

4.1. Antibiotic detection methods in milk

Four method families

4 different methods are currently available to detect antibiotics. They progressively appeared over the last decades (Ref. 7, Ref. 47, Ref. 48, Ref. 29, Ref. 31, Ref. 50):

- **Microbiological tests** (like Delvotest®) consist in detecting an antibiotic presence with a bacterial strain which is inhibited or not by the presence of an antibiotic. To simplify, such tests detect numerous antibiotics at thresholds generally very close to the MRL. They are quality tests (presence or absence) but with a known threshold.
- **Rapid tests** use different technologies. They evidence within a few minutes, but without quantifying, the presence of an antibiotic family. The most often used tests are those in search of betalactam-antibiotics, but similar tests for tetracyclines or other families exist.
- **Specific tests**, using HPLC for example (High Performance Liquid Chromatography), search for a given molecule and quantify it. It is possible to search at the same time for several molecules previously defined.
- **Identification and quantification tests** are currently appearing. They are, for example, based on the LC-MS-MS principle (Liquid Chromatography and Mass Spectrometry). They are often used as confirmation after rapid screening tests.

Definitions

Detection threshold: concentration from which a measuring technique shows the unquestionable presence of a molecule.

Quantification threshold: minimum measurable concentration; often higher than the detection threshold.

Microbiological tests

Microbiological tests, like certain rapid tests, can be performed by non professionals (farmers, truck drivers...), whilst the other tests can only be performed in adapted laboratories with specialized technicians. Microbiological or rapid tests cost a few euros, whereas the other tests cost around 100 euros per searched molecule.

It must be reminded that microbiological tests were developed many years ago to guarantee a milk suitable for processing, with no inhibitors likely to disrupt the lactic ferments. **It is in fact the processing constraints which very early lead the dairy sector to search for antibiotics in milk (Ref. 19).**

These microbiological tests, very easy to use, have been “industrialized” and are massively used at different levels: in laboratories analysing producers’

milk, in dairy industrial laboratories and even on farms.

These tests show the following characteristics:

- Ability to **evidence a very wide diversity of molecules**: the reaction is due to the effect of this substance on the development of a bacterium.
- **Variable sensitivity from one molecule to another**: this sensitivity (detection threshold) here also depends on the sensitivity of the chosen strain to the different antibiotics used.
- Possibility of “additive” effects: even if this is very poorly documented, it is possible the simultaneous presence of different antibiotics lead to an additive (and even synergic) effect on the tested bacterium.

To date, **the main tests used in the world (and the oldest) are Delvotest® (DSM) and BrTest**. Many copies of these tests exist (Copan, Eclipse...). Note that nearly all these tests use the same bacterium: *Bacillus stearothermophilus*. Several tests use other bacteria. It is the case of Valio’s T101 which uses lactic streptococci. As an example, in France, it is estimated that 5 000 000 Delvotest® microbiological tests are performed on producers milk every year (Ref. 21).

Certain countries, in order to improve sensitivity to certain molecules, use a “battery” of strains (Ref. 7). Each strain being more or less sensitive to the different antibiotics to detect, their concomitant use on different Petri dishes enables to detect more molecules as well as provide a first orientation.

Note that the existence of natural inhibitors in abnormal milk can be the cause of positive tests. This is particularly true for milk from cows suffering of mastitis or for colostrum (Ref. 25).

Rapid tests

These tests are defined more by their functionalities than by the various technologies they employ. In fact, all these tests provide in a few minutes quality analysis results (presence or absence), generally for an antibiotic family. These tests are used before all to perform “laboratory tests”: milk collect trucks are tested before being unloaded. A negative result allows unloading. A positive result generally leads to blocking the truck and performing new tests.

In most cases, the tests are only used for searching betalactam-antibiotics. Mainly for two reasons: these antibiotics are the ones most often used on dairy farms and they can contaminate very large volumes of milk (see concepts of dilution and contamination power).

Among the most often used rapid tests for detecting betalactam-antibiotics are (Ref. 6):

Penzym: enzymatic and colorimetric test. An enzyme (DD Carboxypeptidase) is inhibited by the presence of betalactam-antibiotics.

Delvo-X-PressM BL: immuno-enzymatic test based on the dosage by colorimetry of the excess of a given reagent. In the presence of betalactam-antibiotics, a “betalactam-tracer” is formed. Consequently, the quantity of free tracer, able to settle on the reaction tube’s walls decreases.

