PROLONGED PRIMARY INCUBATION IN THE ISOLATION OF ANAEROBIC BACTERIA FROM CLINICAL SPECIMENS*

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SUCCESSFUL isolation of anaerobic bacteria from clinical specimens depends on several factors that influence the viability of these organisms from the time specimens are collected until colonies are picked from primary-isolation media. These factors, which include rapid and efficient transport of the specimen to the laboratory, the use of enriched selective and non-selective media and the use of an efficient anaerobic-jar procedure, have been studied by others (Watt, 1972; Watt and Collee, 1974; Hollander, Flodstrom and Holmberg, 1975; Sapala, Ponka and Neblett, 1975; Sutter, Vargo and Finegold, 1975; Wilkins and Jiminez-Ulate, 1975; Watt, Collee and Brown, 1976). Some workers recognise the need for prolonged incubation for the successful isolation of anaerobes from clinical material (Dowell, 1974; Spaulding, *et al.*, 1974; Stokes, 1975; Sutter, *et al.*, 1975) and it has been shown that prolonged primary incubation may be advantageous for the isolation of anaerobes (Spaulding, *et al.*, 1974; Wren, *et al.*, 1977a; Wren, 1977). However, the role of prolonged primary incubation has not previously been investigated in any detail.

In this paper are presented the results of a study in which clinical specimens were cultured for anaerobic bacteria by two incubation methods to determine whether unbroken primary incubation for 48 h gives better results than a conventional culture system. The value of prolonged initial incubation for bacteriological diagnosis was also assessed.

MATERIALS AND METHODS

Conventional incubation (CONV). Pus was collected into sterile screw-capped bottles and delivered immediately to the laboratory and a large loopful was spread on Columbia Blood Agar (Oxoid) and MacConkey agar for aerobic incubation and on Columbia blood agar and kanamycin brucella agar for anaerobic incubation; details of these media have been published previously (Wren, 1977). In the latter part of the study kanamycin brucella agar was replaced by nalidixic acid-Tween (NAT) medium (Wren, 1978) as the selective medium for anaerobes.

Aerobic and anaerobic plates were examined after overnight incubation at 37°C, after 48 h and finally after 5 days. Anaerobic jars were of stainless steel (Stuart Scientific, Croydon, Surrey) fitted with a modified catalyst sachet (Baldwin, 1975). They were set up in the manner recommended by Watt and Collee (1974) with a gas mixture of 90% H₂ and 10% CO₂.

Gram-stained smears were made from all pus samples for microscopical examination.

Forty-eight hour incubation (FEH). Pus was cultured as in the CONV system with the difference that anaerobic plates were incubated for 48 h before their initial examination. The plates were then reincubated and finally examined on the 5th day.

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Identification of anaerobic bacteria was made on the basis of bacterial morphology in gram-stained smears prepared from colonies on blood agar and from cooked-meat glucose cultures, phase-contrast microscopy (Wren, 1977), fermentation patterns (Holdeman and Moore, 1973), antibiogram patterns (Sutter *et al.*, 1975; Wren, Eldon and Dakin, 1977) and, in the latter part of this study, gas-liquid chromatography.

Gas-liquid chromatography (GLC) analysis of short-chain fatty acids produced as end products of metabolism was done on 48-h cultures in cooked-meat glucose medium with a Pye series 104 chromatograph with flame-ionisation detectors. The columns were packed with either Chromosorb 101 or Carbowax 20 M-TPA on Gaschrom Q. Peaks were identified by comparison with known standards. Fatty acids were extracted by the method of Holdeman and Moore (1973).

Identification of aerobic bacteria was by standard methods (Cowan, 1974).

RESULTS

The origins of the 86 specimens examined are listed in table I. When examined by the methods described here, 52 of the samples yielded a mixture of aerobes and anaerobes while the remainder yielded only anaerobes and in five of these a pure culture of a single anaerobe. When both systems were used, a total of 76 aerobic and facultatively anaerobic bacteria were isolated together with 279 strict anaerobes. The aerobic bacteria were: *Escherichia coli* 33 strains, *Klebsiella aerogenes* 13, *K. oxytoca* 2, *Proteus mirabilis* 11, *P. morgani* 2, *P. vulgaris* 4, *Citrobacter freundii* 6, *Serratia marcescens* 3, *Aeromonas hydrophila* 1 and *Pseudomonas aeruginosa* 1. The anaerobic bacteria that were isolated are listed in table II. Anaerobic gram-negative rods accounted for 54.1%, anaerobic cocci for 31.9% and gram-positive rods for 14.0% of the total anaerobic flora.

