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Sequence-Based Identification of Microbial Pathogens: a Reconsideration of Koch's Postulates

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INTRODUCTION

Life has changed since the 1880s when Robert Koch elucidated his guidelines, later to be called Koch's postulates, for determining whether a microorganism is the cause of a disease. The horse-drawn buggy bumping over dirt roads has been replaced by the computer-assisted automobile speeding along paved highways. It would be absurd to expect modern cars to abide by traffic rules and standards designed for horse-drawn carriages. Yet, many continue to hold Koch's postulates as the unchanging standard for determining causation in medicine, despite a revolution in biotechnology and leaps in medical knowledge. Recent findings based on the application of new technologies, especially in the fields of microbiology and infectious disease, demand a renewed dialogue on proof of causation and revised guidelines for defining a causal relationship between a microbe and a disease.

Since the 19th century, advances in knowledge have included the discovery of viruses, chlamydiae, and rickettsiae as new classes of microbes that cannot be propagated in pure culture but that require other cells for reproduction. The spectrum of bacterial, fungal, and protozoan pathogens has been expanded with improved culture techniques and the development of advanced imaging methods. For example, light microscopy has been refined with the use of immunohistochemical or immunofluorescent stains to detect specific molecules in the host or pathogen. Laser scanning confocal microscopy represents a further quantum improvement in z-axis spatial resolution and signal quantification. Transmission and scanning electron microscopy has revealed microorganisms with unprecedented clarity. Serological assays offer an independent, but indirect approach to the clinician for diagnosing disease in individual patients and for studying the epidemiology of microbes in host populations. But the most revolutionary advance in biomedical science since the time of Koch is the discovery of nucleic acids as the source of genetic information and as the basis for precise characterization of an organism. The ability to detect and manipulate these nucleic acid molecules in microorganisms has created a powerful means for identifying previously unknown microbial pathogens and for studying the host-parasite relationship.

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HISTORY

The Dutch botanist van Leeuwenhoek (1632–1722) revealed the diversity and ubiquity of the microbial world through improvements in microscopy. He made miniature lenses with ×200 magnification and 1.5-μm resolution that rival the capability of modern light microscopes (107). Microscopic evidence provided support for the emerging germ theory of disease in the 19th century. However, advances in technology were not met by the requisite advances in theory of disease causation until the late 19th century. The 17th century physiologist William Harvey described (6) the prevailing conditions: "The too familiar vice of the present age is to obtrude as manifest truths, mere fancies, born of conjecture and superficial reasoning, altogether unsupported by the testimony of sense." The proliferation of identifiable microbes led to the proliferation of candidate pathogens and claims that they caused human disease. Amidst this chaos, a scientific challenge arose: how to distinguish between pathogenic microbes and commensal organisms.

Medical research and thought in the 19th century was dominated by anatomic pathologists such as Rudolf Virchow who

looked to the internal structure and function of the body to explain disease. The German anatomist Friedrich Jakob Henle shared this view, but he also believed in the possibility of external factors contributing to disease. Henle speculated that many diseases may be caused by microorganisms, an idea which he advanced in his book *On Miasmata and Contagie* in 1840 (44). Although cognizant of the vast potential of microbes to produce disease, he thought that proof of causation required more than the simple association of microbes with diseased hosts. Henle noted, "one could prove empirically that (the organisms) were really effective only if one could isolate...the contagious organisms from the contagious fluids, and then observe the powers of each separately." Henle's entreaties towards scientific rigor went mostly ignored by the medical community, however, because no practical method existed for separating most microbes by culture. Henle continued his research in anatomy at the University of Gottingen but did not focus on infectious causes of disease (11).

In 1862, Robert Koch enrolled at the University of Gottingen to study medicine. Koch developed a close friendship with Henle; however, the amount of influence that Henle exerted on the subsequent development of Koch's postulates for disease causation is debated (11). It was here that Koch developed skills in scientific research, pathology, and microscopy. Bacteriology was in its infancy. Koch eventually set up a country medical practice, complete with a microbiology research laboratory, and proceeded to elucidate the life cycle of the anthrax bacillus. Koch was a prolific investigator who developed new staining methods to visualize bacteria in histologic specimens and solid-medium culture techniques for growing bacteria in the laboratory. Using this new technology, Koch established bacterial causes for anthrax, tuberculosis, and wound infections (60).

One of Virchow's pupils, Edwin Klebs, was a contemporary of Koch who shifted his focus from the internal causes of disease favored by his mentor to the external causes of disease, including infections. His early research concerned wound infections. In 1877, Klebs proposed two pathways that could be followed in order to investigate the significance of microbes in disease production. Each pathway was considered valid (11): "(i) if organisms that are well characterized and found exclusively in the given disease process are identified, anatomical evidence can be conclusive; (ii) if the form of the organisms provides no certain point of departure, it can be decisive to convey the disease by means of organisms that have been isolated and cultivated outside the body."

It is clear that Klebs had some influence on Koch's thinking about disease causation, since these two scientists attended the same meetings and Koch made reference to the work of Klebs in his own papers. Koch proceeded to discover the cause of tuberculosis through the development of new culture and staining techniques for mycobacteria in 1882. In 1905, Koch was awarded the Nobel prize for his development of tuberculin and his contribution to bacteriology. However, many people remember Robert Koch not so much for his technological advances in the laboratory as for his thoughtful discussion of disease causation by microbes.

The reasoning behind Koch's ideas on disease causation are probably best represented in his paper "Die Aetiologie der Tuberculose" read before the Physiological Society in Berlin on 24 March 1882 (52).

> On the basis of my numerous observations I consider it established that, in all tuberculous affections of man and animals there occur constantly those bacilli which I have designated tubercle bacilli and which are distinguishable from all other microorganisms by characteristic properties. However, from the mere coincidental relation of tuberculous affections and bacilli it may not be concluded that these two phenomena have a causal relation, notwithstanding the not inconsiderable degree of likelihood for this assumption that is derivable from the fact that the bacilli occur by preference where tuberculous processes are incipient or progressing, and that they disappear where the disease comes to a standstill.
>
> To prove that tuberculosis is a parasitic disease, that it is caused by the invasion of bacilli and that it is conditioned primarily by the growth and multiplication of the bacilli, it was necessary to isolate the bacilli from the body: to grow them in pure culture until they were freed from any disease-product of the animal organism which might adhere to them: and, by administering the isolated bacilli to animals, to reproduce the same morbid condition which, as known, is obtained by inoculation with spontaneously developed tuberculous material.

Koch went on to relate his successful isolation of the tubercle bacilli from human patients and animals with tuberculosis, describing the properties of the bacteria on solid culture media and the histologic appearance of the organism. He also presented the details of seven experiments in which he isolated the tubercle bacilli from diseased patients, grew the bacteria in culture for months, and then inoculated culture material into various animals with the production of disease that resembled tuberculosis. Control animals inoculated with serum remained healthy.

KOCH'S POSTULATES

Koch's postulates derived from his work on infectious diseases such as anthrax and tuberculosis. These guidelines were an attempt to establish a standard for evidence of causation in infectious disease. The standard was intended to convince skeptics that microbes can cause disease and to push microbiologists to use more rigorous criteria before claiming a causal relationship for a microbe and a disease. Koch's postulates can be summarized from his presentation before the Tenth International Congress of Medicine in Berlin in 1890 (53, 95).

(i) The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease.
(ii) The parasite occurs in no other disease as a fortuitous and nonpathogenic parasite.
(iii) After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew.

If these three conditions were satisfied, Koch stated (53, 95), "the occurrence of the parasite in the disease can no longer be accidental, but in this case no other relation between it and the disease except that the parasite is the cause of the disease can be considered." Some reviewers have added a fourth postulate consisting of a requirement to reisolate the microbe from the experimentally inoculated host. Although logically consistent with Koch's other conditions, this fourth condition appears not to have been emphasized or required by Koch. The critical elements of Koch's postulates include a specific association of the microbe with the disease state; scientific concordance of microbiological, pathological, and clinical evidence; isolation

of the microbe by culture on lifeless media; and reproduction of disease by inoculation of the cultured organism into a host.

These stringent criteria provide a framework for thinking about the proof of microbial disease causation. For diseases like tuberculosis, these postulates have been quite successful. Koch was able to visualize *Mycobacterium tuberculosis* in diseased tissue from patients, to grow the bacteria in culture, and to reproduce the disease in animals upon inoculation from pure culture. Animals and people without disease were found not to have *M. tuberculosis* in tissues. However, even Koch was aware of the limitations imposed by these postulates. He believed that cholera and leprosy were caused by specific visible microbes, but he could not fulfill all of the postulates for disease causation. Although *Vibrio cholerae* was isolated from patients with cholera in the time of Koch, it was also isolated from healthy subjects, thereby defying the specificity of association demanded by Koch's second postulate (29). Scientists have been no more successful today than a century ago in culturing the etiologic agent of leprosy, *Mycobacterium leprae*. The inability to isolate *M. leprae* in pure culture prevents the fulfillment of Koch's third postulate. Nonetheless, Koch stated (95): "Therefore, we are justified in stating that if only the first two conditions of the rules of proof are fulfilled, i.e., if the regular and exclusive occurrence of the parasite is demonstrated, the causal relationship between parasite and disease is validly established."

