WHAT ARE ANAEROBIC COCCI?

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At present, the classification of obligately anaerobic cocci of clinical interest is in a state of confusion. Different authorities classify these organisms into different species, even different genera, and some workers allow inclusion of micro-aerophilic or CO₂-requiring strains (see Smith, 1975). The absence of clear guidelines for characterisation is a constant source of discouragement to the clinical microbiologist and this has held back studies on the role of these organisms in health and disease.

As part of a study of methods for the isolation and characterisation of obligately anaerobic cocci of clinical interest, we found it necessary first to develop a clear definition that separates the obligately anaerobic cocci from micro-aerophilic or CO₂-requiring strains. The present paper sets out the evidence for a provisional definition of "anaerobic cocci" that might allow studies on these organisms to be more soundly based.

MATERIALS AND METHODS

Strains. The following strains, representative of various Hare groups and labelled anaerobic cocci, were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London NW9 5HT: Hare Group I, no. NCTC9801; Group III, nos. NCTC9803 and NCTC9814; Group IV, nos. NCTC9804 and NCTC9815; Group V, nos. NCTC9805 and NCTC9816; Group VIa, nos. NCTC9806 and NCTC9817; Group VIb, no. NCTC9807; Group VIIa, no. NCTC9808; Group VIIb, nos. NCTC9809 and NCTC9819; Group VIII, nos. NCTC9810 and NCTC9820; and Group IX, nos. NCTC9811 and NCTC9821.

Twenty-one strains of cocci were received from Dr P. N. Edmunds and colleagues, Victoria Hospital, Kirkcaldy; two strains were received from Dr F. J. Bone, Dumfries Royal Infirmary; one strain each of *Peptostreptococcus anaerobius*, *Peptococcus asaccharolyticus* and an uncharacterised "anaerobic streptococcus" were received from Dr A. T. Willis, Public Health Laboratory, Luton; one strain of *Veillonella parvula* and one of *V. gazogenes* were received from Dr W. P. Holbrook, Manchester; 108 strains of cocci were isolated from clinical specimens at the Central Microbiological Laboratories, Edinburgh; and 90 strains were isolated from the genital tracts of women attending a family planning clinic in Edinburgh. A total of 226 strains were examined in the first instance. The test strains were subcultured in cooked-meat broth (CMB).

Gram staining. Smears of solid and liquid cultures of the test strains were stained by Kopeloff and Beerman's modification of Gram's method (see Cruickshank *et al.*, 1975, p. 35).

Culture media. Cooked-meat broth was prepared as described by Cruickshank *et al.* (1975, p. 122), but the broth component was Nutrient Broth No. 2 (Oxoid); immediately before use it was steamed at 100°C for 30 min. to remove dissolved air and then cooled rapidly to 37°C. Nutrient Broth No. 2 was also used as a diluent in MIC studies. Horse blood agar (HBA) was prepared as follows. Agar water was prepared by dissolving 120 g

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Agar No. 3 (Difco) in 6 litres of tap water by autoclaving at 121°C. Nutrient broth was prepared by dissolving 320 g Proteose Peptone No. 3 (Difco), 320 g Lab Lemco powder, and 160 g sodium chloride in 2 litres of tap water by steaming. Six litres of agar water were added to 2 litres of nutrient broth, the pH was adjusted to 7.3, and the molten medium was dispensed in 500-ml amounts and autoclaved at 121°C for 20 min. These aliquots were re-melted by autoclaving, and after cooling to approximately 50°C, horse blood (10%) was added; after thorough mixing, molten medium was poured into plates. *Columbia blood agar* (CBA) plates were prepared with Columbia Blood Agar Base No. 2 (1.2%) enriched with horse blood (10%). This was supplemented with neomycin and menadione (final concentrations of 70 µg per ml and 1 µg per ml respectively) to give *neomycin blood agar*. The blood and supplements were added after autoclaving. *Heated blood agar* was prepared by heating horse blood (5%) in Columbia Blood agar Base to 75°C for 10 min. before pouring. *Non-fermenting (NF) medium* (Watt, to be published) was prepared as follows: 20 g Proteose Peptone No. 3 (Difco), 10 g Yeast Extract (Oxoid), 5 g sodium chloride, 2.5 g sodium succinate and 12 g agar were added to 1 litre distilled water, steamed until dissolved (approximately 30 min.), and then autoclaved. The pH was adjusted to 7.1 before pouring.

