Effect of the growth of anaerobic bacteria on the surface pH of solid media

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SUMMARY Changes in surface pH occurring after varying periods of anaerobic incubation were measured for a total of 23 test solid media. There was little change in the surface pH of uninoculated plates, but plates inoculated with *Bacterioides fragilis* showed a striking fall in pH, to pH 5 in the case of some of the test media. The problems of controlling the surface pH of solid media are discussed and possible methods of control are considered.

Anaerobic incubation (in an atmosphere containing both carbon dioxide and hydrogen) leads to a fall in the surface pH of solid media.¹ We have shown that this fall due to the incubation environment is overshadowed, certainly in the case of *Bacterioides fragilis*, by a fall in pH due to bacterial growth.² Our original observations were based on findings with a single medium, and we have therefore studied a wide variety of media and measured the surface pH changes occurring with inoculated and uninoculated plates. We have also assessed visually the surface growth of the test organisms on the various test media and ranked the media in terms of their ability to sustain good surface growth as judged by colony size.

Material and methods

TEST MEDIA

The test media used are listed in Tables 1 and 2. All of the media were made up according to the manufacturers' instructions and supplemented with horse blood to give a final concentration of 10% in the poured plates. The media were divided into two groups: susceptibility test media and general culture media. Plates of Diagnostic sensitivity test agar (Oxoid) were included with each group as a control.

TEST ORGANISMS

The following organisms were used: *B fragilis*, NCTC 9343; anaerobic coccus, laboratory number ACK; *Clostridium perfringens*, laboratory number C1; *B melaninogenicus*, laboratory number M30. All strains were maintained in cooked meat broth

and checked for purity. Each plate (see below) was spread with 0.03 ml of a 1/10 dilution of a 40 h cooked meat broth culture of the test organism (1/100 dilution for *B fragilis*).

INOCULATION OF TEST MEDIA

For a given medium, six plates were seeded with each of the test organisms (resulting in 24 plates in all) and six plates were retained as uninoculated controls. The 30 plates were then divided into three groups, each containing 10 plates (two plates for each of the four test organisms and two uninoculated controls). One group of plates was incubated anaerobically for 20 h, one for 40 h, and one for five days.

DETERMINATION OF SURFACE pH

The surface pH of the plates was determined using a Pye Unicam pH meter combined with a flat combination surface pH electrode (Orion Research). The pH of the uninoculated plates was read before and after incubation and that of the inoculated plates after incubation. Preliminary experiments showed that surface pH values rose during the first 20 min after removal of a given plate from the anaerobic jar but stabilised thereafter. All plates were therefore left for 30 min on the bench after incubation before the pH was determined.

ASSESSMENT OF COLONY SIZE

Plates of each of the test media were inoculated with each of the test organisms to give discrete colonies and incubated anaerobically for 20 h, 40 h, and 120 h. After incubation, the plates were examined visually and surface growth (in terms of colony size) was ranked for each medium and for each test organism. The results for the three incubation times

Table 1 Surface pH changes and relative colony sizes of susceptibility test media