The limitations of Koch's postulates, evident in the 1800s, are even more pronounced today. Organisms such as *Plasmodium falciparum* and herpes simplex virus or other viruses cannot be grown alone, i.e., in cell-free culture, and hence cannot fulfill Koch's postulates, yet they are unequivocally pathogenic. Similarly, certain microbes such as human immunodeficiency virus (HIV) exhibit a host range that is restricted to humans; they cannot produce typical disease in other hosts, thereby making impossible or unethical the final fulfillment of the third postulate. Furthermore, how does one meet criteria for causation when a pathogenic microbe is also capable of a carrier state (e.g., *Neisseria meningitidis*), causing disease in one individual and not in another? In contrast to the beliefs of Koch and those of his era, we are well aware today that microbial pathogens often cause subclinical infection. For example, the vast majority of patients exposed to *M. tuberculosis* will simply develop a silent infection accompanied by microscopic forms of pathology, marked by the presence of a positive tuberculin skin test, and will not go on to develop active disease. The presence of tubercle bacilli in healthy subjects or subjects with an unrelated disease would seem to violate Koch's second postulate. What of the microbe that produces distant injury by release of a toxin or injury that occurs via immune mechanisms well after disappearance of the causative agent? What of the microbe that can switch on or off disease-producing genes? What of the bacteria that require coinfection with a bacteriophage or acquisition of extrachromosomal DNA to be able to cause disease (e.g., *Corynebacterium diphtheriae* and enterotoxigenic *Escherichia coli*) or the virus (hepatitis D virus) that relies on a second virus (hepatitis B virus) to provide the necessary structural components for reproduction in human tissue (i.e., polymicrobial causation)? How does one incorporate host factors into the equation of causation, such as immunological status, physiology, and genetic variability (42)? How does one incorporate environmental factors (e.g., the roles of vectors and reservoirs in virulence) into the equation of causation? These features of microbial pathogenesis and of microbial pathogens do not integrate well with the paradigm provided by Koch's postulates.

Considerations of host, environment, microbial adaptation, and the complexities of host-parasite relationships suggest that we change our perspective on microbial causation. Definitions should address the difference between "necessary" and "sufficient"; that is, the presence of a microbial pathogen or its products (at some point in time) may be necessary but not sufficient to produce disease in a given host. As Alfred Evans noted (30), "...failure to fulfill the Henle-Koch postulates does not eliminate a putative microbe from playing a causative role in a disease. It did not at the time of Koch's presentation in 1890 and it certainly does not today. Postulates of causation must change with the technology available to prove them and with our knowledge of the disease."

CAUSATION THEORY

Koch's postulates were formulated in an attempt to create guidelines for proving disease causation by microbes, formalizing how we think about the connection between cause and effect in medicine. Can causation be proven through scientific observation or experimentation? Although mathematical relationships can be proven by deductive logic, biological relationships can only be inferred from empirical observations. This type of evidence frequently adopts a statistical form. As Marley and McMichael remind us, causation is not observable: only the events that suggest a link between cause and effect are observable (68). In his *Treatise of Human Nature* written in 1739 (49), the British philosopher and skeptic David Hume wrote, "We have no other notion of cause and effect, but that of certain objects which have always been conjoined together . . . we cannot penetrate into the reason of the conjunction." From assembled observations are created explanatory theories which are modified as new observations are made. This process is the basis for inductive reasoning (68).

Unfortunately, Koch's postulates have frequently been applied to issues of causation with a mathematical zeal that is not warranted in the biological world. A microbe that fulfills Koch's postulates is most likely the cause of the disease in question. A microbe that fails to fulfill Koch's postulates may still represent the etiologic agent of disease or may be a simple commensal. The power of Koch's postulates comes not from their rigid application but from the spirit of scientific rigor that they foster. The proof of disease causation rests on the concordance of scientific evidence, and Koch's postulates serve as guidelines for collecting this evidence.

REVISIONS OF KOCH'S POSTULATES

The fundamental limitations of Koch's postulates are no more apparent than when applied to obligate parasites. Since viruses propagate by usurping cellular machinery, they cannot be propagated in pure (lifeless or cell free) culture and therefore cannot fulfill Koch's postulates. The dogmatic insistence that viruses fulfill traditional Koch's postulates probably impeded the early understanding of viral pathogenesis, as observed by the virologist Thomas Rivers in his Presidential Address before the Society of American Bacteriologists in 1936. He stated (95), "It is unfortunate that so many workers blindly followed the rules, because Koch himself quickly realized that in certain instances all the conditions could not be met. . . . Thus, in regard to certain diseases, particularly those caused by viruses, the blind adherence to Koch's postulates may act as a hindrance instead of an aid." Recognizing the weight of evidence supporting the notion that viruses cause specific diseases, and the inability of Koch's postulates to incorporate this evidence, Rivers proposed his own postulates to establish a causal relationship between a virus and a disease (95): (i) a

TABLE 1. Hill's epidemiologic criteria for causal association[a]

Causal criterion	Causal association
Strength of association	What is the relative risk?
Consistency of association	Is there agreement among repeated observations in different places, at different times, using different methodology, by different researchers, under different circumstances?
Specificity of association	Is the outcome unique to the exposure?
Temporality	Does exposure precede the outcome variable?
Biological gradient	Is there evidence of a dose-response relationship?
Plausibility	Does the causal relationship make biological sense?
Coherence	Is the causal association compatible with present knowledge of the disease?
Experimentation	Does controlled manipulation of the exposure variable change the outcome?
Analogy	Does the causal relationship conform to a previously described relationship?

[a] Modified from reference 45.

specific virus must be found associated with a disease with a degree of regularity; and (ii) the virus must be shown to occur in the sick individual not as an incidental or accidental finding but as the cause of the disease under investigation.

Rivers' postulates differed from Koch's postulates in that the pathogenic virus did not need to be present in every case of the disease, the possibility of a viral carrier state was recognized, and the requirement for propagation of the pathogenic virus in media or cell culture was abandoned. Rivers proposed several approaches to fulfilling the second postulate, which distinguished between causation and simple association. The pathogenic virus should be present at the proper time in specific lesions. Disease should be produced with some regularity by serial inoculation of infected material (tissue, blood, or exudate) "free from ordinary microbes or rickettsiae" into susceptible hosts, incorporating appropriate controls (95). The production of specific antibodies in response to a viral infection provides further evidence of a pathogenic role, especially in concert with other evidence. Rivers recognized multiple potential pitfalls, including the difficulty of determining the presence of a virus in a tissue sample for animal inoculation and the potential for coinfection with other nonpathogenic viruses or viruses persisting in a carrier state, leading to misinterpretation of animal experiments. Nevertheless, Rivers' challenge to the classical Koch's postulates provided a new way of thinking about disease causation.

Technological advances led to the discovery of hundreds of new viruses over the next two decades. Some of these viruses were found typically to establish chronic or latent infections in humans, challenging the approaches to proof of causality. Heubner in 1957 (47) wrote, "In addition to many opportunities for spurious etiologic associations provided by the simple chance occurrence of numerous ubiquitous and prevalent viruses, some representatives of these agents are demonstrably persistent in the human host for weeks or months . . . and simultaneous multiple viral infections are extremely common." How, then, does one establish a causal link between a virus and a disease? Heubner proposed a list of suggestions based on Koch's and Rivers' postulates which he called a "Bill of Rights for Prevalent Viruses comprising a guarantee against the imputation of guilt by simple association," consisting of nine points (47). Heubner interjected epidemiology into the criteria used to judge disease causation and refined the previous postulates so that they fit viral pathogenesis. Immunologic criteria, such as the development of specific antibodies or the prevention of disease by specific vaccine, were considered to be important corroborating evidence of causation. Recognizing the perils of strict adherence to a set of rules, he presented his suggestions as guidelines, as opposed to formal postulates.

Sir Austin Bradford Hill also discussed epidemiologic criteria for distinguishing between disease causation and association, here in reference to environmental and occupational medicine, in his President's Address before the Royal Society of Medicine in 1965 (45). He proposed that one consider nine factors in evaluating a possible causal relationship (Table 1). As others before him had done, he argued that no set of criteria can provide absolute proof of causation but that guidelines can, and should, be used to weigh evidence.

Serological, or immunological, criteria became more widely applied to investigations of microbial disease causation with the purification of viral antigen and detection of specific antibody. The relationship between Burkitt's tumor-associated herpes-type virus (Epstein-Barr virus [EBV]) and infectious mononucleosis was clarified, and causality was assigned, on the basis of the population-based features of seroreactivity to EBV antigen (43). Accordingly, Alfred Evans proposed his "Elements of Immunological Proof of Causation" that were derived from experience with EBV (29):

(i) antibody to the agent is regularly absent prior to the disease and exposure to the agent;
(ii) antibody to the agent regularly appears during illness and includes both immunoglobulin G and M classes;
(iii) the presence of antibody to the agent predicts immunity to the clinical disease associated with primary infection by the agent;
(iv) the absence of antibody to the agent predicts susceptibility to both infection and the disease produced by the agent;
(v) antibody to no other agent should be similarly associated with the disease unless it is a cofactor in its production.

