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E-Mail :
editor.ijasem@gmail.com
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Evaluation of Liquid Media for Growth of *Helicobacter pylori* isolated from antral biopsies of duodenal ulcer patients

Wani Imtiyaz[1] and Mir Mohsin[2]

[1]Adesh Institute of Allied & Healthcare Professionals, Adesh University, Bathinda (Pb.) India

[2]Adesh Institute of Pharmacy & Biomedical Sciences, Adesh University, Bathinda (Pb) India

Abstract

Helicobacter pylori a fastidious microorganism has been routinely isolated and grown on solid media. We have succeeded in obtaining growth of this organism in several broth media in large volumes, including Cryptic soy broth, Mueller-Hinton broth, brucella broth, brain heart infusion broth, and Columbia broth. Growth was tested in the media with and without supplementation. Growth was obtained after incubation under microaerobic conditions and with Co_2 enrichment. Growth in a stationary system versus that in an agitated system was evaluated. Results showed that *H.pylori* can be grown in any of the liquid media tested except buffered yeast extract—α-ketoglutarate if serum is added. No growth was observed on buffered yeast extract-α-ketoglutarate even with serum and other supplementation. Growth of *H.pylori* in most of the liquid media with supplements was improved if the culture was incubated in a CO_2 atmosphere.

Introduction

Helicobacter pylori is a fastidious microorganism which has been shown to play a major role in the etiology of type B gastritis (1, 4). This microorganism can be cultured from biopsy specimens on selective and nonselective solid media (3). Growth of the organism in liquid media, however, has not been established. There are reports indicating that this organism grows in liquid media under microaerophilic conditions, but only in small volumes (2, 5, 7, 9). Large-scale growth of *H.pylori* by using GasPak jars or gas-generating chambers on a shaking platform is rather cumbersome.

The objective of the study reported here was to facilitate culture of *H.pylori* in large volumes for physiological studies.

MATERIALS AND METHODS

Bacterial strains: Strain ATCC 13629 (Institute of Microbial Technology, IMTECH, Delhi) was used in the initial stages of the study. Subsequently, a set of strains of *H. pylori,* ATCC 43504, was obtained from IMTECH, Chandigarh, Punjab was also investigated. In addition, three strains freshly isolated from human biopsy specimens and in low passage number were also included. Stock cultures were stored at —70°C in horse blood.

Media and supplements. All media except blood agar base no. 2 were from Difco (Detroit, Mich.); blood agar base no. 2 was from Oxoid (Hampshire, England). All supplements were obtained from Sigma (St. Louis, Mo.) Horse serum and horse blood were from Remel (Lenexa, Kans.).

Growth on solid medium. *H. pylori* strains were grown on blood agar plates supplemented with 8% horse blood. Plates were placed in anaerobic jars with CampyPak Plus (BBL Microbiology Systems, Cockeysville, Md.). The jars were incubated at 37°C for 72 h. Both the urease test and microscopic examination were done at the beginning of each experiment.

Growth in broth media: Growth of *H. pylori* in tryptic soy broth (TSB), brucella broth (BB), Columbia broth (CB), Mueller-Hinton broth (MHB), brain heart infusion broth (BHIB), and buffered yeast extract—α-ketoglutarate (BYEn) were tested. These media were used with and without supplements. Supplements added in later experiments included 10% horse serum (HS), 10% fetal calf serum IFCS), 0.1% yeast extract (YE), and 1% hemin.

Two sets of liquid media were prepared; one set was sterilized by autoclaving (121°C, 15 lbs./in^2, 15 min), and the other set was sterilized by filtration by using the Nalgene filtration system (Nalgene Company, Rochester, N. Y.). IN. *pylori* cultures grown on blood agar for 3 days were used as inocula. For each liquid medium, a suspension of cultures was made in the same liquid medium, and 1 ml of this was inoculated into 10 ml of each medium. That is, 10 ml of a medium was transferred to a 50-ml tissue culture flask, providing an initial concentration of 10^6 to 10^7 cells per ml. The flasks were divided into two sets. One set was incubated in anaerobic jars fitted with loose caps and containing CampyPak Plus (BBL). These jars were incubated at 37°C, with shaking (100 rpm). Another set of cultures was incubated horizontally in an incubator with 10% CO and 100% humidity; the jars containing these cultures were also fitted with loose caps. Growth was measured daily for up to 5 days.

Large-volume growth of H. pylori: Strains of *H.pylori* were first cultured in 5 ml of MHB and TSB amended with 10% FCS and incubated horizontally at 37°C for 4 days in an atmosphere of 10% CO_2 The cultures were transferred to tissue culture flasks containing 20 ml of the same medium and were incubated for up to 4 days, at which time the volume was increased by the addition of 40 ml of fresh medium to the cultures and the cultures were reincubated. The cultures were subsequently transferred to 1-liter flasks containing 100 ml of fresh medium and were reincubated. After that, the volume of the cultures was further increased to 600 ml. After plating to test for purity, the urease reaction and microscopic observation were done at each stage of the process to test for growth. Fresh medium was always prewarmed to 37°C before use.