Bêtastar: test based on the use of a specific receptor related to gold particles. These receptors migrate differently on a strip depending on whether they are linked to betalactam-antibiotics or not.

Snap: uses an immuno-enzymatic method. Receptors can link either to the antibiotic (if it is present) or to the antibiotics fixed to the test surface.

Charm II: an analyzer uses an immunocompetition reaction between the searched molecule and a molecule marked with C14 or H3. It is a competition test measured by radioactivity. An important investment is required.

Many publications on these tests are available (Ref. 47, 6).

Physicochemical tests

The 1980s: chromatography, UV-Visible and fluorometry

These tests developed in the 1980s thanks to the advances of electronics and information technology, allowing the development of more and more performant High Performance Liquid Chromatography (HPLC), UV-Visible detection system, and fluorimetric systems.

These methods have very low detection limits: the results obtained enable to settle on the sample’s conformity to the MRL. The limit of these methods is their spectrum: as many methods are needed as there are antibiotic families. Certain antibiotics are difficult to identify due to their inability to absorb within the UV.

Beginning of the 1990s: coupling of liquid chromatography and mass spectrometry

Coupling liquid chromatography (LC) and mass spectrometry (MS) combines the separation power of the liquid chromatography and the structural information on the analysis given by the spectrometer. These new techniques (“Thermospray”, “Particle Beam”) allowed to dose molecules that do not absorb the UV such as erythromycine.

The liquid chromatography coupling with mass detector was improved in the middle of the 1990s.

The arrival of new technologies (atmospheric pressure sources: electrospray = ESI, atmospheric pressure chemical ionization APCI) significantly widened the application field. The advantages of these techniques LC/MS and LC/MS/MS justify their use for routine analyses in official veterinary drug residue control laboratories (Ref. 14).

Official method, interprofessional method, private method

It is important to distinguish the methods according to their aims and not to their nature. The official methods are recognized by a country or a group of countries to ensure food product monitoring either for inspection purposes or for surveillance plan purposes.

For example, the official detection method for inhibitors in milk in France consists in two steps: a first screening with an acidification test (*Streptococcus thermophilus*) and a second screening for confirmation with three Petri dishes (*Bacillus cereus*, *Bacillus stearothermophilus*, *Bacillus megatherium*) (Ref. 19 ; Decree 2 sept 1983 – Official Gazette 6 oct. 1983).

The interprofessional methods are recognized both by a group of suppliers (milk producers...) and a group of purchasers (dairy companies...). Thus they are contractual methods which can just as well fix thresholds different from MRL. These analyses are meant above all to set the price of milk.

Private methods are used either internally within a company (control upon receipt, final control...) or between a supplier and a client.

4.2. Antibiotic residue detection and natural inhibitors

The microbiological tests presented above consist in the evaluation of a bacterium's ability to develop in presence of the matrix to test (milk, meat, eggs...). Yet these bacteria can be sensitive to inhibitor substances naturally present in the samples to test.

As regards to milk, it is necessary to take into account two situations when the milk can contain natural inhibitors. The colostrum, secreted by the udder during the first days after calving, has a composition very different from that of milk: very high content in immunoglobulin, presence of many leukocytes (white line cells ...). Several authors have shown that tests made on colostrum could lead to false positive results. However, this phenomenon mainly concerns the milk from one animal and not the whole herds' milk. As for residue detection, it is therefore better to wait at least four days after calving. As a reminder, in France, the colostrum period is of seven days. For several dry off treatments, in case of very short dry periods or abortion, antibiotics residue traces are found during several days, and even several weeks. This is why the withdrawal time after calving must necessarily be superior to fourteen days in case of a shortened dry period.

Another case of presence of natural inhibitors is mastitis. Indeed, in such a case, the secretion can be highly modified with a rush of cells coming from the blood (leukocytes) and different substances. Here also false positive results (in the absence of antibiotics) have been recorded. Note however that on one hand the milk from cows suffering from mastitis must not be delivered for consumption, and on the other hand it is rare that a cow suffering from mastitis is not under an antibiotic treatment.

The question of the existence or not of false positive results on milk from cows having only subclinical mastitis is regularly asked. The studies carried out show that, except in cases corresponding to very high cellular variations, false positive results are very rarely found (Ref. 28).

For milk, as for meat, incorrect results due to natural inhibitors must not be mistaken for those due to badly kept samples.