Another challenge to previous concepts of causation emerged with discovery of slow virus infections of the nervous system. The agents of kuru and Creutzfeld-Jacob disease could not be seen, grown in the laboratory, or monitored by serological response. Not only do these agents fail to fulfill Koch's postulates, but they fail to meet many of the criteria for disease causation by viruses suggested by Rivers and Heubner. In 1974, Johnson and Gibbs proposed three criteria for linking a slow virus with a disease process that incorporated in situ detection of the causative agent and microbiologic-histologic correlations (50): (i) consistency in the transmission of the disease to experimental animals or in the recovery of the virus in cell cultures; (ii) either serial transmission of the clinicopathologic process, using filtered material and serial dilutions to establish replication of the agent, or consistent demonstration of the recoverable agent in the diseased tissue and in appropriate cells within the lesions, by electron microscopy, immunofluorescence, or other methods; (iii) parallel studies of normal tissue or tissues of patients with other diseases to establish that

the agent is not an ubiquitous agent or a contaminant. These criteria have been met for agents such as those producing kuru, Creutzfeld-Jacob disease, subacute sclerosing panencephalitis (measles), and progressive multifocal leukoencephalopathy (JC papovavirus).

The role of microorganisms in carcinogenesis has received increasing attention as chronic or persistent infections and the attendant chronic inflammatory response become better understood. Long-term relationships between pathogen and host pose additional difficulties for proof of causation. Some of these difficulties and issues include the long incubation period between exposure and disease, low frequency of outcome (neoplasia), the confounding role of endogenous viruses and other host factors, and the absence of relevant disease models (29). Despite these obstacles, evidence that certain viruses, for example, play a pivotal role in the production of neoplasms has accumulated. Multiple lines of evidence link EBV with Burkitt's lymphoma and the human papillomavirus with cervical cancer. Both seroepidemiological and virologic criteria have been applied to these and other virus-associated disease phenotypes. The latter criteria include evidence of in situ viral multiplication or gene expression; presence of intact virus, viral genome, or products in affected tissue; demonstration of viral capability for malignant transformation of cells in vitro; and reproduction of tumors in an animal model by using purified virus or viral products (29). Arguments for a causal role of *Helicobacter pylori* in certain forms of gastric cancer have been primarily based on seroepidemiological data (73, 80, 81). The strength of these arguments derives from calculations of relative risk, the consistency of the associations, and temporality (see Hill's criteria in Table 1 and Evans' elements given above). Further assessments of causality will be based on the results of intervention studies.

Koch's postulates have been modified by many investigators working outside the traditional infectious disease paradigm. These investigations have concerned the causative roles of environmental toxins (40), organic dusts (25), human serum cholesterol (9), and autoimmunity (41) in human disease. In addition to gene products and other molecules, the logic that is inherent in Koch's original postulates can be applied to the association between microbial genes and virulence-related microbial phenotypes (32). In this setting, the postulates offered by Falkow (32) emphasize constancy of association and evidence that the gene is necessary, rather than sufficient for pathogenicity (i.e., inactivation of the gene leads to a measurable loss of microbial virulence).

In his review "Causation and Disease" (29), Evans documented the evolution of thought on causal theory in medicine following the enunciation of Robert Koch's postulates. Evans developed a set of criteria for causation based on modern technology, improved understanding of pathogenesis, and an appreciation of the limitations of the original Koch's postulates. This unified concept of causation was intended to apply to acute and chronic diseases with diverse etiologies.

(i) Prevalence of the disease should be significantly higher in those exposed to the putative cause than in control cases not so exposed.

(ii) Exposure to the putative cause should be present more commonly in those with the disease than in controls without the disease when all risk factors are held constant.

(iii) Incidence of the disease should be significantly higher in those exposed to the putative cause than in those not exposed, as shown in prospective studies.

(iv) Temporally, the disease should follow exposure to the putative agent with a distribution of incubation periods on a bell-shaped curve.

(v) A spectrum of host responses should follow exposure to the putative agent along a logical biologic gradient from mild to severe.

(vi) A measurable host response following exposure to the putative cause should regularly appear in those lacking this before exposure or should increase in magnitude if present before exposure.

(vii) Experimental reproduction of the disease should occur in higher incidence in animals or humans appropriately exposed to the putative cause than in those not so exposed; this exposure may be deliberate in volunteers, experimentally induced in the laboratory, or demonstrated in a controlled regulation of natural exposure.

(viii) Elimination or modification of the putative cause or of the vector carrying it should decrease the incidence of the disease (e.g., control of polluted water or smoke).

(ix) Prevention or modification of the host's response on exposure to the putative cause should decrease or eliminate the disease (e.g., immunization or drug).

(x) The whole thing should make biologic and epidemiologic sense.

GENOTYPE-BASED MICROBIAL IDENTIFICATION

The emergence of technology that grants ready access to nucleic acid sequences and the conceptual advances that allow inference of evolutionary relationships from certain sequences have brought about the identification and detection of novel and previously uncharacterized microorganisms. The petri dish and traditional tissue stains have been supplanted by nucleic acid amplification technology (98, 99) and in situ oligonucleotide hybridization (1, 27) for "growing" and "seeing" some microorganisms. The power of these techniques has opened a new window on the diversity of environmental and human-associated microorganisms (35, 37, 87, 88, 111, 112). It has also led to an explosion of amplified sequences purportedly derived from uncultivated or fastidious microbes that are associated with pathology or disease. The quest to find relevance in these sequences demands a reassessment of our analysis of disease causation proof; in this context, Koch's original postulates seem even less applicable. One of the most problematic situations arises from the amplification of phylogenetically informative microbial sequences directly from diseased host tissue (91).

There is a growing trend towards reliance on genotypes for microbial characterization. Genotypes are more specific and are more easily quantified and standardized among different organisms than are the phenotypic markers used traditionally. Various conserved genetic sequences have been evaluated as representations of the entire genome and its evolutionary history. Among these, 16S (and 16S-like) and 18S (and 18S-like) rRNA sequences are found among all cellular life and reliably predict organismal phylogeny (79, 115). For these reasons, they have become the most common amplification targets for broad-range microbial identification. These arguments are also self-fulfilling: as the database of such sequences becomes increasingly robust, their usefulness and attractiveness increase. However, there are other broadly conserved, phylogenetically useful sequences, such as those encoding elongation factor G and proton-translocating ATPases (79).

In the 1980s, universal or broadly conserved 16S rRNA sequences were proposed as targets for rapid sequence determination or gene amplification (7, 16, 61, 114). This concept

