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Emerging bacterial pathogens: the past and beyond

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Abstract

Since the 1950s, medical communities have been facing with emerging and reemerging infectious diseases, and emerging pathogens are now considered to be a major microbiologic public health threat. In this review, we focus on bacterial emerging diseases and explore factors involved in their emergence as well as future challenges. We identified 26 major emerging and reemerging infectious diseases of bacterial origin; most of them originated either from an animal and are considered to be zoonoses or from water sources. Major contributing factors in the emergence of these bacterial infections are: (1) development of new diagnostic tools, such as improvements in culture methods, development of molecular techniques and implementation of mass spectrometry in microbiology; (2) increase in human exposure to bacterial pathogens as a result of sociodemographic and environmental changes; and (3) emergence of more virulent bacterial strains and opportunistic infections, especially affecting immunocompromised populations. A precise definition of their implications in human disease is challenging and requires the comprehensive integration of microbiological, clinical and epidemiologic aspects as well as the use of experimental models. It is now urgent to allocate financial resources to gather international data to provide a better understanding of the clinical relevance of these waterborne and zoonotic emerging diseases.

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Introduction

With the discovery of penicillin by Alexander Fleming in 1928 and scientific progress that followed during the 20th century, it was thought that bacterial diseases would be easily controlled [1]. However, since the 1950s, physicians have been faced with emerging and reemerging infectious diseases (EIDs), which have brought significant public health and financial challenges. As an example, in 2010, specialists struggled with a mysterious clinical picture that associated a severe inflammatory syndrome with vascular events such as venous thromboembolisms or transient ischemic attacks. At least ten cases were described, especially in

patients with either autoimmune diseases or haematologic malignancies [2]. Despite multiple investigations, no microbiologic agent was identified, and no clinical improvement was observed on various antibiotic regimens until a eubacterial 16S RNA PCR followed by genome sequencing revealed the presence of *Neoehrlichia mikurensis*. This strict intracellular bacterium is related to *Ehrlichia* spp., the agent of human ehrlichiosis, and is emerging as a tick-borne zoonotic pathogen. Patients were subsequently switched to doxycycline therapy and rapidly improved.

EIDs represent infections that have recently appeared among humans or that are rapidly spreading among humans in terms of incidence or geographical distribution [3]. Though known to be an issue for millennia, there has lately been an increased interest from the scientific community, and EIDs are now considered to be a major microbiologic public health threat [4].

In this review, after an historical review, we will first explore the factors associated with emergence of bacterial pathogens; next we will address the approaches that may be used to

confirm their pathogenic role in humans; and finally we address future challenges.

History

During the last 40 years, at least 50 emerging infectious agents have been identified [5]; approximately 10% of them are bacterial agents [6]. Similar to *N. mikurensis*, some of these show distinct clinical pictures and require specific diagnostic tools and particular antibiotic treatments.

In Table 1, we present 26 major emerging bacterial pathogens identified during the last 50 years. We decided to include only new genera and species belonging to a previously characterized genus only when it caused a clinical entity distinct from the other species in the genus (i.e. *Chlamydia pneumoniae*). The list is thus far from being complete, and it does not include all newly discovered pathogenic species. For example, before 1984, only eight species of the genus *Rickettsia* were known to be pathogenic in humans, two of the typhus group and six of the spotted fever group. Currently, at least 25 species of the spotted fever group are recognized, most of which are pathogenic to humans or strongly suspected to be [7–10]. In addition, new virulent strains of known species have been discovered such as the enterohemorrhagic *Escherichia coli* (O104:H4), which caused a large outbreak of hemolytic uremic syndrome in 2011 in Germany and was associated with sprout consumption [11,12].

Why Do Pathogens Emerge?

