Current and Past Strategies for Bacterial Culture in Clinical Microbiology

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(continued)

SUMMARY

A pure bacterial culture remains essential for the study of its virulence, its antibiotic susceptibility, and its genome sequence in order to facilitate the understanding and treatment of caused diseases. The first culture conditions empirically varied incubation time, nutrients, atmosphere, and temperature; culture was then gradually abandoned in favor of molecular methods. The rebirth of culture in clinical microbiology was prompted by microbiologists specializing in intracellular bacteria. The shell vial procedure allowed the culture of new species of *Rickettsia*. The design of axenic media for growing fastidious bacteria such as *Tropheryma whipplei* and *Coxiella burnetii* and the ability of amoebal coculture to discover new bacteria constituted major advances. Strong efforts associating optimized culture media, detection methods, and a microaerophilic atmosphere allowed a dramatic decrease of the time of *Mycobacterium tuberculosis* culture. The use of a new versatile medium allowed an extension of the repertoire of archaea. Finally, to optimize the culture of anaerobes in routine bacteriology laboratories, the addition of antioxidants in culture media under an aerobic atmosphere allowed the growth of strictly anaerobic species. Nevertheless, among usual bacterial pathogens, the development of axenic media for the culture of *Treponema pallidum* or *Mycobacterium leprae* remains an important challenge that the patience and innovations of cultivators will enable them to overcome.

INTRODUCTION

As proposed by Robert Koch, a pure culture is the foundation of all research in infectious diseases (1, 2). The first isolation of a bacterium enables the design of experimental models to analyze virulence and to complete Koch's criteria, thereby establishing a link between microorganisms and infectious diseases (3). Bacterial culture also enables the study of the antibiotic suscepti-bility of bacteria and is the first step in establishing recommendations for effective treatment (4, 5). Obtaining a pure bacterial culture also enables genome sequencing of these strains (6, 7) and proteomic studies to highlight specific proteins and analyze their antigenicity by immunoproteomic techniques, eventually facilitating the production of these proteins, which serve as antigens for serologic tests (8). Finally, pure bacterial culture enables manipulation and transformation by adding or deleting genes to analyze the cause of virulence and antibiotic resistance and the invasive potential of bacteria. However, over the last 30 years, the same progress observed with molecular biology has not emerged with culture in clinical microbiology (9).

Bacterial culture is frequently more difficult and often requires more training than molecular techniques. Consequently, the number of microbiologists specializing in anaerobic bacteria has declined steadily for 30 years, and currently, there are few specialists compared with the number of specialists during the 1970s. Renewed interest in bacterial culture was initiated in large part by clinical microbiologists (10–12) specializing in intracellular bacteria. They have developed axenic media, which are sterile media containing no living organism except the one being cultivated, to culture extremely fastidious bacteria (11, 13, 14). We propose here, after a brief report of early strategies of culture, a comprehensive review regarding past and current culture techniques used for the culture of fastidious bacteria. In an additional review, we elaborate on the progresses allowed by new identification methods and the application of all these advances through the example of the study of human gut microbiota by culturomics (15).

EARLY STRATEGIES AND GENERAL PRINCIPLES

The first culture media were developed empirically, using environmental components. Overall, the choice of nutrients, atmosphere, temperature, and time of incubation are the 4 primary elements that determine the growth of bacteria (16).

Nonselective Culture Media

Nonselective culture media contain no inhibitors and should permit the growth of most of the microorganisms present in the clinical samples studied. Meat infusions or heart or brain extracts were the initial substrates used empirically. Yeast extracts remain among the major components of several culture media (17). Vegetable components can also be used (18).

Peptones, which are carbohydrate-free sources of nutrients, defined as soluble products from the enzymatic hydrolysis of proteins, are more often used as nutrient additives in culture media. Diverse enzymes can be distinguished, and the diverse substrates are meat, casein, soya, and gelatin.

Solid agar and coagulated serum. Clinical microbiology was revolutionized by the invention of the petri dish (19), which, because of the use of a transparent lid, has allowed us to observe colonies and to limit contamination. The addition of kitchen components, such as gelatin or agar, has led to the design of solid culture media (20) and, consequently, the possible description of bacterial species in pure culture. The use of solid culture media and petri dishes was probably comparable to the progress by Koch and Pasteur in modern clinical microbiology. Other solidifying components can be used, such as coagulated eggs, as in Lowenstein-Jensen medium, which is used for *Mycobacterium* culture (21). Coagulated serum can also be used, notably in Loeffler medium, which was designed for performing *Corynebacterium* culture (22).

Enriched media. Enriched media are designed to facilitate the growth of fastidious microorganisms. The primary enrichment component is blood, which provides hemin and other nutrients. This component is frequently added to agar media in several commercial media to notably increase the growth of anaerobic bacterial species (23). Blood agar, which was initially accidentally used for *Mycobacterium tuberculosis* culture, was also shown to be a cost- and time-effective method, with better growth than with the egg-based agar reference medium (24, 25).

Selective Culture Media

In clinical microbiology, a challenge has been to isolate the pathogenic microorganisms from complex microbiota in pure culture (26).

Organic and inorganic components and minerals. Deoxycholic acids are frequently used as Gram-positive bacterial inhibitors. Bile salts are also used as Gram-positive inhibitors, while crystal violet inhibits the growth of Gram-positive bacteria (26). Bismuth sulfite agar uses the ability of bismuth to inhibit most Gram-positive and Gram-negative commensal organisms for the culture of enteric bacilli (26). Finally, Chapman agar is based on the ability of staphylococci to grow in culture media with a high NaCl concentration (7.5%) (26).

Antibiotics and antiseptics. Antibiotics are not specific to bacterial species and generally inhibit the growth of many bacterial genera or species. As examples, ANC (nalidixic acid and colimycin) medium promotes the specific growth of Gram-positive bacteria, and *Campylobacter* blood agar is an enriched selective medium that contains 5 different antimicrobial agents. Antibiotics are used in *Mycobacterium* culture media primarily as a cocktail that includes polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (27). Antiseptics, such as bromocresol purple, were used previously in agar culture media to select *Enter-*

obacteriaceae in particular, with different uses, such as the isolation of injured coliforms from drinking water (28).

Sample Decontamination

Another strategy is to decontaminate the samples to decrease the rapid overgrowth common in commensal bacteria (21). The *N*-acetyl–L-cysteine–NaOH method was initially used for culturing *Mycobacterium* spp. (29). Chlorhexidine (30) was used to decontaminate the sputum of cystic fibrosis patients, with the aim of culturing nontuberculous mycobacteria (31). El Khechine et al. used chlorhexidine to decontaminate stool samples before *Mycobacterium* species culture (32). This noninvasive method obviates the requirement for gastric aspiration without a difference in pulmonary tuberculosis diagnosis (33). Finally, lytic phages have been used to decontaminate the normal flora from sputum before *M. tuberculosis* culture to replace the use of antibiotics (27, 34).

Temperature and Atmosphere Control

Temperature. Most of the species implicated in clinical microbiology are mesophilic bacterial species, and these species grow at medium temperatures of 25°C to 45°C. In clinical microbiology, one of the most famous examples of the requirement for the use of an adapted temperature for growth was highlighted by *Rickettsia felis* (35). After failing to grow the bacterium in human embryonic lung (HEL) cells at 37°C, researchers suspected that temperature growth was critical because this factor had been reported for many arthropod-borne microorganisms (36–39). Finally, the first cell culture of *R. felis* was performed, using XTC2 cells obtained from *Xenopus laevis* oocytes growing at 28°C, which were usually used for arboviruses (39, 40).

Atmospheres. (i) Aerophilic and anaerobic conditions. Various atmospheres can be used in clinical microbiology. To describe microbes from human feces, roll tubes (defined precisely in the section on anaerobic methods, below) were previously designed (41). Nottingham and Hungate first used a nonselective medium but a stringent atmosphere consisting of 80% H_2 and 20% CO_2 to isolate nonidentified methanogenic *Archaea* from humans (42).

(ii) Microaerophilic conditions. When the concentration of oxygen required to obtain growth is relatively low, the bacterial species are considered microaerophiles, such as *Campylobacter* spp., which can cause human infections involving primarily the gastrointestinal tract (43). Most *Campylobacter* species require a microaerobic atmosphere containing ~5% O_2, 10% CO_2, and 85% N_2 for optimal recovery. Recently, a microaerophilic atmosphere demonstrated better efficiency than aerobic conditions in promoting *Mycobacterium* culture and was proposed as a routine condition for laboratories performing these cultures (44).

Incubation Time

Most clinical pathogens grow easily over 24 to 48 h in plate media (45), but several bacterial species require a much longer time, whereas most routine laboratories maintain cultures within 5 days. As a common example, *Helicobacter pylori*, the bacterium causing most gastrointestinal ulcers, requires a longer incubation period. Petri dishes with bacterial cultures were inadvertently left by B. Marshall in the incubator for 5 days (46). Thereafter, he observed small colonies constituting the first successful culture and isolation of a bacterium from the stomach of a patient suffering from gastritis. The association between the bacterium and gastritis was met with great skepticism by the scientific commu-

nity, and to confirm this association, Marshall inoculated himself by drinking a solution containing vast amounts (10^9) of the bacterium. He developed acute gastritis, confirming Koch's postulates that the bacterium was the causative agent (3).

As another example, human infections caused by *Bartonella* species can cause trench fever; cat scratch disease; and, under particular conditions, bacillary angiomatosis, peliosis hepatitis, endocarditis, or chronic lymphadenopathies (47–49). Usually, the growth of *Bartonella* spp. is slow, requiring 12 to 14 days when blood agar is used, with certain isolates sometimes requiring longer incubation periods of >45 days (48). *Bordetella pertussis* colonies are visible in 3 to 4 days, (18), and *Legionella pneumophila* colonies usually appear on day 3 (50). Fecal samples should be incubated for at least 3 days to culture *Campylobacter* spp. (43). For the isolation of aerobic actinomycetes, such as *Nocardia* spp. and *Actinomyces* spp., an incubation time ranging from 2 to 3 weeks is recommended (51). Finally, for routine clinical microbiology, anaerobic species cultures should be incubated for at least 5 days (45).

Improving Collection and Transport Time to the Laboratory

The viability of organisms depends on several factors, such as transport time, storage period, temperature (52), as well as specific storage systems to ensure that the microorganism's viability is maintained, although clinical or research samples should ideally be cultivated immediately after sampling. The use of an inefficient sampling device will lead to a misidentification of pathogens due to contaminant growth resulting from oxygen exposure or from an extremely small fraction of bacteria being recovered. Because of their low cost, utility, ease of use, and availability, swabs are still used to collect and transport various sample types (53, 54). New swab types have been designed (55) to improve specimen collection, such as the new nylon-flocked swab with Amies liquid medium, which is more effective in bacterial recovery and which provides for more efficient release than classic swabs (54).

SPECIFIC STRATEGIES FOR FASTIDIOUS BACTERIA

Mycoplasma

Mycoplasma spp. do not have cell walls (56) and do not stain by Gram coloration. In 1898, Nocard and Roux reported the first culture of *Mycoplasma mycoides* subsp. *mycoides*, which is the agent of bovine pleuropneumonia (57), 15 to 20 days after inoculation of a semipermeable collodion sac. In 1960, *Mycoplasma mycoides* was successfully cultured in a medium that included a heat-stable defatted serum protein fraction, cholesterol, both saturated and unsaturated fatty acids, serum albumin, glycerol, and high concentrations of DL- or L-lactate and glucose (58, 59). For diverse strains of *Mycoplasma* (known at that time as pleuropneumonia-like organisms [PPLOs]) (60), a variety of substances were used as supplements, including blood serum or ascitic fluid (61), lipid extracts of egg yolk, cholesterol (62), lipoprotein, lecithin, and acetate (63, 64). The Eaton agent (*Mycoplasma pneumoniae*) was first cultured in a cell-free medium consisting of 70% Difco PPLO agar, 10% of 25% boiled yeast extract, and 20% unheated horse serum (65). *M. pneumoniae* was successfully isolated directly from patients with atypical pneumonia by using this medium and adding penicillin, amphotericin, and thallous acetate (66). Chanock et al. proposed an effective medium (65), and Tully et al. then developed the refined SP4 medium in 1979, which be-

came the most widely used broth and agar medium for culturing of *M. pneumoniae* from clinical samples (67). This medium contains tryptone, peptone, PPLO broth, heat-inactivated fetal bovine serum, yeast extract, Yeastolate, and CMRL 1066 medium (67). *Ureaplasma urealyticum* (also called T-strain mycoplasma for tiny colonies) causes genital infections in humans. The total growth of *Ureaplasma urealyticum* was directly correlated with urea concentrations, with a maximum yield of organisms being observed with a 32 mM urea concentration. Finally, the addition of SP4 medium, glucose, urea, or arginine, depending on the suspected *Mycoplasma* species, was proposed. Indeed, common human mycoplasmas were then distinguished by 4 biochemical tests: glucose oxidation, arginine deamination, urea hydrolysis, and methylene blue reduction or growth inhibition.

