Usefulness of Adult Bovine Serum for *Helicobacter pylori* Culture Media

Keigo Shibayama,1* Mitsuaki Nagasawa,2 Takafumi Ando,3 Masaaki Minami,3 Jun-ichi Wachino,1 Satowa Suzuki,1 and Yoshichika Arakawa1

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo, Japan1

Department of Laboratory Medicine, National Defense Medical College Hospital, Tokorozawa, Japan2

Department of Gastroenterology, Nagoya University Graduate School of Medicine, Nagoya, Japan3

*Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1, Gakuen, Musashimurayama, Tokyo 208 0011, Japan. Phone: +81-42-561-0771. FAX +81-42-561-7173

E-mail: keigo@nih.go.jp

Running title

Evaluation of different serum for *H. pylori* culture
Abstract

Fetal bovine serum (FBS) and adult bovine serum (BS) exhibited bactericidal activity against *Helicobacter pylori* at various levels, which were higher in BS than in FBS. The bactericidal activity was inactivated by heat-treatment at 56°C for 30 min. Our results demonstrated that heat-treated BS is a useful serum source for *H. pylori* culture medium.
*Helicobacter pylori* is usually grown on media that contain blood or serum. Horse blood is most commonly used for agar plates because of its high growth-supporting ability. For liquid culture fetal bovine serum (FBS) is used in most studies. In vitro growth is largely affected by culture media, and the serum used is one of the most influencing factors. Difference of growth-supporting ability of media would be of deep concern in both clinical and research settings, in which stable bacterial growth is required. In addition, because of economical reason, less expensive serum that gives optimum growth is desired. In this study we examined the growth-supporting property of different serum lots. We show that adult bovine serum (BS), which is far less expensive than FBS, could be a useful serum source for *H. pylori* culture media after heat-treatment.

The growth-supporting property of 3 FBS lots and 2 BS lots was compared with *H. pylori* 26695 (American Type Culture Collection, CRL-700392). The serum-containing liquid media and agar media were made with 10% serum in Brain Heart Infusion (BHI)(DIFCO) and Brain Heart Infusion agar (BHI agar)(DIFCO), respectively. Heat-treatment of serum was performed by incubating serum at 56°C for 30 min. *H. pylori* was grown in an incubator (Model 7000, NAPCO), in which atmosphere was controlled at 5% O₂, 12% CO₂, 95% humidity, and temperature 37°C.

For examination with liquid media, *H. pylori* was inoculated to each medium at concentration of 6.5 x 10⁶ CFU/ml in 10 ml of medium in 9cm dish and grown with rotary shaking at 60 r.p.m. The growth was monitored by measuring optical density (O.D.) at wavelength of 600 nm and optical path length of 10 mm with a
spectrophotometer (Model U-3010, HITACHI, Japan). Measurement of bacterial growth by O.D. should be applicable when bacterial cells are dispersed homogenously in culture. *H. pylori* strains often form aggregation in liquid culture, which impairs reproducible quantification of bacterial cells. In this experiment all strains used grew homogenously in liquid culture. When untreated serum was used (indicated by dotted line in Fig. 1), there was obvious growth inhibition at various levels. While two FBS lots (F1 and F2) yielded growth, no growth was obtained with one FBS lot (F3) and two BS lots (A1 and A2). When heat-treated serum was used (indicated by solid line in Fig. 1), efficient growth was observed with all lots examined. With heat-treated serum F3, doubling time of the bacteria was estimated to be approximately 3.1 h. These results indicate that each untreated serum contains different level of growth-inhibiting activity which is inactivated by heat treatment. With serum F1, the growth by untreated serum was slightly delayed when compared with that by heat-treated serum, but the gradient of the plot was almost the same, suggesting that the inhibition of the growth is due to reduction of live bacteria in the initial stage rather than suppression of the multiplication process during bacterial growth. Complement is a major bactericidal substance contained in serum, and complement-mediated bactericidal activity against *H. pylori* has been reported (6, 8). The characteristics of the bactericidal activity observed in this experiment agree with the general property of complement. In media with untreated serum F3, A1, and A2 all inoculated cells would have been inactivated, resulting in complete inhibition of growth. Since initial bacterial load was 6.5x10^6 CFU/ml in 10 ml of medium with 10% serum, actual bacterial killing was estimated to be more than
6.5x10⁷ CFU bacteria per ml of serum. The bactericidal effect was further confirmed with 2 additional strains, *H. pylori* NCTC11637 (ATCC 43504) and *H. pylori* 60190 (ATCC 49503). Untreated and heat-treated serum A2 was tested with these strains in the same manner. While no growth was obtained by untreated serum, efficient growth was obtained with heat-treated serum, consistent with the results of *H. pylori* 26695. These results indicate that even highly bactericidal serum lots yield efficient bacterial growth for *H. pylori* if heat treatment is performed in advance. Bacterial cultures of *H. pylori* 26695, NCTC11637, and 60190, with O.D. of 1.0, contained 2.3x10⁸, 1.1x10⁸, and 0.9x10⁸ CFU/ml of bacterial cells, respectively, suggesting that the growth of these strains was at almost similar levels in the liquid media examined.