Blood. In the early part of the study, defibrinated horse blood was supplied by Oxoid; latterly it was supplied by Gibco Bio-Cult Laboratories, Washington Road, Paisley. Out-dated human blood was supplied by the Blood Transfusion Service; each 500-ml volume of the human-blood preparation consisted of 430 ml of whole blood and 70 ml of water containing 2 g disodium citrate and 1.7 g dextrose.

Haemolysis. Haemolysis on Columbia Blood Agar (with 10% horse or human blood) was checked after incubation for 48 h.

Chemicals. Menadione was supplied by Sigma, London, and sodium succinate by Hopkin and Williams, Chadwell Heath, Essex.

Anaerobic jar procedure. The standard anaerobic procedure of Collee *et al.* (1972) was followed, with Baird and Tatlock (BTL) anaerobic jars. Preliminary subculture of strains was performed in jars equipped with one catalyst sachet; more demanding studies (see below) were performed in jars equipped with three sachets. All cultures were incubated in 90% H₂ with 10% CO₂ at 37°C.

Incubation in carbon dioxide. Plates were incubated in an incubator that was continually flushed with a gas mixture of 10% CO₂ in air.

Gases. Cylinders of a special gas mixture of H₂ 90% with CO₂ 10% were supplied by British Oxygen Company Ltd. Cylinders of CO₂ gas were supplied by Distillers Company Ltd.

Metronidazole sensitivity tests

Disk sensitivity tests. For preliminary testing, 10 colonies from a culture of the test strain on solid medium were spread evenly over the surface of a horse blood agar (HBA) plate with a standard loop. A 5-µg metronidazole disk was added and the zone of inhibition measured after incubation for 48 h.

For detailed testing, duplicate plates of NF medium were seeded with 0.02 ml inocula of a 48-h CMB culture of the test strain and spread with sterile spreaders of standard size. A 5-µg metronidazole disk was applied to each seeded plate and the zone of inhibition measured after incubation for 48 h.

Determination of minimum inhibitory concentration (MIC). Two-fold dilutions of a filter-sterilised solution of metronidazole were prepared in pre-steamed nutrient broth and added to molten NF medium at approximately 50°C, together with 10% horse blood. After careful mixing, plates were poured. The range of final concentrations in the solid medium was 0.02 to 10 µg metronidazole per ml. Antibiotic plates were seeded with the test strains, with a multiple inoculator delivering an inoculum of approximately 10⁵ c.f.u. Duplicate plates, together with control plates (without antibiotic) were seeded in parallel; appropriate control strains were also put up in parallel (see below). The plates were incubated under anaerobic conditions for 48 h at 37°C and the MIC was taken as the lowest concentration that totally inhibited bacterial growth.

Control strains. *Bacteroides fragilis* no. NCTC9344 and an anaerobic coccus (lab. no. A/C 44) were used as controls for the antibiotic sensitivity tests, and *B. fragilis*, no. NCTC9344, was used as a control to check that satisfactory anaerobic conditions were achieved.

Metronidazole. Metronidazole powder of known potency was supplied by May and Baker Ltd. Disks of metronidazole (5 µg), manufactured by Mast Laboratories, were also supplied by May and Baker.

RESULTS

Clinical isolates

We investigated a total of 205 clinical isolates. All of the strains were cocci that, on primary culture, had grown under anaerobic conditions but failed to grow aerobically. They were screened in our laboratory as follows.

