Nucleic Acid *In-situ* Hybridization Detection of Infectious Agents

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abstract

Limitations of traditional culture methods and newer polymerase chain reaction (PCR)-based methods for detection and speciation of infectious agents demonstrate the need for more rapid and better diagnostics. Nucleic acid hybridization is a detection technology that has gained wide acceptance in cancer and prenatal cytogenetics. Using a modification of the nucleic acid hybridization technique known as fluorescence *in-situ* hybridization (FISH), infectious agents can be detected in a variety of specimens with high sensitivity and specificity. The specimens derive from all types of human and animal sources including body fluids, tissue aspirates and biopsy material. Nucleic acid hybridization can be performed in less than one hour. The result can be interpreted either using traditional fluorescence microscopy or automated platforms such as microarrays. This paper demonstrates proof of concept for nucleic acid hybridization detection of different infectious agents. Interpretation within a cytologic and histologic context is possible with fluorescence microscopic analysis, thereby providing confirmatory evidence of hybridization. With careful probe selection, nucleic acid hybridization promises to be a highly sensitive and specific practical diagnostic alternative to culture, traditional staining methods, immunohistochemistry and complicated nucleic acid amplification tests

Keywords: Detection, diagnostics, DNA, infection, infectious, *in-situ* hybridization, laboratory, leishmania, mycobacteria, mycobacterium, micro-organisms, molecular, mycobacteria, mycoplasma, nucleic acid, RNA, pneumonia, trypanosome, trypanosomiasis, tuberculosis

1. INTRODUCTION

Each year, two-thirds of the world's human population is afflicted with life-threatening infectious diseases. In addition, the agriculture industry loses millions of dollars due to infectious diseases that target animals, requiring thousands to be sacrificed. Existing detection technologies are often insensitive, much of the time leaving the caretaker without a secure diagnosis or treatment plan. Early and accurate diagnosis is important in the successful treatment of many diseases such as tuberculosis.[1]

Current diagnostic techniques are limited for many infectious agents. Traditional culture techniques, while are often highly sensitive, can take a very long time to grow. This is especially problematic in the diagnosis of tuberculosis where a delay in diagnosis can result in the spread of the infection and an increase in morbidity. In addition, it is often not possible in the standard clinical laboratory to culture many infectious agents, such as *Entameba histolytica*, a common cause of diarrhea in travelers. Many infectious agents can be identified using monoclonal antibodies, however, these antibodies often suffer from poor specificity such as in the diagnosis of mycoplasma ("walking") pneumonia. Similarly, in amebiasis antibody tests do not differentiate between *E. histolytica* and *E. dispar,* the former of which causes disease and the latter of which does not.

Polymerase chain reaction (PCR)-based methods have increased sensitivity over traditional staining methods. These nucleic acid "amplification" techniques, however, can have a significant false-positive rate caused by cross-contamination or residual DNA from a prior infection. In addition, endogenous inhibitors of the PCR reaction are often present within specimen samples. For example, in the Gen-Probe Amplified Mycobacteria test, a test used to quickly verify the diagnosis of tuberculosis in patients with acid fast bacilli identified in their sputum, up to 80% of the patients have identifiable

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endogenous inhibitors.[2] Up to 5% of the patients demonstrate a false-negative test. Variable sensitivities, the significant rate of false-positive results, and the highly technical aspects of the PCR technique currently limit its clinical utility and widespread use.[3,4] This is especially true for detection in formalin-fixed, paraffin-embedded tissue.[5-9]

Clearly, better diagnostics need to be explored for detection and speciation of infectious agents. Nucleic acid *in-situ* hybridization (ISH) is being used with increasing frequency for a variety of applications. Figure 1 below illustrates ISH.

