# ANAEROBIC BACTERIA

## Role in Disease

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# ANAEROBIC BACTERIA:

## Role In Disease

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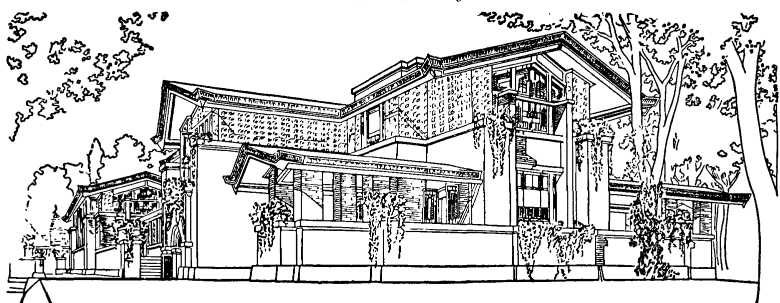


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# FOREWORD

The genesis of this series, *The American Lecture Series in Clinical Microbiology,* stems from the concerted efforts of the Editor and the Publisher to provide a forum from which well-qualified and distinguished authors may present, either as a book or monograph, their views on any aspect of clinical microbiology. Our definition of clinical microbiology is conceived to encompass the broadest aspects of medical microbiology not only as it is applied to the clinical laboratory but equally to the research laboratory and to theoretical considerations. In the clinical microbiology laboratory we are concerned with differences in morphology, biochemical behavior and antigenic patterns as a means of microbial identification. In the research laboratory or when we employ microorganisms as a model in theoretical biology, our interest is often focused not so much on the above differences but rather on the similarities between microorganisms. However, it must be appreciated that even though there are many similarities between cells, there are important differences between major types of cells which set very definite limits on the cellular behavior. Unless this is understood it is impossible to discern common denominators.

We are also concerned with the relationships between microorganism and disease—any microorganism and any disease. Implicit in these relations is the role of the host which forms the third arm of the triangle: microorganism, disease and host. In this series we plan to explore each of these: singly where possible for factual information and in combination for an understanding of the myriad of interrelationships that exist. This necessitates the application of basic principles of biology and may, at times, require the emergence of new theoretical concepts which will create new principles or modify existing ones. Above all, our aim is to present well-documented books

which will be informative, instructive and useful, creating a sense of satisfaction to both the reader and the author.

Closely intertwined with the above *raison d'etre* is our desire to produce a series which will be read not only for the pleasure of knowledge but which will also enhance the reader's professional skill and extend his technical ability. *The American Lecture Series in Clinical Microbiology* is dedicated to biologists—be they physicians, scientists or teachers—in the hope that this series will foster better appreciation of mutual problems and help close the gap between theoretical and applied microbiology.

This book represents the published proceedings of an International Conference on Anaerobic Bacteria. It includes major aspects of the bacteriological and clinical considerations of the organisms and disease entities of what constitutes a most important segment of infectious diseases today. The Conference was held at the Center for Disease Control in Atlanta, Georgia, in November 1972, and was jointly sponsored by Emory University, The Upjohn Company, and the Center for Disease Control. The stimulus for the Conference came from the increasing isolation of anaerobic bacteria from clinical specimens and a mounting awareness of their association with significant numbers of infectious and other diseases. The Conference provided a meaningful interchange of information on the bacteriology and clinical relevance of anaerobes with a clear indication that this interchange paved the way for a better understanding of the microorganisms and the diseases they cause. There was no question but what the proceedings needed to be published in a permanent form so that the information could be widely disseminated and shared with clinicians and microbiologists throughout the world. It seemed only proper that *The American Lecture Series in Clinical Microbiology* serve as the vehicle for this purpose. One need only to glance at the Contents to realize the extent of coverage of the subject and the expertise of the authors in their presentations dealing with this most intriguing area of infectious disease. This book is designed not only to share the data and information that were presented, but also to provide direction

for additional research leading to improved diagnoses and therapy. If these objectives are met (and all indications are that they will be), then this book will have well served its purpose.

