A study of the oxygen and carbon dioxide requirements of thermophilic campylobacters

FJ BOLTON, D COATES

From the Public Health Laboratory, Preston Infirmary, Meadow Street, Preston, Lancashire PR1 6PS

SUMMARY The oxygen and carbon dioxide requirements of different biotypes of thermophilic campylobacters were investigated by means of (a) quantitative studies, and (b) total growth studies. Oxygen tolerance of the five test organisms differed markedly and varied with the carbon dioxide concentration. At most carbon dioxide concentrations tested, *Campylobacter jejuni* strains NCTC 11168 and NCTC 11392 tolerated 21% oxygen (growth reduced), *C. coli* NCTC 11353 tolerated 15% oxygen (growth reduced), and *C. jejuni* ATCC 3036 and (nalidixic acid resistant thermophilic campylobacter) NCTC 11352 tolerated 10% oxygen (growth not reduced). Total growth studies indicated that 10% oxygen was the optimal concentration for growth of the five test organisms. All exhibited a requirement for carbon dioxide, and only *C. jejuni* strains NCTC 11168 and NCTC 11392 tolerated its absence (growth reduced), when the oxygen concentration was low. The studies indicated that atmospheres containing 5% to 10% oxygen and 1.0% to 10% carbon dioxide are suitable for growth of the various biotypes of thermophilic campylobacters.

The oxygen and carbon dioxide concentrations produced in anaerobic jars by variations of the evacuation-replacement technique were determined and suitable practices identified.

Although it is known that campylobacters are microaerophilic, little information is available on the optimum and range of oxygen and carbon dioxide concentrations required for culture of the different biotypes. Kiggins and Plastringe¹ reported that an atmosphere of 5% oxygen and 10% carbon dioxide in nitrogen was optimal for *Campylobacter fetus* (*Vibrio fetus* of bovine origin), and Dekeyser *et al*² and Skirrow³ found that similar mixtures were suitable for *C. jejuni* and *C. coli*.

Several methods of producing a microaerobic atmosphere suitable for campylobacters have been examined. Butzler & Skirrow⁴ drew a partial vacuum (500 mm Hg) in a carbon dioxide incubator or anaerobic jar (without catalyst) and refilled with a 15% carbon dioxide/85% nitrogen or hydrogen mixture (evacuation-replacement technique). Other workers have evacuated to different negative pressures and refilled with different gas mixtures. DeBoeck⁵ incorporated the aerotolerant supplement (a mixture of ferrous sulphate, sodium matabisulphite and sodium pyruvate) of George *et al*⁶ into Butzler's medium⁴ and cultured specimens in a candle jar. A more convenient method is to use

one of the commercial gas generating envelopes recently introduced. In a previous study⁷ we analysed the oxygen and carbon dioxide concentrations produced by these methods and tested the suitability of the atmospheres for culture of the different *Campylobacter* biotypes.⁸ The present study was carried out to determine the optimum and range of oxygen and carbon dioxide concentrations required for culture of the different biotypes.

Material and methods

TEST ORGANISMS AND MEDIA

The test organisms used were *Campylobacter jejuni* biotype 1 (8) NCTC 11168 and ATCC 3036, *C. jejuni* biotype 2 (8) NCTC 11392, *C. coli* NCTC 11353, and nalidixic acid resistant thermophilic campylobacter (NARTC) NCTC 11352.

The organisms were grown on Columbia agar (Oxoid CM331) containing 5% horse blood for 24 h at 42°C in a microaerobic atmosphere produced in an anaerobic jar (catalyst removed) by evacuation to 500 mm Hg and refilling with 10% carbon dioxide in nitrogen. Bacteria were harvested into 0.1% peptone water (Oxoid L37) and the suspensions standardised to approximately 2×10^8 colony forming

units (CFU) per ml with a Perkin-Elmer model 6/20 spectrophotometer at a wavelength of 450 nm. Ten-fold dilutions over six steps were made in 0.1% peptone water.

The recovery medium used in tests was nutrient broth No 2 (Oxoid CM67) containing 2% New Zealand agar and 5% lysed horse blood, prepared as plates or slopes in bijoux bottles. Plates were dried at room temperature.

JARS

Anaerobic jars (Baird & Tatlock) were used without catalysts. An adjustable needle valve was fitted to each lid and side arm for input of gas mixtures and removal of gas samples respectively.