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Agitated system: In comparative experiments, flasks inoculated with *H.pylori* were incubated on a shaking platform at 100 rpm, either under microaerobic conditions, using anaerobic jars and CampyPak Plus (BBL), as described above, or in a 10% CO_2 atmosphere in a CO_2 incubator.

Enumeration of *H. pylori:* Viable counts expressed as CFU per milliliter were obtained from the experiments performed in triplicate on prewarmed 8% horse blood agar plates. For this, decimal dilutions were made (up to 10*') by using TSB as the diluent. A portion of 100 til/ml from each sample was added to each plate (three plates for each sample), and the plates were incubated in jars with CampyPak plus. Colonies were counted after 72 h of incubation at 37°C.

Acridine orange direct counts (AODCs) were carried out by epifluorescence microscopy after acridine orange staining and filtration through a 0.2-tim-pore-size filter (Millipore Corporation, Bedford, Mass.) by the method of Hobbie et al. (6).

For absorbance measurements, cultures were transferred to spectrophotometer cuvettes and the A_{450} was read by using an Ultraspec 2 instrument (LKB). The blank for each culture measurement was an uninoculated culture of the same medium, which was incubated under the same conditions.

Purity control was carried out at each stage of growth by examination of colony morphology; Gram staining; and oxidase, catalase, and urease testing.

RESULTS

The data that were obtained by using different liquid media, with and without supplementation, for growth of *H. pylori* are summarized in Table 1 for strain RSB6; results for this strain are representative of those obtained with an additional 10 strains of *H. pylori.* A comparison of microaerobic and CO_2-enriched incubation conditions, with humidity control, was done. Although moderate growth was obtained in TSB, BHlB, and MHB without supplementation under microaerobic conditions, media supplemented with 10% HS provided vigorous growth, even under COC-enriched conditions (Table 1). Therefore, FCS or HS was found to be essential for good growth in all of the liquid media examined in this study. Results of the experiments done with both filtered and autoclaved media indicated that the method of sterilization did not affect growth.

H. pylori cultures in liquid media develop clumping of up to 50 to 100 cells. CFU measurements, therefore, may not reflect the growth of colonies from single cells. CFU counts were compared with direct total counts by acridine orange staining and photometric measurement at A_{450}. Results of viable counts, AODCs, and absorbance measurements (Table 2) showed a good correlation for the methods used in this study; however, because of the clumps that formed, the CFU count may not be the result of counting single cells in liquid media, as indicated above.

BHIB plus 10% FCS plus 0. 1% YE was used to study the effect of the initial inoculum size of *H. pylori* CIP 101260, using 1 x 10^4, 5 x 10^4, and 1 x 10^5 cells per ml, for growth in a stationary liquid culture system (Fig. 1). Significant differences were observed between time 0 and 24 h. Bacterial cell concentrations were identical after 72 h. This observation was also not *H.pylori* strain dependent.

H.pylori, like other gram-negative bacteria, is known to exhibit a round body morphology under adverse microenvironmental conditions. We studied this phenomenon in the physiological experiments described above. The coccoid forms made up ca. 10% of the population (after inoculation from a plate) in the early log phase of growth and decreased to less than 2% during the log phase of growth. In the late stationary and starvation phases of growths coccoid cells became predominant. Having shown in previous studies (10, 11) that coccoid forms of *H.pylori* are a dormant, metabol-

TABLE 1. Growth of *H. pylori* RSB6 in liquid media with and without supplementation

Medium	Growth[a]:	
	Under CO_2	In jars
TSB		++
TSB + 0.I% (YE)		++
TSB + 1% hemin		
TSB + 0.l% YE + 1% hemin		
TSB + 10% HS	++++	+++
TSB + 10% HS + 0.1% YE	++++	++++
BB		
BB + 0.1% YE		
BB + 1% hemin		
BB + l0% HS	+++	+++
BB + 0.1% YE + 1% hemin		
BB + 10% HS + 0. 1% YE	+++	+++
CB		
CB + 0.1% YE		++
CB + 1% hemin		
CB + 0.1% YE+1% hemin		
CB + l0% HS	++++	+++
CB + 10% HS + 0.1% YE	++++	+++
MHB		++
MHB + 0.L% YE		++
MHB + 1% hemin		
MHB + 0.1% YE + 1% hemin		
MHB + 10% HS	++++	+++
MHB + 10% HS + 0.1% YE	++++	+++
BHIB	++	++
BHIB + 0.1% YE	++	++
BH1B + 1% hemin		
BHIB + 0.1% YE +1% hemin		
BH1B + l0% HS	++++	++
BHIB + l0% HS + 0.1% YE	++++	++
BYEα		
BYEα + 0.1% YE		
BYEα + l% hemin		
BYEα + 0.ho YE + 1% h		
BYEα + l0% HS	—	+
BYEα+ l0% HS + 0.l% YE	—	+

"Growth was assessed by measurement of the A_{450} daily for up to 5 days. Slight growth (+) corresponded to ca. less than 10' cells per ml. Moderate growth (+ +) corresponded to ca. 10' cells per ml. Good growth (+ + +) corresponded to ca. 10^9 cells per ml. Full growth (+ + + +) corresponds to ca. 10^{10} or more cells per ml. Hemin did not enhance growth. and when it was added to TSB, CB. M HB. and BHlB, growth was not detected.

