration. One hundred microliters from each dilution was placed for isolation onto each of eight media. The plates were incubated in 100% humidity at 37°C for up to 7 days in a microaerophilic gas mixture composed of 10% CO₂, 5% O₂, and 85% N₂ (Campy-Pak; Unipath S.p.A., Garbagnate Milanese, Milan, Italy). The agar plates were checked for growth from day 3 through day 7. An isolate was identified as *H. pylori* on the basis of positive catalase, oxidase, and urease reactions, typical colony morphology (small, round colonies), and the presence of characteristic curved gram-negative bacilli on Gram-stained smears. Suspect colonies were confirmed as *H. pylori* by using Api-Campy (BioMerieux Italia S.p.A., Rome, Italy). *H. pylori* NCTC 11638 served as a control strain.

Media. Eight different media were compared for primary isolation of *H. pylori*. For all media, defibrinated sheep blood and defibrinated horse blood were acquired from Biolife Italiana S.r.l. (Milan, Italy); 2,3,5-triphenyltetrazolium chloride and antibiotic powders of known potency included in egg yolk emulsion agar (EYE; cefsulodin, trimethoprim, vancomycin, and amphotericin) were purchased from Sigma Chemical Co. (Milan, Italy). The rest of the agar base and supplements were purchased from Unipath (Milan, Italy). All media were stored under identical conditions at 4°C. No plates were more than 1 week old at the time of use.

(i) **Selective media.** Four selective media were used. EYE (31) contained Columbia agar, 10% egg yolk emulsion, 1% Vitox, and 40 mg of 2,3,5-triphenyltetrazolium chloride per liter with an antibiotic supplement (5 mg of cefsulodin, 5 mg of trimethoprim, 6 mg of vancomycin, and 6 mg of amphotericin B per liter). Modified Thayer-Martin medium (MTM) (23) contained GC agar base, 10% soluble hemoglobin powder, and GC supplement (10 g of yeast

* Corresponding author. Mailing address: Dipartimento di Scienze Biomediche, Università "G. D'Annunzio," Via dei Vestini 31, I-66100 Chieti, Italy. Phone: (39) 871-355283. Fax: (39) 871-355282.

TABLE 1. Qualitative growth of *H. pylori* isolated from biopsy specimens on direct culture

Qualitative growth	No. (%) of <i>H. pylori</i> -positive cultures recovered on the following media ^a							
	SK	EYE	MTM	DENT	MCHOC	BHIA	TSA	BRUC
+++	26 (40)	58 (54)	43 (52)	31 (37)	45 (47)	18 (46)	25 (39)	16 (46)
++	18 (28)	38 (35)	29 (34)	25 (30)	29 (30)	10 (23)	19 (29)	3 (9)
+	15 (23)	12 (11)	9 (11)	18 (22)	18 (20)	9 (23)	18 (28)	12 (36)
±	6 (9)	0	3 (3)	10 (11)	3 (3)	3 (8)	3 (4)	3 (9)
Total	65 (100)	108 (100)	84 (100)	84 (100)	95 (100)	40 (100)	65 (100)	34 (100)
Recovery rate (%) ^b	57	95	74	74	83	35	57	30

^a Values in parentheses are the percentage of *H. pylori*-positive cultures recovered only on that medium.

^b Percentage among a total of 114 strains isolated.

extract, 1.5 g of dextrose, 0.15 g of NaHCO₃, 3 mg of vancomycin, 7.5 mg of colistin methane-sulfonate, 12,500 IU of nystatin, and 5 mg of trimethoprim per liter). Skirrow's medium (SK) consisted of Columbia agar, 7% laked horse blood, Campylobacter growth supplement, and Campylobacter selective supplement (10 mg of vancomycin, 2,500 IU of polymixin B, and 5 mg of trimethoprim per liter). Dent's medium (DENT) (5) consisted of Columbia agar, 7% laked horse blood, and Dent's supplement (10 mg of vancomycin, 5 mg of trimethoprim, 5 mg of cefsulodin, and 5 mg of amphotericin B per liter).

