Use of Internal Controls in Clinical PCR Assays

KJ Mutton & M Guiver
HPA NW & Clinical Virology
Manchester Royal Infirmary
Internal controls detect inhibitors of amplification

- False negative PCRs
  - Failure of one or more reagents
  - Failure of amplification
    - Failure of thermal cycling
    - Inhibition of amplification
  - Failure of reporting of reaction
Recent events highlight risks of false negative results

- **False negative qualitative HCV PCR result**
- **In house HCV PCR assay**
  - used for confirmation of initial anti-HCV Ab reactivity
  - long established assay
  - No internal amplification control

- Discrepancy noted 23/4/10 in renal patient
  - Negative in qualitative HCV PCR
  - previously Positive

- **Sample inhibitory** in commercial quantitative PCR
  - 635,118 IU/ml
  - two other samples qualitative PCR negative
Laboratory lookback shows extent of false negative results

90 Qualitative PCR Negative Antibody Positive samples retested by commercial assay
- 79 (88%) Negative PCR by quantitative assay
- 11 (12%) Positive (3 with VL >50000 IU/ml).

153 samples re-tested retrospectively using an internal control of amplification
- 3 (2%) were found to give complete inhibition
- further 21 (14%) were partially inhibitory.
  - Dilution of serum 1 in 10 diluted out the inhibitors in most cases
  - No changes in sequence affecting primer/probe sites

- 12-16% false negative PCR rate
- Potential failure to treat active HCV cases
Commercial assays tend to include IC

- **Commercial**
  - Regulation
  - High volume – developmental/returns

- **In house**
  - Historical – gel based
  - Evolution of tests – increased clinical utility
  - Inertia on redeveloping old assays – development priority
  - Effort
  - Cost
  - Consensus/strategy
    - Organizations
    - Validation may not show need eg tma / csf
Assessing extent of IC problem

**Questionnaire 1**

- 10 HPA regional/collaborating laboratories

- to audit HCV confirmation strategies
  - VSOP5

- to assess in house assay use and internal controls
  - hepatitis C
    - other hepatitis virus PCRs
  - other
    - bacterial
    - fungal
    - parasitic
Questionnaire 1

• **Qualitative HCV PCR?**
  2 in house real time
  1 in house block based
  2 commercial real time

• **With internal amplification control**
  1 in house RT
  2 commercial

• **any in house assays without inhibitor controls?**
  Yes  7
  No   3

Questionnaire 2

HPA Reference Laboratory PCRs
Questionnaire 3

• CVN/HPA Questionnaire
• information on in house assay IC use
• 12 responses, most from larger Virology centres

Only 1 (SVC) had IC in every assay
Range of assays performed

- **Viral**
  - (HCV)
  - HIV, HBV
  - adenovirus, HSV, VZ, CMV, EBV
  - respiratory panels
  - norovirus, astrovirus, sapovirus, rotavirus
  - enterovirus, parechovirus
  - BK and JC polyomaviruses
  - measles
  - genotyping HCV etc

- **Bacterial**
  - 16S (1), *S.aureus* PVL (1), *T.pallidum* (2)

- **Parasitic**
  - *Toxoplasma gondii* (1)

- **Fungal**
  - *Aspergillus* (1), *Pneumocystis jirovecii* (2), *Candida* spp
Examples show variation in IC use and type

- **CMV** 6 assays 6 IC
  - BMV 1
  - Murine CMV 1
  - Phocine herpes 1
  - Exogenous synthetic DNA oligonucleotide 3

- **HSV** 7 assays 5 IC
  - BMV 1
  - Murine CMV 1
  - Exogenous synthetic DNA oligonucleotide 3
  - Endogenous RNase P 1

- **EBV** 3 assays 2 IC
  - Murine CMV 1
  - Endogenous RNaseP 1

- **Adenovirus** 9 assays 5 IC
  - Mengovirus cDNA 1
  - Murine CMV 2
  - Exogenous synthetic DNA oligonucleotide 1
  - Endogenous RNAse P 1
Examples show variation in IC use and type

- **RSV** 6 assays 5 IC
  - Mengovirus RNA 1
  - BMV 2
  - MS2 phage 1
  - Phocine distemper 1

- **Parainfluenza** 6 assays 5 IC
  - Mengovirus RNA 1
  - BMV 2
  - MS2 phage 1
  - Phocine distemper 1

- **Influenza**
- **Influenza H1 N1**
Stage of addition of IC

<table>
<thead>
<tr>
<th>Virus</th>
<th>Whole process</th>
<th>Amplification only</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>HSV</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>influenza</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>norovirus</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
IC: separate target or multiplexed with test target

<table>
<thead>
<tr>
<th></th>
<th>separate</th>
<th>multiplex</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>HSV</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>influenza</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>norovirus</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>
monitoring IC performance

<table>
<thead>
<tr>
<th></th>
<th>Interface to QC software</th>
<th>Interface to LIMS</th>
<th>Manual record</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>HSV</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>influenza</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>norovirus</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Validation of test run:
IC used as Pass/Fail

e.g. cutoff mean CT of IC + 3SD
Conclusions – further consideration

• Need for internal amplification controls
  – Range of assays still lack IC
    • Improving
    • prioritise assays/specimen types
    • revalidation of assay required if internal control added

  – Range of approaches

  – **Standardisation**?
    • consider rationalizing range of internal controls being used
      – **Consensus**
      – Better standardisation
      – Reduced cost
      – Improved quality

    • consider IT requirements for monitoring
    • consider
      – whole process controls
      – PCR only controls
      – endogenous gene assessing sample adequacy
        » Hierarchy of ‘adequacy’ of control

• **EQA/IQA**
  – ensure *all* assays are adequately tested