FASFC ACCEPTANCE CRITERIA FOR MICROBIOLOGICAL INHIBITOR TESTS: FULFILLMENT BY NEW TESTS

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Abstract
The Belgian Federal Agency for the Safety of the Food Chain (FASFC) fixed acceptance criteria for microbiological inhibitor tests to be used for the testing of ex-farm milk on inhibitors as part of the regulatory quality programme. It concerns criteria regarding general test specifications, the test repeatability, the detection capabilities and the test robustness.
Validation studies according to Commission Decision 2002/657/EC of newly developed microbiological inhibitor tests were performed at ILVO-T&V in order to check if they meet the criteria and hence could be added to the list of accepted tests. Following microbiological inhibitor tests were involved in the study: Delvotest SP-NT Accelerator, Eclipse 50, Charm Blue Yellow II, Eclipse 3G and Delvotest T.

Introduction
In accordance with Corrigendum to Regulation (EC) No 853/2004, a representative number of raw milk samples, collected from milk production holdings by random sampling, must be checked for that the raw milk placed on the market is not containing antibiotic residues in a quantity that exceeds the levels authorized (MRLs) under Council Regulation (EEC) No 2377/90 (now repealed by Commission Regulation (EU) No 37/2010), or that the combined total of residues of antibiotic substances does not exceed any maximum permitted value. To fulfill this requirement, in most European countries, farm milk is screened for antimicrobials as part of a regulatory quality programme by means of a microbiological test.
In Belgium, in the regulatory quality programme the CMT (Copan Milk Test; DSM-Food Specialties) is presently (May 2012) used as screening test. The milk control stations can choose a screening test among the tests mentioned in the list of accepted tests of the Belgian Federal Agency for the Safety of the Food Chain (FASFC). On October 15, 2010 the FASFC published on their website acceptance criteria for screening tests for antimicrobials. Since the first publication, new versions were published on August 9, 2011 and December 13, 20112 (Anon., 2011).

Approval criteria for microbiological tests for screening of antibiotics and chemotherapeutics in raw milk by the Belgian milk control stations
- The reading of the test result must be instrumental; for visual tests, instrumental reading must be in accordance with visual colour interpretation.
- The total test time should not exceed 4 hours.
- The test must screen a large spectrum of antibiotics. In terms of detection capability:
- 85% of the following beta-lactams must be detectable at their respective Maximum Residue Limit (MRL) (Commission Regulation (EU) No 37/2010): benzylpenicillin, ampicillin, amoxicillin, cloxacillin, nafcillin, cefalexin, ceftiofur, desfuroylceftiofur, cefquinome, cefazolin, cephaipirin, desacetyl cephaipirin, cefoperazone and cefalonium.
- 75% of the following sulfonamides must be detectable at their respective MRL or Recommended Concentration for Detection (Anon., 2007): sulfadiazine, sulfadoxine, sulfadimethoxine and dapsone.
- 100% of the following tetracyclines must be detectable at two times their MRL: chlortetracycline and oxytetracycline.
- 35% of the following substances must be detectable at three times their MRL: spiramycin, tylosin, spectinomycin, dihydrostreptomycin, gentamicin, neomycin, kanamycin, marbofloxacin, danofloxacin, enrofloxacine, colistin, rifaximin, lincomycin, pirlimycin, clavulanic acid and trimethoprim.

The substances mentioned above are the pharmacologically active substances of all brand/trade names with a registration in Belgium for use on lactating cows as of August 5, 2010 (Anon., 2010) and the prohibited substance dapsone.

- The test must be robust, may not be disturbed by certain microflora, lipolysis or a high cell number, e.g., unless the testing procedure used is adapted to prevent that false-positive results lead to penalties.
- The shelf life of the reagents shall not be less than 3 months, the detection capability must be stable during shelf life and batches may not be significantly different (25% of the CCβ). The variation is tested on a penicillin, a sulfonamide and a tetracycline.
- The manufacturer of reagents should have a sufficient manufacturing and storing capacity and be able to guarantee „in time“ delivery.
- The supplier should provide information on the quality control of the distinct batches.
- The incubation period should be mentioned on each batch and has to be checked for a penicillin, a sulfonamide, a tetracycline and a negative milk sample.
- The test should be validated by the National Reference Laboratory (NRL) according to Commission Decision 2002/657/EC.
- The test shall be approved by the FASFC upon assessment of a validation file compiled by the NRL. After approval, any change in the nature of products and in the protocol shall be clearly indicated and notified to the competent authority (FASFC), in order to be re-validated by a NRL.

Materials and methods
Different microbiological inhibitor tests were validated at ILVO-T&V to check if the tests meet the FASFC approval criteria. It concerns following tests: Eclipse 50 (ZEU-INMUNOTEC S.L.), Delvotest Accelerator (version 2011) (DSM-Food Specialties), Charm Blue Yellow II (Charm Sciences Inc.), Eclipse 3G (ZEU-INMUNOTEC S.L.) and Delvotest T (DSM-Food Specialties). All tests were used as defined by the kit manufacturers.
Following parameters were checked in the validation studies:

Detection capability
For testing of the detection capability, residue-free raw milk was spiked with the compounds at target concentration (FASFC-criteria). Each concentration was at least 20 times tested in a time period of at least three days. The test is considered as able to
screen the compound at target concentration when at least 19 times upon 20 tests a positive result was obtained.

