Enliven Archive
Intensifying Thoughts

Research Article | www.enlivenarchive.org | Enliven: Microbes and Microbial Techniques

ISSN: 2576-2540

Development of Techniques for Isolating Microorganisms

Kesen Ma[1*], Sarah D. Kim[1]

[1]Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

[*]Corresponding author: Kesen Ma, Department of Biology, University of Waterloo, 200 University Avenue West, Waterloo, Canada, Tel: 1-519-888-4567 x33562; Fax: 1-519-746-0614; E-mail: kma@uwaterloo.ca

Received Date: 14th September 2018
Accepted Date: 10th October 2018
Published Date: 12th October 2018

Citation: Ma K, Kim SD (2018) Development of techniques for isolating microorganisms Enliven: Microb Microbial Tech 5(1): 001.

Abstract

Isolation of pure cultures is a fundamental technique in microbiology, and has been employed for centuries in laboratories across the globe. Several commonly used methods have contributed to the successful isolation of microbial species to date. Microorganisms are present in all types of environments with various chemical and physical requirements, but only a very tiny portion of them has been isolated as pure culture, and the vast majority have yet to be isolated and characterized. Microbial pure cultures are crucial for obtaining a full understanding of their biochemical and biophysical attributes. Future development of new techniques for isolating pure microbial cultures is required, which is an essential step for achieving further advancement of microbiological research and applications.

Introduction

For over a century, microbiologists have sought to develop techniques for isolating microorganisms from various environments. Ranging from the early works of Robert Koch and his plating techniques [1], to the more modern approaches using optical tweezers [2], the realm of microbial isolation has broadened with aims of identifying the vast repertoire of microbes existing in the world. "Microbial isolation" refers to the techniques and methods used to obtain pure cultures of microorganisms and is crucial to the foundation of microbiology. Isolating pure cultures, especially those of novel species, allows researchers to gain more information on the biochemical routes and physiological structures within these organisms. Currently, there are approximately 30,000 microbial species [3] belonging to more than 2,500 genera which have been isolated and characterized [4] since the first discovery of microbes, which were observed using a microscope more than three centuries ago [5]. This number, however, is only about 0.000003% of the total microbial species on earth, which is estimated to be approximately a trillion microbial species [6]. Microorganisms not only play an essential role in our ecosystems, but also in a human body where bacterial number is almost equal to that of the human cells [7]. Due to the general size in micrometer scales, a pure culture of a microbial species is required for an understanding of its full physiological capacities. Our knowledge of the metabolic diversity of microorganisms is quite limited because of the challenges that exist in isolating pure cultures. There is a lack of proper methods as well as unculturability of fastidious organisms making it difficult for researchers to make considerable progress in isolating new cultures [8]. Reviewing methods and techniques that have been used for isolating microbes is an important step for the development of more efficient ones. This paper outlines the most commonly used methods for microbial isolation, describes the major methods/techniques from liquid, solid media to laser tweezer, discusses challenges for culturing the uncultured microbes from mesophiles to extremophiles, and provides future perspectives.

Development of Microbial Isolation Techniques and Challenges

From the first documentation of bacterial isolation [9] to the introduction of solid media plating by the "father of culture media" Robert Koch [10], microbial isolation techniques have gone a long way to develop into the techniques that are commonly used today.

Koch extended the development of solidified media to the discovery of the widely employed streak plate method. Despite this breaking discovery, there were some challenges associated with using this gelatin mixture; mainly, on warm days the media would liquefy, and some bacteria were capable of digesting the gelatin [10]. Fortunately, a year later in 1882, by coincidence Fanny Eilshemius provided an insightful piece of information that revolutionized the culturing of microbes on solid media. Eilshemius introduced Koch to agar-agar, a seaweed product from Indonesia that was capable of staying solid and was tolerant of most digesting bacteria [11].

Microbial isolation is an important facet to any field of science however, the progress in isolating pure cultures has been quite slow [8]. There are many factors that influence the culturability of a particular community of microbes. The techniques and tools used to isolate these microorganisms heavily depend on the environment the organisms are living in, such as soil versus water, aerobic versus anaerobic organisms, temperature ranging from mesophiles to hyperthermophiles, as well as their biochemical/biophysical requirements, to name a few. Among the many challenging factors, temperature, oxygen, and nutrient requirements are further described in the following.

