SUMMARY OF LA LETTRE DE CECALAIT, N° 37 (2nd quarter 2001)

(Translation : A. BAPTISTE, Correction : H. LAMPRELL)

The detection of inhibitors in milk

Antimicrobials (antibiotics and sulpha drugs) have been applied in the treatment of dairy cows almost eversince they had been developed in the 30s-40s [18], mainly for the prevention or treatment of mastitis [15]. However, when residues remain in milk, technological problems may occur during cheese or yoghurt manufacture. They may also be hazardous to public health. Therefore, Maximum Residue Limits (MRLs) have gradually been fixed in the European regulation and still are. The detection of residues of antimicrobials or other inhibitors has thus become necessary : numerous tests have been developed and there are still advances in that field. They cover all steps concerned by residue control : some tests are for detection, others for confirmation, others for detection or identification of specific antimicrobials. Their principles are then very different. For confirmation or identification of inhibitors, there are immunoenzymatic assays, radioimmunoassays, chemical, enzymatic, chromatographic, electrophoretic but also microbial methods.

However, the detection of inhibitors is mainly based upon microbial screening methods, aimed at showing the inhibition of the growth of a test microorganism.

Inhibitors are substances, acting at the molecular level by certain biochemical pathways, and thus stopping or inhibiting the growth of one or several bacterial groups. They may be disinfectants, cleaners, preservatives, pesticides, some food additives and veterinary drugs, including anticoccidial drugs and mostly antibacterials…

↳ Antibacterials

They are mostly antibiotics and chemotherapeutic agents, especially sulpha drugs (sulfonamides). The latter, which appeared in the 30s to treat infectious diseases, are synthetic antibacterials. But, antibiotics are substances produced by some microorganisms, inhibiting the growth of other microorganisms and even their own growth. This activity was first observed in the XIXe century, but it became was mostly studied in the 30s-40s, following the discovery of penicillin, and then of a lot of other substances. New natural antimicrobials were systematically seeked. They have been used in human therapeutics since the early 40s and were soon introduced in veterinary medecine, but were also used as feed additives. In dairy farms, they are mainly applied in mastitis therapy (due to *Staphylococus aureus*, and other Gram+ or Gram- pathogens), preventive drying-off treatments and some non-mammary pathologies (locomotory or pulmonary pathologies) [8].

With microbial research and semi-synthetic work, there is now a very large array of available antibiotics. They can be separated into 10 to 12 groups :

- β-lactams, themselves separated into subgroups, including penicillins,cephalosporins…
- tetracyclines,
- amphenicols, mostly chloramphenicol,
- aminoglycosides, for instance neomycin, streptomycin and its analogues,
- macrolides, for instance erythromycin,
- polypeptides, for instance, bacitracin,
- lincosamides,
- ansamycins, for ex. rifamycin,.

and many others.

β-lactams are the prevailing type used in breeding [7]. However, recent surveys show a rise in the proportion of other antibiotics (20% of positive samples or more : aminoglycosides, tetracyclines, macrolides [7], sulpha drugs [14], alone or in combination with a β-lactam [15]).

Milk from cows treated with antibacterials must be discarded during and after the treatment, during a withdrawal period recommended by the manufacturer. However, residues of antibacterials may remain in the collected milk, mainly because [8,13] :

- of the (often) accidental non-respect of withdrawal time,
- of the non-respect of recommendations for drug administration,
- of accidental contamination during milking,
- of abnormally long excretion time of the drug with some ill animals,
- of early calving,
- of the presence of antibacterials in feed, though dairy cattle feed should, in principle, not contain these substances.

↳ Undesirable residues

In the case of presence of residues, technological and public health problems may occur. From a technological point of view, they have high adverse effects in milk fermentation processes, resulting in severe consequences in cheese or yoghurt production. [13]

From the point of view of public health, the risks to be taken into account are pharmacological-toxicological, microbiological (risk of favouring resistant or pathogenic microorganisms in the intestinal flora) or allergies. They are evaluated in the course of toxicological studies, which lead to the determination, for each antibacterial :

- of the acceptable daily intake (ADI *or DJA in french*), then,

- of the Maximal Residue Limit (MRL), in milk and other foodstuffs.

  NB : MRLs are concepts used in Codex and European Union texts; whereas in the USA, FDA refers to a neigbouring concept : safe/tolerance level. These values may differ for a same substance.
- and at last, of the withdrawal time [12].

The detection of antibiotic and sulpha drug residues in milk thus comprises these two different aspects. From a technological safety point of view, milk payment on the basis of quality is, in most countries, very disadvantageous for the producer in case of inhibitors presence. From the standpoint of public health, for years, the only existing MRL dealt with penicillin [12]. However, in 1990, in the European Union, a community procedure for the fixation of MRLs in foods of animal origine was laid down and since then, MRLs are gradually fixed for all veterinary drugs. (see below).

