K.UPENDR, *IJRRPAS*, 2011, Volume-1 Issue-4, Page-313-322.

ISSN:2249-1236

INTERNATIONAL JOURNAL OF RESEARCH AND REVIEWS IN PHARMACY AND APPLIED SCIENCES

Research Article

Received: 13-11-2011 *Accepted: 26-11-2011* *Published: 29-11-2011*

EVALUATION OF ADVANCED DISEASE DIAGNOSTIC METHODS BY USING NUCLEIC ACIDS

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ABSTRACTPathogenic organisms are transmitted to the host organism through all possible connected pathways, and cause severe disease state in the new host. Commonly occurring curable infectious diseases still impose the greatest health impacts on a worldwide perspective. Several technologies are used for diagnosing the disease state in that particular host. Golden standard for diagnostics of pathogenic bacteria has long been culture able Medias. This method was used from the ancient days to diagnose a disease. Environmental biologists have estimated that less than 1% of all bacteria are culture able and it takes lots of time to get conformation about the disease, severity and causative organism. And these are the cost effective techniques. Genomic-based approaches offer the potential to identify all microbes from all the biological kingdoms. Nucleic acid based pathogen diagnostics has evolved significantly over the past decades. Novel technologies offer increased potential in sensitivity, specificity, decreased costs and parallel sample management. However, most methods are confined to core laboratory facilities. To construct an ultimate nucleic acid based diagnostic for use in areas of need, potential frontline techniques need to be identified and combined.

The research focus of this work has been to develop and apply nucleic acid based methods for pathogen diagnostics. Methods and assays were applied to the two distinct systems i) screening for antibiotic resistance mutations in the bacterial pathogen *Helicobacter pylori* appears to be an important risk factor for stomach ulcers, gastritis and possibly stomach cancer and ii) genotype determination of the cancer causative Hepatitis B and C virus (HBV & HCV). The first part of the study included development of rapid, direct and multiplex Pyro sequencing nucleic acid screenings. With improved methodology in the sample preparation process, we could detect an existence of multiple co-infecting HBV & HCV genotypes at greater sensitivities than previously described, when using the same type of methodology. The second part of the study focused on multiplex nucleic acid amplification strategies using Molecular Inversion Probes with end-step Pyro sequencing screening.

Key words: Helicobacter pylori, Hepatitis B virus, Hepatitis C virus, Pyro sequence screening, Molecular inversion probes.

1. INTRODUCTION

The major groups of microbial pathogens are bacteria, viruses, parasites, fungi, and prions. Viruses are the most abundant biological entity on our planet, representing an estimated total of 3% of all predicted proteins. Pathogenic organisms are transmitted to the host organism through all possible connected pathways and cause a myriad of diseases or illnesses, commonly occurring curable infectious diseases still impose the greatest health impacts on a worldwide perspective. To measure the global annual human burden of morbidity and mortality caused by a specific disease, the World Bank and World Health Organization (WHO) established the disability-adjusted life-years (DALY) concept, combining the loss involved with premature death and impacts in healthy life caused by disabilities.

2. REVIEW OF LITERATURE

2.1 PATHOGEN DIAGNOSTIC METHODS

Pathogen diagnostic methodology can be divided into four categories:

i) Syndromic management diagnostics, comparing patients phenotypes to that of characteristic disease states,

ii) Microbe visualizable detection methods, including microbial cultivations24, and/or light-microscopic examinations,

iii) Protein detection methods, such as antibody-antigen detection in the Enzyme-Linked Immuno sorbent Assay (ELISA), and iv) nucleic acid based diagnostics, screening for the source codes of infecting agents.