Temperature Dependency

Microorganisms grow in a wide range of temperatures, ranging from extremely hot to extremely cold environments. Depending on the temperature optima (T_{op}), they can be classified as the following: psychrophiles ($T_{op} \leq 15$ ˚C but $T_{max} < 20$ ˚C), mesophiles ($T_{op} = 20 \leq 45$ ˚C), thermophiles ($T_{op} = 45 \leq 80$ ˚C), and hyperthermophiles ($T_{op} > 80$ ˚C) [12]. With such strict temperature requirements, it comes to no surprise that very specific methods must be used to isolate each. To maintain proper temperatures, incubation systems are used (such as wet and dry incubation tanks), with the appropriate vessel used for containment of the organism.

Thermophiles require high temperature conditions for cultivation thus, traditional plating methods cannot be used as agar would melt and water vaporize upon incubation [13]. A popular solution for plating thermophiles/hyperthermophiles using solid media (see section: *Solid Media*) is to use a modified solidifying agent such as GELRITE [13] or Phytagel [14]. Both GELRITE and Phytagel are gelling agents, naturally derived as a fermentation product of *Pseudomonas* spp., used specifically for culturing thermophilic microorganisms [13,15]. GELRITE proves to be successful in that a number of thermophilic strains have been isolated including: *Bacillus acidocaldarius, Bacillus stearothermophilus, Thermus thermophilus, Thermus aquaticus* [15]. It has been observed that GELRITE provided more clear and thermo stable gel results [15] as compared to agar-based gels proving it is a "good" alternative to agar-based gels. Another alternative to GELRITE for the isolation of thermophilic strains is Phytagel, which has been reported to solidify slower than GELRITE [14]. Researchers have successfully isolated *Thermococcus litoralis* and *Pyrococcus furiosus* on Phytagel, both which are hyperthermophiles with optimum growth temperatures above 85 °C [14].

Oxygen Requirements

Microorganisms can also be categorized depending on their oxygen requirements, or lack thereof. Those that require oxygen for growth are known as strict aerobes, while those that cannot grow in the presence of oxygen are known as strict anaerobes. Some anaerobes can be classified as moderate or micro aerotolerant [16]. Those that grow in the presence and absence of oxygen are facultative. There are no special techniques to cultivate aerobic microbes thus, isolation is relatively straightforward as air is more readily available. Anaerobic microorganisms on the other hand, are markedly more difficult to cultivate and depending on the strictness of oxygen tolerance, require a complex system that is completely free of oxygen. For both aerobes and anaerobes, a mixture of supplements are required for growth and cultivation, however, anaerobic media must include a carbon source, electron acceptor, an electron donor, and often includes trace elements [17]. A reducing agent (such as sodium sulfide) is also commonly incorporated prior to inoculation which thereby reduces oxygen tension within the vessel [16]. When working with anaerobes, a redox indicator (such as resazurin) is often used to detect the presence of oxygen in the media. In a laboratory setting, anaerobic organisms are commonly grown in glass vessels sealed with butyl caps, with oxygen-free gas used to sparge out any oxygen present in the vessel [18]. Other incubation systems for cultivation include the use of the Hungate method, also known as the roll tube method, where an oxygen-free gas flow is utilized to replace atmospheric oxygen inside the tube [19]. Another example of a commonly used system is an anaerobic chamber, which allows anaerobes to be cultivated directly within the chamber thus, never being exposed to oxygen. It is crucial that all methods use careful aseptic technique to minimize exposure to oxygen when cultivating anaerobes thereby allowing for successful growth and isolation of the desired organism. Although growing anaerobes is relatively feasible, the difficulty of maintaining an anaerobic environment makes the process of isolating of pure culture of anaerobes quite slow [16].

