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Factors Affecting Growth and Susceptibility Testing of *Helicobacter pylori* in Liquid Media

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In order to increase the database for in vitro growth and/or susceptibility testing in liquid media, we evaluated the growth of *Helicobacter pylori* in broth media containing 5% sheep blood. We also compared the effect of bismuth on the growth of *H. pylori* in broth media containing 10% fetal calf serum with the effect on growth in media containing 0.5% starch. In contrast to the result seen with agar, we found that sheep blood, whether whole or laked, inhibited the growth of *H. pylori* in broth media. In addition, we found that bismuth inhibited growth in media with starch but that this inhibition was negated in media with serum.

Helicobacter pylori is associated with gastroduodenal disorders because of its frequent isolation from the gastric and duodenal mucosae of patients with gastritis and peptic and duodenal ulcers (1, 2, 7). During the past 10 years, this organism has been the focus of many in vitro studies evaluating growth requirements as well as susceptibilities to various agents. Most of these studies have been conducted with agar media. In order to increase the database for liquid media, we compared the growth of *H. pylori* in broth supplemented with starch alone with that in broth containing starch plus laked or whole sheep blood. We also compared growth in broth supplemented with fetal calf serum (FCS) with growth in broth containing starch. In addition, we evaluated the inhibitory effects of bismuth and ampicillin on the growth of *H. pylori* in broth containing FCS versus broth containing starch.

In all studies, the inocula were prepared by using methodology developed for time-kill kinetic studies (5). Briefly, cation-adjusted Mueller-Hinton broth (CAMHB) (18 ml; Difco Laboratories, Detroit, Mich.) and FCS (2 ml; Sigma Chemical Co., St. Louis, Mo.) were aseptically added to 50-ml sterile bottles (Becton Dickinson and Co., Sparks, Md.). Sterile rubber stoppers (The West Co., Phoenixville, Pa.) were inserted, and aluminum seals were hand crimped tightly to prevent air leakage. The media were inoculated with a tuberculin syringe, and the bottles were flushed with a mixture of three gases (10% CO_2, 5% O_2, and 85% N_2) and shaken continuously at 150 rpm during incubation at 37°C. In order to assure logarithmic-phase growth, cells were initially grown for 24 h and then passed and grown for an additional 16 h. On the second day, most of the cells used for the inocula showed a typical curved morphology when viewed by phase microscopy and many were motile.

We first compared the growth of *H. pylori* in broth medium supplemented with starch alone with that in broth medium supplemented with starch and sheep blood. Bottles were prepared as described for the inoculum preparation but contained CAMHB with either 0.5% starch or starch and 5% whole sheep blood (Editek Inc., Burlington, N.C.) or 5% laked sheep blood. Inoculation and other procedures were as described above.

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Table 1 summarizes the effects of whole and laked sheep blood on the growth of the American Type Culture Collection (ATCC) type strain and four clinical isolates of *H. pylori*. All strains showed growth in medium supplemented with starch alone. However, either whole or laked sheep blood clearly inhibited growth of all strains tested. It should be noted that *H. pylori* fails to grow in unsupplemented basal media, such as CAMHB (personal observation and reference 3).

We next evaluated the inhibitory effects of bismuth and ampicillin on the growth of the ATCC 43504 strain and one clinical isolate of *H. pylori* in broth media (CAMHB) containing one of two different supplements, 10% FCS and 0.5% starch. In order to evaluate the potential role of bismuth cation, a second set of bottles was also supplemented with 1.25 mg of fetuin (Sigma) per ml, a negatively charged protein which chelates cations. The media were inoculated and bacterial growth was established as described for the inocula. At 0, 3, 7, and 24 h, 20-μl samples were removed and serially diluted for colony counts (5). By a surface colony count method, samples were dropped in duplicate onto medium supporting optimal growth, i.e., campylobacter base agar supplemented with whole sheep blood, horse serum, cholesterol, and cations (4). These cultures were incubated under microaerobic conditions at 37°C for 4 to 5 days, and then results were read with a magnifying lens. The lowest level of cell detection was 1,500 CFU/ml.