However, in most cases, there is a gap between the concentrations of residues regarded as "technologically safe " and those fixed considering public health [12]. Till then, constant development of new tests or of existing tests in order to improve their sensitivities have been observed.

A survey of the recent litterature thus shows that more than a hundred articles deal with the detection of antibacterials or the specific substance/group detection. They propose advances in existing methods as well new methods. Some also consider the sensitivity of present official methods regarding MRLs or compared to advanced methodology [6, 14].

Concerning the legal situation, things changed mostly regarding MRLs. The initial text is regulation 2377/90 from 26/6/1990 laying down a community procedure for the establishment of maximum residue limits of veterinary medical products in foodstuffs of animal origin. Its annexes, empty at the beginning, were meant for :

- " the list of substances where MRLs are fixed (annex I),
- the list of substances which do not require a MRL (annex II),
- the list of substances where preliminary MRLs are fixed (annex III),
- the list of substances where no MRL can be fixed " (annex IV).

The MRLs of veterinary drugs, including antibacterials, are gradually fixed in annex I, after a toxicological study under the responsability of the Scientific Veterinary Committee, considering the ADI and a safety factor. In case these studies are not finished yet, a preliminary MRL may be fixed in annex III, for a maximal duration of 7 years (a 5 year period with possibly 2 extra years). At the end of the evaluation, either a MRL is fixed in annex I; or it does not appear necessary and the substance belongs then to annex II. At last, for some substances, it appears that their residues, " whatever limit may be hazardous ". Therefore, no MRL can be fixed and these substances, listed in annex IV should not be used. For antibacterials, this is the case for chloramphenicol and dapsone.

Following this procedure, annexes were filled with 5 to 10 annual amending regulations. The last compendium of amending regulations was issued in March 1999 : regulation n° 508/1999. But since then, more than twenty new amendments supplemented the lists ! The legislation in force can, however, be consulted on the European Union web site : http://europa.eu.int/eur-lex/en/lif/dat/1990/fr_390R2377.html.

Official methods, even issued after regulation 2377/90 did not change that much. They are different among countries, but mainly based upon microbial inhibitor tests. For the European Union, the methods in force are described in :

- decision 91/180 for the detection in raw and heat-treated milk (pages 39-47), [2]
- regulation n° 213/2001 for skim milk powder. (page 79) [5].

In France, screening and confirmation methods were established in 1983 [1]. Nevertheless, for milk payment, the legal situation is changing, as the Goverment, following an Interprofessionnal demand, approved a modification of the principle of the method. The new screening technic, based upon the use of *Bacillus stearothermophilus* (see below) will be enforced at the beginning of 2002.

The vast array of available tests can be separated into two large families. The first aims at the detection of the broadest possible spectrum. These tests are used for screening purposes, in milk payment schemes or self-controls in farms or dairies. They are microbial inhibitor tests, where the inhibition of the growth of a test microorganism is observed. Below, we shall describe their common features as well as their differences.

The other family of tests aims at the specific detection of a substance or groups of substances. They are used for confirmation of presumptive positive samples or for detection or identification or even quantification of specific antibacterial substances. Some of these tests are also microbial, but then using different agar diffusion methods. However there are numerous more sophisticated methods, depending on the kind of antibacterial substance under study : enzymatic and immunoenzymatic methods, radioimmunoassays, chromatographic and electrophoretic methods… These tests are mainly meant for further analysis of presumptive positive samples after screening..

↳ Microbial inhibitor screening tests

➲ COMMON FEATURES

The milk sample to be tested is added to or placed on a growth medium containing the test microorganism, characteristic of the microbial test. If the sample contains antibacterials, normal growth is inhibited, which is revealed by different means, depending on the test.

The test microorganisms generally belong to the genus *Bacillus* or *Micrococcus* or *Streptococcus*... They must be sensitive to a broad spectrum of antimicrobials : different antibiotic groups and sulpha drugs. Indeed, a satisfactory screening method should be able to detect the broadest variety of antimicrobials. Microbial tests should also share the following features, given in the description of the «ideal » microbial inhibitor test [18] :

- Considering detection possibilities :

to allow the detection of a broad spectrum of antimicrobials at limits in correspondence with MRLs,

- From the practical standing point :
  to be cheap, fast, easy to perform, not to require educated persons or sophisticated equipment, possibly suitable for mechanized analysis ,
- in order to avoid false positives :
  to be of low susceptibility to natural inhibitors of milk, such as lysozyme or lactoferrin,
- in order to avoid false positives and/or false negatives :
  to be of low susceptibility to interference factors due to sample microflora or to test procedure (incubation conditions, sample volume …etc),
- considering analytical performance :
  to be repeatable and reproducible.