Oligotrophy

It is crucial that microorganisms are cultivated with a media composition that will provide the essential growth nutrients the microbes need to survive and proliferate. A carbon source is typically used for an enrichment culture regardless of any other condition(s). The vast majority of microorganisms present in the world are oligotrophic [8] (ie. organisms that can live in environments with low nutrient concentrations), meaning media compositions must be carefully constructed to support their growth. Oligotrophs have a relatively smaller cell size compared to other microbes, so they are often filtered through an appropriately sized membrane filter (such as 0.2 µm) with a solution containing nutrients at very low concentrations (a few mg per liter). Note that low concentrated solutions are required for isolation using this method, as it limits the growth of undesirable heterotrophs while allowing the targeted oligotrophs to thrive. Those samples are diluted to a very low cell density (a few cells per ml) in a culture medium then incubated, often for several weeks or longer. Following incubation, a higher (up to a few magnitudes of the initial) cell density is considered positive meaning pure culture may be obtained. This method is known as the "dilution to extinction approach" [20]. One challenge with this approach is that it often is difficult to solely isolate the microorganism(s) of interest, especially when the numbers for all organisms are relatively equal [20]. Therefore, for this method to be effective the density of the microorganism of interest must be in excess. In terms of equipment, specialized containers must be used for oligotrophs as they are commonly small in cell number [8]. A micro-petri dish, for example, is a useful system for cultivating those organisms as it contains a series of million wells, with efficient oxygen and nutrient transfer capabilities [8].

Commonly used Methods for Microbial Isolation

Since the introduction of the agar plate, methods for microbial isolation have developed for a broader range of microbes to be isolated. As previously mentioned, microbes have vastly different attributes which dictate the methods that can and cannot be used for their isolation. This section includes a non-extensive list of major methods and techniques commonly used for microbial isolation.

Liquid Media

As mentioned above, an important component for microbial isolation is the composition of the growth media used. Before Koch's discovery of solid media for isolation, microbes had been and are still widely cultivated on liquid media. There are a variety of methods that can be used to isolate microbes on liquid media, with the most commonly used techniques involving modifications to media constituents (see: *Selective vs. Non-Selective*) and isolation through a series of controlled dilutions (see: *Serial Dilution Method*).

Selective vs. Non-Selective

Culture media can be categorized as either non-selective/non-discriminatory growth of microorganisms or selective/intentional addition and/or substitution of media constituents allowing for selective growth of target microorganism(s). In non-selective growth media, yeast extract tends to be a major component [16]. Non-selective growth media are commonly used when working with a large community of diverse microbes. Selective culture media are more commonly used for the isolation of pure cultures, and incorporates the use of added constituents, such as organic/inorganic compounds, that allow for the proliferation of a single or small set of target microbes [16]. Enrichment media are also commonly used for the selective enrichment of desired microorganisms [21], which can either stimulate or inhibit growth of particular microbes [20].

Serial Dilution Method

Microorganisms undergo a series of dilutions, transferring small aliquots of culture per transfer to ultimately have a pure culture by the final dilution. Joseph Lister was the first to successfully perform the serial dilution method on a bacterium that spoils milk [9]. Further developments of the serial dilution method are now commonly employed for pure culturing, and include the following: dilution to extinction approach, concentration to extinction approach, toxicity to extinction approach, dilution to stimulation approach [20]. A challenge for the serial dilution method is that population densities for the microbe of interest must be significantly higher than that of the surrounding community as much of the microorganisms are diluted out during this process, including the organism(s) of interest.

Solid Media

Koch's discovery of agar solid media is a milestone in microbial isolation, and has fostered the development of various techniques such as the streak plate method, spread plate method, and pour plate method. These techniques have been described in further detail below.

Streak Plate Method:

The streak plate method, if not the most commonly used method for microbial isolation, involves the use of agar solid media and a sterilized inoculating loop or needle. A small amount of mixed culture is obtained using the inoculated loop/needle, and is carefully streaked across the surface of the agar. A single colony on the agar plate is comprised of millions of microbes and is visible to the naked eye [22]. The quadrant method is a common streaking pattern used, whereby the culture is streaked across the agar by four "quadrants". In the first quadrant, the culture is spread in a rapid movement across one-quarter of the agar plate. The plate is turned 90°, and the flame-sterilized loop is streaked across the agar starting from the halfway point of the first quadrant into an empty second quadrant. This is repeated to get a total of four quadrants on the plate, and will result in single pure culture cells in the fourth quadrant [22].