Growth-supporting property of different serum lots was further examined with agar medium. BHI agar medium containing either FBS F3, heat-treated FBS F3, BS A1, or heat-treated BS A1, and a commercial selective agar medium NISSUI Plate Helicobacter (Nissui, Japan) were tested for *H. pylori* growth. Horse blood agar medium was used as a reference. The bacterial suspension of which O.D. was 1.0 was diluted 10⁴-fold with phosphate buffered saline, and 25 µl of the diluted suspension was spread on each agar medium. Number of colonies on each plate was counted after 4 days. The result is shown in Fig. 2. The growth-inhibitory effect was evident with BS but not with FBS. While both untreated and heat-treated FBS efficiently yielded colonies, the number of colonies obtained with untreated BS was significantly lower compared with horse blood agar plate (132.3±7.6 versus 574.0±116.3; *P*<0.01). When heat-treated BS was used, the number of colonies became approximately 10-fold higher compared with
untreated BS (944.7±123.6 versus 132.3±7.6; P<0.01). The difference in the number of colonies between untreated and heat-treated BS A1 media was approximately 8 x 10^2, as estimated from the data shown in Fig. 2. Since the agar plate was made with 2 ml of serum in 20 ml of medium, actual killing of bacteria is estimated to be approximately 4 x 10^3 CFU per ml of serum, which is considerably lower compared with that observed by liquid culture. It is suggested that while complement is able to bind efficiently to bacterial cells in liquid medium, agar medium might be less feasible for complement molecules to migrate and reach bacterial cells which exist on the surface of agar, thus resulting in reduction of actual bactericidal activity. The activity of complement contained in serum F3 might be insufficient level to exert bactericidal activity with agar medium. Our results indicate that heat-treated BS, which is usually unpopular as a serum source for *H. pylori* culture media, is a useful serum source for *H. pylori* culture. Colonies on NISSUI Plate Helicobacter were fewer compared with horse agar plate (58.7±48.9 versus 574.0±116.3; P<0.01). NISSUI Plate Helicobacter plates were kept sealed at 4ºC after purchase until use, however, the surface of the medium seemed somewhat dry when the bacterial suspension was inoculated. This might lead to the formation of the fewer colonies in comparison with other agar media which were prepared and used freshly, since *H. pylori* requires high humidity for growth.

Usefulness of heat-treated BS was further examined with clinical specimens. Selective BHI agar (S-BHI agar) medium was made with 10% heat-treated BS and following concentration of antibiotics, 10 µg/ml of vancomycin, 5 µg/ml of trimethoprim, 5 µg/ml of amphotericin B, and 2.5 units/ml of polymyxin B. S-BHI agar
medium was tested for *H. pylori* recovery from biopsy specimens in two clinical laboratories. Either of Horse blood agar plate or NISSUI Plate Helicobacter was used as the reference medium. Microaerophilic condition was generated in a special jar with a gas-generating kit AnaeroPouch Helico (Mitsubishi-Gas Chemical, Japan), and conventional incubator was used. Inoculation was done by smearing both S-BHI agar plate and reference agar plate with single biopsy specimen. Urease test was performed for all isolates for identification. The addition of antibiotics in the medium did not cause significant reduction of colony formation when examined with *H. pylori* 26695 (data not shown). When horse blood agar plate was used as the reference medium, the results completely matched. S-BHI agar medium was able to detect *H. pylori* from all positive specimens (9 of 9 specimens), but from none of negative specimens (0 of 5 specimens).

When selective medium NISSUI Plate Helicobacter was used as a reference medium, *H. pylori* growth was observed from 35 of 39 positive specimens, no growth was obtained from 4 positive specimens by S-BHI agar medium. From 3 of the 4 specimens only a few colonies grew on NISSUI Plate Helicobacter, suggesting that the false negative result was ascribed to a very small number of bacteria contained in biopsy specimens rather than aptness of the medium. From one specimen hundreds of colonies grew on NISSUI Plate Helicobacter. In consideration of other observations which show the efficient growth-supporting ability of heat-treated BS, this false negative result might be ascribed to a technical error which could have taken place through the procedure, such as inappropriate medium preservation, gas leak, or insufficient humidity.

There was no negative specimen by NISSUI Plate Helicobacter at this experiment.
Taken together, the results indicate that heat-treated BS is useful for primary culture from clinical specimens as well.

To date a number of media containing serum, blood, blood derivatives, or egg yolk have been developed for *H. pylori* culture (2, 5, 12). Comparison studies have accordingly been done on various agar media for primary isolation using biopsy specimens (3, 4, 7, 9-11). For liquid culture it was reported that a lysed human erythrocytes-supplemented medium supported a remarkably rapid growth with a doubling time of 50 min. (1).

For practical use, materials to be used should generally be available and medium should be feasible for preparation. From view of these points, heat-treated BS is a favorable material. In this study we showed that heat-treated BS is a useful serum source for both liquid and agar media for *H. pylori* culture. BS is usually far less expensive than FBS. In addition, BS can be stably preserved for a long time, in contrast to defibrinated blood which should be consumed in a short time. Heat-treated BS would thus be a cost-effective choice as a serum source for *H. pylori* culture medium.

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REFERENCES


Figure legends

FIG. 1. Growth curve of *H. pylori* 26695 in BHI medium supplemented with FBS F1, FBS F2, FBS F3, BS A1, and BS A2. Results are representative of three independent experiments.

FIG. 2. Colony formation of *H. pylori* 26695 on different agar media. HB, horse blood agar plate; NISSUI, NISSUI Plate Helicobacter; BS, BHI agar with untreated BS; H-BS, BHI agar with heat-treated BS; FBS, BHI agar with untreated FBS; H-FBS, BHI agar with heat-treated FBS. The number of colonies on each agar plate was counted, and results were expressed as the mean plus standard deviation (SD) from three plates.
FIG. 1
FIG. 2

Number of colonies

HB  NISSUI  BS  H-BS  FBS  H-FBS