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Growth of *Campylobacter pylori* in Liquid Media

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Until recently, broth cultivation techniques for *Campylobacter pylori* were unavailable. We developed a method to cultivate bacterial cells within 24 h in liquid media. Cultivation in broth depended on the adequate dispersion of appropriate gases. A static broth at 37°C in a GasPak jar (BBL Microbiology Systems, Cockeysville, Md.) with a CampyPak (BBL) envelope did not support growth after 5 days of incubation. A broth placed in a flask on a Gyrotory water bath shaker (150 rpm; New Brunswick Scientific Co., Inc., Edison, N.J.) fitted with a gassing hood connected to a gas mixture of 10% CO_2, 5% O_2, and 85% N_2 supported good growth. An initial inoculum of 10^5, 10^3 to 10^4, or 10^2 CFU/ml resulted in $\geq 10^8$ CFU/ml after incubation for 24, 48, or 72 h, respectively. Under these conditions, the bacteria grew as motile, spiral bacilli rather than the oval and coccal bacilli occasionally reported. Several bases supported good growth when supplemented with serum. For the determination of basal growth conditions, brucella broth base was used. Fetal calf serum (1%) provided maximum growth. Vitox was not necessary for growth and did not augment growth. *C. pylori* grew over a wide optimal pH range of 5.5 to 8.5.

Campylobacter pylori is a newly recognized agent. Laboratory methods for culture, storage, susceptibility testing, and characterization of strains are not well established. Little information is available on the virulence properties of the bacterium. *C. pylori* is a microaerophilic, gram-negative, spiral bacterium which requires extended incubation periods at 37°C for culturing (2–4, 10, 12, 14). Standard enteric media do not support the growth of the bacterium; rather, complex nutrients are required for culture propagation. In general, broth cultivation is difficult.

C. pylori is associated with ulcerative and nonulcerative gastritis in humans (1, 7, 8, 10, 11, 13, 14, 16, 18, 19). Although the role of the bacterium in upper gastrointestinal tract disease is uncertain, evidence is accumulating which suggests that *C. pylori* is an etiologic agent of human gastritis. The bacterium is observed in more than 70% of patients with gastric pathology and is rarely observed in healthy volunteers. Patients with gastric pathology and *C. pylori* have significant anti-*C. pylori* antibody levels. In contrast, healthy blood donors rarely have significant titers (1, 5, 6, 9, 10, 15).

The concept of infectious gastritis is novel. Antimicrobial therapy, in place of or in combination with antacid or H_2 antagonist therapy, may prove more beneficial than current therapy. Therefore, to support the development of antimicrobial therapy for infectious gastritis, methods for culturing the bacterium were developed. An adequate selective enrichment medium is useful for the implementation of clinical studies. In this report, a method to enrich media for the growth of *C. pylori* is described.

MATERIALS AND METHODS

Bacterial strains. A single human isolate of *C. pylori* TX30A, obtained in 1985 from G. E. Buck, was used for most studies. Recent human gastric isolates (obtained from 1986 to 1987) from Peru (strains 99B, 117A, and 130B) and the United States (strains TX38 and 86-324) in addition to archive strains from Australia (NCTC 11638) and England (strains 60190 and 26695) were also studied. Stock cultures were stored at −50°C in whole rabbit blood.

Cultivation on agar media. Organisms were grown routinely on GCHI chocolate agar (Remel Media Laboratories, Lenexa, Kans.) supplemented with trimethoprim (5 mg/liter), vancomycin (10 mg/liter), polymyxin B sulfate (2,500 IU/liter), and amphotericin B (2 mg/liter). Plates were incubated at 37°C for 3 to 5 days in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) with catalysts and CampyPaks (BBL). The gas-generating envelopes were replaced every 48 h.

Direct examinations. Wet mounts were made to confirm the morphology of strains under phase-contrast microscopy. Dried preparations were stained by a modified Gram stain method using 0.3% carbol fuchsin as the counterstain.

Broth cultivation. In preliminary experiments, 10 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.), brucella broth (Difco), or Iso-Sensitest broth (Oxoid Ltd., Basingstoke, England) supplemented with 10% fetal calf serum (FCS) (GIBCO Laboratories, Grand Island, N.Y.) and 1% Vitox (Oxoid) was placed into 50-ml Erlenmeyer flasks with a porous stopper or loosely fitted screw cap. Flasks were inoculated to 10^5 CFU/ml (1.0 ml of 10^6 CFU/ml diluted in 9 ml of medium) and incubated at 37°C in a GasPak jar with a CampyPak. Flasks were observed for growth for up to 48 h. In subsequent experiments, 10 ml of brucella broth supplemented with FCS or Vitox or both (as specified in each experiment) was placed in a sterile 50-ml Erlenmeyer flask with a porous stopper or loosely fitted screw cap. Flasks were inoculated with *C. pylori* and incubated on a Gyrotory shaking platform (model G76/D water bath shaker with a 13-mm shaking orbit; New Brunswick Scientific Co., Inc., Edison, N.J.) at 150 rpm under an enclosed hood flushed with a gas mixture of 10% CO_2, 5% O_2, and 85% N_2. Growth was enumerated after 24 to 72 h by the plate count technique. Serial dilutions were made in normal saline and plated in duplicate onto GCHI agar. Colonies were counted after 72 h of incubation at 37°C in a microaerobic environment.

