



The
Dark Art
of Blood
Cultures

EDITED BY Wm. Michael Dunne, Jr.
and Carey-Ann D. Burnham

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To Linda and Ryley for keeping us sane.

Editors' Disclaimer

While references are used extensively to support observations, published data, clinical trials, and laboratory comparisons of blood culture systems, culture media, protocols and procedures, etc., it is inevitable that personal opinions and small biases might have unintentionally snuck in to the final copy. The editors were very attuned and sensitive to this possibility and made every attempt to avoid blatant conflicts of interest without interrupting the independence of the authors. All of the individuals and commercial entities that have contributed to the advancement of the science of blood cultures have had a significant influence on the field and we thank them all.

Contents

Contributors ix

Preface xi

About the Editors xiii

- 1 Historical Perspectives on the Art and Science of Blood Culture 1**
Wm. Michael Dunne, Jr.
- 2 Conventional Blood Culture Methods 21**
Robyn Atkinson-Dunn and Wm. Michael Dunne, Jr.
- 3 Lysis-Centrifugation Methods of Blood Culture 39**
Erin McElvania TeKippe and Morgan A. Pence
- 4 Bactec Blood Culture Systems 59**
Robin R. Chamberland
- 5 The bioMérieux BacT/Alert: Automation at Last in the Black Box 85**
Bradley Ford and George Kallstrom
- 6 TREK Blood Culture Systems 113**
Neil W. Anderson and Melanie L. Yarbrough
- 7 Molecular Methods for Detection of Pathogens Directly from Blood Specimens 137**
Mark D. Gonzalez and Robert C. Jerris
- 8 Pediatric Blood Cultures 151**
Paula Revell and Christopher Doern

- 9 Epidemiology of Bloodstream Infections 163**
Allison R. McMullen, Craig B. Wilen,
and Carey-Ann D. Burnham
- 10 Best Practices in Blood Cultures 183**
Robert J. Tibbetts and Barbara Robinson-Dunn
- 11 Processing Positive Cultures 207**
Matthew L. Faron and Nathan A. Ledebøer
- 12 Fungal and Mycobacterial Blood Cultures 245**
Robert S. Liao and William Lainhart
- 13 The Bacterial Blood Microbiota/Microbiome 277**
Eileen M. Burd and Lars F. Westblade
- 14 Postmortem Blood Culture 297**
Robin R. Chamberland and Carl O. Deetz
- Index 309**

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Preface

Many years ago, we began kicking around the possibility of devoting an entire text to the subject of blood culture technology and the continuing evolution that has occurred over the past decades (and centuries if you start with historical perspective). As with any idea, there is often an energy of activation to get the project underway but once that occurred, we were able to gain the support of those involved.

This project was a team effort, and each of the chapters was authored and/or coauthored by the fellows of the Medical and Public Health Microbiology training program established at Washington University School of Medicine and Barnes-Jewish Hospital in 2001. We are equally proud of each of the fellows, and any proceeds generated from this publication will be funneled back into the training program to help keep it alive. We are also thankful for those contributors who worked with former or current fellows to generate some of the chapters presented in the text. The title and cover we selected for this book underscores the slightly warped sense of humor we have integrated into the training program over the years, and even today, there are a number of inside jokes shared by this family as it continues to grow. In that vein (there's the first pun), a good microbiologist who recovers *Eikenella corrodens* from a blood culture will always ask about bite marks. It is our hope that this compendium of all things blood culture will also continue to expand with future editions and will always include graduates of the Washington University program both before and after they leave to direct clinical microbiology laboratories of their own. We might have not provided a complete evaluation of all the technologies that have come and gone over the years, but we'll stick with it (second pun).

WM. MICHAEL DUNNE, JR.
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About the Editors



Wm. Michael Dunne, Jr., PhD, is currently a senior research fellow for bioMérieux, Inc. in Durham, North Carolina. Prior to joining bioMérieux, he was Medical Director of the Diagnostic Microbiology Laboratory at Barnes-Jewish Hospital and Professor of Pathology and Immunology, Molecular Microbiology, Pediatrics, and Medicine at Washington University School of Medicine in St. Louis from 2000–2011 and remains on the faculty there. During that time, he established an internationally-recognized training program in medical and public health microbiology, which is still active under the direction of Carey-Ann Burnham. He has previously served as Medical Director of Microbiology Laboratories at Henry Ford Hospital (Detroit, Michigan), Texas Children’s Hospital (Baylor College of Medicine, Houston, Texas) and Children’s Hospital of Wisconsin (Medical College of Wisconsin, Milwaukee, Wisconsin, where he had received his PhD in 1982). Dr. Dunne is a Diplomate of the American Board of Medical Microbiology and a Fellow of the American Academy of Microbiology, the Infectious Diseases Society of America, the Pediatric Infectious Diseases Society, and the Canadian College of Microbiology. He served as a senior editor of the *Journal of Clinical Microbiology* for ten years and remains on the editorial board. He is also an Adjunct Professor of Pediatrics at Duke University School of Medicine, Durham, North Carolina. He has authored or coauthored over 170 peer-reviewed publications and 10 chapters, in addition to this book.