Albert Balows, Ph.D.
*Editor*

# PREFACE

The decision to publish the proceedings of the International Conference on Anaerobic Bacteria was made once it became apparent that the nature and scope of the material presented by the distinguished group of speakers would be in large demand, not only by those who attended the Conference, but also by clinicians and microbiologists who daily face the problem of anaerobic bacterial infections. The Editors soon recognized that their responsibilities were many and diversified. The publication of these proceedings which represents the culmination of the Conference, was accomplished with the excellent support and collaborative efforts of many individuals. There are three who are particularly deserving of mention because, without their "behind the scenes" efforts, the Conference and this published account of that Conference would have been most difficult to achieve and assuredly would not have had the measure of success that it has been accorded.

To Mrs. Ruby W. Caplan of the Center for Disease Control we express our sincere thanks and gratitude for the excellent manner in which she managed the entire Conference. To Mrs. Diana Schellenberg of The Upjohn Company who served as the Editors' editor with her mastery and skill in handling the manuscripts we are most grateful and thankful. We are indebted to Dr. Dwight W. Lambe, Jr., of Emory University for his capable assistance in the planning of the Conference and in handling the fiscal aspects of the Conference.

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RAYMOND M. DEHAAN
V.R. DOWELL, JR.
LUCIEN B. GUZE

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# ANAEROBIC BACTERIA:

## Role In Disease

CHAPTER I

# ANAEROBIC BACTERIA PERSPECTIVES

Albert Balows

It is my task to introduce the subject of this Conference, anaerobic bacteria, by placing it in proper perspective. This can best be done by paraphrasing a comment in a recent editorial on anaerobic infections: medicine and microbiology must synergize lest, once again, all the world become anaerobic (Medeiros, 1972).

Not too many years ago, we had no great problems in recognizing anaerobic diseases: botulism, tetanus and gas gangrene were distinct clinical entities, each caused by specific sporeforming clostridia. Today, the situation has taken a 180-degree turn. Anaerobic bacteriology is literally in a renaissance period of development, and with it comes a new approach to the clinical relevance of infections caused by anaerobes. Improvements in technology during the past 10 years have permitted more definitive bacteriologic and clinical studies which continue to emphasize the importance of anaerobes in human and animal disease. These studies have dispelled certain misconceptions—so prominent in the literature—in relation to the disease potential of these anaerobes that are indigenous to man. When we recall that the microbiota of man is heavily favored for the anaerobes (by factors of 10 : 1 in the vagina, oral cavity, and skin to as much as 1,000 : 1 in the large intestine), it is no wonder that life-threatening diseases caused by one or more of the endogenous anaerobes are more common than those due to anaerobic bacteria of exogenous origin.

Technological achievements make it possible to cultivate virtually all anaerobes that may be present in a clinical specimen. This is accomplished by using improved methods of specimen collection, coupled with one or more anaerobic

systems and improved isolation media. A wide variety of differential media and sophisticated end-product determinations facilitate the classification of these anaerobes. More recently, several laboratories have directed their efforts toward the development of *in vitro* antimicrobic susceptibility techniques to aid in the selection of appropriate therapy.

At the outset of this Conference, we should note that some indigenous anaerobes appear to be frequently associated with human disease, whereas others, although present in large numbers in the normal microflora, seldom, if ever, are involved in human infections (Table I-I). For example, all

TABLE I-I

GENERA OF ANAEROBIC BACTERIA

| *Frequently encountered in significant infections* | *Seldom, if ever, encountered in significant human infections* |
|---|---|
| *Actinomyces* | *Acidaminococcus* |
| *Arachnia* | *Borrelia* |
| *Bacteroides* | *Butyrivibrio* |
| *Bifidobacterium* | *Lachnospira* |
| *Clostridium* | *Lactobacillus* |
| *Eubacterium* | *Leptotrichia* |
| *Fusobacterium* | *Ruminococcus* |
| *Peptococcus* | *Succinimonas* |
| *Peptostreptococcus* | *Succinivibrio* |
| *Propionibacterium* | |
| *Treponema* | |
| *Veillonella* | |

five subspecies of the *Bacteroides fragilis* group may be present in large numbers in normal gut flora. We rarely, if ever, isolate subspecies *ovatus* from clinical specimens associated with human infection, but the remaining four subspecies are isolated with almost regular frequency. To emphasize this point, let me show you a list (Table I-II) of anaerobic bacteria most frequently submitted to the CDC laboratories for identification (Dowell and Hawkins, 1972). These isolates are received from laboratories across the country, so this list is representative of those anaerobes most frequently isolated in the *average* hospital laboratory.