Why do bacterial pathogens keep emerging? Antigenic drift due to random mutations is a common mechanism in the emergence and spread of viral diseases such as severe acute respiratory syndrome and HIV. Unlike viruses, bacteria possess a more stable genome, and thus bacterial divergence after random mutations is less common. Therefore, are we truly confronted with new pathogenic species and strains, or are we simply confronted with the endless biodiversity of the prokaryote world? Retrospectively, it seems that most EIDs are due to bacteria that have long been present in our environment [3] but that humans have only been recently exposed to or that we were unable to detect so far. With that in mind, three main aspects need to be discussed to understand the dynamics of bacterial diseases emergence: (1) development of new diagnostic tools, (2) increase in human exposure to bacterial pathogens and (3) emergence of more virulent bacterial strains and opportunistic infections.

Development of new diagnostic tools

Identification of a bacterium traditionally depends on culture. However, traditional axenic culture media, as invented by Pasteur, are quite limited and do not allow culture of all bacteria. In addition, differentiation between species might not be possible based only on culture properties. Isolation of recent emerging bacteria was achieved through improvement in traditional culture techniques and development of cell cultures, molecular techniques and implementation of mass spectrometry in microbiology.

Culture. Adjunction of specific antibiotics or selective substrates to broad-spectrum media, as well as optimization of culture duration and temperature, or preinoculation filtration and plate centrifugation, have significantly improved the efficiency of traditional culture [13]. Thus, prolongation of the incubation up to 12 weeks (instead of 6 weeks) allowed the recovery from patients' blood of various *Mycobacterium*, especially *M. genavense*, that would otherwise remain undetected [14]. Additional examples of such improvements are the isolation of the enterohemorrhagic *E. coli* using a sorbitol–MacConkey media [15] and the culture of *Campylobacter* spp. or *Helicobacter* spp. using a selective antibiotic-containing media [16,17]. Similarly, specific media have also been developed, such as Kelly media allowing the culture of *Borrelia* spp. [18]. Finally, cell culture played a significant role in the identification of emerging bacteria, as many of the recently discovered species are strict intracellular bacteria. Historically, these bacteria were recovered using animal models or embryonated eggs. Various cell culture models can be used, including mammalian cells, such as HEL cell lines, which enabled the recovery of *Tropheryma whippelii* from a cardiac valve biopsy sample [19], and monocyte cell lines, which were used to isolate *Ehrlichia* spp. [20]. Nevertheless, because bacteria often present host restriction, nonmammalian cell models such as amoebae have been used. Amoebae are extremely useful to discover new microorganisms, either through amoebae co-culture or amoebal enrichment, especially in highly contaminated samples such as water or sputum [21]. Indeed, most amoebae feed on other bacteria, and amoebal co-culture are therefore less subject to contamination. This technique has enabled us to isolate various *Chlamydia*-related bacteria, such as *Parachlamydia acanthamoebae* [22], *Estrella lausannensis* [23,24] and *Criblamydia sequanensis* [25]. Culture with arthropod cell lines is a promising model to help identify arthropod-transmitted zoonotic agents. In addition to allowing the recovery and identification of otherwise uncultivable bacteria, cell culture provides a higher sensitivity than traditional culture. For example, the isolation of *Bartonella quintana* from a skin biopsy sample was only possible by culture with endothelial cell lines [26].

