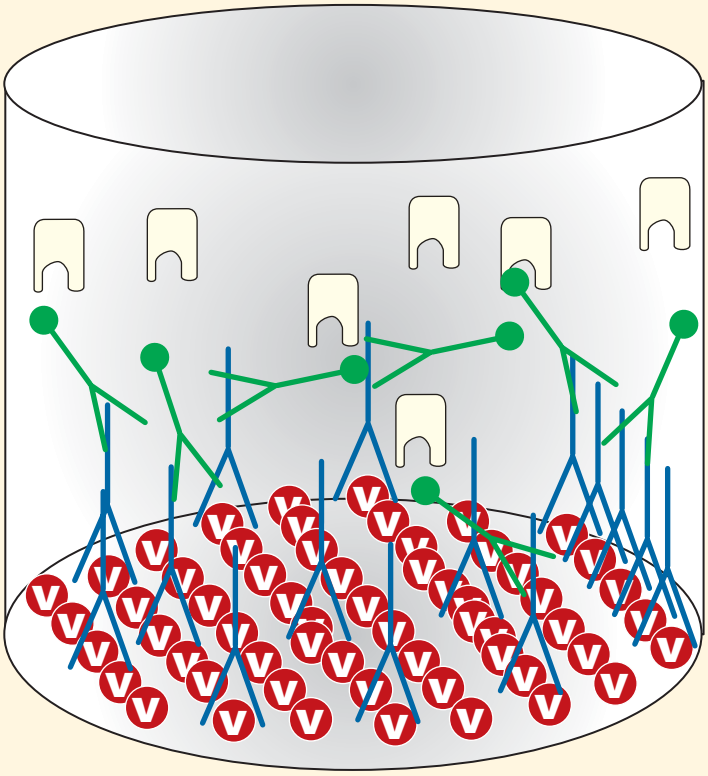
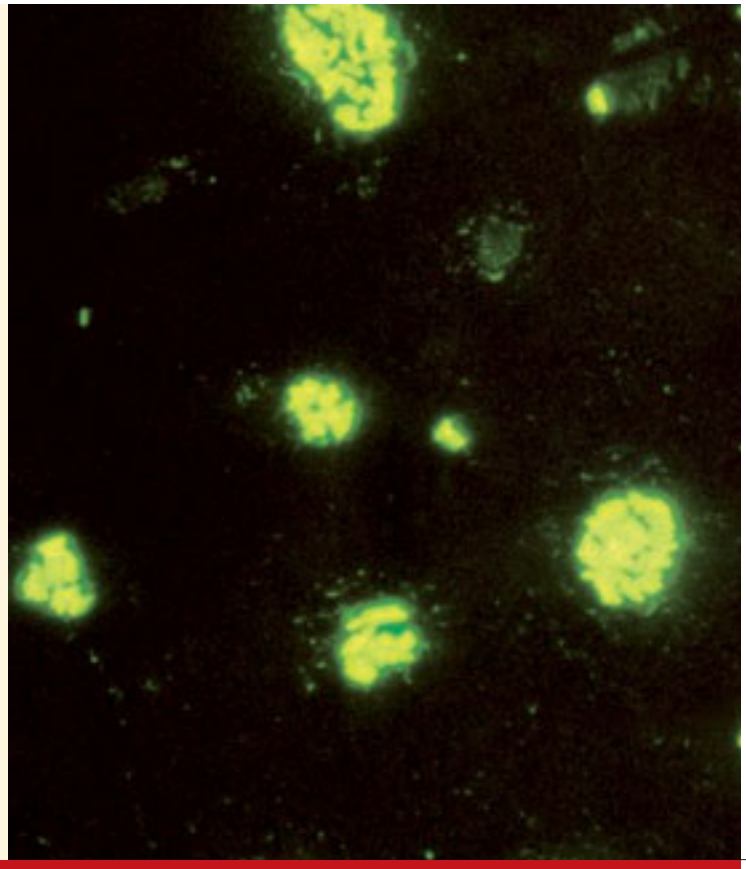
