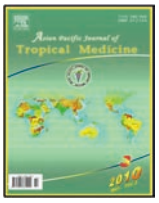
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm



Document heading

Use of reducing agents for the aerobic growth of *Campylobacter jejuni*

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ARTICLE INFO

Article history:
Received 4 November 2009
Received in revised form 17 January 2010
Accepted 10 February 2010
Available online 20 March2010

Keywords:
Camylobacter jejuni
Microaerophilic
Reducing agents
Lid agar technique

ABSTRACT

Objective: To produce a technique for the growth of *Campylobacter jejuni* in aerobic condition **Methods:** Different combinations of reducing agents were tested in brucella broth and the growth turbidity was compared with tubes containing normal broth only. Microaerophilic environment was also provided in a petri plate seeded with *Campylobacter* culture by pouring 3 different concentrations (10%, 5% and 2.5%) of five reducing agents along with bacto-agar in the lid which was used to cover and seal the culture plate. Six reducing agents were also added in broth in concentration of 0.25 mg/mL of each with different combinations. **Results:** In lid agar technique, *Campylobacter jejuni* growth appeared in all three concentrations of reducing agents, that is 10%, 5% and 2.5% after 24 hours of incubation but the best results were observed in 10% concentration. The colonial and morphological characters were not affected when the organisms were grown by this technique. **Conclusions:** It was found that reducing agents enhance the growth of *C. jejuni/coli*. In combination of $FeSO_4$, Na_2CO_3 with H_3BO_3 worked as ideal mixture for the aerobic growth of *Campylobacter*. This technique is more economical as compared to commercially available media in the market and can be used for the oral facultative and microaerophilic bacterial growth in laboratory.

1. Introduction

Campylobacter is now recognized as a major cause of gastroenteritis[1]. It is Gram-ve, microaerophilic bacteria belonging to genus *Campylobacter* and family *Spiralaceae*. Optimal growth occurs at 42 ℃ in an atmosphere of 5% oxygen, 10% carbondioxide and 85% nitrogen[2]. It is postulated that microaerophilic bacteria are more sensitive than other oxygen dependent bacteria to toxic forms of oxygen (super anions, peroxide etc.). To overcome this difficulty, different techniques were developed. Johan H. Brewer et al. in 1986 described a simple method for the cultivation of microaerophiles and carbondioxide requiring organisms[3]. In this technique they used the reducing agents with agar, heated and poured into petridish lid and after solidifying inverted on a petridish base having any type of medium seeded with culture.

We modified their technique with different reducing agents and their combinations. Growth medium was also supplemented with different reducing agents to determine the effect on the growth of *Campylobacter*.

2. Material and methods

2.1. Preparation of mixture for lid agar technique

Three different concentrations of five reducing agents including boric acid (H_3BO_3, Prolab), Sodium carbonate(Na_2CO_3, Merck), sodium bicarbonate($NaHCO_3$, Merck) sodiumdithionite ($Na_2S_2O_4$, Merck) and ascorbic acid (Merck) were used (10 g, 5 g, 2.5 g in 1:1 ratio, that is 0.5 g of each reducing agent). The solutions were prepared in distilled water. 2 g of bacto agar (Difco) was added to the salt solution after which this mixture was autoclaved at 121 ℃ for 15 minutes and poured on the lid of petri dish and allowed to solidify. Plates inoculated with culture were inverted on the lids and incubated aerobically at 42 ℃ for 24, 48, and 72 hours.

2.2. Combined effect of reducing agents in broth medium

10 × solution of ferrous sulphate ($FeSO_4$) was prepared by adding 2.5 mg in 10 mL brucella broth (Difco). For working solution 1 mL of 10 × solutions was added to 9 mL of brucella broth. Ferrous sulphate and its concentration were kept constant while the second reducing agent was different. The concentration of each was 2.5 mg/10 mL. Solutions of these reducing agents were prepared fresh and kept in refrigerator at 8 ℃ in dark bottles. Duplicate tubes with brucella broth of different combinations of reducing agents were inoculated with culture (adjusted spectrophotometrically, 5 unit Mc Farland). One set of tubes was incubated aerobically and the other microaerobically kept at 37 ℃ for over night. Control objectives which grew without reducing agents were also tested. The next day, turbidity was observed and optical density was taken with the help of spectrophotometer.

