

Enhanced cultivation of *Helicobacter pylori* in liquid media

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Abstract

Aims—To evaluate a technique for culture of *Helicobacter pylori* in large quantities of liquid media and to determine the factors that could influence the results.

Methods—Fifteen clinical isolates of *H pylori* and a reference strain of *H pylori* NCTC11637 were used to evaluate a method to cultivate the organism in 100 ml liquid medium comprising brain heart infusion broth with 5% horse serum and 0.25% yeast extract. Tissue culture flasks containing the inoculated liquid medium were placed in a CO₂ incubator with 5% CO₂ for 2 hours and then incubated in a shaking incubator at 120 rpm.

Results—All the clinical isolates and the reference strain grew in the broth, although only a moderate growth of the reference strain occurred. Inoculum size significantly influenced the kinetics of growth of *H pylori* in the liquid medium. Vancomycin, nalidixic acid, and amphotericin B, used to suppress contamination, did not affect growth of *H pylori* in the medium. CO₂ was essential for *H pylori* to grow or survive in the liquid medium. Incubation with CO₂ in a CO₂ incubator for 30 minutes or 2 hours did not affect the results.

Conclusions—*H pylori* can be cultivated in large quantities of brain heart infusion broth with 5% horse serum and 0.25% yeast extract. Initial inoculum concentrations influence the kinetics of *H pylori* growth in the liquid medium. Vancomycin, nalidixic acid, and amphotericin B can be used as selective antimicrobial agents. CO₂ is essential for initial growth of *H pylori* in liquid media. The findings in this study may provide a useful, reproducible, and simple method for biochemical, molecular, and physiological studies of *H pylori*, when those require large quantities of the organism.

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Helicobacter pylori colonises human gastric epithelium and causes acute and chronic gastritis.¹ It also has an important role in the development and recurrence of peptic ulcer disease.² Isolation and subculture of *H pylori* on various selective and non-selective solid media have been established.³⁻⁷ Growth of

this organism in small amounts of liquid media has been achieved.^{4,5,8-13} Cultivation of *H pylori* in large quantities of liquid media, however, essential for biochemical and physiological studies, is rarely reported.¹⁴⁻¹⁶ The aim of this study was to evaluate a method for the culture of *H pylori* in large quantities of liquid media and to determine the factors that may influence the results.

Methods

In initial experiments a clinical isolate of *H pylori* IRL92589 was used to establish suitable culture conditions. Three other clinical isolates of *H pylori*, IRL92587, IRL92661, IRL92664, and a reference strain NCTC 11637 (National Collection of Type Cultures, Public Health Laboratory, London, England) were added in a formal experiment for evaluation of reproducibility and feasibility of this method. Eleven other clinical isolates were also used. The isolate IRL92589 was used in all of the studies.

Solid media were used for growth of strains before inoculation of liquid media. *H pylori* isolates and strain NCTC 11637 were subcultured on chocolate agar plates (Columbia agar base, LAB M, Topley House, Wash Lane, Bury, BL9 6AU, England) with 7% horse blood. The plates were incubated in gas jars at 37 °C for three days immediately after flushing with CO₂. Colonies were examined using the urease test and Gram stain for confirmation of identity.

Liquid media used for a series of studies were based on brain heart infusion broth (Oxoid, Unipath Ltd, Basingstoke, Hampshire, England) with 5% horse serum (Oxoid) and 0.25% yeast extract (Oxoid). Cultures from chocolate agar plates were inoculated into 5 ml aliquots of nutrient broth to achieve graded concentrations of bacterial cells, determined by comparator turbidity tubes (McFarland No 0.5—No 5). Each suspension (1 ml) was transferred to a 200 millilitre tissue culture flask (Sterilin Ltd, Hounslow, Middlesex) containing 100 ml prewarmed liquid medium. The culture flasks were shaken gently and placed in a CO₂ incubator (with 5% CO₂) for 2 hours with loosely fitted caps. The flasks were removed, caps tightened, and then incubated on a shaking platform (Model G25, New Brunswick Scientific Co. Inc. Edison, New Jersey, USA) at an angle of about 20° with a rotation speed of 120 rpm. The cultures were examined daily for viable cell counts, and the urease test