TABLE 1. Major emerging bacterial pathogens of the last 50 years

Year	Bacterial species	Diseases	Comments	Transmission	Antibiotic treatment ^a	References
1973	<i>Campylobacter</i> spp.	Diarrhea		Zoonosis (poultry, cattle, uncooked meat, unpasteurized milk)	Unnecessary in most cases (macrolides, quinolones)	[17,74]
1974	<i>Clostridium difficile</i>	Pseudo-membrane colitis; toxic megacolon	Commonly associated with antibiotic use	Part of normal flora	Vancomycin	[75,76]
1974	<i>Streptococcus bovis</i> group	Endocarditis	Commonly associated with adenocarcinoma of colon and chronic liver diseases	Part of normal flora and/or zoonosis (contaminated food)	β-Lactam	[77–79]
1976	<i>Legionella pneumophila</i>	Lung infection		Amoebae in water	Azithromycin, respiratory quinolones	[80,81]
1976	<i>Capnocytophaga canimorsus</i>	Sepsis	In asplenic patients, hepatic diseases, alcohol abuse	Zoonosis (dogs)	β-Lactam–β-lactamase combinations, cephalosporin, carbapenem	[82]
1981	<i>Staphylococcus aureus</i> toxin	Toxic shock syndrome	Associated with tampon use	Skin and mucous membrane colonization	Vancomycin + clindamycin	[83]
1982	<i>Escherichia coli</i> O157:H7	Hemorrhagic colitis, hemolytic uremic syndrome	Known as 'hamburger disease'	Zoonosis (contaminated food)	Not required	[84]
1982	<i>Borrelia burgdorferi</i>	Lyme disease		Zoonosis (Ticks)	Doxycycline, amoxicillin	[85]
1983	<i>Chlamydia pneumoniae</i>	Lung infection	First isolated in 1965 in context of trachoma vaccine trial in eye	Person to person	Macrolides, doxycycline	[86]
1983	<i>Helicobacter pylori</i>	Gastric ulcers	Associated with higher risk of gastric adenocarcinoma and lymphoma	Person to person	PPI + clarithromycin + amoxicillin/metronidazole	[16]
1986	<i>Rhodococcus equi</i>	Pneumonia in immunosuppressed		Zoonosis (herbivores, especially horses)	Multidrug therapy due to resistance	[87]
1987	<i>Ehrlichia chaffeensis</i>	Human ehrlichiosis		Zoonosis (ticks)	Doxycycline	[32,67]
1990s	Non-diphtheria <i>Corynebacterium</i> spp.	Endocarditis in immunosuppressed, patients with underlying valve disease or prosthetic valve; other invasive infections	Most important: <i>C. amycolatum</i> , initially confounded as <i>C. xerosis</i> , <i>C. striatum</i>	Part of normal flora	β-Lactam + glycopeptides; if resistant, vancomycin	[57,58,87–90]
1990s	Spotted fever group <i>Rickettsia</i> spp.	Spotted fever rickettsiosis	Notably <i>R. africae</i> , <i>R. helveticae</i> , <i>R. slovaca</i> , <i>R. mongolotimonae</i>	Zoonosis (ticks)	Doxycycline	[8,9,91–93]
1990	<i>Anaplasma phagocytophilum</i>	Human granulocytic anaplasmosis	Previously thought to be <i>Ehrlichia</i> spp.	Zoonosis (ticks)	Doxycycline	[94,95]
1991	<i>Tropheryma whippelii</i>	Whipple disease		?	Ceftriaxone followed by trimethoprim–sulfamethoxazole	[30,31]
1992	<i>Vibrio cholerae</i> O139	Diarrhoea		Contaminated water	Not required	[96]
1992	<i>Bartonella henselae</i>	Cat-scratch disease, bacillary angiomatosis	Initially named <i>Rochalimaea</i>	Zoonosis (cats)	Generally not required in immunocompetent patients	[97,98]
1992	<i>Aerococcus</i> spp.	UTI, endocarditis	Mainly <i>A. urinae</i> and <i>A. sanguincola</i> ; especially in elderly or patients predisposing factors such as diabetes, urinary catheters	Part of the normal flora (?), person-to-person transmission	β-Lactam, glycopeptides,	[99,100]
1995	<i>Wolbachia</i> spp.	Associated with onchocerciasis and lymphatic filariasis	Indirectly acts as endosymbionts of filarial nematodes, increasing their pathogenicity	Filarial nematodes	Doxycycline with or without antifilarial treatment	[101–103]
1997	<i>Simkania negevensis</i>	Lung infection		?	Macrolides, doxycycline	[104]
1997	<i>Actinobaculum schaalii</i>	UTI	First considered as a contaminant; especially in elderly or patients with predisposing factors such as diabetes, urinary catheters	?	β-Lactam, glycopeptides,	[105]
1997	<i>Parachlamydia acanthamoebae</i>	Lung infection	Isolated from water of humidifier involved in epidemic of fever in Vermont	Amoebae in water (?)	Macrolides, doxycycline	[106]
2007	<i>Waddlia chondrophila</i>	Miscarriages		?	Macrolides, doxycycline	[107]
2007	<i>Alloscardovia omnicolens</i>	UTI	Especially in elderly or patients with predisposing factors such as diabetes, urinary catheters	?	β-Lactam, cotrimoxazole, glycopeptides, fluoroquinolones	[108]
2010	<i>Neoehrlichia mikurensis</i>	Neoehrlichiosis: systemic inflammatory response; vascular and thromboembolic events	More frequent among immunocompromised patients	Zoonosis (ticks)	Doxycycline	[109–111]