Carey-Ann D. Burnham, PhD, completed her PhD in Medical Sciences at the University of Alberta in Edmonton, Alberta, Canada and her postdoctoral training in Medical and Public Health Microbiology at Washington University under the direction of Wm. Michael Dunne, Jr. Currently, Burnham is an Associate Professor of Pathology & Immunology, Molecular Microbiology, and Pediatrics at Washington University in St. Louis School of Medicine. Burnham has a keen interest in education and is the Program Director for the Medical and

Public Health Microbiology Fellowship at Washington University, the co-editor of Medical Microbiology Question of the Day, and the section editor of “The Brief Case” for the *Journal of Clinical Microbiology*. Burnham’s research program focuses on development of diagnostic assays for infectious diseases and the transmission and epidemiology of antimicrobial resistance in bacteria. Burnham has authored or coauthored more than 100 peer-reviewed publications in addition to numerous invited articles and book chapters.

Historical Perspectives on the Art and Science of Blood Culture

1

Wm. Michael Dunne, Jr.^{1,2,3}

From an historic point of view, one can divide the evolution of blood culture technology into four distinct “archeological” periods that I refer to (tongue in cheek) as the “Manualithic” (pre-1970), the “Bactecene” (1970 to 1990), the “Continuous Monitorassic” (1990 to 2000), and the “Ampliaissance” (post-2000) ages (Fig. 1). Each of these denotes a quantum shift in the use of technology to increase the sensitivity and decrease the time-to-detection of microorganisms in blood. While the rate of improvement in both speed and sensitivity has clearly reached steady state over recent years, and the ultimate goal of directly detecting microorganisms causing bacteremia/fungemia/sepsis from blood has not yet been achieved, progress is most definitely moving forward and the tools being developed for that purpose are impressive. However, before we find out how far we have come, it is best to review where the concept of finding microorganisms in blood began, and some historians would conclude that it started with Athanasius Kircher (Fig. 2).

Kircher was an ordained Jesuit priest whose life nearly spanned the entire 17th century (1602 to 1680). He is referred to as a polymath/savant and his life story reads more like Indiana Jones and the Arc of the Covenant (1). Kircher was a scholar and true renaissance man who became well versed in linguistics, astronomy, magnetism, volcanology, archeology, and acoustics, just to name a few of his scientific hobbies. When the bubonic plague struck Rome in full force in 1656, Kircher extended his credentials to include

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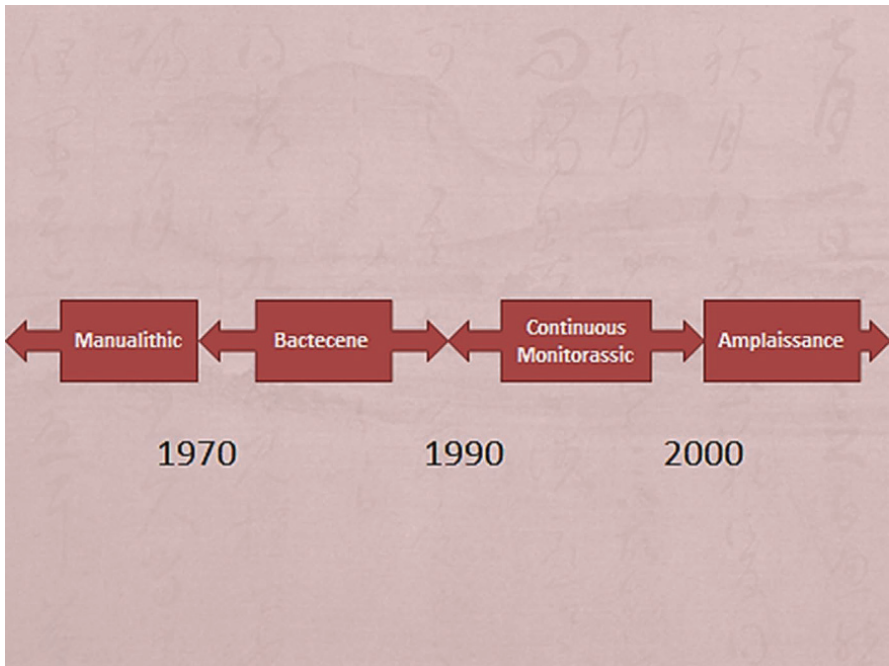


Figure 1 The archeological periods of blood culture evolution.

ad hoc pathologist and infectious diseases researcher (1, 2). Using a primitive microscope, he described observing tiny worms or *vermes* (*contagia animate*) in the “verminous blood of fever patients” and became the first to develop a germ theory of disease, which was published 2 years later (*Scrutinium physico-medicum contagiosæ luis*. Romæ: Typis Mascardi, 1658; references 2, 3). In this treatise, he describes the disease thusly: “*Pestis est flagellum and sagitta Dei ob peccata hominibus immissa*,” which loosely translates into “plague is the whip and arrows of an angry God used to punish sinful humans.” From a purely microbiological standpoint, two words in this description jump out: *pestis* and *flagellum*. Of course, the former became the species name for the causative agent of bubonic plague. However, the latter was inaccurate, because *Yersinia pestis* is nonmotile and lacks flagella. He further stated that “vinegar, milk, and the blood of those sick with fever are full of worms invisible, however, to the unaided eye. That the matter is not otherwise than I have said, the putrid blood of those afflicted by fevers has fully convinced me; I have found it, an hour or so after letting, so crowded with worms as to well high dumbfound me; and I had even been persuaded forthwith that man



