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Improved Growth of *Campylobacter pylori* in a Biphasic System

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The recovery of *Campylobacter pylori* from clinical specimens is difficult, even when done with an optimal medium, atmosphere, and temperature. The growth of this organism was investigated by comparing a biphasic system with broth culture. The effects of gyration, inoculum, and pH were studied. Brucella agar and broth supplemented with 2.5% fetal bovine serum were used. Growth in the biphasic system was an average of 2 log units (7×10^8 versus 5×10^6 CFU/ml) greater than that in the broth system ($P < 0.01$), and this occurred 12 to 24 h sooner in the biphasic system. When gyration was added, an average of 1 log unit of growth improvement was seen in comparable systems. Improved growth was also seen with low inoculum levels, in which stationary-phase cells in the broth system reached 10^5 CFU/ml compared with 10^7 CFU/ml in the biphasic system. At the three pH ranges studied, growth was best at pH 8 to 9 (6×10^9 CFU/ml), averaging 2 log units greater growth than that at pH 6 to 7 and 4 log units greater growth than that at pH 4.5 to 5.5 ($P < 0.01$). The improved recovery of the organism for low inoculum levels in a biphasic system may be important for long-term storage and clinical isolation.

Since *Campylobacter pylori* was first isolated in 1983 by Marshall and Warren (15), efforts have been focused on studying the classification and potential pathogenesis of this organism in gastrointestinal diseases. This organism has been implicated as the causative agent in nonulcer dyspepsia (24), duodenal ulcers (1, 2, 4, 9, 15), and gastritis and gastric ulcers (1, 2, 5, 9, 13, 15, 19, 23). However, it is not clear whether this organism contributes to the pathogenesis of peptic ulcer disease or dyspepsia since there is a positive correlation between the incidence of the organism and increasing age of patients (7, 12, 20), and many normal individuals have *C. pylori* present in their gastrointestinal tracts.

Several attempts have been made to develop methods for rapid detection; culture, serology (enzyme-linked immunosorbent assay, complement fixation, and immunoblot), urease, the [^{13}C]urea breath test, and electron microscopy have been used; however, diagnosis is contingent on isolation of the organism (6–8, 10, 14, 16, 17, 20, 21, 25). Cultivation is difficult, requiring incubation periods of up to 7 days under microaerophilic conditions (10, 11, 15, 18). Morgan et al. (18) have studied growth in brucella broth supplemented with fetal bovine serum (FBS) and have shown that there is an increased yield of organisms in gyrated media.

We report here the improved growth of organisms in a biphasic system. This biphasic system is superior to currently used stationary cultures and broth cultures with gyration. It offers the advantages of convenience and clinical application and allows the addition of antibiotics directly into the system to obtain pure cultures of *C. pylori*.

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MATERIALS AND METHODS

Bacterial strains. *C. pylori* 84-182, 87-2 and 87-91, which were recovered from human gastric biopsy specimens, were obtained from M. Blaser and B. Dunn (VAMC, Denver, Colo.). Stock cultures were stored at −70°C in brucella broth (Difco Laboratories, Detroit, Mich.) with 20% glycerol and 1% FBS. FBS (lots B79607, B79306, and A72601; Armour Pharmaceutical Co., Kankakee, Ill.) was heat inactivated at 56°C for 30 min prior to use.

Media preparation. Biphasic systems were made by using an agar slant with overlying broth in a 2:1 volume ratio, respectively. Autoclaved or filter-sterilized brucella broth (filter pore size, 0.22 μm) supplemented with 2.5% heat-inactivated FBS was poured over the agar slant to a level approximately just below the agar slant tip when the flask was in the horizontal position (Fig. 1).

Cultivation. Organisms were thawed from −70°C and inoculated into prewarmed (37°C) biphasic systems. *Campylobacter* cultures were grown at 37°C under microaerophilic conditions of 5% O_2, 10% CO_2, and 85% N_2 by using 50-ml sterile glass Ehrlenmeyer flasks or 75- and 25-cm^2 plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.). Ehrlenmeyer flasks were stoppered with sterile gauze plugs, and tissue culture flasks were loosely capped to allow gas exchange. Agar plates and small flasks were kept in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) for gassing and incubation. If gyration was used, the tissue culture flasks were gassed and sealed with rubber stoppers before inoculation. Samples were removed with a syringe (Fig. 1). Pure cultures were obtained on primary isolation medium modified by the addition of antibiotics (10 μg of vancomycin per ml, 10 μg of trimethoprim per ml, and 2 μg of amphotericin B per ml). Inoculum size and growth curve data were determined by colony plate counts on bacteria harvested from broth cultures and biphasic broths at 24-h intervals for 72 h and serially diluted in peptone-saline. Fractions of 0.01 ml were plated onto brucella agar (supplemented with 2.5% FBS) in triplicate, colony counts were performed, and values were averaged to obtain each datum

