MycoTOOL
A Commercial Mycoplasma PCR Test according E.P. 2.6.7

Markus Klinkicht
MycoTOOL test
A Commercial Mycoplasma PCR Test according E.P. 2.6.7

Introduction

Generic Method Validation

Implementation of a Ready-To-Use Test-Kit

Product-specific Method Validation

Summary
MycoTOOL test

Acceptance by Authorities - Current Status (2)

- In 2008 the Ministry of Health, Labour and Welfare of Japan (MHLW) and the EMEA approved a pharmaceutical product of Bayer Health Care using a homebrew Mycoplasma PCR Test following the same underlying concept.

- In July 2009, the European Medicines Agency (EMA) approved a pharmaceutical product from Roche in combination with the MycoTOOL: The MycoTOOL PCR assay is approved to replace both traditional mycoplasma test methods.

The EMEA has informed the Commission on 25 June 2009 that the CHMP recommends the following variation to the marketing authorisation file:

Scope of the variation introduced by the procedure EMEA/H/C/278/II/46
Changes in the analytical methods or testing location of specific tests for the control of the drug substance:
- Replacement of the test for mycoplasma by a PCR method at Penzberg and Genentech sites.
MycoTOOL test
Acceptance by Authorities - Current Status (3)

- December 2009: Preliminary assessment report of the German Health Authority (BfArM) (rapporteur) with result “approvable” for a Type II Variation for a Biological (Variation on new cell banking system and analytical changes, e. g. Mycoplasma-PCR)

- Assessor’s comment:
  - Using a NAT for mycoplasma determination as an alternative to the cell culture method and the indicator cell culture method is in accordance with the respective monograph of the Ph.Eur. From the validation data provided it can be concluded that the proposed mycoplasma PCR complies with the specific requirements of the Ph.Eur., chapter 2.6.7, and is suitable for mycoplasma determination in the unprocessed … bulk.
  - The cell culture method and the indicator cell culture method are used as confirmatory tests in case of PCR detection of bacterial DNA. Both methods are performed in accordance with the Ph.Eur. chapter 2.6.7. The validation data indicate that both methods meet the predetermined acceptance criteria and are suitable for detection of mycoplasmas in the unprocessed bulk of …. The change is considered acceptable.

- January 2010: Preliminary assessment report of the Spanish Health Authority (rapporteur) with result „approvable“ for a Type II Variation of a Biological follows the assessment of BfArM (Variation on new cell banking system and analytical changes, e. g. Mycoplasma-PCR for the production of the intermediate X)
2010: Authorization of the Federal Agency for medicines and health products (Belgium) to use Mycoplasma-PCR for clinical trials:

Unofficial translation

Conform article 12 of the Law of 7 May 2004 concerning experiments on the human person, I have decided to authorize the above mentioned clinical trial. However, the points as mentioned in annex are to be followed up.

ANNEX

QUALITY

There are no major concerns arising from the quality assessment of the investigational medicinal product. Therefore, we have no objections against the start of the Clinical Trial.
MycoTOOL test

Acceptance by Authorities - Current Status (6)

- 2010: Authorization of the MHRA (England) to use Mycoplasma-PCR for clinical trials
Customer can refer to the Roche method validation data and decrease effort of validation

Validation of a NAT-based *Mycoplasma* assay according European Pharmacopoeia

Sven M. Deutschmann*, Holger Kavermann, Yvonne Knack

Roche Diagnostics GmbH, Quality Control, Nonnenwald 2, 82377 Penzberg, Germany

**E.P. 2.6.7 Guidelines, Validation of NAT detection of Mycoplasmas, Scope, page 8**

"...Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can replace validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user..."
MycoTOOL test

Introduction – Steps to Validation of MycoTOOL

• Genentech – Test design, published 2004 ¹)

• Transfer of test to Roche Pharma, 2007

1. Roche homebrew – Generic method validation

   MycoTOOL kit - Development

2. MycoTOOL kit - Bridging study (comparability of homebrew and MycoTOOL test)

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MycoTOOL test
Validated according E.P. 2.6.7 – Sensitivity (1)

Sensitivity / Detection Limit: *M. orale* (as an example)

