Principles of Nucleic Acid Hybridization and Comparison with Monoclonal Antibody Technology for the Diagnosis of Infectious Diseases

STEPHEN C. EDBERG, Ph.D., A.B.M.M.

Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut

Received March 21, 1985

Until the 1980s the diagnosis of specific etiologic agents of infectious diseases rested with their isolation in vitro and identification by analysis of their phenotypic characteristics. In the 1970s the concept of a microbial species evolved from phenotypic analysis to nucleic acid homology. Currently, nucleic acid sequences specific for a given species are being isolated and amplified and utilized not only to identify the pathogen after it has been grown in vitro but also elucidate it directly in biological material. The procedures for making nucleic acid hybridization probes are analogous to the generation of monoclonal antibody tests. Currently, research and development are centered in choosing the particular nucleic acid to analyze, establishing the most efficient vector system for amplifying the nucleic acid, generating an efficient means of selecting the particular nucleic acid fragment specific for the microorganism, and in measuring the hybridization reaction. While immunological techniques have been utilized in the clinical laboratory for over thirty years, the means of detecting nucleic acid hybridization reactions are just beginning to be usable in the clinical diagnostic laboratory. Much of nucleic acid hybridization research is proprietary, and a particular challenge is to develop a means whereby information can be used for the progress of science as a whole when generated by private ownership.

THEORETICAL BASIS OF NUCLEIC ACID HYBRIDIZATION

The chemical basis for nucleic acid hybridization assays rests in the reversible helix-coil transition of the nucleic acid molecule. Nucleic acids will associate as double-stranded molecules or disassociate into single-stranded polymers conditioned on the physico-chemical parameters of temperature, base pair composition [1], ionic strength of the milieu [2], and the concentration of denaturing agents [3]. The conditions which permit us to manipulate the association and disassociation of the nucleic acid polymer strands are being intensely investigated in order to develop nucleic acid hybridization (NA hybridization) probes that can be applied to the diagnosis of human infectious diseases.

Much of the theoretical and applied research of NA hybridization technology concerns factors affecting the stringency of the interaction of the nucleic acid strands. Complementary single-stranded nucleic acid associates according to second-order kinetics, the major rate-determining parameter being the number of complementary sequences between the polymer strands [4]. The amount of non-homology that can be tolerated while still achieving association between the two strands under particular conditions is known as stringency. Stringency may be manipulated by varying the

Presented at a symposium on “Rapid Methods for Immunodiagnosis of Infectious Diseases,” at the annual meeting of the American Society for Microbiology, Las Vegas, Nevada, March 5, 1985

Address reprint requests to: Dr. Stephen C. Edberg, Dept. of Laboratory Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510

Copyright © 1985 by The Yale Journal of Biology and Medicine, Inc.
All rights of reproduction in any form reserved.
temperature, the time, and the ionic strength of the milieu. High stringency implies a great deal of specificity between nucleic acid hybrids with large numbers of base pair matches; low stringency implies a relatively small number of base pair matches, yet enough to yield a double-stranded moiety.

For the diagnosis of human microbial diseases, NA hybridization reactions occurring at conditions requiring high stringency will detect closely related pathogens. Conditions that utilize low stringency, even with the same NA hybridization probe, can detect an entire genus or family of microorganisms. For example, a nucleic acid probe at high stringency conditions may detect only Herpes simplex virus type I, whereas at low stringency the entire genus of herpes virus may be detected [5].

Recombinant nucleic acid technology is being used in three major ways. First, one can isolate specific DNA fragments from a complex mixture of DNA molecules and amplify them in milligram quantities; the diagnosis of infectious diseases falls in this category and will be considered in detail. Second, one can alter DNA molecules by inserting or deleting restriction endonuclease recognition sites; in effect, one can create new genes. Third, one can synthesize large amounts of peptides or proteins in bacteria or eukaryotic cells.

PROCEDURE FOR MAKING NA HYBRIDIZATION PROBES

There are four essential steps in producing an NA hybridization probe [6]. A specific nucleic acid fragment from the microbial pathogen must first be isolated; this fragment must be specific to the organism under study. Second, the fragment has to be inserted into a vector, or vehicle; this vector must be one that can accept foreign DNA and replicate in a living cell; semi-synthetic plasmids have been developed for this purpose. Third, the vector must be introduced into a host organism with the host not only accepting the vector but replicating it when the host itself replicates. Fourth, one must screen large numbers of cells to capture the desired recombinant clone. Table 1 presents the essentials steps in the cloning protocols of both NA hybridization and monoclonal antibody technologies.

A finding that allowed the development of NA hybridization probes was the discovery of restriction endonucleases. Restriction endonucleases are enzymes that specifically cut nucleic acid sequences within the polymer molecule. Type I restriction endonucleases are complex proteins of two to eight subunits that require ATP,
NA HYBRIDIZATION AND MONOCLONAL ANTIBODY

TABLE 2  
Commonly Employed Restriction Endonucleases in the Diagnosis of Infectious Diseases

| Enzyme Name | Species | Sequence Attacked |
|-------------|---------|------------------|
| EcoRI       | *Escherichia coli* (with plasmid R1) | 5'-G-A-A-T-T-C-3' |
| Bam HI      | Bacillus amyloliquefaciens | 5'-G-G-A-T-C-3' |
| Hind III    | *Hemophilus influenzae* | 5'-A-A-G-C-T-T-3' |
| Hpa I       | *Hemophilus parainfluenzae* | 5'-G-T-T-A-A-C-3' |
| Hae III     | *Hemophilus aegypticus* | 5'-G-G-G-C-3' |
| Hinc        | *Haemophilus influenzae Rf* | 5'-G-T-Py-Pu-A-C-3' |
| Hinf I      | *Haemophilus influenzae Rf* | 5'-G-A-N-T-C-3' |
| Alu I       | *Arthrobacter luteus* | 5'-A-G-C-T-3' |

N-adenosyl-L-methionene, and magnesium. These enzymes hydrolyze randomly. Type II restriction endonucleases are simple proteins that require only magnesium for function. These cut double-stranded nucleic acid polymers at defined points. Type II restriction endonucleases are the kind utilized in nucleic acid hybridization research [5].

Restriction endonucleases are produced by microorganisms to eliminate foreign DNA from themselves. The host protects itself from its own restriction endonuclease by methylating the base pair target sequence [7]. There are now several hundred restriction endonucleases available from commercial sources. Examples of the commonly employed restriction endonucleases used for the diagnosis of infectious diseases and the specific sequences they attack are presented in Table 2. Knowing the specificities of the enzymes, one may choose restriction endonucleases to yield nucleic acid fragments of known size distributions from the microbial pathogen. Practically, one grows the microbial pathogen in large numbers, removes its nucleic acid by a combination of physical (sonication) and chemical (detergents and alkali) means, and cuts the nucleic acid into small pieces with specific restriction endonucleases. The small pieces are separated from each other by electrophoresis on gel and column chromatography. The investigator chooses those pieces based on size distribution or other particular parameters for insertion into a cloning vector [5,8].