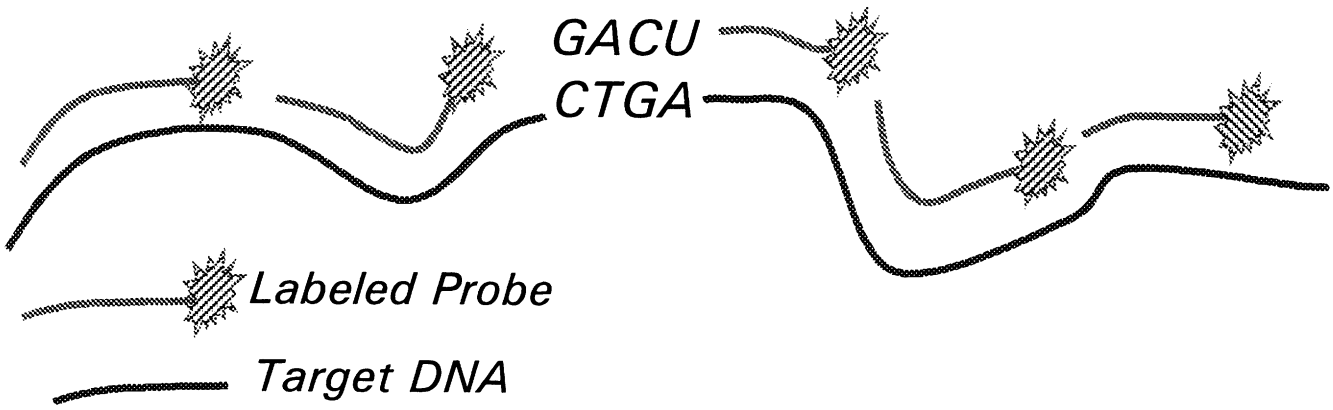

Figure 1. In-situ Hybridization (ISH). The figure shows how labeled DNA probes specifically hybridize to target DNA through traditional Watson-Crick base-pairing. The tag on the DNA probe can be seen through either secondary labeling (i.e. biotin-avidin interaction) or through direct labeling with a molecule, often a fluorochrome, that can be detected with an instrument plaform (*i.e.*, fluorescence microscope).

ISH offers multiple advantages including detection of hybridization without the use of radionucleotides and simultaneous detection of multiple DNA targets using multiple DNA probes labeled with different fluorochromes, each with separable emission spectra. With an appropriate counterstain, correlation of hybridization signals with cytologic and histologic features is possible. This correlation provides additional confirmation of a positive test. Fluorescence ISH (FISH) detection has an added advantage in that the background signal is reduced such that single infectious agents can be detected with certainty. This review demonstrates proof of concept for the detection of various infectious agents in different specimen samples.

2. TECHNIQUE

2.1 Specimens

A variety of specimens sources have been examined as follows:

- Body fluids including sputum and stool
- Biopsy material--formalin-fixed, paraffin-embedded biopsy tissue sections. All clinical specimens presented in this review were obtained in accordance with guidelines established by the University of California, San Francisco and the University of New Mexico Committees on Human Research. Tissue from mice was obtained under guidelines established by the University of New Mexico Committee on Animal Tissue Research.

2.2 Probe generation

The DNA probe for each infectious agent was derived from different sources. All probe demonstrated in this review was derived from the entire genome of the infectious agents. The genomic DNA was obtained from culture of different infectious agents. The DNA was purified and directly labeled using nick translation (Gibco BRL, Gaithersburg, MD). The labeling method was performed according to the manufacturers' instructions with modifications of the reagents to incorporate FITC-

dUTP (Boehringer Mannheim Corp., Indianapolis, IN), SpectrumRed-dUTP (Vysis, Inc., Downers Grover, IL) or Alexa532-dUTP (Molecular Probes, Inc., Eugene, OR) instead of biotin-dUTP. An internal control targeting the chromosome 8 alpha satellite probe directly labeled with FITC was used on human samples (Vysis, Inc.).

2.3 Specimen Preparation

Body fluid smears were dispersed and filtered as needed. Smears were placed on commercially available "positively charged" slides commonly used in immunohistochemistry. The smears were fixed in freshly prepared Carnoy's solution (methanol:acetic acid 3:1). The slides were washed in water and dehydrated through a series of 70%, 85% and 100% graded ethanol baths.

