

Evaluation of Techniques for Isolation, Subcultivation, and Preservation of *Helicobacter pylori*

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With sheep blood agar (SBA), Belo Horizonte medium, and Brussels campylobacter charcoal agar, 104 strains of *Helicobacter pylori* were detected in 309 gastric biopsies. Each medium revealed only 69 to 71% of the strains. Ten strains grew solely on SBA, and four strains each grew on Belo Horizonte medium and Brussels campylobacter charcoal agar. Subculturing of 50 fresh *H. pylori* isolates on SBA revealed a progressive reduction in growth with increasing passage. Thirty strains stopped growing between passages two and seven. Four strains survived more than 20 passages. The preservation of fresh *H. pylori* isolates at -193 and -70°C and by lyophilization was compared by use of 10% porcine mucin solution, fetal calf serum, and a commercial cryopreservative fluid. Of 30 strains, 77 to 90% could be recultivated on SBA after preservation at -70°C in all three storage media. The data indicate that for the primary isolation of *H. pylori*, not only one selective medium but several selective media with different antibiotic supplements plus at least one nonselective medium should be used to yield the highest culture rates. Frequent subculturing of *H. pylori* on SBA selects strains which may not be representative of clinical isolates. Storage of fresh *H. pylori* isolates at -70°C in 10% mucin solution is a simple and effective preservation procedure.

A large number of different agar media for recovering *Helicobacter pylori* have been described (1, 6). There are two main types of media: (i) nonselective media based on nutrient agar such as brain heart infusion agar or brucella agar supplemented with 5 to 10% sheep or horse blood, serum, activated charcoal, or soluble starch (2, 7-9) and (ii) selective media based on supplemented nutrient agar containing antibiotics (3, 7-9) and 2,3,5-triphenyltetrazolium chloride (5, 10). Only minimal work has been conducted to establish which of these media is best for isolating the organism (1). Likewise, it is not known whether media which are suitable for the primary isolation of *H. pylori* are useful for repeated subcultivations, e.g., to establish a collection of viable stock cultures. Furthermore, even though several preservation mixtures have been described (4, 6, 11, 13), exact data comparing the efficiency of various preservation procedures are still lacking.

The object of this study was to compare selective and nonselective media for primary isolation, subcultivation of fresh isolates, and different preservation procedures.

MATERIALS AND METHODS

Isolation and identification. Gastric biopsy specimens were kept in transport medium consisting of brain heart infusion broth (CM 225; Oxoid, Basingstoke, England) with 5% Fildes extract (SR 46; Oxoid) and brought to the laboratory on the day of endoscopy. Equal portions of the specimen were streaked on the media outlined in Table 1. The plates were incubated at 37°C for 4 to 5 days in a microaerophilic gas mixture composed of 8 to 10% CO_2 , 5 to 7% O_2 , and 83 to 87% N_2 (Anaerocult C; E. Merck, Darmstadt, Federal Republic of Germany) in GasPak jars (BBL). An isolate was identified as *H. pylori* on the basis of small translucent (blood agar) or golden pigmented (Belo Horizonte agar and Brussels campylobacter charcoal agar) colonies; gram-negative curved or spiral cells; and production of urease,

oxidase, and catalase. *H. (Campylobacter) pylori* ATCC 43504 served as a control strain.

Subcultivation. Colonies of primary isolates of *H. pylori* were subcultured on sheep blood agar at intervals of 4 to 5 days. Several colonies were streaked to areas approximately 1 cm in diameter on three different plates. The incubation and identification procedures were the same as for primary isolation. To prevent death of the cultures upon exposure to air, the inoculations were done within 30 min. Subcultures were repeated until growth was no longer visible on all three inoculated plates or until more than 20 passages were reached.

Preservation. Primary isolates of *H. pylori* were propagated on blood agar for 4 to 5 days. A saline suspension equivalent to a McFarland no. 6 standard (ca. 3×10^8 CFU/ml) was made from the culture material. Fifty microliters of the suspension was introduced into 0.5 ml of each of the following storage media: 10% porcine stomach mucin (M-1778; Sigma) in distilled water, fetal calf serum (no. 47900; Serva, Heidelberg, Federal Republic of Germany), and cryopreservative fluid from a commercial preservation system (Protect; Technical Service Consultants, Bury, Lancashire, England). Inoculated vials with mucin and fetal calf serum were kept at -193°C (liquid nitrogen) and -70°C and were lyophilized. Vials with cryopreservative fluid were kept at -193 and -70°C but were not freeze-dried because preliminary experiments showed that a dry state could not be reached. The samples were stored for at least 4 weeks. Recultivation was performed on blood agar by spotting the storage media and the freeze-dried preparations, which were reconstituted with 0.5 ml of distilled water, in 0.1-ml portions on three plates. Incubation and identification of reconstituted strains were carried out as for primary isolation.