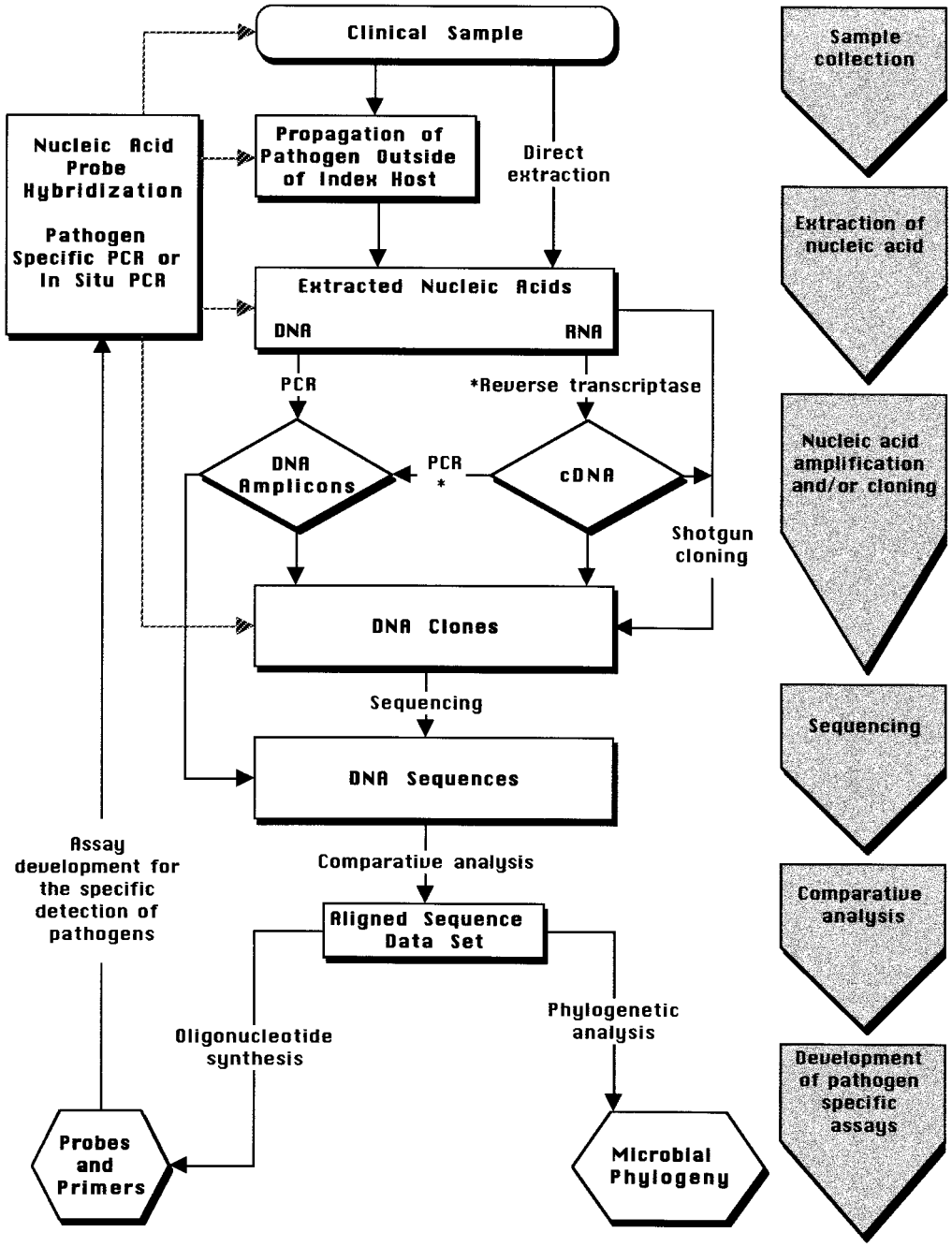

FIG. 1. Flow diagram demonstrating various approaches for the identification and detection of microorganisms in clinical samples using sequence-based technology. The combination of the two steps denoted by an asterisk (*) is known as RT-PCR. Modified from reference 1 with permission of the publisher.

led the way to rapid identification of fastidious or uncultivated bacterial pathogens on a genus-specific level (5, 8) and domain (Bacteria)-wide level directly from infected human tissue (91, 104). With this type of approach, one obtains highly specific information about the evolutionary history of a putative microbial pathogen and hence its taxonomic position relative to those of other known organisms. In addition, one obtains sequence information from which specific primers and probes can be designed (Fig. 1). For example, in 1990 the visible bacilli associated with bacillary angiomatosis (BA) could not be cultivated and remained unidentified. With broad-range 16S rDNA primers, a partial gene sequence was amplified from digested BA tissue (91). Sequence analysis suggested that this bacillus was closely related to *Rochalimaea* (*Bartonella*) *quintana*. With specific primers designed from this sequence, three other independent BA tissues were found to harbor the same organism, subsequently designated *Rochalimaea* (*Bartonella*) *henselae*, and tissues without this disease tested negative. These results were supported by investigators who cultivated both *Bartonella henselae* and *Bartonella quintana* from the tissue or blood of patients with BA or bacteremia and confirmed species identification with DNA sequence (54, 64, 90). Culti-

vation of *B. henselae* subsequently led to the generation of specific antisera and immunologic detection of the organism in diseased tissue (85, 106), although there is still no available animal model with which to induce BA experimentally.

In practice, one must test the ability of any sequence information to discriminate between taxons and clones, especially since the latter may be more pertinent to questions regarding pathogenicity (89). Nonetheless, the power of such an approach is obvious, especially with regard to uncultivated microbial pathogens (87). However, with only amplified sequence available, the biological role or even existence of these inferred microorganisms remains unclear. And the absence of a purified intact microorganism prevents experimental reproduction of disease (Koch's third postulate). Studies of environmental microbial communities illustrate the dimension of such problems. 16S rRNA or rDNA sequences obtained directly from thermal springs, ocean water, and soil reveal a degree of microbial diversity well beyond that which was previously suspected on the basis of laboratory cultivation (35, 62, 102, 111, 112). To date, few if any of these hundreds of sequences correspond to known, cultivated microorganisms. Problems similar to these were alluded to by earlier workers in the field of microbial disease causation and have been described above. However, certain aspects of PCR-amplified, tissue-based microbial identification methods pose added difficulties for proving causation.

The extreme sensitivity of nucleic acid amplification techniques enhances the possibility that one will detect clinically irrelevant "contaminant" or "endogenous" target sequences. This is especially true with clinical samples that contain relatively few pathogenic microorganisms and has always been a problem to a lesser extent with laboratory cultivation. PCR has revealed for the first time the low levels of microbial DNA contamination in most laboratory reagents and may well reveal a previously unsuspected degree of intrinsic microbial contamination in many human anatomic sites. When one considers the ease and accessibility of amplification and DNA sequencing methods and the difficulties in eliminating contamination, it seems likely that the number of new claims for clinically associated microbial sequences will soar. Furthermore, when a sequence is amplified from a digested sample in a tube, the sequence information becomes anatomically disconnected from the disease process, and its relevance becomes more difficult to ascertain. In situ nucleic acid techniques offer the opportunity to establish this connection.

One of the first applications of in situ nucleic acid techniques to the study of an infectious process in intact tissue concerned human papillomavirus infection. Hybridization of radioactive RNA and DNA probes confirmed viral type-specific associations with various forms of cellular pathology (koilocytosis, dysplasia, and frank neoplasia) and allowed subcellular localization of viral genome and mRNA (108, 109). Earlier demonstrations of human papillomavirus viral types in tissue-derived DNA with Southern methods were strengthened by this demonstration of the direct association of specific viral gene expression with cytopathic effects. With the finding of certain human papillomavirus types such as 16 and 18, before the development of cellular dysplasia and with further information about the interaction of purified recombinant human papillomavirus type 16 and 18 gene products E6 and E7 with host cell cycle regulatory proteins, Hill's causal association criteria of temporality and biological plausibility could be addressed (15, 56, 100, 101).

In situ nucleic acid techniques have advanced since the mid-1980s. In situ PCR amplification of target sequence prior to probe hybridization improves the sensitivity of microbial detection. In this fashion, Haase et al. were able to increase the sensitivity of lentiviral DNA detection in cells by 2 orders of magnitude (39); however, the results with the use of this technique by different investigators are variable. Fluorescence labeling and confocal laser microscopy enhance the ease and sensitivity of in situ oligonucleotide probe detection. These techniques have been applied to 16S rRNA-based in situ detection of uncultivated bacteria and can be used to quantitate and follow the relative abundance of any given microorganism within its natural ecologic niche (1, 23, 36). rRNA is present in several orders of magnitude greater copy number per cell than rDNA (i.e., there is intrinsic amplification), and it is relatively stable in comparison with mRNA. Thus, this experimental approach permits a physical association to be made between a specific amplified sequence used to infer the presence of a putative pathogen and a visible microbial structure within host tissue. Preliminary results suggest that *B. henselae* can be detected directly in BA tissue lesions with in situ 16S rRNA oligonucleotide hybridization (Fig. 2) (92).

Representational difference analysis (RDA) is another technique that has been used for the detection and sequence-based characterization of microorganisms that cannot be separated from host cells and tissue (63). RDA relies on subtractive and kinetic enrichment to isolate rare DNA fragments that are present in only one of two otherwise identical populations of DNA (Fig. 3). The DNA population that contains the target DNA fragments of interest is termed the "tester" population. Adaptors that encode PCR primer sequences are ligated to these fragments. Excess "background" DNA ("driver") without adaptors hybridizes to DNA molecules that are common to both populations, producing hybrids that contain only one PCR priming site. These hybrids undergo only linear amplification during PCR. Unique (target) tester DNA fragments contain an adaptor on each DNA strand and therefore undergo exponential amplification during PCR, leading to enrichment. This approach has remarkable power for detecting small differences between complex genomes. Single copies of a viral genome (e.g., adenovirus) can be detected and isolated within a background of human chromosomal DNA (63). The disadvantage of this approach for identifying microbial pathogens is that only small (<1 kbp) random fragments from the microbial genome are obtained; the sequence information contained within these fragments may not be sufficient for the identification of an organism with a relatively complex genome (e.g., a bacterium or fungus). Nevertheless, an investigation of Kaposi's sarcoma (KS) tissues with RDA revealed a novel sequence with significant similarity to known herpesviruses (12) (see below). Many of the issues relevant to proof of causality for uncultivated microbial agents described above also apply to RDA-based findings; however in theory, any sequence identified with RDA has already been selected on the basis of its absence or relative scarcity in normal host tissue.

SEQUENCE-BASED DETECTION OF MICROORGANISMS IN HUMAN DISEASE: EVIDENCE OF CAUSATION?