Golden standard for diagnostics of pathogenic bacteria has long been cultureable Medias. Environmental biologists have estimated that less than 1% of all bacteria are cultureable. Genomic-based approaches offer the potential to identify all microbes from all the biological kingdoms. Standard methodology for diagnostic of viruses compromises screens for specific protein markers and or nucleic acid signatures. Nucleic acid based diagnostics has evolved significantly over the past decades. Novel technologies offer increased potential in sensitivity, specificity, decreased cost, and parallel sample management, but are mostly confined to core laboratory facilities, and thus difficult to implement in clinical environments. To construct an ultimate nucleic acid diagnostic for use in areas of need, potential frontline techniques need to be identified and combined. The four fundamental processes for nucleic acid diagnostics include biomarker identification, nucleic acid extraction, selected target amplification, and target validation. The key principal exploited is complementary base pairing; combining a synthetic nucleic acid strand with a signature sequence that can be designed to fish for a complementary target strand. Integrating the basic steps into a one-reaction assay would be a significant accomplishment towards an ASSURED diagnostic method. Analogous diagnostic methodology can be applied for both RNA and DNA detection, with a seemingly endless spectrum of methods and applications. There are also

promising developments towards interdisciplinary methodology usage, such as the Proximity Ligation Assay (PLA), where antibody-antigen interaction is detected via coupled nucleic acid amplification. Alternatively, direct antibody detection of nucleic acid hetero duplexes was developed with the Hybrid Capture assay (HC).

2.2 GENETIC BIOMARKER SELECTION

The multidisciplinary field of bioinformatics emerged as an important discipline shortly after the development of the first generation DNA sequencing technologies. Historic approaches to finding biomarkers were based on wet-lab processes focusing on a limited number of candidates, using prior knowledge about gene functions, unique proteins, or toxins.

3. AIM AND OJECTIVES

To diagnose the Cancer causing micro organisms by using the different nucleic acid based pathogen diagnostic methods. With a bacterial and viral model, we investigated two distinct questions involving pathogen diagnostics. In the *helicobacter pylori* model, we targeted mutation sites highly correlated with antibiotic resistance. While in the Hepatitis B & C virus (HBV & HCV) models, we sought methods capable of genotyping variant genomes.

4. METERIALS AND METHODS

4.1 MODEL ORGANISMS

Helicobacter pylori (H. pylori)

Helicobacter pylori (H. pylori) are a small, Spiralshaped, Gram-negative bacterium that has beenimplicated in the pathogenesis of gastritis, pepticulcer and gastric cancer of humans. Severaltechniques have been used for the detection of H.pylori, different sensitivity and specificity. The routine invasive diagnostic assays consist of culture, urease tests, and histological examination of gastric biopsy sections requiring endoscopy. Urea breath tests and serologic detection of antibodies are used as non-invasive methods.

Hepatitis B: (HBV)

Type B hepatitis is the most wide spread and the most important type of viral hepatitis. HBV is a 42nm DNA virus with an outer envelope and an inner core which is belongs to hepadnaviridae family. Hepatitis B may cause chronic hepatitis and in some cases can lead to full-blown liver failure and death. Transmission of hepatitis B virus occurs through blood and body fluid exposure such as blood, semen, vaginal secretions or saliva. A vaccine for hepatitis B does exist and is now widely used for routine childhood immunization (Richard *et al.,* 1999).

Hepatitis C: (HCV)

Hepatitis C is a blood borne infection very rarely it can also be passed through other body fluids, such as through, having unprotected sex. Drug users sharing needles are particularly at high risk, but also anyone whose blood has come into contact with the blood of someone infected with hepatitis C. the virus is not transmitted through normal social contact, such as hugging, kissing, sharing kitchen utensils infection (Cliver *et al.,* 1997).

4.2 NUCLEIC ACID EXTRACTION

4.2.1 DNA extraction For H. pylori

The genomic DNA from the gastric tissue, isolated culture and bile samples was isolated *as per* the standard protocol previously described[16]. In case of bile sample, briefly 450 µL of the sample was diluted with equal volume of PBS and centrifuged at 15 000 g for 20 min. The supernatant was discarded and the pellet was again subsequently mixed with 250 µL of the PBS and DNA isolated by modifi ed cetyl trimethyl ammonium bromide (CTAB) method. The DNA was extracted and preserved at -20oC until amplification was performed. Appropriate care was taken during extraction to remove the PCR inhibiting substances present in the bile[17]. Briefly as *Helicobacter* DNA was isolated from an unusual source, there is possibility of existence of specifi c inhibitors and competing substrates**.** For such situations, dilution of inhibited samples provides a rapid and straightforward way of permitting amplification. This dilution exploits the sensitivity of PCR by reducing the concentration of inhibitors relative to target DNA.