Bacterial DNA can be utilized directly in a nucleic acid hybridization probe assay. From viruses and eukaryotic pathogens, however, one must make complementary DNA (cDNA). RNA is generally cloned indirectly. RNA can be converted into DNA utilizing a reverse transcriptase enzyme from retroviruses. Utilizing the RNA as a template, single-stranded cDNA is produced. The RNA can be eliminated from cDNA in the mixture utilizing RNase. A complementary second strand of DNA may be made to the cDNA, using DNA polymerase. This second strand of DNA is a DNA copy of the original RNA polymer. The double-stranded cDNA can then be utilized to produce NA hybridization probes. Table 3 presents the essential steps in the synthesis of complementary DNA [5].

The field is at present in a state of flux concerning which type of nucleic acid to use as a probe target and which to use as the probe. Most work has been done with DNA hybridization, but a growing body of evidence indicates that for clinically useful tests RNA may be preferable. DNA has the major limitation in that a particular specific sequence may be present only once in a given cell. Ribosomal RNA or mRNA may have a sequence repeated many times in the cell. Theoretically, therefore, the
sensitivity of an RNA method should be greater than a DNA hybridization method [8].

Recently Brenner reported the ability to specifically detect Legionella, utilizing ribosomal RNA probes [Brenner DJ: Abstracts of the Annual Meeting of the American Society for Microbiology, 1985]. He found that probes to the ribosomal RNA of Legionella were not only specific but, because of the physical properties of ribosomal RNA, the hybridization procedure could take place within one hour. He utilized a commercially produced probe (Gen-probe, Irvine, CA).

Also in a state of flux is the means of selection of the fragments. Two possibilities have been investigated: cloning of all fragments from a pathogen ("shotgun cloning") and pre-selection. Shotgun cloning methods were first used and are still the most common. Many fragments of similar size distribution from the microbial pathogen are inserted into vectors. Each vector is cloned and amplified, and each clone must be tested for specific utility. Current research is directed at largely eliminating this tedious task by pre-selecting specific fragments from the restriction endonuclease digest before they are inserted into the cloning vectors. Most intensely being studied are affinity chromatography methods to pre-select desired fragment(s). The desired fragment(s) stick to the column while unwanted fragments pass through it. The proper fragment(s) are eluted from the column to be inserted into the vector.

The cloning vector is either a plasmid, bacteriophage, or a genetically engineered plasmid known as a cosmid. The nucleic acid must be inserted into a vector with both the vector and the nucleic acid retaining function. The vectors are extra-chromosomal and can be transferred to a host organism. Each time the host organism replicates once, the vector replicates at least once. Table 4 presents the common cloning vectors utilized for the construction of NA hybridization probes for the diagnosis of infectious diseases [5, 8].

The nucleic acid fragment is inserted into the vector, utilizing specific restriction endonucleases. The vector contains single recognition sites for one or more restriction endonucleases. The common vectors have been genetically engineered and have been completely sequenced. Each vector has a particular selection marker so that there is a signal to detect if the insertion has been successful. A vector with both plasmid and microbial DNA is known as a chimera. For example, the vector plasmid pBR322 contains genes that confer resistance to the antibiotics ampicillin and tetracycline. If one chooses a restriction endonuclease that cuts the plasmid in the tetracycline resistance gene to insert the nucleic acid from the microbe here, tetracycline resistance is lost. Accordingly, once this plasmid is transferred to a host bacterium, successful insertions will be observed by resistance only to ampicillin. Unsuccessful insertions will be resistant to both ampicillin and tetracycline.

| Step | Process | Mechanism |
|------|---------|-----------|
| Cell lysate | Detergent/pH | Disruption |
| Total cellular RNA | RNA selection | Precipitation |
| Total mRNA | Bind 3' polyadenylate tail | Poly dT binding |
| cDNA | cDNA-mRNA complex | Reverse transcriptase |
| Single-stranded cDNA | RNA destruction | Selective digestion of RNA |
| Double-stranded cDNA | Reverse transcription | Enzyme linking |
TABLE 4
Commonly Utilized Cloning Vectors

| Vector Name | Selection Marker                      | Source                                           | Use                                                                 |
|-------------|---------------------------------------|-------------------------------------------------|----------------------------------------------------------------------|
| pBR322      | Ampicillin tetracycline resistance     | Laboratory construct of three naturally found plasmids | Expression in E. coli, used for constructing new cloning vectors; e.g., pSP64 and pUC |
| pSP64       | Ampicillin resistance                  | Laboratory construct                           | For producing RNA hybridization probes in vitro                      |
| M13 series  | Beta-galactosidase                     | Natural                                         | DNA sequencing and DNA hybridization probes                         |
| pUC series  | Ampicillin resistance and beta-galactosidase | Laboratory construct                         | DNA sequencing and expression in E. coli                          |

Practically, one selects a restriction endonuclease to cut the particular vector in a specific spot. The nucleic acid is inserted into this sequence and the vector reassociated with a DNA ligase. The vector is then inserted into a receptive host.

The host ordinarily utilized to make NA hybridization probes for the diagnosis of infectious diseases is *Escherichia coli*, the most commonly utilized being *E. coli* K12. This *Escherichia coli* is sensitive to all commonly utilized antibiotics, accepts the vector efficiently, and replicates the vector at least once every time it multiplies. In the cloning protocol, the susceptible *E. coli* has available to it two vectors: a chimera with a successful insertion and a reannealed vector that did not accept a nucleic acid piece. The vectors are mixed in liquid growth medium with the host *E. coli*, with three possible outcomes: an *E. coli* with no vector, an *E. coli* with a reannealed vector, and an *E. coli* with a chimeric vector. The mixture is spread over the surface of an agar medium to select only the *E. coli* with chimera. Colonies of transformed bacteria, each *E. coli* in the colony containing at least one copy of the chimeric plasmid, are grown in large numbers. The cells are harvested and the copies of the original microbial nucleic acid released, utilizing specific restriction endonucleases [5,8].

The cloned microbial DNA must be labeled to be utilized as a nucleic acid hybridization probe. Traditionally, labeling has occurred by a nick translation process [9,10]. Single-stranded nicks are introduced into double-stranded DNA, utilizing DNAase I. These nicks are subsequently repaired, utilizing a DNA polymerase I in the presence of 32P-labeled deoxyribonucleotide triphosphates. Activity levels of 10^8 counts per minute per microgram of DNA can be obtained. Single-stranded DNA probes capable of detecting 0.1 picogram of target DNA result. The minimum sensitivity of an individual nucleic acid hybridization probe will depend on the size of the genomic target and the percentage of homology between the probe and the target.