PPI, proton pump inhibitor; UTI, urinary tract infection.

^aSuggested as examples of commonly used antimicrobial therapy. It should be adapted to local guidelines and resistance.

Molecular techniques and metagenomics. PCR has long been used to detect bacteria in various specimens. However, in the 1980s, universal primers targeting the 16S rRNA gene were developed [27–29] and have enabled the identification of various emerging bacteria such as *T. whipplei* [30,31], *Ehrlichia chaffeensis* [32] and, as mentioned, *N. mikurensis* [2]. Such primers amplify most bacteria present in a clinical or environmental sample, and subsequent sequencing of the amplicons allows species determination. In addition, development of next-generation sequencing, based on pyrosequencing (Illumina; 454) or proton sequencing (Ion Torrent) [33] has largely facilitated broad sequencing of PCR products, offering a complete view of the microbiome present. PCR-based and direct metagenomics studies are now widely used to study flora modifications associated with diseases such as inflammatory bowel disease and ecosystem modifications. As an example, a recent study analysed the gut flora of preterm babies with necrotizing enterocolitis and found a strong association with the presence of *Clostridium butyricum* [34].

Broad-range PCR is especially useful to diagnose bacterial infections in otherwise sterile body sites, such as blood, heart valves, joints, central nervous system and pleura, but cannot be used for nonsterile samples such as sputum, feces or vaginal specimens. Order- or family-restricted PCRs such as the recently developed pan-*Chlamydiales* PCR are an alternative to overcome this limitation. This PCR has been used to detect emerging pneumonia-associated pathogens [35] as well as to demonstrate the common presence of *Chlamydiales* in ticks [36].

To summarize, molecular amplification techniques are extremely powerful to identify unknown bacteria. First, they overcome the culture limitations of fastidious organisms. Second, they are highly sensitive, with a detection limit as low as five copies, and are therefore extremely useful to detect bacteria after empirical antibiotic treatments or in cases of latent infections, such as Q fever in cattle, for which PCR has been shown to be extremely useful to identify animals with active shedding [37]. Finally, they enable identification of a better taxonomic affiliation, and many emerging bacteria have been classified or reclassified after analysis of their 16S RNA gene.

Mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) was initially used in clinical chemistry and was then adapted to identify bacteria by using an acidic matrix specifically extracting small basic proteins such as ribosomal proteins. Since 2010, this technique has been used in microbiology laboratories to detect bacteria from clinical samples, with excellent results in terms of specificity and speed compared to culture [38]. This technique allowed the easier identification of emerging urinary pathogens such as *Aerococcus* spp. or *Actinobaculum* spp. [39,40]. MALDI-

TOF may also be applied to strict intracellular bacteria and provides some phenotypic information essential for thorough polyphasic taxonomic affiliation [24].

