Onderstepoort J. vet. Res., 54, 553–556 (1987)

A COMPARATIVE STUDY OF THE GROWTH OF *CAMPYLOBACTER FETUS* STRAINS IN LIQUID MEDIA

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ABSTRACT

VAN DER WALT, MARTHA L., 1987. A comparative study of the growth of *Campylobacter fetus* strains in liquid media. *Onderstepoort Journal of Veterinary Research*, 54, 553–556 (1987).

The growth of *C. fetus fetus*, *C. fetus venerealis* and *C. fetus venerealis* biotype *intermedius* was examined in 10 liquid media. From the data obtained, a 10 % inoculum size and an oxygen level of 6 % seemed imperative for consistent growth, especially for the *C. fetus venerealis* strain. A lowered redox potential obtained by the addition of 0,1 % cysteine-HCl to the media was stimulatory. The medium which yielded the best growth was the one described by Dennis & Jones (1959). The fastidious *C. fetus venerealis* strain yielded maximum values of 0,5 % packed cell volumes after 48 h cultivation in a microaerophilic atmosphere on this medium. The other strains yielded higher values.

INTRODUCTION

The group of organisms known as *Campylobacter fetus* consist of 2 subspecies, *C. fetus fetus* and *C. fetus venerealis*. Intermediate to the 2 subspecies is a biochemical variant known as *C. fetus venerealis* biotype *intermedius*. All 3 of these organisms can cause bovine genital campylobacteriosis, which is preventable by immunization (Garcia, Eaglesome & Rigby, 1983). The Veterinary Research Institute, Onderstepoort, currently produces a vaccine which contains 2 *C. fetus fetus* strains and a *C. fetus venerealis* biotype *intermedius* strain. The vaccine *C. fetus venerealis* strain does not grow adequately on the medium employed for the other 3 strains. The aim of this study was to critically evaluate the growth supporting properties of different media and to define conditions that would consistently give acceptable yields of *C. fetus venerealis* as well.

C. fetus organisms are obligate aerobes, as they need oxygen for their respiratory metabolism (Smibert, 1984). Energy for growth is obtained from the tricarboxylic acid cycle, while no carbohydrates are used. However, the organisms are microaerophilic (Smibert, 1984) in that the oxygen concentration in air (20 %) is toxic for them. Oxygen levels of 6 % appear to be optimal. Even at optimal oxygen levels the organisms are still slow growers. Long incubation periods and high inoculum levels are required to obtain enough cells for vaccine production.

A number of different liquid media have been described for the high bulk growth of *C. fetus* by various authors (see Materials and Methods). The media consisted mainly of peptone-yeast extract bases, while some contained organic acids and/or reducing agents as well. Of these media, 10 which were developed by various workers over the last 3 decades, were used in this study.

MATERIALS AND METHODS

Media

The media studied are listed in Table 1. They were prepared as described by the various authors. To distinguish the different media, they were named according to their authors or origins: Botha's (Cameron, 1982), Onderstepoort vaccine medium (OPVM) (Cameron, 1982), Brewer's thioglycollate medium (Simon, 1976), Berg's (Berg & Firehammer, 1978), Dennis' (Dennis & Jones, 1959), Clark's (Clark, Dufty & Monsborough, 1972), Robertstad's (Robertstad & Morrison, 1957), Bryner's[1], Manclark's (Manclark & Picket, 1960) and McCoy's (McCoy, Doyle, Burda, Corbeil & Winter, 1975). The major components of the media is given in Table 1.

Cultures

The strains used in the study were as follows: *C. fetus fetus* strains 68/4 and 7572, *C. fetus venerealis* strain 796/1 and *C. fetus venerealis* biotype *intermedius* strain 873/5. Strain 796/1 is presently not included in the vaccine.

Experimental procedures

Growth from 48 h blood agar plates, incubated at 37 °C in anaerobic jars containing a microaerophilic atmosphere of 6 % O_2, 10 % CO_2 and 84 % N_2, was harvested in phosphate buffered saline (PBS) pH 7,2. The suspended cells were used to inoculate tubes of Albimi Brucella broth[2] containing 0,16 % agar. The tubes were incubated for 48 h at 37 °C in an aerobic atmosphere. The growth obtained from 3 tubes was used to inoculate Erlenmeyer flasks containing 30 mℓ of the medium to be examined. These flasks were incubated for 24 h stationary at 37 °C followed by 24 h on a gyratory laboratory shaker. The growth in these flasks was used to inoculate 150 mℓ of medium in Erlenmeyer flasks as a 5 % or a 10 % inoculum. Incubation was as for the smaller flasks. All experiments were repeated 3 times.

