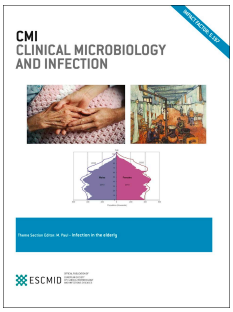
Title: Emerging bacterial pathogens: the past and beyond.
Authors: Vouga M, Greub G
Journal: Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases
Year: 2016 Jan
Volume: 22
Issue: 1
Pages: 12-21
DOI: 10.1016/j.cmi.2015.10.010

Accepted Manuscript



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PII: S1198-743X(15)00909-X

DOI: 10.1016/j.cmi.2015.10.010

Reference: CMI 410

To appear in: *Clinical Microbiology and Infection*

Received Date: 3 September 2015

Revised Date: 29 September 2015

Accepted Date: 5 October 2015

Please cite this article as: Vouga M, Greub G, Emerging bacterial pathogens: past and Beyond…, *Clinical Microbiology and Infection* (2015), doi: 10.1016/j.cmi.2015.10.010.

EMERGING BACTERIAL PATHOGENS: PAST AND BEYOND…

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RUNNING TITLE

Emerging bacterial pathogens

ABSTRACT

Since the 1950's, medical communities have been facing with Emerging and Re-emerging infectious diseases (EIDs) and they are now considered as the main microbiological 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 EIDs of bacterial origin; most of them originated either from an animal and are considered as 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 due to sociodemographic and environmental changes and 3) Emergence of more virulent bacterial strains and opportunistic infections, especially affecting immunocompromised populations. Precise definition of their implication in human diseases is challenging and requires the comprehensive integration of microbiological, clinical and epidemiological aspects, as well as the use of experimental model. 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.

INTRODUCTION

With the discovery of Penicillin by Henri Pasteur in 1928 and the many scientific progress that followed during the 20th century, it was thought that bacterial diseases would be easily controlled [1]. However, since the 1950's, physicians have been facing with Emerging Infectious Diseases (EID) and Re-Emerging Infectious Diseases, 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 suffering from either autoimmune diseases or hematologic malignancies [2]. Despite multiple investigations, no microbiological agent was identified and no clinical improvement was observed on various antibiotics regimen, until an eubacterial 16sRNA 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 switch to doxycycline and rapidly showed significant improvements.

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

In this review, after an historical back-sight, we will first explore the factors associated with emergence of bacterial pathogens, secondly, the approaches that may be used to confirm their pathogenic role in humans and, finally, future challenges.

HISTORY

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

In table 1, we present 26 major emerging bacterial pathogens identified during the last 50 years. We decided to include in this table only new genera and species belonging to a previously characterized genus only when the later caused a clearly distinct clinical entity than the other species in the genus (i.e *Chlamydia pneumoniae*). Thus, the list is far from being complete and does not include all newly discovered pathogenic species. For example, before 1984, only 8 species of the genus *Rickettsia* were known to be pathogenic in human, two of the typhus group and 6 of the spotted fever group (SFG). Currently, at least 25 species of the SFG 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 enterohemorragic *Escherichia coli* (O104:H4) who 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 of viral diseases such as SARS and HIV. Opposite to viruses, bacteria possess a more stable genome and, thus, bacterial divergence following random mutations is less common. Therefore, are we truly confronted to new pathogenic species/strains or are we simply confronted to the indefinite 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, 3) Emergence of more virulent bacterial strains and opportunistic infections.

1. Development of new diagnostic tools

Identification of a bacterium traditionally depends on culture. However, traditional 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 a) Improvement of traditional culture techniques and development of cell culture b) Molecular techniques and c) Implantation 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 pre-inoculation 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. genaveniae*, that would otherwise remain undetected [14]. Additional examples of such improvements are the isolation of the enterohemorrhagic *Escherichia 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 the 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 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: mammalian cells, such as HEL cell lines, which enabled the recovery of *Tropheryma whipplei* from a cardiac valve biopsy [19] and monocytes cell lines, which were used to isolate *Ehrlichia* spp. [20]. Nevertheless, since bacteria often present host restriction, non-mammalian 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 they are, therefore, less subject to contamination. This technique has enabled to isolate various *Chlamydia*-related bacteria, such as *Parachlamydia acanthamoebae* [22], *Estrella lausannensis* [23,24] or *Criblamydia sequanensis* [25]. Culture with arthropods cell lines is a promising model to help identify arthropods-transmitted zoonotic agents. In addition to allow 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 was only possible using culture with endothelial cell lines [26].