Research is being conducted to eliminate radiolabeling by replacing 32P with a non-radioactive signal. The first non-radioactive signal to achieve widespread use was the synthesis of biotin-labeled analogues of deoxyribidine triphosphate and uridine triphosphate. Biotin was covalently linked to the C5 position of the pyrimidine ring through an allylamine linker arm [11]. It was found that the attachment of biotin to the nucleic acid did not affect the association properties. Linker arms of various lengths were employed to attach biotin to nucleic acid, the most useful being a length of approximately 11 units (see Fig. 1). Table 5 demonstrates the differences between radiometric and non-radiometric, biotin-labeled probes [12].
Nucleic acid hybridization probes labeled with biotin can be detected by either immunoochemical or enzymatic methods. The strong avidity of biotin for avidin has been most successfully exploited. This protein has an avidity of $K_d = 10^{-15}$, with four binding sites for biotin [13]. One or more of the avidin binding sites can be labeled with an enzyme such as horseradish peroxidase [14] or alkaline phosphatase [12]. If the biotin-labeled NA hybridization probe attaches to microbial nucleic acid, biotin will be available for reaction with avidin. If one adds avidin to which an enzyme has been attached, and subsequently a colorimetric substrate for the enzyme, the interaction of the probe with microbial nucleic acid can be directly visualized. The sensitivity of biotin-labeled NA hybridization probes has recently been increased significantly, utilizing polymerized alkaline phosphatase attached to avidin [15].

Another strategy to detect the interaction of the NA hybridization probe with microbial nucleic acid polymer is the utilization of antibody specific to the nucleic acid complex. Although patients with systemic lupus erythematosus have anti-nuclear antibodies, the direct antibody detection of nucleic acid complexes has not proven efficacious. Recently a technique has been developed to label nucleic acid with a hapten [16]. Antibody to the hapten is used to detect the hybridization. Tchen et al. [17] found that guanine residues in nucleic acids could be labeled with N-acetoxy-N-2-acetylaminofluorene (AAF) and its 7-iodo (AAIF) derivatives (see Fig. 2). These

![FIG. 1. Attachment of biotin to nucleic acid, utilizing linker arms of various lengths.](image)

### TABLE 5

| Step                  | Radiometric | Non-Radioactive (Biotin)                  |
|-----------------------|-------------|-------------------------------------------|
| Choice of signal      | $^3P$       | Biotin:Avidin:Enzyme                      |
| Insertion of signal   | Nick translation | Covalent enzymatic linking of biotin to pyrimidine ring |
| Reading of signal     | Direct      | Indirect                                  |
| Developer             | None        | Avidin to which an enzyme is attached     |
| Measurement           | Radioactive counts (generally film exposure) | Detection of enzyme activity by addition of substrate |
| Stability             | $^3P$ half-life 14.3 days | $-20^\circ C$ for two years |
| Precautions           | Radioactivity | None                                      |
AAF or AAIF derivatives could be directly detected in an immunochemical sandwich-type assay. If the hapten-labeled probe attached to microbial nucleic acid, the hapten was exposed. Antibody labeled with a signal, such as an enzyme, fluorescent dye, and so on, bound to the hapten and yielded a positive test (see Fig. 3).

Recently, a procedure to produce large quantities of RNA hybridization probes, which are ten times more sensitive than DNA hybridization probes, was published [18,19,20]. The method utilized the RNA vector SP6. Chemically, DNA/RNA hybrids are more temperature-stable than DNA/DNA hybrids. Accordingly, RNA hybridization probes can be utilized under higher stringency conditions than those which permit DNA polymers from associating. Working under these different physico-chemical conditions permits the detection of smaller numbers of pathogens.

Other strategies to label nucleic acid have been explored but as yet have not proven clinically efficacious. Signal molecules have been introduced into nucleic acid, utilizing nucleotide analogues that function as substrates for nucleic acid polymerases [11,12]. Also, glycosylated T4 bacteriophage DNA, whose carbohydrate-modified nucleotides can act as natural signals, have been synthesized [21].

The future research and development of NA hybridization probe analysis of microbial pathogens will be concentrated in exploiting colorimetric methods. Color has obvious advantages over radioactivity, not the least being it can be packaged for markets ranging from large laboratories to private, over-the-counter tests. Colorimetric methods are approaching the sensitivity of radiometric procedures [22]. The time required for color development has been significantly shortened in the last years, from 24 or more hours to same-day readability. By polymerizing the alkaline phosphatase attached to avidin, color could be measured within two hours [15,23].
After the nucleic acid has been captured from the microbial pathogen, inserted into a vector, amplified in *E. coli*, and labeled, the last step in the diagnosis of an infectious disease using an NA probe is the assay itself. Current assays utilize solid support hybridization methods. By analogy to immunological techniques, these will be referred to as heterogeneous methodologies because several washing steps are required to remove bound from unbound reactants. Homogeneous techniques, in which the reaction occurs in a liquid medium without the requirement to separate the bound from the unbound, have not as yet been developed for NA hybridization.

There are four basic assay techniques in use. The most extensively studied is the dot, or dot-blot, hybridization technique. This method employs a nitrocellulose filter paper support. A sample from the patient containing the infectious microbe is disrupted to release its nucleic acid. The nucleic acid is bound in a spot on nitrocellulose by a combination of physical (heating) and chemical (salts, detergents) means. The labeled NA hybridization probe is added to the spot under particular stringency conditions. If there is complementary nucleic acid from the patient's sample, the labeled nucleic hybridization probe will be bound; if there is no complementary nucleic acid on the spot, the hybridization probe will not be bound and can be easily washed away. A substrate to the label on the NA hybridization probe is added and the reaction measured. If the label is $^{32}\text{P}$, the measurement involves the overnight development of photographic film. If the label is an enzyme, the measurement may be either by densitometry or by direct visualization [12].

Varying the arm length of the biotin-labeled target, the concentration and types of signals, and the utilization of polymerized alkaline phosphatase, dot hybridization procedures can detect as little as 1–2 picograms of target sequence (refer to Table 6) [15,22,23]. The clinical utility of dot hybridization analysis has been demonstrated with hepatitis B virus [24,25], cytomegalovirus [26], adenovirus [27], and herpes virus [28]. Other viruses and microbial pathogens are currently under clinical investigation [8]. Of particular interest is the ability to elucidate viruses that cannot be cultured directly from clinical samples.

