Caught between two tools: Proof of concept of the new ISO 16140-6 for the validation of confirmation methods and harmonized study design with the AOAC Guidelines

Erin Crowley (USA), Imola Ferro (NL), Wilma Jacobs (NL), Daniele Sohier (DE), Paul in’t Veld (NL), Joost Witsenburg (NL), Members of MicroVal Technical Committee
<table>
<thead>
<tr>
<th>General Committee</th>
<th>Food Safety Authorities</th>
<th>Certification and standardization bodies</th>
<th>Laboratories and users</th>
<th>Manufacturers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FVST</td>
<td>AOAC</td>
<td>ADRIA* Campden BRI*</td>
<td>3M* bioMérieux* Bio-Rad</td>
</tr>
<tr>
<td></td>
<td>NVWA*</td>
<td>NMKL Loyd’s</td>
<td>Nestlé* RIVM* Q-Laboratories*</td>
<td>Biotecnu Bruker*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Merck R-Biopharm</td>
</tr>
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<td>Bio-Rad Biotecnu</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bruker* Merck</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Neogen*</td>
</tr>
</tbody>
</table>

*Organizations involved in the ISO working group on the ISO 16140 series
International organization for the validation of alternative methods in food and water microbiology
1. The ISO16140-6 in the ISO 16140 series
2. Confirmation versus Identification
3. The ISO 16140-6 technical rules
4. MicroVal pilot studies for confirmation methods
5. Caught between two tools...
6. Take-home message
ISO 16140 for the validation of alternative (proprietary) methods

Timeline (1/2)

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003</td>
<td>Publication of the ISO 16140 standard - Protocol for the validation of alternative methods</td>
</tr>
<tr>
<td>2006</td>
<td>Revision of the ISO 16140</td>
</tr>
<tr>
<td>2016</td>
<td>Publication</td>
</tr>
</tbody>
</table>
  - ISO 16140–part 1: Vocabulary |
  - ISO 16140–part 2: Protocol for the validation of alternative (proprietary) methods against a reference method |

1. Alternative (proprietary) methods for the detection and enumeration of specific microorganisms are validated according to the ISO 16140 standard since 16 years.

2. This ensures the recognition of the validated methods by regulation bodies (e.g. EU 2073/2005, FDA) and facilitate the accreditation process by the end-users.

3. These alternative methods enable usually time- and cost-saving, and are easy-to-use.

What about the validation and recognition of confirmation and typing methods?
ISO 16140 for the validation of alternative (proprietary) methods

Timeline (2/2)

- **2003**  
  Publication of the ISO 16140 standard - Protocol for the validation of alternative methods

- **2006**  
  Revision of the ISO 16140

- **2016**  
  Publication
  - ISO 16140–part 1: Vocabulary
  - ISO 16140–part 2: Protocol for the validation of alternative (proprietary) methods against a reference method

- **2019**  
  **FDIS of the ISO 16140-part 6**: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures

- **2019**  
  FDIS of the ISO 16140-part 4 and 5 respectively on in-house method validation and factorial design
  
  Pre-FDIS of the ISO 16140-part 3 on method verification
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Confirmation versus Identification

2.17 - confirmation procedure or test

procedure or test which is carried out to verify a presumptive result

*Note 1 to entry: Not all methods have a confirmation procedure*

The expected outcome of a confirmation test is a binary scheme, e.g., “positive” or “negative” = “Salmonella spp isolate or non-Salmonella spp. isolate”

2.30 - identification procedure or test

procedure or test yielding the identity of the analyte

The expected outcome of an identification test is an open scheme, e.g. Salmonella spp. or Klebsiella oxytoca
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The sample is a microbial isolate on a specific culture medium.

Therefore, the culture media tested during the validation shall be clearly defined.

Photo kindly provided by Bio-Rad
### General principles (2/4)

**Workflow**

<table>
<thead>
<tr>
<th>Screening step, i.e. detection or enumeration</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Reference method, e.g. ISO method</td>
</tr>
<tr>
<td>• Alternative method validated according to the ISO 16140-2 standard</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isolation on defined culture media</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>XLD</strong></td>
</tr>
<tr>
<td>✓</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Confirmation or Typing</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Reference method protocol, e.g. ISO method</td>
</tr>
<tr>
<td>• Proprietary protocol tested during the ISO 16140-2 validation of the screening method</td>
</tr>
<tr>
<td>• Alternative method validated according to the ISO 16140-part 6 standard for defined culture media</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>XLD</strong></th>
<th><strong>Hektoen</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>✓</td>
<td>✗</td>
</tr>
</tbody>
</table>
ISO 16140-6 study