(A) Each strain was subcultured on to heated blood agar and incubated for 24 h at 37°C in an atmosphere of 10% CO₂ in air.

(B) Two plates of horse blood agar (HBA) were seeded with each strain; one was incubated aerobically, the other anaerobically (see *Methods*) for 24 h at 37°C.

(C) The sensitivity of each strain to metronidazole was determined by a disk diffusion test on HBA, with a 5-µg disk of metronidazole.

The results (table I) show that 175 of the 205 strains grew under anaerobic conditions only, and were sensitive to metronidazole. Thirty were resistant to metronidazole and three of these grew under anaerobic conditions only, 10 grew aerobically and the remaining 17 grew in 10% CO₂ in air.

TABLE I
Gaseous requirements and metronidazole sensitivity of 207 clinical isolates of cocci

Number of strains	*Growth on solid media in			Susceptibility to metronidazole†
	air	air plus 10% CO ₂	90% H ₂ plus 10% CO ₂	
10	+	+	+	R
17	—	+	+	R
3	—	—	+	R
175	—	—	+	S

* All strains incubated overnight at 37°C.

+ = Visible surface growth; — = no growth.

† R = resistant, i.e., zone of inhibition < 15 mm after incubation for 24 h; S = sensitive: zone of inhibition > 15 mm after incubation for 24 h.

A representative collection of 91 of our metronidazole-sensitive strains, together with the three apparently anaerobic metronidazole-resistant ("AAMR") strains were tested further as follows.

(1) Each strain was subcultured on to heated blood agar, and the plates were incubated for 7 days in 10% CO₂ in air at 37°C.

(2) The disk tests for sensitivity to metronidazole were repeated with the procedure (see *Methods*) in which standard inocula were tested on NF medium.

(3) The MIC of metronidazole was determined for each strain.

(4) Metronidazole-resistant strains were also tested for their ability to grow on plates of NF medium containing 500 μg metronidazole per ml.

The results (table II) show that all three of the AAMR strains grew within 7 days on heated blood agar in 10% CO_2 in air. These AAMR strains were able to grow satisfactorily in the presence of metronidazole at a concentration of 500 μg per ml. The remaining 91 strains did not grow in 10% CO_2 even after incubation for 7 days and all were sensitive to metronidazole; of these, 90 strains had MIC values that did not exceed 2.5 μg per ml, and one strain had an MIC of 5 μg per ml.

TABLE II

Growth and susceptibility to metronidazole of 94 clinical isolates of cocci initially considered to be anaerobic

Number of strains	Growth on solid media in air plus 10% CO_2 *	MIC of metronidazole (μg per ml)
3	+	> 500
90	—	2.5 or less
1	—	5

* Plates incubated for up to 7 days (see *Methods*).

Reference strains

A total of 21 reference strains tested included 17 NCTC strains, one strain of *Peptostreptococcus anaerobius* and one strain of *Peptococcus asaccharolyticus* from Dr Willis, and two strains of *Veillonella* spp. from Dr Holbrook. Preliminary testing (see above) showed that the four defined strains from Dr Willis and Dr Holbrook were obligate anaerobes, and were sensitive to metronidazole. Of the 17 NCTC strains, 13 appeared to be obligate anaerobes; one strain grew aerobically and three strains grew in 10% CO_2 in air. Of the 13 obligate anaerobes, all but one (NCTC9819) were sensitive to metronidazole. All of the four "non-anaerobic" strains were resistant to metronidazole (see table III).

All of the strains were tested further, as outlined in (1)–(4) above. The results showed that strain no. NCTC9819 was not an obligate anaerobe, but grew in 10% CO_2 after incubation for 7 days. This strain, and the other four "non-anaerobic" strains, were capable of growth on media containing 500 μg metronidazole per ml and incubated anaerobically. The remaining 12 obligately-anaerobic NCTC strains were all sensitive to metronidazole (MIC values less than 2.5 μg per ml). The two strains of *Veillonella*, the strain of *Peptostreptococcus anaerobius* and the strain of *Peptococcus asaccharolyticus* were all obligate anaerobes and were sensitive to metronidazole.