TABLE I-II

ANAEROBIC BACTERIA FROM HUMAN INFECTIONS MOST FREQUENTLY SUBMITTED TO THE CDC ANAEROBE LABORATORY: 1962–1972

1. Clostridia
   - *C. bifermentans*
   - *C. butyricum*
   - *C. cadaveris* (*C. capitovale*)*
   - *C. innocuum*
   - *C. limosum* (*Clostridium* sp CDC group P-1)
   - *C. perfringens*
   - *C. ramosum* (*Catenabacterium filamentosum, Bacteroides terebrans*)
   - *C. septicum*
   - *C. sordellii*
   - *C. sporogenes*
   - *C. subterminale*
   - *C. tertium*
2. Nonsporeforming gram-positive bacilli
   - *Actinomyces israelii*
   - *Actinomyces odontolyticus*
   - *Actinomyces naeslundii*
   - *Arachnia propionica* (*Actinomyces propionicus*)
   - *Bifidobacterium eriksonii* (*Actinomyces eriksonii*)
   - *Eubacterium alactolyticum* (*Ramibacterium* species)
   - *Eubacterium lentum* (*Corynebacterium diphtheroides*)
   - *Eubacterium limosum*
   - *Propionibacterium acnes* (*Corynebacterium acnes*)
   - *Propionibacterium granulosum* (*Corynebacterium granulosum*)
3. Nonsporeforming, gram-negative bacilli
   - *Bacteroides fragilis* ss. *fragilis* (*B. fragilis*)
   - *Bacteroides fragilis* ss. *thetaiotaomicron* (*B. variabilis*)
   - *Bacteroides fragilis* ss. *vulgatus* (*B. incommunis*)
   - *Bacteroides melaninogenicus* ss. *asaccharolyticus*
   - *Bacteroides melaninogenicus* ss. *intermedius*
   - *Fusobacterium mortiferum* (*Sphaerophorus ridiculosis*)
   - *Fusobacterium necrophorum* (*Sphaerophorus necrophorus*)
   - *Fusobacterium nucleatum* (*Fusobacterium fusiforme*)
4. Anaerobic cocci
   - *Peptococcus* sp. CDC group 2
   - *Peptostreptococcus* sp. CDC group 1
   - *Peptostreptococcus* sp. CDC group 2
   - *Peptostreptococcus* sp. CDC group 3
   - *Veillonella alcalescens*
   - *Veillonella parvula*

* Former name.

From the bacteriologic viewpoint, we have made considerable progress; the mystique of the anaerobic bacteria is not nearly so overwhelming as it was a dozen years ago. We also have developed a clearer understanding of the clinical importance of these anaerobes. Bacteremia caused by *Bacteroides fragilis* is an established clinical entity, with a consequence of which most clinicians are well aware. A striking association

of *Clostridium septicum* with certain malignancies, such as leukemia and various types of carcinoma, has been established. *Propionibacterium acnes,* a well recognized member of the normal cutaneous flora and frequently discarded by the clinical laboratory as a "diphtheroid," has been definitely incriminated as an etiologic agent of subacute bacterial endocarditis and may also be involved in some cases of chronic meningitis and chronic actinomycotic-like illnesses.

This Conference stems from the desire to reappraise our observations and data so that we can better assess the role of anaerobic bacteria in disease and the directions future investigations should take.

## REFERENCES

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# PART I

## NOMENCLATURE, TAXONOMY AND GENERAL METHODOLOGY

## CHAPTER II

# COLLECTION OF CLINICAL SPECIMENS AND PRIMARY ISOLATION OF ANAEROBIC BACTERIA

V.R. DOWELL, JR.

