Application of Molecular Microbiology Methods in a Pathology Laboratory

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Why detect microorganisms?

- Resistance to antivirals is becoming more common eg HIV resistance to AZT requires antiviral susceptibility testing

- Biological - infected cell culture titrated with drug dilutions eg HSV

- Molecular - point mutations in the nucleotide sequence of target DNA eg HBV

Why diagnose continued -

- Clinical diagnosis of once common diseases such as measles, mumps, rubella, may be difficult due to atypical presentation in immunocompromised or under immunised patients

- Immunocompromised rely on diagnosis because

  - Develop diseases from common latent viruses eg herpes viruses (CMV, HSV, VZV)
  - Result in considerable morbidity and mortality
  - Volume of knowledge on viral diseases is expanded with these patients
Tests performed in a large molecular diagnostic lab

<table>
<thead>
<tr>
<th>Organism</th>
<th>Number per month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory Viruses</td>
<td>1000</td>
</tr>
<tr>
<td>Chlamydia/gonorrhoea</td>
<td>2500</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>250</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>100</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>650</td>
</tr>
<tr>
<td>Herpes</td>
<td>700</td>
</tr>
<tr>
<td>Human Papilloma virus</td>
<td>150</td>
</tr>
<tr>
<td>Other (e.g., N. meningitidis, enterovirus)</td>
<td>1350</td>
</tr>
<tr>
<td>Viral culture</td>
<td>33</td>
</tr>
</tbody>
</table>

Tests are performed with either commercial or “in-house” assays

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PCR in a diagnostic laboratory

- Very sensitive (1 copy – 10 copies of DNA)
- Can detect organisms that cannot be isolated
- Rapid (TAT = < 24 hrs)

Disadvantages

- Technically demanding
- Expense
- Risk of contamination (therefore strict control procedures)
- Requires extensive QC

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Application of PCR in the Diagnostic Lab

**Commercial vs “In-House” assay ?**

Factors influencing the amplification reaction

- Nature of the target gene
- Primer characteristics
- Reagent concentrations
- Reaction conditions
- Equipment

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Steps involved in Developing a PCR Protocol

- Identify the target
- Literature search - suitable target gene
- Search gene databases for primer regions
- Design and test primers
- Optimise the PCR variables
- Validate the test

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Parameters to be Optimized

- Equipment (cycler, tubes)
- Cycling profiles (time, temp, no of cycles)
- Primer design (target area, 2° structures)
- Reagent conc. (Mg, dNTP, Taq, primers)
- Co-solvents (DMSO, detergents, BSA)
- Size/structure of amplicon/target DNA
- Reaction volume

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Sequence Information

Genbank
EMBL
PCR Primers - Design rules

- Target length 100 to 400 bp optimal (depending on type of specimen e.g. Formalin fixed tissue)
- Computerized oligonucleotide selection

Parameters to be Optimized

- Mg** concentration, 0.5 to 3.0 mM (1.5).
  Increase Mg** lowers stringency
- dNTP’s 20 to 200 uM (200 um)
  If dUTP used needs to be 3X > concentration than others, also [Mg**] needs to be increased (2.5 mM)

Reaction optimization

- Annealing temperature (5°C less than melting temperature) 2 or 3°C higher if co-solvents used (DMSO or formamide)
- Use rule of thumb, move up 2 degrees at a time until sensitivity drops off
- Optimization steps should use sample type to be tested

Primer Sequence composition

- G+C content should approximate target
- Runs of AAA…., GGG….etc. avoided
- 3’ end recognized by polymerases - avoid high G+C (stable base pairing), T at end more tolerant of mismatches
- avoid self complementarity at 3’ end and hairpin structures

Laboratory Validation

- Test PCR against positive DNA (synthetic).
  Determine analytical sensitivity (Limit of Detection)
- Test PCR against known positive/negative samples (50) Determine Clinical Sensitivity
- Test PCR against a panel of unrelated organisms (N >100). Determine Specificity
- Test PCR against 200 clinical specimens, also tested by other methods. Correlate results.
- Release as a validated PCR assay

- Agarose Gel Detection

- Solid Phase Hybridisation and Colour Detection

- Real-time Detection

Plate Hybridisation
Real time probes

The red circle represents the quenching dye that disrupts the observable signal from the reporter dye (green circle) when it is within a short distance.