(ii) **Nonselective media.** Four nonselective media were used. Brucella agar (BRUC) was made from brucella medium base, 1% Vitox, and 5% sheep blood. Trypticase soy agar (TSA) consisted of Trypticase soy agar and 5% sheep blood. Brain heart infusion agar (BHIA) contained brain heart infusion agar and 7% defibrinated horse blood. Modified chocolate agar (MCHOC) contained Columbia agar, 1% Vitox, and 5% sheep blood.

Evaluation criteria. All of the agar plates were also monitored for contaminants as well as semiquantitative estimation of the growth of *H. pylori*. Data were recorded as follows: abundant growth (+++), $\geq 10,000$ CFU; good growth (++) , $1,000 \leq$ CFU $< 10,000$; moderate growth (+), $100 \leq$ CFU $< 1,000$; scanty growth (\pm), < 100 CFU. To compare the sensitivities of culture and histologic methods, we considered *H. pylori* to be present if either culture or histology result was positive.

RESULTS

Over a study period of 5 months, 666 gastric mucosal biopsy specimens were obtained from 222 consecutive dyspeptic patients (142 males and 80 females) undergoing endoscopy. Overall, by using all eight media, *H. pylori* was detected in 114 patients (76 males and 38 females), yielding an isolation rate of 51% (66.6% for males and 33.4% for females).

Table 1 indicates the qualitative growth of *H. pylori* on each of the eight media used. None of the media by itself gave maximum recovery: EYE gave the highest isolation rate (108 of 114 plates; 95%), followed by MCHOC (83%), DENT (74%), MTM (74%), SK (57%), TSA (57%), BHIA (35%), and BRUC (30%). The recovery rate obtained with EYE was significantly higher ($P < 0.01$) compared with those obtained with DENT, MTM, SK, TSA, BHIA, and BRUC; the difference was not statistically significant ($P > 0.05$) for EYE compared with that for MCHOC. EYE gave the highest percentage (89%) of isolates giving abundant ($\geq 10,000$ CFU) or good ($1,000 \leq$ CFU $< 10,000$) growth, followed by MTM (86%) and MCHOC (77%).

The recovery rate and the qualitative growth of *H. pylori* with all possible combinations of two media are presented in Table 2. The combination that appeared to be the most effective for the primary isolation of *H. pylori* was EYE (selective medium) plus MCHOC (nonselective medium), which yielded a maximum recovery rate of 100% (114 of 114). This combination also gave the highest percentage (88%) of isolates giving abundant ($\geq 10,000$ CFU) or good ($1,000 \leq$ CFU $< 10,000$) growth, followed by EYE-MTM (84%) and MTM-MCHOC (81%).

Table 3 indicates the qualitative growth of contaminants associated with each of the eight media. Among the selective media, MTM gave the lowest contamination rate (11%; 12 of

114 plates), a value significantly different from those obtained with SK, EYE, MCHOC, BHIA, TSA, and BRUC ($P < 0.01$). Among the nonselective media, BRUC gave the lowest rate (39 of 114 plates), followed by BHIA, MCHOC, and TSA.

One hundred twelve patients were concordantly positive for *H. pylori* by both culture and histologic examination; 2 patients were positive by culture only, 3 patients were positive by histology only, and 105 patients were concordantly negative by both culture and histology. Culture performed with EYE-MCHOC had a 97% (114 of 117) sensitivity, compared with a 98% (115 of 117) sensitivity for histologic examination.

DISCUSSION

Primary isolation of *H. pylori* from a biopsy specimen is a difficult process: in specialized laboratories, isolation rates of 75 to 90% can be achieved (8, 20). This may be due to the fastidious nature of *H. pylori* and to a number of factors that are hard to control (patchy distribution of the organism on the gastric mucosa, contamination of biopsy forceps, ingestion of anesthetic, presence of oropharyngeal flora, loss of viability of the organisms during transportation, etc.) and that are, altogether, responsible for a poor negative predictive value associated with culture of *H. pylori*. For these reasons, although culture has been considered the "gold standard" for the diagnosis of *H. pylori* infection by various investigators (15, 18, 27), culture is now usually used only in the research setting. However, the need for a high *H. pylori* recovery rate from gastric biopsy specimens is increasing among practicing clinicians. In fact, treatment of *H. pylori* infection is a first-line approach for the treatment of patients with *H. pylori*-positive gastric and duodenal ulcers and is increasingly used for other conditions such as dyspepsia. Yet, treatment efficacy may be reduced by the presence of primary resistance to commonly used antibiotics, such as metronidazole (10). In addition, it is generally accepted that widespread use of anti-*H. pylori* treatments in general practice will lead to the emergence of resistance to multiple antibacterial agents among *H. pylori* isolates. Thus, we anticipate that sensitivity testing of antibiotics will be largely required in treatment failures or before initiating therapy in patients harboring *H. pylori* infection.