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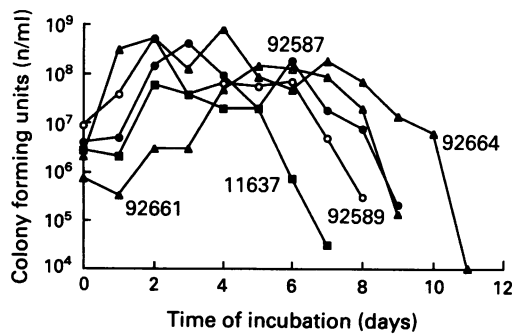


Figure 1 Growth of four clinical isolates and a reference strain NCTC 11637 in the liquid medium.

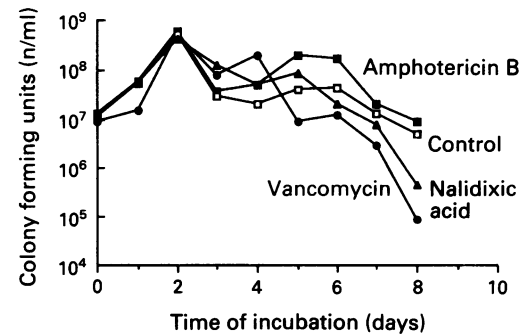


Figure 3 Effect of selective antibiotics on growth of isolate IRL92589 in the liquid medium.

and Gram stain were also used. Oxidase and catalase tests were also used to outrule contamination.

Dilutions of 10^{-1} , 10^{-3} , and 10^{-4} were made from the tissue culture flasks by suspending the culture in nutrient broth. The suspensions were inoculated with a $1 \mu\text{l}$ calibrated loop on to chocolate agar plates in triplicate. The plates were incubated as described above and colonies counted after five days of incubation. The mean value of three readings was calculated and expressed as colony forming units per millilitre (cfu/ml).

Bacterial cells of isolate IRL92589 from chocolate agar were suspended into nutrient broth to a turbidity of McFarland No 4. Four 10-fold dilutions were then made to yield five inoculum concentrations in the liquid media (from about 10^3 to 10^7). The suspensions were inoculated into the broth and incubated as described above.

A suspension (1 ml) (McFarland No 4) of isolate IRL92589 was inoculated into four culture flasks containing 100 ml of the liquid medium and also 6 mg/l vancomycin (Sigma Chemical Co. Ltd, USA), 20 mg/l nalidixic acid (Sigma), 4 mg/l amphotericin B (Sigma), or no antimicrobial agent, respectively. The culture flasks were incubated as described above.

Another suspension (1 ml) (McFarland No 4) of isolate IRL92589 was inoculated into three culture flasks. Subsequently, two flasks were kept in the CO_2 incubator with loosely fitted caps for 30 minutes or 2 hours, respectively. The flasks then were removed and

incubated in the shaker at 120 rpm as described before. The third flask was placed in the shaking incubator without CO_2 , as a control.

Results

In the initial study *H pylori* isolate IRL92589 grew well in a tissue culture flask containing 100 ml brain heart infusion broth supplemented with 5% horse serum and 0.25% yeast extract. This was when the flask was exposed to 5% CO_2 for about 2 hours and shaken at 120 rpm at 37°C . Subsequently, three other clinical isolates and a reference strain were evaluated in the same manner. The results for the five strains are shown in fig 1. The reference strain NCTC11637 performed less satisfactorily in terms of the maximum growth achieved than the clinical isolates, all of which reached 10^8 cfu/ml during the period of incubation. All the other 11 clinical isolates grew well in the medium (data not shown).