4.2.2 *DNA extraction* for HBV

For phenol-chloroform DNA extraction, a 125-ml aliquot of serum was incubated at 708C for 2 h in the presence of 400 mg of proteinase K ml21, 1% sodium dodecyl sulfate, 2.5 mM disodium EDTA, and 25 mM sodium acetate. The suspension was sequentially extracted with phenol and then chloroform. The DNA was precipitated with 0.3M sodium acetate and then with absolute ethanol, washed with 70% ethanol, vacuum dried, and then dissolved in 50 ml of best-quality water (BQW).

4.2.3 R*NA extraction* for HCV

500µl of solution D, 150µl of serum sample, 50µl of sodium acetate, 500µl water saturated phenol, 200µl of chloroform: isoamyl alcohol (24:1) was taken in a fresh eppendorf tube and vortex for 40sec. immediately kept it in the ice for 20 minutes. Centrifuge the tube at 12,000rpm for 15 minutes at 4°c. The supernatant was collected in a fresh eppendorf tube and equal volumes of chloroform: isoamyl alcohol was added. Centrifuge the tube at 12,000rpm for 15 minutes. Supernatant was collected in a fresh eppendorf tube and 1ml of chilled isopropanol was added, and then placed at -20°C for overnight. The RNA sample in Isopropanol was sediment at 10,000g for 20 minutes. The RNA pellet was resuspended in 80% ethanol, sediment, and air dried (15 minutes), and dissolved in 20µl of DEPC treated water at 65°C for 10 minutes.

4.3 AGAROSE GEL ELECTROPHORESIS

0.4gr of the agarose gel is prepared by weighing the above ingredients except Ethidium bromide. Boil in order to dissolve agarose in oven to get clear solution. It is left at room temperature until it reaches 50°C or tolerable temperature. Immediately add 4µl of Ethidium bromide. Dispense the solution in to the present boat with comb. Leave it at room temperature until it solidifies. Remove the blocker or tape immediately and carefully. Add tank buffer up to the limit. Remove the comb so that wells are formed. Product is treated with loading dye and glycerol.

Then the product with loading dye and glycerol is loaded into the wells. After electrophoresis, the gel is taken out and observed under U.V to trace the DNA and RNA fragments.

4.4 MOLECULAR DIAGNOSTICS OF ORGANISMS

Molecular diagnostic approaches have utilized the discovery of genetic enzyme systems involved in nucleic acid replication and repair. As an example, the discovery of the PCR became recognized as a highly sensitive technique for detection and amplification of target sequences in nucleic acids. The extremely high affinity and specificity of nucleic acid hybridization have allowed for the development of nucleic acid assays that exceed the sensitivity of antibody based technologies.

4.4.1. ***Nucleic acid diagnostics for H.Pylori DNA***

Five microliters of each DNA solution were subjected to a two-step nested PCR using two primer pairs from the urease structural gene of the H. pylori genome (23). The outer primer pair was 5'-GCCAATGGTAAATTAGTTCC-3' and 5'-CTCCTTAATTGTTTTTACAT-3', and amplified a 411-bp product from urease A gene (nucleotides 304 to 714). The amplification cycle consisted of 40 cycles at 96°C for 30 seconds, 56°C for 15 sec- onds and 74°C for 30 seconds. The final cycle included extension for 10 min at 74°C to ensure full extension of the product. Negative reagent control reactions were performed with each batch of amplifications, consisting of tubes containing distilled water in place of the DNA samples. After PCR, 1 microliter of the reaction mixture was transferred to the second round reaction mixture containing 0.6 μM of each inner primer and the same buffer as in the first round. The nested inner primer pair was 5'-AGTTCCTGGTGAGTTGTTCT- 3' and 5'-AGCGCCATGAAAACCACGCT– 3', and amplified a 361-bp product from urease A gene (nucleotides 318 to 678). The amplification cycle of the second round PCR was the same as in the first round. Ten microliters of the first and second round PCR products were electrophoresed on a 2 % agarose gel containing 0.5 μg of ethidium bromide per ml.