Test medium (manufacturer, cat no)	Duration of incubation (h)	Surface pH* of				Relative surface growth over 24 h†	
		Uninoculated control	Media seeded with†				
			<i>B fragilis</i>	<i>Peptococcus</i> sp	<i>C perfringens</i>		<i>B melaninogenicus</i>
Sensitest agar (Oxoid, CM409)	0	7.4	—	—	—	4	
	20	7.2	5.6	7.2	6.5		7.25
	40	7.15	5.4	7.1	6.6		6.3
	120	7.2	5.4	6.95	6.6		5.4
Isosensitest agar (Oxoid, CM471)	0	7.4	—	—	—	9	
	20	7.3	5.5	7.4	6.2		7.4
	40	7.4	5.3	7.5	6.4		6.25
	120	7.45	5.2	7.5	6.5		5.2
Diagnostic sensitivity test agar (Oxoid, CM261)	0	7.4	—	—	—	1	
	20	7.35	5.8	7.4	7.2		7.3
	40	7.2	5.7	7.2	7.2		6.1
	120	7.1	5.8	7.1	7.4		5.9
Wilkins–Chalgren agar (Oxoid, CM619)	0	7.2	—	—	—	5	
	20	7.3	5.2	7.35	7.3		7.35
	40	7.4	5.2	7.5	7.4		6.2
	120	7.2	5.3	7.5	7.3		5.3
Mueller–Hinton agar (Oxoid, CM337)	0	7.5	—	—	—	10	
	20	7.45	5.7	7.5	7.5		7.45
	40	7.3	5.5	7.5	7.5		7.4
	120	7.2	5.65	7.3	7.5		5.55
Wilkins–Chalgren agar (Mast, DM235)	0	7.3	—	—	—	2	
	20	7.3	5.85	7.4	7.7		7.2
	40	7.2	6.5	7.4	7.65		6.1
	120	7.0	7.1	7.3	7.8		5.85
SAF agar (Mast, DM215)	0	7.5	—	—	—	12	
	20	7.4	7.2	7.5	7.95		7.4
	40	7.3	7.1	7.5	7.9		7.1
	120	7.35	7.1	7.7	8.05		6.9
Mueller–Hinton agar (Mast, DM170)	0	7.65	—	—	—	3	
	20	7.55	5.35	7.55	7.25		7.6
	40	7.25	5.2	7.45	7.15		5.7
	120	7.25	5.4	7.4	7.1		5.5
Sensitest no 1 agar (Gibco, 152–4370)	0	7.4	—	—	—	11	
	20	7.25	5.45	7.3	7.0		7.25
	40	7.2	5.3	7.2	6.95		5.2
	120	7.15	5.6	7.2	7.1		5.1
Sensitest no 2 agar (Gibco, 152–4375)	0	7.5	—	—	—	5	
	20	7.4	5.0	7.4	6.25		7.45
	40	7.3	4.8	7.15	6.2		5.2
	120	7.2	5.0	6.85	6.5		5.1
Mueller–Hinton agar (Gibco, 152–3300)	0	7.3	—	—	—	7	
	20	7.2	5.4	7.4	6.8		7.3
	40	7.15	5.2	7.3	6.7		6.0
	120	7.2	5.5	7.5	6.9		5.25
Mueller–Hinton agar (Difco, 0252)	0	7.4	—	—	—	7	
	20	7.3	5.3	7.4	7.15		7.3
	40	7.2	5.25	7.35	7.5		5.9
	120	7.3	5.5	7.35	7.15		5.65
Sensitest agar (Lab M, Lab 12)	0	7.5	—	—	—	13	
	20	7.4	6.2	7.45	7.65		7.48
	40	7.2	6.0	7.45	7.65		7.3
	120	7.3	5.9	7.4	7.7		6.4

*pH values expressed as mean of six readings from duplicate plates.

†*B fragilis* (NCTC 9343); *Peptococcus* sp (lab no ACK); *C perfringens* (lab no CI); *B melaninogenicus* (lab no M 30).

‡Shown as ranking for surface growth (see Material and methods).

§Surface growth was poor.

were combined to give an overall ranking for each of the test media. The test media were divided into two groups for the purpose of ranking: susceptibility testing media and general culture media. Rankings were derived for each group (Tables 1 and 2).

ANAEROBIC PROCEDURE

The procedure was as previously described,³ except

that the gas mixture was hydrogen 10%, carbon dioxide 10%, nitrogen 80%.

Results

SUSCEPTIBILITY TEST MEDIA

The starting pH values of fresh media ranged from 7.2 (Wilkins-Chalgren agar Oxoid) to 7.65

