Comparison of solid media for cultivation of anaerobes

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Abstract

Two commercial agar media for the cultivation of anaerobes were compared with four other media for their ability to support the growth of a wide range of anaerobes from clinical specimens of subgingival plaque. Fastidious anaerobe agar (FAA, Lab M) and anaerobe agar (GAA, Gibco) allowed better growth of the pure cultures than the other media. FAA recovered the highest numbers of bacteria from subgingival plaque specimens which were composed predominantly of anaerobes. GAA performed poorly with these samples. It is concluded that FAA seemed to be superior to the other media tested for the cultivation and recovery of anaerobes.

The importance of obligately anaerobic bacteria in infection is increasingly being recognised. Isolation techniques have improved with the widespread introduction of anaerobic cabinets and the use of transport media. Nutritionally rich culture media, designed specifically for the cultivation of anaerobes, have also become available. The evaluation of new media for anaerobes, however, is difficult in the clinical laboratory. The numbers and range of anaerobes in clinical specimens may have been depleted by poor collection techniques or inadequate or delayed transport before receipt in the laboratory, or both. Differences among media may therefore go undetected. Pure cultures of standard strains for culture collections can also be used to evaluate media. While this may be useful for the detection of nutritional deficiencies of media for particular groups of organisms, standard strains, having been subcultured several times and survived to be catalogues, are more adaptable than fresh isolates.

In advanced periodontal disease the microflora of deep periodontal pockets is composed predominantly of obligate anaerobes,¹ and up to 325 species may be represented.² Therefore, subgingival plaque makes an excellent source of clinical material for the evaluation of new culture media for anaerobes.

In this study two media designed specifically for the cultivation of anaerobes were compared with four other media for their ability to allow the growth of standard strains and freshly isolated of anaerobes and to recover anaerobes from subgingival plaque.

Methods

The following standard strains were used: *Actinomyces viscosus* NCTC 10951, *Actinomyces odontolyticus* NCTC 9935, *Bacteroides intermedius* NCTC 9936, *Fusobacterium nucleatum* NCTC 10562, *Peptostreptococcus anaerobius* NCTC 11460, *Peptostreptococcus asaccharolyticus* NCTC 11461 and *Wolinella recta* NCTC 11459.

The fresh isolates tested were recovered from subgingival plaque from periodontal pockets more than 6 mm in depth in patients with chronic periodontitis and identified using schemes contained in the *Manual of Clinical Microbiology*.³ Strains used were as follows: *Bacteroides intermedius* (n = 2), *Bacteroides veroralis* (n = 1), *Bacteroides oralis* (n = 1), *Fusobacterium nucleatum* (n = 5), *Fusobacterium varium* (n = 1), *Fusobacterium alocis/sulci* (n = 2), *Porphyromonas gingivalis* (n = 4), *Wolinella* spp (n = 2), *Selenomonas sputigena* (n = 1), *Veillonella parvula* (n = 2), *Actinomyces israelii* (n = 3), *Actinomyces viscosus* (n = 1), *Propionibacterium acnes* (n = 3), *Eubacterium aerofaciens* (n = 1), *Eubacterium alactolyticum* (n = 2), *Peptostreptococcus micros* (n = 4), and *Peptostreptococcus anaerobius* (n = 2).

The media tested were as follows: fastidious anaerobe agar (FAA, Lab M, Bury, England), anaerobe agar (GAA, Gibco, Paisley, Scotland), Columbia agar (Oxoid, Basingstoke, England), Columbia agar with 0.5% yeast extract (Difco, West Molesey, England), and 5 mg/l haemin and 10 mg/l vitamin K (E Col) brain heart infusion agar (Oxoid) and Wilkins Chalgren agar (Difco). All media were supplemented with 5% defibrinated horse blood (Tissue Culture Services, Buckinghamshire, England).