The results shown in Table 2 indicate that both strains grew in the broth medium containing starch, although neither strain grew as well as it did in the medium with FCS. More importantly, however, at 24 h bismuth inhibited the growth of *H. pylori* in medium containing starch, but little or no inhibition occurred in medium containing serum (the curve for the ATCC strain is shown in Fig. 1). The inhibitory effect observed with bismuth was virtually negated when fetuin was added to the starch medium. In the absence of bismuth, fetuin did not significantly affect the growth of *H. pylori*, nor was any difference in growth observed between bottles containing ampicillin and FCS and bottles containing ampicillin and starch (data not shown).

The results of this study are important because few reports have described the growth of *H. pylori* in liquid media. Our finding that sheep blood, laked or whole, inhibited growth does not support an earlier report that [in general] blood will enhance the growth of *H. pylori* in liquid media (6). The source (species of animal) of the blood may be critical, as preliminary

TABLE 1. Effect of starch alone versus starch plus whole or laked sheep blood on growth of *H. pylori* in broth

Strain or isolate no.	Time (h)	Change in $\log_{10}$ CFU/ml after incubation with following supplement(s) relative to that at time zero[a]		
		STA	STA + WSB	STA + LSB
ATCC 43504	3	+0.4	>−2.8	+0.8
	7	+0.6	>−2.8	>−2.8
	24	+1.3	−2.6	−2.6
1	3	+0.1	>−3.0	>−3.0
	7	+0.4	>−3.0	>−3.0
	24	+2.0	>−3.0	>−3.0
2	3	+0.2	−2.2	−1.9
	7	+0.5	>−2.9	>−2.9
	24	+0.9	>−2.9	−2.6
3	3	0	−1.7	−1.8
	7	+0.3	>−2.9	>−2.9
	24	+0.5	>−2.9	>−2.9
4	3	+0.3	>−3.5	>−3.5
	7	+0.3	>−3.5	>−3.5
	24	+1.7	>−3.5	>−3.5

[a] Abbreviations: STA, 0.5% starch; WSB, 5% whole sheep blood; LSB, 5% laked whole sheep blood.

data obtained in our laboratory suggest that laked horse blood does, in fact, support the growth of *H. pylori* in liquid media. At 24 h, CAMHB plus 5% laked horse blood (with or without starch) yielded optimal growth of more than 10^8 CFU/ml for each of three strains tested (the ATCC strain and two clinical isolates).

The inhibition of *H. pylori* growth in broth by sheep blood differs markedly from what is observed with agar. Agar supplemented with 5 or 10% whole sheep blood is able to support good growth of *H. pylori* (2a, 4, 8). In our studies, the defibrinated whole sheep blood samples that were added to either broth or agar (4) were obtained from the same manufacturer and prepared in the same manner. Buck and Smith (3) reported that although hemin per se was not required for growth, *H. pylori* did appear to require constituents beyond the usual

TABLE 2. Effect of starch versus FCS on antimicrobial activity of bismuth on *H. pylori* in broth medium

Strain or isolate no.	Supplement[a]	Time (h)	Change in $\log_{10}$ CFU/ml after incubation with indicated component(s) relative to that at time zero			
			None (control)	Fetuin[b]	Bismuth[c]	Bismuth + fetuin[b,c]
ATCC 43504	FCS	3	+0.2	+0.2	+0.1	+0.2
		7	+0.9	+0.7	+0.6	+0.6
		24	+1.9	+2.1	+0.2	+0.5
	STA	3	+0.2	+0.2	+0.2	+0.2
		7	+0.9	+0.7	+0.3	+0.4
		24	+1.7	+1.9	>−3.1	+0.6
2	FCS	3	+0.3	+0.3	+0.5	+0.4
		7	+0.8	+0.8	+0.5	+0.4
		24	+2.1	+2.2	+0.6	+0.9
	STA	3	+0.3	+0.3	+0.4	+0.4
		7	+0.6	+0.6	+0.4	+0.4
		24	+1.2	+1.7	>−3.1	+0.4