Flasks were incubated in an aerobic atmosphere, or put into anaerobic jars, which were subsequently gassed to obtain an atmosphere of 6 % O_2, 10 % CO_2 and 84 % N_2. Aerobic incubation and a 5 % inoculum were first used, but as very poor growth resulted, a 10 % inoculum and a microaerophilic atmosphere were used instead. Later, the influence that the different inocula and oxygen tensions had on growth was examined.

Growth obtained from the 150 mℓ of media was measured as per cent packed cell volumes (PCV) in Hopkin's tubes[3], which were centrifuged for 1 h at 1 000 *g*. Purity of inocula and final growth were checked by Gram-stained smears.

Redoxpotential (Eh) determinations

As many of the media contained a reducing agent, the Eh of the various media was examined by adding redox indicators. Methylene blue or resazurin were added at 0,001 % concentrations. Methylene blue changes from blue to colourless at Eh values of 11 mV and resazurin at

Received 26 June 1987—Editor

[1] National Animal Disease Centre, U.S.A., Working document

[2] Difco Laboratories, Laboratory and Scientific Equipment Co. (Pty) Ltd, P.O. Box 45125, Mayfair, Johannesburg 2108

[3] Kimble, Laboratory and Scientific Equipment Co. (Pty) Ltd, P.O. Box 45125, Mayfair, Johannesburg 2108

TABLE 1 Comparative composition of the 10 media examined

Medium	Medium components in g/ℓ			Reducing agent
	Peptone	Yeast extract	Succinate	
Botha's	20	5	—	—
OPV	20	5	—	—
Brewer's	10	—	—	0,1 % thioglycollate
Bryner's[1]	20	5	2	0,1 % thioglycollate
Dennis'	10	5	2	0,1 % cysteine-HCl
Clark's	30	5	13	0,02 % cysteine-HCl
Robertstad's	+		—	0,005 % glutathione
Berg's	+	5	2	0,025 % thioglycollate
Manclark's	30	—		0,02 % cysteine-HCl
McCoy's	+			*

\+ Brucella broth is used as the base

* 12,5 mg glutathione, 37,5 mg thioglycollate, 200 mg cysteine-HCl per litre

[1] Contains 5 g/ℓ NaCl and 1 g/ℓ $MgCl_2$ as well

−51 mV (Costilow, 1981). Eh measurements were conducted in uninoculated media under aerobic and microaerophilic atmospheres.

RESULTS

Aerobic incubation and 5 % inoculum

The aerobic incubation of flasks and the addition of a 5 % inoculum to the 150 mℓ quantities of media resulted in very poor, inconsistent growth for the strains on the media of Botha, OPV, Brewer and Berg (Table 2). The *C. fetus fetus* strain 7572 grew the best of the 4 strains, in this first part of the experiment where aerobic incubation was used (Table 2).

Microaerophilic incubation and a 10 % inoculum

The use of an atmosphere with a lowered oxygen tension (6 % O_2) and the use of a larger inoculum, resulted in consistent and reproducible growth for the 4 strains studied (Table 3).

TABLE 2 % PCV obtained after growth of the *C. fetus* strains in an aerobic atmosphere and with the use of a 5 % inoculum

Medium	Strains			
	796/1	873/5	68/4	7572
Botha	NG	0,5	0,6	0,55
OPV	0,2	NG	NG	NG
Brewer	NG	NG	NG	NG
Berg	NG	NG	NG	0,2
Dennis	NG	NG	NG	NG

NG = no growth

TABLE 3 % PCV obtained after growth of the *C. fetus* strains in a microaerophilic atmosphere with the use of a 10 % inoculum

Medium	Strains			
	796/1	873/5	68/4	7572
Botha's	0,4	0,5	0,5	1,0
OPV	0,6	1,0	0,5	0,3
Brewer's	NG	NG	NG	NG
Berg's	0,2	0,4	0,6	0,6
Dennis's	0,5	0,5	0,5	0,5
Clark's	0,4	0,9	0,5	1,0
Robertstad's	0,3	0,7	0,5	0,8
Bryner's	0,4	0,2	0,5	0,6
Manclark's	0,5	0,2	0,4	0,7
McCoy's	0,2	0,3	0,5	0,4

NG = no growth

The growth of the *C. fetus venerealis* strain 796/1 was taken as the criterion to judge the ability of a medium to yield acceptable growth. The OPV medium yielded the highest % PCV value for strain 796/1, but on later repetitions this strain grew less well on this medium. If the PCV values for all 4 strains together were taken as the criterion Dennis' medium gave the best results (Table 3).

