Creation of a New Synthetic Medium for Culturing *Helicobacter Pylori*

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We developed a scheme of consecutive replacement of complex components of a known *Brucella* medium containing peptones and blood with simple analogs and created a synthetic medium for *Helicobacter pylori* culturing. *H. pylori* cells require hemic iron for their growth; an appreciable increment in biomass was ensured by hemoglobin, but not simpler hemocontaining compounds (hemin and cytochrome C). Glutamine (20 g/liter) was used as the main nitrogen-containing component, and other amino acids were added in trace amounts. Adhesion was provided by adding agarose gel (0.1%) also promoting the increase in biomass. The proposed medium of a certain chemical composition differs from the known foreign analogs by the presence of hemocontaining component (hemoglobin), short period of exponential growth, and appreciable accumulation of cell protein.

Key Words: *Helicobacter pylori; synthetic media; culturing*

Ample data obtained since the discovery of *H. pylori* inhabiting the gastroduodenal mucosa confirm their opportunistic nature. The detection of these bacteria in the majority of normal subjects [1] suggests a certain ecological function of these bacteria, but the diagnosis of helicobacteriasis remains an important problem.

Nutrient media and culturing processes serve the basis for the creation of diagnostic test systems.

Creation of media containing no high-molecular-weight compounds of unknown composition will make it possible to obtain extracellular antigenic proteins requiring no special purification. The proposed synthetic media for culturing of *H. pylori* [5,10,11] contain crude serum albumin and do not ensure an appreciable increment in the biomass.

The aim of our study was to create synthetic media for *H. pylori* culturing ensuring rapid growth and accumulation of biomass.

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MATERIALS AND METHODS

The study was carried out on *H. pylori* strains from the collection of I. I. Metchnikov Institute of Vaccines and Sera. The bacteria were inoculated in solid Columbia agar base medium (ICN) with 5% human donor blood and antibiotic solution [2]. After inoculation the dishes were put into an anaerostate with a gaseous mixture consisting of 10% carbon monoxide, 6% oxygen, and 84% nitrogen and incubated for 2-7 days at 37°C. The resultant cultures were identified by the type of colonies, Gram staining, cell mobility and morphology in an unstained smear, and by qualitative reactions for urease, catalase, and oxidase activities [3]. Inoculation material for culturing in liquid media was prepared as follows: the colonies were washed, suspended in sterile saline (1:20), the cells were precipitated by 20-min centrifugation at 5000 rpm and the precipitate was resuspended in sterile saline (1:2).

Culturing in liquid media was carried out in 100 and 500 ml flasks containing 10-15% media. Saline base (except phosphates) and some other organic components were put into the flasks in solutions, after

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which the flask was bubbled with the above-mentioned gaseous mixture. The flasks were then closed with silicone stoppers and aluminium screw caps, sterilized for 30 min at a pressure of 0.5 atm. Sterile vitamin and antibiotic solutions, blood, hemocontaining additives, phosphates, and inoculation material were added with syringes after sterilization. The flasks were incubated at 37°C in a thermostat in a stationary mode. The minimum oxygen level was attained by pricking (after 24 h) into the silicone stopper a syringe needle (0.6 mm in diameter) with a sterile cotton tampon, after which culturing was continued.

Concentration of the biomass was evaluated by optical density of cultures on a KF-77 photoelectrocolorimeter (Zalimp) at λ=535 nm in cuvettes with 10-mm optical distance.

Protein content was evaluated by the method of Lowry. Hemoglobin solutions were prepared by suspending in distilled water (1:6), 15-min centrifugation at 5000 rpm, and filtration of the supernatant through a bacterial filter with 0.22-μ pores. Electrophoretic purity was evaluated as described previously [7] in 12.5% polyacrylamide gel in the presence of sodium dodecylsulfate. Hemoglobin concentration in the medium and culture fluid was estimated by the calibration curve plotted using hemoglobin solution in water (abscissa: hemoglobin protein content; ordinate: absorption at 550 nm on an SF-26 spectrophotometer, LOMO). Low-molecular-weight hemoglobin fraction was isolated on a 2.5×40 cm column packed with Sephadex G-100 gel (Serva) using potassium phosphate (0.05 M) eluting buffer (pH 7.0).

Amino acid analysis was carried out on a 2021 Maxicoldlab analyzer (LKB) at Institute of Biophysical Medicine (Pushchino-on-Oka). Pearson linear correlation coefficient and other characteristics were evaluated using Microsoft Excel software.

