A Comparative Study of *Helicobacter pylori* Growth on Different Agar-based Media

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**Background/Aims:** Optimal culture conditions for *Helicobacter pylori* have not been established. We compared the effectiveness of four different agar-based media for the growth of *H. pylori*.

**Materials and Methods:** G27, ATCC #43504 and 60190, and primary cultured strains were used. *H. pylori* strains were cultured for four days under four culture conditions: chocolate agar, Thayer-Martin (TM) agar containing vancomycin-colistin-nystatin inhibitor (VCNI), Brucella agar, and brain heart infusion (BHI) agar containing 5% horse blood and IsoVitaleX (BBL™ BD, USA). Culture of cells in each medium was repeated fourteen times. The growth of *H. pylori* was measured by using a spectrophotometer.

**Results:** TM, Brucella, and BHI agars showed mean absorbance values of 0.099, 0.059, 1.410, and 0.913, respectively. These values were significantly different (*P* = 0.030). After post-adjustment by Bonferroni correction, similar growth was noted for in chocolate, Brucella, and BHI agars; however, TM agar significantly suppressed *H. pylori* growth compared with Brucella agar (*P* = 0.031).

**Conclusions:** Chocolate, Brucella, and BHI agars provided effective culture conditions for the growth of *H. pylori*. TM agar containing VCNI suppressed the growth of *H. pylori* and other organisms. (Korean J Helicobacter Up Gastrointest Res 2017;17:208-212)

**Key Words:** Agar; Culture; *Helicobacter pylori*; Media

**INTRODUCTION**

*Helicobacter pylori* is a gram-negative microorganism that grows in a microaerophilic environment. *H. pylori* was first identified approximately two decades ago by Marshall and Warren, who found it in the stomach of patients with chronic gastritis and gastric ulcers that were not thought to be caused by microbial infections. Accumulating evidence suggests that *H. pylori* is a major factor in the development of gastric cancers, thus its eradication will be a main preventive strategy of gastric cancer.

This microorganism, however, is difficult to culture in vitro. Blood or serum added to media is recommended for maximizing the success of the culture; thus, various agar media containing bovine blood or serum have been used to cultivate *H. pylori*. Other typical supplements added to growth media include: lyzed erythrocytes or hemin, IsoVitaleX (BBL™ BD, Sparks, MD, USA), β-cyclodextrin, charcoal, cholesterol, and ferrous sulphate plus sodium pyruvate and/or mucin.

There is currently no standard medium that provides a significant growth advantage *in vitro* for maintaining the growth of *H. pylori*. *H. pylori* has been demonstrated to grow well in several nutrient-rich media, such as chocolate media, Brucella media, and brain heart infusion (BHI) media. However, there are no studies that compare the effectiveness of these media by repeated experiments.

Therefore, we aimed to compare and contrast four agar-based media (chocolate media, Brucella media, BHI media and Thayer-Martin [TM] agar) for allowing efficient growth of *H. pylori* and to find the most suitable one for laboratory use.

**MATERIALS AND METHODS**

1. Preparation of agar-based media

Two of the agar-based media, chocolate agar and TM agar, were purchased from Hanil Komed (Seongnam, Korea). Two of the agar-based media, chocolate agar and TM agar, were purchased from Hanil Komed (Seongnam, Korea).
Korea). Improved brucella agar containing 5% horse blood was prepared in our laboratory using brucella broth (BBL™ BD), IsoVitaleX Enrichment (BBL™ BD) and whole defibrinated horse blood (Hanil Komed). Improved BHI agar containing 5% horse blood was also prepared in our laboratory using BHI broth (Bacto™ BD), IsoVitaleX Enrichment and whole defibrinated horse blood (Hanil Komed). The compositions of the four different agar-based media are summarized in Table 1.

2. Bacterial strains and culturing procedure

A total of four *H. pylori* strains were used in this experiment: G27 (kindly provided from Prof. Nayoung Kim, Seoul National University, Korea), ATCC #43504 (obtained from the American Type Culture Collection [ATCC]), 60190 (originally from ATCC #49503), and a strain from primary cultured samples. The G27, ATCC #43504 and 60190 strains were stored at \(-70^{\circ}C\) in 200 \(\mu\)L of brucella broth (BBL™ BD) containing 10% fetal bovine serum and supplemented with 10% glycerol (Cat. G5516; Sigma, St. Louis, MO, USA).