Increase in human exposure to bacterial pathogens

Our environment represents an indefinite reservoir of prokaryote species, some of which play a potential pathogenic role in humans. Most recent emerging bacterial diseases derive from animals and are therefore considered as zoonoses (Table 1).

Zoonotic agents can be transmitted to humans through direct contact, bites or scratches, arthropod vectors, consumption of contaminated food and contact with carcasses or feces-contaminated environmental sources such as water or soil [41]. An alternate important reservoir for prokaryotes is water sources, notably through amoebae-contaminated water. Finally, many bacteria with a potential pathogenic role are part of the normal flora in humans. During the last century, major sociodemographic and environmental changes have disrupted the dynamic equilibrium that exists among humans, prokaryotes and their environment and has led to an increase in human exposure to some environmental pathogenic species, as well as person-to-person transmission of commensal bacteria.

Sociodemographic changes. Successful emergence of novel infectious agent generally requires their rapid dissemination among human populations. Therefore, the increase in population density has been a significant factor in disease emergence, as illustrated by plague epidemic in the 14th century or the dissemination of scrub typhus, caused by *Orientia tsutsugamushi*, among Allied troops during World War II. Currently the increasing density of the population, especially in hospital settings, and the increasing use of invasive procedures have increased healthcare-associated infections, such as *Clostridium difficile* infections, which now represent a significant public health challenge [42].

In addition, today's populations have not only increased in number but have also increased the speed and rate by which they move across the earth, enabling rapid spatial dissemination of pathogens. This is explained by globalization, which has led to a dramatic increase in international trade and commercial transportations, population migration and a reduction in travelling expenses, which has increased the number of leisure travels to exotic destinations. This is further accompanied by an increase in merchandise and alimentary products' movements, potentially bringing with them tropical diseases. An excellent example is provided by the reemergence of cholera (*Vibrio cholerae* O1) in South America in 1991, which is thought to be linked to the bilge water dumped by an Asian merchant ship off the Peruvian coast, with subsequent infection of over 1.4 million people over 6 years [43]. Thanks to international travel,

cases were then reported in the United States, and in 1992, 75 out of 336 passengers of a plane returning to Los Angeles from Argentina were infected as a result of the presence of *V. cholerae* in the seafood salad served onboard, prepared by a Peruvian caterer [44,45].

Additionally, since the 1950s, leisure activities have increased as a result of an increased interest in self-development enabled by more flexible working hours and higher wages. Outdoor activities such as hiking are now common and put the population at risk of arthropod-transmitted diseases, such as Lyme disease [46], spotted fever [47] or *Chlamydia*-related bacterial infections, as shown by two recent studies [36,48]. Similarly, people possess more pets—not only cats and dogs but also reptiles, exotic fish and guinea pigs—which are reservoirs for a further variety of bacterial pathogens [49].

Modern convenience has led to the dissemination of air-conditioning systems and humidifiers, which both contain stagnant water and produce aerosols. As shown by the *Legionella* outbreak in 1976, these increase the risk of infection by amoebal-resistant microorganisms (*Legionella*, mycobacteria), and such systems may be a reservoir for emerging respiratory pathogens, such as *Parachlamydia acanthamoebae* or *Simkania negevensis* [50,51].

Environmental changes. Over the last 50 years, we have been faced with climate changes which have significantly modified our ecology. For example, today's warmer winters tend to increase the rodent populations in the summer, leading to increased contact with humans [3]. Climate changes probably played an important role in the emergence of *V. cholerae* O139 in Bangladesh in 1992. Marine life, such as algae or copepods, acts as a reservoir for *V. cholerae* spp. They subsist in a dormant form, but then under favorable conditions such as warming, they reactivate and propagate among marine species [52]. In addition, warming also increases algae blooms, with which epidemics of cholera seem associated [53]. Congruently, *V. cholerae* O139 first appeared in coastal zones, and the heavy monsoon that occurred in 1993 might have increased its dissemination [52].