The following additives were used: bovine serum (Biomed Firm), BSA (Reanal), aminokrovin — nonhemic erythrocyte content hydrolysate — and human donor blood (Moscow Center for Blood Transfusion), hemin chloride (ICN), cytochrome C (ICN), bovine hemoglobin (ICN; No. 151235), equine hemoglobin (Reanal), glutamine (Reanal; chemically pure), agarose (Serva; research grade). Constant components of the media were amino acid solutions (ICN; Nos. 1601149 and 1681049), 1 ml/liter; vitamin solution (ICN; No. 1601449), 1 ml/liter; trace element solution according to the traditional Pfenning recipe, 2 ml/liter; salts (g/liter): NaCl (5), $(NH_4)_2SO_4$ (0.5), $MgSO_4 \times 7H_2O$ (0.5), $CaCl_2 \times 2H_2O$ (0.4), K_2HPO_4 (4), KH_2PO_4 (1); solution of antibiotics (mg/liter): vancomycin (10.0), trimetoprim lactate (5.0), and polymyxin B (2.5).

RESULTS

Composition of multicomponent media and methods of *H. pylori* culturing were described previously [6, 12]. Complex nutrient media were used in these studies: cardiocerebral extract, *Brucella broth*, Muller—Hinton broth, and soybean broth with blood or serum. Complete *Brucella broth* served the base for our medium; it contained peptones (meat peptone and casein peptone) as sources of carbon and nitrogen and blood

TABLE 1. Blood Components, Nitrogen-Containing Components, and Solid Phase Used for *H. pylori* Culturing in Liquid Medium

Medium; blood and its components		Source of amine nitrogen	Presence of solid phase	Maximum increment of biomass, OD 535
Complete	blood 1%***	Casamino acids*	—	0.660
	serum 1%	Casamino acids	—	0.015
	aminokrovin 1%	Casamino acids	—	0.005
Semisynthetic	hemin***	Casamino acids	—	0.015
	hemin	Casamino acids	+	0.032
	cytochrome C***	Casamino acids	—	0.030
	cytochrome C	Casamino acids	+	0.100
	blood 1%	Glutamine **	—	0.125
	blood 1%	Glutamine	+	0.260
Synthetic	hemoglobin***	Glutamine	—	0.290
	hemoglobin	Glutamine	+	0.660
	cytochrome C	Glutamine	+	0

Note. *Casamino acid concentration 40 g/liter; **glutamine concentration 20 g/liter; ***concentration of all hemic components was equalized by the content of hemic iron by measuring optical density of solution at λ=550 nm. Here and in Table 2: OD: optical density.

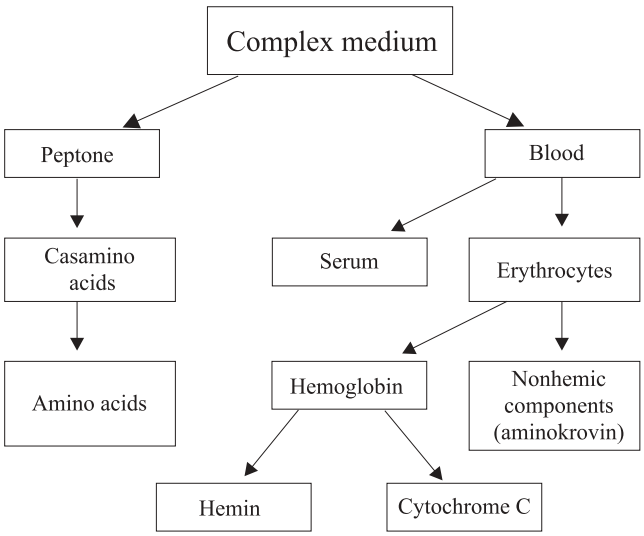

Fig. 1. Algorithm for creation of synthetic medium for *H. pylori* on the basis of complete medium.

as the source of organic iron. An algorithm protocol (Fig. 1) was used for logical simplification of this medium in order to create the synthetic medium.

Two cultures most actively growing in solid medium were selected from the collection of *H. pylori* strains. These cultures (Nos. 2 and 10) were cultured for 5 days in 2 modified media; one of them contained only meat peptone (2.3%) as the source of carbon and the other only casamino acids (2.3%); blood content was 5%. The medium with casamino acids (with higher content of free amino acids) proved to be preferable; culture No. 2 grew better in both substrates than culture No. 10. Hence, casamino acids were selected as the main source of carbon and energy and culture No. 2 was selected of the two cultures.

Usually 5-7% blood is added to the medium for *H. pylori* culturing. The study of blood concentrations from 0.25 to 5% in the presence of 40 g/liter casamino acids revealed an appreciable increment in the biomass in the presence of 1-5% blood. Bacterial growth was notably limited in the presence of blood concentrations below 1%, and hence, this concentration was used in further experiments.