Pour Plate Method

Diluted mixed culture is added to a semi-liquid agar mixture, then poured onto a plate resulting in a uniformly spread out solid medium composed of mixed culture within and on the surface of the agar. This method incorporates both the serial dilution method (see: *Serial Dilution Method*) and the streak plate method (see: *Streak Plate Method*), where culture is diluted directly in the melted agar, then poured out onto a plate to solidify. Although this is a widely used and successful method for isolating pure cultures, there are disadvantages, namely, the culture of interest must be able to withstand the temperature of the melted agar, which reaches between 42 - 45 °C [23,24]. Another challenge to this method is that pure cultures must be "dug out" from the solidified agar, which may potentially cross-contaminate with other cultures present in the medium.

Spread Plate Method

The spread plate method is similar to the streak plate method, but there is no use of a sterilized inoculating loop or needle. Diluted samples (0.1 – 1 ml) in sterilized tubes are spread over the top of a solidified agar plate resulting in an evenly spread out mixture of colonies on the surface [22]. There are two techniques to performing the spread plate method, which are spread-plating with a turntable and spread-plating with glass beads. With the first method, a sterilized glass rod is used to evenly spread the sample of culture placed on the surface of the agar. The agar is then inverted and incubated for a set amount of time. The second method, also known as the "Copacabana method" [22], involves the use of sterilized glass beads. A drop of diluted culture is placed on the agar plate that contains the glass beads, the lid is closed, and plate is gently shaken to allow the beads to distribute the sample across the agar. The agar is again inverted and incubated for a set amount of time.

Mechanical Techniques

With the ever-growing advancement in science and technology, the development of new methods are emerging. These include the use of mechanical, or physical, systems which play out of the conventional media-incorporated methods. Often times mechanical methods are more ideal for isolation when cultures are limited to plating techniques that will work.

Differential Centrifugation

Differential centrifugation is a common technique used to isolate pure cultures, employing a form of gradient (such as sucrose) to separate mixed cultures [25]. Varying concentrations of gradient will be mixed in with the mixed cultures, then centrifuged at an appropriate speed. Cultures will then be extracted depending on the layer in which they are expected to reside in. This method has proven to be successful, for example, researchers found traditional plating techniques were unsuccessful in isolating *Nocardia* spp. in soil however, differential centrifugation in sucrose was able to isolate the culture [25].

Micromanipulation Techniques

Previously, micro-needles or micro-capillaries were commonly used to isolate cultures, however, there were several disadvantages to using micro-needles/micro-capillaries in the past including the lack of proper magnification to be able to see single cells [2]. A capillary tube with a beveled tip connected to a pressure device allows for the aspiration of culture. Another micromanipulation technique used are optical tweezers, which utilizes infrared laser beams and has been successful in isolating hyperthermophilic bacteria and archaea [26]. The optical tweezer method involves the use of a neodymium laser and a microscope, with movement controlled using a computer [2]. The pure culture is separated from mixed cultures through the direct separation/manipulation using the laser.

Magnetic Separation Techniques

An intriguing technique for pure culture isolation involves the use of magnetism to separate magnetic from non-magnetic components. In some cases, cultures will contain trace amounts of magnetic particles thus modification is not necessary [27]. For example, a freshwater spirillum containing chains of magnetite crystals was successfully isolated from a swamp using a magnetic field [33]. In most other cases, cells must be tagged using a magnetic label to allow for magnetic manipulation; labels can be either magnetite, maghemite, ferrite, or chromium, to list a few [27,29]. In terms of the separator systems themselves, batch or flow-through magnetic separators are most commonly used. Batch magnetic separating systems consist of a removable magnetic plate to perform the separation, whereas flow-through magnetic separators incorporate cells into a liquid suspension which is run through the separator [28].

Unculturable Cultures

As previously mentioned, the progress of isolating new cultures is slow; one reason possibly being that the vast majority of microbes in the world are unculturable within the laboratory setting [21,30,31]. In the laboratory setting, it is often difficult to replicate and maintain the strict conditions/requirements for growth (such as strict nutrient requirement and pH optimum), which is likely the reason for why certain cultures are deemed "unculturable" [32,33].

