

# MONITORING ANTIBIOTICS IN MILK - THE CHANGING WORLD OF TEST METHODS

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## SUMMARY

For many years, the UK operated a national approach to the detection of antibiotics in milk, the Delvotest P (Gist-brocades BV, The Netherlands) being, initially, the only test listed in the Joint Committee *Code of Practice for the Assessment of Milk Quality* (the 'Blue Book'). Thus, the Delvotest P was accepted nationally for the examination of tanker milks and became known as the 'UK Industry Standard' test. In 1992, the LacTek  $\beta$ -lactam test (Idexx Laboratories, USA) became the first 'rapid' test to be incorporated into the Blue Book but, since deregulation in 1994, several new rapid tests have appeared that differ significantly in sensitivity as well as in the range of substances detected. Although the newer tests are not yet listed in the Blue Book, their presence within the dairy industry, raises the question, "Are we now spoiled for choice?" and there is considerable debate over the advantages and limitations of different proprietary tests. To enable objective comparisons to be made, the International Dairy Federation has produced two guideline documents for the evaluation of tests that detect antibiotics in milk, the first of which has been adopted by ISO as an International Standard (1). In England & Wales, the Milk Quality Forum and the Dairy Industry Federation commissioned the Hannah Research Institute to assess independently the test manufacturers' claims. This work has formed the basis for revising the Blue Book list of tests and the final report of the Hannah work (2) has recently been issued (28<sup>th</sup> July). At the time of writing, however, the revised list of Blue Book 'acceptable' tests remains eagerly awaited.

## PRINCIPLES AND PRACTICE OF ROUTINE TESTING FOR ANTIBIOTICS

The 'traditional' tests for antibiotics in milk, known as 'microbial inhibitor' tests, involve incubating a susceptible organism in the presence of the milk sample. In the absence of an antibiotic, the organism grows and can be detected visually either by opacity of the agar growth medium or by a colour change resulting from acid production. In the presence of an antibiotic, or any other inhibitor, the organism fails to grow and a zone of inhibition or lack of a colour change is observed. Such tests are exceptionally sensitive to  $\beta$ -lactam antibiotics, though they can also be made to detect sulphonamides and other antimicrobials. They are generally reliable and cost-effective but require incubation for several hours before the result can be visualised.

The desire for a more rapid result has promoted the development of tests that employ the 'immune receptor' test principle, which is a variation of the well-established enzyme-linked immunosorbent assay (ELISA). Essentially, a specific target antibiotic group is captured by immobilised antibodies, or by a broader-spectrum receptor such as a bacterial cell. Most tests involve a competitive principle in which antibiotic in the sample competes with an internal antibiotic standard for the immune receptor. The antibody-antibiotic complex is then usually linked to an enzyme that catalyses a colour or fluorescence reaction and a comparison of the intensity of the 'test' reaction with that of a 'control' determines whether the sample is positive or negative. Because of their competitive

principle, a low intensity usually means 'positive' whilst a high intensity is regarded as 'negative'. Immune receptor tests can be made quantitative but are generally used to provide a 'pass/fail' result. They are generally more expensive than microbial inhibitor tests but only detect substances that react immunologically with the immobilised receptor and they provide a result in less than 10 min.

The commercially available immune receptor tests employ several variations of capture mechanism and colour reaction but most possess the common features of an immunological reaction coupled with a change in colour (or fluorescence). There are, however, two exceptions. The Penzym test (UCB Bioproducts, Belgium) employs the inhibition of an enzyme reaction (DD-carboxypeptidase activity), instead of an immune reaction, to detect the presence of a  $\beta$ -lactam and it visualises this by a colour change. Conversely, the Charm II assay (Charm Sciences Inc., USA) employs an immune reaction to bind the antibiotic to a microbial receptor but detects this complex using a low-level  $^3\text{H}$  or  $^{14}\text{C}$  radio-label, instead of an enzyme reaction.