Anaerobes

Anaerobes are generally widespread, can be found in the environment, and are members of the normal human flora (68–70), but only a few species are frequently encountered in significant human infections (71). The sensitivity of anaerobes to oxygen differs depending on the species (72, 73). Loesche et al. (72) classified bacteria into three different categories according to their oxygen sensitivity: strict (bacteria cannot grow on medium with a partial O_2 pressure [pO_2] of >0.5%) (73, 74), moderate (bacteria can grow in the presence of oxygen levels of between 2 and 8%) (72), and microaerotolerant (growth occurs in the presence or absence of oxygen in the medium; however, maximal growth occurs at intermediate oxygen levels) (72).

Culturing of strictly anaerobic bacteria in the absence of oxygen requires specific bacteriological techniques, which could explain the low frequency of isolation in many laboratories (75, 76). Therefore, strictly anaerobic bacteria require complex media with many supplements for growth (77–81). Hungate revolutionized the culture of anaerobic species by cultivating extremely oxygen-sensitive microorganisms, such as sulfate-reducing bacteria and methanogenic *Archaea* (76).

Brief history of earlier methods. The equipment required for maintaining reduced oxygen tension (77, 78) is simple and inexpensive. First, most of the oxygen is removed and is replaced by a suitable gas source.

(i) Physical reduction of oxygen tension. The more commonly used technique for obtaining free O_2 medium consisted of using carbon dioxide or hydrogen through or over the surface of the medium to replace oxygen (82). Additionally, the candle technique was commonly used to cultivate anaerobes (83–85). This technique consisted of burning a candle inside a sealed jar to replace the oxygen with CO_2, which resulted in the production of 3% CO_2 under standard conditions (84).

(ii) Chemical reduction by reducing agents. Strictly anaerobic bacteria grow in an almost total absence of oxygen, which is often toxic (70). These bacteria must be grown in a reducing atmosphere, in which energy is produced by fermentation or anaerobic respiration (86). Chemical techniques were based on reducing oxygen tension by adding reducing agents such as thioglycolate, glutathione, cysteine-HCl, sodium sulfide (Na_2S) (82), or sodium carbonate-oxalic acid (84). Ascorbic acid-supplemented medium can also be used as an anaerobic medium. Recently, La Scola et al. successfully performed an aerobic culture of 6 strictly anaerobic species, including *Fusobacterium necrophorum*, using Schaedler agar supplemented with high-dose ascorbic acid or glutathione

and with pH adjustment to 7.2 (87), suggesting substantial perspectives in routine bacteriology.

Constituents of anaerobic media. Freshly prepared, highly enriched, and properly stored medium has been essential to enhance anaerobic bacterial growth (88, 89).

(i) Major and minor constituents of suitable anaerobic media. Anaerobic media must contain carbon sources, electron acceptors, and donor elements (90). The major constituents of suitable growth medium are represented by macroelements and metals in sufficient quantities (90), because the typical composition depends on a percentage of microbial dry mass (91). The addition of minor constituents (trace elements) is not required because most microorganisms use a unique carbon source to grow. Moreover, it is difficult to demonstrate which growth factor is required for an organism to allow better growth and which factor is essential (90). Nevertheless, a few bacteria require specific focus and particular constituents, such as many *Bacteroides* strains, which require the addition of vitamin K_1 and hemin to the medium for growth (92).

(ii) Growth factors. Growth factors of undefined composition, such as yeast extract and pyrimidines (93), peptone (93), Casitone, Casamino Acids, or clarified rumen fluid, are still preferably added to culture media (90). These fluids contains volatile fatty acids and heme, which are not commonly found in the extracts or hydrolysates added to classical media (94). These fluids have been supplemented with glycerol, Trypticase, hemin, and mineral solutions 1 and 2 (modified 98-5 medium) (95) and sometimes with sterilized fecal extract (92) to allow higher percentages of anaerobe recovery than with other analyzed media (95, 96). Further studies were performed in humans, and human sterilized fecal samples have been used as a nutrient source to provide specific anaerobe growth (42, 97).

Anaerobic incubation systems used to increase the ability to cultivate anaerobes. Clinical laboratories may prefer to use anaerobic jars and anaerobic chambers rather than roll tubes because of the delay and because of the complexity of this method. The Hungate technique may be used particularly for research activity (98).

(i) Anaerobic jars. Currently, the GasPak system produces an atmosphere containing ~10% CO_2 with sachets containing a dry powder or pellets of sodium borohydride and sodium bicarbonate, which react with water to produce hydrogen gas and carbon dioxide. The hydrogen produced then reacts with oxygen gas on a palladium catalyst, allowing greater water production to remove the oxygen gas (74). Anaerobic jars do not allow a continuously anaerobic atmosphere from sample reception to seeding to be established, and their use is inadequate for cultivating certain strictly oxygen-sensitive bacteria (72).

(ii) Roll tube method. The Hungate method, which is based on the use of roll tubes, was introduced to prepare an anoxygenic medium for methanogen cultivation (99, 100). The principle is based on replacing atmospheric oxygen with other gases, such as N_2, CO_2, H_2, or other mixtures with adjusted pH (101), using an anaerobic glove box. After incorporation of all the elements, the medium is mixed and then boiled in an oxygen-free nitrogen atmosphere in the presence of resazurin as an indicator of the presence of oxygen. After resazurin reduction, the medium is cooled under nitrogen at room temperature. The flask is then capped and transferred to the anaerobic glove box, where the medium is distributed into serum vials or Hungate tubes. The containers are sealed with plastic stoppers, and the media are then sterilized in an autoclave before inoculation (76). The isolated colonies then form

on the walls of the tubes after several days or weeks of incubation (101). The roll tube method greatly improves anaerobic growth (102) and remains the reference method for methanogens and archaea; however, this technique remains time-consuming and rather complex for large-scale studies due to the requirement for the use of roll tubes instead of petri dishes and cannot be used in clinical laboratories (99).

(iii) Anaerobic chambers. The best method to ensure anaerobe viability is to incubate anaerobic organisms directly in an anaerobic chamber (103, 104), never allowing exposure of the sample to air (105). This method, which is usable in both research and clinical laboratories because it does not require special training (106), is inexpensive. A prereduced medium with low redox potential is used to seed the samples.

Perspectives on anaerobic culture. The human gut microbiota is the human site that contains a higher concentration of anaerobes (9). In future research projects, it may be important to continue to characterize human anaerobic microflora by high-throughput sequencing, to identify anaerobic clusters, and to design new anaerobic strategies that use antioxidant agents (87) that allow the cultivation of uncultivated anaerobic bacteria. This approach will clearly revolutionize the culture of anaerobic bacteria in routine bacteriology and in culturomics studies.

Spirochetes

Although most spirochetes are free-living chemoorganotrophs, spirochetes of the genera *Borrelia*, *Treponema* (*Spirochaetaceae*), and *Leptospira* (*Leptospiraceae*) are major human pathogens (107). Many members of the genus *Brachyspira* are important veterinary pathogens but occasionally have been reported to be associated with human pathology (108–110). *Spirochaetes* are often not visible by Gram staining, requiring dark-field microscopy or special coloration (111). Molecular identification is usually based on group-specific *flaB*, *ospA*, *ospB*, and *ospC* genes and *rrf* (5S)-*rrl* (23S) intergenic spacer amplification (112). Among conservative housekeeping genes, *rpoB* could be a useful tool (113).

All borreliae are fastidious organisms and chemoheterotrophic and use carbohydrates, amino acids, long-chain fatty acids, or long-chain fatty alcohols as carbon and energy sources. Depending on the species, growth occurs under anaerobic, facultatively anaerobic, microaerophilic, or aerobic conditions (114). Because of the lack of or limited biosynthetic potential (the ability to elongate long-chain fatty acids to synthesize most amino acids, enzyme cofactors, and nucleotides), complex nutritional requirements are needed for spirochete cultivation.

Leptospira interrogans was previously considered the only species of this genus that is pathogenic in humans; however, a phylogenetic analysis of all isolated strains showed that among 22 currently recognized species (http://www.bacterio.net/leptospira.html), leptospirosis-causing bacteria belong to at least 11 different species (115, 116) (see Table 2). Leptospirae can be isolated by inoculating ~100 μl of a patient's blood in solid or semisolid oleic acid, which is an albumin-containing medium. Several media are available, namely, EMJH (117), polysorbate medium (118), and LVW agar (119). The presence of rabbit serum, polysorbates, and vitamins is essential.

Four different *Treponema* spp. are human pathogens, including 3 subspecies of *T. pallidum* (*T. pallidum* subsp. *pallidum* [syphilis], subsp. *pertenue* [yaws], and subsp. *endemicum* [nonvenereal epidemic syphilis]) and the pinta agent *T. carateum*. None of these pathogens has yet been successfully cultivated in axenic medium,
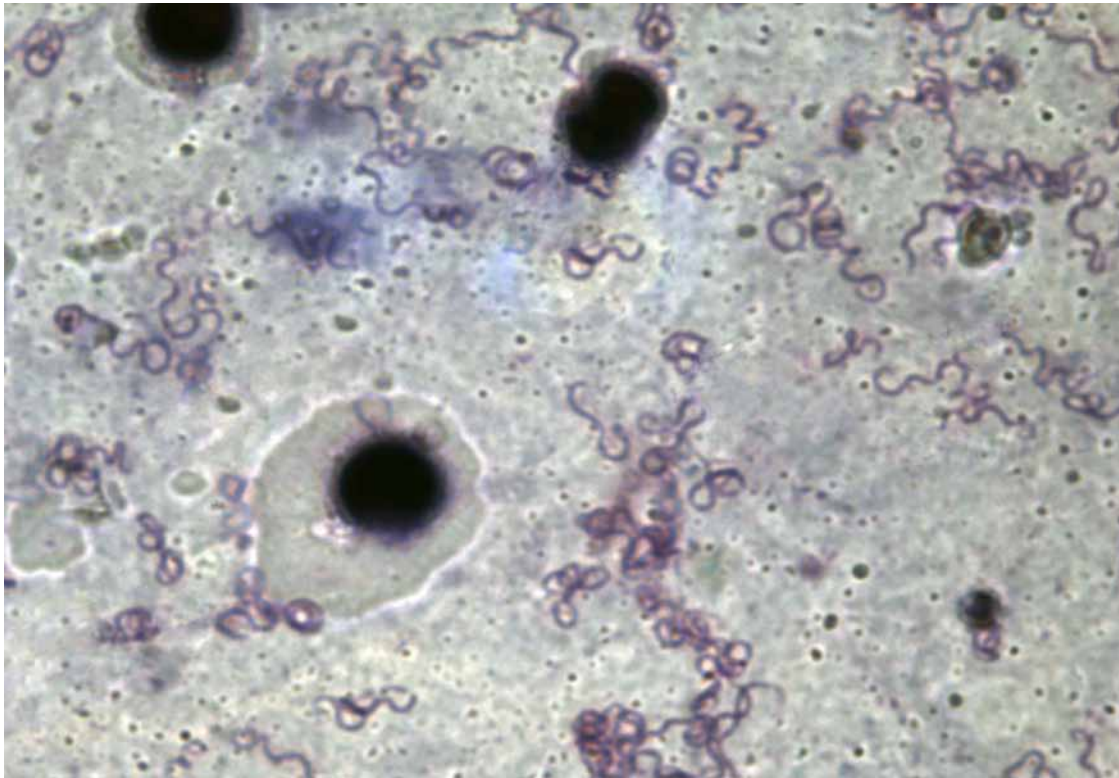
FIG 1 Thick smear of mouse blood showing *Borrelia hispanica* strain OM003 (Giemsa staining). Magnification, ×900.

and isolation in pure culture is not a diagnostic option. *Treponema pallidum* subsp. *pallidum* can be propagated only in laboratory animals (rabbits) by intratesticular, intradermal, intravenous, or intracisternal inoculation (120). *T. pallidum* grows slowly, with a doubling time of 30 to 33 h (107), and a mean of 10^{10} bacteria has been harvested from the testis of a rabbit. *Treponema paraluis-cuniculi*, which is the causative agent of rabbit venereal spirochetosis, is genetically closely related to *T. pallidum* (121) and easily infects laboratory rabbits. The closely related agents of yaws and nonvenereal epidemic syphilis share similar features; however, their culture has been even less studied. *T. carateum* has also never been isolated; moreover, there are no successful animal model. These organisms are among the last few as-yet-uncultured major human pathogens. Similarly, among the numerous *Treponema* phylotypes found in the oral cavity, only 10 different species have been cultivated, with *Treponema denticola* being the most extensively studied (122). The development of an axenic culture of pathogenic *Treponema* remains a challenge for microbiologists.