Influence of inoculum size and oxygen tension

To determine the influence different oxygen concentrations and inoculum sizes had on growth, the growth of strains 796/1 and 7572 were examined on Dennis' medium (Table 4). A lowered oxygen potential was essential for the growth of the *C. fetus venerealis* strain 796/1 and it improved the growth of *C. fetus fetus* 7572. Irrespective of the size of the inoculum, strain 796/1 was never capable of growing in an aerobic atmosphere, while strain 7572 was able to grow aerobically if a 10 % inoculum was used (Table 4). In the microaerophilic atmosphere, the larger inoculum improved the growth of strain 796/1, but not of strain 7572.

TABLE 4 The influence of the amount of oxygen and the size of the inoculum on the % PCV of strains 796/1 and 7572 grown in Dennis' medium

% oxygen	% inoculum	Strains	
		796/1	7572
20	5	NG	NG
20	10	NG	0,1
6	5	0,1	0,4
6	10	0,5	0,4

NG = no growth

Redox potential determinations

The 2 redox indicators used revealed differences in the Eh-values of the various media studied (Table 5). For most of the media, Clark's and Manclark's being exceptions, the atmospheric conditions did not have an influence on the Eh of the medium. The microaerophilic atmosphere showed a lowering of the redox potential of Clark's and Manclark's media. All the media except the 3 which had cystein as reducing agents (Table 5) had Eh values > 11 mV. Dennis' medium had the lowest redox potential, i.e. lower than −51 mV, even in the aerobic atmosphere. Clark's medium had Eh values below −51 mV in the microaerophilic atmosphere, but in the aerobic atmosphere a value of 11 mV was recorded. In the microaerophilic atmosphere Manclark's medium recorded an Eh of 11 mV.

DISCUSSION

The various authors have used different parameters to express the yield from the media used. Cell counts were used (McCoy *et al.*, 1975), optical density measurements (Robertstad & Morrison, 1957) and yield in g

TABLE 5 Eh determinations of the liquid media

Medium	% added of reducing agent	Eh in mV according to colour change			
		Microaerophilic atmosphere (6 % O_2)		Aerobic atmosphere (20 % O_2)	
		Methylene blue	Resazurine	Methylene blue	Resazurine
Botha's	—	>11	−51	>11	> − 51
OPV	—	>11	−51	>11	> − 51
Robertstad's	0,005 % glutathione	>11	−51	>11	> − 51
Brewer's	0,1 % thioglycollate	>11	ND	>11	ND
Berg's	0,025 % thioglycollate	>11	−51	>11	ND
Bryner's	0,1 % thioglycollate	>11	−51	>11	> − 51
Dennis's	0,1 % cysteine-HCl	>11	−51	<11	< − 51
Clark's	0,02 % cysteine-HCl	>11	−51	~11	> − 51
Manclark's	0,02 % cysteine-HCl	~11	−51	>11	> − 51
McCoy's	*	>11	−51	>11	> − 51

ND = not done

* = 12,5 mg glutathione, 37,5 mg thioglycollate, 200 mg cysteine-HCl per litre

cells/ℓ medium (Manclark & Picket, 1960; Dennis & Jones, 1959; Clark *et al.*, 1972). In this study, the parameter of volume cells/100 mℓ medium (% PCV) was used. Percentage PCV values are comparable, although not accurately, to the values obtained by Clark *et al.*, 1972), Manclark & Picket (1960), and Dennis & Jones (1959). It is difficult to compare the methods used by the other authors to % PCV. The % PCV of 0,5, obtained by the *C. fetus venerealis* strain in this study, appears to be lower than the cell yields obtained by Dennis & Jones (1959), Clark *et al.*, (1972) and Manclark & Picket (1960). This can be attributed to differences in experimental procedures and strains used.

A fermenter-type cultivation system where the medium can be continuously gassed was not used in this study. In such a system the gas is fed directly into the medium containing vessel and better gas diffusion to the medium occurs. This results in better growth of the organisms to be cultivated. The system used in this study was one where flasks were put into anaerobic jars which were then gassed. Gas diffusion to the medium in this system is not as effective as that of the fermenter system. It is believed that even better cell yields of *C. fetus venerealis* 796/1 or other *C. fetus venerealis* strains could be obtained if the organisms are grown in a system which is continuously gassed with gas mixture in which the oxygen level can be controlled. In this way the gas mixture can be altered. During the exponential growth phase *Campylobacter* needs more oxygen than during the lag phase (Clark *et al.*, 1972). A static atmosphere as used in this study may in fact retard growth when the organisms are actively growing and oxygen supplies are smaller (Dennis & Jones, 1959).