The commercial availability of microbial inhibitor and immune receptor tests has created a significant dilemma for the dairy industry. Although  $\beta$ -lactam antibiotics are the most commonly used antimicrobials in veterinary medicine, especially for intramammary administration, preparations containing sulphonamides, tetracyclines or other antimicrobials are also available. Microbial inhibitor tests have a broad spectrum and can therefore detect substances other than  $\beta$ -lactams; however, they are not specific for antibiotics and there are occasional reports of positive reactions associated with other inhibitors such as lactoferrin, lysozyme or sanitisers. Conversely, immune receptor tests are specific for a particular antibiotic group or even a specific substance. Most of these tests are capable of detecting the  $\beta$ -lactam group although at least 2 commercially available immune receptor tests are specific for penicillins and would fail to detect the presence of a cephalosporin. Although immune receptor tests for sulphonamides and tetracyclines are also commercially available, these tests need to be undertaken separately, with obvious adverse implications for cost and convenience.

The UK dairy industry currently employs essentially two (but, increasingly, three) levels of testing for antibiotics. 1) A farmer quality payment scheme was first introduced in England & Wales by the Milk Marketing Board in 1982. Bulk tank samples were tested weekly and the test results were therefore retrospective. Thus, there was (and still is) no commercial need for a rapid result, the main requirements of a test being low cost, broad spectrum and reliability. 2) Tanker milks are also usually tested on arrival at the dairy where there is a desire to accept or reject the consignment before off-loading. Similarly, for silo samples, it is desirable to obtain the test result before the milk is released into production. In this situation, a rapid result is essential and consequently many dairies have chosen to sacrifice a broad spectrum and, to some extent, cost for the benefit of speed. 3) Since deregulation, tests for antibiotics have begun to be applied at the individual cow level, notably for freshly calved animals that have received dry cow therapy. The ideal test for this situation would possess a broad spectrum, speed and low cost; however, no test currently achieves all three attributes and the farmer is thus faced with a choice between speed and broad spectrum. This approach to the control of antibiotics in the milk supply has received international approval and is embodied in the IDF Integrated Detection System for Antimicrobials (3).

The diversity of tests now available and the fragmentation of the UK dairy industry since deregulation have created a significant dilemma. Firstly, different tests may be applied by purchaser and supplier, since there is no longer a standardised, national approach and there is an increased

danger that conflicting results might be obtained between two tests undertaken on the same consignment of milk. The supplier-customer relationship often defines which tests are to be used, to ensure that consistent results are obtained by both parties, an aspect that is becoming increasingly complex as the movement of milk across national boundaries expands. Thus, tests may be employed simply because they are used by an important customer. Secondly, dairies must decide whether they should screen milk supplies for the widest possible range of substances using a test where the result is obtained retrospectively or whether they should use a rapid test to detect only those antimicrobials most commonly encountered. It seems that some UK dairies have taken the former approach whilst others have adopted the latter. However, most dairies employ the Delvotest as the 'definitive test' and may confirm rapid test results by re-testing a positive sample with the microbial inhibitor test.

Three additional factors further complicate the situation. 1) The EU Maximum Residue Limits (MRL) for veterinary medicinal residues in milk apply to an ever-expanding list of substances. Milk processors have a responsibility to ensure that their milk supplies do not contain any of these substances at levels that exceed the MRLs, yet test methods cannot keep pace with the rate at which the list is expanding. 2) The UK Food Safety Act defines the concept of 'Due Diligence' under which, in the event of legal proceedings, a milk processor must demonstrate that all reasonable precautions had been taken. Since some antibiotic groups are much more commonly found than others, is it necessary to screen milk for all antimicrobial groups? 3) Both the dairy industry and the test manufacturers have precious little specific information on the market breakdown for veterinary medicinal preparations. The information that does exist appears largely to be anecdotal making it difficult for test manufacturers to design appropriate tests and difficult for dairies to choose the test that best fits their needs.