The clinical utility of dot hybridization has significantly progressed, especially in the ability to manipulate nucleic acids and eliminate contaminating proteins and other inhibiting substances from specimens. Traditionally, NaCl was utilized as part of the stringency conditions to fix DNA to nitrocellulose. It was found that RNA which lacked poly(A) did not bind to nitrocellulose. Subsequently it was discovered that changing the salt from NaCl to NaI allowed mRNA to fix specifically to nitrocellulose [29,30]. Not only did mRNA bind, but DNA and many proteins did not stick to nitrocellulose. The NaI seemed to promote mRNA nitrocellulose interaction, solubilize the pathogenic cell, and did not promote the interaction of DNA with nitrocellulose. Adding or subtracting detergents such as brij-35 and DOC allowed one to affix selectively either DNA or mRNA to nitrocellulose [29,30].

Sandwich hybridization techniques are currently under development and have been utilized to detect virus from clinical specimens (Fig. 3). This methodology involves the production of two non-complementary nucleic acid sequence reagents. These reagents are produced from adjacent sites on the microbial pathogen's genome. The first nucleic acid sequence is immobilized on to the solid nitrocellulose support and serves as a target sequence to interact with nucleic acid from the microbial pathogen. The second sequence is labeled and serves as the nucleic acid detector probe. Like dot hybridization, nucleic acid is first extracted from the clinical sample. The extracted nucleic acid
NA HYBRIDIZATION AND MONOCLONAL ANTIBODY

TABLE 6
Relative Sensitivity of Biotin-Specific Reagents in Detecting Biotin-Labeled Polynucleotides Bound to Nitrocellulose Filters

| Bio-DNA Target | Detector Reagents | Substrates | Detection Limits (pg Target Sequence) |
|----------------|-------------------|------------|--------------------------------------|
| 1. Bio-4       | Anti-biotin IgG + FITC-2° Ab | —          | 2,000–4,000                          |
| 2. Bio-11      | Anti-biotin IgG + FITC-2° Ab | —          | 500–1,000                            |
| 3. Bio-4       | Anti-biotin IgG + HRP-2° Ab | DAB/EAC    | 500–1,000                            |
| 4. Bio-11      | Anti-biotin IgG + HRP-2° Ab | DAB/EAC    | 150–200                              |
| 5. Bio-4       | ABC (avidin DH-Bio HRP) | DAB/EAC    | None detected                        |
| 6. Bio-11      | ABC                | DAB/EAC    | 75–150                               |
| 7. Bio-16      | ABC                | DAB/EAC    | 75–150                               |
| 8. Bio-16      | Anti-biotin IgG + Bio-2° Ab + ABC | DAB/EAC | ≥100–200                             |
| 9. Bio-16      | ABC + Bio-DNA + ABC | DAB/EAC    | ≥100–200                             |
| 10. Bio-16     | ABAP (Avidin-Bio Alk. Phos.) | NBT + BCIP | 20–30                                |
| 11. Bio-16     | poly ABAP (avidin-poly Bio Alk. Phos.) | NBT + BCIP | 1–2                                  |

Abbreviations: Bio-4-dUTP, Bio-11-dUTP, and Bio-16-dUTP—analogs of TTP that contain a biotin molecule linked to the C-5 position of the pyrimidine ring through linker arms that are 4, 11, and 16 atoms long, respectively. Bio-4-DNA, Bio-11-DNA, and Bio-16-DNA—DNA probes prepared with Bio-4-, Bio-11-, or Bio-16-dUTP analogues, respectively. NBT—nitro blue tetrazolium. BCIP—5-bromo-4-chloro-3-indolyl phosphate. DAB—3,3′-diaminobenzidine. EAC—ethyl aminocarbazole. ABAP—complexes of avidin and biotinylated alkaline phosphatase polymers. NaCl/ Cit—standard saline citrate (0.15 M CaCl/0.015 M sodium citrate, pH 7.0). From [11]

is mixed with the probe nucleic acid and hybridized against the target sequence, which has been bound to nitrocellulose paper. Particular stringency conditions are obeyed. If the microbial pathogen nucleic acid is complementary to both the probe reagent nucleic acid and the filter-bound nucleic acid, a sandwich will be formed and the complex detected by the addition of a developer to the probe label [31]. Utilizing this technique, as little as 8 × 10⁻¹⁸ mols (approximately 5 × 10⁶ molecules) of DNA from adenovirus can be detected [32]. This level of detection is comparable to radioimmunoassay, and the time of detection, approximately 20 hours, is significantly less than the days required for viral culture.

In 1975 Southern described a method for analyzing specific DNA fragments from a complex mixture. This method has become standard to establish the specification of nucleic acid sequences. After restriction endonuclease digestion, the mixture is separated by agarose gel electrophoresis. The DNA fragments are transferred by either blotting or electroelution to nitrocellulose paper. Nucleic acid hybridization probes are added to the nitrocellulose paper to establish identity. The major limitation of the Southern technique for clinical use is that it requires large amounts of DNA and restriction endonucleases and is quite time-consuming. A modification of the Southern technique to analyze RNA, commonly known as the “Northern” technique, has become standard to establish the specificity of RNA [12]. The Northern technique has not yet been utilized in the clinical setting.

The last major heterogeneous technique utilized to detect infectious agents by NA hybridization is in situ hybridization. Here, human tissue is treated with a combination of dilute detergents, mild acid, and proteases on a nitrocellulose support. This treatment fixes the cells in their natural configuration while allowing the introduction of a labeled NA hybridization probe. Analogous to the fluorescent antibody detection
of microbial pathogens within tissue, in situ hybridization allows the direct visualization of nucleic acid sequences as they occur in vivo. This method has proven particularly useful in detecting viruses from tissue specimens. Brigati et al. [22] were able to detect parvovirus, polyomavirus, herpes simplex virus, adenovirus, and retrovirus genetic material from infected cell cultures. They further demonstrated the presence of herpes virus and adenovirus DNA in paraffin-embedded autopsy tissue; they utilized a biotin-labeled DNA probe. Myerson et al. could detect cytomegalovirus in open lung biopsies within 24 hours. They constructed their probe from seven different cytomegaloviruses to ensure that no member of this genus would be missed [33,34].

In situ hybridization will most likely find its greatest applicability in the research environment. It is too labor-intensive and yields too little quantitative information for clinical utility.

CLINICAL UTILITY OF NUCLEIC ACID HYBRIDIZATION PROBES

From the first description of type II restriction endonucleases in 1975 until the early 1980s, the clinical utility of NA hybridization probes was in the developmental stages. Within the last three years, methods have been developed to translate the basic research to tests which can be utilized in the clinical laboratory. The technology is rapidly developing with any given procedure and limits of detection likely to become rapidly historical.