TABLE III

Gaseous requirements and metronidazole sensitivity of 21 reference strains of anaerobic cocci

NCTC number or label	*Growth on solid media in			Susceptibility to metronidazole†
	air	air plus 10% CO ₂	90% H ₂ plus 10% CO ₂	
9801	—	—	+	S
9803	—	—	+	S
9804	—	—	+	S
9805	—	—	+	S
9806	—	+	+	R
9807	+	+	+	R
9808	—	—	+	S
9809	—	+	+	R
9810	—	—	+	S
9811	—	—	+	S
9814	—	—	+	S
9815	—	—	+	S
9816	—	—	+	S
9817	—	+	+	R
9819	—	‡	+	R
9820	—	—	+	S
9821	—	—	+	S
<i>Peptostreptococcus</i> <i>anaerobius</i>	—	—	+	S
<i>Peptococcus</i> <i>asaccharolyticus</i>	—	—	+	S
<i>Veillonella</i> <i>parvula</i>	—	—	+	S
<i>Veillonella</i> <i>gazogenes</i>	—	—	+	S

* All strains incubated overnight at 37°C.

† R = resistant: zone of inhibition less than 15 mm after incubation for 48 h; S = sensitive: zone of inhibition greater than 15 mm after incubation for 48 h.

‡ This strain was subsequently shown to grow in 10% CO₂ in air after prolonged incubation (see text).*Haemolysis on blood agar*

None of the 191 obligately-anaerobic metronidazole-sensitive cocci (clinical isolates or reference strains) showed any haemolysis on horse or human blood agar. Three of the CO₂-requiring metronidazole-resistant clinical isolates showed good haemolysis on horse-blood agar, as did strain no. NCTC9807, an aerobic organism.

Disk sensitivity tests

Differentiation between metronidazole-sensitive and metronidazole-resistant strains was clear-cut on the basis of disk sensitivity testing. All resistant organisms had inhibition zones of less than 10 mm, whereas sensitive strains gave zones of 20 mm or more.

Gram reaction

Of the 196 strains of obligately anaerobic cocci (175 clinical isolates and 21 reference strains), many showed considerable variability when stained by Gram's

method; six were consistently gram-negative, 150 were consistently gram-positive, and the remaining 40 strains were gram-variable. The morphology of the strains was not characteristic: only 40 strains showed typical streptococcal morphology whilst the remainder appeared in clumps, with short chains, in gram-stained smears. In many cases, there was considerable pleomorphism.

DISCUSSION

There has been great interest in recent years in the role of anaerobic bacteria in human health and disease, yet this interest has largely been confined to the clostridia and to the gram-negative anaerobic bacilli. The obligately anaerobic cocci have received little attention, in spite of several reports attesting their importance in man (e.g. Pien, Thompson and Martin, 1972; Lambe, Vroon and Rietz, 1974).

The reason for the scanty attention received by these organisms may lie in the confusion surrounding their classification. In recent years, differing and conflicting schemes for the classification of anaerobic cocci have been proposed by several workers including Prévot and Fredette (1966), Holdeman and Moore (1972), and Buchanan and Gibbons (1974). The confused field has been reviewed by Smith (1975). The situation is further complicated: many cocci that on primary culture only grow anaerobically may grow well in 10% CO₂ in air on subculture, and some of the classification schemes allow inclusion of micro-aerophilic or CO₂-requiring strains, e.g., *Peptostreptococcus intermedius* (Smith, 1975).