Table 2 Surface pH changes and relative colony sizes of anaerobic culture media

Test medium (manufacturer, cat no)	Duration of incubation (h)	Surface pH* of					Relative surface growth overall‡
		Uninoculated control	Media seeded with†				
			<i>B fragilis</i>	<i>Peptococcus</i> sp	<i>C perfringens</i>	<i>B melaninogenicus</i>	
Blood agar (Oxoid CM55)	0	7.3	—	—	—	—	13
	20	7.5	7.45	7.65	7.75	7.5	
	40	7.6	7.65	7.9	7.9	7.35	
	120	7.6	7.7	7.9	7.9	6.9	
Blood agar no 2 (Oxoid, CM271)	0	7.3	—	—	—	—	9
	20	7.2	6.25	7.3	7.4	7.3	
	40	7.3	6.7	7.5	7.6	6.7	
	120	7.25	6.9	7.5	7.6	6.2	
Columbia blood agar (Oxoid, CM331)	0	7.4	—	—	—	—	6
	20	7.3	5.9	7.4	7.4	7.3	
	40	7.25	5.7	7.7	7.5	6.4	
	120	7.25	5.8	7.6	7.5	5.9	
Schaedler agar (Oxoid, CM437)	0	7.5	—	—	—	—	8
	20	7.5	5.6	7.5	5.7	7.5	
	40	7.5	5.2	7.55	6.0	7.6	
	120	7.6	5.3	7.5	6.1	5.1	
Brucella agar (Oxoid, CM691)	0	7.25	—	—	—	—	11
	20	7.3	5.65	7.4	7.25	7.35	
	40	7.5	5.8	7.6	7.5	6.1	
	120	7.5	5.9	7.8	7.5	5.75	
Anaerobic blood agar (Gibco, 152-0205)	0	7.2	—	—	—	—	4
	20	7.15	5.0	7.2	5.65	7.1	
	40	7.0	4.8	7.1	5.7	5.05	
	120	6.95	4.9	7.2	6.2	4.9	
Columbia agar (Gibco M10800)	0	7.15	—	—	—	—	9
	20	7.1	5.9	7.3	7.1§	7.2§	
	40	7.05	5.55	7.4	6.95	6.4	
	120	7.0	5.7	7.45	7.35	6.0	
Schaedler agar (Gibco, 152-4270)	0	7.45	—	—	—	—	6
	20	7.35	5.1	7.4	5.9	7.45	
	40	7.2	4.7	7.15	5.9	7.2	
	120	7.15	4.8	7.2	6.1	5.1	
Columbia agar (Mast, DM115)	0	7.45	—	—	—	—	1
	20	7.3	5.6	7.45	7.5	7.3	
	40	7.25	5.9	7.5	7.0	7.0	
	120	7.2	6.0	7.4	6.85	6.85	
Columbia agar (Difco, 0792)	0	7.4	—	—	—	—	2
	20	7.25	5.5	7.25	6.8	7.2	
	40	7.1	5.4	7.25	6.85	5.8	
	120	7.1	5.6	7.2	6.9	5.0	
Columbia agar (Lab M, Lab 1)	0	7.6	—	—	—	—	5
	20	7.45	5.8	7.6	7.5	7.2	
	40	7.3	5.6	7.6	7.45	6.2	
	120	7.25	5.6	7.4	7.5	5.85	
Anaerobic identification base (Lab M, Lab 66)	0	7.25	—	—	—	—	12
	20	7.2	6.7	7.4	7.45§	7.25	
	40	7.1	6.55	7.45	7.4	7.0	
	120	7.1	6.3	7.2	7.6	6.85	
Diagnostic sensitivity test agar (Oxoid, CM261)	0	7.4	—	—	—	—	(used as control) 3
	20	7.5	5.75	7.45	7.5	7.5	
	40	7.3	5.8	7.1	7.25	6.25	
	120	7.3	5.8	7.15	7.3	6.0	

*pH values expressed as mean of six readings from duplicate plates.

†*B fragilis* (NCTC 9343); *Peptococcus* sp (lab no ACK); *C perfringens* (lab no CI); *B melaninogenicus* (lab no M 30).

‡Shown as ranking for surface growth (see Material and methods).

§Surface growth was poor.

(Mueller-Hinton agar, Mast) (Table 1). After incubation uninoculated plates of most media showed a fall in pH, although, even after 120 h incubation, the total pH fall rarely exceeded 0.3 of a pH unit.