- Validated sensitivity: 1 cfu/mL

<table>
<thead>
<tr>
<th>Spike</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Σ</th>
<th>acc. criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
<td>24/24</td>
<td>passed</td>
</tr>
<tr>
<td>100 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
<td>24/24</td>
<td>passed</td>
</tr>
<tr>
<td>10 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
<td>24/24</td>
<td>passed</td>
</tr>
<tr>
<td>1 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
<td>24/24</td>
<td>passed</td>
</tr>
<tr>
<td>0.1 cfu/mL</td>
<td>8/8</td>
<td>6/8</td>
<td>8/8</td>
<td>22/24</td>
<td>failed</td>
</tr>
<tr>
<td>0.01 cfu/mL</td>
<td>7/8</td>
<td>6/8</td>
<td>7/8</td>
<td>20/24</td>
<td>failed</td>
</tr>
</tbody>
</table>

**Note:**
- 8/8: 8 out of 8 spiked samples were positive
- All corresponding positive and negative controls fulfilled the acceptance criteria
### Summary of the results of the generic method validation

<table>
<thead>
<tr>
<th>Validated: ≥ 23 out of 24 replicates to be positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species</strong></td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>A. laidlawii*</td>
</tr>
<tr>
<td>M. arginini</td>
</tr>
<tr>
<td>M. fermentans</td>
</tr>
<tr>
<td>M. hominis*</td>
</tr>
<tr>
<td>M. hyorhinis</td>
</tr>
<tr>
<td>M. orale</td>
</tr>
<tr>
<td>M. pneumoniae</td>
</tr>
<tr>
<td>M. salivarium</td>
</tr>
<tr>
<td>S. citri</td>
</tr>
<tr>
<td>M. gallisepticum</td>
</tr>
<tr>
<td>M. synoviae</td>
</tr>
</tbody>
</table>

* Result from bridging study with MycoTOOL test

- This validation for a CHO cell line required more than 17,000 PCR reactions.
- Nine isolates were validated at a 95% confidence level (≥23 out of 24 have to be positive).
- A tenfold dilution series of 1000 CFU/ml to 0.01 CFU/ml was performed.
- Each replicate was done in quadruplicate PCR, and 96 PCR reactions were performed per concentration. The established sensitivity is 1 CFU/ml.
Introduction

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MycoTOOL test

Weaknesses of the “Homebrew”-Assay

- **Reagents:**
  - 25 Reagents or solutions from 5 different suppliers with the risk of lot-to-lot variability
  - Extreme labor- and cost-intensive procedure
    - to prepare the solutions
    - to qualify so called “critical” reagents
      - 16 reagents / solutions defined as „critical“ reagents / solutions
      - Special SOP (45 pages) for the preparation and qualification of these critical reagents / solutions

- **Procedure:**
  - Psoralen / UV-treatment to prevent DNA-contaminations - “volunteers” (critical to standardize)
  - Risk of carry-over contamination
  - Post-PCR-staining of the amplicons (potential harmful)
MycoTOOL test

Challenge

Develop DNA depleted „ready to use“ reagents
**MycoTOOL test**

*Increased Sensitivity compared to Homebrew*

A bridging study comparing the Roche homebrew Test to the MycoTOOL test demonstrates higher sensitivity

<table>
<thead>
<tr>
<th>Validated sensitivity: ≥ 23 out of 24 replicates to be positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>A. laidlawii</strong></td>
</tr>
<tr>
<td><strong>M. hominis</strong></td>
</tr>
</tbody>
</table>
MycoTOOL test

Increased Specificity compared to Homebrew

A bridging study comparing the Roche homebrew Test to the MycoTOOL test demonstrates higher specificity

<table>
<thead>
<tr>
<th>Species</th>
<th>ATCC</th>
<th>Roche Homebrew test [CFU/ml]</th>
<th>MycoTOOL test [CFU/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus</td>
<td>4356</td>
<td>n.d.</td>
<td>+</td>
</tr>
<tr>
<td>S. bovis</td>
<td>35034</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. sporogenes</td>
<td>11437</td>
<td>+</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
MycoTOOL test

