CHAPTER

6

FUTURE PREVENTIVE MEDICINE

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Traditional methods of detecting infectious disease agents have relied on recovering the inciting organisms from tissue samples, excretions or secretions, or indirect demonstration that an organism has been present in the body through the detection of a host immune response (principally antibody production).

All of these detection systems have inherent problems. Techniques that detect the presence of an organism (eg, electron microscopy, antigen ELISA, antigen immunodiffusion assays and histopathology) are either non-specific, relatively insensitive or both.

Many microbes cannot be propagated *in vitro* and are present in low numbers in secretions or excretions, making their antemortem detection difficult. *In vitro* propagation may require weeks, reducing the clinical value of the information obtained.

Documenting an infection through the host's production of antibodies requires a functional immune system. Additionally, paired serum samples collected two weeks apart must be tested to demonstrate a four-fold increase in antibody titer. The accurate detection of an infection, based on an acute and convalescent serum sample, is effective for documenting active infections, but the information is obtained too late to influence disease management. Determination of antibody titers may also be ineffective in detecting subclinical carriers, latently infected animals or slow infections.

Nucleic acid amplification and detection technologies will continue to improve and will compensate for many of the problems associated with other diagnostic techniques. Every clinician should have a rudimentary understanding of the methodologies, applications and problems associated with these test systems. Nucleic acid probe technology is currently being used to detect microorganisms, determine gender and detect genetic abnormalities.

Overview of DNA and RNA

The ability to selectively amplify and detect nucleic acid from pathogens is based on the fact that unique sequences of DNA or RNA are present in all living organisms. As a review, DNA is a polymer made of four units (bases): adenine (A), guanine (G), cytosine (C) and thymidine (T). RNA is a polymer made of four bases: adenine, guanine, cytosine and uracil (U). In the cell, the replication of DNA or RNA is catalyzed by specific enzymes (polymerases). Specifically derived heat-stable polymerases can be used *in vitro* to reproduce nucleic acid.

DNA generally exists in a double-stranded form (some viruses have single-stranded DNA or RNA). When double-stranded DNA is heated, the individual strands will separate (melt) from each other. When the strands are allowed to cool, the individual strands will rebind (reanneal) to their complementary strand of DNA, so that adenine from one strand binds to thymidine on the other strand, and cytosine on one strand binds to guanine on the other strand (Table 6.1).

If two single strands of DNA (for example, a synthetically produced DNA probe and a target sequence of pathogen DNA) bind together, the process is called hybridization (Figure 6.1). This hybridization process is the basis of using pathogen-specific DNA sequences (probes) to detect the presence of an organism's nucleic acid in samples collected from a host.

TABLE 6.1 Complimentary Binding of DNA and RNA Bases

For DNA	For RNA
A is complementary (binds to) **T**	**A** is complementary (binds to) **U**
C is complementary (binds to) **G**	**C** is complementary (binds to) **G**

If two strands of single-stranded DNA with the complimentary sequences;
Single strand 1 = 3′ **TACGGACCTTACG** 5′
Single strand 2 = 5′ **ATGCCTGGAATGC** 3′
are mixed together under the correct conditions they will bind together (hybridize) to form a double-stranded molecule.
Double stranded = 3′ **TACGGACCTTACG** 5′
5′ **ATGCCTGGAATGC** 3′
If two strands of single-stranded DNA that do not have complimentary sequences;
Single strand 1 = 3′ *TAC***GGACCTTA***CG* 5′
Single strand 2 = 5′ *ATG***TTAAGGCG***GC* 3′
they will not bind together (hybridize) and will remain as two single stranded molecules
Single strand 1 = 3′*TAC***GGACCT***TACG* 5′
Single strand 2 = 5′ *ATG***TTAAGG***ATGC* 3′

FIG 6.1 DNA hybridization process.