TABLE I
Sources of clinical material

Clinical condition	Number of samples
Peritonitis	23
Thoracic empyema	16
Appendix abscess	7
Perineal abscess	6
Pelvic abscess	5
Paracolic abscess	4
Uterine sepsis	3
Cholecystectomy wound	3
Ischio-rectal abscess	3
Scalp abscess	2
Gastrectomy wound	2
Retrogastric abscess	1
Retroperitoneal abscess	1
Abdomino-perineal resection wound	1
Subphrenic abscess	1
Ovarian abscess	1
Brain abscess	1
Neck abscess	1
Laparotomy wound	1
Bartholin's abscess	1
Bladder abscess	1
Caesarean-section wound	1
Leg ulcer	1

TABLE II
Anaerobic species isolated

<i>BACTEROIDES</i>	122	<i>PEPTOSTREPTOCOCCUS</i>	22
<i>B. fragilis</i>	47	<i>Pst. anaerobius</i>	14
<i>B. melaninogenicus</i>	25	<i>Pst. micros</i>	3
<i>B. oralis</i>	13	<i>Pst. productus</i>	3
<i>B. corrodens</i>	6	<i>Pst. species</i>	2
<i>B. putredinis</i>	3		
<i>B. praeacutus</i>	3	<i>PEPTOCOCCUS</i>	55
<i>B. capillosus</i>	3	<i>Pc. asaccharolyticus</i>	15
<i>B. bivius</i>	3	<i>Pc. prevoti</i>	15
<i>B. disiens</i>	2	<i>Pc. magnus</i>	12
<i>B. splanchnicus</i>	2	<i>Pc. saccharolyticus</i>	3
<i>B. species</i>	15	<i>Pc. species</i>	10
<i>FUSOBACTERIUM</i>	29	<i>CLOSTRIDIUM</i>	7
<i>F. nucleatum</i>	12	<i>Cl. perfringens</i>	2
<i>F. mortiferum</i>	6	<i>Cl. sporogenes</i>	2
<i>F. russi</i>	4	<i>Cl. septicum</i>	1
<i>F. necrophorum</i>	3	<i>Cl. bifermentans</i>	1
<i>F. necrogenes</i>	2	<i>Cl. species</i>	1
<i>F. gonidiaformans</i>	1		
<i>F. naviforme</i>	1	<i>NON-SPORING GRAM POSITIVE RODS</i>	
		<i>Eubacterium lentum</i>	8
<i>VEILLONELLA</i>	12	<i>Eubact. combessi</i>	3
<i>V. parvula</i>	12	<i>Eubact. alactolyticum</i>	2
		<i>Propionibacterium acnes</i>	11
		<i>Propion. sp.</i>	5
		<i>Bifidobacterium adolescentis</i>	3

Anaerobic gram-negative rods

A total of 98 anaerobic gram-negative rods were isolated by the CONV system and 90 of these were isolated within 48 h. In contrast the FEH system yielded 151 strains and 126 of these were detectable at the initial 48-h reading. Closer inspection of the species within this group shows that the majority required at least 48 h before becoming visible on plating media. This was true for all fusobacteria, *Bacteroides melaninogenicus*, *B. corrodens* and other members of the genus *Bacteroides*. Thus, nine of 48 strains of *B. fragilis* required incubation for at least 48 h and three of these required the full 5 days of incubation. Of particular significance was the requirement for 48-h incubation by two-thirds of fusobacteria, while the remainder required incubation for 5 days.

Anaerobic cocci

By the CONV system 53 anaerobic cocci were isolated and 52 of these were found at 48 h; by the FEH system 75 strains were isolated at the initial 48-h examination and another 14 strains on the 5th day. Though most anaerobic cocci were isolated within 48 h, only 58.3% of *Veillonella* strains were isolated by this time.

Gram-positive rods

Only seven strains of clostridia were isolated and both systems yielded all seven—six strains within 48 h and one after 5 days.

TABLE III

Isolation of anaerobes from clinical specimens by the conventional (CONV) and 48-h (FEH) incubation systems

Organism	Total number of strains isolated	Number of strains isolated					Only by FEH (% of total)
		by CONV system at			by FEH system at		
		24 h	48 h	5 days	48 h	5 days	
<i>B. fragilis</i>	47	38	4	2	44	3	3 (6.4)
<i>B. melaninogenicus</i>	25	4	12	3	20	5	6 (24.0)
<i>B. corrodens</i>	6	0	1	0	5	1	5 (83.3)
<i>Bacteroides</i> sp	44	7	16	0	38	6	21 (47.7)
<i>Fusobacterium</i> sp.	29	0	8	3	19	10	18 (62.1)
<i>Peptostreptococcus</i> sp.	22	6	9	0	20	2	7 (31.8)
<i>Peptococcus</i> sp.	55	17	18	1	48	7	19 (34.5)
<i>Veillonella</i> sp.	12	0	2	0	7	5	10 (83.3)
<i>Clostridium</i> sp.	7	6	0	1	6	1	0 (0)
<i>Eubacterium</i> sp.	13	2	2	0	10	3	9 (69.2)
<i>Propionibacterium</i> sp.	16	0	2	2	8	8	12 (75.0)
<i>Bifidobacterium</i> sp.	3	1	2	0	3	0	0 (0)
Total	279	81	76	12	228	51	110 (39.4)

