MODERN MOLECULAR TOOLS AND TECHNIQUE FOR DETECTION OF WATER BORNE PATHOGENS

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DISEASES ARE CAUSED BY:

- FUNGI
- BACTERIA
- VIRUSES
- NEMATODES
- MYCOPLASMA
- OTHERS
  ORGANISM S
ARE WE SOFT TARGETS?

- Food production and distribution networks are susceptible to contamination with human pathogens.
- Pathogens produce animal and human toxins.
- They may have latent infection.
- Utmost and ideal sanitation is not possible.
- Environmental conditions cannot be manipulated.
- Prevention is better than cure.
- Water is one of the major sources of contaminants of life.
- Need of constant vigilance for every pathogen.
INTRODUCTION

Pathogen: A Micro-organism causing disease

Waterborne diseases:

Contaminated surface water sources and large poorly functioning municipal water distribution systems contribute to transmission of waterborne bacterial diseases.
COMMON SYMPTOMS OF WATERBORNE DISEASES

- Profuse watery diarrhea
- Diarrhea
- Abdominal cramping
- Nausea
- Vomiting
- Fever
- Headaches
WATERBORNE DISEASE CAUSING AGENTS OR PATHOGENS

**Bacteria**
- *Campylobacter jejuni*
- *Vibrio cholerae*
- *Shigella*
- *Campylobacter*
- *Francisella tularensis*
- *Legionella pneumophila*
- *Salmonella*
- *Toxigenic Escherichia coli*
- *Yersinia enterocolitica*
- *Helicobacter pylori*
- *Mycobacterium avium*

**Protozoa**
- *Toxoplasma gondii*
- *Cyclospora cayetanensis*
- *Naegleria fowleri*

**Viruses**
- *Hepatitis E*
- *Rotavirus*
A stream at Yellowstone

Brush your teeth!
In nature most bacteria do not exist in pure culture

Many microorganisms are associated with surfaces in complex multispecies communities called BIOFILMS

Many important microbial processes cannot be performed by a pure culture:

- Anaerobic digestion of organic matter: Syntrophic* juxtaposition of H₂-producing acetogens and H₂ consuming methanogens

- Degradation of xenobiotic compounds (Herbicides, nitrate esters etc.)


\[
\text{NH}_4^+ \rightarrow \text{NO}_2^- \quad \text{NO}_2^- \rightarrow \text{NO}_3^- 
\]

*Syntrophy: A nutritional situation in which two or more organisms combine their metabolic capabilities to catabolize a substance not capable of being catabolized by either one alone.
Biofilm formation:

Attachment | Colonization | Growth

Planktonic cells → BULK FLUID → Sessile cells → SURFACE

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P. Dirckx
# Incidence Statistics for Types of Waterborne Diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Incidence Estimate</th>
<th>US people estimate</th>
<th>Statistic Used for Calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>cholera</td>
<td>approx 1 in 45,333,334 or 0.00% or 5 people in USA</td>
<td>5</td>
<td>6 annual cases notified in USA 1999 (MMWR 1999)</td>
</tr>
<tr>
<td>typhoid fever</td>
<td>approx 1 in 786,127 or 0.00% or 346 people in USA</td>
<td>346</td>
<td>346 annual cases notified in USA 1999 (MMWR 1999)</td>
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<tr>
<td>dysentery</td>
<td>N/A</td>
<td>N/A</td>
<td>No information</td>
</tr>
<tr>
<td>Amebic dysentery</td>
<td>N/A</td>
<td>N/A</td>
<td>No information</td>
</tr>
<tr>
<td>Brainerd diarrhea</td>
<td>approx 1 in 54,400 or 0.00% or 5,000 people in USA</td>
<td>5,000</td>
<td>estimated 5,000-8,000 patients (DBMD)</td>
</tr>
</tbody>
</table>
DISEASE SEVERITY

Chronic infection

Disease

Acute Infections

Severe Disease

Mild-Moderate Disease
USE OF MOLECULAR DIAGNOSTICS IN DISEASE MANAGEMENT

- Identification of disease causing entities
  (microorganisms),
- To detect pests and pathogens causing diseases, as a tool for managing
- For pathogen detection in water sources and reservoirs, for certification purposes and
- To monitor pesticide and other organic chemical contamination (natural toxins and disease causing organisms) in water/food.
What’s out there?
NEED OF DIAGNOSTICS

- Disease surveillance
- Disease forecasting
- Understanding of the patho-system
- Monitoring of disease epidemics/outbreak
- Helping in water quality certification aspects for declaring it pathogen free
- Establishment of sanitation and other regulatory issues
BASIS OF DIAGNOSIS

WHAT IS SPECIFIC TARGET?