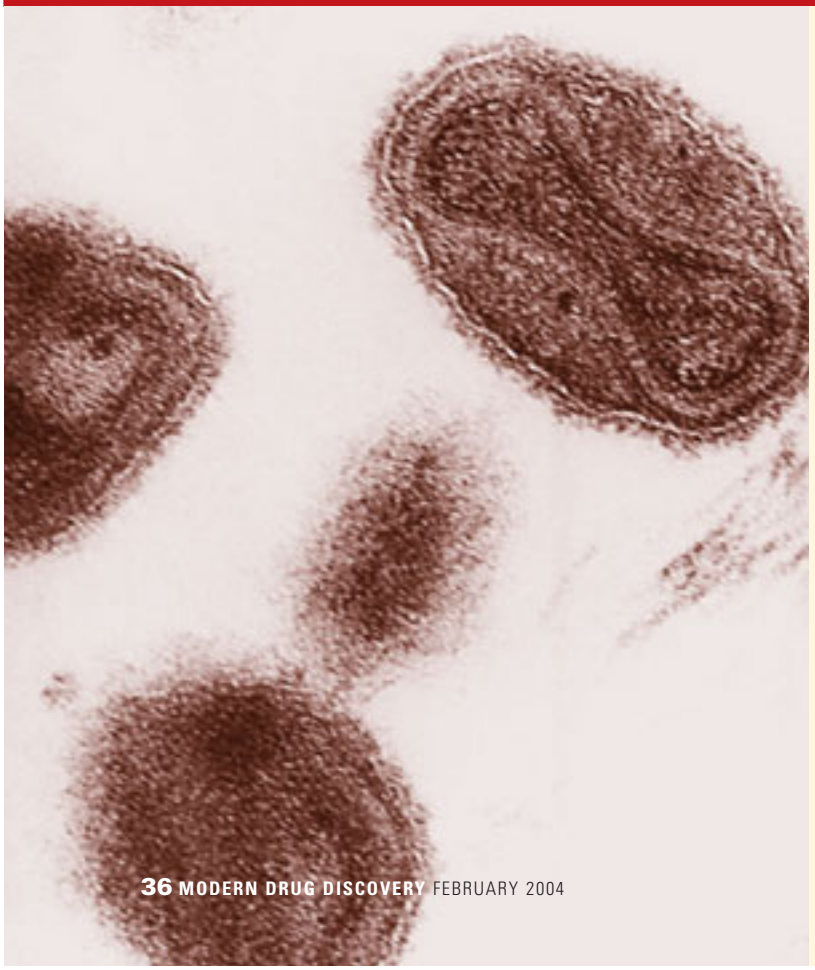
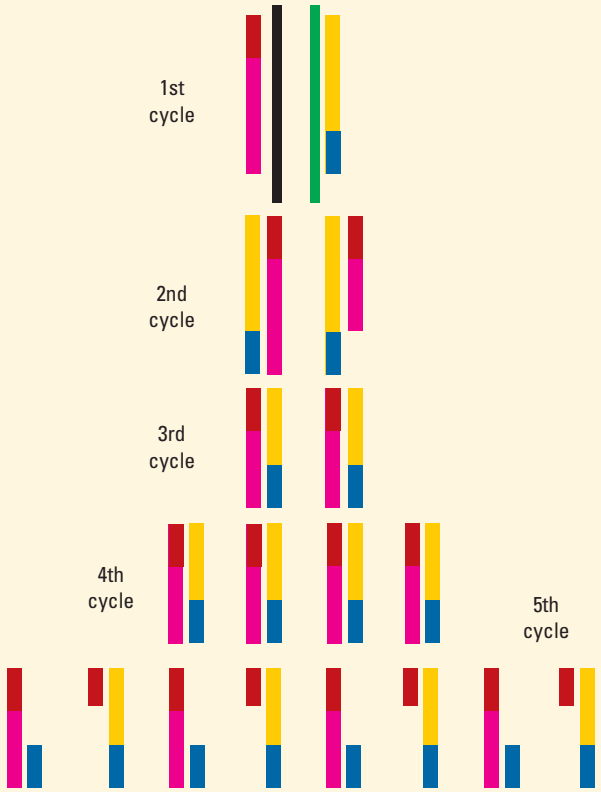