Easy further steps of confirmation or identification, using the same test, added with certain substances would also seem desirable [18].

➲ HOW TO OBSERVE GROWTH INHIBITION

Depending on the tests, test microorganism cultures are either ready to use, in microwell-plates for instance, or to be prepared using specific strains, in specific media. Contact with the milk sample is either by diffusion in agar media, or direct in liquid medium. In this latter case, preliminary heating of the sample is usually part of the procedure in order to inactivate natural milk inhibitors [9]. If the tested sample contains antibacterials, the growth of the test microorganism is inhibited. Following the tests, this may be revealed by :

- in disk assays on agar media, observation of an inhibition zone around the disk impregnated with the sample. This zone is clearer than the rest of the agar ; in some tests, indicators may increase the colour difference.
- observation of the change, or not, of colour of a pH indicator. Indeed, normal growth results in the acidification of the medium, causing the pH indicator colour to change. The presence of antibacterials inhibits the growth, resulting in less acidification and no colour change. More seldom, the acidification, or not, is followed by titration.
- On a near principle, observation of the change, or not, of colour of a redox indicator, as normal growth also results in the reduction of the redox indicator.
- Still on near principles, some tests use the change, or not, of colour of a chromogenic substrat specific of an enzyme synthetized during normal growth.

➲ MICROBIAL INHIBITOR TESTS

Tables 1 to 4 in La Lettre de CECALAIT try to review (**not exhaustively**) the different microbial inhibitor tests which can be used for the screening of milk samples.

They are widely based upon the descriptions given in the compendium of methods issued by the IDF in its 1991 bulletin n° 258 [9]. The IDF special issue on the same topic published in 1995 [10], especially its articles [16] and [18] was also very useful.

The different tests are classified according to the test microorganism they use. Some of them have been developed since then in order to improve their sensitivity or their easy use : so there are several variances of the test, though the principle remained unchanged. On the contrary, some became obsolete. Only a strict survey of scientific and technic litterature, but also of the test available in suppliers' catalogues could allow an exhaustive and updated compendium. This was not our point.

Very great care must be taken regarding the sensitivity figures. References for sensitivities are not thoroughly documented. Furthermore, results may be affected by milk compositional variations, especially when antibiotic concentrations are close to the detection limit of the test. However, methods where agar is involved seem less influenced.

Tables show that spectra and sensitivities differ depending on the test microorganism.

Tests using *Bacillus stearothermophilus* appear to be very sensitive for β-lactams. Their sensitivity for tetracyclines or sulpha drugs seems to be more dependent on the concept of the test (incubation conditions, presence of additives…) [16]. The detection of some aminoglycosides and macrolides is more difficult. Chloramphenicol is even more difficult to detect. In France, when this forbidden substance is suspected, more severe and more sophisticated controls, using specific physico-chemical methods, may be used.

Tests using *Streptococcus salivarius ssp. thermophilus* detect sensitively β-lactams, tetracyclines and macrolides, the detection of aminoglycosides, sulpha drugs and chloramphenicol is more difficult [16].

The Arla test, using *Bacillus subtilis* is particularly sensitive for tetracyclines [7].

Anyhow, no test can claim an equivalent sensitivity to all antibacterial groups nor fulfill all UE-MRLs [16]. It even seems that such a test will probably never exist [7], [18].

↳ In conclusion

Microbial inhibitor tests are necessary for the screening of antibacterial residues in milk. Choosing in the array of available tests depends on practical aspects (available kit, easy performance) as well as on the seeked substances or the expected analytical performances. However, despite their broad spectrum, none of them have the same sensitivity for all antibacterial groups and can fulfill all MRLs (These are not the same among residues and may change as regulations are amended, but are all very low).

Therefore specific and sensitive confirmation tests are needed. Moreover, increasing the sensitivity of tests or developing new ones still remains interesting. They must however comply with guidelines gradually laid down by IDF / ISO experts [18], standardized since 1999 by IDF, and soon standardized by ISO [11]. These guidelines describe a procedure for the evaluation and the validation of tests, which principally will allow the comparison of results of different origin.

Meanwhile the survey of the evolution of veterinary medicine therapeutic pratics and of breeding pratics is necessary in order to anticipate probable residues.

Thanks to J.P. Moretain at AFSSA Fougères, H. Damour, director of Cecalait and P. Bouriot, farmer in the Franche-Comté area.

The list of abbreviations and bibliographic references are in « La Lettre de CECALAIT »