The changes in the surface pH of uninoculated plates were completely overshadowed by the changes that occurred with inoculated plates. In the case of plates seeded with *B fragilis* the surface pH

after 20 h was usually <6 and after 40 h incubation the pH usually fell to values below 5.5. In one case the pH fell to 5 after 20 h and remained at this low level after 120 h. (Sensitest no 2 agar, Gibco.) There were exceptions, however: some media showed a very small fall in pH—for example, SAF agar, Mast—while others showed a rise after 120 h—for example, Wilkins-Chalgren agar, Mast.

As far as the other test organisms were concerned the results varied. For plates seeded with the anaerobic coccus there was little or no change in surface pH compared with uninoculated plates. For the *C perfringens* strain most media showed a fall in pH (less than that with *B fragilis*), while in the case of the *B melaninogenicus* strain the pH was little changed after 20 h incubation but was much reduced after 40 h and further reduced after 120 h incubation.

As might be expected, there was in general a direct relation between the degree of fall in the surface pH and the quality of surface growth as assessed visually. Thus media showing little or no fall in surface pH gave poor surface growth. SAF agar (Mast), which showed little pH change, was ranked 12th out of 13 overall. The reverse did not necessarily apply, however: the medium ranked first in terms of colony size (DST agar, Oxoid) showed a moderate fall in pH in comparison with that seen with the other susceptibility test media.

ANAEROBIC CULTURE MEDIA

The starting values for uninoculated media ranged from 7.15 (Columbia agar, Gibco) to 7.6 (Columbia agar, Lab M), (Table 2). Again, the small fall seen with uninoculated media was dwarfed by the large falls in surface pH seen with plates seeded with *B fragilis*, the surface pH falling to values of below 5 after 40 h incubation (Anaerobic blood agar, Gibco; Schaedler agar, Gibco). Again, there was little or no fall in pH in the case of plates seeded with anaerobic cocci (some plates showed a slight rise) and variable results in the case of plates seeded with *C perfringens* or *B melaninogenicus*: some media showed a pronounced fall—for example, Schaedler agar, Gibco—while others showed little or no change—for example, Anaerobe identification base, Lab M. As in the case of the susceptibility test media, plates seeded with *B melaninogenicus* showed a late fall in surface pH, occurring after 120 h incubation.

There was, in general, an inverse relation between the fall in surface pH on plates inoculated with *B fragilis* and the quality of surface growth. Media showing little or no fall in surface pH gave poor surface growth. For example, Blood agar (Oxoid) showed a slight rise in surface pH but was assessed as giving the poorest surface growth. Media giving good surface growth showed striking falls in surface pH with *B fragilis*—for example, Columbia agar, Difco.

Discussion

Little attention has been given to the study of changes in medium pH under anaerobic conditions

or to the relation of such changes to bacterial growth. Several authors have commented on the effect of the incubation environment on surface pH, especially in relation to the susceptibility of anaerobes to erythromycin,^{4,5} but there are few or no data on non-gaseous factors. We have compared the surface pH changes due to bacterial growth on a variety of media used for anaerobic microbiology and prepared according to the manufacturers' instructions with added blood. By leaving the plates to equilibrate with the room environment after removal from the anaerobic jar we sought to exclude any temporary changes resulting from gaseous interactions. Although some changes in medium pH occurred on uninoculated plates, these were small compared with those associated with bacterial growth.

The present study has shown that the surface growth of *B fragilis* produces dramatic changes in surface pH, often falling by 2 pH units or more. Although little or no effect was noted in the case of the anaerobic coccus or clostridium, a similar but delayed change was seen in the case of *B melaninogenicus*, presumably because of its slow surface growth.

Such large changes in surface pH may have profound implications for the susceptibility testing of *Bacteroides* strains against pH labile antibiotics such as erythromycin. The interaction between organism and antibiotic is complex, but as the pH falls the activity of the erythromycin also falls.^{5,6}

For the susceptibility testing of anaerobes there is a requirement for a medium that gives good growth of the test strain, without any change in surface pH. Unfortunately, the present study has shown that those sensitivity test media that show the least change in surface pH are those that give poor surface growth, as assessed visually. The best of the test media in terms of surface growth was the one that we use routinely (DST agar, Oxoid); this gave only a moderate fall in surface pH.