INFECTION





PHOTOS: CENTERS FOR DISEASE CONTROL AND PREVENTION

DETECTION

Improved molecular techniques help researchers diagnose microbial conditions.

BY RANDALL C. WILLIS

If the recent outbreaks of Norwalk-like virus on cruise ships and the perpetual fear of the release of biological weapons on an unsuspecting public have done nothing to calm an anxious populace, they have drawn to the fore concerns over the effectiveness and accuracy of our various methods of detecting microbial infections. Whether caused by contaminated well water, *Salmonella*-laced egg salad, or drug-resistant microbes borne and bred in hospital hallways, infection is a widespread problem, both in the United States and worldwide (see box, "Petting zoo perils").

Although medical science continues to make strides in the treatment of many microbial species, some microbes appear to be quite tenacious. In a recent report, researchers from the Centers for Disease Control and Prevention (CDC, *www.cdc.gov*) followed the infection incidence of several organisms in the U.S. populace over the last few years (Figure 1, *1*). They found that although the numbers are improving for several diseases, the results are less promising for others. Although the researchers cautioned that this data could be the result of various factors (e.g., symptoms, public awareness, and control measures), given these statistics, it is not surprising that corporations and government health officials have put much effort into microbiological testing.

Clinical testing

The two big challenges to large-scale testing of clinical samples are time and sensitivity. Traditional methods require that the microbes be cultured and characterized for a variety of metabolic and physical markers. This process can take days to weeks depending on the organism.

For example, a *Salmonella* detection test includes a bacterial pre-enrichment step that takes 16–20 h, a *Salmonella*-specific enrichment that takes another 24 h, and a final identification step in which cultures are streaked onto selective media, which can take 24–48 h. If the results are positive, they must then be confirmed by subcultivation and serological testing. Thus, this assay can take anywhere from 3 to 6 days, and during this time, the people who are being tested for possible food poisoning can become even sicker or die. It is therefore preferable to have an assay that can locate and identify the offending microbes in hours, not days.

In addition, the assay must be very sensitive, because the tested samples might have no more (and possibly less) than one cell per milliliter or gram of starting material. Although this does not sound like much, infection with *Listeria* can result from as few as 10 cells.

To overcome these problems, researchers have developed a variety of molecular techniques that identify the various sources and forms of microbial infection.

Biological methods

In the fight to detect bacteria, some researchers use the bacteria's own enemies against them—bacteriophages (or phages), viruses that attack bacteria. Like antibodies that target specific antigens, phages target specific bacterial species and sometimes particular strains. Thus, clinicians have used phage-based microbial typing assays for several years to identify microbes in clinical samples.

For example, Francis Drobniewski and colleagues at King's College Hospital (*www.kcl.ac.uk*) and the Laboratório Análises Clínicas (Lisbon) recently developed a modified phage-based assay for detecting mycobacteria from sputum (*2*). In this assay, the phages attack and are internalized by *Mycobacterium tuberculosis* found in sputum samples. The sample is then treated with a virucide to eliminate free phage particles, and the infected cells are combined with a culture of *M. smegmatis* sensor cells. The phages reproduce in the *M. tuberculosis* cells and are released into the medium, where they attack the *M. smegmatis* cells. This secondary infection causes cell lysis, which is detected by a colorimetric dye. According to the researchers, the test allows them to detect as few as 60 *M. tuberculosis* cells/mL of sputum. Furthermore, the assay can be completed in 24–48 h, almost a week faster than the more traditional assay on solid media (*3*). But two days can be a long time while waiting for clinical results.

The AK Phage system, developed by researchers at Alaska Food Diagnostics (*www.alaskafooddiagnostics.com*), uses a series of phages to identify contaminating bacteria in food products. In the presence of the appropriate virus, the bacteria lyse, releasing an enzyme called adenylate kinase (AK). The released enzyme then reacts with a second enzyme (luciferase) that has been added to the buffer. Luciferase is a protein from fireflies, and when activated by AK, it emits light, which is monitored by a detector. Using the AK Phage system, and over a sample preparation time of 8 h, researchers can detect fewer than 100 bacteria in a sample.