DNA Probe Technology

Use of DNA Probes

The ideal diagnostic test would provide maximum sensitivity (no false negatives), specificity (no false positives) and rapid results. Advances in the understanding of molecular biology have lead to the development of techniques that allow for the amplification (increasing quantities) and detection of organism-specific nucleic acid sequences. These test systems are based on the use of organism-specific DNA probes, and this new generation of tests most completely meets the requirements of an ideal method of detecting and identifying an organism (Figure 6.2).

The sensitivity and specificity of nucleic acid probe technology, the speed of obtaining results and the fact that the process can be used to detect organisms that will not replicate *in vitro*, may ultimately lead to nucleic acid probes replacing culture techniques as the gold standard in detecting pathogens.

With some microorganisms, DNA probes may be useful in detecting active as well as subclinical infections. The identification of subclinically infected animals requires that the reservoir site for the infectious agent be identified, and that samples be collected from the appropriate site at the correct stage of the infection. Depending on the organism and the host, these reservoir sites may be blood cells, hepatocytes, enterocytes, neurons or possibly any cell within the body. Samples that might be effective in detecting subclinical carriers could then include whole blood, liver biopsies or excrement (Figure 6.3).

Given the correct conditions, synthesized DNA probes will bind to specific, complementary target DNA (in a diagnostic test this is pathogen DNA), and the hybridization that occurs can be detected by using a number of visual indicator systems (eg, color change systems, radiographic sensitive systems).

An oligonucleotide DNA probe is a short sequence of nucleotide bases that is designed to detect a complementary strand of nucleic acid in a diagnostic sample

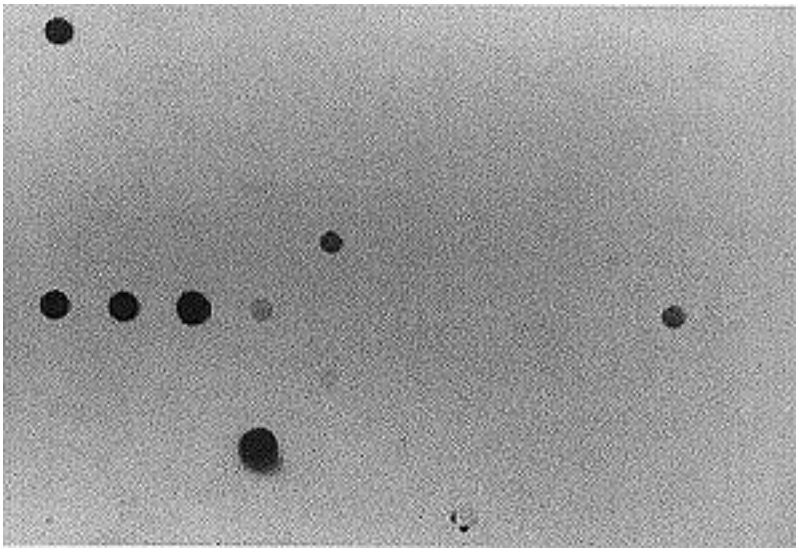

FIG 6.2 The 96-well format is used to test for the presence of PBFD virus nucleic acid in birds' white blood cells using viral-specific DNA probes. Each black dot represents a positive test (courtesy of Avian Research Associates).

FIG 6.3 For a DNA probe to be effective in detecting a subclinical infection in a bird, it is necessary to know where the pathogen is located in the body so that the correct sample can be collected and tested. In this case, viral-specific DNA probes were used to compare cloacal swabs and serum for detecting the presence of polyomavirus. Lane 1 is a control. The white bands present in the cloacal swab samples indicate the presence of polyomavirus nucleic acid. The absence of bands in the serum samples indicates that polyomavirus nucleic acid could not be detected in the serum of birds that were shedding polyomavirus.

(eg, tissue, sputum, blood, feces). For a DNA probe to be specific, it must be developed from a known genomic sequence of the organism in question. A probe designed from unknown sequence can create errors in interpretation.