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 have been developed [27–29] and have enabled the identification of various emerging bacteria such as *T. whipplei* [30,31], *E. chaffeensis* [32] and, as mentioned, *N. mikurensis* [2]. Such primers amplify most bacteria present in a clinical or environmental sample and the subsequent sequencing of the amplicons allows species determination. In addition, development of Next-Generation Sequencing (NGS), 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 ecosystems modifications. As an example, a recent study analyzed the gut flora of preterm babies with Necrotizing Enterocolitis (NEC) and revealed a strong association with the presence of *Clostridium butyricum* [34].

Broad-range PCR are 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 non-sterile 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 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 5 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 a better taxonomic affiliation and many emerging bacteria were classified or reclassified after analysis of their 16s RNA gene.

Mass spectrometry. MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry) was initially used in clinical chemistry and was then adapted to identify bacteria by using 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 term of specificity and time gain 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 MS may also be applied to strict intracellular bacteria and provides some phenotypic information essential for thorough polyphasic taxonomic affiliation [24].

2. Increase in human exposure to bacterial pathogens

Our environment represents an indefinite reservoir of prokaryote species among which some carry a potential pathogenic role towards humans. First, as shown in table 1, most recent emerging bacterial diseases derive from animals and are therefore considered as zoonoses.

Zoonotic agents can be transmitted to humans either through i) direct contacts ii) bites or scratches iii) arthropods vectors iv) consumption of contaminated food and v) 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 between humans, prokaryotes and their environment and led to an increase in human exposure to some environmental pathogenic species, as well as person-to-person transmission of commensal bacteria.

a. 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 diseases emergence as illustrated by the epidemic of plague in the 14th century or by the dissemination of scrub typhus, caused by *Orentia tsutsugamushi*, among allied troop during World War II. Currently, the densification of the population, especially in hospital settings, and the increasing use of invasive procedures have increased health care-associated infections, such as *Clostridium difficile* infections, which now represent a significant public health challenge [42].

In addition, nowadays, 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 migrations and the reduction in the traveling 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 re-emergence of cholera (*Vibrio cholerae* O1) in South America, in 1991, which is thought to be linked to the bilge water dumping of an Asian merchant ship off the Peruvian coast with subsequent infection of over 1.4 million people over 6 years [43]. Owing to international travels, 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 due to the presence of *V. cholerae* in the seafood salad served onboard, prepared by a Peruvian caterer [44,45].

Additionally, since 1950's leisure activities have increased due to an increased interest in self-development enabled by more flexible working hours and higher wages. Outdoors activities such as hiking are now common and put population at risk of arthropods-transmitted diseases, such as Lyme disease [46], Spotted Fever [47] or *Chlamydia*-related bacteria infections, as shown by two recent Swiss studies [36,48]. Similarly, people possess more pets, not only cats and dogs, but also reptiles, exotic fishes and guinea pigs, which are reservoir of 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 micro-organisms (legionella, mycobacteria) and such systems might be the reservoir of emerging respiratory pathogens, such as *Parachlamydia acanthamoebae* or *Simkania negevensis* [50,51].

b. Environmental changes. Over the last 50 years, we have been facing with some climatic changes, which have significantly modified our ecology. For example, warmer winters,