But in vivo systems such as this can be difficult to use, requiring precise culturing conditions that can greatly affect the accuracy of their quantitation; and there is always the fear of false-positive or negative results caused by sample contamination with a secondary microbe. For this reason, most research efforts have been applied to in vitro systems.

PHOTOS: CENTERS FOR DISEASE CONTROL AND PREVENTION

Antibodies

Because of their great molecule-binding specificity, antibodies have been frequently used in the development of infection testing, usually through a method called the enzyme-linked immunosorbent assay (ELISA, *4*). In these assays, samples are divided into the wells of a multiwell dish, which carry antibodies directed against specific microbial antigens. After the microbes are allowed to interact with these capture antibodies, the wells are rinsed, and a solution containing a second antibody that also binds to the microbe is added to the wells. This detection antibody is typically tagged with an enzyme that reacts with a substrate in the buffer to cause a color or luminescent change.

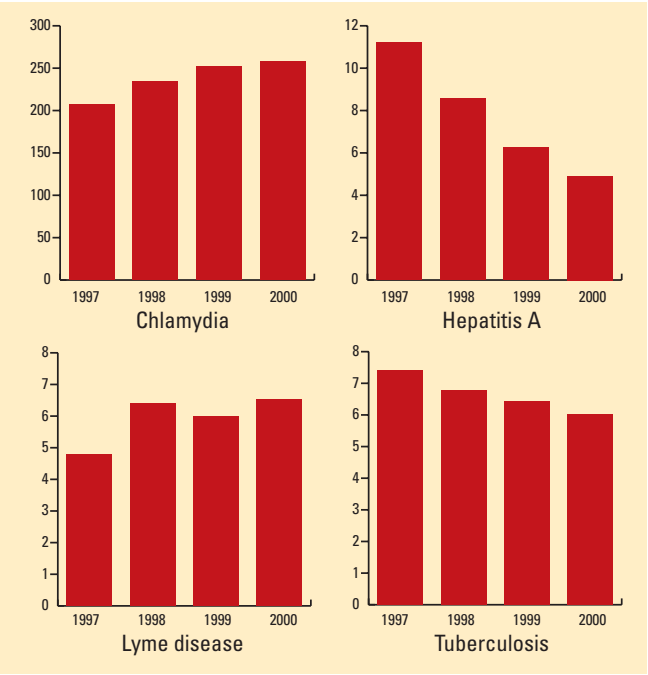


Figure 1. Incidence of disease per 100,000 people in the United States. (Adapted from a U.S. National Center for Health Statistics table, www.cdc.gov/nchs/data/hus/tables/2002/02hus053.pdf.)

IGEN International, a subsidiary of Roche Holdings Ltd., developed the Path*IGEN* system, which is targeted at *Salmonella*, *Listeria*, *Campylobacter*, and *E. coli*. The assay requires a brief (6- to 48-h) incubation of the test sample in culture medium, but in this case, the capture antibodies are conjugated to magnetic beads rather than the multiwell plastic. Upon binding to the beads, the bacteria are bound by a second antibody that carries a ruthenium-based tag (the ORI-TAG). The bead–bacterium–antibody complex is then attracted to an electrode that alters the chemistry of the ORI-TAG such that it reacts with tripropylamine and upon excitation, emits light that is measured by a photodetector. Using the system, researchers can detect as few as 10^3–10^4 cells in 6–48 h.