Isolation must, therefore, be performed within the natural environment of these cultures. For cultures that cannot be successfully grown through cultivation in media broth or plating techniques, other tools are employed for isolation. One method for culturing unculturable microbes is through the use of diffusion chambers, as Kaeberlein et al. did in the isolation of aerobic organo heterotrophs present in intertidal marine sediments [31]. In this study, the researchers designed a diffusion chamber which provided cultures with chemical components aimed to simulate their natural environment [31]. Through this method, Kaerberliein et al. were able to successfully cultivate previously deemed unculturable culture [31]. Another method incorporates the idea of co-culture dependence, where some cultures cannot grow unless they are close to other cultures from the same environment due to the required growth factors provided by one another, such as complementing auxotrophies among others [33]. Microbial symbiosis is very common in nature, but is also a challenge for isolating pure culture [33,34].

Conclusion and Critical Outlook

Microbial isolation has consistently been an important area of microbiology. There has been a long history of microbial isolation, which gives rise to the common methods that are employed in laboratories across the globe as well as new techniques that have developed and proven to be successful [2,25,36]. Despite it being considered a more "traditional" approach, plating methods (such as the streak plate method) are commonly used for the isolation of cultures. Plating methods have shown their potential and are successful at isolating pure culture, however, it is a relatively tedious and time-consuming method [8,16]. There are challenges for each of the methods for example, the issues of non-selectivity in the serial dilution method and the temperature requirements for the pour-plate method (limited to non-heat sensitive organisms). Nevertheless, this has been and is still a reliable and feasible method for microbial isolation and has become a "household name" in the realm of microbiology. New methods (such as the optical tweezer) provides a solution to the problems associated with the traditional methods, with most involving non-invasive contactless separation of cells. A major challenge with these methods, however, is that they are quite costly and require a certain amount of training/knowledge to implement [36]. Despite this, micromanipulation techniques are rapid, efficient, and accurate in the isolation of pure cultures.

New techniques in microbial isolation are still urgently needed as only a very small fraction of all microbial species have been isolated and characterized. There are still many difficulties with unculturability, especially with fastidious cultures. In fact, it has been recognized that the vast majority of microbial species on earth belong to the category of "unculturable" thus, further development and advancements of tools/techniques are essential. The isolation of pure cultures will continue to provide researchers with an incomparable amount of knowledge for novel species regarding their lifestyles, biochemical/biophysical features, and other information surrounding these organisms. Further investigation is required for the isolation, identification, and cultivation of uncultured cultures, which will revolutionize the field of microbiology.

Acknowledgements

This work was supported by research grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) to KM.

References

1. Koch R (1881) Zur Untersuchung von pathogenen Organismen. Mitth. a. d. Kaiserl. Gesundheitsampte 1: 1-48. Cited in Milestones in Microbiology: 1556 to 1940, translated and edited by Thomas D. Brock, ASM Press. 1998, 101.
2. Keloth A, Anderson O, Risbridger D, Paterson L (2018) Single Cell Isolation Using Optical Tweezers. Micromachines 9: 434.
3. Dykhuizen D (2005) Species Numbers in Bacteria. Proc Calif Acad Sci 56: 62-71.
4. Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany, Prokaryotic Nomenclature Up-to-Date [September 2017].