The results of the present study indicate that, among the eight media tested, EYE is the most sensitive for the primary isolation of *H. pylori*, yielding a recovery rate of 95%. Our results conflict with those of Hachem et al. (11). Since EYE and DENT possess identical antibiotic supplements, the different isolation rates between these two media (95% for EYE versus 74% for DENT) may be attributable to the richer growth supplement (egg yolk emulsion with 1% Vitox) in EYE. Furthermore, the red color induced by triphenyltetrazolium chloride included in EYE made the *H. pylori* colonies

TABLE 2. Qualitative growth of *H. pylori* on all possible combinations of media

Medium combination	No. of <i>H. pylori</i> -positive cultures/total no. of cultures (%)	No. (%) of plates on which growth was recorded according to qualitative growth of <i>H. pylori</i>					Total
		+++	++	+	±		
EYE + MCHOC	114/114 (100)	101 (50)	77 (38)	20 (10)	3 (2)		201
EYE + MTM	108/114 (95)	93 (49)	66 (35)	23 (12)	8 (4)		190
DENT + EYE	111/114 (97)	88 (46)	63 (33)	30 (16)	9 (5)		190
MTM + MCHOC	107/114 (94)	87 (49)	57 (32)	27 (16)	6 (3)		177
SK + EYE	111/114 (97)	83 (48)	56 (32)	27 (16)	6 (4)		172
EYE + TSA	110/114 (96)	82 (48)	56 (32)	30 (18)	3 (2)		171
DENT + MCHOC	101/114 (88)	75 (42)	54 (30)	36 (21)	12 (7)		177
EYE + BRUC	107/114 (94)	72 (51)	41 (29)	24 (17)	3 (3)		140
DENT + MTM	105/114 (92)	74 (44)	53 (32)	27 (16)	12 (8)		166
SK + MCHOC	101/114 (88)	70 (44)	47 (29)	33 (21)	9 (6)		159
MCHOC + TSA	95/114 (83)	69 (44)	47 (29)	36 (23)	6 (4)		158
EYE + BHIA	98/114 (86)	75 (51)	47 (32)	21 (15)	3 (2)		145
SK + MTM	93/114 (81)	69 (47)	46 (31)	24 (16)	9 (6)		148
MTM + TSA	96/114 (84)	68 (47)	46 (31)	27 (18)	6 (4)		147
SK + DENT	93/114 (81)	57 (39)	44 (29)	33 (22)	15 (10)		149
DENT + TSA	90/114 (79)	56 (38)	43 (29)	36 (24)	12 (9)		147
MCHOC + BHIA	95/114 (83)	62 (47)	38 (28)	27 (21)	6 (4)		132
MTM + BHIA	84/114 (74)	61 (50)	37 (30)	18 (15)	6 (5)		122
MCHOC + BRUC	92/114 (80)	59 (46)	32 (25)	30 (24)	6 (5)		127
SK + TSA	93/114 (82)	51 (39)	36 (28)	33 (26)	9 (7)		129
MTM + BRUC	90/114 (79)	58 (50)	31 (27)	21 (18)	6 (5)		116
DENT + BHIA	90/114 (79)	49 (40)	34 (28)	27 (22)	12 (10)		122
DENT + BRUC	90/114 (79)	46 (40)	28 (24)	30 (26)	12 (10)		116
SK + BHIA	71/114 (62)	44 (42)	27 (26)	24 (23)	9 (9)		104
TSA + BHIA	78/114 (68)	43 (42)	27 (26)	27 (26)	6 (6)		103
SK + BRUC	84/114 (74)	41 (42)	21 (21)	27 (28)	9 (9)		98
TSA + BRUC	71/114 (62)	40 (41)	21 (22)	30 (31)	6 (6)		97
BRUC + BHIA	50/114 (44)	33 (46)	12 (17)	21 (29)	6 (8)		72