Electron microscopy. The surfaces of subculture plates with no visible growth were rinsed with 1 ml of 0.9% NaCl. The fluid was filtered through 0.8- μm -pore-size membrane filters (Millipore) and concentrated to 0.1 ml with collodion bags (SM 13200; Sartorius, Göttingen, Federal Republic of Germany) and polyethylene glycol 20,000 (Serva). The prep-

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TABLE 1. Compositions of sheep blood agar, Belo Horizonte agar (10), and Brussels campylobacter charcoal agar (5) used for the isolation and subcultivation of *H. pylori*

Ingredient	Quantity in:		
	Sheep blood agar	Belo Horizonte agar	Brussels campylobacter charcoal agar
Blood agar base (CM 55; Oxoid)	40 g		
Brain heart infusion broth (CM 225; Oxoid)		37 g	37 g
Agar (no. 1614; Merck)		10 g	10 g
Distilled water	900 ml	900 ml	860 ml
Sheep blood (FSR 1055; Oxoid)	100 ml	100 ml	
Horse serum (29-211-54; Flow)			100 ml
Yeast extract (25%) (30-00-49; Flow)			40 ml
Activated charcoal (L9; Oxoid)			2 g
2,3,5-Triphenyltetrazolium chloride (no. 8380; Merck)		40 mg	40 mg
Amphotericin B (Squibb)		5 mg	10 mg
Vancomycin (Lilly)		6 mg	10 mg
Nalidixic acid (Serva)		20 mg	
Trimethoprim (Hoffmann-La Roche)			5 mg
Cefsulodin (Grünenthal)			5 mg

arations were negatively stained with 4% phosphotungstic acid and examined for phages with a Zeiss EM 10 electron microscope.

RESULTS

H. pylori was recovered from 104 of 309 gastric biopsy specimens examined when the cultures on the nonselective blood agar and the two selective media, Belo Horizonte agar and Brussels campylobacter charcoal agar, were evaluated together. However, none of the three media revealed all 104 isolates (Table 2). The low rate of isolation of the nonselective medium was mainly caused by the overgrowth of *H. pylori* with other bacteria. Suppression of the growth of *H. pylori* was the main cause of the low rate of isolation of the selective media. Of the 104 *H. pylori* isolates, 10% grew solely on blood agar and not on the selective media. Conversely, 4% of the isolates found on either selective medium were not found on the other media.

Fifty *H. pylori* isolates from gastric biopsies were randomly selected for repeated subcultivations on blood agar. Approximately four strains were lost on each subculture up to seven passages, and thereafter two strains failed to grow on each subculture. Four strains survived more than 20 subcultures. Gram stains of cultures before each passage showed no significant morphological alteration or transformation of cells to coccoid forms. The urease, oxidase, and catalase activities as well as the colony appearance remained unchanged regardless of whether the strains grew in the next

subculture or not. Electron microscopic examinations of the inocula from the subculture plates without growth did not reveal phages which could be responsible for lysis of the inocula.

The preservation studies showed that freeze-drying in 10% mucin solution or fetal calf serum followed by storage at room temperature harms *H. pylori*; none of the strains could be recultivated after that preservation procedure. Storage in 10% mucin solution, fetal calf serum, or cryopreservative fluid at -193 or -70°C kept 23 to 27 (77 to 90%) of the strains viable. Preservation at -193°C was not more successful than that at -70°C . Strains kept in 10% mucin solution developed larger colonies on the recultivation plates than did strains stored frozen in the other two media. Considering effectiveness and practicability, storage at -70°C in 10% mucin solution was the preferred preservation procedure.

DISCUSSION

With nonselective media, the isolation of *H. pylori* is impeded by exogenous contaminants and, more frequently, endogenous contaminants which are present in biopsy specimens (3, 5, 7-9). When biopsy specimens were cultured on sheep blood agar, contaminants obscured the growth of *H. pylori* in 22 (21%) of 104 culture-positive specimens. Other workers have reported overgrowth on nonselective media in 22 (23%) of 97 (5) and in 8 (23%) of 35 (3) specimens containing *H. pylori*.