5. Karamanou M, Poulakou-Rebelakou E, Tzetis M, Androutsos G (2010) Anton van Leeuwenhoek (1632-1723): father of micromorphology and discoverer of spermatozoa. Rev Argent Microbiol. 42: 311-314.
6. Locey KJ, Lennon JT (2016) Scaling laws predict global microbial diversity. Proc Natl Acad Sci U. S. A. 113: 5970-5975.
7. Sender R, Fuchs S, Milo R (2016) Revised estimates for the number of human and bacteria cells in the body. PLoS Biol 14: e1002533.
8. Overmann J, Abt B, Sikorski J (2017) Present and future of culturing bacteria. Annu Rev Microbiol 71: 711-730.
9. Microbiology: Past, present, and future (2009) Nature Reviews Microbiology 7, 174.
10. Sandle T (2011) History and development of microbiological culture media.The Journal: Institute of Science and Technology: 10-14.
11. Geeta S (2009) Principles of microbiology. Tata McGraw-Hill Education.
12. Tse C, Ma K (2016) Growth and metabolism of extremophilic microorganisms. In Biotechnology of Extremophiles: Edited by P. H. Rampelotto. Springer: 1-46.
13. Kazemi MJ, Kargar M, Nowroozi J, Sepahi AA, Doosti A, et al. (2017) Optimization and Comparison of Different Procedures of Preparation GELRITE and Starch as Solidifying Agents for Culturing Indigenous Thermoacidophilc Microorganisms and How to Keep them at the Temperature of 70ºc. Biosciences Biotechnology Research Asia 14: 63-68.
14. Lin CC, Casida LE (1984) GELRITE as a Gelling Agent in Media for the Growth of Thermophilic Microorganisms. Appl Environ Microbiol 47: 427-429.
15. Shungu D, Valiant M, Tutlane V, Weinberg E, Weissberger B, et al.(1983) GELRITE as an Agar Substitute in Bacteriological Media. Appl Environ Microbiol 46: 840-845.
16. Lagier JC, Edouard S, Pagnier I, Mediannikov O, Drancourt M, et al. (2015) Current and past strategies for bacterial culture in clinical microbiology. Clin Microbiol Rev 28: 208-236.
17. Plugge CM (2005) Anoxic media design, preparation, and considerations. Methods Enzymol 397: 3-16.
18. Mielenz JR (2009) Biofuels: methods and protocols. Humana Press Inc.
19. Speers AM, Cologgi DL, Reguera G (2009) Anaerobic cell culture. Curr Protoc Microbiol Appendix 4: Appendix 4F.
20. Lee DJ, Show KY, Wang A (2013) Unconventional approaches to isolation and enrichment of functional microbial consortium–a review. Bioresour Technol 136: 697-706.
21. Basu S, Bosen C, Ojha N, Das N, Das J, et al. (2015) Evolution of bacterial and fungal growth media. Bioinformation 11: 182-184.
22. Sanders ER (2012) Aseptic laboratory techniques: Plating methods. J Vis Exp 63: e3064.
23. Buck JD, Cleverdon RC (1960) The spread plate as a method for the enumeration of marine bacteria. Limnol Oceanogr. 5: 78-80.
24. Hoben HJ, Somasegaran P (1982) Comparison of the Pour, Spread, and Drop Plate Methods for Enumeration of Rhizobium spp. in Inoculants Made from Presterilized Peat. Appl Environ Microbiol 44: 1246-1247.
25. Yamamura H, Hayakawa M, Iimura Y (2003) Application of sucrose-gradient centrifugation for selective isolation of Nocardia spp. from soil. J Appl Microbiol 95: 677-685.
26. Johnstone KI (1973) Micromanipulation of Bacteria. The Cultivation of Single Bacteria and Their Spores by the Agar Gel Dissection Technique. Churchill Livingstone, Edinburgh.
27. Šafařík I, Šafaříková M (1999) Use of magnetic techniques for the isolation of cells. J Chromatogr B Biomed Sci Appl 722: 33-53.
28. Blakemore RP, Maratea D, Wolfe RS (1979) Isolation and pure culture of a freshwater magnetic spirillum in chemically defined medium. J Bacteriol 140: 720-729.
29. Olsvik O, Popovic T, Skjerve E, Cudjoe KS, Hornes E, et al. (1994) Magnetic separation techniques in diagnostic microbiology. Clin Microbiol Rev 7: 43-54.
30. Šafařík I, Šafaříková M (2004) Magnetic techniques for the isolation and purification of proteins and peptides. Biomagn Res Technol 2: 7.
31. Kaeberlein T, Lewis K, Epstein SS (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science 296: 1127-1129.
32. Vartoukian SR, Palmer RM, Wade WG (2010) Strategies for culture of 'unculturable'bacteria. FEMS Microbiol Lett 309: 1-7.
33. Stewart EJ (2012) Growing unculturable bacteria. J Bacteriol 194: 4151-4160.
34. Wells J, Varel V (2011) Symbiosis of plants, animals, and microbes. In: W.G Pond, F. W. Bazer, and B.E. Rollin (ed.) Animal Welfare in animal agriculture: husbandry, stewardship and sustainability in animal production. CRC Press New York P: 185-203.
35. Frohlich J, Konig H (2000) New techniques for isolation of single prokaryotic cells. FEMS Microbiol Rev 24: 567-572.
36. Sassera D, Epis S, Pajoro M, Bandi C (2013) Microbial symbiosis and the control of vector-borne pathogens in tsetse flies, human lice, and triatomine bugs. Pathog Glob Health 107: 285