easier to spot on EYE than on the other media. Unlike the contaminants, *H. pylori* colonies show a characteristic golden shine when viewed with reflected light. The isolation rate of 57% yielded by SK is not comparable to those reported in previous studies (5, 16, 28), which reported values ranging from 94 to 97%. It is possible that these differences could be a result of methodological variability, slight differences in medium components, and the freshness of the media; differences between isolates from different geographical areas also possibly exist. In addition, among the selective media, SK yielded the highest number of contaminated plates. The low selectivity of this medium may be due to the absence of an antifungal agent in its composition, unlike the other selective media. Among these, MTM was the most selective, confirming that the antimicrobial supplement present in this medium (vancomycin, colistin, trimethoprim, and nystatin) limits the overgrowth by flora of the upper respiratory tract (23). Our modified chocolate agar yielded a good rate of 83%, comparable to

those obtained with classic chocolate agar in other studies (5, 7, 28). Other nonselective media yielded the lowest rates because of the abundant growth ($\geq 10,000$ CFU) of contaminants (especially *Proteus* spp., *Pseudomonas aeruginosa*, *Streptococcus* spp., and *Candida* spp.) that obscured the growth of *H. pylori* (Table 3). In the primary isolation of *H. pylori*, it is very important to consider the qualitative growth of contaminants isolated on each medium. In fact, when the growth of contaminants was scanty (<100 CFU), it did not interfere with *H. pylori* isolation. Conversely, growth of *H. pylori* could not be detected on the plate in the presence of a high number of contaminants. With respect to this point, the DENT medium has always shown a moderate growth (<1,000 CFU) of contaminating microorganisms; 64% of contaminated EYE plates showed scanty growth. BHIA gave a recovery rate of 35%, which was significantly lower than that obtained by Hachem et al. (11) (96%).

The maximum recovery rate (100%) was obtained only by

TABLE 3. Qualitative growth of contaminants isolated from biopsy specimens on direct culture

Qualitative growth	No. (%) of contaminated plates recorded for each medium ^a							
	SK	EYE	MTM	DENT	MCHOC	BHIA	TSA	BRUC
+++	3 (11)	3 (12)	3 (25)	0	9 (20)	15 (35)	16 (32)	18 (46)
++	0	0	3 (25)	0	9 (20)	3 (7)	9 (18)	6 (15.5)
+	9 (32)	6 (24)	6 (50)	10 (62.5)	9 (20)	6 (14)	9 (18)	6 (15.5)
±	16 (57)	16 (64)	0	6 (37.5)	18 (40)	19 (44)	16 (32)	9 (23)
Total	28 (100)	25 (100)	12 (100)	16 (100)	45 (100)	43 (100)	50 (100)	39 (100)
Contamination rate (%) ^b	25	22	11	14	39	38	44	34

^a Values in parentheses are the percentage of contaminants isolated only on that medium.

^b Percentage among a total of 114 *H. pylori*-positive plates.

using a combination of a selective medium and a nonselective medium in parallel (EYE and MCHOC). Furthermore, this combination of media gave the highest percentage (88%) of isolates with abundant or good growth.

Histologic examination and culture by using EYE-MCHOC are methods of equally high sensitivity for the detection of *H. pylori* in gastric biopsy specimens. Thus, the culture method performed with an appropriate combination of media (selective and nonselective) could be considered the gold standard for the diagnosis of *H. pylori* infections; nevertheless, this issue needs to be further explored in comparison to other diagnostic technique such as the ¹³C-urea breath test (17).

Our data indicate that use of selective and nonselective media in parallel is superior to the use of one medium alone, according to Krajden et al. (16) and Tee et al. (28). We recommend the combined use of EYE and MCHOC to obtain maximum recovery of *H. pylori* from gastric antral biopsy specimens with only a slight increase in costs.

This recommendation is particularly relevant since culture of *H. pylori* is becoming increasingly important to the search for efficient antimicrobial combinations that eradicate this bacterium from the stomach.

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