Similarly, modifications of our environment brought by industrialization, such as deforestation and reforestation or the development of dams and agriculture, change ecosystems and their relations with humans. Dams increase arthropod populations; cultivated land attracts animals; and it is well known that the emergence of Lyme disease was associated with the reforestation of some periurban regions [54].

Emergence of more virulent bacterial strains and opportunistic infections

In the last 20 years, medical communities have been faced with the apparition of multidrug-resistant species, such as

methicillin-resistant *Staphylococcus aureus*, multidrug-resistant or extensively resistant tuberculosis, vancomycin-resistant enterococci, extended-spectrum β -lactamase *E. coli* and carbapenemase-encoding Gram negative bacteria. As a result of their rapid dissemination among hospitalized patients and the general population, these may be considered emerging pathogens and require significant attention. Nevertheless, this major public health issue is outside the scope of the present review. In addition, there have been significant concerns about the development of virulent laboratory bacterial strains and bioterrorism, especially after the 2001 anthrax attack. Although these aspects need to be taken into account when discussing emerging bacterial diseases, they remain extremely rare and have been recently reviewed elsewhere [55].

More importantly, atypical syndromes due to commonly inoffensive bacteria have appeared among vulnerable populations, such as the potentially lethal bacillary angiomatosis caused by *Bartonella henselae* or *B. quintana* in HIV patients, which are often paucisymptomatic in the general population. Over the last 30 years, there has been an increase in the number of patients with impaired immune systems. Recent medical advances partly contribute to this phenomenon by enabling higher survival rates of patients with cancer, with chronic diseases such as renal insufficiency and diabetes, or transplant therapies. Additional contributing factors include population ageing and, on the opposite end of the spectrum, a higher rate of preterm babies as well as, the HIV epidemic and common use of immunosuppressive therapy in the management of autoimmune diseases. Cases of invasive infections, such as sepsis or endocarditis, caused by non-diphtheria *Corynebacterium* spp. are additional illustrations [56,57]. These bacteria are normal residents of the skin and mucosa and are therefore often thought of as being a contaminant when found in cultures, thus delaying diagnosis. This can further affect medical management, as many of these microorganisms, such as *Corynebacterium amycolatum*, are multidrug resistant [57,58]. Additionally, such patients may be at risk of severe infections due to environmental bacteria, such as *Capnocytophaga canimorsus*, that has now emerged as a cause of septicaemia in splenectomized or cirrhotic patients bitten by dogs [59].

“New” Does Not Mean Pathogenic

Recent advances in microbiologic diagnosis have enlarged the number of identifiable prokaryotes, making it more difficult to determine the ones that are pathogenic from beneficial or harmless microbes. Congruently, the GenBank database reports an increase in bacterial nucleotides sequences submitted per year of 21% [60], and one can understand why some authors fear an “epidemic of emerging infectious diseases” [61].

TABLE 2. Historical principles established to determine microbial disease causation^a