Primary cultured strains were obtained from gastric biopsy specimens collected from three patients referred for endoscopies to the Center for Gastric Cancer, National Cancer Center, Goyang, Korea. We received informed consent from each patient who underwent the endoscopic biopsy procedure. This study was approved by the Institutional Review Board of the National Cancer Center, Korea (NCC2016-0271). This study conforms to the ethical standards of the Institutional Review Board of the National Cancer Center, Korea and the Helsinki Declaration of 1964 and later versions. Informed consent was obtained from all the patients before the procedure. The biopsy samples were placed in a sterile tube in containing 1.5 mL of transport medium (brucella broth) and were then immediately processed to isolate bacteria. The delay between the removal of the specimens and the inoculation onto the isolation media did not exceed 3 hours. After being transported to the laboratory, the biopsy samples were homogenized by grinding in a glass grinder. The homogenized biopsy specimens were streaked onto *H. pylori* isolate agar containing: GC base media, 5% fresh sheep blood, 1% IsoVitaleX, 5 \(\mu\)g/mL vancomycin and 1 \(\mu\)g/mL amphotericin B. The plates were incubated in 10% \(CO_2\) and 96~100% humidity at 37\(^{\circ}\)C for three to four days. Suspected *H. pylori* colonies originating from human biopsy samples were identified by morphology and a rapid urease test (Pronto Dry; Pronto Dry; Gastrex Sarl, Gilly les Citeaux, France).

All *H. pylori* strains, including the primary cultured strain, were first grown on chocolate agar plates, then they were collected using autoclaved cotton swabs before being inoculated equally onto four different agar plates. The plates were incubated in 10% \(CO_2\) and 96~100% humidity at 37\(^{\circ}\)C for four days. The concentration of \(O_2\) was kept constant using an AnaeroPack-MicroAero (Mitsubishi

| Table 1. The Composition of Four Different Agar-based Media for the Growth of *Helicobacter pylori* |
|-----------------------------------------------|
|                                              |
| Chocolate agar | Thayer-Martin agar | Brucella agar, improved | Brain heart infusion agar, improved |
|----------------|--------------------|-------------------------|--------------------------------------|
| Enzymatic digest of casein (g)                | 7.5                | 7.5                     | 10.0                                 |
| Enzymatic digest of animal tissue (g)         | 7.5                | 7.5                     | 10.0                                 |
| Sodium chloride (g)                           | 5.0                | 5.0                     | 5.0                                  |
| Sodium bisulphide (g)                         |                    |                         | 0.1                                  |
| Monopotassium phosphate (g)                   | 1.0                | 1.0                     | 2.5                                  |
| Dipotassium phosphate (g)                     | 4.0                | 4.0                     |                                      |
| Disodium phosphate (g)                        |                    |                         |                                      |
| Corn starch (g)                               | 1.0                | 1.0                     |                                      |
| Dextrose (g)                                  |                    |                         |                                      |
| Yeast extract (g)                             |                    |                         |                                      |
| Agar (g)                                      | 10.0               | 10.0                    | 10.0                                 |
| Horse blood (mL)                              | 50                 | 50                      | 50*                                  |
| IsoVitaleX (mL)                               | 10                 | 10                      | 10*                                  |
| Vancomycin-colistin-nystatin inhibitor (mL)   |                    |                         | 10*                                  |

*Added supplements to media.
Gas Chemical, Tokyo, Japan).

We assayed the G27 and ATCC #43504 strains in quadruplicate, while the 60190 strain and the primary cultured sample were assayed in triplicate, such that 14 plates of each medium were used. The experiment was repeated using freshly made media for each replicate. All of the agar plates were monitored for contamination using semi-quantitative estimation. In cases where one or more plates were contaminated, the entire media set was excluded from data collection.

### 3. Measurement of optical density

After four days of growth on agar plates, each *H. pylori* strain was suspended in 200 μL of brucella broth. The suspension was then diluted tenfold. Absorbance was measured using a UV/Vis spectrophotometer (Beckman Coulter, Pasadena, CA, USA) with optical density (OD) measured at 600 nm.

### 4. Statistical analysis

We assumed that the growth difference between the chocolate agar and the brucella agar was three-fold, and was repeated fourteen times for each group of media to have a power of 80%. Kolmogorov-Smirnov tests were used to assess the normality for comparisons between media or strains. All distributions of comparative groups showed normality. Data are presented as the mean±standard error. A two-way ANOVA test with post hoc analysis using Bonferroni’s correction was utilized to analyze the significance of differences among the four culture media, considering the interaction effect of synergies between the media and strains. Strains were classified into two types, ‘freezing strains’ included G27, 60190, and ATCC #43504, vs. the ‘primary culture strain’. We also used Student’s t-test to make comparisons between each medium. The difference between the groups was considered significant when the P value was <0.05. Statistical analyses were performed using the PASW Statistics ver. 18.0 (IBM Co., Armonk, NY, USA).

**RESULTS**

The mean absorbance values were 0.99, 0.60, 1.41, and 0.91 for chocolate agar, TM agar, improved brucella agar and improved BH II agar, respectively (Table 2). The brucella agar showed the highest growth among four different agars (Fig. 1). A two-way ANOVA test revealed a difference of *H. pylori* growth among the four culture conditions (P=0.031). However, no significant difference was observed when the brucella agar was compared with the other agars using post hoc analysis, except the TM agar (P=0.003). The TM agar provided the lowest growth among the four media tested. Using Student’s t-test, the TM agar showed statistically significant lower growth compared with the chocolate agar (P=0.034) and with the brucella agar (P=0.001).