Tissue sections were incubated in undiluted xylenes (Sigma, St. Louis, MO) twice for 10 minutes at room temperature, and then for 30 min at 56^{0}C to remove paraffin and then washed in 100% ethanol. Different tissue sources required different protease digestion times. All tissue was incubated with 1 M NaSCN for 10 minutes at 80^{0}C to begin protein digestion and to reduce protease treatment time. After a wash in water, the tissue was digested with a protease. If a tissue had not been previously used, different sections were treated with different digestion times to optimize the digestion.

2.4 Hybridization

All specimens were hybridized using the labeled-genomic probe. Slides were placed in 70% formamide (Ultrapure Grade, Gibco BRL, Gaithersburg, MD), 2X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% polyoxyethylenesorbitan monolaurate [Tween 20] (Sigma, St. Louis, MO) for up to 1 hour at room temperature, drained, warmed to 70^{0}C, and incubated in 70% formamide/2X SSC, pH 7 for 5 min at 72^{0}C to denature DNA. Slides were cooled to 37^{0}C and blotted dry.

Thirty to 50 µl of a hybridization solution containing the labeled probe (at a final concentration of 0.67 ng/µl of probe in 50% formamide/2X SSC, pH 7.0) was denatured at 72^{0}C for 5 minutes and then dropped onto the slides. Slides were coverslipped, sealed with rubber cement, and incubated overnight at 37^{0}C. The following morning, the rubber cement was removed, coverslips floated off in 1X SSC at room temperature, slides warmed to 60-70^{0}C and washed in 1X SSC for 5 min at 72^{0}C, 2X SSC for 5 min at 37^{0}C, 2X SSC for 5 min at room temperature, and distilled deionized water for 5 min at room temperature. Slides were dehydrated for 2 min each in a series of 70%, 85% and 100% graded ethanol baths and dried completely at 37^{0}C. Slides were counterstained with propidium iodide (0.2 g/ml) in an antifade solution (Sigma; 1% p-phenylenediamine dihydrochloride in 90% glycerol/PBS, pH 7.0), coverslipped, and the edges of the coverslip sealed with clear nail polish.

2.5 Microscopic examination

The hybridized slides were microscopically examined with an epifluorescence Olympus microscope equipped with single and dual band pass filters that allow signal detection within a histologic context. Images were captured using a cooled CCD camera attached to an imaging system (Vysis QUIPS). The slides were scanned using a final 100X magnification, and signals confirmed at 400X to 1000X magnification.

3. RESULTS

The protocol reproducibly yielded bright fluorescent signals. Different samples must be optimized for the treatments times. Some specimens required significant protease treatments (up to 60 minutes) while other specimens required no protease treatment at all. The results for each infectious agent are reported as follows:

3.1 *Mycobacterium tuberculosis*

Hybridization to tissue sections from an infected lymph node in which acid fast bacilli were identified on an acid fast stain demonstrated single bacilli and clusters of bacilli especially within the necrotic areas of the lymph node. (See Figure 2 below) This tissue had been formalin-fixed. Attempts to hybridize to cytologic preparations (sputa and cultured bacilli) that were not formalin-fixed (*e.g.*, smears of concentrated mucolysed sputa) required additional treatment. Successful hybridization for non-formalin-fixed specimens required incubation in 10% neutral buffered formaldehyde for at least 30 minutes, treatment with an undiluted organic solvent (*i.e.*, xylenes, benzene and hexane), incubation in a heated sodium

thiocyanate solution, and protease digestion. Hybridization was not adversely affected if specimens were fixed in formalin for > 12 hours. As for organic solvent treatment, all undiluted solvents tested were equally effective and heating was not necessary. Treatment with sodium thiocyanate had the added benefit of reducing the time for protease digestion.

3.2 *Mycoplasma pulmonis*

Hybridization was performed on necropsy specimens from mice infected with *Mycoplasma pulmonis*. (See Figure 2 below) Sections from the lungs showed abundant organisms within the bronchioles, especially along the edge of the bronchiole. The bacteria were present within the cytoplasm of the pneumocytes lining the bronchioles. Single bacteria were identified within the lumens of terminal alveoli.