- The probe consists of two types of fluorophores.
- The quencher (Q) fluorophore reduces the fluorescence from the reporter (R) fluorophore.
- It does this by the use of Fluorescence Resonance Energy Transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton. The reporter dye is found on the 5' end of the probe and the quencher at the 3' end.

- The reporter dye is released from the extending double-stranded DNA created by the Taq polymerase.
- Away from the quenching dye, the light emitted from the reporter dye in an excited state can now be observed.

FRET (Fluorescence Resonance Energy Transfer) using adjacent hybridization probes

Adjacent Hybridisation Probes

Unlike taqman which only uses a single probe this format uses 2 probes. The 2 probes align on specific PCR product. This allows the energy from the fluorophore on the first probe to be transferred to the fluorophore on the 2nd probe. It is this energy transfer and subsequent fluorescence which is monitored by the instrument. Essentially as the PCR product accumulates the fluorescence which is emitted by the reaction mix increases.

Biological Safety Cabinet
Laboratory design for Molecular Assays

Physically separate areas are required to reduce the risk of cross-contamination and carry-over contamination

Dedicated, separate, clean and contained

ONE WAY WORKFLOW OF STAFF AND SPECIMENS

1. Mastermix preparation (positive pressure)

2. Extraction area (positive pressure)

3. Amplification (negative pressure)

4. Post PCR (sequencing, nested PCR) (negative pressure)

What are the guidelines for the operation of a molecular diagnostic facility?

NATA: National Association of Testing Authorities

NPAAC: National Pathology Accreditation Advisory Council

Based on Health Insurance Act 1973

Laboratory Accreditation Standards and Guidelines for Nucleic Acid Detection and Analysis (draft Nov 2004)

To provide uniform standards nationally based on good laboratory practice

International Standards

AS ISO/IEC 17025:1999 General requirements for the competence of testing and calibration laboratories

ISO 15189:2003 Medical laboratories – Particular requirements for quality and competence

Extraction of Nucleic Acid

MagnaPure
table-top instrument for batched extraction of nucleic acids (32 per run)

Large footprint

CAS-1200 Robot

Automated aliquotting of mastermixes, extracts, loading

X-tractorGene: 96 well capacity

Small footprint
COBAS AMPLICOR
automates the amplification and
detection steps of the Polymerase Chain
Reaction (PCR) testing process on a
single instrument. It combines thermal
cycler, automatic pipettor, incubator,
washer and reader

Utilizes the AmpEraser® contamination control system. AmpEraser®
uracil-N-glycosylase) chemically inactivates amplified material and prevents
it from contaminating future reactions

Assays performed
- Cytomegalovirus (CMV) quantitative
- Gonorrhoea
- Hepatitis C (qualitative and quantitative)
- HIV viral load (quantitative)
- Contains internal control to detect inhibition

PRACTICAL APPLICATION
Monitoring CMV disease in transplant patients,
particularly Bone Marrow Transplant recipients.

1. Early detection of disease progression to
   apply appropriate drug therapy
2. Detect ganciclovir drug resistance

Prevention of amplicon contamination
Use of uracil-N-glycosylase enzyme
removes uracil residues from DNA preventing its use as a hybridization
target
- Perform PCR with dUTP substituted for dTTP

In subsequent PCR reactions:
- Add 1 unit of UDG per 100 uL PCR reaction (1 unit = catalyzes the
  release of 1 nmol of free uracil in one hour at 37˚C from 3H-poly-dU)
- Incubate all PCR reactions at 37˚C prior to starting cycling reactions to
  allow enzyme to function
- The enzyme will be destroyed when the PCR reaction is heated to 94˚C
- Only effective with low level contamination

TaqMan 48
Has two thermocycler segments,
and can load up to two different
assays on board simultaneously

Assays
- Hepatitis B quantitative assay
- Chlamydia assay

Light Cycler II
quantification of amplification
products in real time, using
two fluorescence-based
methods and six different
detection channels.