Many different approaches and arguments have been proposed in the course of sequence-based detection of putative microbial pathogens in an attempt to address causality. Some of these studies are summarized below. In most of these cases, a causal relationship based on Koch's original postulates has not been firmly established, especially for those agents that cannot be cultivated in the laboratory. Instead, various forms

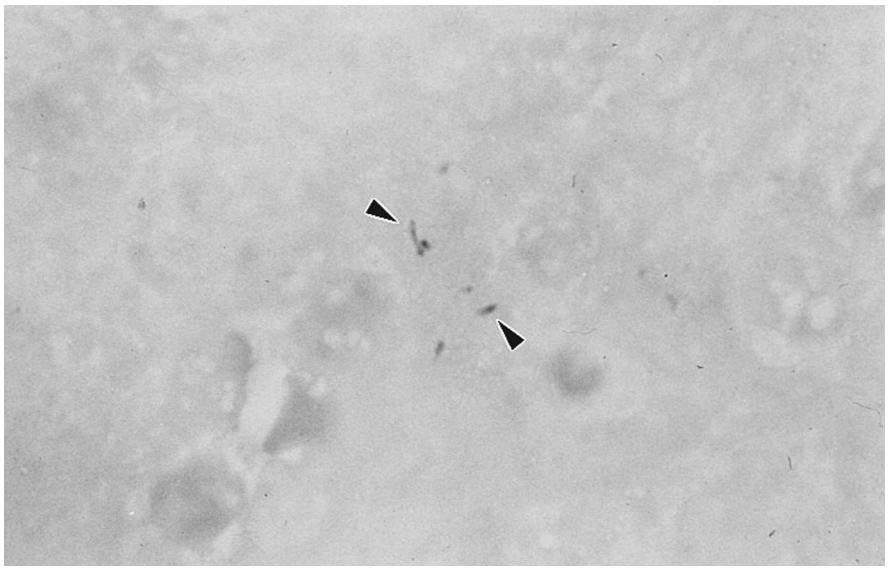

FIG. 2. In situ oligonucleotide detection of a cluster of *B.* (*Rochalimaea*) *henselae* organisms in a tissue lesion from a patient with BA. A specific oligonucleotide probe was designed from the *B. henselae* 16S rDNA sequence and linked to biotin. Bound probe (arrowheads) was detected with a streptavidin-alkaline phosphatase conjugate. Original magnification, ×1,000. Photo courtesy of Donald Regula, Department of Pathology, Stanford University.

of evidence that together offer the groundwork for further consideration of causation proof have been compiled.

Whipple's Disease

Ever since its first description in 1907, two forms of evidence have suggested that the etiology of Whipple's disease is bacterial. First, a monomorphic (but uncultivated) bacillus is consistently seen within areas of pathology in all affected tissues by electron microscopy (14, 24, 105, 116). Second, patients respond favorably to treatment with antibacterial drugs. Disappearance of the bacilli accompanies clinical improvement; reappearance of the bacilli presages clinical relapse (110). Sequence-based identification of the presumed causative bacillus was reported by two groups (93, 113). In one investigation, a partial bacterial 16S rDNA sequence was amplified from a tissue with classical histologic features of this disease, using broad-range bacterial PCR primers (113). From the analysis of serial dilutions of the DNA extracted from this tissue, the investigators estimated that the original biopsy contained about 10^7 organisms, a microbial load too high to represent simple colonization in the upper gastrointestinal tract; however, the specificity of this finding was not tested with negative or other positive tissues. In this situation, the concordance between the observed tissue burden of organisms as determined by microscopy and the measured tissue burden of unique microbial nucleic acid as estimated by PCR was proposed as evidence of a causal association (113). In the second study, a similar but more complete 16S rDNA sequence was associated with five independent cases of disease but not with 10 control patients who had no evidence of Whipple's disease (93). Several independent amplification reactions with different pairs of broad-range bacterial 16S rDNA primers yielded sequences from the same tissue with identical overlapping segments. On the basis of unique phylogenetic relationships inferred from the amplified sequence, and the unusual clinical and morphologic features of the disease and bacillus, respectively, a new genus and species name, "*Tropheryma whippelii*," was proposed for this organism (93) (Fig. 4). Although the number of patients studied was small, the data favored a specificity of association demanded by Koch's first two postulates. Examination of additional Whipple's disease clinical samples confirms the presence of the same or a nearly identical 16S rDNA sequence (66, 70, 72, 86, 94). To strengthen these data, one would like to demonstrate specific hybridization of a *T. whippelii* 16S rDNA probe with visible bacilli in Whipple's disease tissues. It is argued that data on anatomic localization of amplified sequence should be included in proposals for new sequence-based taxons (74). Additional evidence for causality may be provided by quantitative PCR demonstration of a (gene) dose-response (disease) relationship, particularly in the context of disease treatment.

Human Ehrlichiosis

Another successful application of sequence-based technology in determining disease causation was demonstrated by the search for the agent(s) of human ehrlichiosis. In 1986, Maeda et al. reported the case of a 51-year-old man who complained of 5 days of fever, headache, malaise, and myalgia (65). The patient was noted to be confused, with mild hepatitis, acute renal failure, anemia, and thrombocytopenia. Two weeks before, the patient had been bitten by ticks while in Arkansas. A presumptive diagnosis of Rocky Mountain spotted fever was made, and the patient slowly responded to chloramphenicol followed by doxycycline. Review of the blood smear revealed inclusions within circulating leukocytes, predominantly monocytes and lymphocytes. Curiously, all serologic tests were negative except that for *Ehrlichia canis*, a species found in dogs. Electron micrographs confirmed the presence of *Ehrlichia*-like intraleukocytic inclusions (morulae). This report suggested that *E. canis* or a closely related organism might be a human pathogen. Between 1987 and 1993, 299 cases of human ehrlichiosis were reported (31); leukopenia, thrombocytopenia, and elevated liver transaminase levels are common laboratory findings in this illness (34). From a 21-year-old army reservist from Ft. Chaffee, Ark., with a febrile illness, an *Ehrlichia*-like organism was propagated in a canine macrophage cell line (21). This culture material, as well as blood samples from two patients, including the index case, served as a target for bacterial broad-

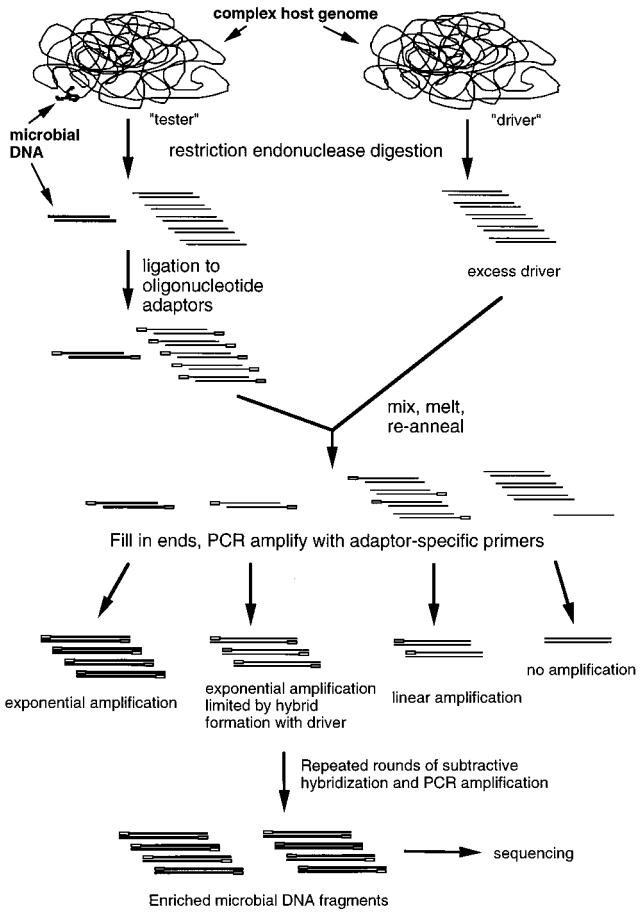

FIG. 3. Simplified diagram of RDA. The tester and driver genomes are first converted to less complex "representations" by restriction endonuclease digestion and an initial round of PCR amplification (not shown). The products tend to be smaller than 1 kb, and although only a small portion of the genome is represented, different endonucleases can be used to sample other parts of the genome. Oligonucleotide adaptors are ligated to the 5′ ends of the tester fragments only. Tester and driver DNA are then mixed, melted, and reannealed in the presence of excess driver DNA. PCR amplification of this mixture with adaptor-specific primers results in preferential amplification of self-reannealed tester DNA because adaptor priming sequences are present on both DNA strands. Driver DNA which is complementary to tester DNA competitively inhibits amplification of sequences common to both populations by preventing the self-reannealing which is necessary for PCR-mediated logarithmic expansion of amplicons. The unique target DNA sequences present only in the tester population are left alone to self-reanneal and amplify exponentially.

range PCR amplification of 16S rDNA. Analysis of the amplified 16S rDNA sequence indicated that this agent of human ehrlichiosis was a previously uncharacterized *Ehrlichia* species, most closely related to *E. canis*; it was designated *E. chaffeensis* (2). A review of human ehrlichiosis cases confirms a high degree of serologic cross-reactivity between *E. canis* and *E. chaffeensis* (21, 34). All of 19 cases of human ehrlichiosis with positive *E. chaffeensis* serology have yielded *E. chaffeensis*-specific sequences with specific 16S rDNA PCR primers (3, 31).