Figure 2 Cornelis Bloemaert II, *Portrait of Athanasius Kircher*, 1664/1678. Kircher was a Jesuit priest who launched the notion that certain diseases were caused by microscopic organisms carried in the blood and published his germ theory of disease in 1658 amid the plague epidemic in Rome. The quotation loosely translates to “Plague is the whip and arrows of an angry God used to punish humans.” Note the Latin words *pestis* and *flagellum* in the quotation that had unintended contemporary application in current bacteriology. Reprinted from reference 1, with permission.

both alive and dead swarms with numberless but yet invisible worms; and that this may be the meaning of the words of Job: ‘I have said to corruption, thou art my father; to the worms, my mother and my sister.’” As fate would have it, the microscope Father Kircher used likely lacked the resolution to visualize *Y. pestis*. What he actually observed remains a mystery, but it was probably clumps of red blood cells (3, 4). However, his speculation that bubonic plague was indeed blood borne and caused by a microorganism was entirely spot on, albeit perhaps a lucky guess (2, 4). He also opined that infectious diseases were not transmitted through the air but rather by person-to-person contact or via another living thing. Kircher also unintentionally invented infection prevention by offering ways of avoiding disease through piety, repentance, and acts of mercy which did not preclude leaving the city, having adequate ventilation, or using odorous substances (2, 4). In fact, it was not until nearly 25 years later that Antonie van Leeuwenhoek had

developed a microscope with sufficient resolution (most likely in combination with some form of dark-field illumination) to observe a microorganism the size of *Y. pestis* (5, 6). So in a sense, Father Kircher was the father of the concept of blood-borne disease.

In a continuation of the theory that blood harbors agents of infection, Professor Jean Antoine Villemin (Fig. 3), a French physician, reported to the Académie de Médecine in 1868 that the injection of blood, sputum, or granulomatous material from animals or humans with tuberculosis into the veins of laboratory animals (rabbits, dogs, cows, etc.) generated lesions in the lungs, bone, and viscera of these animals that were indistinguishable from those produced by active infection (7). Furthermore, Dr. Villemin stressed that the lesions thusly generated via venous inoculation were quite distinct from embolic lesions generated by the inoculation of inert materials such as dust or even pus from other infectious processes. In the strictest sense, Villemin had just described a “biological blood culture,” i.e., the use of laboratory animals to recover agents of blood-borne infection.

At the turn of the 20th century, just prior to the outbreak of World War I, the process of culturing blood from septic patients was already a complex

Figure 3 Dr. Jean Antoine Villemin, a French physician, reported to the Académie de Médecine in 1868 that the injection of blood, sputum, or granulomatous material from animals or humans with tuberculosis into the veins of laboratory animals (rabbits, dogs, cows, etc.) generated lesions in the lungs, bone, and viscera of these animals that were indistinguishable from those produced by active infection.



affair beginning with preparation of the skin surface prior to drawing a sample (8). It was recommended that preparation for a blood draw should take place at least an hour prior to collection by using green soap, alcohol, and bichloride of mercury in succession to cleanse the skin as one would do to prepare for surgery. Blood should be collected using a sterilized syringe (30 min of boiling or hot-air chamber) using a linen bandage as a tourniquet. Approximately 10 ml was the target collection volume, and this could be mixed with sterile 2% ammonium oxalate to prevent clotting so that “transfers can be made more leisurely to culture media.” In cases where transportation to the laboratory might be delayed, cultures could be prepared at bedside (point-of-care system?). Recommended media combinations consisted of glucose-meat-infusion agar and broth; the former to be melted and combined with blood (0.25 to 1.0 ml) to prepare pour plates. Three flasks containing between 100 and 150 ml of broth were to be inoculated with “varying” volumes of blood, but one flask should be at high dilution. It was further suggested to add powdered calcium carbonate to one flask to ensure a neutral pH, thus supporting the growth of either pneumococci or streptococci that are “sensitive to acid” (do not tell oral streptococci this!) and to “develop and retain their vitality.” It was recognized at this point in time that contaminants posed a challenge to interpretation, but one could look at the frequency of isolation (one flask or one plate only) or location (near the edges or on the surface of the pour plate) as an indication of contamination. It was also considered a good idea to regard all staphylococci recovered from blood with suspicion. *Staphylococcus albus* (now *S. epidermidis*/coagulase-negative *Staphylococcus* spp.) should most often be deemed a skin contaminant, whereas the isolation of *Staphylococcus pyogenes aureus* from a single blood culture should be substantiated with a second (8). Patients with suspected typhoid fever prompted an entirely different blood culture strategy that was based on clinical investigations of Coleman and Buxton, Conradi, Schottmueller, and Castellani, as discussed in reference 9. For this method, flasks containing 20 ml of a mixture of ox bile, glycerin, and pepton (peptone) were inoculated with 3 ml of blood and incubated for 18 to 24 h. At that time, samples were subcultured to lactose-litmus agar, and isolated colonies were identified using agglutination or additional biochemical tests. This protocol produced a positivity rate of 89% during the first week of illness, 73% during the second, 60% during the third, 38% during the fourth, and 26% thereafter. In the eighth edition of the *Manual of Bacteriology*, Muir and Ritchie promote two approaches for the culture of blood depending on the organism(s) being sought (10). For enteric bacilli, it was

recommended to mix 5 ml of blood with 10 ml of sterilized ox bile or 50 ml of a 0.5% solution of bile salts in a 1% solution of sodium citrate and incubate the mixture. When examining for other organisms such as streptococci, however, it was suggested that 5 to 10 ml of blood be added to a large volume of liquid medium (e.g., 100 ml)—an interesting observation considering that the recommended blood-to-broth ratio in the absence of neutralizing resins remains the same even today. They also mention the use of trypsin added to broth to neutralize the antibacterial properties of blood and to prevent clotting (Douglas and Colebrook cited in the chapter but no reference provided). The trypsin/broth blood culture medium could be made ahead of time and stored in the ice chest for several weeks.