Considering the high rate of contaminants on nonselective media, selective media with antibiotics are thought to be mandatory for the detection of *H. pylori* (1, 6). The rate of contamination on Belo Horizonte agar and Brussels campylobacter charcoal agar was greatly reduced in comparison with that on sheep blood agar, but the rate of isolation of *H. pylori* was not proportionally increased. The selective media inhibited the growth of contaminants as well as the growth of *H. pylori* isolates at rates of 19 and 27%, respectively. The more pronounced inhibitory effect of Belo Horizonte agar may have been due to nalidixic acid, which has been suggested to inhibit 14% of *H. pylori* isolates at a concentration of 20 mg/liter (3). However, even Brussels campylobacter charcoal agar, the antibiotic mixture of which has been claimed to be noninhibitory for *H. pylori* (3, 5) and sufficiently suppressive for contaminants (3), yielded a culture

TABLE 2. Rate of efficiency of agar media for the isolation of *H. pylori* from 104 gastric biopsy specimens from which *H. pylori* grew on at least one of the three media

Agar	Rate of efficiency (%) of agar for:		
	Growth of:		No visible growth
	<i>H. pylori</i> ^a	Other microbes	
Blood	71 (10)	21	8
Belo Horizonte	71 (4)	2	27
Brussels campylobacter charcoal	69 (4)	12	19

^a Values in parentheses are percentages of *H. pylori* strains isolated only on that medium.

rate no higher than did Belo Horizonte agar and blood agar because of *H. pylori* inhibition and overgrowth of contaminants.

The present data do not support the recommendation that cefsulodin medium can be used alone, without a nonselective medium, for the primary isolation of *H. pylori* (3). Quite to the contrary, a nonselective medium should be used, as 10 of 104 *H. pylori* strains could be isolated only on sheep blood agar. Selective media with different antibiotic combinations should also be used. With the combination of Belo Horizonte medium and Brussels campylobacter charcoal medium, 30 (29%) of 104 isolates were detected in addition to the isolates detected on the nonselective medium. Therefore, the use of nonselective and selective media in parallel is superior to the use of one medium alone (8).

It has been reported that subculturing of single colonies of *H. pylori* is difficult but can be achieved if one colony is subcultured in an area only 1 cm in diameter (6). In addition, prolonged culturing should give rise to the emergence of coccoid forms, which are difficult to propagate, possibly reflecting a transformation in response to unfavorable conditions or nutrition (6). Despite the avoidance of these known obstacles to subcultivation, only a minority (8%) of *H. pylori* isolates were maintained viable by repeated subculturing on sheep blood agar. No morphological or biochemical signs of starvation, i.e., alteration of colony appearance, increase in coccoid forms, or loss of oxidase, catalase, or urease, which would explain the discontinuation of growth were observed. In electron microscopic investigations, phages have been found in *H. pylori* strains; they may have been induced by subculturing and storage (12). However, in the 46 strains which stopped growing before passage 20, no lytic phages were found electron microscopically. In further experiments on other *H. pylori* strains, phages were not induced by heat, UV light, or mitomycin C (7a). It is therefore improbable that phages were responsible for the discontinuation of growth. The limited viability of *H. pylori* strains during subcultivation hampers the setting-up of viable strain collections and includes the risk of confining detailed microbiological investigations to easily subcultivable strains. Such strains are the minority of clinical isolates and may lack distinctive characteristics of the majority, e.g., virulence factors.

To circumvent frequent subculturing, it is necessary to preserve strains for long-term storage. Freeze-drying harms *H. pylori* (6), as the present data confirm. Lyophilizing without freezing by use of 20% L-glutamic acid solution has been recommended (6); however, this method is too cumbersome for routine usage (13). Subculturing of strains in liquid broth before storage (4) is a procedure prone to contamination and is time-consuming as well. More practicable methods are storage at -70°C in 1% peptone water containing 25% glycerol (6), in defibrinated horse blood (13), in tryptone soy broth containing 15% glycerol (11), or in a commercial cryopreservative fluid (11). All of these recom-

mendations, however, are based on laboratory experience and not on the systematic investigation of large numbers of strains. The investigation of 30 fresh isolates of *H. pylori* showed that the most simple and economical preservation procedure was the storage of *H. pylori* in 10% mucin solution at -70°C . Additionally, routine use of that procedure showed that the preservation time could be extended beyond 6 months without a significant loss of viability.

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