The five inoculum concentrations were used to determine the kinetics of growth of *H pylori*. Different log phases of growth and different survival times appeared according to different inoculum concentrations (fig 2). With inocula of 10^6 and 10^7 cfu/ml, maximum growth was achieved after two days of incubation; maximum growth was delayed when the inoculum concentrations decreased. With an inoculum of 10^3 cfu/ml, maximum growth occurred after 10 days of incubation. The survival times of *H pylori* were shorter with the increased inoculum concentrations. With inocula of 10^6 and 10^7 cfu/ml, the viable counts dropped below 10^4 cfu/ml after nine days of incubation, while with inocula of 10^3 - 10^5 cfu/ml, the times were 13 to 15 days.

Vancomycin, nalidixic acid, and amphotericin B did not influence the growth of *H pylori* in the liquid medium (fig 3). The pattern of growth in fig 3, showing two peaks, is similar to that in fig 2. With inoculum of 10^7 cfu/ml, the first peak of growth appeared after two days of incubation and the second after six days.

Incubation with CO_2 for 30 minutes and 2 hours did not affect the growth of *H pylori* in the liquid medium. Without incubation in CO_2 , however, *H pylori* survived in this

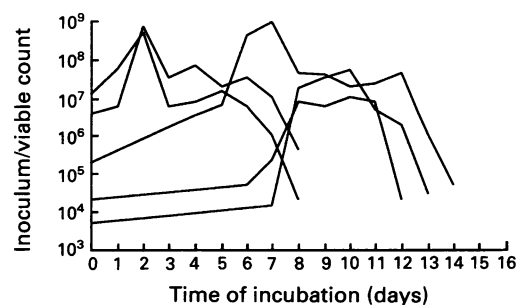


Figure 2 Growth of isolate IRL92589 with different inoculum concentrations in the liquid medium.

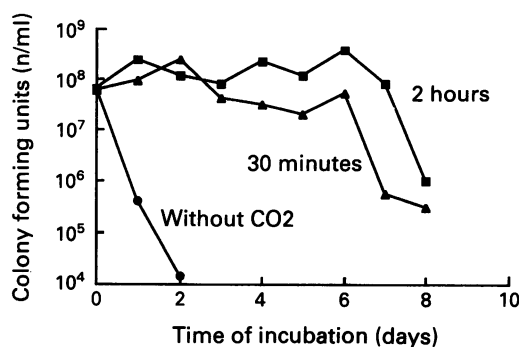


Figure 4 Effect of incubation time in CO₂ on growth of isolate IRL92589 in the liquid medium.

medium for less than two days and viable bacterial cells were decreased considerably (fig 4).

Discussion

H pylori is a microaerophilic organism which does not grow in poor conditions. Many methods for the isolation and subculture of this bacterium on different solid media have been established, but cultivation of this organism in broth, especially to achieve large quantities, still presents problems. Previous studies have shown that growth of *H pylori* is achieved in Brucella broth supplemented with 1% to 10% fetal calf serum.^{12,14} Fetal calf serum or horse serum is essential for optimal growth in tryptone soya broth, brain heart infusion broth, and Mueller-Hinton broth, whether in small or large volumes of liquid media. Tissue culture flasks were first applied by Shadowen *et al*¹³ to culture the organism in a biphasic system (an agar slant with overlying broth in a 2:1 volume ratio, the flask being incubated horizontally), but this system is unsuitable for large volume growth of *H pylori* and is more applicable to recovery of *H pylori* from clinical material. Shahamat *et al* reported a method for growth of large quantities (600 ml) of *H pylori* by several cycles of reincubation in tissue flasks (at least five times, four days each), either by transferring the cultures into new flasks containing an additional volume of liquid media or by adding a measured amount of the media into the flasks containing the cultures, and finally transferring these into 1-L flasks.¹⁴ Unfortunately, the authors did not give the results of their method for large volume cultures. Drawbacks of this method are the prolonged procedure, cumbersome technique, and the hazard of contamination during the reinoculations. Hudson *et al* described a continuous culture technique using a chemostat apparatus, which is more technically demanding, thus limiting its routine use.¹⁵ Secker *et al* also successfully applied gas-permeable Lifecell tissue culture flasks for large quantity cultivation of *H pylori*.¹⁶ Contamination is a hazard during the multiple samplings required unless performed in a laminar flow cabinet. In the present study all the 15 clinical isolates and a reference strain of *H pylori* grew well in the