## **COMMERCIALY-AVAILABLE MICROBIAL INHIBITOR TESTS**

The Delvotest (Gist-brocades BV, The Netherlands) is the best known microbial inhibitor test but it is less widely recognised that several versions of this test exist. The first version to be developed, in the 1970s, was the Delvotest P, designed to detect  $\beta$ -lactams. The target organism, *Bacillus stearothermophilus*, is encapsulated in an agar medium containing a pH indicator, a nutrient tablet and the milk sample both being dispensed onto the agar surface. The 'ampoule version' is designed for individual tests or small-scale testing whilst a micro-titre plate version is designed for mass testing where 96 tests can be undertaken simultaneously. A negative result is indicated by a colour change from purple to yellow, due to acid development during incubation at 64°C for 2½ hours. The Delvotest P has been used throughout the world and has a sensitivity to penicillin G of 0.005 IU/ml although the Delvotest P kits distributed within the UK have historically been selected especially to meet the UK's unique demand for a sensitivity to 0.006 IU/ml penicillin G!

A more recent development, the Delvotest SP, is capable of detecting a wider spectrum of substances, notably sulphonamides, but also has increased sensitivity to tylosin, erythromycin, neomycin, gentamicin, trimethoprim and other antimicrobials. The Delvotest SP appears identical to the Delvotest P, the only difference being the need to incubate the Delvotest SP for 2¾ hours. The Delvotest SP is sold throughout the world and, universally, has a sensitivity to penicillin G of 0.003-0.004 IU/ml.

The Delvotest Cow Test was introduced into the UK in 1994 for testing individual animals as well as bulk tank milk and is identical to the ampoule version of the Delvotest P, differing only in its packaging. However, as the UK dairy industry is now beginning to change to the use of Delvotest SP (see below), an 'SP' version of the Delvotest Cow Test has recently become available.

Finally, a fourth version of the Delvotest, the Delvotest MCS test, is soon to be launched in the UK. This test is similar to the micro-titre plate version of the Delvotest SP but has the nutrients included in the agar which makes the addition of a nutrient tablet unnecessary but gives the test materials a shorter shelf life. This test is aimed at the high-volume, quality testing laboratory (or 'Milk Control Station') market where low cost and simplicity are vital and reduced shelf life is not an inconvenience.

Although the Delvotest is by far the most widely used microbial inhibitor test in the UK, three similar tests, manufactured by Charm Sciences Inc. (USA), are also available. The Charm AIM-96 test is a micro-titre plate test, similar to the Delvotest and capable of detecting  $\beta$ -lactams, sulphonamides, tetracyclines, macrolides and aminoglycosides in 96 samples simultaneously. Unlike the Delvotest, however, it employs a liquid medium instead of agar. The inoculated micro-titre plate is incubated on a heating block, programmed to provide a time-temperature profile suited to the batch of *Bacillus stearothermophilus* spores being used; the incubation period is typically 3-4 hours, at the end of which a blue-yellow colour change indicates that a sample is negative. The Charm Farm test is a 'test-tube' version of the AIM-96 test, designed for on-farm use and employs the same microbial inhibitor principle with a colour change. There are two versions of this test: the Charm Farm Test-'Vial' and the Charm Farm Test-'Mini Vial' that both employ larger quantities of test medium than the AIM-96 test but are designed for fewer samples.

In addition, there are several other microbial inhibitor tests, produced by several companies. These include the Brilliant Black Reduction Test, the Valio T101 test, the Copan microbial inhibitor test, the Lumac rapid antibiotic test, the Arla micro test and the Biosys bioluminescence method. However, these tests are either not available in the UK or have not yet captured the interest of the UK dairy industry.

## **COMMERCIALY-AVAILABLE IMMUNE RECEPTOR TESTS**

Probably the most widely used immune receptor test in the UK is the LacTek test for  $\beta$ -lactams (Idexx Laboratories, USA). The test has a test tube format that is suited to laboratory use and the test takes 7 minutes to complete. The milk sample and a competitive enzyme tracer are added to an antibody-coated test tube and bind to the tube surface whilst the tube is shaken at room temperature, any antibiotic in the milk competing with the tracer for the surface receptor. The tube is washed and a colour developer is added to visualise the surface-bound complex. The colour intensity is measured in a spectrophotometer and compared with that of a penicillin 'standard', an intense colour indicating that the sample is negative, a pale colour indicating a positive result. The LacTek  $\beta$ -lactam test is highly specific for penicillins and does not detect cephalosporins. Several lesser-known versions of the LacTek test are also available that separately detect ceftiofur, tetracyclines, sulphamethazine, gentamicin and the banned substance, chloramphenicol.