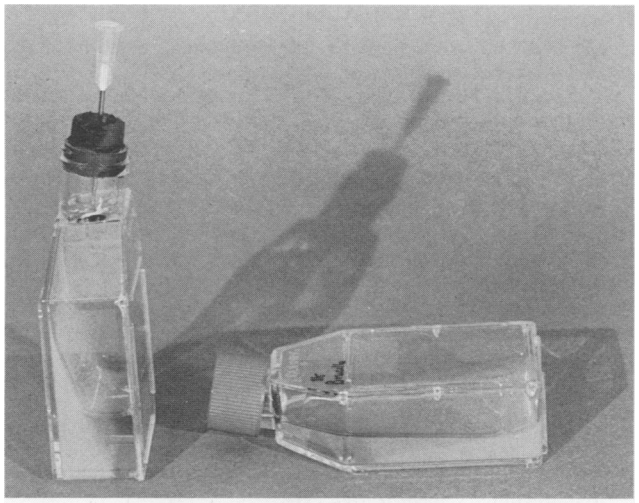

FIG. 1. Photograph of the biphasic system. Inoculation and sampling are done with a needle through the rubber stopper.

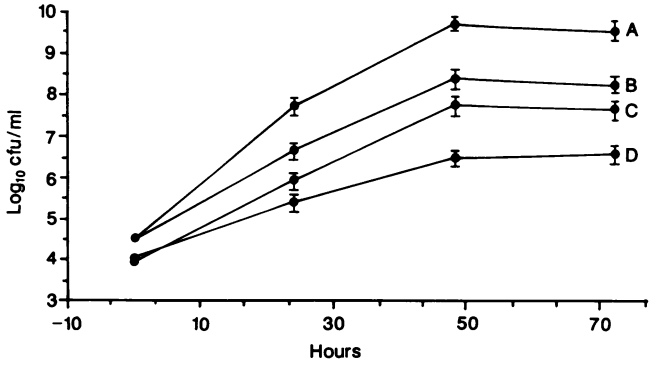

FIG. 2. *C. pylori* kinetics of growth with and without gyration in broth and biphasic culture systems. In a stationary culture, the biphasic system improved growth by a factor of 100. Gyration improved growth by more than a factor of 10 in comparable systems. Curves: A, Biphasic system with gyration; B, biphasic stationary system; C, broth system with gyration; D, stationary broth system ($P < 0.01$). Vertical bars represent standard deviations ($n = 12$).

point. The influences of inoculum size and pH were assayed similarly. All experimental cultures were paired in duplicate, repeated six times, and incubated either in stationary cultures or gyrated at 150 rpm in a gyratory incubator-shaker (Lab-line model 3525 with a 1-inch [2.54 cm] throw; Lab-Line Instruments, Inc., Melrose Park, Ill.). Data were averaged to produce growth curves; however, all raw numbers were used for statistical analysis. For some experiments sterile brucella broths containing 2.5% FBS were adjusted to average pHs of 5 ± 0.5, 6.5 ± 0.5, and 8.5 ± 0.5 by using 1 N citric acid or 1 N NH_4OH. The pH-adjusted broths were poured over the brucella agar slants to produce pH-adjusted biphasic systems. In addition to the growth curve data (as described above), media were evaluated for pH changes at 0 and 72 h in the pH experiments.

Statistical analysis. All results were analyzed by using standard deviation, the Fisher exact test, the Scheffe F test, and the Dunnett *t* test, with *P* values used for the analysis of significance.

RESULTS

Gyratory versus stationary cultures in broth and biphasic systems. Broth and biphasic cultures were compared in both the gyrated and stationary states in six experiments (Fig. 2). *C. pylori* grown in a biphasic system yielded an average of 2 log units greater of growth (7×10^8 versus 5×10^6 CFU/ml) than if it was grown in broth ($P < 0.01$). There was no significant difference between inoculum levels compared with $P < 0.01$ at harvest counts at 72 h, suggesting a more rapid growth rate in the biphasic system. When gyration was added, an average of 1 log unit of growth improvement was seen in comparable systems (Fig. 2 and 3). Overall, there was a difference of 3 log units of growth (5×10^9 versus 5×10^6 CFU/ml) when the biphasic system with gyration was compared with the stationary broth culture system ($P < 0.01$).

Recovery from low inoculum cultures and freezer storage. In the six experiments done with low inoculum levels (100 to 200 CFU/ml), our data showed that an average peak of 10^7 CFU/ml occurred in the biphasic stationary system by 72 h (Fig. 3). We subsequently used inocula as low as 50 CFU/ml and achieved the same results (data not shown). At the lower inoculum, there was no growth in the broth system without gyration. Growth in broth may be dependent on the diffusion of the overlying gaseous mixture into the broth. In more recent trials we found that a broth layer that was less than 3 cm in height improved recovery from cultures with lower inocula; however, the overall yield was limited in this setting. When bacteria were removed from −70°C and placed directly into the biphasic system, recovery was consistently reliable.