Organisms were grown for 48 hours anaerobically on Columbia agar with 5% defibrinated horse blood and then harvested into Ringer's solution and the turbidity adjusted to the McFarland No 1 standard. Suspension (10 µl) was inoculated on to a whole plate of each test medium and regressed spread. Plates were incubated for five days at 37°C in an anaerobic cabinet (Don Whitley MR III, Don Whitley Scientific, Shipley, England) and examined daily for growth. Growth was scored as follows: - = no growth, + = poor growth, ++ = moderate growth, +++ = good growth.

Thirty samples of subgingival plaque were obtained from periodontal pockets more than 6 mm in depth from patients with chronic

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Table 1 Growth of standard strains on six media

	48 hours						120 hours					
	WC	Col	E Col	BHI	FAA	GAA	WC	Col	E Col	BHI	FAA	GAA
<i>A viscosus</i> 10951	+++	+++	++	++	+++	++	+++	+++	+++	+++	+++	+++
<i>A odontolyticus</i> 9935	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>B intermedius</i> 9336	++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
<i>F nucleatum</i> 10562	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>P asaccharolyticus</i> 11461	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>P anaerobius</i> 11460	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
<i>W recta</i> 11459	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

WC—Wilkins Chalgren agar; BHI—Brain heart infusion agar; Col—Columbia agar; FAA—Fastidious anaerobe agar; E Col—Enriched Columbia agar; GAA—Gibco anaerobe agar.

— = no growth; + = poor growth; ++ = moderate growth; +++ = good growth.

periodontitis. Samples were taken using a small spoon excavator, immersed in 2 ml reduced transport medium⁴ and taken immediately to the laboratory. Samples were dispersed by vortex mixing for 30 seconds after the addition of 0.25 ml mesh glass beads (BDH, Poole, England). The test media were inoculated using a spiral plater (Spiral Systems, Don Whitley Scientific) and incubated for seven days in the anaerobic cabinet. An extra Columbia agar plate was incubated in air +10% carbon dioxide. After incubation the plates were counted and the results expressed as colony forming units (cfu)/specimen and anaerobe count: air + carbon dioxide count.

The counts obtained from the clinical specimens were compared using paired *t* tests and Wilcoxon paired tests on log-transformed data. Two-way analysis of variance was performed on the untransformed data.

Results

GROWTH OF PURE CULTURES

The results of growth of standard strains at 48

and 120 hours is shown in table 1. No differences were detected among media; all strains grew moderately by 48 hours and good growth was seen by 120 hours. In contrast, differences were seen among media in the growth of the fresh isolates of *F nucleatum*, *P gingivalis*, *A israelii*, *E aerofaciens*, *E alactolyticum* and *P anaerobius* (table 2). The *F nucleatum* strains showed moderate or good growth on FAA and GAA at 48 hours but only poor or moderate growth on the other media. Growth of all four *P gingivalis* strains could only be detected on brain heart infusion agar at 48 hours while no strains gave detectable growth on Wilkins Chalgren agar or Columbia agar at that time. At 120 hours good growth of all strains was seen on E Col, brain heart infusion agar, FAA and GAA. GAA permitted good growth of all *P acnes* strains at 48 hours while strain 1305 was undetectable on the other media at this time. By 120 hours good growth of all strains was seen only on FAA and GAA. The *Eubacterium* species tested exhibited similar results, good growth at 120 hours being confined to FAA and GAA. *P*