We are at present engaged on a study of the isolation, characterisation and antibiotic susceptibility of obligately anaerobic cocci of clinical interest. In the early stages of our study, it was necessary to formulate a definition that included all of the obligately anaerobic strains but excluded any strains of cocci that were not obligate anaerobes. As we collected more strains, several points became clear in our attempts to define anaerobic cocci. (1) Many apparently anaerobic strains grew well in 10% CO₂ on subculture, although some required incubation for up to 7 days to develop visible colonies on solid media. (2) All obligately anaerobic strains appeared sensitive to metronidazole in a disk diffusion test. (3) All aerobic or CO₂-requiring strains were resistant to metronidazole.

The results of more detailed studies on a large number of clinical isolates and reference strains confirm these preliminary findings. All of the obligately anaerobic cocci (both gram-positive and gram-negative strains), were sensitive to metronidazole *in vitro*, both in disk diffusion tests and in MIC determinations. All of the remaining cocci grew in 10% CO₂ within 7 days and were resistant to metronidazole. These latter strains may be of as much importance in human health and disease as the obligately anaerobic strains, but they form a separate and clearly distinguished group.

One of the problems involved in the classification of obligately anaerobic cocci is the absence of a satisfactory collection of reference strains. Our results

show that of the 17 strains of "anaerobic cocci" lodged by Hare, used in his early scheme of classification (Thomas and Hare, 1954) and listed in the 1974 NCTC catalogue, only 12 behave as obligate anaerobes in our hands. These strains are sensitive to metronidazole. The remaining five strains all grow in 10% CO₂ within 7 days and are resistant to high concentrations of metronidazole. There is a clear need for a well-documented reference collection of obligately anaerobic cocci derived from clinical isolates, with agreed biochemical reactions, to form a basis for comparative studies.

We have found that obligately anaerobic cocci have little or no characteristic colonial appearances. They exhibit variable staining patterns and pleomorphism in Gram-stained smears, are non-haemolytic and have a wide range of antibiotic susceptibility patterns (Watt, in preparation). In the present paper we have avoided the use of any of these parameters in putting forward the provisional definition of anaerobic cocci as "Cocci that grow well under satisfactory conditions of anaerobiosis and do not grow on suitable solid media in 10% CO₂ in air even after incubation for 7 days at 37°C."

This definition sets out clear criteria for basic studies on these organisms—criteria that are valid for gram-positive and gram-negative cocci of clinical interest. The definition has the practical disadvantage that an incubation period of 7 days is required before a given strain can be regarded as an anaerobic coccus; this is too long for the practising clinical microbiologist. The use of metronidazole sensitivity testing (a procedure that can be completed within 24 or 48 h) provides both valuable chemotherapeutic information and an effective screening procedure. In our experience, anaerobic cocci are all sensitive to metronidazole. Accordingly, at the time of writing, all metronidazole-sensitive strains can be assumed to be anaerobic cocci, and all resistant strains must be checked further; we have not yet encountered a metronidazole-resistant anaerobic coccus. Clearly it would be unwise to rely solely upon metronidazole sensitivity in the definition of anaerobic cocci; with increasing clinical use of the drug there is a possibility that resistance may develop amongst obligately anaerobic bacteria. The combination of a basic definition with a useful screening test seems to cover this possibility, and we follow attempts to determine the mode of action of the drug with interest (see Edwards, 1977).

The clinical microbiologist, faced with terminological confusion, often resorts in despair to the use of the term "anaerobic streptococci" to cover all obligately anaerobic cocci isolated from clinical samples, yet many strains fail to show streptococcal morphology and may not be obligate anaerobes. We submit that the term "anaerobic cocci" for organisms that conform to our working definition provides a more sound basis for characterisation in the diagnostic laboratory and for further studies of these organisms in human health and disease.

SUMMARY

Criteria are suggested for the definition of "anaerobic cocci" as a general term to include all obligately anaerobic cocci. Such a definition draws a clear distinction between obligately anaerobic and micro-aerophilic strains and might

form a basis both for characterisation of these organisms in the diagnostic laboratory and for further studies on their taxonomy and pathogenicity.

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