ABSTRACT: *Techniques for collection of clinical specimens and primary isolation of anaerobic bacteria are described briefly. The aspects emphasized are (1) proper selection of specimens to avoid contamination with normal flora and consequent erroneous results; (2) collection of specimens under anaerobic conditions; (3) use of fresh or prereduced primary isolation media; and (4) selective isolation procedures.*

Anaerobic bacteria associated with human infections are widely distributed in nature. Their habitats include soil, water, and the oral cavity, gastrointestinal tract, genitourinary tract, and skin of man and lower animals (Rosebury, 1962; Smith and Holdeman, 1968; Willis, 1969). Although there are a number of diseases involving anaerobic bacteria from exogenous sources (Table II-I), endogenous infections involving these microorganisms (Table II-II) are much more common. Factors commonly predisposing to endogenous anaerobic infections include surgery, malignancy, diabetes

TABLE II-I

DISEASES INVOLVING ANAEROBIC BACTERIA OF EXOGENOUS ORIGIN

| |
|---|
| Foodborne illnesses |
| Botulism |
| *Clostridium perfringens* gastroenteritis |
| Wound Infections |
| Tetanus |
| Myonecrosis ("gas gangrene") |
| Crepitant cellulitis |
| Benign superficial infections |
| Infection involving human or animal bite |
| Botulism |
| Septic abortion (contaminated instruments) |

TABLE II-II

ANAEROBIC INFECTIONS OF ENDOGENOUS ORIGIN

| |
|---|
| Central Nervous System: |
| Brain abscess, meningitis |
| Dental, ENT, Respiratory: |
| Peridontal infection, otitis media, pharyngitis, tonsillitis, sinusitis, pulmonary abscess, pneumonia, empyema, "actinomycosis" |
| Intra-abdominal: |
| Appendicitis, diverticulitis, colitis, malabsorption disease; post-surgical infection including cellulitis, myonecrosis, and tetanus; abscess of any organ, peritonitis |
| Genitourinary: |
| Endometritis, salpingitis, ovarian abscess, infection of Bartholin's gland, urethritis, nephritis, abscess of kidney |
| Other: |
| Bacteremia, subacute bacterial endocarditis, osteomyelitis, perirectal abscess, decubitus ulcer, etc. |

mellitus, arteriosclerosis, alcoholism and antibiotic, immunosuppressant, corticosteroid or X-irradiation therapy (Bornstein *et al.*, 1964; Felner and Dowell, 1971).

As discussed in previous publications, proper selection and collection of specimens; culture of the material as soon as possible after collection; use of fresh, properly reduced media; and provision of adequate anaerobic conditions are some of the more important considerations in the isolation of anaerobic bacteria (Dowell and Hawkins, 1968; Dowell, 1970).

## COLLECTION OF SPECIMENS

Proper selection and collection of specimens are extremely important for laboratory confirmation of anaerobic infections. Otherwise, culture results may be misleading or meaningless. Since various anaerobic bacteria are present in large numbers in the normal flora of the oral cavity and gastrointestinal tract and some inhabit the genitourinary tract and skin (Dowell, 1970; Finegold, 1970), specimens likely to be contaminated with these microorganisms should not be cultured. These include the following:

1. Throat or nasopharyngeal swabs.
2. Sputum or bronchoscopic specimens.
3. Feces or rectal swabs.
4. Voided or catheterized urine.

5. Vaginal or cervical swabs (not collected by visualization via a speculum).
6. Material from superficial wounds or abscesses not collected properly to exclude surface contaminants.
7. Material from abdominal wounds obviously contaminated with feces, e.g. open fistula. On the other hand, *all* body fluids and tissues from sites not contaminated with normal flora should be cultured for anaerobic bacteria (Sutter *et al.*, 1972). Examples of acceptable clinical specimens for laboratory diagnosis of anaerobic infections are listed in Table II-III.

Exposure of clinical specimens to oxygen is probably one of the major reasons some laboratories have little success in cultivation of anaerobes. The most suitable samples for isolation of anaerobic bacteria are aspirated materials and tissue biopsies; swab samples are much less satisfactory (Dowell and Hawkins, 1968). When a sample is collected with a needle and syringe, the syringe should be cleared of air and the fluid injected immediately into a sterile *gassed out* tube or vial (Holdeman and Moore, 1972; Sutter *et al.*, 1972) to minimize exposure to oxygen. If it is not possible to culture tissue samples immediately, these should also be placed in an anaerobic environment until processed. A miniature anaerobic jar utilizing a 35-mm film container and steel-