TABLE IV

Comparison of CONV and FEH systems

Clinical condition	Bacteriological findings achieved by the	
	CONV system	FEH system
Septic laparotomy wound	No growth	<i>B. fragilis</i>
Thoracic empyema	<i>E. coli</i>	<i>E. coli</i> , anaerobic non-sporing gram-positive rods
Perineal abscess	No growth	<i>B. fragilis</i>
Peritonitis	Microaerophilic streptococcus	Microaerophilic streptococcus, <i>F. necrophorum</i> , <i>Bacteroides</i> sp., anaerobic non-sporing gram-positive rods
Peritonitis	No growth	<i>F. necrophorum</i>
Endometritis	No growth	<i>B. fragilis</i>
Brain abscess	<i>E. coli</i> ; faecal streptococci	<i>E. coli</i> , faecal streptococci, <i>F. nucleatum</i> , <i>Pst. micros</i>
Uterine sepsis (post partum)	No growth	<i>F. nucleatum</i>

The CONV system yielded nine of 11 strains of non-sporing gram-positive rods within 48 h while the FEH system yielded 21 strains at 48 h and another 11 after 5 days; of these strains, 16 were members of the genus *Propionibacterium*.

Comparison of CONV and FEH systems (table III)

From both incubation systems a total of 279 anaerobes were isolated. However, only 169 (60.6%) of these strains were isolated by the CONV system even after incubation for 5 days. Extension of the initial incubation period to 48 h yielded an additional 110 strains (39.4%) from the same group of specimens.

DISCUSSION

Major problems associated with the isolation of anaerobic bacteria from clinical specimens are that these organisms are highly susceptible to oxygen during the early stages of growth (Smith, 1975), and that they grow slowly.

Factors affecting the isolation of anaerobes may be the presence of antimicrobial agents in the specimen, exposure of the specimen to atmospheric oxygen during collection and transport, overgrowth of aerobes and facultative aerobes, and desiccation especially if the specimen is received on a swab. Earlier studies in this laboratory have shown that unbroken primary incubation for 48 h may be an important phase in the cultural process (Wren, *et al.*, 1977a; Wren, 1977). The present study shows that only 29% of the cultivable anaerobic flora in a group of specimens can be isolated after incubation for 24 h and 60% after 48 h by the CONV system. Extension of the primary incubation to 48 h allowed isolation of 81.7% of anaerobes at the first examination. This lends support to the findings of the earlier studies that the incubation of anaerobic plates for an unbroken 48 h is of value. Although the majority of anaerobic strains may be isolated in 48 h, incubation for a total of 5 days is still necessary for the isolation of certain gram-negative anaerobes and the non-sporing gram-positive rods. Throughout the range of non-sporing anaerobic genera, isolation rates are improved by prolongation of primary incubation.

The premature opening of anaerobic jars in the CONV system resulted in the loss of 40% of the total anaerobic flora, this loss being greater for some genera than for others; such organisms may be more easily damaged by oxygen and prolonged incubation may be necessary for their "resuscitation" and initiation of growth. Alternatively the developing colonies of such strains may be more sensitive to early exposure to oxygen.

These findings have important clinical implications because in the case of eight patients who provided specimens for this study a bacteriological misdiagnosis, indeed in five cases no bacteriological diagnosis at all, would have been made had the CONV system been used alone. In these eight cases anaerobic bacteria could be isolated only by the FEH system. Of particular importance was the presence of clinically significant anaerobes, such as *B. fragilis*, in pure culture in three of these cases, and fusobacteria in pure culture in two cases and

as the predominant organism in another two (table IV). Although derived from only a small number of patients, these results have far-reaching implications because the majority of these isolates were from abdominal sites, which are known to be those most often leading to anaerobic septicaemia (Sonnenwirth, 1974a; Wren, *et al.*, 1977). These patients would have received or continued to receive inadequate therapy had the results of only the CONV system been available.

The conclusions to be drawn from these results are that the yield of anaerobic bacteria from clinical specimens is more representative of the bacterial flora in the original lesion when the initial period of incubation of primary anaerobic plates is extended to 48 h. This appears to reduce the effects of adverse environmental factors on the specimen. Many anaerobic species will not be isolated on media that are examined prematurely, and this may lead to a misleading bacteriological diagnosis or failure of diagnosis altogether.

SUMMARY

The value of prolonged primary anaerobic incubation for the isolation of anaerobic bacteria from clinical material was assessed by comparison of two incubation systems. Continuous incubation of primary anaerobic plates for 48 h was superior to conventional methods in that more anaerobes were isolated. The results indicate that in some cases prolonged incubation may be the only method of achieving the correct bacteriological diagnosis, particularly when relatively oxygen-sensitive strains are involved or when organisms may be damaged by exposure to oxygen during transport.

Use of extended primary incubation provides a clearer picture of the microbial flora of the original lesion and reveals that some anaerobic genera may be more commonly involved than is at present recognised.

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