HOW WILL YOU IDENTIFY A SPECIFIC TARGET?

WHAT IS THE BASIS OF IDENTIFICATION?

PRESENCE OF UNIQUENESS

BASED ON PHENOTYPIC AND GENOTYPIC CHARACTERISTICS

PRESENCE OF UNIQUE MOLECULAR MARKERS

EPISEMANTIC COMPOUNDS:
SECONDARY METABOLITES SUCH AS ALKALOIDS FLAVANOIDS, GLYCOSIDES, BAKOSIDES TERPENOIDS, FATTY ACID COMPOSITIONS (FAME), SUBSTRATE UTILIZATION (BIOLOG)

SEMENTIC COMPOUNDS:
SENSE CARRYING MOLECULES SUCH AS GLYCOPROTEINS PROTEINS, ENZYMES, ISOENZYMES NUCLEIC ACIDS SEROLOGICAL MARKERS, MULTILOCUS ENZYME PROFILES (MEPs)
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ATTRIBUTES TO BE CONSIDERED IN THE SELECTION OF DIAGNOSTIC METHOD

**Sampling plan:**
Randomization numbers to be drawn per predefined lot

**Sample preparation:**
Non-destructive diagnostic methods,
Destructive diagnostic methods,
Handling before examination, Transportation, Incubation

**Biological amplification:**
Extraction and isolation, Culture in case of pathogen, preparation of macerate and dilutions, composition of extraction fluid

**Enumeration procedure:**
Qualitative test, Quantitative test

**Operation:**
Simple, Fast and Rapid

**Target:**
Molecular entities
WHAT ARE THE MOLECULAR TARGETS?

- Cell
- Nucleus
- Chromosome
- Protein
- Gene (mRNA), single strand
- Gene (DNA)
**DIAGNOSTICS IS OF PARAMOUNT IMPORTANCE**

- **CONVENTIONAL DIAGNOSTIC METHODS**
  
  Detection based on microscopic and morphological analysis

- **LIMITATIONS**
  
  - Prevention is better than cure
  - Specificity
  - Sensitivity
  - Time consuming
  - Interference in samples
  - Subjective in nature

- **HOW WILL YOU OVERCOME THESE LIMITATIONS?**

- **USE OF MOLECULAR DIAGNOSTIC METHODS:**
  
  - Antibody-based analysis
  - Nucleic acid-based analysis

**PREVENTION IS BETTER THAN CURE**

**NEED OF CONSTANT VIGILANCE FROM SEEDLING TO HARVESTING STAGE OF CROPS**
BASIC PARADIGMS INVOLVED IN DETECTION METHODS

PROTEIN / ANTIGEN OR NUCLEIC ACID (DNA OR RNA)

TAGGED MOLECULES

DETECTION
WHY DO WE REQUIRE MOLECULAR DETECTION?

- DEVELOPMENT OF DIAGNOSTIC REAGENTS
- MANAGEMENT OF WATER-BORNE PATHOGENS
- IDENTIFICATION AND CHARACTERIZATION OF PATHOGENS
- UNDERSTANDING THE MOLECULAR MECHANISMS ASSOCIATED WITH PATHOGENESIS
SUPERIORITY OF MOLECULAR DIAGNOSTICS OVER CONVENTIONAL TECHNIQUES

- **Rapidity:**
  - Quick identification of disease causing entities,
  - Fastest means of detection

- **Specificity:**
  - Capacity to differentiate,
  - High resolution is easily achievable

- **Sensitivity:**
  - Minimum threshold to be detected
  - High sensitivity guaranteed and
  - Very low amount of sample is required

- **Stringency:**
  - Enhanced at very low extra cost

- **Commercialization:**
  - Commercial kits are cost effective and user friendly,
  - Adaptable for scanning of very large samples

- **Ingeniousness:**
  - Less labor intensive,
  - Established methodology eliminates the dependence on skilled personnel

- **Investment:**
  - Single investment pays off for a long run
MOLECULAR TECHNIQUE FOR DETECTION OF WATERBORNE PATHOGENS

- Immunological detection
- Nucleic acid based detection
TWO SPECIFIC ATTRIBUTES OF THESE TECHNIQUES

**SENSITIVITY**

**SPECIFICITY**

**SPECIFICITY:** CAPACITY TO DIFFERENTIATE

**SENSITIVITY:** MINIMUM THRESHOLD TO BE DETECTED
ANTIBODY BASED DIAGNOSTICS

- Generation of specific antibodies against the target molecules
- Use of specific antibodies as probe for detection of target molecule/organism in question