The genus *Borrelia* is essentially composed of the Lyme disease group and the relapsing fever group (123–125). Many *Borrelia* species, particularly those species of the Lyme disease group, are routinely cultured in liquid Barbour-Stoenner-Kelly II (BSK-II) medium under microaerophilic conditions (126, 127) or its variations, such as BSK-H (128), at 30°C to 34°C. Despite the existence of other media such as MPM (129), inoculation of clinical samples (blood or skin lesion biopsy specimens) or triturated ticks in BSK medium remains the most reliable approach for isolating Lyme disease borreliae. Successful culture of these fastidious organisms has also been achieved in BSK medium solidified with 1.5% agarose (130). Contaminants can be eliminated by adding nalidixic

acid and 5-fluorouracil (126, 131). These organisms grow slowly, dividing every 8 to 12 h during the exponential growth phase. Primary strains from clinical samples and ticks can take as long as several weeks to grow; however, culture-adapted isolates can reach cell densities of 10^7 to 10^8 cells per ml after cultivation *in vitro* for 5 to 7 days.

Isolation by animal inoculation was the first and most reliable method for the isolation of relapsing fever borreliae. BSK medium incubated at 35°C was also used to isolate *Borrelia recurrentis* from the serum of a patient with louse-borne relapsing fever (132). One bacterium is sufficient to produce infection in laboratory animals, including mice, rats, rabbits, guinea pigs, and monkeys (133), but the mouse is the most susceptible animal, producing high-level borreliemia (reaching the maximum at 3 to 6 days) (134). However, within 10 to 14 days postinoculation, when bacteria disappear from the blood, the bacteria can still be found in the reticuloendothelial system or in other organs of infected animals, primarily in brain, where borreliae persist for a longer time (133, 135). The genome of *Borrelia miyamotoi* was obtained from *Borrelia* cells present in the blood of infected mice (136).

Similar to its use on Lyme disease borreliae, BSK-II medium was also used for isolating and culturing *Borrelia duttonii* from patients' blood specimens (137) and for culturing *Borrelia crocidurae* from ticks in Mali (138). Successful isolation was achieved by inoculating blood specimens at room temperature, with subsequent blind subculture every 3 days in fresh medium at 33°C. Bovine serum albumin fraction V complement seems to be an extremely important component of this medium. *Borrelia hispanica* is particularly difficult to isolate in axenic medium (124), and animal inoculation remains the method of choice (Fig. 1). For

TABLE 1 Culture strategies for isolating spirochetes related to human pathology

Family	Genus and species	Pathogenicity for humans	Culture method(s)[a]
Brachyspiraceae	*Brachyspira aalborgi*	Colonizes human gut, most likely associated with diarrhea	Tryptose soy blood agar plates + 10% calf blood, 38.5°C in an anaerobic atmosphere (95% H_2–5% CO_2)
	Brachyspira pilosicoli	Colonizes human gut, most likely associated with diarrhea	Tryptose soy blood agar plates + 10% calf blood, 38.5°C in an anaerobic atmosphere (95% H_2–5% CO_2) + BHIS broth or HS broth containing carbohydrates
	Brachyspira hominis	Colonizes human gut, most likely associated with diarrhea	Not yet isolated
Leptospiraceae	*Leptospira interrogans, L. kirschneri, L. noguchii, L. borgpetersenii, L. weilii, L. santarosai, L. alexanderi, L. alstonii, L. kmetyi, L. broomii, L. fainei*	Human pathogens, leptospirosis	Nonsolid or semisolid (0.1–0.2% agar) oleic acid and albumin-containing media at 30°C; some fastidious strains can require 6 mo of incubation
Spirochaetaceae	*Treponema pallidum* subsp. *pallidum*	Syphilis	Intratesticular inoculation of clinical samples or bacterial strains in a rabbit model
	Treponema pallidum subsp. *pertenue*	Yaws	Intratesticular inoculation of clinical samples or bacterial strains in a rabbit model
	Treponema pallidum subsp. *endemicum*	Nonvenereal epidemic syphilis	Intratesticular inoculation of clinical samples or bacterial strains in a rabbit model
	Borrelia carateum	Pinta	Not yet isolated
	Borrelia burgdorferi, B. afzelii, B. garinii, B. valaisiana, B. lusitaniae, B. bissettii, B. spielmanii	Lyme disease	BSK-II, BSK-H, MKP, MPM media; microaerophilic conditions at 30°C–34°C
	Borrelia miyamotoi	Unnamed fever	BSK-II medium at 31°C, severe combined immunodeficient mice[b]
	Borrelia recurrentis	Louse-borne relapsing fever	BSK medium, susceptible animals (mice, rats, rabbits, guinea pigs, and monkeys)
	Borrelia duttonii	Tick-borne relapsing fever, East Africa	BSK medium, susceptible animals (mice, rats)
	Borrelia crocidurae	Tick-borne relapsing fever, West Africa	BSK medium, susceptible animals (mice, rats)
	Borrelia hispanica	Tick-borne relapsing fever, North Africa, Europe	Only mouse model
	Borrelia hermsii	Tick-borne relapsing fever, North America	BSK medium, mouse model
	Borrelia turicatae, B. parkeri	Possible tick-borne relapsing fever, North and South America	BSK medium
	Borrelia persica	Tick-borne relapsing fever, Central Asia	Guinea pig and mouse models; after sensitization, BSK medium
	Borrelia microti	Possible tick-borne relapsing fever, Asia	Mouse model
	Borrelia baltazardii, B. caucasica, B. latyschewii	Possible tick-borne relapsing fever, Asia	No culture available, laboratory animals

[a] BHIS, brain heart infusion salt; HS, heart infusion plus serum; MKP, modified Kelly-Pettenkofer.
[b] See reference 136.

Borrelia hermsii, a combination of both methods is applicable: inoculation in mice followed by cultivation of isolated *Borrelia* in BSK medium (139) (Table 1).

The development of optimized culture medium for borreliae and axenic culture of spirochetes remains a challenge; thus, increasingly, new methods are appearing in the literature. Recently, the growth of *Leptospira* and *Borrelia* was shown to be supported by a versatile, axenic medium composed of Vero cell extracts (140).

Mycobacteria

Mycobacteria are environmental organisms that can act as opportunistic pathogens, with a few notable exceptions that are host adapted and responsible for severe infections, including tuberculosis (*M. tuberculosis* complex), leprosy (*Mycobacterium leprae*), and Buruli ulcers (*Mycobacterium ulcerans*). Mycobacterial cul-

tures have long been regarded as requiring specialized laboratories. Recent data have indicated that mycobacteria are in no way particular regarding their manipulation in routine clinical microbiology laboratories. Concerning isolation, routine sheep blood medium sustains the isolation and growth of the vast majority of mycobacteria encountered in clinical microbiology laboratories, including *M. tuberculosis*, *Mycobacterium avium*, and *Mycobacterium intracellulare* (24, 141, 142), with the notable exception of *M. leprae*, which cannot be propagated outside animals (143) but can be cultured in the footpads of immunocompromised mice. In addition, sheep blood agar and sheep serum agar media have also been used to analyze *in vitro* susceptibility to antibiotics (144). Incubation temperature is another key factor for the successful culture of mycobacteria. Although 37°C is the standard incubation temperature used for most human pathogens, the ability of *M. leprae* to be cultured in the footpads of mice indicates that this

TABLE 2 Growth characteristics of obligate and opportunistic mycobacteria frequently encountered in clinical microbiology, including optimal temperatures[a]

Pathogen	Amoebal culture	Blood component requirement	Axenic culture growth	Nonconventional temp (°C)	Optimal growth in a microaerophilic atmosphere	Reference(s)
Obligate						
M. tuberculosis complex	+	+	+		+	24, 150
M. leprae	+	−		<30		151
M. ulcerans	+		+	30	+	334
Opportunistic						
M. marinum	+		+	<30		271
M. xenopi	+		+	45		149
M. abscessus complex	+		+			148
M. massiliense	+		+			148
M. avium complex	+		+			142
M. tiburgii	NR		−	NR		153
M. fortuitum complex	+					148
M. genavense	NR	+	+		+	146
M. haemophilum		+	+	42		146
M. mucogenicum complex				30		
M. aubagnense				30		148
M. phocaicum				30		148
M. porcinum complex						148
M. kansasii	+					148
M. simiae	+					146
M. malmoense	+			24–42		148
M. szulgai	+			25–37		148

[a] NR, not reported.

species requires a low temperature of 28°C for growth. This finding is consistent with the clinical observation that leprosy develops in the nasal mucosa, superficial nerves, and the skin, which are body regions with temperatures of <37°C. Other pathogens, including *Mycobacterium marinum* and its derivative, *M. ulcerans*, as well as *M. haemophilum*, require a lower temperature of 30°C (145), and others, such as *Mycobacterium genavense* (146) and *Mycobacterium xenopi*, grow better at temperatures as high as 45°C. Similarly, the atmosphere of incubation warrants further evaluation. Mycobacteria have been reported to be aerobic; however, we recently observed that *M. tuberculosis* grew better in microaerophilia, which is defined as 2.5 to 5% oxygen tension, than in a conventional 5% CO_2 atmosphere (44). The fastidiousness of *M. tuberculosis* complex mycobacteria has further challenged growth detection techniques. In liquid medium, growth is routinely detected with automated instrumentation by significant changes in oxygen and carbon dioxide concentrations; in solid medium, colonies are routinely monitored by the naked eye, with difficulties in distinguishing colonies from inoculation debris. The natural autofluorescence of some mycobacteria can help in the detection of microcolonies. We observed 943- $\pm$ 51-μm-diameter colonies containing $6.4 \times 10^5 \pm 3.5 \times 10^5$ mycobacteria by the naked eye, whereas by autofluorescence, we detected 103- $\pm$ 19-μm-diameter microcolonies containing $7.9 \times 10^3 \pm 5.3 \times 10^3$ mycobacteria. Such microcolonies are identifiable by using the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) technique, thus allowing culture-based diagnosis of mycobacterial infections as a routine task (147).

We also observed that most opportunistic mycobacterial pathogens are indeed amoeba-resistant organisms (148), as described comprehensively below. Interestingly, various amoebae are cul-

tured at various temperatures, which allows the modulation of the incubation temperature to isolate mycobacteria such as *M. xenopi* (149). We further showed that *M. tuberculosis* complex mycobacteria are also intra-amoebal organisms (150), as previously shown for the other two major obligate pathogens, *M. leprae* (151) and *M. ulcerans* (152). Therefore, almost all the opportunistic and obligate mycobacterial pathogens are, in fact, intra-amoebal mycobacteria, suggesting that this system could be used in cases of as-yet-uncultured opportunistic pathogens such as *Mycobacterium tilburgii* (153) (Table 2).

Automatic systems using Middlebrook broth have revolutionized the routine culture of mycobacteria, yet parallel inoculation of solid media is still recommended to fill the gaps with automatic detection. Rapid progress in colony imaging, coupled with MALDI-TOF MS (147), could render broth culture obsolete. Solid media in miniaturized formats adapted to automated scanners could become the standard for culture and first-line antibiotic susceptibility testing (M. Drancourt, unpublished data). Axenic culture of as-yet-uncultured pathogens is another exciting challenge, as is the discovery of new opportunistic pathogens using the extended spectrum of axenic cell extract-containing media, such as that used for *Mycobacterium bovis* (120), and cell-based systems, including protozoa (150), offered by culturomics (154).