At the onset of World War I, Mildred Clough, a physician/microbiologist at Johns Hopkins University, first reported on the use of a lysis-centrifugation procedure for the recovery of *Mycobacterium tuberculosis* from the blood of patients with miliary tuberculosis (11). Ten milliliters of blood was lysed with distilled water and the lysate was “centrifugulized” for 1.5 h at high speed. The sediment was then washed over the surface of blood agar slants and incubated. It was noted that colonies of *M. tuberculosis* could sometimes be visualized within 7 to 10 days, which, even by today’s standards, would be considered rapid.

One of the more important developments in modern blood culture technology occurred during the 1930s and 1940s and concerned the recognition of “Liquoid” (Hoffman-LaRoche; sodium polyanethol sulfonate) as a potent anticoagulant, anticomplement, and antiphagocytic agent. This substance was found to be useful as a blood transport medium (thus avoiding complicated bedside inoculation of media), an additive to liquid blood culture media, and indeed a self-contained culture medium that could be subcultured periodically during incubation to solid, agar-based media (12). Initially used at a concentration of 0.16%, later experiments suggested that concentrations higher than 0.05% could inhibit the growth of certain organisms (13). Liquoid essentially replaced saponin and trypsin as a blood culture anticoagulant over the next few decades, although the most effective final concentration and its use for the recovery of anaerobes remained an issue of some debate (12).

At about this same time, an interesting protocol for the recovery of streptococci was reported (14). This method (credited to Cecil without reference) called for the collection of 20 ml of blood in two tubes that were allowed to clot overnight in the icebox. The clots were then disrupted with a glass rod and placed in 50 ml of beef heart infusion broth and incubated at

37°C for a month. Subcultures were made every fifth day to blood agar and broth. The authors noted that the risk for contamination was high.

By the end of World War II, blood culture techniques would be fairly recognizable by clinical microbiologists today with a few exceptions. Blood samples were to be collected when patients exhibited temperature spikes, although the logistics of doing so would make timing a nightmare (15). Several (number not stated) specimens should be collected at hourly intervals to increase the likelihood of obtaining a positive result. Special consideration should be given to the choice of vein for collection. Drawing from a vein draining an infected area should be used whenever feasible. Blood collection from the femoral artery was believed to be better than venous blood for culture, although this too was disputed. If blood cultures proved negative, one could try sternal marrow as an alternative. It was recognized that pneumonia and meningitis were associated with bacteremia in about 50% of cases and duplicate bottles should be obtained in cases of subacute bacterial endocarditis, puerperal sepsis, and subacute focal infections, one bottle each being incubated aerobically and anaerobically. The latter was always to be included for patients with postsurgical infections using thioglycollate broth. Once collected, blood cultures should be examined once daily for 3 weeks. If *Brucella* spp., *Neisseria gonorrhoeae*, or *Neisseria meningitidis* infection was suspected, the atmosphere of the bottles should include 10% CO₂.

To avoid contamination, the following process was suggested. Blood should not be collected within 8 h of any intravenous injection. All electric fans should be turned off and windows and doors closed. The best vein for the infectious process at hand should be selected and a sterile towel placed under the collection site. Wash the site with soap or tincture of green soap in a circular motion. Remove the soap with a sterile gauze. Apply tincture of iodine or merthiolate over the area and cover with a sterile gauze until dry. Wash this off with 95% alcohol and allow to dry under a sterile gauze or towel. At bedside, withdraw about 15 ml of blood and inoculate a bottle containing 150 ml of dextrose beef infusion hormone broth with 5 ml of the total volume collected. Place another 5 ml in a citrated tube and mix to prevent clotting. In the laboratory, prepare two pour plates with 1 and 2 ml of citrated blood mixed with melted beef infusion agar cooled to 45°C and poured into sterile petri plates. Allow to harden, and label the volume of blood in each (these will be used to estimate the level of bacteremia). Place another 2 ml of blood into 25 ml of thioglycollate glucose broth for anaerobes. Prepare a Spray dish. (Note: a Spray dish is like a pineapple upside-down cake. It is a pour plate with the agar-blood mix in the top of the petri

dish, which is then covered by the bottom of the dish and inverted.) A mixture of sodium bicarbonate, sulfuric acid, and water is added to the bottom to generate CO₂ for cases of suspected *Brucella* spp., *N. gonorrhoeae*, or *N. meningitidis* infection. The plate is then sealed with paraffin and incubated. All cultures are incubated at 37°C for 24 h, examined, and an initial report generated based on Gram stains of the broth cultures which are subsequently subcultured to blood agar slates. This process is repeated every 2 to 3 days for 10 to 21 days until being reported as negative.