ANTIBODIES AS REAGENTS:

Target specific antibodies can be generated through:

- Conventional antibodies approach (Use of antisera)
- Monoclonal antibodies approach (Hybridoma Technology)
- Recombinant antibodies approach (Engineered antibodies)
- Phage display antibodies
- Plantibodies
Immunoassays can be in a number of method formats:

- Enzyme linked immunosorbent assay (ELISA)
- Radioimmunoassay (RIA)
- Immunodiffusion
- Immunoblotting
- Latexagglutination
- Countercurrent immunoelectrophoresis (CIE)
- Immunofluorescence and immunogold labelling methods
IMMUNO ASSAY SYSTEM

- Multiwell ELISA
- Dot Blot ELISA
- Tissue Print ELISA
- Electro Blot Immunob assay
- Immunofluorescent Assay
- Rapid ImmunoFilter Paper Assay
- Immunogold Labelling
- Radio Immunoassay
- Delayed Enhanced Lanthanide Fluorescence Immunob assay
- Chemiluminescence Vassay
- Bio Luminescence Immunob assay
- Substrate Labelled Fluorescence Immunob assay
COUNTERCURRENT IMMUNOELECTROPHORESIS (CIE)

**Negative CIEP result**

+ | -
---|---

Positive control | Suspect reaction | Negative control

- | Ab | Ag |

**Positive CIEP result**

+ | -
---|---

Positive control | Suspect reaction | Negative control

- | Ab | Ag |
LATEX AGGLUTINATION
IMMUNOMAGNETIC SEPARATION

1. Mycobacterial target cells
2. Other microorganisms
3. Antibody-coated magnetic beads
4. Mycobacteria bound to the magnetic beads
5. Magnet applied and contaminants removed by washing
6. Magnet removed, solid phase resuspended and used to inoculate mycobacterial culture media
Principles of Enzyme Linked ImmunoSorbent Assay (ELISA)

Wells of ELISA plate coated with antigen → Primary antibody reacted with antigen → Reagent added that is converted to colored product by enzyme color

Competitive ELISA
Primary antibody reacted with bound antigen and varying amounts of "free" antigen → Primary antibody bound to antigen detected using secondary antibody that is coupled to an enzyme such as peroxidase → color
RADIO IMMUNOASSAY

1. Radiolabelled Antigen ($^{125}$I)
2. Incubate
3. Wash
4. Measure

- Specific Autoantibody
- Radiolabelled Complex
IMMUNOFLUORESCENCE

Fluorescein-labelled Ab

serum

[Diagram showing immune response]
IMMUNO FLUORESCENT STAINING TEST (IFST)

IMMUNOBLOTTING

Nuclear antigens separated on agarose gel by an electric field
SUMMARY

- Water borne disease diagnosis and pathogen detection approaches require substantial levels of specificity, sensitivity, and speed.

- With the miniaturization of assay volumes, as tests are adapted to microchips, multiple sampling of a specimen will become very important.

- Using modified ELISA protocols, samples can be assayed directly in the field.

- The immunological tools can be useful for the study of disease initiation and pathogenesis of diseases.

- On site screening and user friendly tests can be useful in detection of water borne pathogens, quality analysis & certification and other sanitation regulation.
DNA BASED MOLECULAR DETECTION

DNA and RNA hybridization based
  Nucleic acid / proteins hybridization
  SOUTHERN BLOT
  NORTHERN BLOT
  DOT / SLOT BLOT
  MICRO ARRAY/ BIOCHIPS BASED

Polymerase chain reaction based
  STANDARD PCR
  RT-PCR
  NESTED / TAIL PCR
  ARDRA
  BIO-PCR
  ANCHOR / RACE / SLIC / UP / AP PCR
  ITS- PCR
  REAL TIME PCR / MULTIPLEX PCR
Water

Molecular

Culture based

Phenotype

DNA $C_{tr}$ plots

Tree

41 biotypes, 80% similarity

Five dominating biotypes, 43% of the strains

Torsvik et al., 1990

Water

All isolates

E. coli

Strain 23A

41 biotypes, 80% similarity

Torsvik et al., 1990
DNA/RNA PROBES BETTER
IDENTIFICATION OF PATHOGENESIS GENE,
ACTIVE /LATENT VIRAL INFECTION,
MUTATION DETECTION VARIETAL IDENTIFICATION
GENE REARRANGEMENT AGRONOMIC TRAITS