Table 2 Growth of fresh isolates on six media

Species	Isolate No	48 hours						120 hours					
		WC	Col	E Col	BHI	FAA	GAA	WC	Col	E Col	BHI	FAA	GAA
<i>B intermedius</i>	1351	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++
	2002	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
<i>B veroralis</i>	1379	—	—	—	—	+	+	+++	+++	+++	+++	+++	+++
<i>B oralis</i>	2003	++	+++	++	++	+++	++	+++	+++	+++	+++	+++	+++
<i>F nucleatum</i>	574	+	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++
	2004	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
	585	+	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++
	2005	++	++	+	++	+++	+++	+++	+++	+++	+++	+++	+++
	639	+	+	++	+	+++	+++	+++	+++	+++	+++	+++	+++
<i>F varium</i>	1369	++	+	+	++	++	++	+++	+++	+++	+++	+++	+++
<i>Fusobacterium</i> spp	1359	+	+	+	+	+	++	+++	+++	+++	+++	+++	+++
	1358	+	+	++	++	++	+++	+++	+++	+++	+++	+++	+++
<i>P gingivalis</i>	641	—	—	+	+	—	+	++	+	+++	+++	+++	+++
	1360	—	—	+	+	—	—	++	+	+++	+++	+++	+++
	576	—	—	—	+	—	—	++	+	+++	+++	+++	+++
	2001	—	—	—	+	—	—	++	+	+++	+++	+++	+++
<i>Wolinella</i> spp	2009	++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++
	663	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
<i>S sputigena</i>	450	+	—	+	++	+	+	+++	+++	+++	+++	+++	+++
<i>V parvula</i>	500	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	447	+++	++	++	+++	+++	++	+++	+++	+++	+++	+++	+++
<i>A israelii</i>	658	+	+	+	+	+	+	+++	+++	+++	+++	+++	+++
	647	+	—	+	—	—	—	+++	+++	+++	+++	+++	+++
	2006	+	++	++	++	++	++	+++	+++	+++	+++	+++	+++
<i>A viscosus</i>	2007	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
<i>P acnes</i>	1305	—	—	—	—	—	—	+	+	++	++	+++	+++
	2008	++	+	+	++	+	+++	+++	+++	+++	+++	+++	+++
	1392	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
<i>E aerofaciens</i>	1376	—	—	—	—	—	+	+	+	++	++	+++	+++
<i>E alactolyticum</i>	1337	+	+	+	+	+	+	+++	+++	+++	+++	+++	+++
	617	+	+	+	+	++	++	+++	+++	+++	+++	+++	+++
<i>P micros</i>	1345	+	++	+	+	++	++	+++	+++	+++	+++	+++	+++
	1348	++	++	++	++	+++	++	+++	+++	+++	+++	+++	+++
	2011	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++
	2012	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++
<i>P anaerobius</i>	794	—	—	—	—	+	++	++	+	+	++	+++	+++
	580	—	—	—	+	+	++	++	+	+	+++	+++	+++

WC—Wilkins Chalgren agar; BHI—Brain heart infusion agar; Col—Columbia agar; FAA—Fastidious anaerobe agar; E Col—Enriched Columbia agar; GAA—Gibco anaerobe agar.

— = no growth; + = poor growth; ++ = moderate growth; +++ = good growth.

Table 3 Recovery of bacteria from subgingival plaque on six media. Total anaerobic count (CFU $\times 10^4$ /specimen)

Specimen No	Media							Highest anaerobic/BA count
	WC	Col	E Col	BHI	FAA	GAA	BA*	
1	66	170	99	—	67	53	67	2.5
2	170	180	220	120	180	52	29	7.5
3	180	220	190	170	460	140	29	15.8
4	210	210	260	250	320	150	320	1.0
5	73	42	160	120	130	56	30	5.3
6	8.5	9.9	20	16	22	10	10	2.2
7	2.3	1.7	1.5	1.3	1.6	1.8	0.3	7.2
8	310	210	3.9	280	260	190	270	1.4
9	46	47	67	65	70	53	89	0.8
10	6.7	6.9	6.2	7.8	8.0	5.8	4.5	1.8
11	160	220	390	350	430	190	170	2.5
12	310	340	660	400	1100	69	14	78.6
13	46	86	140	56	110	25	4.4	31.8
14	450	510	440	460	1000	230	210	4.8
15	50	66	80	69	87	270	2.1	128.6
16	370	220	380	310	350	240	240	1.6
17	300	300	500	290	270	200	50	10.0
18	26	39	32	20	89	13	5.4	16.5
19	100	99	300	190	310	110	74	4.2
20	420	410	550	260	590	250	74	7.8
21	270	230	250	210	350	110	1.6	218.8
22	510	610	750	450	530	300	72	10.4
23	530	790	1000	490	1000	320	170	5.9
24	170	170	130	120	190	100	6.7	28.4
25	240	230	280	170	230	80	130	2.2
26	350	260	330	340	430	190	78	5.5
27	530	310	840	370	890	330	58	15.3
28	720	62	70	58	110	140	9.9	14.1
29	170	210	360	180	260	97	62	5.8
30	450	300	540	330	610	180	120	5.1

*BA: Columbia agar incubated in air + 10% carbon dioxide.

anaerobius was detectable only at 48 hours on FAA, GAA, and brain heart infusion agar (one strain), and good growth of both strains at 120 hours occurred on FAA and GAA alone.