PRODUCTION OF DIFFERENT MICROAEROBIC ATMOSPHERES

Gas mixtures containing <0.5% to 21% oxygen and 0 to 15% carbon dioxide were made up in 20 l plastic bags from oxygen, carbon dioxide and nitrogen in cylinders (BOC special gases). After gas analysis the mixtures were introduced into jars that had been evacuated to 650 mm Hg and which contained cultures of test organisms. A gas sample from each jar was taken for analysis and where necessary a second evacuation-replacement cycle was carried out.

ANALYSIS OF GAS SAMPLES

Gas samples were removed from the jars using a vacuum pump which was connected directly to the analysers. Carbon dioxide was determined with a Lira Infrared Analyser, model 303 (Mine Safety Appliances Company, Pittsburgh, Pennsylvania, USA) set at a range of 0 to 10% and accurate to 0.1%. Concentrations >10% were determined with Draeger gas detection equipment. Oxygen was determined with an oxygen analyser, model OA272 (Taylor Servomax, Sybron Corporation, Rochester, New York, USA) set at a range of 0 to 25% and accurate to 0.25%.

GROWTH OF TEST ORGANISMS IN DIFFERENT MICROAEROBIC ATMOSPHERES

Quantitative studies

The ability of the various atmospheres to support the growth of small numbers of test organisms was studied quantitatively using the method of Miles *et al.*⁹ Plates of recovery medium were inoculated with the 10^{-5} and 10^{-6} dilutions of standardised suspensions using a 50-dropper pipette and incubated in the test jars for 48 h at 42°C. A control set of plates was incubated in a jar evacuated to 500 mm Hg and refilled with 10% carbon dioxide in nitrogen (routine laboratory procedure). Counts were made and a growth index calculated by dividing the counts

obtained on the test plates by those on the control plates.

Total growth studies

The total growth of test organisms produced in the various atmospheres was determined by measuring the optical density of harvested suspensions. Two slopes of recovery medium were inoculated using a 1/300 ml loop with the 10^{-3} dilution of a standardised suspension and incubated with caps loosened in the test jars for 48 h at 42°C. After incubation 1 ml of 0.5% formalin in 0.1% peptone water was pipetted onto each slope and also onto an uninoculated slope (for blanking the spectrophotometer). Growth from each of the inoculated slopes was harvested and 0.2 ml of each suspension pipetted into the well of a Linbro, flat-bottomed microtitre plate. The optical density of each suspension, relative to the blank, was measured using a microelisa mini reader model MR90 (Dynatech Instruments Ltd.) at a wavelength of 490 nm, and the mean calculated.

INVESTIGATION OF ATMOSPHERES PRODUCED BY DIFFERENT EVACUATION-REPLACEMENT TECHNIQUES

Jars were evacuated to 400, 500, 600 and 700 mm Hg and refilled with mixtures of 5%, 10%, 15% and 20% carbon dioxide in nitrogen. Gas samples were then taken for analysis. All experiments were done in duplicate.

Results

QUANTITATIVE STUDIES

The Table gives the growth indices obtained for the test organisms in atmospheres of varying oxygen and carbon dioxide concentrations. Differences between the mean counts obtained in the test and control atmospheres were analysed by a two sample *t* test for each strain separately at the 5% level of significance. Growth indices significantly different from 1.00 are asterisked in the Table.

In the absence of carbon dioxide *C jejuni* ATCC 3036, *C coli* and the NARTC strain all failed to grow on the test plates; *C jejuni* strains NCTC 11168 and NCTC 11392 grew in atmospheres containing 1% and 5% oxygen but the growth indices were significantly less than 1.00.

In atmospheres containing less than 0.5% oxygen *C jejuni* NCTC 11168 and ATCC 3036 grew satisfactorily in carbon dioxide concentrations ranging from 2.5% to 15%, and the other test organisms in carbon dioxide concentrations of 1% to 15%. In atmospheres containing 1% oxygen, all of the test organisms grew satisfactorily in carbon dioxide concentrations ranging from 1% to 15%. In atmos-

Growth indices of test organisms (counts obtained in test atmospheres/counts obtained in the control atmosphere) cultured in atmospheres of varying oxygen and carbon dioxide concentrations.