Scott's method simplified things a bit (16). It used 2-oz.-square, clear glass bottles with a screw-cap top and a rubber septum underneath. The aerobic bottle consisted of 15 ml of melted Trypticase soy agar dispensed into the bottle, which was turned on its side and cooled. Then, 10 ml of Trypticase soy broth was added to the bottle to create a modified Castaneda bottle (Fig. 4). Various iterations of this type of a system would be seen in later years, e.g., the Septi-Chek system from Becton Dickinson (Sparks, MD). The anaerobic bottle consisted of 25 ml of thioglycollate broth that had been autoclaved and stoppered to remove O₂. The aerobic/anaerobic bottles were inoculated with 5 and 2 ml of blood, respectively (the difference



Figure 4 The advent of the modern blood culture set of E. G. Scott (16) consisting of a thioglycollate anaerobic bottle and a broth/agar slant combination bottle for aerobes fashioned after the *Brucella* bottle designed by Castaneda (17). The bottle set on the left demonstrates no growth in either bottle, while the set on the right shows growth in both. Reproduced from reference 15, with permission.

in volume was not elucidated). *p*-Aminobenzoic acid (PABA) and penicillinase could be added if the patient was receiving sulfonamides or penicillin, respectively (first antimicrobial removal system?). The bottles were again incubated at 37°C and examined at 24 h and every 2 days thereafter. The agar side of the aerobic bottle could be “washed” with the blood/broth mixture if no growth was observed and the atmosphere of the bottle could be recharged by venting in the presence of 10% CO₂. Most isolates were recovered in 24 to 72 h unless antibiotics were on board. This method was also amenable for bedside inoculation.

If the blood sample clotted, serum was removed and the clot was placed in the barrel of a 10- to 20-ml syringe. The plunger was replaced, and the clot was forced through the tip of the syringe into a 100-ml bottle containing 50 ml of 0.12% beef infusion agar (semisolid).

Recognized contaminants of the day included diphtheroids (19% of the total) and skin cocci (*S. albus*, 24% of the total). Scott reported an overall contamination rate of 4.7% using his system, which included diphtheroids, *S. albus* (*S. epidermidis* and coagulase-negative staphylococci), *Escherichia coli*, and *Proteus* spp. One has to wonder about the latter two being designated contaminants.

Other standard blood culture media of the mid-20th century included hormone broth with 0.2% glucose, peptone colloid medium, tryptose phosphate broth, and Kracke's blood culture media designed to fix complement (15).

In all fairness, the first description of a biphasic liquid-solid medium interface was made by Castaneda in 1947 (17), and specifically designed for the recovery of *Brucella* species from blood. But, with time, it gained a more general appeal for the recovery of most organisms, including fungi and intracellular organisms causing bacteremia. Unfortunately, the popularity of biphasic blood culture bottles was offset by the complexity of producing them.

Advances in blood culture technology progressed at a glacier pace over the next decade, but two notable events paved the way for more significant developments down the road. The first of these seems like a non sequitur, but bear with me, because the connection will be made later. Levin et al., in a 1962 *Science* article, described a system developed by the National Aeronautics and Space Administration (NASA) by which microbial life in Martian soil could be detected using radiospinometry (18). The system was to be loaded on board a small probe called “Gulliver,” which was intended to be delivered to the surface of Mars. Once there, the probe would launch two

cylindrical projectiles attached by 23 feet of wire onto the surface and reel the projectiles back into the probe, collecting soil samples in the cylinders in the process (Fig. 5). The contents would then be emptied into a microbial growth chamber containing a defined broth medium supplemented with glucose- ^{14}C (uniformly labeled) and formate- ^{14}C . If microorganisms were present in the sample and they utilized glucose or formate as a carbon source for growth, detectable levels of $^{14}\text{CO}_2$ would be generated and detected (after precipitation as barium carbonate) by a solid-state beta detector coated with barium hydroxide and situated directly above the growth chamber. Field tests on terra firma showed that the system worked with a wide variety of aerobic, microaerophilic, and anaerobic microorganisms. The system was also equipped with a nonlabeled CO_2 -generating system that permitted flushing of the growth chamber prior to inoculation to eliminate any labeled gas that was generated during the long trip to Mars. This same approach would appear later as a microbial growth detection system specifically applied to the detection of microorganisms in blood cultures.

Similar to the Gulliver story, the second involved the development of a "rapid" blood culture method and modeled the way for future advancements years later. Winn et al. (19) described a novel membrane filtration blood culture technique that was more extensively evaluated later by Finegold et al. in 1969 (20). Although quite labor intensive, the method demonstrated the value of separating microorganisms from whole blood via dextran sedimentation of erythrocytes followed by differential capture of bacteria and yeasts on a filter and direct plating on solid media. The process allowed for removal of antimicrobial substances, promoted more rapid growth, provided discrete colonies for quantitation and rapid identification, and, hence, better discrimination between potential contaminants and significant isolates. It also allowed for the recognition of multiorganism bacteremia. Despite being more rapid than standard broth cultures (average time-to-detection 28 h versus 61 h for broth), the latter proved to be more sensitive for overall detection of microorganisms in blood.