214 experienced nowadays, tend to increase rodent populations in the summer, increasing their
215 contacts with humans [3]. Climatic changes have probably played a significant role in the
216 emergence of *V. cholerae* O139, in Bangladesh, in 1992. Marine life, such as algae or copepods,
217 acts as a reservoir for *V. cholerae* spp., in which they can subsist in a dormant form; under
218 favorable conditions such as warming, they can reactivate and propagate among marine spp.
219 [52]. In addition, warming also increases algal blooms, with which epidemics of cholera seem
220 associated [53]. Congruently, *V. cholerae* O139 first appeared in coastal zones and the heavy
221 monsoon that occurred in 1993 might have increased its dissemination [52].
222 Similarly, modifications of our environment brought by industrialization,, such as deforestations
223 and reforestations or development of damsand agriculture, change ecosystems and their relations
224 with humans. Dams will increase arthropods populations, cultivated lands attract animals and it
225 is well known that the emergence of Lyme disease was associated with the reforestation of some
226 peri-urban regions [54].
227

3. Emergence of more virulent bacterial strains and opportunistic infections

In the last 20 years, medical communities have been facing with the apparition of multidrug resistant species such as Methicillin-Resistant *S. aureus* (MRSA/MSSA), Multidrug or Extensively Resistant Tuberculosis (MDR-TB/XDR-TB), Vancomycin Resistant *Enterococcus* (VRE) and Extended Beta-lactamase *E.coli* (ESBL). Due to their rapid dissemination among hospitalized patients and the general population, these may be considered as emerging pathogens and require significant attention. Nevertheless, this major public health issue is outside the scope of the present review and due to the complexity of the phenomenon, it will not be discussed here. In addition, there have been significant concerns about the development of virulent laboratory bacterial strains and bioterrorism, especially after the 2001 Anthrax attack. Though, these aspects need to be taken into account when discussing emerging bacterial diseases, they remain extremely rare and were recently reviewed [55].

More importantly, atypical syndromes due to commonly inoffensive bacteria have appeared among vulnerable populations, such as the potential lethal bacillary angiomatosis caused by *B. henselae* or *B. quintana* in HIV patients, which are often asymptomatic in the general population. Over the last 30 years, there has been an increase in the number of patients with impaired immune systems. The recent advances in medicine partly contribute to this phenomenon enabling higher survival rates of patients with cancer, chronic diseases, such as renal insufficiency and diabetes, or transplant therapies. Additional contributing factors comprise population ageing and, on the opposite, a higher rate of preterm babies, the epidemic of HIV, and common usage of immunosuppressive therapy in the management of autoimmune diseases. Cases of invasive infections, such as sepsis or endocarditis, caused by non-diphteria *Corynebacterium* spp. are additional illustrations [56,57] These bacteria are normal resident of

the skin and mucosa and are, therefore, often thought as being a contaminant when found in cultures delaying the diagnosis. This can further have a significant impact on medical management as many of them, such as *C. 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 septicemia in splenectomized or cirrhotic patients bitten by dogs [59].

NEW DOES NOT MEAN PATHOGENIC

The recent advances in microbiological diagnosis have enlarged the number of identifiable prokaryotes enhancing the difficulty to determine the one that are pathogenic from all beneficial or harmless microbes. Congruently, the DATABASE GenBank 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]. Historically, the confirmation of the pathogenic role of a microorganism required the fulfillment of 4 criteria, established by Koch in 1890 [62] (exposed in table 2). However, these postulates are limited when considering opportunistic infections, uncultured organisms, toxin-related pathologies and more recently microbial-associated neoplasia (HPV, EBV, *H. pylori*) or auto-immune diseases (Reiter’s syndrome). Some authors suggest that they have became obsolete and have proposed additional criteria (see table 2) [63–65]. As mentioned, molecular studies have helped identifying causative agents of emerging diseases, especially for organisms with fastidious growth requirements and one can ask whether culture is still required. However, PCR identify both living and dead bacteria, as well as bacterial fragments. Moreover, due to its high sensitivity, it is subject to samples’ contaminations during the process of samplings, 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 have established some principles to guide the use of molecular studies (Table 2) [63]. 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 were brought by the visualization of typical morulae within leucocytes and serological evidence [67]. Similarly, the excellent antibiotic response strongly

supports the role of *N. mikurensis* in cases described earlier. With that in mind and the already proposed criteria (table 2), we recommend the following elements, exposed in table 3, to be taken into account to determine the pathogenic role of a recently isolated bacterium. However, with the more complex nature of recent infectious diseases, it is hopeless to think that every criterion can be strictly met and we should not expect absolute comparisons such as absent or present, but refer to relative differences that make an epidemiological 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 apparition of IgM [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 epidemiological 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 studies [70–72]. Finally, certain bacteria present some host restriction and the development of experimental animal is not possible.