Test organism	% oxygen	% carbon dioxide					
		0	1	2.5	5	10	15
<i>C jejuni</i> NCTC 11168	<0.5	Not done	0.53*	0.89	1.03	0.84	1.10
	1	0.07*	1.21	1.18	1.16	1.17	1.16
	5	0.06*	0.99	0.98	0.99	0.98	0.98
	10	0	1.13	1.15	1.12	1.10	1.15
	15	0	0.35*	0.58*	0.60*	0.71*	0.76
	21	0	0.34*	0.41*	0.39*	0.30*	0.28*
<i>C jejuni</i> ATCC 3036	<0.5	Not done	0.53*	0.98	1.15	1.02	0.93
	1	0	1.02	1.02	1.01	0.98	0.97
	5	0	0.96	0.81	0.78	0.96	0.63*
	10	0	0.97	0.95	0.79	0.78	0.51*
	15	0	0	0	0	0.49*	0
	21	0	0	0	0	0	0
<i>C jejuni</i> NCTC 11392	<0.5	Not done	1.02	0.96	1.17	1.16	1.02
	1	0.61*	0.92	0.91	0.92	0.89	0.85
	5	0.75*	0.95	1.01	1.05	1.03	0.88
	10	0	1.03	0.98	1.15	1.11	0.86
	15	0	0.81	0.82	0.68*	0.72*	0.44*
	21	0	0.57*	0.50*	0.48*	0.38*	0
<i>C coli</i> NCTC 11353	<0.5	Not done	1.02	1.09	1.09	1.09	1.05
	1	0	0.98	0.96	0.96	0.92	0.92
	5	0	0.78	0.79	0.97	1.01	0.90
	10	0	1.02	0.85	0.95	0.86	0.88
	15	0	0	0.89	0.29*	0.32*	0.12*
	21	0	0	0	0	0.08*	0
<i>NARTC</i> NCTC 11352	<0.5	Not done	0.99	1.00	1.01	0.88	1.02
	1	0	1.02	0.97	0.82	0.77	0.79
	5	0	1.01	1.10	1.06	1.35†	1.10
	10	0	1.03	0.91	0.86	0.95	0.88
	15	0	0	0	0	0	0
	21	0	0	0	0	0	0

*Growth index significantly less than 1.00 (5% level).

†Growth index significantly greater than 1.00 (5% level).

0: No growth of test organism on test plate.

pheres containing 5% to 10% oxygen. *C jejuni* strains NCTC 11168 and NCTC 11392, *C coli* and the NARTC strain all grew satisfactorily in carbon dioxide concentrations ranging from 1% to 15%, and *C jejuni* ATCC 3036 in carbon dioxide concentrations of 1% to 10%. The growth index of the NARTC strain was significantly greater than 1.00 when grown in an atmosphere containing 5% oxygen and 10% carbon dioxide. In atmospheres containing 15% oxygen growth on the test plates was generally much reduced or absent; *C jejuni* NCTC 11168 grew satisfactorily in 15% carbon dioxide, *C jejuni* NCTC 11392 in 1% and 2.5% carbon dioxide, and the *C coli* strains in 2.5% carbon dioxide. In atmospheres containing 21% oxygen in all cases growth on the test plates was either very much reduced or absent.

TOTAL GROWTH STUDIES

Figure 1 shows the total growth patterns of the five test organisms on slopes of recovery medium after incubation in atmospheres containing oxygen con-

centrations ranging from 1% to 21%, and carbon dioxide concentrations ranging from 0% to 15%.

Figure 1a demonstrates that in the absence of carbon dioxide *C jejuni* NCTC 11168 grew poorly or not at all, depending on the oxygen concentration. However, in the presence of 1% to 15% carbon dioxide the total growth was good and tended to increase slightly with carbon dioxide concentration. With regard to oxygen concentration, the total growth was good over the range 1% to 21% with a peak at 10%. An atmosphere containing 5% to 15% carbon dioxide and 10% oxygen was optimal.

Figure 1b demonstrates that the total growth patterns of *C jejuni* ATCC 3036 in different concentrations of carbon dioxide were variable. The organism failed to grow in the absence of carbon dioxide, and only grew in the presence of 15% oxygen when the carbon dioxide concentration was 10%. An atmosphere containing 1% to 5% carbon dioxide and 10% oxygen was optimal.

Figure 1c demonstrates that the total growth patterns of *C jejuni* NCTC 11392 were similar to those

Fig. 1 Total growth patterns of test organisms on slopes after incubation in atmospheres of varying oxygen and carbon dioxide concentrations.

of *C. jejuni* NCTC 11168; an atmosphere containing 2.5% to 10% carbon dioxide and 10% oxygen was optimal.