DISEASE MOLECULAR MARKERS

ANTIBODIES BETTER
DISEASE DIAGNOSTICS AND DETECTION
DIFFERENTIAL DIAGNOSIS
QUALITY ANALYSIS OF WATER
VIRULENCE ANALYSIS OF PATHOGENS
ELICITOR ANALYSIS INVOLVED IN DEFENCE

COMPARISION OF DIAGNOSTIC USES OF NUCLEIC ACID PROBES AND ANTIBODIES
16S rRNA, the molecule of choice

1. Found in every living cell and genetically stable.

2. Length, long enough (~1,500 nt), contains independently evolving domains, i.e. variable regions.

3. Shorter than the 23S (~2,900 nt), easier to sequence.
Nucleic acid based detection is depend upon several types of nucleic acid interaction:

- DNA:DNA interaction
- DNA:RNA interaction
- RNA:RNA interaction

DNA:RNA and RNA:RNA hybrids are somewhat more thermally stable than DNA:DNA duplexes, but RNA molecules are quite labile at alkaline pH.
Nucleic acid hybridization techniques can also be exploited as diagnostic tools.

Example - southern and northern blots

DNA / RNA electrophoresed

Transferred to membranes

Immobilized/ cross linked to membrane

Probed with labelled nucleic acids on the basis of complementarity
DOT BLOT AND SLOT BLOTS

IT IS AN ADVANCED VERSION OF NUCLEIC ACID HYBRIDIZATION BASED DETECTION

- Samples are directly transferred to the membranes with the aid of blotting apparatus
- No need of electrophoresis
- Short efficient and convenient method of measuring transcripts genes or specific DNA sequences
- But information regarding size is not possible
- Ex: tomato spotted wilt virus TSWV tomato & tobacco

SAP TREATMENT

CRUDE SAP IMMOBILIZATION

HYBRIDIZATION WITH PROBE ANALYSIS
**MICRO ARRAY/ BIOCHIPS**

- Microarray or biochips consists of optically flat glass plates containing 96 or more densely packed probe arrays.
- Both nucleic acids and antibody-based probes are commercially available.
- Chip-based approaches are very flexible, robust, and have potentials to reduce unit costs.
- Provides tremendous capacity for long-scale accurate and reliable testing.
- This format is compatible with automated robotic systems.
- Still further scope for miniaturisation.
- Even total components of single cells could be analysed in one lot and at minimal cost.
- Minimum risk of cross-contamination and false positive results.
- Ex - 96 well flat glass ELISA is commercially available---------------------------
  "HIGH THROUGH PUT MICRO ARRAY ELISA"
  Each well contains 4 identical 36 element array
  =144 elements cell⁻¹ = total 144x96 element per plate.
Oligonucleotide-Based Microarrays
cDNA MICROARRAY

DNA clones

PCR amplification purification

robotic printing

hybridize target to microarray

test

reference

reverse transcription

label with fluor dyes

excitation

laser 1

laser 2

emission

computer analysis
INTRODUCTION: development

- 1985: SAIKI et. al. prenatal diagnosis of sickle cell anaemia (publ. account)
- Automation Taq pol (Thermus aquaticus 72 °C optm.)
**STANDARD PCR**

**METHODS:**

**A: Preparation of nucleic acid**
- Any suitable extraction method—SDS/CTAB / PHENOL – CHCl3
- Any tissue living or dead/ fossilised
- Purification— CsCl / proteinase / RNase

**B: PCR parameters**
- Primer designing—seq knowledge— specificity
  - GC-content > 60-70%
  - Bp- 16-30
  - Complementarity—none

**Reaction components**
- Taq DNA polymerase
- MgCl2
- Primers
- PCR buffer(Tris +/- MgCl2 Ph-8.0/KCl/DTT)
- Template
- MilliQ grade water

**Thermocycling conditions**
- Denaturation 94°C—2.9’
- Annealing 35-55°C—0.5-3
- Extention 72°C—1-5’
- Denaturation 94°C—1-3’
- Final extention 72°C—5-10’