SUBGINGIVAL PLAQUE SPECIMENS

The mean anaerobe:air + carbon dioxide ratio was 21.5, indicating the high proportion of anaerobes in these specimens (table 3). FAA produced the highest anaerobic count from 17 of the 30 specimens, followed by E Col ($n = 10$), GAA ($n = 2$), Columbia agar ($n = 1$), Wilkins Chalgren agar ($n = 1$) and brain heart infusion agar ($n = 0$). Paired t tests and Wilcoxon tests showed highly significant differences ($p < 0.001$) between log-transformed counts for FAA compared with Wilkins Chalgren agar, Columbia agar, brain heart infusion agar and GAA. No significant difference was seen between FAA and E Col. Two-way analysis of variance confirmed highly significant differences among the media, FAA and E Col giving the highest counts and GAA the lowest.

Discussion

The media chosen for this study were used for the primary isolation of anaerobes with the exception of Wilkins Chalgren agar which was formulated for antimicrobial susceptibility testing of anaerobes.⁵ The poor performance of this medium in supporting the growth of fresh isolates suggests that a more nutritious medium should be proposed for the reference method of susceptibility testing.

This study showed that for the cultivation of pure cultures of anaerobes FAA and GAA were superior to the other media tested, while FAA and E Col gave the highest recoveries from clinical specimens composed predominantly of anaerobes. FAA has been formulated

specifically for the optimal cultivation of anaerobes and contains several ingredients which have been reported to enhance the growth of anaerobes. Starch is included to absorb toxic products⁶ and sodium pyruvate is present to neutralise hydrogen peroxide and as an energy source for *Veillonella* spp.⁷ The medium also contains arginine which has been reported to stimulate the growth of *Eubacterium* species and sodium bicarbonate for its buffering properties.⁸ Haemin and vitamin K are growth factors required by many *Bacterioides* species⁹ and are included together with the reducing agent cysteine hydrochloride, which has also been shown to stimulate directly the growth of some anaerobes.¹⁰

The poor performance of GAA in the recovery of organisms from clinical specimens was unexpected because it afforded good growth of all the pure cultures tested. Inspection of the plates after 48 hours' growth, however, showed a pronounced discolouration of the plates. This colour change might have been caused by acid production due to the inclusion in the media of 0.5% glucose. This observation has been reported previously with GAA.¹¹ Facultative organisms present in the sample may have produced acid products which subsequently inhibited the growth of slower growing anaerobes. Lactic acid, in particular, has been shown to be inhibitory to Gram negative anaerobes.¹²

The fresh isolates and subgingival plaque specimens tested in this study had been taken from the mouth and thus, it might be argued, the conclusions reached may not be applicable to the cultivation and recovery of clinically important anaerobes from other body sites. In particular, the range of organisms found in subgingival plaque might differ considerably from those found in clinical specimens. The

groups of anaerobes found in clinical specimens but not normally found in the mouth—fragilis group *Bacteroides* and *Clostridia* spp—are generally less nutritionally demanding, however, than the other groups of anaerobes isolated from man, including those found in the mouth. Therefore the results of this study may have relevance for all body sites. Furthermore, higher isolation rates of anaerobes resulting from the use of improved media may result in the finding of positive associations between previously uncultivated anaerobes and disease states. A recent example is the frequent isolation of *Eubacterium nodatum*¹³ from infections associated with intrauterine contraceptive devices; *E nodatum* was originally described as a fastidious periodontal species.¹⁴ In conclusion, on the basis of the results of this study FAA seems to be a useful all-round medium for the cultivation and recovery of anaerobes.

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