*MycoTOOL Carrier DNA reagent for non cellular matrices (I)*

MycoTOOL Carrier DNA reagent enhances sensitivity

Tris buffer without Carrier DNA

1 2 3 4 5 6 7 8 9 10 11 12 M

1-4: 10cfu/ml A.laidlawii; 5-8: 3cfu/ml A.laidlawii; 9-12: 1cfu/ml A.laidlawii

Tris buffer with Carrier DNA

1 2 3 4 5 6 7 8 9 10 11 12 M
MycoTOOL test

MycoTOOL Carrier DNA reagent for non cellular matrices (II)

MycoTOOL Carrier DNA reagent enhances specificity

1-4: 3cfu/ml *A.laidlawii* spiked to allantoic fluid → sample preparation w/o Carrier DNA;
5-8: 3cfu/ml *A.laidlawii* spiked to allantoic fluid → sample preparation with Carrier DNA;
9-12: allantoic fluid w/o *A.laidlawii* → sample preparation with Carrier DNA;
13-14: 10 cp plasmid control
MycoTOOL test
Covers a broad variety of sample materials

These MycoTOOL test results demonstrate that there is no PCR inhibition. Mycoplasmas are also easily detected in cell-free matrices with the same sensitivity when MycoTOOL Carrier DNA reagent is added during sample preparation.

<table>
<thead>
<tr>
<th>Cellular matrices</th>
<th>A. laidlawii</th>
<th>M. orale</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 CFU/ml</td>
<td>3 CFU/ml</td>
</tr>
<tr>
<td>MycoTOOL test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRC 5 cells</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vero cells</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BHK cells</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human stem cells</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Cell-free matrices

<table>
<thead>
<tr>
<th>MycoTOOL test + MycoTOOL Carrier DNA reagent</th>
<th>Allantoic fluid</th>
<th>Human stem cell supernatant</th>
<th>CHO cell supernatant</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Not validated
Introduction

Generic Method Validation

Implementation of a Ready-To-Use Test-Kit

Product-specific Method Validation

Summary
Sensitivity / Detection Limit Product C (MAB)

- **A. laidlawii**: 1 cfu/ml
- **M. hominis**: 1 cfu/ml

<table>
<thead>
<tr>
<th>Mollicutes Species</th>
<th>Spike</th>
<th>Ferm. Run 1</th>
<th>Ferm. Run 2</th>
<th>Ferm. Run 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. laidlawii</strong></td>
<td>10 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>5 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>1 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td><strong>M. hominis</strong></td>
<td>10 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>5 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>1 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
</tbody>
</table>

**Note:** 8/8: 8 out of 8 spiked samples were positive

All corresponding positive and negative controls fulfilled the acceptance criteria.
### MycoTOOL test

*Product-specific Method Validation IV*

#### Sensitivity / Detection Limit Product D (MAB)

- **A. laidlawii**: 1 cfu/ml
- **M. hominis**: 1 cfu/ml

<table>
<thead>
<tr>
<th>Mollicutes Species</th>
<th>Spike</th>
<th>Ferm. Run 1</th>
<th>Ferm. Run 2</th>
<th>Ferm. Run 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. laidlawii</em></td>
<td>10 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>5 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>1 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td><em>M. hominis</em></td>
<td>10 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>5 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>1 cfu/mL</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
</tbody>
</table>

*Note: 8/8: 8 out of 8 spiked samples were positive*

All corresponding positive and negative controls fulfilled the acceptance criteria.
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MycoTOOL test

Summary

- The first commercial Mycoplasma PCR test used for release testing of approved pharmaceutical products.

- High sensitivity, 1cfu/ml:
  - Purified Mycoplasma free reagents are used
  - The precipitation technique is a highly sensitive technology

- Safety on results:
  - The whole cellular DNA goes into PCR, no risk to loose target DNA
  - Repetitions: two sample preps and 4 PCRs per sample are performed
  - Controls: GAPDH PCR controls lysis of matrix and inhibition

- Broad panel of sample materials:
  - MycoTOOL performs robust on different media, different cell lines and different cellular titers
  - MycoTOOL Carrier DNA reagent serves as sensitivity enhancer (cell free material) and a PCR specifier for difficult matrices
MycoTOOL test

Core Team

- C. Birkner and A. Bartes, R&D, Roche Diagnostics Penzberg
- Y. Ueno, Sales, RDKK Tokyo
- M. Klinkicht, Marketing, Roche Diagnostics Penzberg
- S. Deutschmann, QC, Roche Pharma Penzberg
MycoTOOL test

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