In 1982 Chou and Merrigan reported the detection and quantitation of cytomegalovirus from human urine specimens, utilizing DNA hybridization with 32P [26]. They were able to detect as few as 10^3 viruses per milliliter of urine. Ward and his associates were able to detect herpes simplex virus directly from clinical lesions, utilizing a dot-blot hybridization technique [22]. Torres et al. [66] have detected 10^5 Haemophilus influenzae from cerebrospinal fluids, utilizing 32P-labeled DNA hybridization probes. Redfield et al. [35] quantitated herpes virus from human lesions. Autoradiographs of the hybridization of 32P-labeled nucleic acid hybridization probes of a typical assay are presented in Fig. 4. DNA was extracted by centrifugation at 10,000 g followed by the addition of 0.3 M sodium hydroxide, heating for one hour at 60°C, and the addition of 2 M ammonium acetate. The specimens were dotted on to nitrocellulose paper. The nitrocellulose blots were air-dried and baked for two hours at 80°C before hybridization. The probe was a DNA BAMH1 restriction endonuclease fragment with a molecular weight of approximately 7 x 10^6. It was cloned in plasmid pRB131. Their technique had a sensitivity of 78 percent and a specificity of 100 percent compared to standard viral culture. No hybridization was observed with related viruses.
TABLE 7
DNA Hybridization Assays with Potential for Diagnostic Application

| Pathogen                         | Probe                                      | Specimen Examined       | Reference |
|----------------------------------|--------------------------------------------|-------------------------|-----------|
| Enterotoxin-producing E. coli    | Heat-labile and heat-stable toxin genes    | Stool isolates          | [57, 58]  |
| Gonococcus                       | Gonococcus cryptic plasmids                | Swabs of male urethra   | [28]      |
| Cytomegalovirus                  | Selected viral fragments                   | Urine                   | [26, 60]  |
|                                  |                                            | Buffy-Coat Cells        | [60]      |
|                                  |                                            | Lung Tissue             | [60]      |
| Epstein-Barr virus               | Viral DNA                                  | Various infected lymphoid cell lines | [62] |
| Herpes simplex virus             | Thymidine kinase gene and viral DNA fragments | Viral isolates          | [27]      |
| Adenovirus                       | Two probes used in a double-sandwich assay | Swabs of nasopharynx   | [32]      |
| Hepatitis B virus                | Viral DNA                                  | Stool specimens         | [63]      |
|                                  |                                            | Liver                   | [64]      |
|                                  |                                            | Serum                   | [64, 65]  |

There are no currently available commercial kits to diagnose infectious diseases by NA hybridization, although intense effort is under way to produce them. Table 7 presents hybridization assays that have been utilized to diagnose infectious diseases from clinical specimens under field conditions [8,26,28,57,58,59,60,61,62,63,64,65].

The major hurdle to overcome before NA hybridization is available to the clinical laboratory is the translation of the basic, one-test-at-a-time research into procedures capable of being performed in large numbers with minimal human interpretation. In order for the nucleic acid hybridization techniques to be clinically useful, several modifications from published procedures must occur. First, $^{32}$P must be replaced with a colorimetric system; the colorimetric developer must be rapid enough to ascertain positivity within two to four hours. Second, the nucleic acid hybridization probe must have a sensitivity of $10^3$ organisms/ml; currently, the limit of detection is approximately $10^5$/ml. Third, the hybridization probe, whether DNA or RNA, must be broad enough to detect all members of a given species; for example, the hybridization probe mixture used by Chou and Merrigan [26] to detect cytomegalovirus contained probes against seven different strains of this virus. Fourth, probes must be developed to detect resistance to antibiotics; one must learn both the name of the pathogen and which drugs are active against it. Fifth, homogeneous assays must be developed to replace currently employed heterogeneous technologies; homogeneous assays are much less costly and labor-intensive. Sixth, because a single probe can detect only one parameter, instruments must be developed to automate the very large number of procedures that must be performed on each clinical specimen. If, for example, one wishes to examine cerebrospinal fluid, one would have to test probes for a large number of bacteria, viruses, and yeasts plus many antibiotic resistance factors; this large battery of tests virtually precludes manual performance.

**MONOCLONAL ANTIBODIES**

It is beyond the scope of this paper to present in detail the manner by which monoclonal antibodies are produced [41]. Table 1 presents the essential steps in
monoclonal antibody production and compares them with the parallel steps used to produce NA hybridization probes. The reader is referred to the review by Engleberg and Eisenstein [8] for further details.

Although the technology to produce in large amounts monoclonal antibodies against selected antigens dates from the mid-1970s, immunochemistry as a science dates to the 1930s. By the time means to make monoclonal antibody was discovered, the knowledge to purify and produce antibody-based laboratory kits was well established. Accordingly, while there is still much to be learned about the translation of research data to clinical applicability, monoclonal antibody technology is in a significantly more advanced state than NA hybridization. Commercial kits utilizing monoclonal antibodies for the diagnosis of infectious diseases became available in 1983 [42].

Analogous to NA hybridization, both the strength and the weakness of monoclonal antibody technology lie in its extreme specificity. A monoclonal antibody against an epitope in a highly variable portion of a pathogen's surface, like hybridization done under extreme stringency conditions, will not likely recognize related members of the same species. Therefore, both technologies can require multiple monoclonal clones to detect a particular species of pathogen [43].

A second limitation of monoclonal antibody technology lies in the reactivity of the antibody itself. The combination of antigen with antibody is a primary reaction, which we do not directly measure. We assay a secondary product of this interaction such as precipitation, agglutination, and the like. It has been found that the avidity constants of monoclonal antibodies to epitopes are of a relatively low order of magnitude, thus limiting the types of detection systems one may employ. In the same vein, a particular monoclonal antibody may react well in one secondary system, such as a radioimmunoassay, but not at all in another system, such as complement fixation. This limitation is particularly important for the clinical laboratory because one ideally would want to develop a central technology, such as enzyme-linked immunosorbent assay (ELISA), to process the large number of tests required with monoclonal screenings.

Third, unlike NA hybridization, where one can select a particular base pair sequence specific for an organism, microbes may have large numbers of different antigens capable of eliciting monoclonal antibody responses. While these large numbers of epitopes enhance the likelihood that one could be found specific for a given species, it also increases the likelihood that two or more species may share a particular antigenic determinant. Fourth, because these antigenic sites are so small, the theoretical possibility exists that they may be more prone to phenotypic variation. This variation could occur not only by mutation but also because of the growth environment, nutrient mix, atmospheric conditions, and the loss or acquisition of plasmids. Fifth, antigens useful for in vitro testing may not be expressed or may be hidden in vivo [43].

A wide variety of heterogeneous and homogeneous immunological assay procedures have been developed in the last ten years for both polyclonal and monoclonal antibody technology. For the diagnosis of infectious diseases, the first widely utilized technique for the detection of pathogens in body fluids was counterimmunoelectrophoresis, developed in the early 1970s. This procedure was replaced by latex particle agglutination and co-agglutination tests in the early 1980s. Latex agglutination utilizes polystyrene beads to which a monoclonal antibody is adsorbed in the Fc region. Co-agglutination utilizes Staphylococcus aureus that has protein A on its surface. Protein A binds the Fc portion of IgG. With both latex and co-agglutination
techniques, the body fluid is mixed with a suspension of the antibody-coated particles and, if antigen is present, agglutination occurs in minutes. The reactions are read manually. Clinical studies utilizing particle agglutination technology have recently been published and appear to be between five and ten times more sensitive than counterimmunoelectrophoresis methods. Counterimmunoelectrophoresis, which has been well studied with many bacterial pathogens for a decade, can detect between $10^4$ and $10^5$ bacteria/ml (refer to Table 8) [42].