Archaea

Phylogenetic classification based on the sequence of the 16S rRNA gene divide archaea into four phyla: the *Euryarchaeota* and *Crenarchaeota* (155–157), which include most cultivated archaea, as well as two new phyla, the *Nanoarchaeota* and *Korarchaeota*, which have a single cultivated species each. Methanogenic *Archaea* belonging to the phylum *Euryarchaeota* showed great adaptation to the human gut (158–164). *Euryarchaeota* and *Thaumarchaeota*
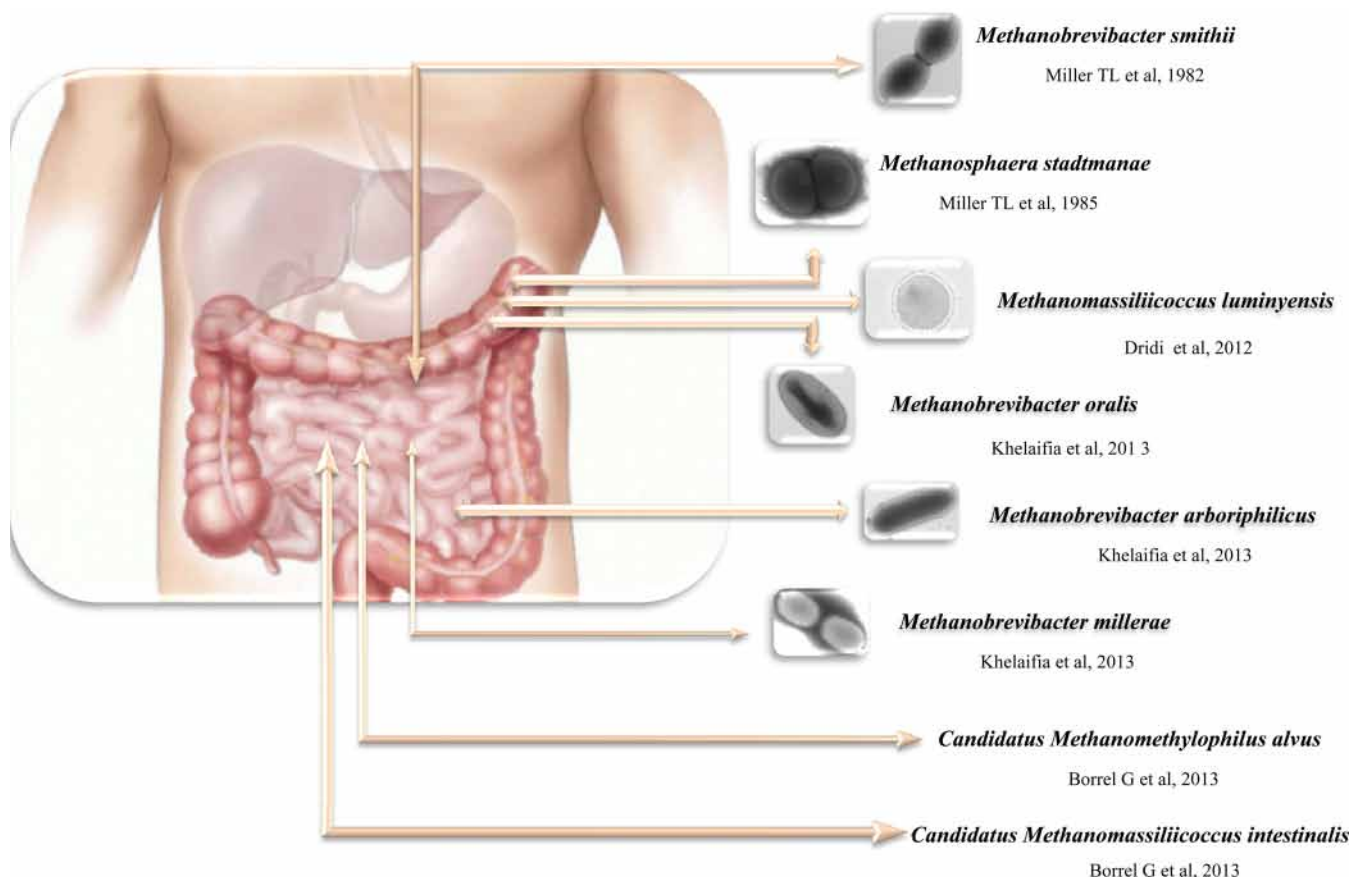
FIG 2 Archaeal species detected in or cultured from the human gut (97, 98, 159, 170, 181, 183, 185).

are parts of the skin microbiota (165–167) and the mucosa-associated microbiota of the oral and vaginal cavities (168). *Methanobrevibacter* species, *Methanosphaera* species, and *Sulfolobus* species sequences have been retrieved from coprolites dating from AD 180 to AD 600 (169). However, metagenomic studies, including the Human Microbiome Project, have most likely underestimated the diversity and the density of *Archaea* in microbiota (165) due to limitations of the currently used PCR primers and of archaeal DNA extraction protocols (170). Several studies have consistently observed an age-dependent increase in the prevalence and diversity of gut *Archaea* and methanogens (171–174). An increased prevalence of *Methanobrevibacter* spp. correlated, albeit nonsignificantly, with short-term and long-term carbohydrate ingestion (160), as was the case in anorexic patients (175), whereas the prevalence of *Nitrososphaera* spp. correlated, albeit nonsignificantly, with protein and amino acid ingestion (160). Methanogens in the oral cavity have been associated with periodontitis (176, 177); however, their role, if any, remains unknown.

Among the 20 currently described species among the human microbiota, 3 *Archaea* have been enriched but not obtained in pure culture. Halophilic *Halobacteriaceae* archaea were enriched in colonic mucosal biopsy specimens collected from one patient with inflammatory bowel disease (178). Fluorescence *in situ* hybridization (FISH) confirmed the presence of viable archaeal cells, but this archaeon has not been isolated in pure culture or deposited in public collections (178). Similarly, the use of meth-

anol as the substrate led to the enrichment in stool microbiota of "*Candidatus* Methanomethylophilus alvus" and "*Candidatus* Methanomassiliicoccus intestinalis," a *Thermoplasmatales*-related lineage distantly related to its most closely related species, *Methanomassiliicoccus luminyensis* (179, 180) and *Aciduliprofundum boonei* (159, 174). *M. luminyensis* appears to be the sole cultured representative of a new, seventh order of methylotrophic methanogens (181). Finally, since the seminal isolation of uncharacterized methanogens 45 years ago (42), only 6 human-associated archaeal species have been isolated in pure culture and deposited into collections, all of which are strictly anaerobic. Methanogenic *Archaea* have also been isolated from the digestive tract (Fig. 2). After *Methanobrevibacter smithii* (97, 182), *Methanosphaera stadtmanae* (183) was isolated from the human gut, and *Methanobrevibacter oralis* was isolated from the oral cavity (184). In addition, our laboratory isolated *M. luminyensis* (179), *Methanobrevibacter arboriphilicus*, *Methanobrevibacter millerea*, and *M. oralis* from stool specimens (177). A versatile medium, combined with incubation at 37°C under an atmosphere of 2×10^5 Pa consisting of 80% H_2 and 20% CO_2, permitted the isolation and culture of these methanogens (98). This medium consisted of trace elements, including tungsten (185). Primary isolation was attempted by using the liquid formulation, with cultures being monitored by the CH_4 concentration, which was measured by chromatography, and by microscopic examination of the broth. The colonies were then obtained by subculturing of positive broth onto an agar-based

formulation of the same medium and by using the roll tube technique of Hungate (98, 186). Methanogens are autofluorescent at 420 nm and can be identified by using matrix-assisted laser desorption ionization–time of flight mass spectrometry (187). The availability of colonies allowed antibiotic susceptibility testing, which demonstrated the high natural resistance of *Archaea*, except against imidazole, its derivatives, and fusidic acid (188, 189), and genome sequencing (174, 180, 190). Several archaeal species remaining uncultured have been detected only by molecular tools (159, 161, 162, 165, 178, 191, 192). Thus far, only strictly anaerobic methanogenic *Archaea* have been isolated from humans. Specific strategies could be designed to enlarge the archaeal spectrum from human microbiota, such as halophilic or thermophilic *Archaea*. In addition, in the future, it will be interesting to determine the potential pathogenic roles of *Archaea* in clinical microbiology, particularly in polymicrobial abscesses. We suggest that large archaeal cultures applied to various human samples will partially fill the gap of "microbial dark matter" among the gut microbiota (193).

INTRACELLULAR BACTERIAL CULTURE

Intracellular bacteria are responsible for human diseases with significant morbidity and mortality (194–196). *Rickettsia* spp. and *Orientia tsutsugamushi*, the causative agent of scrub typhus, have tropism for endothelial cells (195). *Coxiella burnetii*, which is the agent of Q fever, replicates in the acidic phagolysosomes of macrophages and monocytes (196). *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*, which cause tick-borne febrile illnesses, replicate preferentially in neutrophils and monocytes, respectively (195). *Chlamydia trachomatis* is the agent of trachoma, lymphogranuloma venereum (LGV), and other sexually transmitted diseases, and *Chlamydophila pneumoniae* and *Chlamydophila psittaci* can cause pneumonia. These cultures, which require suitable techniques, have been performed only in specialized laboratories. Some intracellular bacteria with high potential for infectivity require biosafety level 3 laboratories for their culture. At the beginning of the 20th century, the culture of intracellular bacteria developed considerably by adaptation of the systems used for virus isolation. For a long time, intracellular bacteria were isolated in embryonated eggs and/or with animal inoculation. Thus far, the successful isolation of intracellular bacteria has been based primarily on cell culture systems (197). Recently, the development of axenic medium led to a revival of intracellular pathogen culture (140, 198).

Culture in Embryonated Eggs

In 1931, Goodpasture and colleagues described how fertilized chicken eggs could be used to cultivate some viruses (199, 200). Several years later, Cox injected intracellular bacteria into the yolk sacs of eggs and demonstrated the ability of bacteria to multiply extensively (201). The infections were confined to the yolk sacs and did not extend to adjacent embryonic tissues. For the growth curve of *C. burnetii* in yolk sacs, we observed a lag phase until the fourth day and a period of exponential growth, with maximal infection on the seventh day, followed by a rapid decrease (202, 203). Consequently, the strains were transferred from egg to egg on the third or fourth day, while the embryo was still living or during the 24 h following its death. The incubation temperature and the age of the eggs were critical. The optimal temperature was 35°C, and a higher or lower temperature was deleterious for the

survival of the embryo. Embryos aged 5 or 6 days were preferentially used; the yield was less elevated in older embryos, and younger embryos died prematurely. Virulence and infective titers were maintained through at least several dozen passages in the yolk sac, and this culture system provided higher infective titers than did mammalian tissues. The major inconvenience of this system is its high susceptibility to contamination and variations in its sensitivity according to egg susceptibility and host factors. Moreover, this procedure requires antibiotic-free-diet embryonated eggs from 5 to 8 days of age, which are often difficult to obtain. This culture system was extensively used to isolate and propagate *C. burnetii* and *Rickettsia* spp. as well as to study antibiotic susceptibility (204, 205). Currently, this culture system has been replaced by cell culture systems, which are more convenient to use. Today, only influenza viruses are routinely cultivated in eggs for vaccination production, and *Chlamydia* spp. and *L. pneumophila* are routinely cultivated for producing antigens for immunofluorescence assays.

Animal Inoculation

The susceptibility of animals is dependent on the bacterial species from which the infection originated. Animals for which bacterial multiplication is more important and which present symptoms similar to those of humans are preferentially selected. Despite improvements in culture media, some bacteria remain uncultivable except in animal models, such as *M. leprae* and *T. pallidum*. This technique is expensive, time-consuming, and technically difficult and is applied only in specialized laboratories.

M. leprae differs from other mycobacteria in that this species cannot be cultured *in vitro*, despite many attempts. *M. leprae* propagation has been restricted to animal models, and several attempts have been undertaken in different animals with low body temperatures (206). First, *M. leprae* was propagated in mouse footpads, where 10^6 bacteria were harvested; however, no leprosy-like lesions were produced (207, 208). This yield could be increased by growing the bacteria in immunosuppressed mice (208). The animal most susceptible to *M. leprae* was a natural host for the bacterium, the nine-banded armadillo (*Dasypus novemcinctus*), which exhibited manifestations of leprosy comparable to those in humans (209, 210). In 1971, Kirchheimer et al. successfully cultured *M. leprae* in this exotic-looking house-cat-sized animal, which is present only on the American continents (209). Their low body temperature (32°C to 35°C) and long life span (12 to 15 years) make these animals preferred for propagating *M. leprae* (211). *Dasypus novemcinctus* is the only animal to develop regularly disseminated infections following intravenous inoculation; however, 30% of the infected animals do develop systematic infections. The organs of the reticuloendothelial system are the most affected (208), with bacterial concentrations reaching 10^9 to 10^{11} *M. leprae* bacteria/g (211). The growth of *M. leprae* is extremely slow, with a doubling time of 14 days. Consequently, cultivation in armadillos requires a minimum of 18 months to obtain a sufficient number of bacilli.

T. pallidum culture is possible only after animal inoculation, which also remains the preferred method for isolating some species of *Borrelia* that are difficult to cultivate in cell culture or in artificial medium, as described above (134).