Koch postulates	Bill of rights for prevalent virus	Elements of immunologic proof of causation	Criteria for causation: a unified concept	Molecular guidelines
Koch, 1891 (1) Microbe occurs in each case presenting disease in clinical setting compatible with pathologic changes and clinical picture observed	Huebner, 1957 (1) Virus as real identity: virus must be cultured in animals or cell cultures and be established as clear, distinct microbe in laboratories (1) Nucleic acid sequence belonging to putative microbe should be detected in most patients with disease. Microbial nucleic acids should be preferentially detected in organs specifically affected by disease and not in unaffected organs	Evans, 1974 (1) Specific antibodies to microbe are normally absent before exposure to microbe or development of disease	Evans, 1976 (1) Prevalence of disease should be significantly higher in patients exposed to agent than in unexposed controls	Fredricks and Relman, 1996
(2) Microbe occurs in no other patient as commensal and nonpathogenic agent	(2) Origin of virus: virus should be isolated from patients with disease	(2) Throughout disease course, specific antibodies to microbe of both IgM and IgG classes appear	(2) Exposure to agent should be identified more commonly in patients with disease than in healthy controls provided that all risk factors are held constant	(2) Fewer or no copy numbers of microbe-associated nucleic acid sequences should be detected in patients without disease
(3) When inoculated to animal in pure culture, microbe can induce same disease	(3) Antibody response: specific antibody response should be observed in patients with disease	(3) Presence of specific antibodies to microbe suggests primary infection and immunity to disease	(3) Incidence of disease should be significantly higher in patients exposed to agent than unexposed controls as evaluated by prospective studies	(3) With clinical improvements of disease (e.g. after adequate treatment), copy number of microbe-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, they should increase
(4) Microbe can be reisolated from experimentally infected animal ^b	(4) Characterization and comparison with known agents: virus should be clearly characterized in term of morphology, host cell range, cytopathic effects and immunologic characteristics and compared to other known viral agents (5) Constant association with specific illness: virus should be constantly isolated from patients with disease (6) Studies with human volunteers: inoculation of virus to healthy human beings, with respect to ethical considerations, should reproduce same disease (7) Epidemiologic studies: prevalence in patients versus controls should be investigated through clinical studies (8) Prevention by specific vaccination: specific vaccination against virus should prevent disease	(4) Absence of specific antibodies to microbe suggests susceptibility to infection and disease development (5) No antibodies to other microbes should be similarly associated with disease unless they act as cofactor in their production	(4) Temporally, disease should occur after exposure to putative agent with expected bell-shaped distribution of incubation periods (5) Biologic gradient from mild to severe of host response should be observed after exposure to agent (6) Measurable host response such as antibodies or cancer cells should commonly appear after exposure to putative agent or should increase in magnitude if those were already present before exposure (7) Same disease should occur with higher incidence in appropriately experimentally exposed animals or humans compared to unexposed controls (8) Elimination or modification of putative agent or its vector should decrease incidence of disease (9) Disease should be decreased or eliminated by specific measures increasing host's response upon exposure to agent such as immunization or drug (10) Whole considerations should make biologic and epidemiologic sense	(4) If sequence was already detectable before disease, sequence copy number correlating with severity of disease makes sequence–disease association more likely (5) Type of microbe corresponding to obtained sequence should be congruent with biologic characteristics of that group of microbes (6) Nucleic acid correlates should be searched at tissue level: efforts should be made to demonstrate specific <i>in situ</i> hybridization of microbial sequence in diseased organs, visible microbes and organs where microorganisms are expected to be present (7) Sequence-based evidence for microbial causation should be reproducible

^aAdapted from historical references [62–65,112].

^bThis principle was not part of the initial Koch postulates but was added later by reviewers.

Historically, the confirmation of the pathogenic role of a microorganism required the fulfillment of four criteria, established by Koch in 1890 [62] (Table 2). However, these postulates are limited when considering opportunistic infections, uncultured organisms, toxin-related pathologies and more

recently microbe-associated neoplasia (human papillomavirus, Epstein-Barr virus, *Helicobacter pylori*) or autoimmune diseases (Reiter syndrome). Some authors have suggested that they have become obsolete and have proposed additional criteria (Table 2) [63–65].

TABLE 3. Recommended considerations to determine causative nature of new bacterial disease

1. Isolation of bacteria from patients with investigated disease
 - a. Culture followed by identification (using molecular tests or MALDI-TOF) or molecular evidence of presence of microorganism
 - b. Clinical picture should be clearly defined with laboratory markers, radiologic examinations or interventional procedures
 - c. Quantitative relation between bacterial load, severity and evolution of disease is additional hint supporting role of agent, but not prerequisite.^a Very low bacterial load should raise question of specificity of test and contamination
 - d. When isolated bacteria is presupposed contaminant present in normal flora, it should nevertheless be considered potential etiologic agent provided that it can be isolated from several samples and/or is present in high bacterial load
2. Direct visualization in involved organs
 - a. Electron microscopy, immunofluorescence or *in situ* hybridization techniques
3. Response to adequate antibiotic treatment
4. Development of specific antibody response
5. Epidemiologic data, such as prevalence of bacteria among patients and healthy persons
 - a. Presence of bacteria in samples taken from healthy persons is acceptable, provided that bacterial load or prevalence are lower compared to patients
6. Results from animal model experimentation

MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

^aLow bacterial loads are commonly observed with *Mycobacterium tuberculosis* and *Chlamydia trachomatis* despite their obvious pathogenic role.

As mentioned, molecular studies have helped in identifying the causative agents of emerging diseases, especially for organisms with fastidious growth requirements, and one can ask whether culture is still required. However, PCR identifies both living and dead bacteria, as well as bacterial fragments. Moreover, as a result of its high sensitivity, it is subject to sample contamination during the processes of sampling, extraction or amplification. This aspect has already been questioned in studies evaluating the association between *Chlamydia pneumoniae* and atherosclerosis [66]. To overcome these limitations, Fredricks and Relman [63] have established some principles to guide the use of molecular studies (Table 2). However, the sole isolation of bacterial nucleic acids from a patient does not prove causation of the disease, and additional criteria are required. Indeed, though identification of *E. chaffeensis* was done by PCR, evidence of its pathogenic role was provided by the visualization of typical morulae within leucocytes as well as serologic evidence [67]. Similarly, excellent antibiotic response strongly supports the role of *N. mikurensis* in the cases described earlier. With that in mind, and with the criteria already proposed (Table 2), we recommend that the elements listed in Table 3 be taken into account to determine the pathogenic role of a recently isolated bacterium. However, as a result of the more complex nature of recent infectious diseases, it is hopeless to think that every criterion can be strictly met. We should not expect absolute comparisons such as 'absent' or 'present' but instead refer to relative differences that make epidemiologic sense. For example, the correlation between serology and direct identification of *C. pneumoniae* through PCR is not good [68] and might be explained by a delay (2–3 weeks) in the appearance of

immunoglobulin (Ig) M [69]; the association between pneumonia and *C. pneumoniae* is nevertheless commonly accepted. Similarly, the very high proportion of the population exhibiting a positive serology to *C. pneumoniae* makes it impossible to use IgG for epidemiologic studies investigating possible long-term complications that may be associated with this new pathogen, such as asthma exacerbation or bronchial hyperactivity, as suspected by recent observations [70–72]. Finally, certain bacteria present some host restriction, and the development of an experimental animal model may not always be possible.

Future Challenges

It will be difficult, even hopeless, to control the emergence of new bacterial diseases. However, efforts can be made to rapidly identify the epicenters of potential epidemics using new technologies such as social networks and media in order to prevent the uncontrolled spread of emerging diseases. The example provided by the Haitian cholera outbreak mapping, in 2010, based on social and media reports, is promising [73]. In addition, resources should be allocated to perform clinical studies of quality to precisely define the clinical relevance of recently discovered bacteria, to develop accurate diagnostic tools and to assess the benefits of antibiotic treatments to prevent inadequate antibiotic use. International collaborations are of the utmost importance because globalization has increased infectious disease dissemination; further, such collaborations will help researchers avoid low-powered studies that lead to inconclusive results. It should be emphasized that microbes are not only associated with infectious diseases but, as outlined above, also with noninfectious diseases such as asthma or cancer; therefore, research on emerging pathogens should not only focus on emerging infections but also more broadly on new pathologies that may be associated with these newly discovered bacterial agents. Finally, efforts should be made to increase the general population's knowledge of emerging diseases and to provide a scientifically validated message of the actual risks to ensure that people adequately seek medical care after exposure and comply with general preventive measures.

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Transparency Declaration

All authors report no conflicts of interest relevant to this article.

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