4.4.2. Nucleic acid diagnostics for H.Pylori DNA

PCR Primers. Primers specific for HBV core gene sequences were used for single-round PCR, whereas primers specific for the precore-core region were used for nested PCR,

Amplification. The final PCR mixture contained 0.02 U of DynaZyme DNA polymerase ml21 200 mM each deoxynucleoside triphosphate, 1 mM each primer, 50 mM KCl, 10 mM Tris HCl (pH 8.8), 1.5 mM MgCl2, and 0.1% Triton X-100. For phenol-chloroform and QIAamp DNA extracts, 2.5 ml of target DNA was added to 22.5 ml of the appropriate master mixture. The volume of GeneReleaser used to accomplish cell lysis was compensated by deducting the equivalent volume of BQW from the components of the amplification reagents to maintain. Take the PCR tools from -20ºc and keep in ice box. Briefly spined all tubes for few seconds to settle down all the droplets sticking at the top or sides. Added the following component in to PCR tubes provided. Master mix contain following components, Placed the tubes in thermal cycles and program the number of cycles, the optimized thermal profile is given below.

Step 1: Initial denaturation at 94ºc for 2 minutes.

Step 2: Denaturation at 94ºc for one minute.

Step 3: Annealing at 60ºc for one minute

Step 4: Extension at 72ºc for 1 minute.

. Step 5: Final Extension at 72ºc for 10 minutes.

Step 7: 10ºc forever.

Steps 2,3,4 were repeated for 30 times for the amplification of desired gene.

Collect the tubes from the Thermal Cycler

4.4.3. Qualitative nucleic acid diagnostics for HCV RNA

HCV RNA detection by RT-PCR is widely used to confirm HCV diagnosis and for assessing viremia in patients during and following antiviral therapy. Individual assays comprising RNA isolation, complementary DNA (cDNA) synthesis, PCR amplification and detection of PCR amplicons. The most sensitive and optimized PCR assays report detection of HCV RNA in patient's serum at concentrations of less than 100 copies per ml. Optimization and performance of RT-PCR assays have been reviewed in depth previously.

Quantitative in-house RT-PCR assays have been described in detail. In endpoint dilution, samples (serum, RNA or cDNA) are diluted in series and tested by qualitative PCR or RT-PCR. The last positive dilution (endpoint) is used to calculate starting nucleic acid concentration. This highly sensitive methodology can be used to detect single-copy or absolute cDNA. However, in practice, endpoint PCR is subject to assay variables, making it inappropriate for high-throughput clinical settings. Reliability of results requires a broad dilution series of samples tested in multiple, thereby making the assay labor intensive and costly. In addition the assumption that all steps are equally as efficient is likely not to hold in practice; inefficient cDNA synthesis may underestimate starting RNA concentrations.

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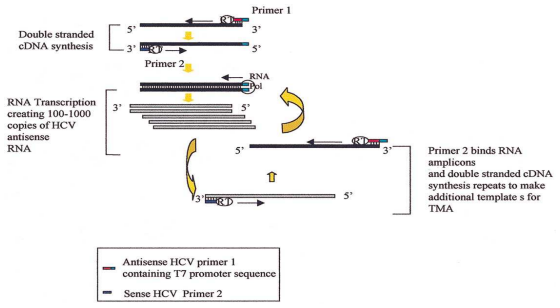
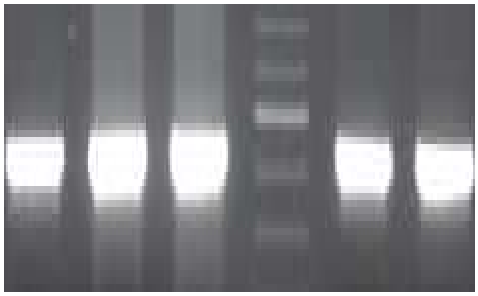