For further simplification of the medium composition it was important to determine the most essential components of the blood and casamino acids for *H. pylori* growth. The main amine and hemic components of media developed during creation of synthetic medium are presented in Table 1. Successive replacement of the blood with simpler analogs in the presence of casamino acids showed that *H. pylori* growth depended on the content of hemic iron in the medium. The serum and aminokrovin contain hemic component of the blood in trace concentrations, and the growth in this case was the minimum. Simple hemocontaining compounds, such as hemin and cytochrome C, also did not ensure appreciable growth of the culture. It was obvious that the blood in the future synthetic medium should be replaced with a more complex analog than cytochrome C or hemin, for example, with hemoglobin.

In order to replace casamino acids with simpler components, *H. pylori* were cultured in a medium containing 40 g/liter casamino acids and 1% blood. Amino acid analysis of the initial medium and culture fluid collected at the end of the active growth phase showed that 27% of total content of amino acids in the initial and final medium was glutamine, while the consumption of the most abundant amino acids varied from 49.2 to 59.1% during growth (Fig. 2).

Hence, only glutamine in *a priori* excessive concentration (20 g/liter) was used in semisynthetic and synthetic media (Table 1), while other amino acids were added in trace amounts as ready solutions.

Accumulation of biomass was appreciably slower during growth in a medium with blood and glutamine (Table 1) in comparison with complete medium, but addition of 0.1% agarose gel as a carrier for cell adhesion promoted the increase in the culture density (with consideration for optical density of agarose).

The use of cytochrome C instead of blood for culturing in a medium with glutamine did not lead to *H. pylori* growth, while replacement of blood with hemoglobin in this medium led to an expected result: a synthetic medium ensuring an increment in *H. pylori* biomass comparable to that in complete medium was

TABLE 2. Relationship between Hemoglobin Concentration in Medium and Accumulation of *H. pylori* after 48 h of Culturing

Initial concentration of hemoglobin in medium, mg/liter	Utilized hemoglobin, mg/liter	Increment of biomass		Yield, mg biomass protein/mg utilized hemoglobin
		by OD at 535 nm	by protein, mg/liter	
696	393	0.45	420	1.07
1392	755	0.66	616	0.82
2784	1631	1.14	1064	0.65
5567	3503	1.34	1251	0.36

TABLE 3. Principal Components and Some Growth Parameters of the Proposed Medium and Known Synthetic Media

Components of media (mg/liter) and culturing parameters		Synthetic medium for *H. pylori*			
		Nedenskov P. (1994)	Reynolds D. J., Penn C. W. (1994)	Testerman T. L. *et al.* (2001)	Proposed medium
Major carbon-containing components					
	sum of amino acids (except glutamine)	1815	2852	453	209
	glutamine	150	374	161	20000
	BSA*	0	5000	2500	0
	hemoglobin	0	0	0	700-5600
	glucose	0	2000	1802	0
Carriers	β-cyclodextrin	0	0	200	0
	agarose	0	0	0	1000
Culturing parameters					
	inoculation dose, CFU/ml	1.6×10^6	1×10^7	1×10^6	1×10^7
	period of exponential growth, h	120	22	24	15
	maximum cell yield, CFU/ml	1.2×10^7	6×10^8	6×10^7	5×10^9-3×10^{10}

Note. *BSA without special purification.

created. Addition of agarose gel further increased the yield of the biomass. The use of bovine and equine hemoglobins led to an appreciable acceleration of the exponential phase and increased the yield of biomass in comparison with semisynthetic medium (Fig. 3). This fact can be explained by the presence of specific antibodies to *H. pylori* in the donor blood, because carriership of these bacteria is sometimes observed in normal subjects. Of the two studied animal hemoglobins, equine was preferable. Comparative electrophoresis of these hemoglobins under denaturing and non-denaturing conditions showed that equine hemoglobin consisted mainly of dimers (molecular weight about 20 kDa), while bovine hemoglobin contained, along with dimers, native tetramer protein (molecular weight 64.5 kDa).

This medium with equine hemoglobin ensured accumulation of biomass of 5 collection *H. pylori* strains (Nos. 1, 4, 10, 31, 41) at the level of 97-128% of that for strain No. 2.

The effect of equine hemoglobin concentration on *H. pylori* growth parameters was studied on strain No. 2; the initial concentrations of the rest components corresponded to the main recipe. Consumption of hemoglobin was estimated as the difference between the initial and remaining hemoglobin. The increment of *H. pylori* biomass increased with increasing hemoglobin content in the medium (Table 2). This relationship