**Test repeatability**
For each test, the repeatability was determined by analysing blank and spiked milk samples *in duplo*.

**Test robustness**
For robustness testing, the impact of a high somatic cell count (between $5 \times 10^5$ and $10^6$ somatic cells per ml and $>10^6$ somatic cells per ml), a high fat content (>6 g per 100 ml) and a low protein content (<2.5 g per 100 ml) of the milk was studied.

**Results and Discussion**

**Detection capability**
The fulfillment of the FASFC criteria for detection capabilities by the different inhibitor tests is listed in Table 1. The Charm Blue Yellow II, Eclipse 3G and Delvotest T are fulfilling the FASFC criteria.

<table>
<thead>
<tr>
<th>Group of anti-infectious agents</th>
<th>Minimal requested % of compounds detected at target conc.</th>
<th>% of compounds detected at target concentration (FASFC criteria)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Eclipse 50</td>
</tr>
<tr>
<td>β-lactams</td>
<td>85</td>
<td>64.3</td>
</tr>
<tr>
<td>sulfonamides &amp; sulfones</td>
<td>75</td>
<td>--</td>
</tr>
<tr>
<td>tetracyclines</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>other</td>
<td>35</td>
<td>--</td>
</tr>
</tbody>
</table>

**Test repeatability**
The test repeatability of the different inhibitor tests for negative (blank) and positive (spiked) milk is listed in Table 2. It should be mentioned that for both Eclipse tests a spectrophotometric reading (absorbances) is applied while for the other tests a flatbed scanner is used for reflectometric reading (z-values or scores).
Table 2. Test repeatability of the different inhibitor tests for blank and spiked milk

<table>
<thead>
<tr>
<th>Test</th>
<th>Blank milk</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low positive</td>
<td>Medium positive</td>
<td>High positive</td>
<td></td>
</tr>
<tr>
<td>Eclipse 50</td>
<td>0.044</td>
<td>0.036</td>
<td>0.040</td>
<td>0.041</td>
</tr>
<tr>
<td>Delvotest Accelerator (Version 2011)</td>
<td>0.44</td>
<td>0.57</td>
<td>---</td>
<td>0.48</td>
</tr>
<tr>
<td>Charm Blue Yellow II</td>
<td>0.77</td>
<td>0.75</td>
<td>---</td>
<td>0.51</td>
</tr>
<tr>
<td>Eclipse 3G</td>
<td>0.050</td>
<td>0.018</td>
<td>0.037</td>
<td>0.040</td>
</tr>
<tr>
<td>Delvotest T</td>
<td>0.46</td>
<td>0.39</td>
<td>---</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Test robustness

For robustness testing, the percentage of false-positive test results due to a high somatic cell count (between $5 \times 10^5$ and $10^6$ somatic cells per ml and $>10^6$ somatic cells per ml), a high fat content ($>6$ g per 100 ml) or a low protein content ($<2.5$ g per 100 ml) was studied. The results are summarized in Table 3. For the CMT, the test presently used, no false positive results were obtained for milk with a somatic cell count between $5 \times 10^5$ and $10^6$ somatic cells per ml and for the milk with a high fat content. Only 1 sample of the group of samples with a somatic cell count $>10^6$ somatic cells per ml caused a false positive result. However, this sample contained $>12$ million somatic cells per ml. The CMT is also sensitive for milk with a low protein content ($<2.5$ g per 100 ml) with a 100% interference.

Table 3. Percentage of false-positive test results due to a high somatic cell count (between $5 \times 10^5$ and $10^6$ somatic cells per ml and $>10^6$ somatic cells per ml), a high fat content ($>6$ g per 100 ml) or a low protein content ($<2.5$ g per 100 g)

<table>
<thead>
<tr>
<th>Milk parameter</th>
<th>Eclipse 50</th>
<th>Delvotest Accelerator (Version 2011)</th>
<th>Charm Blue Yellow II</th>
<th>Eclipse 3G</th>
<th>Delvotest T</th>
</tr>
</thead>
<tbody>
<tr>
<td>somatic cell count:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^5$ - $10^6$ per ml</td>
<td>3.3</td>
<td>20.0/23.8a</td>
<td>6.5/6.1a</td>
<td>15.7a</td>
<td>19.1</td>
</tr>
<tr>
<td>$&gt;10^6$ per ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fat content:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$&gt;6$ g per 100 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>protein content:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$&lt;2.5$ g per 100 ml</td>
<td>50.0</td>
<td>20.0/43.1</td>
<td>12.2/11.1a</td>
<td>nt b</td>
<td>14.9</td>
</tr>
</tbody>
</table>

$^a$ same samples tested in different tests; $^b$ not tested

Conclusions

The FASCC criteria are stressing the kit manufacturers to improve and enlarge the detection capabilities of the microbiological inhibitor tests. On the other side we remark an increased percentage of false positive results due to a high somatic cell count, a high
fat content, a low protein content or higher levels of natural inhibitors for the recently developed tests.

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References
- Anonymous. (2007) CRLs view on state of the art analytical methods for national residue controls plans. CRL guidance paper (December 7, 2007), 1-8