For the clinical microbiologist undertaking susceptibility testing, or for the research worker evaluating new compounds, it is important that all variables in sensitivity testing are controlled.^{7,8} Yet the present study has confirmed our earlier finding that the fall in surface pH associated with growth of some anaerobes is a variable that is not controlled. Although the present study was performed using anaerobic jars, there is evidence that a similar fall in pH occurs in anaerobic incubators.⁹ What steps can be taken to control medium pH? The following points are relevant:

- 1 *The use of buffers.* We have tried both inorganic and biochemical buffers at concentrations up to those that become inhibitory to bacterial growth,

Anaerobes and medium pH

but even at such high concentrations the pH fall is only reduced, not removed (Watt and Brown, unpublished observations).

2 *Design of new media.* It is possible to prepare media on which the surface pH remains constant. We prepared such a pH stable medium (without buffers) in our laboratory; unfortunately, surface growth of anaerobes on the medium was poor and slow to develop and minimum inhibitory concentrations for test antibiotics (including pH stable ones) were considerably reduced. There is a need for good pH stable susceptibility test media.

3 *Addition of blood.* We added blood to all of the test media as we find this necessary for optimal growth of anaerobes. Previous experiments have shown that even doubling the concentration of blood to 20% only marginally reduces the fall in pH.

4 *Use of appropriate control organisms.* The present study has shown that the surface pH changes vary depending on the type of organism concerned, developing more slowly in the case of a slow growing organism. Thus it is important that in susceptibility testing of anaerobes a given test strain is controlled by an organism of a similar type and speed of growth. For example, in our view, it is not acceptable to use a strain of *B fragilis* to control a strain of *B melaninogenicus*.

This study has shown clearly the nature of the problem, but we have not been able to find any practicable solution to it. Buffers do not work even at high, inhibitory concentrations, and sensitivity test media that are pH stable do not give good surface growth. Until satisfactory pH stable media are developed the addition of blood to test media, together with the use of appropriate control organisms, are the only practicable steps that can be taken to reduce the effect of this variable. The surface pH should be monitored so that the conditions of the

tests can at least be accurately recorded, even if they cannot be fully controlled.

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References

- ¹ Ingham HR, Selkon JB, Codd AA, Hale JH. The effect of carbon dioxide on the sensitivity of *Bacteroides fragilis* to certain antibiotics *in vitro*. *J Clin Pathol* 1970;23:254-8.
- ² Watt B, Brown FV. Sensitivity testing of anaerobes on solid media. *J Antimicrob Chemother* 1975;1:420-2.
- ³ Watt B, Brown FV. The comparative activity of cefsulodin against anaerobic bacteria of clinical interest: synergy with cefoxitin. *J Antimicrob Chemother* 1981;7:269-78.
- ⁴ Goldstein EJC, Sutter VL, Kwok YY, Lewis RP, Finegold SM. Effect of carbon dioxide on *in vitro* susceptibility of anaerobic bacteria to erythromycin. *Antimicrob Agents Chemother* 1981;19:335-6.
- ⁵ Hansen LL, Swomley P, Drusano G. Effect of carbon dioxide and pH on susceptibility of *Bacteroides fragilis* group to erythromycin. *Antimicrob Agents Chemother* 1981;20:335-6.
- ⁶ Goldstein EJC, Sutter VL. Effect of carbon dioxide on erythromycin. *Antimicrob Agents Chemother* 1983;23:325-7.
- ⁷ Waterworth PM. Quantitative methods for bacterial sensitivity testing. In: Reeves DS, Phillips I, Williams JD, Wise R, eds. *Laboratory methods in antimicrobial chemotherapy*. Edinburgh, Churchill Livingstone, 1978:31.
- ⁸ Phillips I, Warren C. Anaerobic bacteria. In: Reeves DS, Phillips I, Williams JD, Wise R, eds. *Laboratory methods in antimicrobial chemotherapy*. Edinburgh: Churchill Livingstone, 1978, 94.
- ⁹ Berry PL, Taylor E, Phillips I. The use of an anerobic incubator for the isolation of anaerobes from clinical samples. *J Clin Pathol* 1982;35:1158-62.

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