[a] STA, starch (0.5%). FCS was at 10%.
[b] Fetuin was used at a concentration of 1.25 mg/ml.
[c] Bismuth was used at concentrations of 32 μg/ml for the ATCC strain and 64 μg/ml for isolate 2.

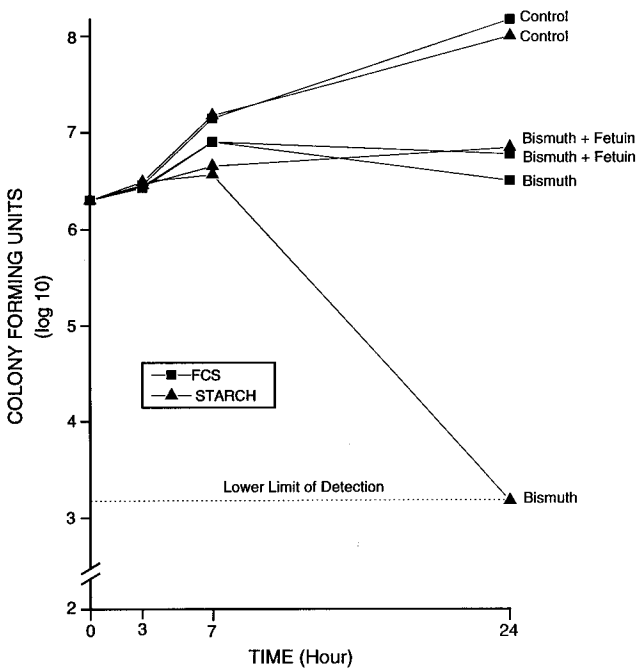

FIG. 1. Effect of broth components, FCS (10%), starch (0.5%), and fetuin (1.25 mg/ml) on the bactericidal activity of bismuth (32 μg/ml).

medium components. They further suggested that the role of certain supplements may actually be to absorb or inactivate toxic factors. Our data are consistent with this suggestion that agar serves as a means of detoxification of growth-related toxins.

A few early reports have indicated that serum also supports the growth of *H. pylori* in liquid media. Morgan et al. (9) reported that optimal growth was achieved with overnight incubation in brucella broth supplemented with 1 to 10% FCS. Buck and Smith (3) studied the effects of different supplements on the growth of *H. pylori* and found that after 3 days of incubation, broth supplemented with 5% horse serum supported slightly more growth of *H. pylori* than did media containing starch. Growth varied with each isolate (two). We had similar results with broth media.

Moreover, our results underscore the need for precautions for in vitro susceptibility testing of *H. pylori*. Although supplementation with FCS per se supports excellent growth (Table 2), FCS also decreases the inhibitory effect of bismuth on *H. pylori*. The mechanism for this inhibition is unknown, but a simple explanation would be that bismuth, a cation, is bound to an anion protein constituent such as fetuin. In our study, the addition alone to starch-containing media of fetuin, an anionic sialoprotein purified from FCS, also negated the inhibitory effect of bismuth.

In summary, either laked or whole sheep blood inhibits the growth of *H. pylori* when added to broth media and therefore should not be used. We also found that while FCS enhances growth, it negates the inhibitory effect of bismuth on growth of *H. pylori* in broth media.

These observations are important because future studies may require in vitro testing of *H. pylori* in broth media. For example, because of stability properties of omeprazole, it is recommended that in vitro susceptibility testing of this agent be performed in liquid media (7a). The use of liquid media for growth of *H. pylori* requires that limitations such as those described be identified.

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