The Delvo X-Press  $\beta$ -lactam II test (Gist-brocades BV, The Netherlands) employs the same principle as the LacTek test and also has a test tube format, suited to laboratory use. The test takes 7 minutes to complete, all incubations and colour measurements being undertaken in an 'Incubator-Shaker-Reader-Printer' instrument. Both penicillins and cephalosporins are detected.

The SNAP  $\beta$ -L test (Idexx Laboratories, USA) was the first of a growing number of tests to employ capillarity to draw the milk sample and test reagents over an immobilised antibody. It consists of a test tube and a disposable plastic unit or 'SNAP' device (rather like an electrical rocker switch) that contains the test reagents. The test is therefore essentially 'dry', making it suitable for laboratory or field use and it has also found some applications for use on tankers. The milk sample is first incubated in a test tube, placed in a heating block, then poured into one end of the SNAP device where it flows along a filter paper strip. After 30s, the device is 'snapped' to allow the colour developing reagents (contained at the opposite end) to flow in the opposite direction. At the end of the test (<10 min.) two colour spots ('control' and 'test') appear in the middle of the device and the intensity of these is compared either visually or in a colour reader. The SNAP test detects both penicillins and cephalosporins and there are separate SNAP tests for tetracyclines, sulphonamides and gentamicin.

The Beta Screen test (Advanced Instruments, USA) is an 'add-on' to the Fluorophos test for phosphatase used in many dairy laboratories and employs a fluorescent end-point. The milk sample and enzyme conjugate are first incubated in an antibody-coated test tube, placed in a heating block. After washing the tube, fluorescence is developed by the addition of reagents and further incubation; finally, the fluorescence intensity is measured in the Fluorophos fluorimeter and compared with that of a penicillin 'standard'. The Beta Screen test takes 10 min.; it is highly specific for penicillins and does not detect cephalosporins.

The Charm II assay (Charm Sciences Inc., USA) is not a single test but a family of separate tests for specific groups of antibiotics, notably  $\beta$ -lactams, sulphonamides, tetracyclines, novobiocin, aminoglycosides and macrolides, as well as various other substances such as chloramphenicol. There are several versions of the test that can detect different substances within an antibiotic group such as the aminoglycosides or macrolides. The Charm II assay is an immune receptor test but is suitable for large laboratories only, requiring a range of laboratory equipment, including a centrifuge and sample mixers to prepare samples as well as a scintillation counter to detect the radio-label. Calibration curves need to be prepared for each analyte and a 'negative control' sample must be tested each day, constraints that may require the laboratory to obtain a source of antibiotic-free raw milk powder.

Several tests for antibiotics have been launched in the UK within the last 18 months. The Beta STAR test (UCB Bioproducts, Belgium) involves a specific  $\beta$ -lactam receptor linked to gold particles. It is a 'dipstick' test that detects penicillins and cephalosporins within 5 min., though extending the incubation to 8 min. makes the test more sensitive. The milk sample is added to a vial containing the test reagents (25 test kit only: for the 100 test kit these are added separately) and incubated, the dipstick is added and incubation is continued. A red 'control' band appears on the dipstick together with a red 'test' band of variable intensity and the latter is compared visually with the former. If the 'test' band is weaker than the 'control' band the result is positive; if the 'test' band gives a stronger reaction, the result is negative.

The Charm MRL test (Charm Sciences, USA) is very similar to the beta STAR test and detects penicillins and cephalosporins in 8 min. The test strip is placed in a heating block, the milk sample is added to an absorbent pad at one end and the test is incubated. Two lines appear on the dipstick, a sample being considered positive if the 'test' line is lighter than the 'control' line. The results can be read visually or using an image reader.

The Penzym test (UCB Bioproducts, Belgium) is newly launched in the UK, though it has been used in Northern Ireland and in mainland Europe for many years. Two versions are available (Penzym and Penzym S) that have different incubation periods and different levels of sensitivity. The test detects  $\beta$ -lactams and is especially sensitive to some cephalosporins. The test is based on the principle that  $\beta$ -lactam antibiotics prevent bacterial multiplication by inhibiting the activity of the enzyme DD-carboxypeptidase. During the test, DD-carboxypeptidase activity liberates D-alanine from an enzyme substrate which is visualised by a colour change; in the presence of antibiotic, no D-alanine can be liberated and no colour change occurs. The Penzym test produces a pink colour when a sample contains no antibiotic whilst a yellow colour is interpreted as 'positive'. In the case of Penzym S, a peach-orange colour is considered to be a negative result whilst a colour with a 'yellow tendency' indicates that the sample contains a  $\beta$ -lactam.