"CO₂-incubated" tissue culture flasks, each containing 100 ml brain heart infusion supplemented with 5% horse serum and 0.25% yeast extract, despite a relatively low growth of the reference strain NCTC11637. With this method, maximum growth of 6.2×10^7 to 5×10^8 was achieved after two to three days (fig 1), which was comparable with those obtained by other methods.¹²⁻¹⁶ Moreover, the method described above is simpler and easier to perform. It can give the required quantities of bacterial growth in a few days and contamination is not a hazard. This method may therefore provide a simple, reproducible method to produce large quantities of *H pylori*.

To our knowledge this is the first report which has assessed *H pylori* growth in liquid media for a prolonged period of time during which the viable count of the organism dropped to lower than 1×10^4 cfu/ml, thus allowing us to examine the kinetics of *H pylori* growth in the liquid media more completely. Based on the data obtained, *H pylori* strains grew and survived in the medium tested for at least seven to 14 days (a viable count lower than 1×10^4 cfu/ml after several days of incubation usually meant that the organism did not survive; data not shown). Many factors might contribute to this, but the initial inoculum had an important role. Inoculum concentrations, as expected and reported,¹⁴ influenced the kinetics of growth of *H pylori*. The greater the inoculum concentration, the shorter the time to the maximum growth, and the shorter the survival time of *H pylori* in the medium (fig 2). Inocula of 10^6 - 10^7 cfu/ml were found to be the optimal because these produced the maximum growth in the shortest time. In our experience the addition of 1 ml of suspension in nutrient broth with a turbidity equal to McFarland No 2 to No 3 into 100 ml brain heart infusion was the optimal inoculum. If cultivation of the organism for more than eight days was needed, however, an appropriate lower inoculum should be used.

Cultivation of *H pylori* in liquid media is difficult, partly because of contamination. Selective solid media with vancomycin, amphotericin B, and nalidixic acid are commonly used in the isolation of *H pylori* from gastric biopsy specimens.^{4,5,10,12} Vancomycin and amphotericin B are also used in broth cultures to control contamination.^{13,16} Their potential effect on growth of the bacterium, however, has not been determined. This study showed that vancomycin and amphotericin B, as well as nalidixic acid, did not affect growth of *H pylori* in the liquid medium (fig 3), suggesting that these antimicrobial agents can be used alone or in combination to suppress contaminating micro-organisms.

CO₂ is essential for primary isolation of *H pylori* from gastric biopsy specimens. This organism, however, can grow on non-selective solid media in an aerobic atmosphere with high humidity once isolated from gastric biopsy specimens (authors' unpublished observations). Previous studies suggested that

10% CO₂ is required for the growth of *H pylori* in liquid media.^{12,14} In the present study CO₂ was confirmed to be necessary to cultivate *H pylori* in the liquid medium and it must be supplied in an even distribution throughout the broth by placing the flask almost horizontally and shaking the flask at 120 rpm during incubation (fig 4). It was found, however, that 5% CO₂ was sufficient for growth of *H pylori* and could be obtained by placing the loosely capped tissue culture flask containing inoculated medium in a CO₂ incubator with 5% CO₂ for only 30 minutes and then tightening the caps.

In conclusion, *H pylori* can be cultivated in large quantities of brain heart infusion broth supplemented with 5% horse serum and 0.25% yeast extract. Initial inoculum concentrations influence the kinetics of growth of *H pylori* in the liquid medium. Vancomycin, nalidixic acid, and amphotericin B do not affect growth of the organism in broth, and can be used as selective antimicrobial agents if contamination is a hazard. CO₂ is essential for initial growth of *H pylori* in liquid media and a concentration of 5% is sufficient. This can easily be obtained by placing a tissue culture flask of the inoculated medium in a CO₂ incubator containing 5% CO₂ for 30 minutes. The findings of this study may provide a useful, reproducible, and simple method for biochemical, molecular, and physiological studies of *H pylori*, when these require large quantities of the organism.

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