TABLE 2. Comparison of viable count, AODC, and absorbance[a]

Time (days)	Optical density	AODC	CFU
0	0.005	7 x 10^6	5 x 10^6
1	0.010	6 x 10^7	2 x 10^7
	0.050	8 x 10^8	1 x 10^8
4	o.ioo	5 x io^9	i x io^9
5	0.500	1 x 10^{10}	8 x 10^9
	1.000	7 x 10^{10}	2 x 10'°

"Growth of *H. pylori* RSB6 was assessed in BHIB amended with 10% horse serum plus 0.1% yeast extract and was incubated at 37°C under CO_2.

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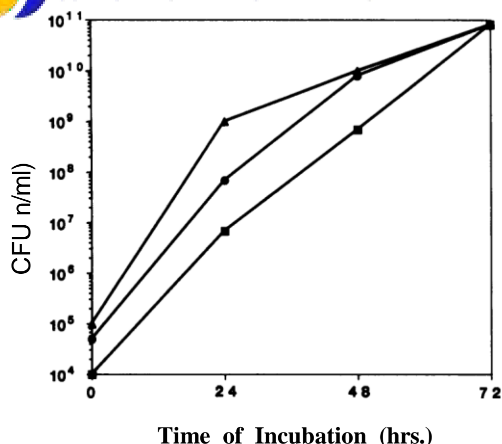

FIG. 1. Influence of initial inoculum size on growth rate of *H. pylori* CIP 101260 cultured in BHIB-10% FCC.1% YE. Growth was monitored in a stationary liquid culture system.

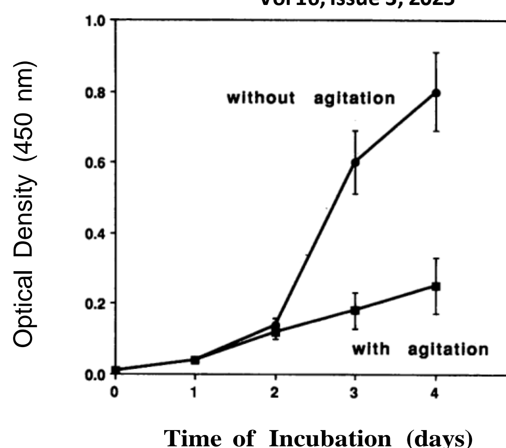

FIG. 3. Comparison between growth in stationary and shaken cultures of *H.pylori* CIP 101260 in BHIB-10% FCC-0.l% YE. Growth in the shaken culture was much less than that in the stationary system.

ically down-regulated, nondividing state of this microorganism, we attempted to optimize the liquid medium, with the intent to decrease the number of dormant (coccoid) cells in the initial inoculum by passaging the cells in liquid media and reinoculating the cells from the previous passage in the early log phase of growth. With the potential adaptation of *H. pylori* CIP 101260 to the medium, the decrease in the numbers of dormant *H.pylori* in the initial inoculum, from ca. 8% (first passage) to less than 1% (fourth passage), may account for the reduction in generation time (Fig. 2). Such preconditioned strains of *H. pylori* are suitable for large-volume growth of the microorganism in fermenters.

To compare the stationary growth system with conditions of growth of *H.pylori* in liquid media on a shaker, as described recently by Morgan et a1. (9), BHIB plus 10% FCS plus 0.1.% YE was used in parallel experiments. As shown in Fig. 3, the agitated system showed only slight growth after incubation for 2 days, in comparison with the stationary system.

DISCUSSION

On the basis of the findings obtained to date, it can be concluded that growth of ff. *pylori* in liquid medium requires FCS or HS, although some strains may grow moderately well in selected liquid media without the addition of serum. Furthermore, growth of *H.pylori* can be obtained in liquid medium in large volumes. An atmosphere of 10% CO_2 should eliminate the requirement for anaerobic jars, Campy-Pak Plus, or a shaking platform to culture *H.pylori.* The findings reported here can be useful in clinical, industrial, and research laboratories that require harvests of large quantities of *H.pylori* cells, which were previously obtainable only by harvesting cells from solid media.

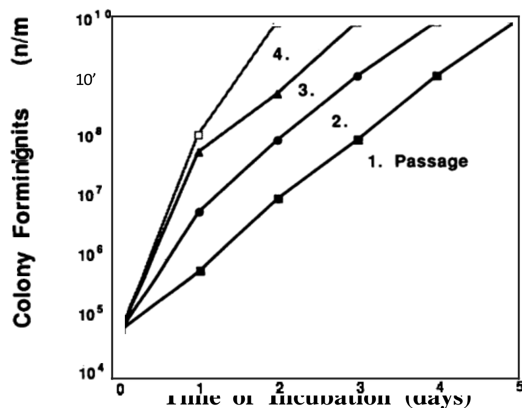

FIG. 2. Adaptation of *H.pylori* CIP 101260 to liquid medium (BHIB, 10% FCS, 0.1% YE). The generation time decreased with multiple passages in liquid medium.

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