Inoculation of animals was also used for cultivating intracellular bacteria before the use of cell culture systems. Guinea pigs and mice are the preferred animals for *C. burnetii* and *Rickettsia* (196, 212). Moreover, some bacteria have been isolated from animals
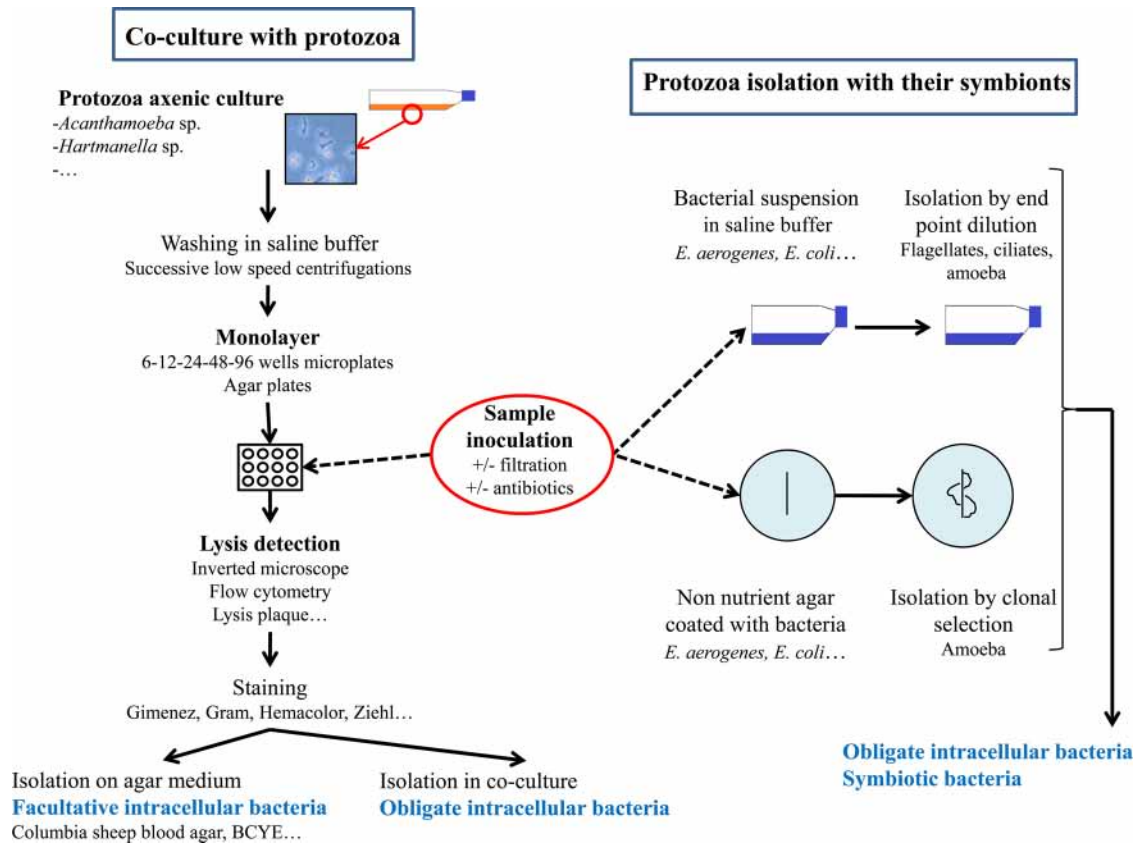
FIG 3 Amoeba culturing methods for recovering amoeba-resisting microorganisms (ARMs). On the left, the method is performed by using laboratory axenic amoebal strains for recovering ARMs from diverse samples. On the right, the amoebae are wild species isolated from the samples, and the ARMs are recovered after amoebal isolation. The circles represent agar plates coated with bacteria, the line is the inoculation point of the sample, and the squiggles represent the growth of the amoebae, following a migration front. BCYE, buffered charcoal-yeast extract.

before being isolated by using cell culture systems. *Rickettsia felis* from fleas was propagated in male Sprague rats before cell culture (213). Similarly, "*Candidatus* Neoehrlichia mikurensis," which was first described in *Rattus norvegicus* rats and in *Ixodes ovatus* ticks, was cultured successfully in rats after intraperitoneal inoculation but has not yet been isolated in cell culture (214). Two major inconveniences of animal inoculation for culture is cross-contamination between infected and uninfected animals and low reproducibility. However, animal models are still useful when attempting to isolate intracellular bacteria from specimens contaminated with other bacteria and when removing contaminated mycoplasmas or microsporidia from cell cultures of intracellular bacteria (215, 216).

Coculture with Amoebae

History. Some bacterial species are capable of resisting digestion after internalization by free-living amoebae and apply this amoeba-resisting property as a survival strategy in nonfavorable environments. These amoeba-resisting microorganisms (ARMs) consist of bacteria that belong to various phylogenetic clades dispersed throughout the prokaryotic tree (217), and some are human pathogens.

T. J. Rowbotham was a pioneer in the isolation of ARMs (218), using an amoebal enrichment method which allowed the isolation of *L. pneumophila* and other nonculturable, protozoonotic,

Gram-negative bacilli, called "*Legionella*-like amoebal pathogens" (LLAPs), from diverse environmental and clinical origins (218–220). Other investigations were performed to investigate the relationship between *Legionellae* species and other protozoa, primarily the ciliate *Tetrahymena* sp., in which *L. pneumophila* is able to survive and acquire high-virulence factors (221, 222).

The investigation of ARMs continued and resulted in the isolation of other microbial types, such as several *Mycobacterium* species (223, 224) and *Chlamydiales* or *Chlamydia*-like species (225). Several viruses were also isolated by using amoebae as culture tools, such as the giant amoeba-associated *Mimiviridae* (226) and their occasionally associated Sputnik virophages (227), *Marseilleviridae* (228), and, more recently, *Pandoravirus* (229).

Notably, protozoa have also been used in the past to isolate and/or to analyze the pathogenicity of strains of bacteria such as *Pseudomonas aeruginosa* (230, 231) and *Cryptococcus neoformans* (232, 233).

Methods. Two types of strategies have been used to isolate these ARMs (Fig. 3).

(i) Coculture with amoebae. The most commonly used method for the isolation of ARMs consists of inoculating samples onto a monolayer of amoebae, usually of the genus *Acanthamoeba*. This method has been largely used and described for isolating bacteria and even giant viruses (234, 235).

TABLE 3 Protists associated with amoeba-resisting microorganisms

Clade	Genus and species	Used for cocultivation and culture	Isolated with ARM(s)
Amoeba	*Acanthamoeba* sp.	Yes (*Babela massiliensis*, etc.)	Yes (*Parachlamydia acanthamoeba*, *Protochlamydia acanthamoeba*)
	Acanthamoeba polyphaga	Yes	Yes
	Acanthamoeba castellanii	Yes	Yes
	Acanthamoeba comandoni		Yes
	Echinamoeba sp.		Yes
	Hartmanella vermiformis	Yes	Yes (*Neochlamydia hartmanella*)
	Naegleria sp.		Yes (*Protochlamydia naeglerophila*)
	Naegleria fowleri	Yes	
	Naegleria fultoni		Yes
	Naegleria australiensis		Yes (*Legionella* sp.)
	Sacchamoeba sp.	Yes	
	Sacchamoeba lacustris		Yes (*Metachlamydia lacustris*)
	Vannella sp.		Yes ("*Candidatus* Mesochlamydia elodeae")
	Dictyostelium discoideum	Yes	
	Vahlkampfia sp.		Yes
Ciliate	*Tetrahymena* sp.	Yes	
	Tetrahymena hyperangularis	Yes (*Legionella* sp.)	
	Tetrahymena tropicalis	Yes (*Legionella* sp.)	
	Tetrahymena pyriformis	Yes (*Legionella* sp.)	
	Paramecium bursaria	Yes	
Flagellate	*Cafeteria roenbergensis*	Yes	
	Trychonympha sp.		Yes (*Endomicrobia*)
	Pyrsonympha sp.		Yes (*Endomicrobia*)

(ii) Isolation of wild amoebae with their symbionts. The second strategy consists of isolating the amoebae present in the sample by using culture on nonnutrient agar layered with a bacterium used as food (usually *Escherichia coli* or *Enterobacter* sp.). After isolation, the amoebae are analyzed for association with eventual ARMs. This method is efficient in isolating strict ARMs (236, 237).

(iii) Protozoa used for ARM culture. The amoebal species used for isolating ARMs are commonly *Acanthamoeba*, although other species have also been found to harbor ARMs. Amoebae and protists from other clades, which are presented in Table 3, have been found to be involved in symbiotic or parasitic associations with bacterial and/or viral ARMs.

Results. Bacteria from many different clades have been isolated from and/or associated with protozoa (217).

(i) *Alphaproteobacteria.* The species *Bartonella rattaustraliani* and *Rhizobium radiobacter* were shown to survive in *Acanthamoeba polyphaga* (238). *Bradyrhizobium* sp. or *Methylobacterium* sp. strains were found by using an amoebal coculture with *A. polyphaga* (235) and *Acanthamoeba castellanii* (223) and had a pathogenic effect on the amoebae (235, 239). Bacteria of the genera *Bosea* and *Afipia* have frequently been isolated from *Acanthamoeba* sp. in hospital networks and could possibly be related to human pathogenicity (219, 231, 235–238).

The species *Sphingomonas koreensis* was found to be associated with *Vanella* sp. In this case, the amoebae were also harboring a new genus of *Chlamydia*-like bacterium, called "*Candidatus* Mesochlamydia elodeae," pathogenic for *Vanella* sp. and maintained in culture in another amoebal species, *Saccamoeba lacustris* (240).

Rickettsia bellii was found to enter and remain stable for 6 weeks within the species *A. polyphaga*. Coinfection with *L. pneumophila* shows that both bacteria colocalize in the vacuoles of the

amoebae, and together with the observation of sequences similar to those of *L. pneumophila* and *Protochlamydia amoebophila* in the genome of *R. bellii*, this feature suggests communication and gene exchanges between bacteria inside the amoeba. Moreover, *R. bellii* has the ability to process gene exchange by conjugation with sexual pili (241).

The bacterium *Odyssella thessalonicensis* is a new, strictly intra-amoebal genus of the *Alphaproteobacteria*, which was isolated from environmental water samples and *A. polyphaga*.

(ii) *Betaproteobacteria.* Several *Betaproteobacteria* have been found to be associated with *Acanthamoeba* sp., for example, *Burkholderia* sp., *Delftia* sp., and *Acidovorax* sp. strains isolated from environmental samples (235, 242).

(iii) *Gammaproteobacteria.* Two species of the genus *Stenotrophomonas* were isolated by using coculture, *S. acidaminiphila* (242) and *S. maltophilia* (235). *S. maltophilia* was also found in association with *Dictyostelium discoideum*, causing infection, invasion, and growth inside the amoeba (243). More recently, two bacterial strains related to the *Stenotrophomonas maltophilia* complex were isolated from two different amoebae, *Acanthamoeba* sp. and *Naegleria* sp. Both amoebae were recovered and infected by the endosymbiont. These bacteria are pathogenic for the amoebae, and coculture leads systematically to the lysis of the amoebae (244). The genus *Aeromonas* has been associated with *Acanthamoeba* sp. (235, 242), has the capacity to multiply in the amoeba, and has cytopathic effects. The species *Aeromonas hydrophila* was found to interact actively with *A. castellanii* and to be able to survive within cysts (245, 246).

Some *Enterobacteriaceae* are also cultivable in several amoeba species. One study focusing on environmental water samples led to the isolation of several strains of *Acanthamoeba*-resisting *Enter-*

obacteriaceae, which are able to multiply within the amoeba but are nonpathogenic, including *Klebsiella* sp., *Enterobacter* sp., *Escherichia coli*, *Morganella morganii*, *Pantoea* sp., *Providentia* sp., and *Serratia* sp., etc. (235, 247); *Yersinia pseudotuberculosis*, *Salmonella enterica* serovar Typhimurium, and *Klebsiella pneumoniae* are able to infect the amoeba *D. discoideum* (243), and some strains of *Enterobacter aerogenes* and *E. coli* also multiplied within *A. castellanii* and survived within cysts (246, 248).

Some *Acinetobacter* species were found in coculture with *A. castellanii* and *A. polyphaga*, with cytopathic effects (235, 242). Additionally *Acinetobacter baumannii* was shown to interact actively with *A. castellanii* (249). For the genus *Pseudomonas*, environmental coculture studies with *Acanthamoeba* led to the isolation of *P. fluorescens* (242) and *P. aeruginosa* (223). *P. aeruginosa* also interacts with the amoeba *D. discoideum* (243). Moreover, this amoeba is now established as a culture model not only for *P. aeruginosa* but also for *L. pneumophila* and *Mycobacterium* sp. (232).