The better growth which resulted from the lowered oxygen level could be expected, as *Campylobacter* is an microaerophile and higher levels of oxygen are toxic (Smibert, 1984). The use of the larger inoculum size of 10 % which resulted in better growth enabled the organisms to adjust more rapidly to their new environment. It is believed that this is achieved by a more rapid lowering of the oxygen tension in the medium than when fewer organisms were used (Smibert, 1984).

C. fetus fetus is adapted to a wide host range while *C. fetus venerealis* occurs only in the bovine genital tract (Véron & Chatelain, 1973). Therefore it is reasoned that *C. fetus venerealis* is a defective mutant of *C. fetus fetus*. *C. fetus fetus* has a faster growth rate than *C. fetus venerealis* (Véron & Chatelain, 1973). This explains why the venereal strain 796/1 and 873/5 grew less well on most of the media than the intestinal strains 68/4 and 7572.

Most of the media described for the growth of *C. fetus* are fairly simple in composition. They consisted mainly of peptone, yeast extract and inorganic salts. Some contained organic acids (Bryner's, Dennis', Clark's, Berg's and Manclark's) and/or reducing agents (Brewer's Bryner's, Dennis', Clark's, Robertstad's and Berg's). *Campylobacter* uses organic acids as energy sources (Alexander, 1957; Ware, 1980) in its aerobic respiration (Smibert, 1984). Although the media which contained organic acids did not yield better growth, it is believed that in a more advantageous oxygen atmosphere these media would give higher cell yields.

All the media contained peptone and most of them yeast extract as well. Both of these substances are rich in amino acids. Only Botha's medium contains additional amino acids. It is believed that each *C. fetus* strain has specific amino acid requirements (Smibert, 1963). Media like those examined, contain a variety of amino acids at low concentrations in the peptone. These media would thus support the growth of any *C. fetus* strain, but would not fulfil the specific amino acid requirements of a specific strain. If the amino acid requirements of the 4 strains studied were known and specific amino acids were added to optimal concentrations for each, it is believed that the strains would grow much better in all the media.

The reducing agents used were cysteine-HCl (Dennis, Clark, Manclark & McCoy's media), thioglycollate (Brewer, Berg, Bryner & McCoy) and glutathione (Robertstad & McCoy). Reducing agents are used to lower the redox potential of media (Costilow, 1981). The redox potential (Eh) of a medium indicates its state of oxidation. It is possible that *C. fetus*, during the initial lag phase in its growth cycle, lowers the Eh to a favourable level before cell dividing commences. *C. fetus venerealis* is capable of lowering the oxygen potential *in utero* (Ware, 1980) and would thus be able to do the same *in vitro*. In a medium which contains a reducing agent, the same degree of lowering of the Eh would not be necessary as with a medium without a reducing agent. Theoretically, organisms can thus start to divide sooner in a medium with a reducing agent. This was not very effectively demonstrated by the data from this study, except for the good growth of strain 796/1 on Dennis' medium, the medium in which the Eh was the lowest.

The amount of oxygen in the atmosphere in which *C. fetus* is grown does have an influence on the Eh of the liquid medium, as was seen by the differences in the Eh values of Manclark's and Clark's media under aerobic and microaerophilic atmospheres. As Dennis' mediums'

Eh was even lower than −51 mV under aerobic atmosphere, the influence of oxygen on this medium could not be demonstrated by the means used in this study. It is believed Dennis' medium would also be more "aerobic" under aerobic conditions. The 3 media which had Eh values below 11 mV all contained cysteine-HC1 as reducing agents. This shows that this reducing agent is capable of lowering the Eh level of a medium considerably. The concentration of 0,1 % as used in Dennis' medium seemed to be the most advantageous for the growth of *C. fetus,* as was observed by Dennis & Jones (1959) as well. The Eh level of a medium is thus controlled not only by the amount of oxygen present in the atmosphere above the medium, but also by the reducing agent present in the medium.

CONCLUSION

Under the conditions used in this study, Dennis' medium was the best to use for the high bulk growth of the *C. fetus venerealis* strain 796/1. The use of a 6 % oxygen level and a 10 % inoculum seemed imperative for the consistent growth of this strain. It is believed that the presence of succinate and cysteine-HC1 as a reducing agent were additional important stimulatory growth factors.

It is believed, however, that the growth of the 4 *C. fetus* strains studied can be improved: (a) by determining the amino acid requirements and adding them to optimum levels for each strain, and (b) by growing the organisms in a fermenter-type system where the gas flow and amount of oxygen in the atmosphere can be controlled to give less oxygen at the beginning of the growth cycle and more when the organisms are actively growing.

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Printed by the Government Printer, Private Bag X85, Pretoria, 0001