The nature of the clinical illness, the presence of leukocytic inclusions, and serologic cross-reactivity with *E. canis* all provide scientific concordance of evidence to suggest a causal role for *E. chaffeensis* in the production of this febrile human illness. The specificity of association of the nucleic acid sequences with diseased patients is also supportive. Arguments for causality are strengthened when microbial genotype (in this case, molecular phylogenetic information) accurately predicts biological and clinical phenotypes. In this case, the inference of an *Ehrlichia* species from 16S rDNA sequence amplified in the setting of a "rickettsia-like" illness and intraleukocytic inclusions is scientifically concordant; the finding of a sequence that suggested the presence of a novel helminth would be discordant. More recently, a second *Ehrlichia* species that is also pathogenic for humans has been detected in the United States (4, 17). The sequence-based phylogenetic relationships of this agent with *E. equi* and *E. phagocytophila* are also concordant with biological phenotype; all three preferentially infect granulocytes.

Hepatitis C

Choo et al. discovered the virus associated with most cases of non-A, non-B hepatitis by using sequence-based technology, with prior knowledge only as to the morphology of this agent, availability of an animal model of infection, and immune sera (19). Viral particles were concentrated from chimpanzee serum with a high infectious titer, and the nucleic acids were extracted and denatured. A cDNA expression library was screened with the serum of a patient with chronic non-A, non-B hepatitis. Of approximately 10^6 clones screened, 1 reactive clone was discovered, and this cDNA was used to identify other overlapping clones in the library. This cDNA did not hybridize to human or chimpanzee DNA. It did hybridize to RNA extracted from the liver of an infected chimpanzee but not to RNA extracted from the liver of an uninfected chimpanzee. Furthermore, the cDNA hybridized to nucleic acid in the serum of the original chimpanzee, and this hybridization was abolished with RNase but not DNase, suggesting that the infectious agent was an RNA virus (19). From overlapping clones, a viral genome was assembled with features closely related to those of the *Flaviviridae*. Recombinant antigen was expressed in yeasts and used as the basis for a serologic test for this newly discovered hepatitis C virus (HCV). A high percentage of patients with chronic posttransfusion non-A, non-B hepatitis were found to be seropositive for HCV (58). The discovery of HCV is an example of the successful combination and interdependence of sequence-based identification with immunologic reactivity in patients as a screen for "meaningful" sequences. Uncharacterized polyclonal antibodies from a patient with the disease in question were used for the initial identification of a sequence that had been transmitted and replicated in a heterologous host. This screen required that a sequence express an antigen that had been previously recognized by patients with the disease. With this and derivative serologic tests and a specific reverse transcriptase PCR (RT-PCR) assay, a strong association has been established between persistent infection and chronic hepatitis (33, 75). RT-PCR utilizes reverse transcriptase to create cDNA from an RNA template such as that found in RNA viruses like HCV. A form of DNA polymerase is subsequently used for amplification of the DNA template by the PCR. In situ techniques confirm that specific HCV sequences and protein can be found in hepatocytes of patients with disease (55, 76).

Hantavirus Pulmonary Syndrome (HPS)

In May 1993, the Indian Health Service reported multiple cases of an unexplained febrile illness with adult respiratory distress syndrome and death in previously healthy patients living in the Four Corners area of the southwestern United States (10, 13, 26). The first clues to the cause of this illness came from serum samples sent to the Centers for Disease Control and Prevention which were found to contain antibod-

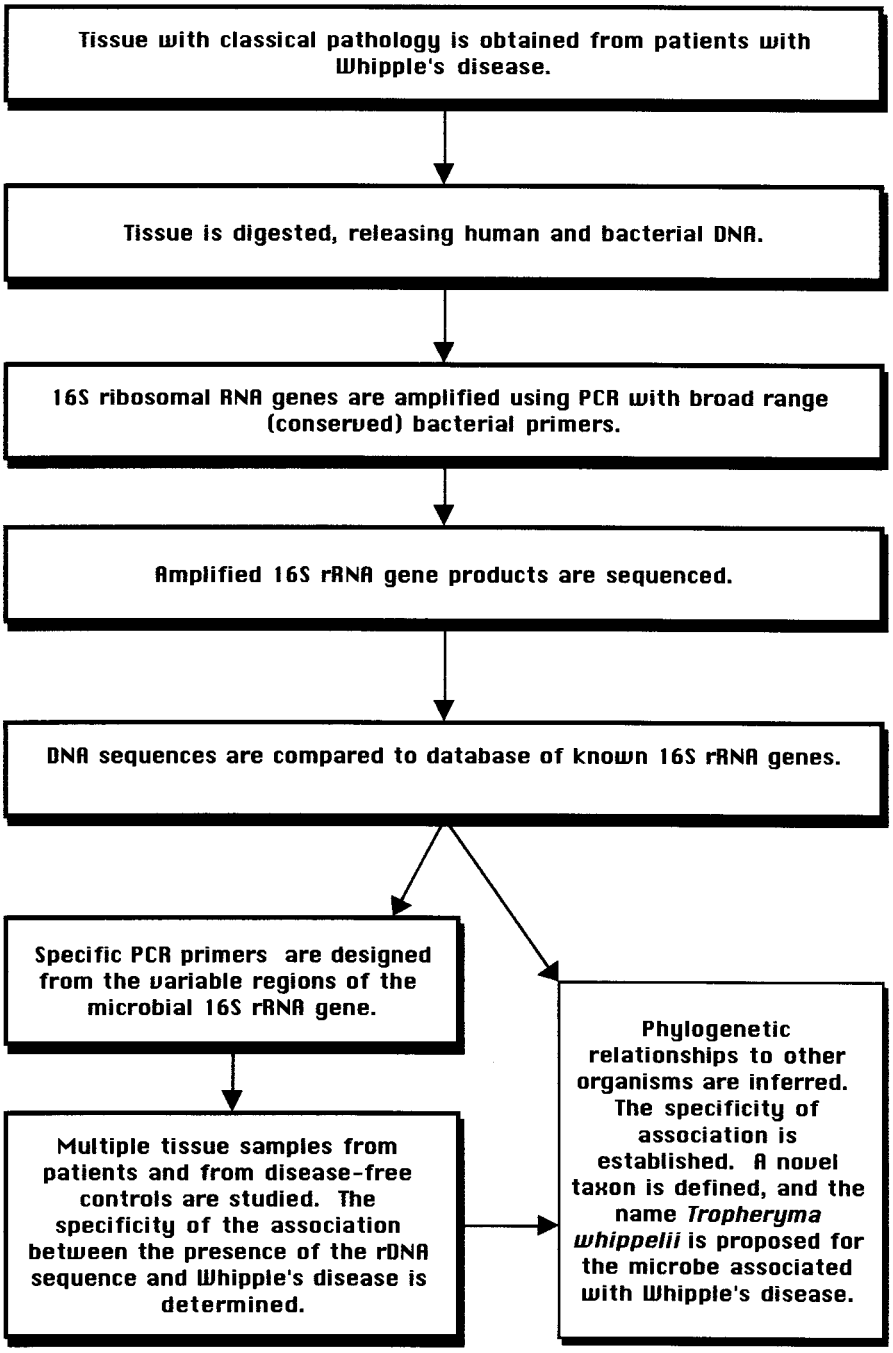

FIG. 4. Flow diagram describing a sequence-based approach taken to identify the uncultivated bacillus associated with Whipple's disease.

ies to several types of hantaviruses (Fig. 5). Surprisingly, hantaviruses were not known to cause pulmonary disease, and no antibodies that recognized known pulmonary pathogens were detected. Immunohistochemical staining of autopsy tissue, using monoclonal antibody directed at conserved hantaviral nucleoprotein segments, confirmed the presence of hantavirus antigen in lung and other types of endothelial cells of these patients (57). This immunological evidence prompted investigators to search for the putative agent in diseased tissues by using a sequence-based approach (77). Hantavirus consensus primers were designed from an area of sequence conservation within the G2 protein-coding region of four diverse, previously characterized hantaviruses. From patient tissues, an RT-PCR assay generated hantaviruslike sequences that were at least 30% different from those previously described. There was significant but less extensive sequence variation among amplicons from different patients. A specific RT-PCR assay confirmed the presence of this virus in 10 of 10 patients studied, with evidence of multiple organ involvement.