FUTURE CHALLENGES

It will be difficult and even hopeless to control the emergence of new bacterial diseases. However, efforts can be made to rapidly identify epicenter of potential epidemics using notably 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 (i) to perform clinical studies of quality to precisely define the clinical relevance of recently discovered bacteria, (ii) to develop accurate diagnostic tools and (iii) to assess the benefits of antibiotic treatments to prevent inadequate antibiotic usage. International collaborations are of utmost importance since globalization has increased infectious diseases dissemination and to avoid low-powered studies leading to inconclusive results. It should be emphasized that microbes are not only associated with infectious diseases, but, as outlined above, also with non-infectious diseases such as asthma or cancers; therefore research on emerging pathogens should not only focus on emerging infections, but more broadly on new pathologies that may be associated with these newly discovered bacterial agents. Finally, efforts should be made to increase general population knowledge on emerging diseases and to provide a scientific-validated message of the actual risks to ensure adequate medical seeking following exposure and compliance to general preventive measures.

ACKNOWLEDGMENTS

We thank Dre. Carole Kebbi-Beghdadi for her precious comments.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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TABLES

Table 1: Major emerging bacterial pathogens of the last 50 years

Abbreviations: PPI, proton pump inhibitor; UTI, urinary tract infection

[1]Suggested as examples of commonly used antimicrobial therapy. It should be adapted to local recommendations