5. RESULTS AND DISCUSSION

Sixty patients (93.8%) were positive for H. pylori by nested PCR, while 34 patients (53.1%) were positive for H. pylori in the first round PCR (Figure 1). Of the 60 nested PCR-positive patients, four (100%) had gastric ulcer, 18 (100%) had duodenal ulcer and 38 (90.5%) had gastritis. All of the four nested PCR-negative patients had gastritis and they also had negative CLO test results. All of the 18 patients with CLO test-negative results were positive by nested PCR and six of them were positive by first round PCR. Fourteen CLO test positive patients were negative by first round PCR. The percentage of nested PCR-positive patients appeared to be higher among patients with both CLO test-positive and first round PCR-positive results. No false positive result was observed in the negative controls in the first and second PCR rounds, and positive control for H. pylori was successfully detected by both the PCR rounds.

361 bps

To optimize the detection of HBV DNA in serum, a pilot experiment was carried out comparing single-round PCR versus nested PCR of phenol-chloroform DNA extracts. HBV DNA could be detected in all HBeAg-positive subjects by using either single-round or nested PCR. Serum samples of patients of this sort would be expected to have more than 106 particles per ml (2). However, single-round PCR was less sensitive in detecting HBV DNA present in the sera of HBeAg-negative subjects. This too would be expected, since sero conversion from HBeAg to anti-HBe can be accompanied by a decrease in virion concentration to between 102 and 106 per ml. Thus, the sensitivity of the method can be increased by the use of nested primers in a double round of PCR, as previously reported by Kaneko et al. However, the sensitivity of the nested PCR is also its potential drawback, because it increases the chances of inadvertent contamination causing false-positive results. Therefore, extreme caution was exercised to avoid cross-contamination



411 bps

Then RFLP analysis was done by using the 5'UTR amplified product. Using Hae III, Mva I, Hinf I restriction enzymes.then finally the digestion products were run in the 1% Agarose gel electrophoresis along with the ladder digested the amplified product. Genotype 1 was common among the chronic hepatitis group and was observed in 4 patients. The remaining patient had genotype 3.

6. CONCLUSIONS

To aptly take advantage of current available treatment options, it is of outmost importance to identify the individuals who require them. Treatment options must be appropriately administered to those who need them to prevent high healthcare costs, unnecessary suffering, and the dominance of resistant organisms. The general goals of diagnostics are clear, but there are many ways to approach the problem. Literature research, assay developments and experiments performed throughout this technical Thesis work have ultimately raised more questions. However, what remains crystal clear is the need of a new diagnostic as presented by the Global Health Diagnostics Forums report.

In this study, we investigated simplex and multiplex nucleic acid amplification and validation strategies for pathogen diagnostics. Simplex analysis schemes have clear disadvantages in complexity readout data, which can be partially compensated by gain in time and cost. Multiplex high throughput strategies offer complex readout data, which can ultimately assist clinicians in gaining a deeper understanding of underlying infectious nature and furthermore generate more relevant biomarkers. The challenges will lie in formatting them into easily executable integrated systems. What is "high-tech" today will be standard tomorrow.

We cannot wait anymore; the day after tomorrow is now. Does the world really need another Molecular Inversion Probe, or is the combined usage of the tools in our current molecular "toolbox" sufficient to meet the demands setup by the Global Health Diagnostics Forum? Hundreds of intellectual property hurdles would create major bottlenecks in the development to implementation stages of fully integrated combined assays. Techniques presented throughout this report all show great promise, and could together be formed into something great. By putting out a helping hand for a fellow man, instead of closing doors with legal documents, I believe we already have what it takes to save the millions of individuals identified by the Global Health Diagnostic Forum.

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