For example, if a specific portion of the polyomavirus genome had bases arranged in the order:

5′...GGTACGTA**ACTTAAGGCCAATTCG**GCCTCGG...3′,

a DNA probe with the sequence

3′ - **TGAATTCCGGTTAAGC** - 5′

would be complementary to this sequence and would bind (hybridize) when the two nucleic acid strands were incubated in the proper conditions. This probe could be used to detect the presence of this specific polyomavirus genome sequence in infected liver tissue, saliva, urine or in a contaminated environment (if the nucleic acid from the virus were present in the sample).

Once a probe has bound to a target DNA molecule, the probe must be able to be detected. This detection can be accomplished by incorporating labels (eg, P^{32}, S^{35}, I^{125}, alkaline phosphatase, digoxigenin, horseradish peroxidase) into the probe. Most commercial probes use alkaline phosphatase, digoxigenin, or horseradish peroxidase to avoid the management problems associated with radioactive isotopes.

For example, if a labelled DNA probe with the sequence

5′ - **AATTCCGG** -3′ Dig
(a digoxigenin labelled probe for mycobacteria)

was mixed into a processed sample containing the mycobacterial nucleic acid sequence

.... 3′ - GACGTA**TTAAGGCC**TAGCAT.... 5′

then the probe would bind (hybridize) to the target sequence. Bound digoxigenin (on the probe) could then be detected by means of a routine series of reaction steps.

If on the other hand, the DNA probe was mixed into a processed sample that contained *E. coli* genome with the sequence

5′ ... AGTAGCCTTAGGAC... 3′

and no mycobacterial nucleic acid sequence (target) then the probe with attached digoxigenin would not bind and would be washed away.

These examples illustrate that the key to a DNA probe detection system is to identify a pathogen-specific nucleic acid sequence and to synthesize nucleic

acid probes to bind specifically to this sequence. The specific nature of the probe prevents cross-reactions with other pathogens, imparts specificity and reduces false-negative results.

Specificity of Nucleic Acid Probes

Nucleic acid probes can be designed to be so specific that they can differentiate between two related organisms that are antigenically similar (induce production of similar antibodies) but have differences in nucleic acid sequence that alter the pathogenicity of the organism. As a hypothetical example, two adenoviruses that are antigenically similar could occur in a bird population. Because they are antigenically similar, these adenoviruses would be difficult to differentiate using an antibody-based diagnostic test. Clinical evidence suggests that in some cases, these adenoviruses are highly pathogenic with high levels of mortality, while in other cases, infected birds develop an immune response and remain subclinical.

FIG 6.4 Pathogen-specific DNA probes can be used to determine if an area is contaminated with nucleic acid from an organism. Lane 1 is a control. The white bands indicate environmental samples that contain PBFD virus nucleic acid. In this case, the home of a client that had a bird with PBFD virus was screened for environmental contamination by the virus. Before cleaning, the enclosure was positive for PBFD virus nucleic acid (lane 3). After cleaning, the enclosure was negative for PBFD virus nucleic acid (lane 9); however, the registers, returns and filter associated with the heating system, as well as the wall and a picture frame in the room where the bird was housed were positive for PBFD virus nucleic acid.

By determining the DNA sequence of virus recovered from different birds, it might be discovered that the virus that causes high mortality has a different nucleic acid sequence than the virus that induces a subclinical infection. This difference might be:

5′ ...ATTG**CCATGGAAT**CCGATT... 3′
for the pathogenic strain and

5′ ...ATTG**TTAGGCTAG**CCGATT...3′
for the nonpathogenic strain.

A DNA probe with the sequence

3′- **AATCCGATC** -5′

could then be used to specifically detect the nonpathogenic strain. A probe with the sequence

3′- **GGTACCTTA** -5′

could be used to detect the pathogenic strain.