Historically, the genus *Legionella* was among the first to be associated with amoebae due to the ecology of these bacteria. *L. pneumophila* can multiply in not only *Acanthamoeba* sp. but also *Hartmanella* and *Dictyostelium* (243) and has the ability to stay in vacuoles of the ciliate *Tetrahymena*. Several other *Legionella* species have been found in association with different amoebae, and some of these species were new, such as the LLAPs *Legionella drozanskii*, *L. rowbothamii*, *L. fallonii* (250), and *L. drancourtii* (251). However, some other previously known *Legionella* species showed abilities to infect and multiply within amoebae (252). In 2004, an *Amoeba proteus* strain was isolated, which harbored a *Legionella*-like endosymbiont, named "*Candidatus* Legionella jeonii," closely related to *Legionella* sp. (253).

Finally, other strictly intracellular *Gammaproteobacteria* have occasionally been shown to be associated with protozoa, such as *C. burnetii*, which is able to survive within *A. castellanii* (254), and *Francisella tularensis*, which is able to survive within cysts of *A. castellanii* (255).

(iv) Epsilonproteobacteria. Within the *Epsilonproteobacteria*, only *Campylobacter jejuni* has been demonstrated to have the capacity to multiply within protozoa, specifically *Acanthamoeba* sp. (256, 257).

(v) Firmicutes. Among the *Firmicutes* group, *Bacillus licheniformis* was shown to be pathogenically associated with *Naegleria fowleri*, with the effects depending on the bacterium/amoeba ratio (258). These different effects were further investigated by using several bacterial strains of the genus *Bacillus* and the amoebae *A. polyphaga* and *D. discoideum* (259).

Strain RN4220 of methicillin-sensitive *Staphylococcus aureus* was shown to be able to be internalized by *A. polyphaga* and to multiply within the amoeba (260), and the species *Staphylococcus pasteuri* and *S. pneumoniae* were isolated in a coculture with *Acanthamoeba* sp. (261). The pathogen *Streptococcus suis* was studied for its pathogenesis and interactions with the amoeba *Dictyostelium discoideum* (262).

(vi) Bacteroidetes. *Flavobacterium johnsoniae*, *Flavobacterium succinicans*, and *Flexibacter canadensis* were isolated by amoebal coculture with *Acanthamoeba* sp. or as symbionts of *Acanthamoeba* sp. (261). The two periodontopathogenic species *Porphyromonas gingivalis* and *Prevotella intermedia* were able to enter and multiply within *A. castellanii* (263). Several species of *Chryseobacterium* were isolated by coculture with *Acanthamoeba* sp. (235,

242), and two strains were described as new species: *Chryseobacterium massiliae* (239) and *C. massiliensis* (235). Other *Proteobacteria* representing new species or genera were isolated for the first time by amoebal coculture: *Cytophaga massiliensis* (235), "*Candidatus* Amoebinatus massiliae" (239), and "*Candidatus* Amoebophilus asiaticus" (264).

(vii) Actinobacteria. Several *Microbacterium* species and other *Actinobacteria*, such as *Rhodococcus equi* and *Rhodococcus erythropolis* (235, 242, 261), have been isolated by coculture with *Acanthamoeba* sp. The primary group of *Actinobacteria* studied for their interactions with protozoa is *Mycobacterium*. Indeed, many rapidly growing mycobacteria were able to be isolated by coculture with *Acanthamoeba* sp. (223, 224, 235, 252) or as natural symbionts in amoebae isolated from contact lens storage cases (265). In 1997, it was demonstrated for the first time that *M. avium* could enter and multiply in vegetative forms and also survive in cysts of *Acanthamoeba* sp. (266, 267). In 2004, *D. discoideum* was demonstrated to be a good model for studying mycobacterial intracellular trafficking using *M. marinum* (268). One study investigated the ability of waterborne *Mycobacterium* to survive within *A. polyphaga* and demonstrated that these bacteria could enter and multiply in the vegetative form of the amoeba, survive in the cyst forms, and resist chlorine treatment while protected inside those cysts (142, 148, 149). Moreover, a link was made between the ability of *Mycobacterium* to survive and multiply within amoebae and the acquisition or enhancement of some virulence traits (269–272). Some pathogenic or slow-growing mycobacteria were also shown to be associated with amoebae, such as the *M. tuberculosis* complex (150) and the pathogenic *M. ulcerans* (152). Another feature of the interactions between *Mycobacterium* and amoebae, the presence of several functional cellulose-targeting genes, was also elucidated recently (150, 273, 274). Another study showed the presence of the *celA* gene, which codes for a cellulose able to degrade carboxymethylcellulose and microcrystalline cellulose, in *Legionella pneumophila* strain 130b (275). The presence of these cellulose genes in the genomes of amoeba-associated bacteria suggests that these proteins could facilitate the growth of these bacteria within amoebae.

(viii) Chlamydia. The association of *Chlamydiales* with amoebae was demonstrated in 1997 with *Chlamydia pneumoniae*, which was able to multiply in *A. castellanii* (276). Recently, *Chlamydophila abortus* was shown to enter and survive within *A. castellanii* but without multiplying (277). Coculture with several amoebae led to the discovery of several new genera, and these findings have extended much of the order *Chlamydiales*. Indeed, since the discovery and description of the new genus *Parachlamydia* in *Acanthamoeba* sp. (278), many other bacteria have been isolated from amoebal cultures, including *Neochlamydia hartmanella* (237), *Protochlamydia amoebophila* (279), *Criblamydia sequanensis* (280), *Protochlamydia naeglerophila* (281), *Metachlamydia lacustris* (282), *Estrella lausanensis* (283), and "*Candidatus* Mesochlamydia elodeae" (240).

Some other *Parachlamydia*-related bacteria isolated in mammalian cells have demonstrated susceptibility to amoebae. *Simkania negevensis* was cultivated successfully in *A. polyphaga* (284, 285), and *Waddlia chondrophila* was cultivated in *A. castellanii*, which enabled antibiotic susceptibility testing (286).

(ix) Endomicrobia. The *Endomicrobia* group is represented by uncultivated bacteria within the candidate phylum termite group I (TG-1) (287, 288). These bacteria were demonstrated to be as-

sociated with termite gut flagellates, primarily the species *Tricho-nympha* and *Pyrsonympha*, and each of the flagellate species harbors phylogenetically distinct species of *Endomicrobia* (288, 289).

(x) TM6 phylum. The TM6 phylum is a putative bacterial phylum represented by the single draft genome of a putative bacterium, JCVI TM6SC1, which was obtained by single-cell genomics and from numerous environmental sequences (290). Amoebal coculture with *Acanthamoeba* sp. recently enabled the isolation of the first effective strain of this phylum, a bacterium that we named *Babela massiliensis*. This bacterium harbors genome features that show high levels of degradation in the cell division machinery, and these features are introduced phenotypically by multiplication in the amoeba that resembles budding, different from classical binary fission (I. Pagnier, N. Yutin, O. Croce, K. S. Makarova, Y. I. Wolf, S. Benamar, D. Raoult, E. V. Koonin, and B. La Scola, unpublished data).

Use in clinical microbiology. Some ARMs have already been implicated in human pathology, primarily *Mycobacterium* spp., *Legionella* spp., and some *Chlamydia* spp. and *Parachlamydia* spp. Amoebal culture and research into ARMs are alternative methods of clinical diagnosis. For example, some *Legionella* species were able to be recovered from clinical specimens only by using amoebal culture (291). Moreover, the first giant virus isolated from a patient sample was a member of the *Marseilleviridae*, called senegalvirus, which was isolated from a stool sample of an asymptomatic patient from Senegal (154, 292). Later, the first human virus of the *Mimiviridae*, LBA111, was isolated from a pulmonary sample (293). Finally, a last giant virus of the *Mimiviridae*, called Shan virus, was isolated from a stool sample of a patient presenting with pulmonary disease (294). Amoebal culture could be useful for clinical diagnosis, primarily to target strictly intracellular ARMs, such as *Chlamydiales* or *Parachlamydiales* species. For example, the species *Protochlamydia naeglerophila*, which was isolated as a symbiont of the amoeba *Naegleria* sp., was found in a pathological sample of a patient with pneumonia by using molecular methods (281), and the species *Waddlia chondrophila* was shown to be able to survive and multiply within human macrophages, suggesting possible implications for human pathology (295, 296).

Perspectives. Culture of microorganisms with protozoa has facilitated several important steps in the comprehension of the microbiological world, and the relationships between protozoa and bacteria were able to be studied more deeply. Amoebal coculture led to the discovery of many new microorganisms, particularly new species such as *L. drozanskii*, *L. rowbothami*, *L. fallonii* (250), and *L. drancourtii* (251). New genera (*Chlamydia*- and *Parachlamydia*-related microorganisms) (282, 283) and new phyla (*Babela massiliensis*) (Pagnier et al., unpublished) or life domains (all amoeba-associated giant viruses) have also been recovered by amoebal coculture. The example of the isolation of *Babela massiliensis* (CSUR P554; GenBank accession number GQ495224 for 16S rRNA) is a good illustration of the increasing importance of microbial culture (Pagnier et al., unpublished). Indeed, many metagenomic approaches led to the discovery of entire unknown groups of microorganisms, and deeper study of these groups has been limited by the nonavailability of real bacterial strains. The use of coculture with amoebae allowed the recovery of a representative member of the putative TM6 phylum, and this finding will lead to other studies elucidating the intracellular way of life of the bacterium. The principle of isolation by coculture can be transferred to other types of protozoa, which could have other host-microorganism spectra. Indeed, culture assays have already been performed with the axenized protists *Vermamoeba vermiformis*, *Dictyostelium discoideum*, *Tetrahymena hyperangularis*, and *Colpoda steinii*. Recently, a high-throughput method was developed by using the amoeba *Acanthamoeba* sp. to isolate giant viruses from >1,000 samples (297) by observation of a lysis plaque on an amoebal monolayer resting on an agar plate (297). Using other protists, lysis due to ARMs can be observed by flow cytometry. These methods have already led to the isolation of new microbial species, such as *Rubidus massiliensis* (CSUR P942; GenBank accession number HG726047 for 16S rRNA), a new *Parachlamydia*-related bacterium, and a new giant virus, associated with *Hartmanella vermiformis*.

Cell Culture

Currently, the most common method for cultivating and isolating intracellular bacteria from clinical samples is cell culture. Two different strategies can be used. First, culture conditions can be standardized to allow the efficient growth of a broad range of bacteria. The second strategy consists of the specific isolation of an already known bacterium with specific adapted culture conditions for bacterial growth optimization. In fact, the type of cells and culture medium used should be adapted specifically, depending on the bacterium being cultivated. The susceptibility of bacteria to different cells lines varies according to their cell tropisms and optimal growth temperatures. Several eukaryotic cell lines can be used, including mammalian and arthropod cell lines. Two types of cells may be used: (i) cells cohering with each other, forming a cell monolayer, and (ii) cells circulating in suspension.

The temperature and atmosphere of incubation are critical and should be adapted. This culture strategy should also be used in the genomic and proteomic era, which requires a large amount of bacteria for large-scale investigations and for antigen production for diagnostic testing. Cell culture systems have also been used to evaluate the antibiotic susceptibility of intracellular bacteria by a plaque assay and a dye uptake assay (298, 299) or with a combination of culture and a PCR assay (5).

Cell lines grown at 37°C. Mammalian cells developing at 37°C are the most often used cells. *C. burnetii* can be grown with a doubling time of 20 h in mouse macrophage-like (P388D1 and J774), murine fibroblast (L929), and kidney epithelial (Vero) cell lines (196, 300–302). Human embryonic lung (HEL) fibroblasts have been preferentially used for routine culture because of their easy maintenance and high level of susceptibility to *C. burnetii* infection. The observed cytopathic effects were the occurrence of large vacuoles in the cytoplasm. *C. burnetii* infection does not destroy host cells, allowing the maintenance of *C. burnetii* for several months in persistent culture (196, 303).