Particle agglutination technology is not amenable to automation and is labor-intensive. Furthermore, the large battery of individual procedures required by monoclonal antibody technology limit its usefulness in the routine clinical laboratory. Accordingly, the research thrust has been to develop instrumented homogeneous enzyme immunoassay tests. Enzyme immunoassays for *Haemophilus influenzae* type b have been developed and can detect 1 ng/ml of the polyribophosphate antigen [44,45,46,47]. ELISA procedures have also been described for other agents of meningitis, including *Streptococcus pneumoniae*, *Neisseria meningitidis*, and group B *Streptococcus* [48,49,50,51]. These assays are in development. Like NA hybridization, workers are experimenting to automate the large number of individual procedures that must be performed on a clinical specimen by monoclonal antibody technology.

Enzyme labels useful for monoclonal antibodies are also useful in the secondary detection systems and automated instruments for NA hybridization. Because the time and intensity of color development is currently the rate-limiting step for both technologies, a large number of enzymes and physico-chemical conditions have been studied. Table 9 presents those enzymes showing the most promise with the conditions under which they are optimally active and the means by which they are detected [52].

The clinical utility of monoclonal antibodies in infectious diseases has centered in five major areas. First, and of most immediate clinical utility, is the identification of specific antigens for diagnostic purposes. A major limitation of monoclonal antibody technology is its inability to recognize antibiotic resistance. Second has been the elucidation of specific antigens to establish if two organisms are the same or different. This application for grouping has been most actively pursued in the field of epidemiology. Third, considerable work has been done on the mechanisms whereby microbial surface structures play roles in virulence. Monoclonal antibodies may be useful in treating a variety of infections and microbial toxicoses [53]. Fourth, monoclonal antibodies have proven useful in studying the transport of materials into and out of cells.
### TABLE 9
Enzymes Commonly Used as Labels for Enzyme Immunoassays

| EIA           | Enzyme                   | Source            | pH Optimum | Specific Activity at 37°C/units mg⁻¹ | $K_m$     | Relative Molecular Mass | End Point                                      |
|---------------|--------------------------|-------------------|------------|-------------------------------------|-----------|------------------------|------------------------------------------------|
| Heterogeneous | Acetylcholinesterase     | Electrophorus electricus | 7.0–8.0    | 1,400                               | 90 µm     | 54,000                 | pH electrode or spectrophotometric              |
|               | Adenosine deaminase      | Calf intestine    | 7.5–9.0    | 200                                 | 60 µm     |                        | ammonia gas-sensing electrode                  |
|               | Alkaline phosphatase     | Calf intestine    | 8.0–10.0   | 1,000                               | 0.2 mM for PNPP | 100,000 | Spectrophotometric or fluorimetric |
|               | Catalase                 | Calf liver        | 6.0–8.0    | 40,000                              | *         |                        | UV absorption or thermometry                    |
|               | B-galactosidase          | Escherichia coli | 6.0–8.0    | 600                                 | 1 mM      | 250,000                | Spectrophotometric or fluorimetric              |
|               | Glucose oxidase          | Aspergillus niger | 4.0–7.0    | 200                                 | $K_m^{\text{GLU}} = 33$ mM; $K_m^{\text{O$_2$}} = 0.2$ mM | 186,000 | H$_2$O$_2$ combines with chromogen |
|               | Peroxidase               | Horseradish       | 5.0–7.0    | 4,500                               | 10 mM     | 40,000                 | H$_2$O$_2$ combines with chromogen              |
|               | Urease                   | Jack beans        | 6.5–7.5    | 10,000                              | 10 mM     | 483,000                | Ammonia reacts with chromogen or gas-sensing electrode |
| Homogeneous   | Acetylcholinesterase     | Electrophorus electricus | 7.0–8.0    | 1,400                               | 90 µm     | 54,000                 | pH electrode or spectrophotometric              |
|               | B-galactosidase          | Escherichia coli | 6.0–8.0    | 600                                 | 1 mM      | 540,000                | Spectrophotometric or fluorimetric              |
|               | Glucose 6-phosphate dehydrogenase | Leuconostoc mesenteroides | 7.8      | 400                                 | $K_m^{\text{GDP}} = 0.1$ mM | 104,000 | Formation of NADH by UV absorption or fluorimetric |
|               | Lysozyme                 | Chicken egg white | 4.5–5.5    | —                                   | *         | 14,500                 | Formation of cell wall fragments (ΔA at 450 nm) |
|               | Malate dehydrogenase     | Pig heart         | 8.5–9.5    | 1,000                               | $K_m^{\text{Mal}} = 0.3$ mM; $K_m^{\text{NAD}} = 0.1$ mM | 70,000 | Formation of NADH |

*$K_m$ depends on substrate.
The clinical utility of monoclonal antibodies in diagnosis has thus far not significantly displaced polyclonal tests. Monoclonal antibody tests are commercially available for the direct detection of group A *Streptococcus* from throat cultures and chlamydia from urethritis. Monoclonals have also been utilized to aid in the identification of microorganisms after they have been grown *in vitro*. Monoclonals have proven more useful than polyclonals for this purpose, particularly for *Neisseria gonorrhoeae*, *Legionella*, chlamydia [8], and herpes [54].

**COMPARISON OF NUCLEIC ACID HYBRIDIZATION AND MONOCLONAL ANTIBODY TECHNOLOGIES FOR THE DIAGNOSIS OF INFECTIOUS DISEASES**

There are no published studies directly comparing a monoclonal antibody and an NA hybridization technique for the diagnosis of the same pathogen in a clinical setting. As mentioned above, polyclonal antibody systems have been utilized to detect pathogens directly from body fluids since the early 1970s. These characteristics are well established [42,55,56]. Monoclonal tests will certainly be at least as sensitive as polyclonal systems. It is too early to predict the sensitivities and specificities of NA hybridization tests when they reach the clinical laboratory. Therefore, monoclonal antibody and nucleic acid hybridization technologies are in unequal states of development. One should expect much greater change in nucleic acid hybridization technology compared with monoclonal antibody techniques. The primary research and development of detection systems for monoclonals lie in the production of simple instruments with appropriate enzyme labels for measuring reactivity. NA hybridization has much further to go. For example, it has not yet been decided which nucleic acid to choose to analyze. Furthermore, the system for releasing nucleic acid from the infecting microbe is not yet satisfactory. Before instrumentation can be developed, these major obstacles must be overcome.