Requirements for growth of *C. pylori* in broth. A series of four experiments was performed to determine whether brucella broth alone, brucella broth plus FCS, brucella broth

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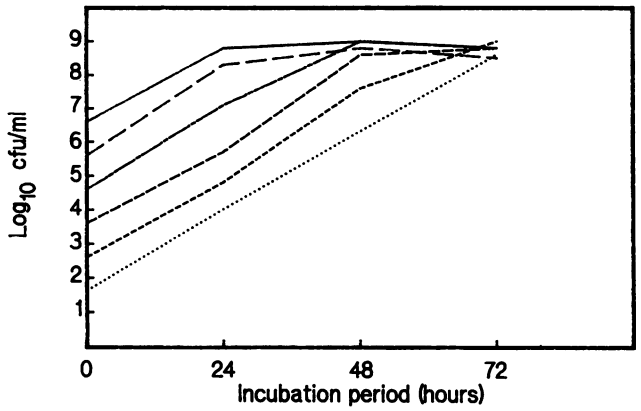

FIG. 1. Kinetics of growth of *C. pylori* in brucella broth. In triplicate experiments, six inoculum levels were inoculated into supplemented brucella broth. Viable cells were enumerated after 0, 24, 48, and 72 h of incubation.

plus Vitox, or brucella broth plus Vitox and FCS would support growth. *C. pylori* TX30A (10^5 CFU/ml, initial concentration) was inoculated into brucella broth, brucella broth plus 1% Vitox, brucella broth plus 10% FCS, or brucella broth plus 1% Vitox and 10% FCS. Viable cells were enumerated after 24 and 48 h of incubation.

Effect of Vitox on growth of *C. pylori* in broth. A series of three experiments was performed to determine whether Vitox was essential for growth of *C. pylori* in brucella broth. *C. pylori* TX30A (10^5 CFU/ml, initial concentration) was inoculated into brucella broth plus 10% FCS with 1% Vitox or without Vitox. After 24 h of incubation, the cultures were serially passaged four times in the same medium. At each interval, viability was determined by the plate count method and morphology was observed by direct examination.

Effect of FCS on growth of *C. pylori*. In four experiments, *C. pylori* TX30A (10^5 CFU/ml) was inoculated into brucella broth containing 10, 5, 1, or 0.1% FCS or no FCS. Viable cells were enumerated after 24 h of incubation.

Kinetics of growth of *C. pylori*. In each of three experiments, six different amounts of *C. pylori* TX30A (10^1 to 10^6 CFU/ml) were inoculated into brucella broth plus 1% Vitox and 10% FCS. Viable cells were enumerated after 0, 24, 48, and 72 h of incubation. A second series of experiments was performed in which nine strains of *C. pylori* (10^5 and 10^2 CFU/ml) were inoculated, incubated, and enumerated as described above.

Effect of pH on growth. The pH of brucella broth with 10% FCS and 1% Vitox was adjusted (with 1 N NaOH or HCl) after autoclaving. The adjusted medium was sterilized by filtration. A pH range of 4.0 to 10.0 in increments of 0.5 pH units was studied. Flasks were inoculated with $\sim 10^5$ CFU/ml. Growth was enumerated after 24 h.

RESULTS

Requirements for growth of *C. pylori* in broth. Initial experiments indicated that brucella broth plus 10% FCS and 1% Vitox supported the growth of *C. pylori* TX30A when flasks were placed on a shaking platform. No growth resulted when flasks were incubated for up to 5 days on a stationary platform. These data indicated that gas dispersion throughout the liquid medium was essential for cultivation of *C. pylori*. Brucella broth with FCS alone supported growth as well as did brucella broth with FCS and Vitox. Cells appeared as spiral bacilli rather than the coccal bacilli sometimes seen on agar cultivation. Brucella broth alone and brucella broth with Vitox did not support growth of *C. pylori* even after a 48-h incubation period. Prolonged incubation (5 days) resulted in a decrease in viability and an increase in coccal forms.

Effect of Vitox on growth of *C. pylori*. To confirm that Vitox was not essential for growth of *C. pylori* TX30A, broth cultures were serially passaged in media with or without Vitox. After four transfers, flasks contained equivalent numbers of viable *C. pylori* cells. In addition, cell shape was the same for cultures grown in either medium. These data confirm that Vitox is not essential for growth of *C. pylori* in broth.