Allows characterization of the
PCR product and genotyping
via melting-curve analysis.

Assays performed
All “in-house” assays with the same cycling conditions
- Bordetella pertussis (whooping cough), CMV (qualitative), Enterovirus,
  HSV1/2, Measles, Mumps, Mycoplasma, Neisserial meningitidis
  (meningococcal), polyoma, Streptococcus pneumoniae, VZV

ON-CALL assays all on this instrument – fast cycling due to rotary format
Characterisation of HSV by melting curve

HSV

DNA pol

Primers common to HSV 1 & 2

Hybridisation probes (to HSV-1)

HSV-1

Amplicon

HSV-2

Mismatch

Rotorgene 3000

4 Channel Multiplexing System

centrifugal design to increase sensitivity and precision

Assays performed

Adenovirus, Bordetella henselae, Chlamydia confirmatory assay, EBV quantitative, GUMP, HHV6, Neisseria gonorrhoea confirmatory assay, Parvovirus B19, Treponema pallidum.
ABI 7500

- High speed thermal cycling in a 96-well format

**Assays performed**

- Multiplex respiratory assay
- Detects: Influenza A and B, Parainfluenza 1, 2 and 3, RSV, Adenovirus and human metapneumovirus

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**Amplification curves of 10 fold target dilutions**

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**Microbial Load Testing**

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**ADVANTAGES OF REAL-TIME PCR**

- Rapid cycling times (1 hour)
- High sample throughput (~200 samples/day)
- Low contamination risk (sealed reactions)
- Very sensitive (3pg or 1 genome eq of DNA)
- Broad dynamic range (10 - 10^{10} copies)
- Reproducible (CV < 2.0 %)
- Allows for quantitation of results
- Software driven operation
- No more expensive than “in house” PCR ($15/test)
Limitations

Failure to detect the organism does not preclude it as the source of disease eg. measles virus, dengue virus only detectable during the acute phases

Has the specimen been collected, transported appropriately?

Is the request appropriate? eg. hepatitis E request requires travel history

Detection of an organism may not be clinically relevant in an immunocompetent patient eg. commensal bacteria

Result Interpretation

*Does detection of DNA indicate infection??*

Normal microbial flora - site dependant
Latent or persistent infections - herpesviruses

**Diagnose current/acute infection by:**

Quantitative PCR - need to determine baseline levels
Detect mRNA - only produced during active replication

Viral Infection Stages

*Sample needs to be taken at correct stage of disease*

<table>
<thead>
<tr>
<th>Disease stage</th>
<th>Virus</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>incubation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>onset</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>acute</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>recovery</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>convalescence</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

*Sample collected from appropriate site*
- nasal swab, CSF, serum, whole blood

Advantages of accurate diagnosis

- discontinue antibacterial therapy
- patient management - less hospital stay
- minimize further lab testing
- appropriate infection control measures
  (eg acute respiratory illness in children)

PCR SUMMARY

Constantly changing technology eg. PCR and gels to real time (2006 on our lab)

Rate limiting step is the extraction procedure

Prevention of amplicon contamination is critical

Require highly skilled staff

Interpretation of the result in combination with clinical information

Variations of PCR in the Diagnostic Lab

The most common variations of standard nucleic acid amplification used in the diagnostic laboratory are:

- Reverse Transcriptase PCR (RT-PCR)
- Nested PCR (n-PCR)
- Multiplex PCR (m-PCR)
- Real-time PCR
- Strand Displacement Amplification (SDA)
- Transcription Mediated Amplification (TMA)
Hydridization Assays
Eg. Human Papillomavirus DNA Assay
HPV DNA + HPV RNA probe cocktail

RNA:DNA hybrid

Chemiluminescence
Alkaline phosphatase conjugated antibody specific for RNA:DNA hybrids
RNA:DNA hybrid
RNA:DNA specific hybrid antibody

Microarrays (Gene Chips)
APPLICATIONS
- Genome mutational analysis
- Multiple drug resistance
- Monitor gene expression in cells
- Pharmacogenomics
- Screening for multiple infectious agents

LAB TOUR ?
Enclosed footwear required