TABLE II-III

EXAMPLES OF ACCEPTABLE CLINICAL SPECIMENS FOR LABORATORY DIAGNOSIS OF ANAEROBIC INFECTIONS

| |
|---|
| CNS: |
| Cerebrospinal fluid, abscess material, tissue biopsy |
| Dental, ENT: |
| Carefully aspirated material from abscesses and biopsied tissue |
| Pulmonary: |
| Transtracheal aspirate, tissue biopsy, direct lung aspirate, pleural fluid, "sulfur granules" from draining fistula |
| Intra-abdominal: |
| Aspirate from loculated abscess, ascitic fluid, tissue biopsy |
| Genitourinary: |
| Urine (suprapubic aspirate), aspirate from loculated abscess, tissue biopsy from normally sterile site, cervical material collected by direct visualization |
| Other: |
| Blood, bone marrow, bile, aspirated "joint" fluid, muscle biopsy from suspected gas gangrene, biopsied tissue from any normally sterile site |

wool immersed briefly in an acidified copper sulfate solution to absorb oxygen has been recommended by Attebury and Finegold for this purpose (Sutter *et al.*, 1972).

If it is absolutely necessary to obtain material with swabs, at least three swabs should be used to provide sufficient material for microscopic examination and culture. These should be processed immediately or placed in prereduced anaerobically sterilized Carey-Blair medium with a *head* of oxygen-free $CO_2$ as recommended by Sutter *et al.* (1972) or in *gassed out* tubes containing oxygen-free $CO_2$ (Holdeman and Moore, 1972; Sutter *et al.*, 1972). Anaerobic transport tubes and vials are now available commercially (Hyland Laboratories, Scott Laboratories). For maximum recovery of anaerobes in the same relative proportions as present in the infected tissue, all clinical specimens except blood samples should be held at room temperature for no longer than 2 hours before processing. The specimens should not be refrigerated, as chilling is detrimental to some anaerobes (Dowell, 1970).

Because of the complexity of the subject, anaerobic blood culture techniques will not be discussed in detail in this report. However, the following points regarding blood cultures should be kept in mind:

1. Before performing the venipuncture the skin must be carefully cleansed and disinfected to avoid contamination with normal skin inhabitants such as *Propionibacterium acnes* (Dowell, 1970; Sutter *et al.*, 1972).

2. Precautions must be taken to expel air from the syringe or collection device to prevent introduction of air into the blood culture system.

3. An adequate volume of blood should be cultured to allow detection of microorganisms in small numbers (at least 5 ml, preferably 10 ml) and the volume of culture medium should be at least 9 to 10 times that of the blood sample.

4. The medium must be nutritionally adequate for the anaerobic bacteria and as fresh as possible. Since some strains of *Bacteroides melaninogenicus* require vitamin K compounds for growth, addition of menadione (vitamin $K_1$) is recommended (Dowell and Hawkins, 1968; Holdeman and Moore, 1972; Sutter *et al.*, 1972).

5. Use of a medium containing polyanetholsulfonate (Liquoid) is advantageous but it should be kept in mind that some anaerobic cocci and *B. melaninogenicus* may be inhibited (Sutter *et al.*, 1972).

6. The medium must be properly reduced and as free of molecular oxygen as possible. Therefore, the medium should contain a reducing agent such as cysteine to lower the oxidation-reduction potential and air should be excluded during storage. This can be accomplished most expediently by preparation of blood culture bottles containing prereduced anaerobically sterilized (PRAS) medium (Holdeman and Moore, 1972; Sutter *et al.*, 1972).

7. PRAS blood culture media are now available commercially (Hyland Laboratories, Scott Laboratories).

## Laboratory Confirmation of Botulism

The most effective means for confirming a diagnosis of botulism is to demonstrate the presence of botulinal toxin in the serum of the patient (CDC, 1970). It is also useful to test stomach contents, feces and suspect foods for toxin and to culture foods, feces, stomach contents and excised tissue (wound botulism) for *Clostridium botulinum*. Specimens to be tested for botulinal toxins and/or *C. botulinum* should be collected and handled as follows:

BLOOD. Collect sufficient blood, before the patient is treated with botulinal antitoxin, to provide 15 to 20 ml of serum. Refrigerate serum at 4°C until examined.