In a hallmark proof-of-principle publication in 1969, Deland and Wagner (21) connected the dots between radiospirometric detection of bacteria on Mars and automated blood culture technology. Using a rudimentary closed-circuit system composed of a culture flask, circulating pump, and an analog ionization chamber connected via Tygon tubing to a readout meter and recorder, they were able to demonstrate active bacterial metabolism in liquid medium through the release of radiolabeled CO_2 (Fig. 6). The culture medium consisted of 20 ml of glucose-free thioglycollate broth supple-

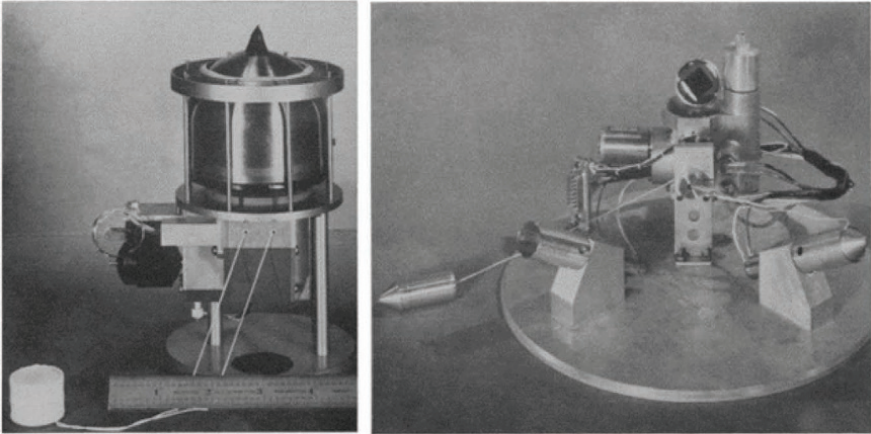


Fig. 6 (left). The first model of Gulliver, designed to probe for microbial life on Mars. Fig. 7 (right). The most recent model of Gulliver. This view shows the projectiles used to carry the soil-sample retrieval lines, the reel-in motor (at left), the broth chamber (at right and center), and the end of the solid detector, resting on the top of the culture chamber. The instrument shown has been used for field testing.

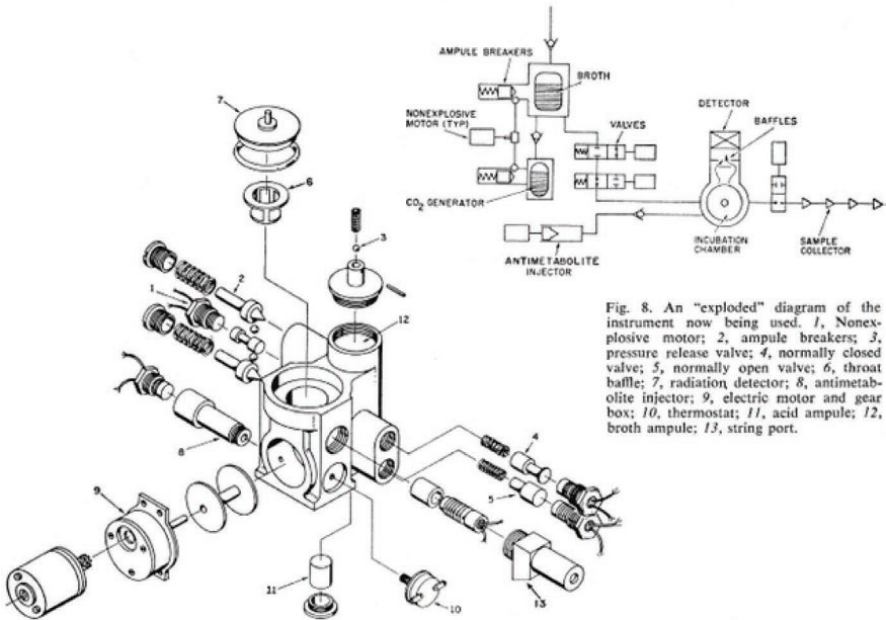


Fig. 8. An "exploded" diagram of the instrument now being used. 1, Nonexplosive motor; 2, ampule breakers; 3, pressure release valve; 4, normally closed valve; 5, normally open valve; 6, throat baffle; 7, radiation detector; 8, antimetabolite injector; 9, electric motor and gear box; 10, thermostat; 11, acid ampule; 12, broth ampule; 13, string port.

Figure 5 Working models (above) and mechanical and schematic views of the Gulliver system, a radiospirometry system designed to collect soil samples on Mars, inoculate broth containing ^{14}C -labeled carbon substrates, and detect the evolution of $^{14}\text{CO}_2$ in an ionization chamber. Reproduced from reference 18, with permission.