From a diagnostic perspective, probes are extremely valuable because they can be developed for pathogens so that they are genera-, species- or strain-specific, depending on which portion of a nucleic acid sequence they are designed to detect. For example, a probe could be developed that would detect any *E. coli* or only an *E. coli* that had a unique biochemical function. In the following example three strains of *E. coli* exist: one is highly virulent, one is considered normal flora in an animal and one is considered an environmental contaminant. The three strains have the nucleic acid sequences:

5′.... GCTATGCTT**AGGCCTTA**....3′ virulent strain

5′.... GCTATGCTT**GCCGATAA**....3′ normal strain

5′.... GCTATGCTT**GATCGGTA**.... 3′ environmental strain.

An oligonucleotide probe with the sequence

3′ CGATACGAA 5′

would detect all three of the *E. coli* organisms because it is designed to be complementary (and will hybridize) to a conserved sequence that is common to all three strains of *E. coli*. A probe with the sequence

3′ **CTAGCCA** 5′

would detect only the strain of *E. coli* considered an environmental contaminant because this sequence is unique to that strain of bacteria (Figure 6.4).

Once a probe has been developed, it can be used to detect nucleic acid that is extracted from a sample and attached to a membrane, or it can be used to detect pathogen-specific nucleic acid in a section of paraffin-embedded, formalin-fixed tissue that has been processed for histopathologic evaluation (*in situ* hybridization).

In situ hybridization using pathogen-specific nucleic acid probes is particularly effective when a pathogen is present in relatively small numbers or produces a lesion that histologically resembles that induced by other pathogens. For example, the intranuclear inclusion bodies caused by polyomavirus can appear morphologically similar to the intranuclear inclusion bodies caused by PBFD virus or adenovirus (see Color 32). *In situ* hybridization using viral-specific DNA probes can quickly and correctly determine which of these viruses induced the identified inclusion bodies. When compared to antibody staining techniques for the identification of pathogens in tissues, nucleic acid probes are more specific and more sensitive than other pathogen detection techniques. They also detect organisms that may have been antigenically altered during processing.

In addition to confirming the presence of a pathogen in tissue, *in situ* hybridization can also be used to detect the type of cell infected and whether the pathogen's nucleic acid is present in the cytoplasm or nucleus of the host cell. This last finding is of particular importance in understanding the replication scheme of many viruses, which can be critical for understanding how infections can be treated or prevented.

Sensitivity of Nucleic Acid Probes

In infected tissues where high numbers of the organism are present, the use of DNA probes to detect the presence of an organism's nucleic acid is fairly straight forward. In contrast, detection of a pathogen in excretions or secretions where numbers of the organism may be small requires further processing. To increase the likelihood of finding an organism in a diagnostic specimen (increased sensitivity), a sample to be tested is often subjected to a group of reactions that will amplify (increase) the number of pathogen DNA molecules in the sample, thus improving the ability of the probe to detect the organism.

The most commonly employed technique for amplifying target nucleic acid is the polymerase chain reaction (PCR) (Table 6.2). Theoretically, when used in combination with pathogen-specific nucleic acid primers, PCR can use one copy of a nucleic acid sequence to produce 1,000,000 copies. The most important component of this process is the pathogen-specific oligonucleotide primers. It is these oligonucleotide primers that allow the process to preferentially increase the number of pathogen nucleic acid molecules without increasing the number of all other contaminating nucleic acid molecules that would be present in a sample.

TABLE 6.2 PCR Amplification of Nucleic Acid

PCR amplification involves three phases:
1) separating (denaturing) ds DNA to create ss DNA
2) binding (annealing) pathogen-specific primers to the ss DNA target (pathogen) nucleic acid
3) synthesizing new strands of target (pathogen) nucleic acid

For example, a fecal sample collected for polyomavirus testing might contain 10 polyomavirus particles, 300 *E. coli*, 10,000 *Staphylococcus* spp., 150 host-derived WBCs (nucleated and containing DNA), 50 host-derived RBCs (also nucleated in birds and containing DNA) and 300 *Candida* spp. It would not be possible to detect only ten copies of the target (polyomavirus) DNA. By using primers designed specifically for the polyomavirus nucleic acid, the amount of target sequence (portion of polyomavirus DNA to be detected) can be increased from 10 copies to 10,000,000 copies, while the contaminating DNA from the *E. coli*, staphylococcus, WBCs, RBCs and candida remain the same and become dilutionally unimportant. The 10,000,000 synthesized copies constitute a quantity that can be easily detected.

