Public Health Research Institute

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Title: Rapid Detection of Pathogens
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Overview

Pathogen identification is a crucial first defense against bioterrorism. A major emphasis of our national biodefense strategy is to establish fast, accurate and sensitive assays for diagnosis of infectious diseases agents. Such assays will ensure early and appropriate treatment of infected patients. Rapid diagnostics can also support infection control measures, which monitor and limit the spread of infectious diseases agents. Many select agents are highly transmissible in the early stages of disease, and it is critical to identify infected patients and limit the risk to the remainder of the population and to stem potential panic in the general population. Nucleic acid-based molecular approaches for identification overcome many of the deficiencies associated with conventional culture methods by exploiting both large- and small-scale genomic differences between organisms. PCR-based amplification of highly conserved ribosomal RNA (rRNA) genes, intergenic sequences, and specific toxin genes is currently the most reliable approach for bacterial, fungal and many viral pathogenic agents. When combined with fluorescence-based oligonucleotide detection systems, this approach provides real-time, quantitative, high fidelity analysis capable of single nucleotide allelic discrimination (4). These probe systems offer rapid turn around time (<2 h) and are suitable for high throughput, automated multiplex operations that are critical for clinical diagnostic laboratories. In this pilot program, we have used molecular beacon technology invented at the Public health Research Institute to develop a new generation of molecular probes to rapidly detect important agents of infectious diseases. We have also developed protocols to rapidly extract nucleic acids from a variety of clinical specimen including and blood and tissue to for detection in the molecular assays. This work represented a cooperative research development program between the Kramer-Tyagi/Perlin labs on probe development and the Perlin lab in sample preparation and testing in animal models.

Experimental Objectives Summary

Specific Aim 1. A Molecular Beacon-based panel will be constructed that includes both broad range pathogen recognition (e.g. all bacteria and fungi), as well as identification of specific pathogens and toxin genes. Universal probes capable of broadly identifying all bacteria will be generated to ensure that a previously unidentified organism is not missed.
To enhance our ability to detect recombinant pathogens we will develop primer sets and molecular beacons specifically designed to detect various virulence determinants.

Molecular beacons were designed to optimize target sequences within multicopy ribosomal genes of various pathogens. The highly conserved rRNA gene (18s) target was used to develop "universal primers" that amplify all bacteria. Each target gene sequence was converted to a molecular beacon. The optimized molecular beacons varied, but a generic molecule contained a 6 nucleotide (nt) arm sequence and a 20 nt probe target recognition sequence. The arm sequences were designed to form a stable stem hybrid at the annealing temperature of the PCR to ensure that

non-hybridized probes remained in a hairpin conformation (no fluorescence). The fluorophore tetrachloro-6-carboxyfluorescein (TET) and quencher 4-(4'-dimethylamino-phenylazo)benzoic acid (DABCYL) (Molecular Probes Inc., Eugene, OR) was covalently attached to 5' and 3' end of the arm sequences. A single color fluorophore was used initially in all molecular beacon assays to optimize conditions that facilitate detection in vitro of organisms (or DNA, depending on availability) in blood, sera and other bodily fluids. Molecular beacons were ordered commercially, and then HPLC purified in our laboratories. For quality control purposes, the fluorescence intensity of each molecular beacon was determined in the presence and in the absence of an excess of perfectly complementary target oligonucleotides over a wide temperature range. A robust signal in real-time assays within a common range with other targeted molecular beacons was a prerequisite for progression to multiplex consideration.

The Kramer-Tyagi labs have concentrated on developing a diagnostic assay that provides early warning of an attack with agents of bioterror, yet is of such general medical use that it will routinely be carried out in hospitals across America. They decided to develop an extremely rapid and sensitive diagnostic screening assay that identifies which bacterial species is causing a fever in a patient. Fever is one of the earliest symptoms of bacterial infection. The new aasays identifies 15 of the most common bacterial species present in a blood sample. These agents include: *Clostridium perfringens, Streptococcus pneumoniae, Klebsiella pneumoniae, Staphylococcus aureus, Acinetobacter calcoaceticus, Haemophilus influenzae, Clostridium difficile, Klebsiella oxytoca, Neisseria gonorrhoeae, Enterobacter cloacae, Campylobacter jejuni, Serratia marcescens, Streptococcus agalactiae, Neisseria meningitides,* and *Staphylococcus epidermidis*. The assay is carried out in a single sealed reaction tube, takes only one hour to complete, and is performed in a commercially available spectrofluorometric thermal cycler that is able to distinguish the signal generated from six differently colored fluorophores. The assay utilizes 15 different highly specific molecular beacons that are each labeled with two differently colored fluorophores selected from a palette of six differently colored fluorophores. Since this assay identifies the causative species in greater than 95% of all people infected with a bacterium, we expect that it will be widely adopted for routine use. They are now extending the assay to include encountered bacterial agents such as *Bacillus anthracis*, *Yersinia pestis*, *Burkholderia mallei*, and *Francisella tularensis*. The assay will contain 20 different species-specific molecular beacons, each labeled with three differently colored fluorophores selected from a palette of six differently colored fluorophores. Widespread use of these "molecular blood cultures" will enable the rapid identification of common infectious agents, while at the same time providing an early warning system to contain the spread of major epidemics caused by agents of bioterror.