Since rodents are the usual hosts of previously characterized hantaviruses, a search for rodent reservoirs of the novel hantavirus was made. Rodent trapping in the Four Corners area

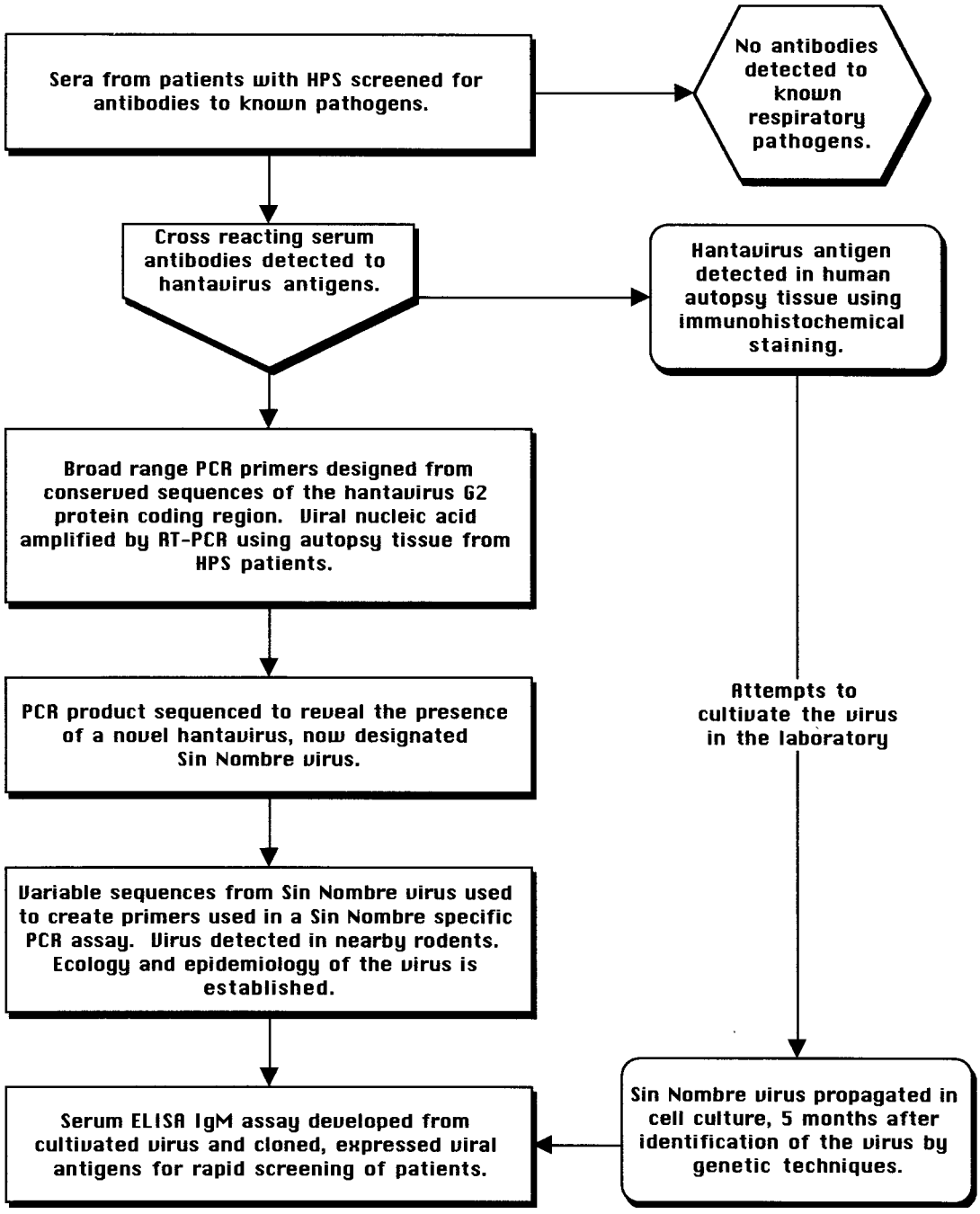

FIG. 5. Flow diagram describing the events leading to the identification of the microbe associated with HPS and the development of specific screening tests. ELISA, enzyme-linked immunosorbent assay; IgM, immunoglobulin M.

revealed that 33% of deer mice examined were seropositive for this group of hantavirus serotypes, and 82% of the seropositive deer mice were also positive for the human pulmonary syndrome-associated hantavirus by PCR. The outbreak of the newly defined HPS was attributed to a 10-fold increase in the deer mouse population in the Four Corners area. Most of the victims had some exposure to rodents, and human illness was associated with rodent exposure by genetic typing of the virus strains in mice and humans. Viruses detected in different geographic regions were distinct. In comparison, sequencing of amplified nucleic acids showed that there was nearly complete identity between virus found in patients and the virus found in mice trapped in or around their homes. It took another 4 months before the hantavirus responsible for HPS, designated Sin Nombre virus (SNV), was finally propagated in culture (28). Initial evidence of disease causation by the SNV included positive patient serologic tests, presence of hantaviral antigen in histologic sections of diseased tissue (57), presence of hantaviral nucleic acid in digested diseased tissue, and epidemiologic agreement with previously described patterns of transmission for this group of viruses (119). Subsequent development of culture methods and specific serology for the SNV

provided data in support of the initial suggestion of causation. Because humans are the only known host in which SNV causes disease, Koch's third postulate has not been fulfilled.

KS

A recent investigation of KS (12) illustrates the ability of RDA (63) to identify rare DNA sequences that are far more abundant in diseased tissue than they are in matched, unaffected tissue. When these sequences share similarity with a known category of infectious agents, as was the case in this study by Chang et al., issues concerning proof of microbial causality are appropriately raised. When these investigators performed RDA (Fig. 3), using tester DNA from KS tissue and driver DNA from uninvolved tissue of the same patient, two amplified products that hybridized specifically with KS tissue DNA were isolated; the sequence of each product predicted expression of polypeptides with significant similarity to herpesviral proteins. Although these predicted polypeptide sequences are similar to proteins encoded by *Herpesvirus saimiri* and EBV, the KS-associated sequences are sufficiently distinct to suggest the existence of a novel member of the herpesvirus family, human herpesvirus 8. A more complete picture of this viral genome seems to confirm these initial impressions.

The specificity of association of the KS-associated herpesviruslike (KSHV) sequence has been addressed by using Southern hybridization methods with labeled DNA amplified by the RDA process and PCR with specific primers designed from one of these original amplified herpesviruslike sequences (12). All 25 KS tissue samples from AIDS patients with amplifiable DNA were found to contain the viral sequence by PCR, while 6 of 39 (15%) lymphoma or normal lymph node samples from AIDS patients were also positive; two of these six patients had concurrent KS at other sites. In contrast, none of 85 tissue samples from non-AIDS patients contained the KSHV sequence. Reproduction of the PCR results in a blinded fashion by an outside laboratory helped to confirm that the association of the viral sequence with KS was not due to laboratory contamination or error. These data support a strong association of amplified sequence with disease. The presence of the KSHV sequence in some AIDS patients without KS is consistent with the biology of most oncogenic viruses and herpesviruses, i.e., a latency period. Despite the suggestive features of the KS-associated sequence, an alternative explanation for these results is that this sequence is a heritable polymorphic DNA marker for KS. This possibility was unlikely, given the selection of original driver and tester DNA from the same person; however, the issue was further addressed by examining the distribution of the viral sequence in various tissues from each of four additional AIDS patients with KS. A gradient of KSHV sequence was discovered, with prevalence greatest in KS lesions themselves, diminishing in adjacent tissue, and lowest in distant tissues such as heart and brain.

More recently, the association of KSHV with KS has been extended by using a KSHV-specific PCR assay to study tissue from HIV-seronegative patients with classic (endemic) KS, as well as KS in AIDS patients and in HIV-seronegative homosexuals (71). KSHV PCR product was found in 10 of 11 tissue samples from patients with AIDS-associated KS, 6 of 6 samples from patients with classic KS, 4 of 4 samples from HIV-negative homosexuals with KS, and only 1 of 21 control samples. Sequencing of the PCR products obtained from the amplification of KS tissues confirmed that the sequences were identical or nearly identical regardless of patient origin. By strengthening the specificity of association between the presence of specific sequence and the presence of disease, these data support the concept that KSHV may play a role in the causation of KS. On the other hand, KSHV PCR product was also found in uninvolved skin from 3 of 14 patients with KS. The presence of KSHV nucleic acid in healthy tissue from KS patients demonstrates the extreme sensitivity of PCR for the detection of microbes and may reflect latent infection with virus, but it also weakens the nucleic acid-disease concordance which forms the basis for proving causality by sequence-based approaches. A report of KSHV sequences in non-KS skin lesions raises further questions as to the specificity of the association (83).

CONFOUNDING ISSUES

Sequence-based approaches to microbial identification and disease causation share some problems with more traditional approaches but also generate some additional problems. Perhaps the most obvious and perplexing issue raised by sequence-based approaches is the absence of a viable or even intact microorganism with which to reproduce disease. Strict adherence to the principle behind Koch's third postulate poses a major difficulty for the evaluation of microorganisms that have not yet been purified or propagated in the laboratory. In the unlikely event that a phylogenetically useful sequence also encodes a virulence factor sufficient for producing disease, expression of this factor from this sequence in a new host might satisfy this principle. In other situations, phylogenetic information from an isolated microbial sequence predicts the laboratory conditions that allow cultivation of the purported pathogen. Although its initial laboratory cultivation was fortuitous, the relationship of *B. henselae* to other bacteria inferred from its 16S rDNA sequence amplified directly from host tissue predicted the similar growth conditions used for propagation of *B. henselae* and *B. quintana* (91, 106).