Table 1 : Major emerging bacterial pathogens of the last 50 years

Year	Bacterial species	Diseases	Comments	Transmission	Antibiotic treatment[1]	References
602						
1973	*Campylobacter* spp.	Diarrhea		**Zoonosis** (Poultry, cattle, uncooked meat, unpasteurized milk)	Unnecessary in most cases (Macrolides; quinolones)	[17,74]
1974	*Clostridium difficile*	Pseudo membrane colitis; toxic megacolon	Commonly associated with antibiotic use	Part of the normal flora	Vancomycin	[75,76]
1974	*Streptococcus bovis* group	Endocarditis	Commonly associated with adenocarcinoma of the colon and chronic liver diseases	Part of the normal flora and/or **Zoonosis** (Contaminated food)	Betalactam	[77,78,79]
1976	*Legionella pneumophila*	Lung infection		Amoebae in water	Azithromycin; respiratory quinolones	[80,81]
1976	*Capnocytophaga canimorsus*	Sepsis	In asplenic patients, hepatic diseases, alcohol abuse	**Zoonosis** (Dogs)	Betalactam-Betalactamase combinations; cephalosporin; carbapenem	[82]
1981	*Staphylococcus aureus* Toxin	Toxic shock syndrome	Associated with tampon use	Skin and mucous membrane colonization	Vancomycin + clindamycin	[83]
1982	*Escherichia coli* O157:H7	Hemorrhagic colitis, Hemolytic uremic syndrome	Known as "Hamburger disease"	**Zoonosis** (Contaminated food)	not required	[84]
1982	*Borrelia burgdoferi*	Lyme disease		**Zoonosis (**Ticks)	Doxycycline; amoxicillin	[85]
1983	*Chlamydia pneumoniae*	Lung infection	First isolated in 1965 in the context of trachoma vaccine trial in the eye	Person to person	Macrolides, doxycycline	[86]
1983	*Helicobacter pylori*	Gastric ulcers	Associated with a higher risk of gastric adenocarcinoma and lymphoma	Person to person	PPI + Clarithromycin + Amoxicillin/Metronidazole	[16]
1986	*Rhodococcus equi*	Pneumonia in immunosuppressed		**Zoonosis** (Herbivores especially horses)	Multidrug therapy due to resistance	[87]
1987	*Ehrlichia chaffeensis*	Human ehrlichiosis		**Zoonosis** (Ticks)	Doxycycline	[32, 67]
1990's	non-diphtheria *Corynebacterium* spp.	Endocarditis in immunosuppressed, patients with underlying valve disease or prosthetic valve; other invasive infections	Most important: *C. amycolatum* initially confounded as *C. xerosis, C. striatum*	Part of the normal flora	Betalactam + glycopeptides; if resistant vancomycin	[57, 58, 87-90]
1990's	Spotted fever group *Rickettsia* spp.	Spotted fever rickettsiosis	Notably *R. africae, R. helveticae, R. slovaca, R. mongolotimonae*	**Zoonosis** (Ticks)	Doxycycline	[8, 9, 91-93]
1990	*Anaplasma phagocytophilum*	Human granulocytic anaplasmosis	Previously thought to be an *Ehrilichia* spp.	**Zoonosis** (Ticks)	Doxycycline	[94, 95]
1991	*Tropheryma whipplei*	Whipple's disease		?	Ceftriaxone followed by trimethoprim-sulfamethoxazole	[30, 31]
1992	*Vibrio cholerae O139*	Diarrhea		Contaminated water	Not required	[96]
1992	*Bartonella henselae*	Cat-scratch disease, bacillary angiomatosis	Initially named *Rochalimaea*	**Zoonosis** (Cats)	Generally not required in immunocompetant	[97, 98]
1992	*Aerococcus* spp.	UTI, endocarditis	Mainly *A. urinae* and *A. sanguinicola* ; especially in elderly or patients predisposing factors such as diabetes, urinary catheters	Part of the normal flora (?), person to person transmission	Betalactam, glycopeptides,	[99, 100]
1995	*Wolbachia* spp.	Associated with onchocerciasis and lymphatic filariasis	Indirectly acts as an endosymbionts of filarial nematodes increasing their pathogenicity	Filarial nematodes	Doxycycline +/- antifilarial treatment	[101-103]
1997	*Simkania negevensis*	Lung infection		?	Macrolides, doxycycline	[104]
1997	*Actinobaculum schaalii*	UTI	First considered as a contaminant ; especially in elderly or patients predisposing factors such as diabetes, urinary catheters	?	Betalactam, glycopeptides,	[105]
1997	*Parachlamydia acanthamoebae*	Lung infection	Isolated from the water of an humidifier involved in an epidemic of fever in Vermont	Amoebae in water (?)	Macrolides; doxycycline	[106]
2007	*Waddlia chondrophila*	Miscarriages		?	Macrolides; doxycycline	[107]
2007	*Alloscardovia omnicolens*	UTI	Especially in elderly or patients predisposing factors such as diabetes, urinary catheters	?	Betalactam, cotrimoxazole, glycopeptides, fluroquinolones	[108]
2010	*Neoehrlichia mikurensis*	Neoehrlichiosis : Systemic inflammatory response; Vascular and thromboembolic events	More frequent among immunocompromised patients	**Zoonosis** (Ticks)	Doxycycline	[109-111]

603 **Table 2: Historical principles established to determine microbial disease causation**

604 This table is adapted from the following historical references [62–65,112]

605

Table 2: Historical principles established to determine microbial disease causation

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

Table 3: Recommended considerations to determine the causative nature of a given new bacterial disease

Table 3 : Recommended considerations to determine the causative nature of a given new bacterial disease

1	**Isolation of the bacteria from patient suffering from the investigated disease** a. Culture followed by identification using molecular tests or MALDI-TOF MS or molecular evidence of the presence of the microorganism b. The clinical picture should be clearly defined with laboratory markers, radiological exams or interventional procedures. c. A quantitative relation between bacterial load, severity and evolution of the disease is an additional hint supporting the role of an agent, but not a prerequisite[1]. Very low bacterial load should raise the question of specificity of the test and contamination. d. When the isolated bacteria is a presupposed contaminant present in normal flora, it should nevertheless be considered as a 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 hybridation techniques.
3	**Response to adequate antibiotic treatment**
4	**Development of specific antibody response**
5	**Epidemiological data, such as prevalence of the bacteria among patients and healthy persons** a. The presence of the 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**

[1]Low bacterial load are commonly observed with *Mycobacterium tuberculosis* and *Chlamydia trachomatis* despite their obvious pathogenic role