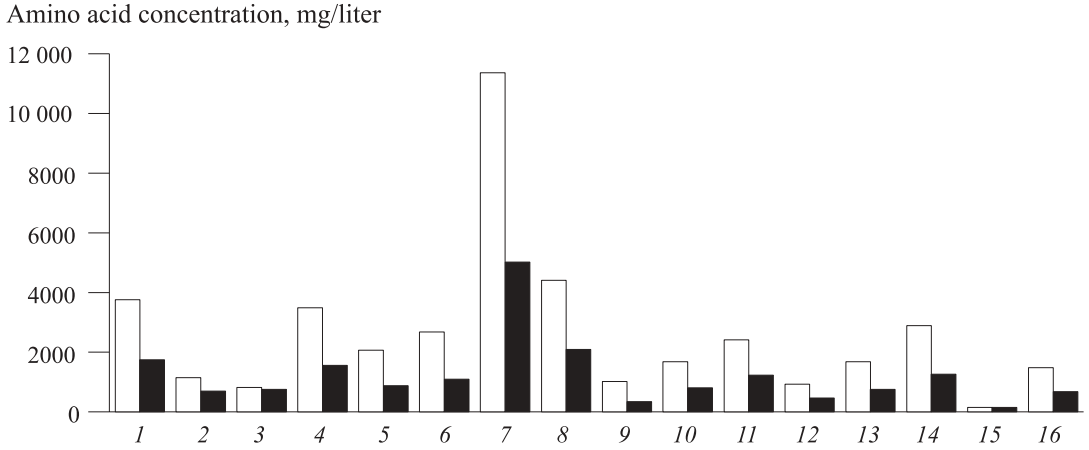

Fig. 2. Consumption of amino acids during *H. pylori* growth in medium with casamino acids (40 g/liter) and blood (1%). Light bars: amino acid content in initial medium; dark bars: amino acids in extracellular lfluid after 7-day culturing. *1*) Lys; *2*) His; *3*) Arg; *4*) Asp; *5*) Tre; *6*) Ser; *7*) Glu; *8*) Pro; *9*) Gly; *10*) Ala; *11*) Val; *12*) Met; *13*) Ile; *14*) Leu; *15*) Tir; *16*) Phe.

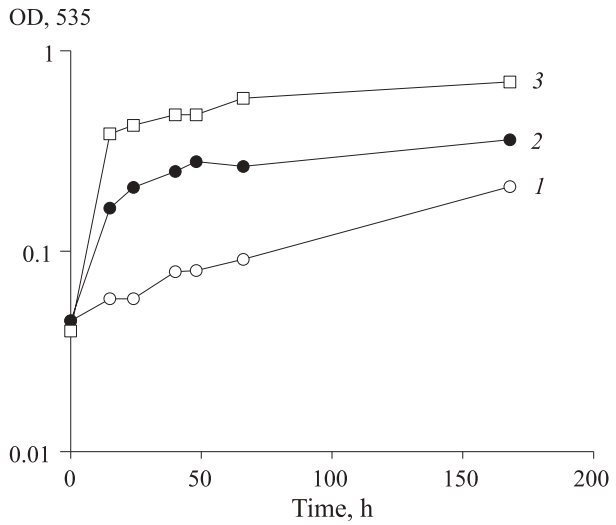

Fig. 3. *H. pylori* growth in semisynthetic medium with blood and in synthetic media with hemoglobin. *1*) blood; *2*) bovine hemoglobin; *3*) equine hemoglobin.

between cell population density and concentration of this or that substrate indicated that this substrate limited the growth [4]. This method for evaluating hemoglobin content (by absorption of cell-free supernatant at 550 nm) indicates that *H. pylori* consumed hemocontaining protein during growth in the "substrate", but not trace amounts. Simple estimations shows that *H. pylori* utilized 1.3-3.5 μg hemic iron (depending on hemoglobin content in the medium) per mg biomass. It is quite logical, because organic iron can be stored in *H. pylori* as an intracellular ferritin pool and utilized for cytochrome synthesis [3].

We compared original synthetic media [11-13] with our new medium by the principal components and some growth parameters (Table 3). The use of glucose is ineffective, but addition of carriers simulating the mucosa of natural bacterial habitat had a positive impact on the growth parameters. The use of electrophoretically pure BSA (molecular weight 67 kDa) did not stimulate the growth of *H. pylori* cells [11]. The growth was stimulated by adding high-molecular-weight components of the serum and BSA (more than 100 kDa). Despite all additives, the authors failed to achieve accumulation of the cell biomass beyond 1 opt. dens. unit. Addition of hemoglobin in a concentration of 14 mg/liter into the medium [10] did not stimulate *H. pylori* growth. Hemoglobin was purified by gel filtration in our experiments and a homogeneous low molecular-weight fraction (20 kDa) was isolated. After addition of this substance into synthetic medium in a concentration of 325 mg/liter (by hemic protein) accumulation of biomass by strain No. 2, evaluated by optical density, was 0.15±0.03.

Our approach to creation of a synthetic medium based on simplification of the known complete nutrient media showed that *H. pylori* requires hemic and protein components of hemoglobin and enabled us to create a medium without high-molecular-weight serum proteins of unknown composition providing better increment in the biomass.

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