A barrier impeding the rapid development of NA hybridization procedures that did not exist in the early 1970s for the development of immunochemical methodologies is the paucity of published information. The majority of work in the NA hybridization field is proprietary and is not being published. It may be that many of the above-mentioned problems have been solved, perhaps solved several times in different places, but are not available to the independent scientist. Progress will be hindered by this lack of public information. The intermediate steps necessary before a final product is generated will have to be discovered, analyzed, and overcome in multiple individual settings. This massive redundancy of scientific investigation is unprecedented.

The technology presented to the clinical laboratory for NA hybridization diagnosis will probably use a combination of nucleic acid probes and monoclonal antibodies. DNA can be labeled with a hapten, following the work of Ward et al. and Tchen et al., to produce the hybridization probe. The probe itself, after reacting with the pathogen’s nucleic acid, can be detected by monoclonal antibody directed to the hapten. Attached to the monoclonal antibody would be an enzyme label, which could be detected in a homogeneous system with inexpensive instrumentation. The procedure, analogous to the standard microtiter tray antibody assays, would be performed in small wells, reducing the cost and quantities of reagents. Each well would detect one genetic element. For each specimen the wells could be inoculated, incubated, and read by the instrument.
The clinical laboratory's choice to use either NA hybridization or monoclonal antibody will not be an either/or one. Some microbial pathogens will probably be more amenable to diagnosis by one technology than the other. The technology that can provide the greatest amount of clinically useful information with the fewest procedures or individual analyses required should achieve primacy.