3.3 Entameba histolytica

Hybridization was performed on a smear of human stool previously diagnosed with *E. histolytica* infection. The ameba had been identified using a traditional microscopic morphologic screen. The hybridization identified single ameba within the smear. (See Figure 2 below)

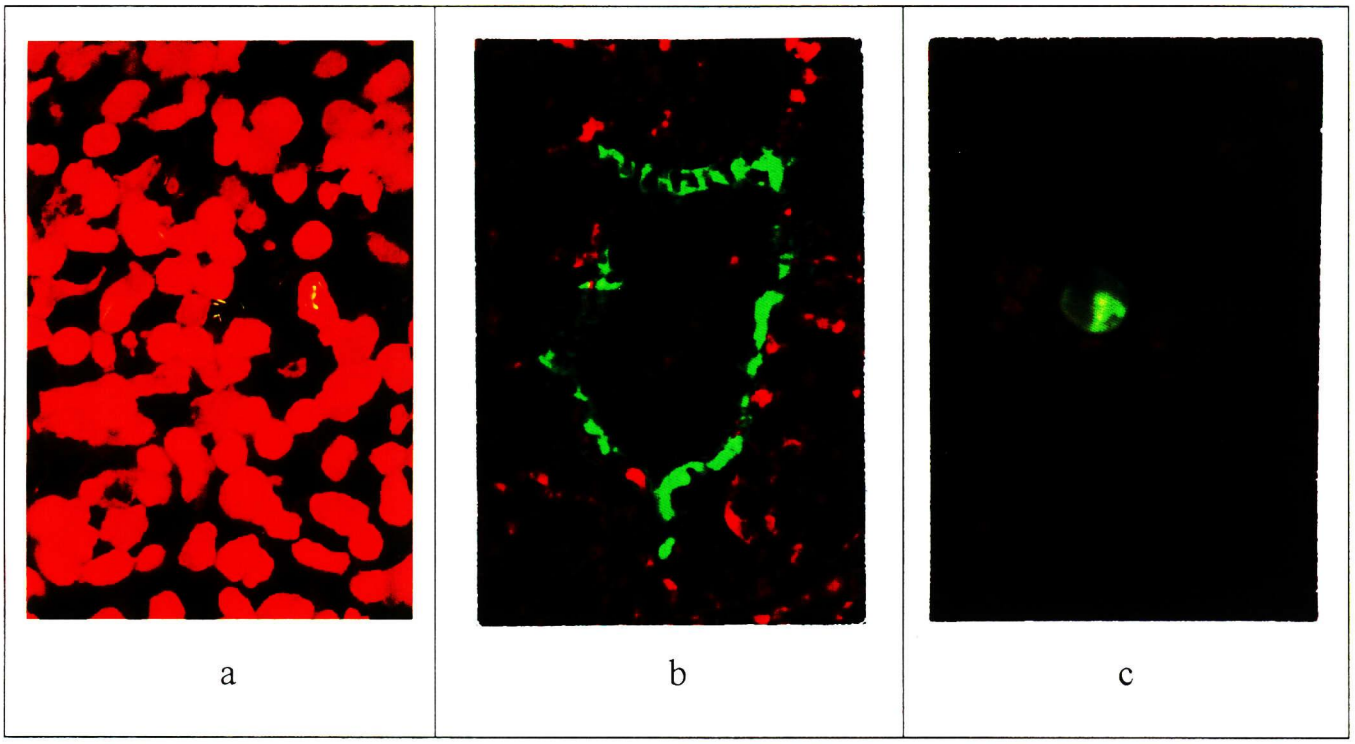

Figure 2. FISH detection of *M. tuberculosis*, *M. pulmonis*, and *E. histolytica* in infected samples.
Figure 2a shows in a formalin-fixed, paraffin-embedded tissue section of a lymph node. Single bacilli (green) are identified within the tissue. The green signal represents hybridization with a probe targeting the entire genome of *M. tuberculosis*. A counterstain with propidium iodide (red) allows interpretation within a histologic context. The lymph node is largely necrotic (1000X).
Figure 2b shows a mouse lung tissue section in which numerous *M. pulmonis* (green) is seen lining a terminal bronchiole. The green signal represents hybridization with a probe targeting the entire genome of the *M. pulmonis*. A counterstain with propidium iodide (red) shows the nuclei of the surrounding pneumocytes, stomal cells and inflammatory cells. (400X)
Figure 2c shows FISH detection of *Entameba histolytica* within a stool sample. The nuclei of the ameba (green) are easily identified. The counterstain with propidium iodide (red) highlights nuclear debris in the specimen from bacteria and sloughed colonic epithelium.