Figure 1d demonstrates that the total growth patterns of *C. coli* NCTC 11353 in atmospheres containing 1% to 15% carbon dioxide were similar over the oxygen range 1% to 10% but diverged at higher oxygen concentrations. The organism failed to grow in the absence of carbon dioxide, and only grew in the presence of 21% oxygen when the carbon dioxide concentration was 10%. An atmosphere containing 2.5% to 15% carbon dioxide and 10% oxygen was optimal.

Figure 1e demonstrates that the total growth patterns of NARTC NCTC 11352 in atmospheres containing 1% to 15% carbon dioxide were similar over the oxygen range 1% to 10%. The organism failed to grow in the absence of carbon dioxide or in the presence of 15% oxygen. An atmosphere containing

2.5% to 10% carbon dioxide and 10% oxygen was optimal.

ATMOSPHERES PRODUCED BY DIFFERENT EVACUATION-REPLACEMENT TECHNIQUES

Figure 2 shows the oxygen and carbon dioxide concentrations produced in anaerobic jars evacuated to different pressures and then refilled with mixtures of 5%, 10%, 15% and 20% carbon dioxide in nitrogen. It can be seen that the oxygen concentration produced in the jars decreased as the evacuation pressure increased. The oxygen concentrations produced with different evacuation pressures were approximately 14% with 400 mm Hg, 9.5% with 500 mm Hg, 5% with 600 mm Hg and 1.5% with 700 mm Hg. Conversely, the carbon dioxide concentrations produced increased as the evacuation pressure increased, and also with the concentration

Fig. 2 Oxygen and carbon dioxide concentrations produced in anaerobic jars by different evacuation-replacement techniques. $\square$ = oxygen concentration range after replacement with 5–20% carbon dioxide in nitrogen mixtures; $\circ$ = carbon dioxide concentration after replacement with 5% carbon dioxide in nitrogen; $\triangle$ = carbon dioxide concentration after replacement with 10% carbon dioxide in nitrogen; $\bullet$ = carbon dioxide concentration after replacement with 15% carbon dioxide in nitrogen; $\blacktriangle$ = carbon dioxide concentration after replacement with 20% carbon dioxide in nitrogen.

of carbon dioxide present in the mixture used to refill the jar. After evacuation to 500 mm Hg, which is usually done,⁴ the carbon dioxide concentrations produced after refilling with different mixtures were approximately 3.25% with 5% carbon dioxide in nitrogen, 4.75% with 10% carbon dioxide in nitrogen, 8% with 15% carbon dioxide in nitrogen, and 10.5% with 20% carbon dioxide in nitrogen.

Discussion

The thermophilic campylobacters are microaerophilic, and many different methods of producing microaerobic atmospheres have been proposed for their isolation. In a previous study⁷ we found that the oxygen and carbon dioxide concentrations present in atmospheres produced by the different methods varied markedly. Hence, in order to ascertain which methods were best, we set out to determine the optimum and range of oxygen and carbon dioxide concentrations required for culture of the different biotypes of thermophilic campylobacters.

The present study has demonstrated that the mic-

roaerophilic nature of different thermophilic campylobacters varies greatly, and that carbon dioxide is important for growth. *C. jejuni* strains NCTC 11168 (biotype 1) and NCTC 11392 (biotype 2) were the most aerotolerant test organisms which grew over the oxygen concentration range <0.5% to 21% but with colony counts generally reduced in $\geq 15\%$ oxygen. *C. coli* NCTC 11353 was the next most aerotolerant test organism which grew over the oxygen concentration range <0.5% to 15% at most carbon dioxide concentrations tested but with colony counts generally reduced in 15% oxygen; slight growth occurred in an atmosphere of 21% oxygen and 10% carbon dioxide. *C. jejuni* ATCC 3036 (biotype 1) was one of the least aerotolerant test organisms which grew well over the oxygen concentration range <0.5% to 10%, but which generally failed to grow in 15% oxygen. NARTC NCTC 11352 was the least aerotolerant test organism which grew well over the oxygen concentration range <0.5% to 10%, but not at all in 15% oxygen. Total growth studies showed that atmospheres of 5% to 10% oxygen and 1% to 10% carbon dioxide produced very good growth of all of the test organisms.