A PCR cycle involves heating the target DNA (from the pathogen in a sample) to cause ds DNA to become ss DNA, thus exposing the target sequence on the pathogen's DNA to the oligonucleotide primers, where they can anneal to prime the generation of new sequence. The temperature of the reaction is then adjusted so that an enzyme (DNA polymerase) will synthesize a new strand of nucleic acid starting from one end (3') of the primer. At a specified time (determined for each pathogen-specific set of primers), the reaction is heated to stop the DNA polymerase and separate the created ds DNA into new target ss DNA.

This process is cyclic and is usually performed 40 times. The synthesized strands of ss DNA serve as new templates for the reaction, and each cycle results in an exponential increase in molecules (ie, one molecule makes two, two molecules make four, four molecules make eight).

From a simplified perspective, the two most critical components for the amplification and detection of nucleic acid from an organism are the pathogen-specific nucleic acid primers used to increase the sensitivity of the test (increases the likelihood of detecting only a few of the organisms) and the pathogen-spe-

cific nucleic acid probes used to ensure the specificity of the test (ensures the amplified sequence is that of the target organism).

Sample Collection

Minimal contamination of a diagnostic sample can be a problem with the amplification step that is used to increase the sensitivity of the test. A knowledgeable clinician can minimize contamination by practicing sound techniques in collecting any samples for DNA probe testing. The same degree of care must be exercised when collecting samples for bacterial culture. For example, if a clinician were testing a bird to determine the presence of PBFD virus in the blood, and the blood sample was collected from a toenail, a positive result may indicate the presence of PBFD virus either in the blood or on the bird's toenail. Washing the bird's nail before collection would not be expected to reduce the potential for contaminating the sample. A blood sample properly collected into a sterile syringe by venipuncture would be less likely to result in a contaminated sample.

Vaccines

Conventional Vaccines

Modified live, killed or subunit vaccines are currently available for use in protecting animals from various infectious diseases. The function of a modified live vaccine is to produce an infection (thus inciting an immune response) without producing disease. Modified live vaccines have inherent risks including possible reversion to a virulent form or an attenuation that alters the antigenicity of the vaccine strain to such a degree that it is not protective against a field strain of a virus. Modified live vaccines may be virulent in animals that are immunosuppressed, may be immunosuppressive themselves, may cause a low level of morbidity that affects reproduction and must be handled with care to prevent inactivation.

Killed vaccines are produced by growing a pathogen *in vitro* and then inactivating it to prevent replication in the vaccinate. These vaccines require exposing the vaccinate to a large dose of antigen and frequently require the addition of harsh adjuvants that can cause unacceptable tissue reactions in the vaccinate.

Subunit Vaccines

To develop a subunit vaccine, the protein from a pathogen that induces a protective immunologic response in the host must be identified. The nucleic acid sequence (gene) that codes for this protein is then inserted (cloned) into a plasmid of an *E. coli* or other organism, which then produces the desired protein. The immunologic protein is then purified away from the producing organism and can be used as a vaccine. Subunit vaccines allow proteins that would protect an animal against different serotypes to be included in the same mixture.

Subunit vaccines represent only the portion of the viral protein that is responsible for eliciting an immune response and are completely safe because the vaccinate is not exposed to the nucleic acid of the pathogen (prevents replication of the organsim in the vaccinate). This prevents potential problems associated with the conversion of attenuated vaccine strains of a virus into a virulent strain. It also eliminates the possibility that a vaccinate may be exposed to a virus that has not been killed.

Several subunit proteins from the same organism can be combined in a vaccine to increase the immunologic response (as is seen with a natural infection) without the risk of inducing disease. In the development of subunit vaccines, it may be advantageous to combine several proteins from the same pathogen in order to stimulate both virus-neutralizing and T-cell immune responses. Subunit vaccines also create the possibility for incorporating several proteins from numerous pathogens into one vaccine.