**Electrophoresis**
**Gel elution**
**Purify amplicons**
**Analysis**
RT PCR

RNA / mRNA

REVERSE TRANSCRIPTASE

cDNA

STD. PCR
MULTIPLEX PCR

STD.PCR

MULTIPLEX PCR

DIFF. SETS OF PRIMERS

c-PRIMERS+AMPLIMERS

CONC. TO BE ADJUSTED

CO-AMPLIFICATION OF DIFF. mRNA GENES
QUANTITATIVE PCR

STD PCR + INTERNAL STD

RELATIVE AMOUNT OF IS AND AMPLICONS

IS-INSERTION / DELETION SEQUENCES

QUANTIFICATION
IMMUNOCAPTURE PCR

SURFACE COATED WITH CORRESPONDING ANTISERA

NUCLEIC ACID IMMOBILIZED ON STERILE SURFACES PREVIOUSLY COATED

STD PCR OR RT PCR
RAPD & AFLP

STD PCR

RAPD
RANDOM BUT CROP SPECIFIC PRIMER

AFLP
RESTRICTION DIGESTION & SPECIFIC AMPLIFICATION PRIMERS
INVERSE PCR

AMPLICIFICATION IN OUTWARD FASHION

PRIMERS ARE DESIGNED ON THE BASIS OF CENTRAL CONSERVED REGIONS

PRIMERS HYBRIDIZE BACK TO BACK

USED FOR WHOLE GENOME OR c DNA SYNTHESIS
ITS PCR

ITS regions contain several tRNA genes and non-coding regions hence it is more variable than 16S or 23S rRNA thus ITS-based identification and detection of soil-borne plant pathogens depends on number & length of amplicons!

Enhanced specificity can be achieved by real-time analysis of ITS PCR amplicons.
BIO PCR

NO NEED OF DNA EXTRACTION / PURIFICATION

COLONY +PCR COMPONENTS

AMPLICONS

ANALYSIS
REALTIME PCR RT / MULTIPLEX

- Binding of primer sets
  - Free quencher

- Exponential increase with time & amplification

- Free flour

- Primer

- Fluorochrome

- DNA / RNA target sequence

- Quencher
REP/ERIC/BOX/PCR

i) REPETATIVE PCR
II) ENT BACTRIA
REPETATIVE INTERGENIC CONSENSUS PCR
III) BOX ELEMENTS PCR

THREE SETS OF PRIMERS SINGLY OR IN COMBINATIONS

IDENTIFY PATHOGENS
DIFFERENTIATE PATHOGENS
GENETIC DIVERSITY ANALYSIS
**RACE / SLIC / TAIL PCR**

i) RANDOM AMPLIFICATION OF c DNA ENDS

ii) SINGLE STRAND LIGATION TO ss c DNA ENDS

iii) THERMAL ASSYMMETRIC INTERLACED PCR

---

FULL LENGTH TARGET c DNA

SLIC----ELIMINATION OF HOMOPOLYMERIC TAILING OF SINGLE SEQ. ANCHOR DIRECTLY ATTACHED TO 3' END SOF SS c DNA

NO NEED OF OLIGOMERIC TAILING

ENRICHMENT OF TARGET SEQUENCES

RACE----VARIATION OF ANCHOR /NESTED PCR
3’RACE-OLIGO dT PRIMER
5’RACE- OLIGO dT&POLY G PRIMERS

TAIL----PRIMER PAIR HAVING DIFF. T M ASSYMMETRIC SYNTHESIS ENRICHMENT OF DESIRED TARGET
REALTIME PCR RT / MULTIPLEX

BINDING OF PRIMER SETS FREE QUENCHER

EXPONENTIAL INCREASE WITH TIME & AMPLIFICATION

FREE FLOUR

PRIMER

FLUOROCHROME

BINDING

DNA / RNA TARGET SEQUENCE
ADVANTAGES OF PCR BASED TECHNIQUES AS DIAGNOSTIC TOOLS

- Detection of very low titres of pathogens
- Generation of pathogen specific clones without purification of pathogen cells
- Detection of unknown multiple and unrelated pathogen in a single reaction
- Identification of pathogens in a mixed infection / disease complex
- Determination of specific sequence information from crude total nucleic acid
- Evaluation of cross protection
- Separation of pathogen from infected plant tissue is not needed
- Reliable results from very small samples
- Unculturable and herbarium species can also be analysed
- Very fast detection of latent infections
- Appears as one time heavy investment but still highly cost effective in long run
- High throughput system could be applied for very large no. of samples
- Highly specific and sensitive technique
- Duration of testing and confirmation is highly reduced
DEMERTS

- Initial lab set up is expensive
- Trained personnel needed
- Possible contamination and false positive is major problem
- Primer designing needs sequence information
- Field application is not possible
CONCLUSION

- A map of disease severity indices reveals information of great economic value.

- Use of Molecular methods for routine diagnosis of water-borne pathogens might become an important tool for disease management.
THANKS