Similarly, Beckman Coulter (*www.beckmancoulter.com*) distributes a bead-bound antibody system that uses a sandwich assay, in which extracted microbe antigens are bound by alkaline phosphatase-conjugated secondary antibodies. After the beads are washed to remove unbound antigen and antibodies, they are exposed to phosphorylated dioxetane. When the alkaline phosphatase

Table 1

Some companies and suppliers involved in infection diagnostics

Company	Website	Assay
Abbott Diagnostics	www.abbottdiagnostics.com	Immunoassay, LCR
Bayer Diagnostics	www.bayerdiag.com	TMA, hybridization
BD Diagnostic Systems	www.bd.com/ds/	Immunoassay
Beckman Coulter	www.beckmancoulter.com	Immunoassay
BioMerieux	http://biomerieux-usa.com	Immunoassay, NASBA
BioStar	www.biostar.com.	Immunoassay
Cepheid	www.smartcycler.com	PCR
Dade Behring	www.dadebehring.com	Immunoassay
Gen-Probe	www.gen-probe.com	TMA
Idaho Technology	www.idahotech.com	PCR
IGEN International	www.igen.com	Immunoassay
Motorola	www.motorola.com	Hybridization
Quest Diagnostics	www.questdiagnostics.com	TMA
Roche Diagnostics	www.roche-diagnostics.com	PCR
ZymeTx	www.zymetx.com	Metabolism

For a more complete listing of companies and diagnostic tests, visit the Infectious Diseases Test Directory of the Association for Molecular Pathology at www.amptestdirectory.org/IDTestDirectory.htm.

cleaves the phosphate group, the dioxetane produces light. In 1999, researchers at the University of Alabama at Birmingham (*www.uab.edu*) described their use of the ACCESS *Chlamydia* immunoassay in analyzing samples from 356 women. They found that the bead-based method was only slightly less sensitive than another method they tested, which was based on the more technically challenging nucleic acid amplification (*5*).

One of the inherent challenges of using an immunoassay, however, is that it does not necessarily indicate the particular strain of the organism, a fact that can be critically important to patient treatment and prognosis. To overcome this challenge, other researchers have focused their efforts on more strain-specific molecules found in each cell: the genome and its resulting RNA transcripts.

Petting zoo perils

Although most foodborne illness stories involve food products after they have left the farm, events at a Pennsylvania dairy farm suggest that it is good to be wary even when the meat is still on the bone.

In September 2000, Montgomery County (PA) health officials noticed a sharp increase in the number of reported cases of *E. coli* O157:H7 infections (*10*). Most of the patients, ranging in age from 1 to 52 years, had recently visited a popular petting farm in the country that also functioned as a dairy farm. Comparison of the infected patients to other farm visitors showed that the victims were more likely to have had direct contact with calves and their environment (e.g., manure-contaminated surfaces) at the farm.

When the cattle were tested for the presence of bacteria, the researchers found that 15% of the herd was colonized and that heifers and calves had a higher incidence of colonization than the older cattle. Pulse-field gel electrophoresis of the bacterial DNA showed that the *E. coli* from 85% of the colonized cattle was the same isolate as that found in the visitors who had complained of being ill.

"Evidence is growing that contact with farm animals and their environment is a substantial contributor to the risk of *E. coli* O157:H7 infection," write the health officials. "This outbreak underscores the need to consider [animal-based] transmission during searches for the source of *E. coli* O157:H7 and other enteric infection and that simple measures such as effective hand washing can make contact with farm animals and their environment safer."

Genomic weaponry

With the advent of genomic technologies, researchers developed new DNA-based tools for identifying microbial contamination and infection (*6*). Most of these tools rely on the various forms of technologies used to selectively amplify a specific fragment of a gene or RNA molecule, including PCR, ligase chain reaction (LCR), transcription-mediated amplification (TMA), and nucleic-acid sequence-based amplification (NASBA). Whereas in PCR, short DNA probes bind to a sample of microbial DNA, which are extended by DNA polymerase and amplified through several rounds of replication, in LCR, the probes themselves are amplified.

In TMA, which is used by companies such as Bayer Diagnostics, the probe binds to a ribosomal RNA target, and reverse transcriptase creates a DNA copy of the RNA molecule. This genelike sequence then serves as the template for RNA polymerase, which rapidly produces thousands of transcripts that become the target for reverse transcriptase. Thus, unlike PCR and LCR, which generate only two copies per reaction cycle, TMA produces thousands of copies per cycle and thus can generate billions of copies per experiment. Similarly, NASBA focuses on the amplification of RNA molecules, but the protocol is much more like that of PCR.