Other Vaccines

Many pathogenic bacteria have been found to have capsular polysaccharides that function as virulence factors and elicit immune responses. For some human pathogens, these capsular polysaccharides have been purified and conjugated to proteins, which elicit immunologic responses and protect the host from the target bacterium. An better understanding of the interaction between bacteria and the host immune system may lead to methods to prevent rather than treat bacterial infections. A similar increase in the knowledge concerning the host immunologic response to parasites will be necessary before parasitic infections can be prevented through vaccination.

Adjuvants

The oil-emulsion adjuvants that are commonly used in mammalian vaccines have been shown to cause severe muscle necrosis in some species of birds (see Figure 32.8). One method of preventing these reactions is to use solid-matrix-antibody-antigen complexes in place of adjuvants. These complexes have been shown to be particularly effective in augmenting the immunologic response induced with subunit vaccines, and are being investigated for use in birds.

Liposomes have shown promise as carriers for immunogenic proteins that can be used for vaccination or immunotherapy. Injected liposomes are rapidly consumed by mononuclear phagocytes, particularly circulating macrophages.[5] In theory, any antigens present in these phagocytized liposomes would be processed and stimulate an immune response.

Antimicrobial Therapy

Conventional antimicrobial therapy depends on using chemotherapeutic agents that selectively interfere with metabolic processes that are unique to bacteria, parasites or fungi, while having little or no effect on the metabolism of the host cell. While this is generally effective for bacteria, parasites and fungi, it is ineffective for most viruses and tumor cells, which use the host cell metabolic pathways for energy production and replication. Antiviral agents are generally designed to prevent uncoating of a virus particle, and thus stop a virus from replicating.

Liposomes have been shown to be effective in transporting agents with immunologic activity against antigen-expressing tumors into the affected cells. For treating cancer, this type of immunotherapy would be far superior to chemotherapeutic methods currently used, because immunotherapy could be targeted specifically for the cancer-producing cells with no effects on normal cells within the affected host. Liposomes can also carry chemical compounds, such as muramyl-tripeptide-phosphatidylethanolamine, which increase macrophage activity.[4]

Monoclonal antibodies have been used as a therapeutic agent for some types of cancers with antigen presenting capabilities. By binding cytotoxic agents to the monoclonal antibodies, high concentrations of therapeutic agents are delivered directly to the affected cells to which the antibodies bind.[1]

Antisense RNA Therapy

In the process of replicating DNA, the cell produces a complementary copy of the DNA in the form of a messenger RNA. This mRNA is then used as a copy to make new DNA molecules. In the 1960's, a concept was developed of inhibiting the replication of DNA by introducing a nucleic acid sequence that would bind to the mRNA and prevent its use as a template for replication. This was termed antisense RNA therapy.

When fully implemented, antisense RNA therapy will revolutionize the way that neoplastic and viral diseases are treated. By binding specifically to mRNA, the antisense RNA would inhibit the replication of cancer cells or viruses, while having no adverse affect on unaffected host cells. Thus, antisense RNA would represent a safe, cell-specific therapy.

As a simplified example, the replication of a DNA virus may involve the initial nucleic acid sequence

5′ ATGCGGCCTTACCATGACAT....3′

The mRNA template for this sequence (in RNA, uracil is found in place of thymidine) would be

3′ **UACGCCGGAA**UGGUACUGUA...5′

If the antisense RNA sequence

5′ - **ATGCGGCCTT** - 3′

was introduced to the infected cell, it could bind to the mRNA sequence and prevent it from serving as a template for viral replication.

To be clinically applicable in the treatment of viral and neoplastic diseases, antisense RNA technology must advance to the point where therapeutic nucleic acid sequences can be introduced to the body in such a way that they enter an affected cell, and subsequently interfere with replication of a virus or neoplastic cell. The use of antisense RNA therapy is likely to evolve into a useful therapeutic regimen over the coming decade.

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