In parallel, the Perlin lab has developed multiplexed panels of probes, as follows: pan-bacterial, pan-fungal, pan-Aspergillus, individual Aspergillus spp., pan-Candida, B. anthracis, Y. pestis, SARS CV, influenza A, influenza B, parainfluenza viruses 1-4, and human metapneumovirus. The respiratory viruses (except SARS) were all combined in a multiplex panel and as were the fungal probes and separately for the bacterial probes. A single panel was established for pan-bacterial and pan-fungal assays. Each pobe was fully validated for specificity and in many cases sensitivity. Typically, 1-5 bacteria and fungi can be detected, while 3-5000 virus particles can be detected.

Specific Aim 2. High throughput sample preparation. Sample processing development is an integral component of a successful diagnostic program.

Rapid nucleic acid extraction from clinical samples will be critical for downstream molecular applications in response to a bioterrorism event. Extraction of genetic materials must be

automated, ultra sensitive and have high throughput capability to process and organize large sample populations. Yet, the detection of nucleic acids from infecting microorganisms or viruses in whole blood, tissues, sputum/BAL, urine, cerebrospinal fluid or amnionic fluid can be influenced by numerous factors. Magnetic bead technology is an ideal choice for nucleic acid isolation and purification because of its greater affinity for nucleic acids than other conventional methods. With the funds from this program, the Perlin Lab purchased a Roche MagNA Pure LC^{TM} to develop protocols for both DNA and RNA from a wide range of pathogenic bacteria, viruses and fungi from clinical samples. We have now routinely protocols for the isolation of such nucleaic acids from a wide range of organisms. In some cases, a pre-lytic step was necessary with *Staphylococcus aureus* (lysostaphin) and fungi (Q-Biogene) . The sample preparation is effective with a wide range of specimen and it has been validated with both human and animal specimes.

Specific Aim 3. Optimization and validation of diagnostic assays in animal models. Assays for detection of microbial nucleic acids will be optimized using samples from experimentally infected animals.

This goal requires that a new molecular detection technique be optimized for both sensitivity and accuracy, and be validated. Ideally, validation of a new diagnostic should occur by statistically demonstrating its equivalence or superiority to conventional detection methodology on clinical samples. Typically, such validations are best determined from clinical specimens obtained from patients in endemic areas of disease. For most select agents, human infections are rare and occur outside the USA, which makes validation on human populations impractical at an early stage of product development. A better solution is to use well-developed animal infection models to both optimize and provide initial validation for new diagnostic tools. The primary advantage of an animal infection model is that infection and progression of disease can be more precisely defined. The goal of optimization studies in animals is to achieve the highest possible level of detection while maintaining fidelity of identification (accuracy) in the absence of false positives. The Perlin lab has established animal models for MSSA, *Candida albicans, Aspergillus fumigatus, Yersinia pestis* and *Baccilus anthracis.* Respiratory delivery of these pathogens is an important part of the model development. Fungi are typically the most difficult organisms to detect, and we have successfully demonstrated that it is possible to extract nucleic acids from mouse blood and tissue (lungs and kidneys) and detect the pathogens by PCR with molecular beacon detection. We typically add an extraction control (known amount of a detectable plasmid) or relate pathogen nucleic acid to total host pathogen genes such as GAPDH. High fidelity real-time probes like molecular beacons can nearly eliminate false positive background levels because of their allele-specificity (1). (i.e. Non-specific amplification of DNA will not lead to productive probe-target hybrids resulting in signal detection.) We have used this property to show detection of drug resistant mutants of *A. fumigatus* and *C. albicans*.

The success of this work has led to new studies and funding from the NIH for continued diagnostic assay development against bloodstream pathogens causing sepsis.