But regardless of which method is used, the sample is filled with multiple copies of microbial nucleic acid that can be measured in toto using gel electrophoresis or in real time using fluorescent tags such as molecular beacons. Thus, clinicians and researchers can detect strong genomic signals from a sample that might have started with a single contaminating organism.

In 2001, Alexandra Clarici and colleagues at Karl-Franzens University (*www.uni-graz.at*) and Roche Diagnostics combined Roche's MagNA Pure LC system for sample preparation with their LightCycler technology for detecting herpes simplex virus (HSV, *7*). By designing a short oligonucleotide (primer) using the sequence from a highly conserved region of the HSV genome, the researchers found they could detect as few as 7 HSV-1 and 3 HSV-2 genomes per assay. Furthermore, the analysis could be completed in as little as 3 h.

In a similar experiment, researchers at Laval University (*www.ulaval.ca*), the Canadian Defence Department (*www.dres.dnd.ca*), and Infectio Diagnostic (*www.infectio.com*) developed a fluorescent PCR-based assay for *Bacillus anthracis* (anthrax) using the Cepheid Smart Cycler (*8*). With either purified spores or cells or in spiked clinical samples, they were able to detect as few as one colony-forming unit per reaction and, by designing primers for highly conserved gene sequences, the researchers achieved 100% specificity for *B. anthracis* over five other nonpathogenic *Bacillus* species.

However, other nucleic acid-based options do not involve enzyme-promoted amplification. In the Versant system produced by Bayer Diagnostics for HIV detection, the viral RNA molecules in infected samples bind to the sides of multiwell plates labeled with sequence-specific oligonucleotides. The bound RNA molecules then hybridize with secondary primers that serve as a template for the binding of alkaline phosphatase-labeled oligonucleotides. The samples are then exposed to a solution of phosphorylated dioxetane, which fluoresces when cleaved. Using this system, clinicians can hope to detect as few as 200 HIV particles/mL of sample.

In a variation on the hybridization-based method, researchers at Motorola developed the eSensor biochip, which is a small circuit board of gold electrodes and single-stranded DNA molecules specific to different microbe genes. When microbial DNA binds the capture probe, it is bound by a signaling probe that carries ferrocene-modified nucleotides. When a voltage is applied to the sensor, the iron atoms are oxidized and the intensity of the current is proportional to the number of bound ferrocene moieties.

So many choices

Of course, the assays just described as well as those listed in Table 1 are only the tip of the clinical iceberg. With so many assays available, which one does a hospital technician choose?

Because they are classified as "biological devices", molecular diagnostic tests are governed in the United States by the FDA, specifically the Center for Biologics Evaluation and Research (CBER, *www.fda.gov/cber*). This group is responsible for the final list of approved assays, and each year, new tests are made available to the public. But availability and regulatory approval are only part of the problem. In many cases, assay cost (and the opportunities for insurance reimbursement) is the main challenge.

"Although most clinicians and microbiologists enthusiastically welcome the new molecular tests for diagnosing infectious disease, the high cost of these tests is of concern," writes Michael Pfaller of the University of Iowa College of Medicine (*www.uiowa.edu*, *9*). "Despite the probability that improved patient outcome and reduced cost of antimicrobial agents and length of hospital stay will outweigh the increased laboratory costs incurred through the use of molecular testing, such savings are difficult to document."

Even though many individual assays may cost only a few dollars to process, the initial expenditures for equipment and infrastructure needed to process and analyze the results can be staggering in small clinical settings. To address this problem, many assay developers are trying to simplify their tests. For example, some of the antibody-based reactions rely on colorimetric detection that can be noted visually in a small plastic device (much like the at-home pregnancy tests), allowing the clinician a rapid, if crude, result. Furthermore, as governments are asked to play a more significant role in public health, the pressures on third-party payers to reimburse patients are expected to increase, and more effort will go into documenting the savings that Pfaller describes.

The technological ability to improve public health is readily available. We now await the societal push.

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