Effect of FCS on growth of *C. pylori*. A series of four experiments was performed to determine the minimum amount of FCS required to support optimal growth of *C. pylori*. Flasks of brucella broth supplemented with 1 to 10% FCS supported growth equally well. A lower concentration (0.1%) did not support growth. Therefore, under our test conditions, 1% FCS was required for optimal growth of *C. pylori* in brucella broth.

Kinetics of growth of *C. pylori*. Six inoculum levels of *C. pylori* were studied over three time periods to determine the rate of cultivation in supplemented brucella broth (Fig. 1). With the two highest inoculum levels (10^5 and 10^6 CFU/ml), maximum growth was achieved after 24 h. With intermediate inoculum levels (10^2 to 10^4 CFU/ml), maximum growth was achieved after 48 h. With as few as 50 CFU/ml, maximum growth was achieved after 72 h. These data demonstrate the enrichment capability of the broth.

To ensure that multiple recent clinical isolates of *C. pylori* would grow equally well in the enrichment medium, nine strains were grown in supplemented brucella broth. All strains grew equally well in controlled studies in which two flasks containing different inoculum levels were enumerated after 24 to 72 h of incubation (Fig. 2). Over 100 recent clinical isolates have been cultivated successfully with 24 to 48 h of incubation in this medium, and no isolate tested so far has failed to grow. However, there is variability among experiments. All strains did not always reach 10^8 CFU/ml after 24 h of incubation; 48 h of incubation was required occasionally to achieve maximum growth. In some experiments, a signif-

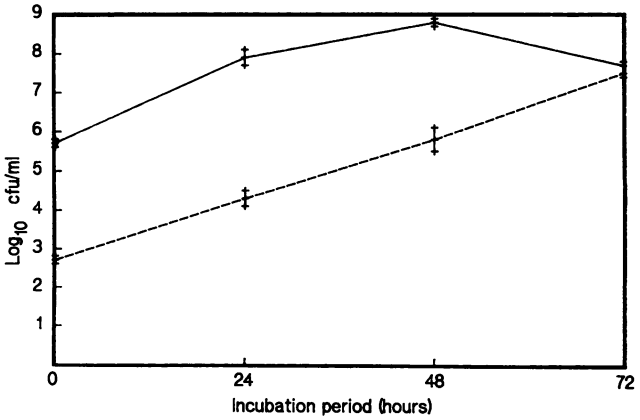

FIG. 2. Kinetics of growth of nine isolates of *C. pylori* in brucella broth. Two inoculum levels were placed into supplemented brucella broth. Viable cells were enumerated after 0, 24, 48, and 72 h of incubation. Vertical lines indicate standard deviations of enumerations done at 24 and 48 h.

icant decrease in viability was noted after 72 h of incubation of flasks with the higher inoculum. This may be due to nutrient deprivation by faster-growing strains.

Effect of pH on growth of *C. pylori*. Cultures of *C. pylori* were inoculated into supplemented brucella broth adjusted to a pH ranging from 4 to 10 to determine the pH tolerance of this bacterium. Maximum growth was achieved over a pH range of 5.5 to 8.5. These data suggest that *C. pylori* is tolerant to wide changes in pH.

DISCUSSION

C. pylori is a newly recognized bacterium with a strong association with human gastritis. Few laboratories worldwide have the ability to isolate and propagate the bacterium. Therefore, standard methodologies for growth, susceptibility testing, and virulence determinations do not exist. Cultivation in broth has proven to be difficult. However, by providing an even distribution throughout the broth of gases which generate the required microaerobic atmosphere, *C. pylori* can readily be grown in a liquid medium. Incubation on a Gyrotory platform flushed with a gas mixture of 10% CO_2, 5% O_2, and 85% N_2 provided adequate atmospheric conditions to support the growth of *C. pylori* in broth. Several basal broth media supplemented with blood products supported the growth of *C. pylori*.

In this report, we describe growth in supplemented brucella broth. Optimal growth was achieved with overnight incubation in brucella broth supplemented with 1 to 10% FCS. The bacterium grew readily over a wide range of pHs, whereas most pathogens have a restricted range within which optimal growth occurs (17). This tolerance may enable the organism to endure the pH changes of the gastrointestinal tract. The ability to tolerate both acid and alkaline environments may be an adaptation by *C. pylori* that allows the organism to colonize the gastric mucosa. The organism is, however, sensitive to the highly acidic conditions of the gastric fluid.

Since brucella broth supplemented with 10% FCS allows the growth of low numbers of *C. pylori*, this medium will be used as the basis to develop a selective enrichment broth. Supplementation with selected antimicrobial agents may enable selective cultivation of *C. pylori* in the presence of contaminating background flora. This technology will be useful in the cultivation of *C. pylori* in specimens from patients.

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