Effects of pH on growth. We measured the effects of various starting pHs of the biphasic system with gyration on the growth of *C. pylori*. Our studies were done in biphasic systems with three different adjusted pHs of 5 ± 0.5, 6.5 ± 0.5, and 8.5 ± 0.5. Cultures were performed in duplicate for six experiments, and growth was determined by colony plate counts. We found that growth was best at pH 8 (Fig. 4). There was an unexplained lag phase in these results during the first 24 h of culture; however, the same peak growth was achieved as was seen in pH-unadjusted medium (Fig. 2).

To assess the ability of this organism to alter the pH of its environment, determinations of pH were made on uninoculated (controls) and inoculated, pH-adjusted (see above) biphasic broth cultures at 0 and 72 h. When grown for 72 h at a starting pH of 5 ± 0.5, *C. pylori* increased the culture

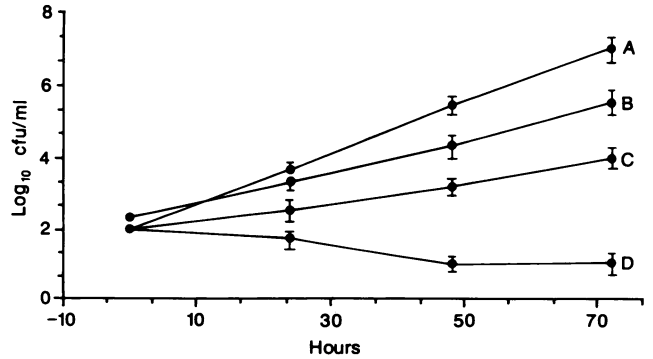

FIG. 3. Kinetics of growth showing that *C. pylori* recovery from low inoculum levels is enhanced with gyration in a biphasic system. Curves: A, Biphasic system with gyration; B, biphasic stationary system; C, broth system with gyration; D, stationary broth system ($P < 0.01$). Vertical bars represent standard deviations ($n = 12$).

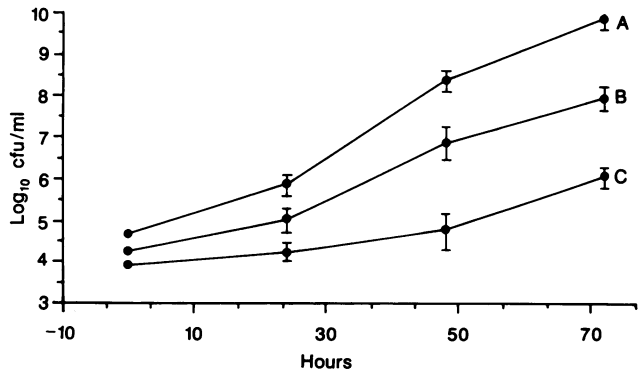

FIG. 4. Growth curve of *C. pylori* at three pH ranges. Growth was best at an alkaline pH. All cultures were done in biphasic systems with gyration. Curves: A, pH 8.5 ± 0.5; B, pH 6.5 ± 0.5; C, pH 5 ± 0.5 ($P < 0.01$). Vertical bars represent standard deviations ($n = 6$).

medium pH to 7 ± 0.5, whereas in other cultures only minor changes in pH were noted. Control media (uninoculated) had less than a 0.05 pH unit change at 72 h. Therefore, *C. pylori* caused an increase in culture medium pH toward the higher, optimum range.

DISCUSSION

Many different media have been studied for the isolation of *C. pylori* (3, 10, 11, 15, 18, 22). Buck and Smith (3) found an increased growth of *C. pylori* by the addition of serum, blood, and corn starch to basal medium, while Morgan et al. (18) found that regardless of inoculum size, *C. pylori* growth in brucella broth was improved by 2 log units with serum supplementation. In comparison, we found that the use of a biphasic system and a serum content of 2.5% improved growth by 4 log units.

Our methods have increased the percentage of recovery from cultures with inoculum levels as low as 50 to 100 bacteria. During growth in the biphasic system, turbidity (suggesting growth) was seen to collect along the interface of the broth and agar slant. When Noble agar was substituted for brucella agar, lower peak growth was achieved. Serial slices of the agar slant stained for *C. pylori* showed that it attached to and penetrated the surface of the agar. These findings suggest that the agar slant plays a role in the adherence and attachment of the organism. It may remove potentially toxic growth products from the environment, provide improved gas diffusion by varying the broth depth, or be a reservoir for nutrients.

Our study revealed that this organism grows best at a neutral-to-alkaline pH range. At acidic pH, stationary-phase growth was achieved 12 to 24 h later and with significantly fewer bacteria per milliliter. It is possible that the ability of *C. pylori* to alter the pH of the medium was a result of urease production, since this brucella medium contained digested animal tissues and likely contained urea. Urease production by *C. pylori* has been described as well (17).

The biphasic medium used in this study offers significant improvement over other methods in the isolation and peak growth of bacteria. It also has the advantage of eliminating the need for gyration. Antibiotics can be added to the system to aid in obtaining pure cultures. This system can easily be applied to the clinical isolation and recovery of *C. pylori*.

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