3.4 Controls

Fluorescently-labeled irrelevant *Leishmania donovoni* whole genomic probe and chromosome 8 alpha-satellite probe did not hybridize to the slides of *M. tuberculosis, M. pulmonis* and *E. histolytica*-infected specimens. The chromosome 8 probe did successfully hybridize to the centromeres of the host nuclei. The hybridization signal with the genomic MTB-FISH probe was diminished when excess unlabeled MTB genomic DNA was added to the hybridization solution. No cross hybridization between the leishmania probe and the entameba stool sample and the mycoplasma-infected mouse lung specimen was observed.

4. DISCUSSION

We have defined the experimental conditions necessary for detection of infectious agents by *in-situ* hybridization (ISH) on a variety of specimen types likely to be encountered in either the clinical and anatomic pathology laboratory. Results from this study suggest at a minimum that ISH could be a viable and rapid method by which many infectious agents could be detected. Of great import is the usefulness of this methodology on specimens for which current diagnostics are limited. These include infectious agents which do not grow well in culture and which cannot be detected with current molecular methods (antibodies of DNA amplification). This technology is especially appropriate for formalin-fixed, paraffin-embedded tissue for which culture is often not concurrently obtained.

In-situ hybridization can be performed in a limited amount of time, often less than one hour. Shorter hybridization periods are easily obtained with increased DNA probe concentration. The performance of FISH as outlined here has a 6-hour turnaround time for results. As such, this turnaround time is not yet competitive with microscopic examination of traditional acid-fast stains on non-paraffin-embedded specimens. A clear-cut advantage to the use of ISH, however, would be for the rapid speciation of mycobacteria should adaptation of a species-specific probe be successful.

Though current reviews of molecular techniques for infectious agent detection project a long-term trend toward detection through nucleic acid *in-situ* hybridization,[9-11] published studies to date have focused largely on viral detection alone.[12-14] The only currently available commercial probes for direct nucleic acid *in-situ* hybridization are for viruses such as Epstein-Barr virus and cytomegalovirus. For bacteria, *Mycobacteria species* have been detected using this technique.[15-16] Clinical application for mycobacteria detection is limited in practical application, though, because the technique does not allow assessment of drug sensitivity. Thus, traditional culture techniques are still required. For protozoa, a single publication for the detection and speciation of the parasite, microsporidia, exists.[17] Thus, little application of *in-situ* hybridization detection of bacteria and protozoa has been reported to date. Finally, peptide nucleic acids, chemically modified proteins that undergo Watson-Crick base pairing, are being applied to infectious agent detection.[18] Peptide nucleic acid hybridization could provide an alternative to DNA probe techniques, but significant issues exist regarding the ability to produce hybridization with little or no background signal.

Newer, more sensitive and cost effective methods for the direct detection of infectious agents must be implemented in accordance with an expected benefit in outcome to the patient. Current data for PCR-based assays indicate that although sensitive, the assays are expensive and relatively labor intensive. Specificity of PCR-based assays can also be problematic, even if there is strict adherence to protocols and laboratory design to avoid cross-contamination of specimens. In addition, endogenous inhibitors of the PCR reaction cause a significant rate of false negative results. In contrast, the processing step for ISH can be automated, and the microscopic examination of the final product uses skills familiar to laboratory technologists and pathologists. With batch hybridizations, the cost of FISH is similar to immunoperoxidase staining. Furthermore, unlike PCR assays, visualization of a positive FISH signal allows interpretation of that finding in the histological or cytological context of the original specimen.

With the future development of species-specific probes, FISH could provide a practical, rapid and relatively inexpensive alternative to traditional staining and culture for the diagnosis of infectious disease in a variety of clinical settings and specimen types.

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