P

- Parkinson's disease, 283, 288
- Parvimonas* spp., 164
- Parvimonas microa*, 45
- Pathogen detection. *See* Molecular methods for pathogen detection
- Pediatric bacteremia, epidemiology of, 151–153
- Pediatric blood cultures
- anaerobic blood culture media, 156–157
 - antibiotic absorbing substances, 156
 - blood culture media, 155–157
 - epidemiology of pediatric bacteremia, 151–153
 - fungal blood cultures, 157–158
 - media options, 155–156
 - specimen collection, 153–155
 - sterile body fluid infections, 159
 - weight- and age-based blood volume recommendations, 154
- Pediatric media, BacT/Alert system, 99–100
- Pediatric Septi-Chek bottles, 28
- Penicillium* spp., 48, 49, 173, 174
- Peptide nucleic acid fluorescent in situ hybridization (PNA FISH), 98
- automated, 224–225
 - fungal blood culture, 253
 - manual, 223–224
- Peptococcus*, 64
- Peptoniphilus* spp., 164
- Peptoniphilus asaccharolyticus*, 45
- Peptostreptococcus* spp., 164
- Peptostreptococcus anaerobius*, 45, 52
- Peptostreptococcus asaccharolyticus*, 45
- Peptostreptococcus magnus*, 45
- Peptostreptococcus micros*, 45
- Plate counts, 203
- Polymerase chain reaction (PCR)
- amplification, 277, 279
 - FilmArray BCID panel, 220
 - IRIDICA BAC BSI assay (IRIDICA), 139, 145–146
 - LightCycler SeptiFast (SF) assay, 138, 139
 - MRSA-specific PCR assays, 225–226
 - rapid identification assays, 218–219, 253–254
 - SepsiTest, 139, 142–143
 - T2Candida Panel (T2C), 139, 146–147
 - VYOO assay, 139, 143–145
- Polymeric resin, BacT/Alert system, 101–102
- Pope, Larry, 92
- Porphyromonas* spp., 164
- Postmortem blood culture
- autopsy, 297–299
 - collection, 301–302
 - decision to collect cultures at autopsy, 299–301
 - interpretation of culture results, 302–304
 - molecular testing for blood pathogens, 304–305
 - relevance of microbiology testing, 206
- Prevotella* spp., 164
- Processing positive cultures, 203, 233–236
- alternative approaches, 226–229
 - automated AST systems, 229
 - direct plating to chromogenic agar, 228
 - direct susceptibilities, 228
 - disk diffusion from blood bottle, 228–229
 - future technology, 229–233
 - Gram stain morphologies, 210
 - immunochromatographic assays, 227
 - laboratory organization, 207–208, 234
 - MALDI-TOF MS detection, 212–216
 - molecular detection, 216–217, 220–222
 - MRSA-specific PCR assays, 225–226
 - peptide nucleic acid fluorescent in situ hybridization (PNA FISH), 223–225
 - rapid diagnostics, 211–229
 - staining techniques and subculture, 208–209, 211
 - structuring laboratory policy, 234
 - see also* Future technology; Rapid diagnostics
- Proof-of-principle publication, 10–11
- Propionibacterium* spp., 173, 304
- Propionibacterium acnes*, 281, 289
- Bactec 301 studies, 65
 - postmortem blood culture, 303
- Proteobacteria*, 282, 283
- Proteus* spp., 9
- Pseudomonas* spp., 14, 27, 40, 42, 282, 283
- Pseudomonas aeruginosa*, 28
- BacT/Alert *vs.* Bactec, 102–103
 - blood culture media, 157
 - bloodstream infection, 163, 164, 166
 - detection assay, 221
 - lysis-centrifugation recovery, 40
 - postmortem blood culture, 302, 304
- Pseudomonas fluorescens*, 281
- Pseudomonas putida*, 281

Pseudozyma spp., 169

Public Health Security and Bioterrorism
Preparedness and Response Act of
2002, 305

Q

Quality Assessment of Diagnostic Accuracy
Studies tool (QUADAS), 138, 142

Quality control
blood culture bottle preparation, 32–33
federal regulation, 190–191

R

Radiospirometry, 10

Raman spectroscopy, 231–232

Raoultella ornithinolytica, 221

Rapid diagnostics, 211–229
alternative approaches, 226–229
automated AST systems, 229
comparing rapid identification assays for
blood culture, 218–219
direct plating to chromogenic agar, 228
direct susceptibilities, 228
disk diffusion from blood bottle, 228–229
immunochromatographic assays, 227
MALDI-TOF MS detection from
positive blood cultures, 212–216
molecular detection from positive blood
cultures, 216–222
MRSA-specific PCR assays, 225–226
peptide nucleic acid fluorescent in situ
hybridization (PNA FISH), 223–225
see also Processing positive cultures

Reller, Barth, 91, 95

Rhizomucor, 170

Rhizopus, 170

Rhodotorula spp., 169, 246

Rhodotorula rubra, fungal culture studies,
73–74

Riemerella anatipestifer, 281

Roche Diagnostics

LightCycler SeptiFast (SF) assay, 138,
141–142

Septi-Chek blood culture system, 25, 27

S

Saccharomyces cerevisiae, 169

Safe Medical Devices Act of 1990, 188

Salmonella, 287

Salmonella enterica subsp. *enterica* serovar
Typhi, 34

Scedosporium spp.
filamentous fungi, 252
fungal bloodstream infections, 168, 170
recovery from blood culture, 173, 174

Science (journal), 9

Scott, E. G., 8–9

Scrutinium physico-medicum contagiosa luis, 2

Sepsis management
molecular methods, 137–138, 147–148
see also Molecular methods for pathogen
detection

SepsiTest (ST)

BLAST tool, 143
pathogen detection, 142–143
test parameter, 139

Septi-Chek blood culture system, 25–27, 26
Pediatric Septi-Chek, 28
performance of, 66–67

Sequencing from blood culture, 232–233

Serratia marcescens, 216

SF. *See* LightCycler SeptiFast (SF) assay

Shewanella, 282

Shigella, 282

Signal Blood Culture System, 71, 113–114

Solid organ transplantation (SOT), 163, 170

Sphingobacteria, 282

Sporosarcina globispora, 281

ST. *See* SepsiTest (ST)