REFERENCES

1. Marmur J, Doty P: Determination of the base composition of DNA from its thermal denaturation temperature. J Mol Biol 5:109, 1962
2. Schildkraut CL, Lifson S: Dependence of the melting temperature of DNA on salt concentration. Biopolymers 3:195, 1965
3. McConoughy JL, Laird CD, McCarthy MJ: Nucleic acid reassociation in formamide. Biochemistry 8:3284, 1965
4. Wetmur J, Davidson N: Kinetics of renaturation of DNA. J Mol Biol 31:349, 1968
5. Cedarbaum SD: Introduction to recombinant DNA. Pediatrics 74:408-411, 1984
6. Grimont PAD, Grimont F, Desplaces N, Tchen P: DNA probe specific for Legionella pneumophila. J Clin Microbiol 21:431-437, 1985
7. Torres AR, Li MK, Ward DC, Edberg SC: Differentiation of Neisseria gonorrhoeae from other Neisseria species by use of the restriction endonuclease HaeIII. J Clin Microbiol 20:687-690, 1985
8. Engleberg NC, Eisenstein BI: The impact of new cloning technique on the diagnosis and treatment of infectious diseases. New Eng J Med 311:892-901, 1984
9. Maniatis T, Jeffrey A, Klieg DG: Nucleotide sequence of the rightward operator of phage. Proc Natl Acad Sci USA 72:1184, 1975
10. Rigby PW, Dieckman PW, Rhodes C, Berg P: Labelling deoxyribonucleic acids to high specific activity in vitro by nick translation with DNA polymerase. J Mol Biol 113:237, 1977
11. Langer PR, Waldrop AA, Ward DC: Enzymatic synthesis of biotin-labelled polynucleotides: Novel nucleic acid affinity probes. Proc Natl Acad Sci USA 78:6633, 1981
12. Unger ER, Leary JJ, Ward DC, Brigati DJ: Application of nucleic acid hybridization in clinical virology. Personal communication
13. Bayer EA, Wilchek M: The use of the avidin-biotin complex as a tool in molecular biology. Methods Biochem Anal 26:1-45, 1980
14. Hsiu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and Unlabelled antibody (PAP) procedures. J Histochem Cytochem 29:577, 1981
15. Leary JJ, Brigati DJ, Ward DC: A sensitive colorimetric method for visualizing biotin-labeled probes hybridized to DNA or RNA on nitrocellulose filters. Chromosomes and Cancer 15:273-290, 1983
16. Lefevre JF, Fuchs RPP, Daune MP: Comparative studies on the 7-ido and 7-fluoro derivatives of N-acetoxy-N-2-acetylaminofluorene: binding sites on DNA and conformational change of modified deoxyribonucleotides. Biochemistry 17:2561-2567, 1978
17. Tchen P, Fuchs RPP, Sage E, Leng M: Chemically modified nucleic acids as immunodetectable probes in hybridization experiments. Proc Natl Acad Sci USA 81:3466-3470, 1984
18. Green MR, Maniatis T, Melton DA: Human B-globulin pre-mRNA synthesized in vitro is accurately spliced in Xenopus Oocyte Nuclei. Cell 32:681, 1983
19. Zinn K, DeMaio D, Maniatis T: Identification of two distinct regulatory regions adjacent to the human B-interferon gene. Cell 34:865, 1983
20. Melton DA, Krieg P, Green MR: Increased sensitivity in Northern and Southern blotting methods using RNA probes synthesized in vitro with SP6 RNA polymerase. Cell, in press
21. Casna NJ, Shub DA: Bacteriophage T4 as a generalized DNA-cloning vehicle. Gene 18:297, 1982
22. Brigati DJ, Myerson D, Leary JJ, Spalholz B, Travis SZ, Fong CKY, Hsiung GD, Ward DC: Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. Virology 126:32-50, 1983
23. Leary JJ, Brigati DJ, Ward DC: Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bioblots. Proc Natl Acad Sci USA 80:4045-4049, 1983
24. Weller IVD, Fowler JF, Monnardino J, Thomas HC: The detection of HBV-DNA in serum by
molecular hybridization: A more sensitive method for the detection of complete HBV particles. J Med Virolology 9:273, 1982
25. Scotto J, Hadchouel M, Hery C, Yvart J, Tiollais P, Brechot C: Detection of hepatitis B virus DNA in serum by a simple spot hybridization technique: comparison with results for other viral markers. Hepatology 3:279, 1983
26. Chou S, Merrigan TC: Rapid detection and quantitation of human cytomegalovirus in urine through DNA hybridization. New Eng J Med 308:921–925, 1982
27. Stanhandske P, Pettersson U: Identification of DNA viruses by membrane filter hybridization. J Clin Microbiol 15:299, 1982
28. Totten PA, Holmes KK, Handsfield HH, Knapp JS, Perine PL, Falkow S: DNA hybridization technique for the detection of Neisseria gonorrhoeae in men with urethritis. J Infect Dis 148:462–471, 1983
29. Gillespie D, Bresser J: mRNA immobilization in NaI: Quick-bLOTS. BioTechniques (November/December):184–192, 1983
30. Bresser J, Doering J, Gillespie D: Laboratory Methods: Quick-blot: Selective mRNA or DNA immobilization from whole cells. DNA 2:243–254, 1983
31. Ranki M, Palva A, Virtanen M, Laaksonen M, Soderlund H: Sandwich hybridization as a convenient method for the detection of nucleic acids in crude samples. Gene 21:75–77, 1983
32. Virtanen M, Laaksonen M, Soderlund H, Palva A, Halonen P, Ranki M: Novel test for rapid viral diagnosis in nasopharyngeal mucus aspirates by means of nucleic-acid sandwich hybridization. Lancet i:381–383, 1983
33. Unger ER, Leary JJ, Ward DC, Brigati DJ: Application of nucleic acid hybridization in clinical virology. Personal communication
34. Myerson D, Hackman RC, Meyers JD: Diagnosis of cytomegaloviral pneumonia by in situ hybridization. J Infect Dis 150:272–277, 1984
35. Redfield DC, Richman DD, Albanil S, Oxman MN, Wahl GM: Detection of herpes simplex virus in clinical specimens by DNA hybridization. Diagn Microbiol Infect Dis 1:117–128, 1983
36. Bauman JG, Wiegant J, van Duijn P: Cytochemical hybridization with fluorochrome labeled RNA. J Histochem Cytochem 29:238, 1981
37. Manuelleidis L, Langer-Safer PR, Ward DC: High resolution mapping of satellite DNA using biotin-labeled DNA probes. J Cell Biol 95:619, 1982
38. Bauman JGJ, Wiegant J, van Duijn P: Cytochemical hybridization with fluorochrome-labelled RNA. III. Increased sensitivity by anti-fluorescein antibodies. Histochemistry 73:181, 1981
39. Grunstein M, Hogness DS: Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc Natl Acad Sci USA 72:396, 1975
40. Andiman W, Gradoville L, Heston L, Neydorff R, Savage ME, Kitchingman G, Shedd D, Miller G: Use of cloned probes to detect Epstein-Barr viral DNA in tissues of patients with neoplastic and lymphoproliferative diseases. J Inf Dis 148:967, 1983
41. Kohler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497, 1975
42. Yolken RH: Use of monoclonal antibodies for viral diagnosis. Curr Top Microbiol Immunol 104:177–195, 1983
43. Blake C, Gould BJ: Use of enzymes in immunoassay techniques: a review. Analyst 109:533–547, 1984
44. Crosson FJ, Winkelstein JA, Moxon ER: Enzyme-linked immunosorbent assay for detection and quantitation of capsular antigen of Haemophilus influenzae type b: Infect Immun 22:617–619, 1978
45. Pepple J, Moxon ER, Yolken RH: Indirect enzyme-linked immunosorbent assay for the quantitation of the type-specific antigen of Haemophilus influenzae b: a preliminary report. J Pediatr 97:233–237, 1980
46. Drow DL, Maki DG, Manning DD: Indirect sandwich enzyme-linked immunosorbent assay for rapid detection of Haemophilus influenzae type b infection. J Clin Microbiol 10:442–450, 1979
47. Wetherall BL, Hallsworth PG, McDonald PJ: Enzyme-linked immunosorbent assay for detection of Haemophilus influenzae type b antigen. J Clin Microbiol 11:573–580, 1980
48. Harding SA, Scheld WM, McGowan MD, Sande MA: Enzyme-linked immunosorbent assay for detection of Streptococcus pneumoniae antigens. J Clin Microbiol 10:339–342, 1979
49. Sippel JE, Voller A: Detection of Neisseria meningitidis cell envelope antigen by enzyme-linked immunosorbent assay in patients with meningococcal disease. Trans R Soc Trop Med Hyg 74:644–648, 1980
50. Polin RA, Kenneth R: Use of monoclonal antibodies in an enzyme-linked inhibition assay for rapid detection of streptococcal antigen. J Pediatr 97:540–544, 1980
51. Yolken RW: Enzyme immunoassays for the detection of infectious antigens in body fluids: current limitations and future prospects. Rev Infect Dis 4:35–68, 1982
52. Porterfield JS, Tobin JOH: Viral and bacterial infectious diseases. Brit Med J 40:283–290, 1984
53. Hansen EJ, Robertson SM, Gulig PA, Frisch CF, Haanes EJ: Immunoprotection of rats against Haemophilus influenzae type b disease mediated by monoclonal antibody against a Haemophilis outer-membrane protein. Lancet i:366–368, 1982
54. Goldstein LC, Corey L, McDougall JK, Tolentino E, Nowinski RC: Monoclonal antibodies to herpex simplex viruses: use in antigenic typing and rapid diagnosis. J Infect Dis 147:829–837, 1983
55. Kaplan SL: Antigen detection in cerebrospinal fluid—pros and cons. Amer J Med (July 28):109–118, 1983
56. Coonrod JD: Urine as an antigen reservoir for diagnosis of infectious diseases. Amer J Med (July 28):85–92, 1983
57. Moseley SL, Huq I, Alim ARMA, So M, Samadpour-Montelevi M, Falkow S: Detection of enterotoxigenic Escherichia coli by DNA colony hybridization. J Infect Dis 142:892–899, 1980
58. Moseley SL, Echeverria P, Seriwatana J, et al: Identification of enterotoxigenic Escherichia coli by colony hybridization using three enterotoxin gene probes. J Infect Dis 145:863–869, 1982
59. Hill WE, Madden JM, McCardell BA, et al: Foodborne enterotoxigenic Escherichia coli: detection and enumeration by DNA colony hybridization. Appl Environ Microbiol 45:1324–1330, 1983
60. Marlowe S, Watkins P, Kowalsky P, Hirsch M, Crumpaker C: Rapid detection of CMV infection and replication by DNA-DNA hybridization. Presented at the 23rd ICAAC, Las Vegas, October 24–26, 1983
61. Spector SA, Rus JA, Spector DH, McMillan R: Detection of human cytomegalovirus in clinical specimens by DNA-DNA hybridization. J Infect Dis 150:121–126, 1984
62. Brandsma J, Miller G: Nucleic acid spot hybridization: rapid quantitative screening of lymphoid cell lines for Epstein-Barr Viral DNA. Proc Natl Acad Sci USA 77:6851–6855, 1980
63. Stanhandske P, Hyypia T, Gadler H, Halonen P, Pettersson U: The use of molecular hybridization for demonstration of adenoviruses in human stools. Curr Top Microbiol Immunol 104:299–306, 1983
64. Brechot C, Hadchouel M, Scotto J, et al: Detection of hepatitis B virus DNA in liver and serum; a direct appraisal of the chronic carrier state. Lancet ii:765–768, 1981
65. Berninger M, Hammer M, Hoyer B, Gerin JL: An assay for the detection of the DNA genome of hepatitis B virus in serum. J Med Virol 9:57–68, 1982
66. Torres AR, Li M, Edberg SC, Ward DC: Unpublished observations, data on file