Culture of *Rickettsia* was first performed by using a primary monocyte cell culture (304). In the 1980s, Vero and L929 cells were used, allowing better and more rapid isolation of rickettsiae than with HEL and MRC5 (human fetal lung fibroblast) cells (305). In fact, *Rickettsia conorii* presented higher multiplication rates in L929 cells than in Vero or 3T3 cells (306). Most of the *Rickettsiae* belonging to the spotted fever group can polymerize actin from cells, to move into the nucleus of the host cell and promote cell-to-cell spreading, which generates rapid cytopathic effects and rapid plaque formation at an optimal growth temperature of 32°C (307). The optimal growth temperature of *Rickettsia* from the typhus group is 35°C. The bacteria invade adjacent cells

only when their multiplication and accumulation in the cytoplasm cause host cell lysis. Consequently, cytopathic effects were less prominent, and the plaques formed were small (307).

L929 cells have been used for culturing *O. tsutsugamushi*, which grew slowly, and 1,000 bacteria were required to invoke cell disruption and propagation in adjacent cells (308). Consequently, a long incubation period is required, which involves difficulties in maintaining the integrity of the cell monolayer and contamination-free medium.

The establishment of *T. whipplei* in culture was reported in 2000, using HEL cells (309), and the bacterium was then cultured from various clinical samples (310). Antibiotics were added to the culture medium to cultivate samples that were naturally contaminated, such as duodenal biopsy specimens or stool specimens (311, 312). The growth of *T. whipplei* was also observed in MRC-5 (313) and HeLa (314) cells.

DH82, which is a continuous canine macrophage cell line (315), is usually used to isolate *E. chaffeensis* and *Ehrlichia canis* (316). However, several other cell lines can be employed, such as mouse embryo, Vero, Buffalo green monkey kidney (BGMK), HEL, HeLa, and L929 cells (315, 317, 318). *E. chaffeensis* induced cytopathic effects on mouse embryo, L929, HEL, and Vero cells and caused macroscopic plaque formation in L929 and mouse embryo cells (318) and microscopic plaque formation in Vero cells (317). *Anaplasma phagocytophilum* has been cultured routinely and with a rapid development of cytopathic effects (319) in HL-60, a human promyelocytic leukemia cell line that grows in suspension culture (320). This cell line was highly susceptible to *A. phagocytophilum* infection. Fresh blood or the leukocyte fraction of EDTA-blood can be inoculated into a 25-cm^2 flask containing a density of 2×10^5 cells/ml. However, the ability of *A. phagocytophilum* to establish *in vivo* infection successfully decreased considerably when it was continually maintained in cell culture (321).

Several mammalian cell lines have been used to propagate *Chlamydia* spp., including mouse fibroblast (McCoy and L cells), human cervical carcinoma (HeLa 229), human laryngeal carcinoma (HEp-2), human epithelial (HL), BGMK, and Vero (322) cell lines. To permit the entry of *Chlamydia* spp. into host cells and the creation of a favorable intracellular environment, several steps in the process are critical for the success of *Chlamydia* species culture (323, 324). A centrifugation step and/or the addition of polycationic stabilizing molecules to overcome the repellent forces between the membrane of the host cell and the bacteria was required, except for *C. psittaci* and the LGV agent. Cycloheximide, which is a selective inhibitor of eukaryotic protein and nucleic acid synthesis, was added to reduce the competition for nutrients between host cells and bacteria, thus enhancing the growth of *Chlamydia* (325). The cultures were incubated for 48 to 72 h at 35°C. McCoy and HeLa 229 cells are the cell lines most commonly used for *C. trachomatis* culture (322, 324), and HL and HEp-2 cells seem to be more sensitive for the recovery of *C. pneumoniae* (323, 326). The cytopathic effect occurs as small, rounded intracytoplasmic inclusions, visualized by immunostaining of cell monolayers.

Cell lines grown at 28°C. Some bacteria require temperatures of <37°C to grow; consequently, the use of cell lines specifically tailored to lower temperatures is required, such as amphibian, tick, mosquito, and fish cell lines. These cell lines have been commonly used for arbovirus culture and allow the isolation of intracellular bacteria, such as *Rickettsia felis*. The first culture of *R. felis*,

which was performed with Vero and L929 cells after incubation at 37°C, was in reality contamination of the culture by *Rickettsia typhi* (213, 327, 328). Thus, the culture of *R. felis* has been credited to Raoult et al. (40), who cultivated this bacterium with success in an amphibian cell line derived from *Xenopus laevis* (XTC2) after 6 days of incubation at 28°C. The bacterium also grows slowly on Vero cells at 28°C and 32°C (40, 329). The growth of *R. felis* in mammalians cell lines is inconstant but was improved by the addition of tryptose phosphate broth in culture media, which seems to be a critical component based on its involvement in the electron transport chain of the Krebs cycle (330). More recently, growth of *R. felis* in *Drosophila melanogaster* S2 cells was reported, with a cell infection rate of 100% after 19 days of incubation at 25°C (331). Other arthropod-borne bacteria, such as *Diplorickettsia massiliensis* (332), *Wolbachia* sp., and *Bartonella bacilliformis* (40), as well as other bacteria such as *Piscirickettsia salmonis* (333) and *M. ulcerans* (334) can also be cultivated more effectively by using this cell line.

Approximately 40 different tick cell lines are available, primarily from ixodid rather than argasid tick species (335). These cells have the advantage of partially reproducing the natural environment of tick-borne pathogens. Most tick cells grow at incubation temperatures of between 28 and 34°C, and acidic or alkaline pH conditions vary according to the cell line. Cells from ticks divide slowly, require up to 1 month between passages, and are generally not strongly adherent, generating partial monolayers or a suspension culture. Tick cell lines have been used successfully for the growth of *A. phagocytophilum*, *Anaplasma marginale*, *Ehrlichia canis*, *Ehrlichia ruminantium*, *Borrelia* spp., *Rickettsia* spp., *Bartonella* spp., and *C. burnetii* (335–340) as well as for the isolation of unknown bacteria, such as a new *Anaplasma* sp. (341), *Borrelia lonestari* (342), and *Rickettsia monacensis* (343). Mosquito cell lines are permissive to multiple arthropod-borne infections and offer the advantage of being easier to culture than tick cell lines. Mosquito cell lines can be passaged in 1 week and grown at room temperature under an ambient atmosphere and have the potential to develop high bacterial titers, and infection is stable in the cells for >40 passages (339). In particular, *Anopheles gambiae* (Sua5B) and *Aedes albopictus* (Aa23 or C6/36) cell lines have been used to grow *R. felis*, *R. montanensis*, *R. peacockii*, and *R. typhi* as well as several strains of the bacterial symbiont *Wolbachia* (37, 339, 344–349).

Few assays have been performed to grow fish-pathogenic bacteria in a fish cell line at temperatures between 21°C and 30°C. *Mycobacterium marinum* was cultivated successfully in a carp monocyte cell line at 28°C (350), and *Piscirickettsia salmonis* was cultivated in a Chinook salmon cell line (351) (Table 4).

Specific devices (shell vial). The shell vial assay is currently the method used in specialized laboratories to isolate fastidious bacteria from clinical samples (Fig. 4). This technique was adapted to bacterial culture in 1989 (352) from a centrifugation-cell microculture system, which was described in 1976 and used for virus isolation (353). The principles of this technique are based on the inoculation of clinical specimens by low-speed centrifugation on confluent cell monolayers seeded onto 1-cm^2-round coverslips and incubated with 1 ml of culture medium in a shell vial. The centrifugation step is critical because this step enhances the attachment and penetration of the bacteria in cells (354). The sensitivity of this cell culture technique is increased by the small surface area of the coverslip, which contains cells that enhance the

TABLE 4 The most susceptible cells line used for the culture of intracellular bacteria[a]

| Bacterium | Cell line(s) (culture temp [°C]) | | Fish and amphibian | Reference(s) |
	Mammalian	Arthropod		
C. burnetii	HEL, Vero (35)	*Ixodes scapularis* cell line-IDE8 (34)		196, 336
Spotted fever group *Rickettsia*	L929, Vero (32)	*Dermacentor* sp. cell lines DAE3 and DALBE3 (34); *Ixodes scapularis* cell line ISE6 (34); *Aedes albopictus* cell linesAa23, AeAl2, C7/10, and C6/36 (22–32); *Anopheles gambiae* cell line Sua5B (22–25)		212, 305, 306, 335, 338, 344, 348
Typhus group *Rickettsia*	L929, Vero (35)	*Aedes albopictus* cell line AeAl2 (28		212, 349
Rickettsia felis	Vero (32)	*Ixodes scapularis* cell line ISE6 (32), *Aedes albopictus* cell lines Aa23 and C6/36 (22–25), *Anopheles gambiae* cell line Sua5B (22–25)	XTC (28)	40, 329, 337, 338, 340, 345
Orientia tsutsugamushi	L929 (32)			308
Anaplasma phagocytophilum	HL60 (37)	*Ixodes scapularis* cell lines ISE6 and IDE8 (34)		319–321, 335, 341
Ehrlichia chaffeensis	DH82 (37)	*Ixodes scapularis* cell line ISE6 (34)		315, 316, 335
Ehrlichia canis	DH82 (37)	*Ixodes scapularis* cell line ISE6/IDE8 (34) *Ixodes ricinus* cell line IRE/CTVM18 (34)		315, 316, 318, 335
Wolbachia pipientis	HEL (28 and 37)	*Aedes albopictus* cell lines Aa23 and C6/36 (26–28)		37, 346, 347
Tropheryma whipplei	MRC5, HEL (35)			309, 310
Chlamydia trachomatis	McCoy, HeLa 229 (35)			322, 324
Chlamydia pneumoniae	HL, HEp-2 (35)			323, 326

[a] Shown are the most susceptible cell lines from mammals, arthropods, fish and amphibians used for culturing of intracellular bacteria, including *C. burnetii*, *Rickettsia* spp., *Ehrlichia* spp., *T. whipplei*, *Chlamydia* spp., and *Anaplasma* spp.

ratio of the number of intracellular bacteria to the number of cells and that allow more efficient recovery. Moreover, the cell type, length, atmosphere, and incubation temperature can be optimized according to the suspected pathogen. Different protocols can be used for the specific isolation of *Rickettsia* spp., *Bartonella* spp., *C. burnetii*, or *Tropheryma whipplei*, and one protocol, which is called "JNSP," is based on the French abbreviation of the sentence "*Je ne sais pas*," which means "I do not know" what I am growing, for unspecific research into other intracellular bacteria (Fig. 4). HEL fibroblasts are used because of their high level of susceptibility to intracellular bacterial infection and their ease of maintenance. To avoid bacterial contamination, antibiotics with no activity against the bacteria of interest can be added. The growth of bacteria is detected by observation of cytopathic effect; acridine orange, Giemsa, and/or Gimenez staining; or immunofluorescence revelation using the patient's serum or sera from immune animals as the primary antibody (197). Definitive identification of the bacteria is performed by PCR. This technique is used routinely in our laboratory and permits the successful isolation of spotted fever group *Rickettsia* (355), *Rickettsia prowazekii* (356), *C. burnetii* (357), *C. trachomatis* (358), and fastidious bacteria such as *Bartonella* sp. (48, 359), *F. tularensis* (360), *Mycobacterium* sp. (359, 361), *L. pneumophila* (362), *Brucella melitensis* (363), *Actinomyces* sp. (197), *Streptobacillus moniliformis* (364), and *Nocardia* sp. (197). The shell vial assay exhibits a low rate of success, but this success can be extremely valuable (197). This technique facilitated the establishment of *T. whipplei* from a cardiac valve of a patient with Whipple's disease endocarditis in the context of JNSP protocols (309). In some cases, this technique is the only technique that allows microbiological diagnosis, and the shell vial system was also used for testing the antibiotic susceptibilities of intracellular bacteria (365, 366).

Axenic Culture Media for Intracellular Bacteria

Knowledge of pathogen physiology and the host cell-pathogen interaction and the use of new tools, such as genomic and tran-

scriptomic analyses, have enabled the study of metabolic pathways, enhancing the success of axenic cultivation (198, 367, 368). The establishment of axenic culture media could have significant effects on the study of their pathogenicity, virulence, and antibiotic susceptibility and on the design of new diagnostic tools (140).