FOOD. Leave unopened containers sealed. Collect others in sterile, unbreakable containers. Refrigerate at 4°C until examined.

GASTRIC CONTENTS, FECES. Collect in sterile, unbreakable containers and refrigerate at 4°C until examined.

EXCISED TISSUE. Collect in sterile *gassed out* tubes if possible and hold under anaerobic conditions at ambient temperature until examined.

If it is necessary to ship the materials to a distant laboratory, place specimens in a leakproof container, wrap with a cushioning material, and pack with ice or refrigerant in a second

leakproof, insulated shipping container and ship by the most rapid means available.

## MICROSCOPIC EXAMINATION OF CLINICAL MATERIALS

Numerous investigators have emphasized the importance of microscopic examination of clinical specimens when anaerobic infections are suspected (Dowell and Hawkins, 1968; Dowell, 1970; Finegold, 1970; Holdeman and Moore, 1972; Smith and Holdeman, 1968; Sutter *et al.*, 1972; Willis, 1969). Microscopic examinations can give immediate presumptive evidence of the presence of anaerobes and aid the physician in his choice of therapy. A Gram-stained direct smear should be examined from all types of clinical materials except blood. In addition to providing information on the cellular character of the specimen and the types of microorganisms present, the Gram-stained smear also aids the microbiologist in his choice of selective media for isolation of anaerobes from polymicrobic infections (Sutter *et al.*, 1972). Microscopic examination of unstained clinical material by regular light, dark-field and phase contrast microscopy is also useful, particularly when spirochetes are suspected (Holdeman and Moore, 1972) and in the examination of "sulfur granules" from patients with suspected actinomycosis. Acid-fast and Giemsa-stained direct smears can also provide useful information (Dowell, 1970).

## PRIMARY ISOLATION

### General Considerations

Media may become inhibitory for anaerobic bacteria if stored in the presence of oxygen (Aranki *et al.*, 1969; Dowell, 1970; Finegold, 1970; Hobbs *et al.*, 1971; Holdeman and Moore, 1972; Killgore *et al.*, 1973; Martin, 1971; Smith and Holdeman, 1968). For this reason, all media used for cultivation of anaerobes should be at optimum freshness; this is particularly true for plating media used for primary isolation.

Also, overheating of media during or after preparation should be avoided (Dowell and Hawkins, 1968; Smith and Holdeman, 1968). Plating media for primary isolation should be prepared on the day of use or freshly prepared media can be stored under anaerobic conditions for a period no longer than two weeks. The media can be stored in an anaerobic jar (Smith and Holdeman, 1968), an anaerobic glove box (Aranki *et al.*, 1969; Killgore *et al.*, 1973), or in an airtight cabinet with a constant flow of carbon dioxide as described by Martin (1971). Liquid media not prepared by the PRAS technique (Holdeman and Moore, 1972) should be stored in tightly capped tubes in the dark at room temperature for no longer than two weeks.

Provided clinical specimens are collected properly and the materials are cultured with fresh, properly reduced media, successful cultivation of anaerobes can be obtained by use of the GasPak anaerobe jar (BBL) or an anaerobic jar with a gas replacement method (Collee *et al.*, 1972; Killgore *et al.*, 1973). The anaerobic glove box system (Aranki *et al.*, 1969; Killgore *et al.*, 1973), and the roll-streak tube system (Holdeman and Moore, 1972) using PRAS media also give excellent results. Since most clinical laboratories do not use an anaerobic glove box or roll-streak systems at present the following isolation procedures are designed for use with anaerobic jars.

## Media

All clinical specimens except blood should be cultured by both direct plating and enrichment procedures, and selective media should be employed if warranted. At a minimum, the following should be inoculated with each specimen:

One tube of chopped meat-glucose medium enriched with hemin-menadione solution (Dowell and Hawkins, 1968);

One tube of thioglycollate broth (BBL 135C or equivalent) enriched with 10 percent V/V sterile rabbit serum and hemin-menadione solution;

Two plates of blood agar containing menadione (Dowell and Hawkins, 1968)—incubate one plate anaerobically and the other in a candle jar or $CO_2$ incubator.