The freezing strains showed lower growth than the primary culture stain (Table 2). A two-way ANOVA test revealed a borderline difference between the two groups (P=0.053). The interaction form of media and the *H. pylori-

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**Table 2. Mean Absorbance of Ten-Fold Diluted Suspensions of Established Strains (G27, ATCC #43504, 69190) and a Sample of a Human *Helicobacter pylori* Strain on Four Different Media**

| Media                        | Strain            | Total (n=14)     | Freezing (n=11) | Primary culture (n=3) |
|------------------------------|-------------------|-----------------|-----------------|-----------------------|
| Chocolate agar               |                   | 0.99±0.48       | 0.91±0.48       | 1.31±0.41             |
| Thayer-Martin agar           |                   | 0.60±0.45       | 0.49±0.39       | 1.01±0.50             |
| Brucella agar                |                   | 1.41±0.68       | 1.32±0.74       | 1.74±0.23             |
| Brain heart infusion agar    |                   | 0.91±0.63       | 0.89±0.70       | 1.00±0.39             |
| Total                        |                   | 0.98±0.63       | 0.90±0.65       | 1.27±0.46             |

Values are presented as mean±standard deviation.

*P < 0.05 by Student’s t-test.

*P < 0.05 by Bonferroni correction.

*P value is 0.053 in a two-way ANOVA between stains. There is no difference between strains and media (P=0.870), but the P value calculated by Student’s t-test is 0.054.
Fig. 1. The average absorbance of ten-fold diluted suspensions of *Helicobacter pylori* on four different media according to *H. pylori* strain. Choco, chocolate agar; TM, Thyer-Martin agar; BHI, brain heart infusion agar; Human, strain obtained from primary gastric biopsy. Two-way ANOVA revealed a significance difference between the four media ($P=0.031$).

*r* strains did not affect the difference between the strains ($P=0.870$).

A total of seven *H. pylori* strain media sets were contaminated; however, the TM agar was never contaminated (chocolate media 3/17, 17.6%; brucella media 5/19, 26.3%; BHI media 6/20, 30.0%).

**DISCUSSION**

The optimal media for growing *H. pylori* have been not well established. Columbia agar, modified chocolate agar or BHI agar supplemented with blood or serum were usually recommended for culturing *H. pylori*.12,13 This study demonstrated that chocolate agar, TM agar, improved brucella agar and improved BHI agar were similarly able to culture the *H. pylori* strains tested. The horse blood and IsoVitaleX were added to brucella and BHI agars, and the supplement effect was eliminated by making the compositions of supplement similar among four media. The growth of *H. pylori* on TM agar in comparison to the brucella or chocolate agars was statistically significant. The low growth on TM agar could have been caused by the vancomycin-colistin-nystatin inhibitor (VCNI) inhibitor, which consists of antibiotics to suppress growth.

In a previous study, the VCNI suppressed the growth of some contaminating organisms but allowed *Neisseria gonorrhoeae* and *Neisseria meningitidis* to grow on TM agar.14 Vancomycin was demonstrated to have an inhibitory effect on *H. pylori* by delaying or suppressing growth.9,15 Colistin and nystatin were reported to inhibit the growth of *H. pylori* at higher concentrations.16 No contamination was observed on TM agar during our study, correlating with other studies that showed a low rate of contamination in the isolation of *H. pylori*.17-19

Previously published studies have reported outcomes that differ from our results. In particular, Brucella agar was not previously shown to support greater *H. pylori* growth compared to other media, such as BHI media.4,17,20 Some studies have shown that chocolate agar supports higher *H. pylori* growth than brucella agar containing supplements.7 However, they did not perform statistical comparisons, therefore they cannot be considered to have produced accurate and consistent results.20 In our study, no difference in *H. pylori* growth was observed among chocolate agar, brucella agar and BHI agar compared using statistical methods. To our knowledge, this study is the first to statistically compare *H. pylori* growth on four different agar based media using repeated cultures.

The variability of growth between strains was not disclosed. The freezing commercialized strains (included in G27, ATCC #43504, and 60190) exhibited a lower growth ability than strains from primary gastric biopsies. This correlated with observations that frozen *H. pylori* did not recover viability as well as those from the natural environment.21 Therefore, the viability of *H. pylori* was not thought to have a synergistic growth effect in combination with the media variable.

There are some limitations of this study. First, it is not known which media component affected the growth of *H. pylori*. A well-designed study has not been published to show the beneficial effect of these components, except blood.5,8 Thus, additional comparative studies are needed to determine which components enhance the growth of *H. pylori*. A second limitation was the relatively small number of replicates used for each culture condition. If repeated with more experiments for each strain and media, the results may be different from the current results.

In conclusion, the chocolate, brucella and BHI agars provided similar culture conditions for the growth of *H.
The TM agar containing the VCNI may suppress the growth of \textit{H. pylori}.

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