3. Results

After 24 hours incubation, in presence of 2.5%, 5% and

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10% reducing agents combination, the *C. jejuni* growth was weak, while in presence of 10%, it was mederate. After 48 hours of incubation, heavy growth of *C. jejuni* was shown in the presence of 10% reducing agent combination, moderate growth in 5% and weak in 2.5%, the morphology (microscopic and colonial characteristics) of these organisms did not seems to be affected by reducing agents. The above mentioned results indicated in the presence of10% reducing agents combination, *C. jejuni* grew well giving pin point isolated colonies.

In order to evaluate the inhibitor or growth enhancing effect of reducing agents, we supplemented muller hinton broth (MHB) with the same reducing agents including ferrous sulphate ($FeSO_4$). Maximum growth of CFU/mL was obtained when 0.25 mg/mL of $FeSO_4$ was added to MHB along with any one of the reducing agents. Heavy growth appeared in broth tube containing 0.5 mg/mL $FeSO_4$ incubated aerobically as compared to the duplicate kept under anaerobic conditions (Table 1). Optical density is given in Table 2.

Table 1

Effect of reducing agents in broth medium (Different combination of two reducing agents keeping $FeSO_4$ as constant).

$FeSO_4$+$Na_2S_2O_4$		$FeSO_4$+Na_2CO_3		$FeSO_4$+$NaHCO_3$		$FeSO_4$+H_3BO_3		$FeSO_4$+Ascorbic Acid		$FeSO_4$+$FeSO_4$	
AA	A	AA	A	AA	A	AA	A	AA	A	AA	A
a.+	++	++	++++	++	+++	+	++	++	+++	++	++++
b.++	+++	+++	++++	+++	++++	+	+++	++	+++	+++	++++

Table 2

Effect of reducing agents in broth medium (optical density) （mg/mL）.

Reducing Agents	$FeSO_4$+Na_2SO_4	$FeSO_4$+Na_2CO_3	$FeSO_4$+$NaHCO_3$	$FeSO_4$+H_3BO_3	$FeSO_4$+Ascorbic Acid	$FeSO_4$+$FeSO_4$
Aerobic	0.4	0.15	0.3	0.1	0.15	0.3
Anaerobic	0.2	0.08	0.07	0.04	0.09	0.1

4. Discussion

Johan H. Brewer[3] reported that microaerophilic organisms can easily grow aerobically by a similar technique, but we modified their technique by using different concentrations and addition of some more reducing agents to the lid agar technique for the aerobic cultivation of *Campylobacter*.

It is believed that iron has an enhancing effect on the growth of *Campylobacter*, but the exact mechanism is not clear[4]. *Campylobacter* is a poor producer of iron chelating substances as such additional exogenous iron is required for its growth. Keeping this point in mind we used $FeSO_4$ along with other reducing agents in broth medium and it was observed that not only the organism can grow aerobically but the growth is also enhanced. Microaerophilism may possibly represent the resultant of several factors and the involvement of iron and iron binding compounds. We propose the working hypothesis that microaerophilic such as *C. jejuni* is unable to synthesize iron binding compounds quickly enough or in amounts sufficient to increase iron availability to the point where the small inocula could grow in 21% oxygen (atmospheric). This situation can be overcome by the addition of $FeSO_4$ along with any other reducing agents. The addition of 0.5 mg/mL $FeSO_4$ may allow the low level of microbial iron binding compounds produced by *C. jejuni* as observed by other scientists[6]. In this case, iron containing enzymes such as cytochromes, catalase, or succinic dehydrogenase are not needed in large concentrations to keep oxygen from reaching the interior of the cell and destroying vital cell components[7].

Additional iron for aerobic growth of *Campylobacter* may also be required for Ferro-superoxide dismutase enzyme activity[8-11].

Incorporation of different combination of reducing agents along with $FeSO_4$ in the growth medium showed much better results as compared to commercially available medium suggesting that reducing agents can be used for the aerobic cultivation of *Campylobacter*. This study also suggests that we can use this technique for the aerobic cultivation of microaerophilic and facultative anaerobic oral bacteria. This technique is more economical as compared to the commercially available media in market.

Conflict of interest statement

We declare that we have no conflict of interest.

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