Coxiella burnetii. *Coxiella burnetii* has been described as being cultivable in an acellular chemically defined medium (11, 369, 370), a medium designed after a comprehensive analysis of the nutrient consumption of *C. burnetii*. First, genomic analysis identified three critical components for this complex *Coxiella* medium (CCM) to sustain the metabolic activity of the bacteria as well as 3 complex nutrient sources (Neopeptone, fetal bovine serum, and RPMI cell culture medium) and a high concentration of chloride and citrate buffer, mimicking the acidic environment of phagosomes (370). A transcriptomic analysis resulted in the conception of ACCM (acidified citrate cysteine medium) enriched with cysteine and Casamino Acids that support the growth of *C. burnetii* by >3 $\log_{10}$ units after 6 days of incubation in a microaerophilic atmosphere (11, 371). This medium was then optimized through the replacement of fetal bovine serum by methyl-β-cyclodextrin and was named ACCM-2; ACCM-2 sustained 4- to 5-$\log_{10}$ growth of *C. burnetii* over 7 days with the addition of moderate shaking (372). Moreover, colonies were observed on ACCM-2 agarose (372). More recently, Singh et al. showed the exponential growth of *C. burnetii* in an empirical medium containing eukaryotic cell extracts (120). With the medium being refreshed every 2 days, low oxygen tension and the presence of small hydrophilic molecules and short peptides were critical for its growth. However, this medium harbors a neutral pH, suggesting that the growth of *C. burnetii* does not require acid activation of its metabolic pathways. The antigenicity and the virulent form (phase I) of *C. burnetii* were conserved, revealing the potential for this culture medium to be used for antigen and vaccine production. Furthermore, this medium permitted the successful isolation of *C. burnetii* from pathological samples.
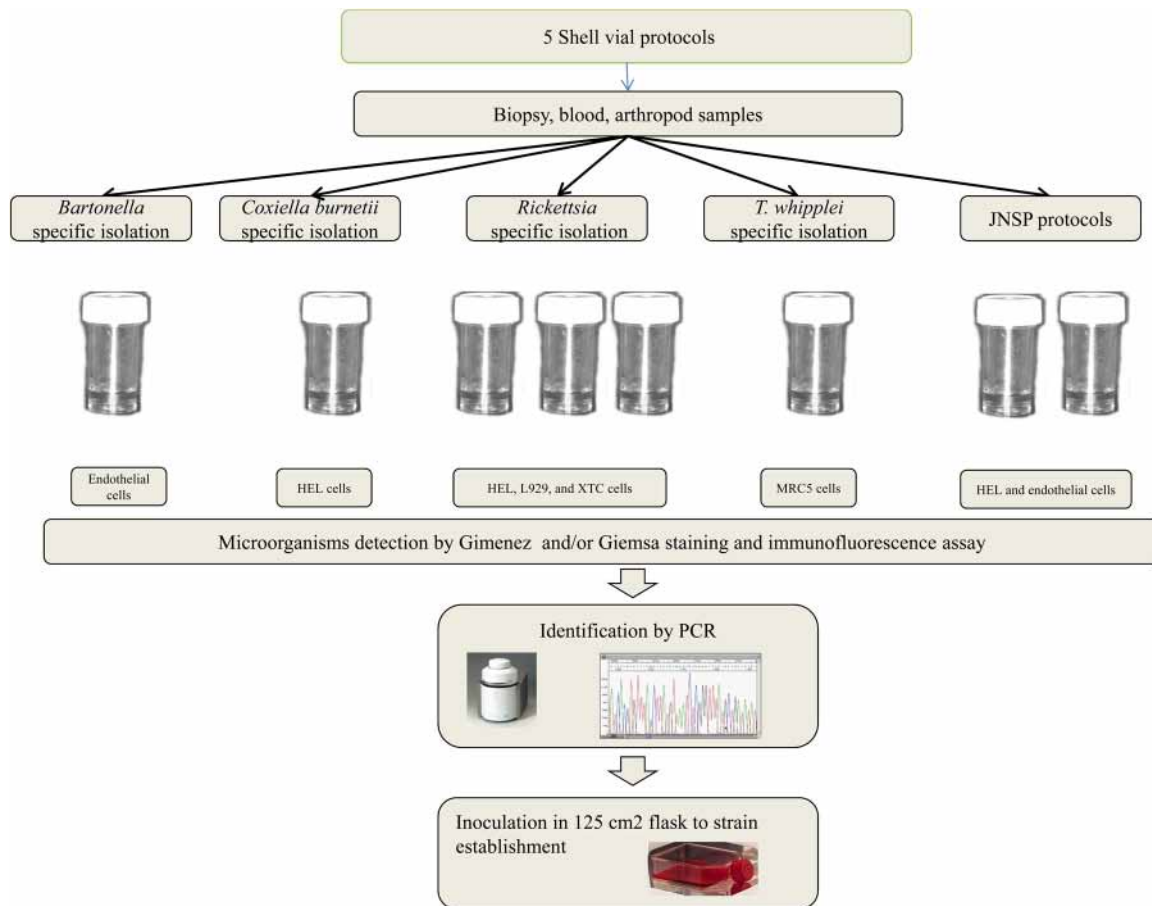
FIG 4 Shell vial system protocols used for specific isolation of *Bartonella*, *Coxiella burnetii*, rickettsiae, or *T. whipplei* and for unspecific research into other intracellular bacteria according to JNSP protocols.

Tropheryma whipplei. Culture of *T. whipplei* permitted the obtaining of the complete genomes of two different strains of *T. whipplei* (7, 313). Genomic analysis revealed a reduced genome size (0.9 Mb) and a specific metabolic deficiency. The genes coding for metabolic pathways for nine amino acids were entirely missing, and the pathways of glutamate and phenylalanine synthesis were nonfunctional (13). An axenic medium compensating for these deficiencies was designed based on these genomic analyses (13, 368). This medium consisted of cell culture medium that provided the missing amino acids, supplemented with 10% fetal calf serum, 1% glutamine, and 1% human nonessential amino acids (13). This approach permitted the successful growth of *T. whipplei* from a great variety of clinical samples as well as antibiotic susceptibility testing (4, 373). The development of an axenic medium permitted the culturing of isolates from sterile and nonsterile sources, such as saliva and stool samples, after a decontamination procedure with glutaraldehyde (312) or with filtration (374, 375).

Chlamydia **species.** *C. trachomatis*, similar to other members of the *Chlamydiaceae* family, presents a biphasic developmental cycle, with important consequences for its metabolic activity and axenic growth. During their biphasic intracellular development cycle, the surviving infectious elementary bodies (EBs) extracellularly differentiate into reticulate bodies (RBs) after entry into the cytoplasm of the host cells, which multiply by binary fission. Two

recent studies demonstrated the ability of EBs to conduct metabolic activity outside the cytoplasm of host cells (198, 376). The development of a host cell-free medium was based on analysis of the *C. trachomatis* genome. A reduced genome (1.04 Mb) and the several missing enzymes and entire metabolic pathways suggested that *C. trachomatis* uses nutrients from host cells for its growth (377). The axenic medium supporting the metabolic activity of *C. trachomatis* consisted of a novel intracellular phosphate buffer and an ion concentration mimicking the composition of the eukaryotic cytoplasm (14). Incubation in a microaerophilic atmosphere and an energy source were critical for the metabolic activity of *C. trachomatis*. EBs have been historically described as metabolically dormant; however, in cell-free media, high levels of metabolic and biosynthetic activity were observed for both EBs and RBs, although the EBs primarily used glucose-6-phosphate, and the RBs used ATP as an energy source. This axenic medium, with further modifications, promises to support the replication of *C. trachomatis* and the study of the metabolism and physiology of *Chlamydiae*.

Empirical broad-spectrum medium. Singh et al. developed an axenic liquid medium based on an extract of eukaryotic cells, allowing the growth of a broad spectrum of bacteria. The eukaryotic cell extract-based universal empirical medium, which was tested for culturing of *C. burnetii* as a proof of concept, supported the growth of other fastidious bacteria and putative bacterial bioter-

rorism agents (120). Rapid growth (<72 h) was observed for *T. whipplei*, *Yersinia pestis*, *Vibrio cholerae*, *Bacillus anthracis*, *Shigella dysenteriae*, *Brucella melitensis*, *Bordetella pertussis*, and *Bartonella henselae*. *F. tularensis* and *M. bovis* were able to grow on this medium in 20 and 8 days, respectively, and *Campylobacter* spp. were able to grow in 5 days under microaerophilic conditions. In addition, this medium was the first to permit the growth of two spirochetes belonging to different genera, *B. crocidurae* and *L. interrogans*, with delays of 14 and 4 days, respectively. However, two limitations of this medium are its inability to sustain the growth of other intracellular bacteria, including *Rickettsia* and *Legionella* species, and its variability, which is caused by the use of uncharacterized biological products. The primary advantage of this empirical medium is its versatility; thus, this medium can be used for many applications, including the isolation and culture of unknown fastidious bacteria from clinical samples, which encourages its use as a replacement for cell-based culture systems. Moreover, this medium offers promising perspectives for culturing other intracellular fastidious bacteria by testing variations in temperature, atmosphere of incubation, and supplementation with nutrients and growth factors required by the bacteria.

Future challenges. We have reviewed older and more current approaches used to cultivate intracellular bacteria. No single approach exists. Older techniques, such as animal inoculation, are still used to propagate some bacteria and to isolate new bacterial species, such as "*Candidatus* Neoehrlichia mikurensis." Cell culture permits the growth of a large number of intracellular bacteria; however, in some cases, cell culture must be more specifically adapted to particular species, mimicking their natural environment to optimize their growth. Future prospects will be based largely on the development of axenic media, facilitating genetic manipulation, and on an understanding of microbial ecology and the pathogenicity of fastidious bacteria. Axenic growth of the historically "uncultivable" *T. pallidum* and *M. leprae* has remained a large challenge for a century. The success of axenic growth of *C. burnetii* and *T. whipplei*, as well as the design of axenic media for the growth of *Rickettsia*, *Anaplasma*, *Ehrlichia*, *Orientia*, and *Chlamydia*, should accelerate soon.

CONCLUSION

Our laboratory (URMITE, Marseille, France), which was created *ex nihilo* in 1984 and which initially specialized in intracellular bacterial culture, has now extended its experience into many fields of clinical microbiology, tirelessly demonstrating the central role of pure culture in understanding infectious diseases. The shell vial procedure and intracellular culture have allowed us to culture 18 different species of *Rickettsia* and have facilitated participation in the description of 7 new bacterial species or subspecies (195, 378–383) and in the first culture of 4 new rickettsial species (40, 384–386). Interestingly, *Rickettsia felis* is now associated with acute febrile illness (387, 388) and with a vesicular fever called "yaaf" in Africa (389). Currently, we have cultured 41 different *T. whipplei* strains from diverse tissues and fluid (310, 312, 374, 375, 390, 391), playing a key role in the understanding of the clinical manifestations caused by this bacterium (194). Coculture with amoebae, which was performed in our laboratory, has allowed us to identify 139 different bacterial species from both clinical and environmental samples, including 10 new bacterial species (224, 235, 239, 251, 261, 392–396). In our laboratory, the great qualities of several "cultivators," technicians, or researchers were identified

and recognized in the names of bacterial species to honor these qualities (i.e., *Rickettsia raoultii*, *Legionella drancourtii*, *Mycobacterium barrassiae*, *Afipia birgiae*, *Bosea vestrisii*, and *Bosea eneae*, etc. [251, 384, 392, 396, 397]), as the names of other species acknowledge several "cultivators," such as *Finegoldia magna*, *Murdochiella asaccharolytica*, or *Gordonibacterium pamelaeae* (398, 399).

Despite the spectacular advances in microbial culture in recent years, there are still unsatisfactory results remaining. A large part of anaerobic bacteria detected by molecular methods remain as yet uncultured. The optimization of transport time and the use of antioxidants will allow a dramatic increase of this repertoire. The development of axenic culture of spirochetes such as *T. pallidum* or *M. leprae* remains an important challenge in clinical microbiology.

Some bacteria have not yet been cultivated due only to the lack of investigators; *Rickettsia raoultii* was cultivated for the first time by Oleg Mediannikov, whereas we failed to culture this strain in the same laboratory for 8 years (384). The patience and energy required for the primary culture of a bacterium are often considerable. Culture is a complex and difficult art, in which the individual qualities of the scientist are not comparable with the individual qualities required by researchers using a more biochemically defined method.

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Didier Raoult, M.D., Ph.D., who specializes in infectious diseases, is a professor of microbiology at the Faculty of Medicine of Marseille, Aix Marseille University. In 1984, he created *ex nihilo* his research laboratory, the Rickettsia Unit. This unit has now become the Research Unit in Infectious and Tropical Emergent Diseases (URMITE), collaborating with the CNRS (National Center for the Scientific Research), the IRD (Institute of Research for Development), and INSERM (National Institute of Health and Medical Research). In 2011, he became the director of the University Hospital Institute Mediterranée Infection, which is a 600-person medical institute focused on infectious diseases. This facility includes the largest diagnostic and research microbiology laboratory in France. As of 2014, Prof. Raoult has published more than 1,900 indexed publications. In the last 30 years, he has cultured approximately 16% of the bacteria isolated for the first time in humans, including *T. whipplei*, in his laboratory and developed a culturomics team in 2011.