- The testing and data interpretation SHALL be conducted by an expert (independent) laboratory
- The data generated by the alternative method are compared to the reference method, e.g. ISO confirmation procedure

Acceptability Limits (AL)

- **maximum positive or negative acceptable difference** between the reference value (or if not known, the accepted reference value) of a sample and an individual result obtained when applying the operating procedure of an analytical method

Interpretation

- Accepted
- Rejected
Method Comparison study on numerous
- target strains = inclusivity testing
- non-target strains = exclusivity testing
To assess the **reliability** of the method

**Expert laboratory** (third party)

Inter-laboratory study with a restricted number of target and non-target strains to assess the **reproducibility** of the method with different operators, instruments, materials

Minimum 10 valid data sets from different collaborators
Content

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## ISO 16140-6
### FOUR Method Comparison Studies

<table>
<thead>
<tr>
<th>Target analytes</th>
<th>Instruments Targets</th>
<th>Culture media</th>
<th>Number of strains</th>
<th>(Strain/culture) conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>MBT MBT smart</td>
<td>5 selective, 1 non selective</td>
<td>150 <em>Salmonella</em> spp. 100 Non <em>Salmonella</em> spp.</td>
<td>6,000</td>
</tr>
<tr>
<td><em>Cronobacter</em> spp.</td>
<td>MBT Steel Targets MBT Biotarget 96</td>
<td>2 selective, 1 non selective</td>
<td>150 <em>Cronobacter</em> spp. 100 Non <em>Cronobacter</em> spp.</td>
<td>3,000</td>
</tr>
<tr>
<td><em>Campylobacter</em> spp.</td>
<td>MBT Steel Targets MBT Biotarget 96</td>
<td>4 selective, 1 non selective</td>
<td>150 <em>Campylobacter</em> spp. 100 Non <em>Campylobacter</em> spp</td>
<td>5,000</td>
</tr>
<tr>
<td><em>Listeria</em> spp. and <em>L. monocytogenes</em></td>
<td>MBT smart</td>
<td>5 selective, 1 non selective</td>
<td>120 <em>Listeria</em> spp.* 100 <em>L. monocytogenes</em> 100 Non <em>Listeria</em> spp.</td>
<td>7,680</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td></td>
<td><strong>21,680</strong></td>
<td></td>
</tr>
</tbody>
</table>

*9 new species*
### ISO 16140-6

**FOUR Method Comparison Studies**

<table>
<thead>
<tr>
<th>Approvals</th>
<th>Inclusivity panel Correct confirmation rate</th>
<th>Exclusivity panel Correct non-confirmation rate</th>
<th>Acceptability Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salmonella spp.</strong></td>
<td>100 % correct confirmation rate</td>
<td>100% correct non-confirmation rate</td>
<td>✅ For all the tested agars</td>
</tr>
<tr>
<td><strong>Cronobacter spp.</strong></td>
<td>100 % correct confirmation rate</td>
<td>100% correct non-confirmation rate</td>
<td>✅ For all the tested agars</td>
</tr>
<tr>
<td><strong>Campylobacter spp.</strong></td>
<td>100 % correct confirmation rate</td>
<td>100% correct non-confirmation rate</td>
<td>✅ For all the tested agars</td>
</tr>
<tr>
<td><strong>Listeria spp. and L. monocytogenes</strong></td>
<td>100 % correct confirmation for Listeria spp. 99.5 %* correct confirmation rate for L. monocytogenes</td>
<td>100% correct non-confirmation rate</td>
<td>✅ For all the tested agars</td>
</tr>
</tbody>
</table>

* *L. marthii* cannot be differentiated from *L. monocytogenes* by the tested technology as mentioned previously by the manufacturer. Note that *L. marthii* isolates do not usually show a halo on Ottaviani & Agosti Agar and on RAPID’L. mono.
ISO 16140-6
FOUR Inter-Laboratory Studies

<table>
<thead>
<tr>
<th>Target analytes</th>
<th>Collaborators</th>
<th>Culture media</th>
<th>Number of strains</th>
<th>(Strain/culture) conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> spp. and other Gram negative</td>
<td>15</td>
<td>2 selective 1 non selective</td>
<td>16 <em>Salmonella</em> spp. 8 Non <em>Salmonella</em> spp.</td>
<td>1,152</td>
</tr>
<tr>
<td><em>Cronobacter</em> spp. and other Gram negative</td>
<td>14</td>
<td>2 selective 1 non selective</td>
<td>16 <em>Cronobacter</em> spp. 8 Non <em>Cronobacter</em> spp.</td>
<td>1,008</td>
</tr>
<tr>
<td><em>Campylobacter</em> spp. and other Gram negative</td>
<td>17</td>
<td>3 selective 1 non selective</td>
<td>16 <em>Campylobacter</em> spp. 8 Campylobacter spp.</td>
<td>1,632</td>
</tr>
<tr>
<td><em>Listeria</em> spp. and <em>L. monocytogenes</em> and other Gram positive</td>
<td>16</td>
<td>3 selective 1 non selective</td>
<td>12 <em>Listeria</em> spp. 16 <em>L. monocytogenes</em> 8 Non <em>Listeria</em> spp.</td>
<td>2,304</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>6,096</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pre-collaborative and collaborative studies = 27,776 data
## ISO 16140-6