Standard operating procedure, blood culture,
34–38

Staphylococcus spp., 282

detection assay, 221
lysis-centrifugation recovery, 40, 42
MRSA-specific PCR, 225–226

Staphylococcus albus, 5, 9

Staphylococcus aureus, 34

Bactec 9000 series, 70–71
Bactec FX studies, 73
blood culture media, 157
bloodstream infection, 163, 164
Difco ESP system, 119
immunochromatographic assays, 227
lysis-centrifugation recovery, 40
pediatric bacteremia, 152–153
pediatric bloodstream infection, 167
pediatric body fluid infection, 159
PNA-FISH, 223, 225
postmortem blood culture, 302, 304, 305

- recovery from blood culture, 173, 174
 recovery with Isolator, 118
 system comparisons, 102–104
 VersaTREK system, 128
- Staphylococcus epidermidis*, 5, 9, 289
 detection assay, 217
 Difco ESP system, 117
 postmortem blood culture, 303
- Staphylococcus pyogenes aureus*, 5
- Stenotrophomonas*, 282, 283
- Stenotrophomonas maltophilia*, 281
- Sterile body fluids (SBF)
 Bactec blood culture systems, 77–78
 pediatric infections, 159
- Sterility control, blood culture bottles, 32, 33
- Streptobacillus*, blood culture media, 156
- Streptococcus* spp.
 BacT/Alert vs. Bactec, 102
 Bactec 9000 series, 71
 Difco ESP system, 119
 intestinal microbiota, 286
 lysis-centrifugation recovery, 40
 PNA-FISH, 223
 recovery of, 6–7
 VersaTREK system, 126
- Streptococcus agalactiae*
 pediatric bacteremia, 152–153
 pediatric bloodstream infection, 167
 pediatric body fluid infection, 159
 postmortem blood culture, 302
- Streptococcus bovis*, 62
- Streptococcus pneumoniae*, 34, 282
 BacT/Alert vs. Bactec, 102
 blood culture media, 157
 bloodstream infection, 163, 166
 Difco ESP system, 117
 infant epidemiology, 153
 lysis-centrifugation recovery, 42, 45
 pediatric bloodstream infection, 167
 pediatric body fluid infection, 159
 pediatric pathogens, 185
 postmortem blood culture, 302, 304
 recovery from blood culture, 173, 174
- Streptococcus pneumoniae* ATCC 49619, 32, 33
- Streptococcus pyogenes*
 bloodstream infection, 163
 pediatric body fluid infection, 159
 postmortem blood culture, 302
- Sudden infant death syndrome (SIDS),
 300–301
- Sudden unexpected death from infectious
 diseases (SUDID), 301
- Sudden unexpected death in infancy (SUDI),
 300–301
- T**
- T2 Biosystems
 fungal blood culture, 254
 T2Candida Panel (T2C), 146–147
- T2Candida panel (T2C)
 pathogen detection, 146–147
 target comparison for, 140–141
 test parameters for, 139
- Thorpe, Thurman, 16f, 85, 87, 88, 91, 92
- TREK blood culture systems
 blood culture workflow on for
 VersaTREK system, 125
 developmental history, 114
 Difco ESP, 115–120
 historical perspective, 113–114
 instrument specifications for VersaTREK
 system, 121–122
 mycobacterial cultures, 258
 performance of VersaTREK system,
 125–129
 REDOX media for VersaTREK system,
 122–125
 VersaTREK system, 120–121
 see also VersaTREK blood culture system
- TREK Diagnostic Systems, 113
- Trichophyton rubrum*, 74
- Trichosporon* spp.
 fungal bloodstream infections, 169
 fungemia, 246
 isolation, 48
- Tropheryma whipplei*, 167, 279, 305
- Turner, James, 16, 91
- U**
- Unexplained deaths due to possible infectious
 causes (UDPIC), 301
- University of Health Sciences (UHS),
 Cambodia, 28
- U.S. Department of Health and Human
 Services, 305
- V**
- Vancomycin-resistant enterococci (VRE),
 166, 187, 228, 235

Van Leeuwenhoek, Antonie, 3
VersaTREK blood culture system, 120–121
 applications other than blood culture,
 131–133
 automated system, 192
 Bactec 9000 series, 72
 Bactec FX studies, 73
 bacterial detection in sterile body fluids,
 131–132
 development history, 114, 114
 example of pressure readings over time,
 127
 instrument specifications of, 121–122
 media components, 124
 mycobacterial culture, 267
 mycobacterial detection and susceptibility
 testing, 132–133
 performance of, 125–129
 photograph, 121
 potential causes of false-negative signals,
 130–131
 potential causes of false-positive signals,
 129–130
 REDOX media for, 122–125
 workflow on, 125, 126
 see also TREK blood culture systems
Vibrio spp., 208, 211

Villemin, Jean Antoine, 4, 4
Volume, blood collection, 22, 184
VYOO assay
 pathogen detection, 143–145
 target comparison for, 140–141
 test parameters for, 139

W

Wampole Isostat Microbial System, 41, 42,
43, 249

X

Xanthomonas (Stenotrophomonas) maltophilia,
recovery with Isolator, 118

Y

Yeast
 fungal bloodstream infections, 168–169
 fungi isolation from positive blood
 cultures, 175
 yeast and yeast-like fungi, 250–252
Yersinia pestis, 2–4, 230, 305

Z

Zygomycetes, 248