In our previous study⁷ we measured the oxygen and carbon dioxide concentrations present in atmospheres produced by different methods of achieving microaerobiosis. An Oxoid BR60 envelope produced 8.5% oxygen and 4.8% carbon dioxide in an Oxoid 3.5 l jar. A BBL CampyPak envelope produced 5.8% oxygen and >10% carbon dioxide in a BBL 2.5 l jar. The present studies show that both of these methods produce atmospheres satisfactory for growth of the different biotypes. The evacuation-replacement technique is widely used to achieve microaerobiosis. Butzler and Skirrow⁴ recommended an evacuation pressure of 500 mm Hg and refilling with 15% carbon dioxide in nitrogen. Our assays have shown (Fig. 2) that this procedure will produce an atmosphere of approximately 9.75% oxygen and 8.0% carbon dioxide which is satisfactory for growth of the different biotypes. Our results show that evacuation pressures of 500–600 mm Hg and refilling with mixtures of 5% to 15% carbon dioxide in nitrogen should produce atmospheres satisfactory for growth of the different biotypes. In our previous study⁷ a candle was found to produce 17.0% to 18.5% oxygen and 1.2% to 1.5% carbon dioxide concentrations in jars of different volume. In the present study it was found that *C. jejuni* strains NCTC 11168 and NCTC 11392 generally tolerated 21% oxygen, *C. coli* NCTC 11353 generally tolerated 15% oxygen, and *C. jejuni* ATCC 3036 and NARTC NCTC 11352 tolerated 10% oxygen. In previous studies with these organ-

isms^{7,10} only *C jejuni* strains NCTC 11168 and NCTC 11392 grew in a candle jar, which conforms with the above findings and demonstrates that a candle jar is only suitable for the growth of relatively aerotolerant strains.

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Requests for reprints to: Mr FJ Bolton, Public Health Laboratory, Preston Infirmary, Meadow Street, Preston, Lancashire PR1 6PS, England.

Letters to the Editor

Pseudohyponatremia and hyperviscosity

Serum from patients with hyperviscosity have low sodium values when analysed by flame photometry.¹ The reason for the spuriously low sodium is related to problems with sample aspiration and dilution by the instrument and also because of the decrease in plasma water due to high protein concentration.¹⁻³ Methods that are available for measuring serum sodium include the traditional technique of flame photometry and more recently the use of an ion-selective electrode. When the serum sample is diluted before analysis by the electrode (indirect potentiometry), the serum sodium values would be expected to be low in the presence of hyper-

proteinaemia and hyperviscosity. With direct potentiometry where no sample dilution takes place, no interference would be expected since the activity of sodium in the water phase only is being measured. The present study was undertaken to determine the magnitude of the decrease in serum sodium in viscous sera using flame photometric and indirect potentiometric measurements for sodium measurement.

Patients and methods

Samples were obtained from patients at the Vancouver General Hospital whose sera when analysed were noted to have increased viscosity at 37°C (Table). The range of serum viscosities was 3.0-17.8

centistokes (normal is <1.8). Serum sodium concentrations were measured in duplicate by flame photometry (FP) (Beckman KLiNa flame: Beckman Instruments Inc, Fullerton, California, indirect potentiometry (IP) Beckman Astra, Beckman Instruments Inc, Fullerton, California) and by direct potentiometry (DP), (Nova-1 Na/K analyser, Nova Biomedical Inc, Newton, Massachusetts). Serum viscosity relative to normal saline was measured using a Cannon Manning Viscometer. The differences in serum sodium obtained between measurements by DP and IP (ΔNa_i) and DP and FP (ΔNa_o) were correlated with measurements of serum viscosity.

Results

Figure 1 shows the correlation ($r = 0.95$, $p < 0.001$) between ΔNa_i and serum viscosity. Figure 2 shows the correlation ($r = 0.71$, $p < 0.01$) between ΔNa_o and serum viscosity. For serum sodium measurements performed by indirect potentiometry, a 2 mmol/l decrease is seen for every one centistoke increase in serum viscosity.

Discussion

Dangerous pseudohyponatremia can occur in patients with hyperviscosity (Table). We wish to draw attention to the fact that the degree of pseudohyponatremia is dependent upon the technique that is used for

Laboratory parameters in the patients with hyperviscosity

Patient	Class and type of paraprotein	Serum viscosity at 37°C	Serum sodium (normal 135-145 mmol/l) measured by		
			Flame photometry	Potentiometry	
				Indirect	Direct
1	IgG κ	15	130	112	142
2	IgM κ	4.8	141	141	150
3	IgM λ	10.2	143	134	150
4	IgM κ	3.3	137	138	143
5	IgG κ	13.3	128	117	144
6	IgG κ	17.8	127	101	143
7	IgG λ	8.8	132	128	146
8	IgM λ	11.1	136	128	145
9	IgM κ	4.9	133	131	144
10	IgG λ	5.7	141	137	148
11	IgM κ	8.9	136	129	146