**FOUR Inter-Laboratory Studies**

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<td><em>Salmonella</em> spp.</td>
<td>100 % correct confirmation rate</td>
<td>100% correct non-confirmation rate</td>
<td>✓ For all the tested agars</td>
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<tr>
<td><em>Cronobacter</em> spp.</td>
<td>100 % correct confirmation rate</td>
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<td>✓ For all the tested agars</td>
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<tr>
<td><em>Campylobacter</em> spp.</td>
<td>100 % correct confirmation rate</td>
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<td><em>Listeria</em> spp. and <em>L. monocytogenes</em></td>
<td>100 % correct confirmation for <em>Listeria</em> spp. and <em>L. monocytogenes</em></td>
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</tr>
</tbody>
</table>
The MALDI Biotyper® (Bruker) is the very FIRST confirmation method certified according to the new ISO/DIS 16140-part 6 standard by MicroVal

for the confirmation of:

✓ Cronobacter spp.
✓ Salmonella spp.
✓ Campylobacter spp.
✓ Listeria spp. and Listeria monocytogenes

The certificates and reports are available on www.microval.org

Certificate N° 2017LR72
Certificate N° 2017LR73
Certificate N° 2017LR74
Certificate N° 2017LR75
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General principles
Two study parts as well

Method Comparison study on numerous
- target strains = inclusivity testing
- non-target strains = exclusivity testing
To assess the reliability of the method

Inter-laboratory study with a restricted number of target and non-target strains to assess the reproducibility of the method with different operators, instruments, materials

Expert laboratory (third party)
Minimum 10 valid data sets from different collaborators
The MALDI Biotyper® (Bruker) has been certified according to the Official Method of Analysis program (OMA) of the AOAC International for the confirmation and identification of:

- *Salmonella* spp.
- *Cronobacter* spp.
- *Campylobacter* spp.
- and other gram-negative bacteria

- *Listeria* spp.
- *Listeria monocytogenes*
- and other gram-positive bacteria

First Action AOAC Official MethodSM 2017.09
First Action AOAC Official MethodSM 2017.10
Confirmation versus Identification

- **ISO 16140-part 1 (2016) definitions**

**2.17 - confirmation procedure or test**

procedure or test which is carried out to verify a presumptive result

*Note 1 to entry: Not all methods have a confirmation procedure*

The expected outcome of a confirmation test is a binary scheme, e.g., “positive” or “negative” = “Salmonella spp isolate or non-Salmonella spp. isolate”

**2.30 - identification procedure or test**

procedure or test yielding the identity of the analyte

The expected outcome of an identification test is an open scheme, e.g. *Salmonella* spp. or *Klebsiella oxytoca*
• ISO 16140-part 1 (2016) definitions

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procedure or test yielding the identity of the analyte

The expected outcome of an identification test is an open scheme, e.g. Salmonella spp. or Klebsiella oxytoca
The MicroVal technical committee has “kindly” reviewed all the identification data of the exclusivity testing. These data are commented in the reports. But these data CANNOT be used for performances assessment according to the currently available ISO standards. There is certainly a need to define new guidelines/rules for the recognition of new identification principles.
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Take-home Message

1. Several members of the MicroVal technical and general committee are strongly involved in the development of the ISO 16140 series.

2. Thanks to this leadership, MicroVal has run the very first studies according to the ISO/DIS 16140-part 6 standard, demonstrating the proof concept for the validation of confirmation methods.

3. The final version of the ISO 16140-part 6 will be published before the end of the year, with no impact of the currently available validation certificates.

4. The certificates and reports of the MALDI Biotyper (Bruker) validation studies are available on www.microval.org.

5. The next step is now certainly to develop guidelines/rules for the recognition of identification methods.
Thank you for your attention

www.microval.org
microval@nen.nl

Erin Crowley (USA), Imola Ferro (NL), Wilma Jacobs (NL), Daniele Sohier (DE), Paul in’t Veld (NL), Joost Witsenburg (NL), Members of MicroVal Technical Committee