Some of the other problems with sequence-based approaches have been mentioned earlier. Nucleic acid amplification methods such as PCR are extremely sensitive, capable of detecting as few as one target molecule. Even after rigorous technical precautions are taken to minimize contamination of PCR reactions (59, 97), primers designed from highly conserved microbial sequences are likely to generate amplified DNA product from most clinical samples due to the presence of microbial DNA contaminating either the sample or the PCR reagents, including *Taq* polymerase (48, 84, 103). The importance of multiple reagent-only controls and clinical control samples analyzed in parallel with disease-associated samples cannot be overemphasized. As with traditional culture techniques, nucleic acid amplification technology has the ability to detect microbes that may behave as "true" or frequent pathogens, transient or permanent commensals, or opportunists that take advantage of preexisting pathology or altered host defenses. The difficulty in making these distinctions is made more challenging by the extreme sensitivity of the amplification technology. For instance, with PCR-based assays, *Mycobacterium paratuberculosis* can be detected in the intestines of a high percentage (72%) of Crohn's disease patients compared with 29% of patients with other gastrointestinal diseases (22). Is *M. paratuberculosis* the cause of Crohn's disease or an opportunist that fills a niche created by the underlying disease process or its treatment (18)?

The problem of interpreting the microheterogeneity found in microbial sequences takes on added significance when sequence information derived directly from host tissues becomes the sole basis for defining the existence of an organism or organisms. Some microbial sequences exhibit much greater variation than do others. Extreme examples of genetic diversity include HIV and SNV, each of which gives rise to closely

related but distinct viruses within individual hosts (HIV quasispecies) and/or between different hosts. In the case of amplified rDNA sequences, possible explanations for microheterogeneity include *Taq* polymerase incorporation errors, variation among different gene copies from the same organism, and the presence of multiple strains or species within the clinical sample (87). Current databases contain an insufficient number of entries with which to define species and other taxon boundaries over a wide range of microorganisms with any sequence. And there is no simple method for integration of immunologic, epidemiologic, pathophysiologic, and clinical data with sequence information for the definition of taxon boundaries. Until these problems are remedied, establishing specific disease associations for microbial sequences with consistent microvariability will be a complicated task.

Because they do not require viable or intact microorganisms, sequence-based identification methods bring some advantages to the study of microbial causation. A number of infectious diseases are associated with dormant or latent organisms. Chronic Lyme arthritis (78, 82) and virus-associated cancers (see above) may be examples. Other diseases may be due to inflammatory processes initiated or perpetuated by microbial components such as cell wall, nonprotein complexes, or membrane-bound DNA (82). Detection of microbial nucleic acids from organisms that are dormant or long dead may help elucidate these types of disease processes. In the case of *Borrelia burgdorferi*-infected laboratory mice, microbial DNA can no longer be detected in tissues by PCR assays soon after initiation of clinically effective antibiotic treatment (67). These findings may not be relevant to all classes of microbial pathogens, e.g., mycobacteria. Detection methods will also produce misleading results when persistent but unrelated nucleic acid and organisms coexist with the pathology under investigation. Of course, the failure to find microbial nucleic acids in diseased tissues does not rule out a microbial etiology (96). For instance, a search for microbial nucleic acid in nervous system tissue from a patient with tetanus will likely yield negative results, because the etiologic agent and source of microbial nucleic acid, *Clostridium tetani*, is located at a distant site and causes disease via release of an absorbed exotoxin with widespread systemic effects.

MOLECULAR GUIDELINES FOR ESTABLISHING MICROBIAL DISEASE CAUSATION

In the absence of a cultivated or purified microorganism, can sequence-based methods for the identification of microbial pathogens provide convincing evidence of disease causation? As originally stated, Koch's postulates cannot be fulfilled in this context because disease cannot be reproduced. Furthermore, when used under conditions of maximal sensitivity, nucleic acid amplification methods will detect the few numbers of pathogenic microorganisms that may occur in the absence of pathology, thereby calling into question the specificity of the parasite-disease association that is demanded by Koch's postulates. Over the years, similar concerns and technological advances have motivated others to offer new interpretations and revisions to these postulates (29). Given the degree to which sequence-based microbial identification methods have had an impact on clinical microbiology and the increasing frequency with which questions of causality arise, we offer some thoughts and guidelines for establishment of causal relationships between microbes and diseases.

(i) A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease. Microbial nucleic acids should be found preferentially in those organs or gross anatomic sites known to be diseased (i.e., with anatomic, histologic, chemical, or clinical evidence of pathology) and not in those organs that lack pathology.

(ii) Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease.

(iii) With resolution of disease (for example, with clinically effective treatment), the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, the opposite should occur.

(iv) When sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship.

(v) The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms. When phenotypes (e.g., pathology, microbial morphology, and clinical features) are predicted by sequence-based phylogenetic relationships, the meaningfulness of the sequence is enhanced.

(vi) Tissue-sequence correlates should be sought at the cellular level: efforts should be made to demonstrate specific in situ hybridization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located.

(vii) These sequence-based forms of evidence for microbial causation should be reproducible.

While the principles behind Koch's postulates still hold, it may not be appropriate to make absolute statements regarding forms of proof. For example, guidelines ii and iii refer to relative numbers of sequence copies rather than to their presence or absence. We think that this proposed language reflects more accurately current concepts regarding the natural ecology of disease-causing microorganisms. Furthermore, strict adherence to every one of these guidelines may not be required for a compelling argument regarding causation. At the same time, the evidence surrounding a particular sequence and the microbe from which it derives should be internally consistent and integrate well with previous observations. Coherence and plausibility are important. Guideline v addresses this issue. The evolutionary relationships established by microbial sequence analysis often suggest testable hypotheses that may then lend support to causality. For example, the presence of cross-reacting antibodies to microorganisms that are related to the putative pathogen on the basis of sequence similarity supports the possibility of a causal relationship.

A review of the sequence-based evidence linking the SNV to HPS may illustrate how these guidelines may be used for establishing causal associations (Fig. 5).

(i) A unique hantaviruslike nucleic acid sequence was detected in 10 of 10 initial patients who met the case definition for HPS (77).

(ii) Hantaviral sequences were not detected in control tissue from patients without HPS (77).

(iii) Evaluation of blood samples from patients with HPS revealed that SNV nucleic acid was present in monocytes or whole blood from patients with active disease but was absent from the blood of most patients in recovery (46).

(iv) There are no published data on the detection of SNV nucleic acid in patients experiencing the prodromal phase of HPS or on the quantitation of SNV nucleic acids in various tissues or patients and the relationship of sequence copy number to disease activity.

(v) The hantaviruslike sequence detected in autopsy tissue from patients with HPS fits well with the serological and immunohistochemical evidence of hantavirus infection also present in these patients (13).

(vi) In situ hybridization studies have physically linked the hantaviruslike sequence to visible areas of cellular pathology, such as the endothelium of the lung microvasculature in patients with HPS (117).

(vii) Subsequent studies have confirmed the specific association of SNV nucleic acids in patients with HPS (118). In addition, rodents captured in the living areas of patients with HPS have been found to carry virus identical to that of the patients and are thought to be responsible for transmission of the virus. SNV from a rodent vector was grown in cell culture and sequenced, confirming the nucleic acid sequence data derived from RT-PCR with clinical samples (28).

FUTURE TRENDS

Fifteen years ago, the identification of the uncultivated Whipple's disease bacillus seemed an insurmountable task. Today, we take for granted the intellectual and technological advances that made this task achievable. There is every reason to think that advancements of the same or greater magnitude will occur in the next 15 years and that they will have a significant impact on our ability to identify and prove microbial causation. Work in three areas is most likely to have this impact. First, the combination of differential gene representation and nucleic acid amplification technologies should facilitate isolation of microbial sequences that are more numerous or preferentially expressed in diseased (versus uninvolved) tissues. Second, the ability to isolate and express sequences that encode clinically relevant antigens will lead to serologic tests for uncultivated microbial agents, as well as the generation of specific antisera that can be used for tissue immunochemical or immunofluorescent staining procedures. For example, one might amplify sequences encoding microbial heat shock proteins from infected host tissue, using known conserved sequences (20, 38), and then express these sequences in vitro, with the goal of detecting a specific immune response to these proteins in the same patients. Detection of this response, or detection of microorganisms in situ with antisera raised against unique heat shock protein epitopes, would help to assign a causal role to this presumed pathogen. Third, animal and cell culture models of human disease are likely to evolve in directions of immediate relevance to microbial disease causation. As an example, human tissues can now be maintained in immunodeficient animals (e.g., SCID-hu mice) (69). These animals can then serve as useful models for (human) host-specific microbial pathogens (51). Further refinements in this methodology may create even more useful systems with which to assess the relevance of newly discovered microbial genomic sequences.

CONCLUDING REMARKS

It is our hope that the guidelines we have proposed will provide a framework for further discussion and debate on microbial pathogenesis and disease causation. We do not believe that every criterion for sequence-based evidence of causation needs to be fulfilled in order to incriminate a presumptive microorganism in disease, just as Koch did not believe in his day that every postulate must be fulfilled to prove causation. Nevertheless, the ability to fulfill some or all of the criteria listed above for sequence-based investigations of microbial causation should provide strong evidence of a clinically important host-parasite relationship. The proof that a microorganism causes a disease can only come from the concordance of scientific evidence that supports this contention. Koch's postulates and their revisions still provide a valid standard for judging disease causation, but it is time that our thinking caught up with our intellectual and technological tools. Thomas Rivers observed (95), "At the time when they were formulated Koch's postulates were essential for the progress of knowledge of infectious diseases; but progress having left behind old rules requires new ones which some day without doubt will also be declared obsolete."

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