Finally, another proprietary test is currently being developed by Idexx Laboratories (USA) though the UK launch date is not yet clear. The Parallax test is designed to detect a range of antimicrobials as well as other substances of interest to the dairy industry, though the exact range of compounds has yet to be announced. Parallax is a laboratory instrument that automates the pipetting, mixing, incubating and reading stages of an immune receptor test by means of a series of pumps, a centrifuge and a fluorescence detector all built into one unit. Each test is presented to the instrument in the form of a disposable cartridge that contains 4 glass capillary tubes, each coated with a different range of antibodies. For antibiotics, two cartridge types are being considered: one with 4 tubes each containing the same range of antibodies (the 'cillins' multi-cartridge) so that 4 different samples can be screened simultaneously and another with 4 tubes containing different antibodies (the 'individual' cartridge) so that a positive sample can be further identified. The instrument has two parts: a 'prep' station and a 'read' station. A cartridge is fitted to the 'prep' station, the milk sample(s) is (are) pipetted into its tray and the instrument is set to run. The milk is mixed with pre-dispensed, dried reagents, any antibiotic competitively binds to the coated tube and the tube is washed. When these operations are complete the cartridge is manually transferred to the 'read station' where it is centrifuged and the fluorescence is measured, a high level of fluorescence indicating that the sample is 'negative'. The test is described as a 'solid-phase fluorescence immunoassay' and takes 4 min. to complete.

## **SENSITIVITY AND SPECIFICITY OF ANTIBIOTICS TESTS**

The UK dairy industry now faces the dilemma that the proprietary tests currently available have yielded a confusing array of detection spectra and test sensitivities. Some sensitivities and specificities for antibiotics tests are given in Table 1. This list is not exhaustive; not all claims made by test manufacturers are listed and most, if not all, tests also detect substances for which no claim is made. The list does, however, encompass many of the substances that may be of concern in milk processing.

Whilst all tests have similar detection limits for penicillin G, there are some wide variations for other  $\beta$ -lactams. The Delvotest P, for example, detects cloxacillin at 25  $\mu\text{g}/\text{Kg}$  which is near the EU MRL of 30  $\mu\text{g}/\text{Kg}$  whilst the LacTek test is much more sensitive (7  $\mu\text{g}/\text{Kg}$ ) and this difference created some discrepancies for dairies and the Milk Marketing Board when the LacTek enter the Blue Book in 1992. Conversely, it recently became apparent that the Delvotest P is sensitive to cephalonium (15-20  $\mu\text{g}/\text{Kg}$ ) whilst the LacTek is not, though there has been no documented evidence of test discrepancies as a result of milk being contaminated with this substance. This debate has further been confused by the fact that no MRL for this substance has yet been set in EU legislation which has led to differences of opinion between the veterinary pharmaceutical companies and the milk processors. With an increasing range of substances for which milk may need to be screened and an expanding choice of proprietary tests, the potential for discrepancies seems likely to rise rather than decline.

## **CURRENT UK SITUATION**

It might be said that the UK dairy industry is on the edge of a precipice. Ever since the Delvotest P was introduced to the UK in the mid-1980s, the dairy industry has had a reliable, consistent and uniform approach to the analysis of milk samples for antibiotics. In addition, the Liaison Chemist Service bridged the gap between farm and dairy for the whole of England and Wales and arbitrated in case of disputes regarding antibiotic contamination of raw milk. Following deregulation, there is no national Liaison Chemist Service and UK dairies are free to choose for themselves which tests they wish to use, though if they wish to meet the Blue Book standard a sample must still fail either the Delvotest P or the LacTek  $\beta$ -lactam test.

Most of the major dairy companies have already chosen to replace the Delvotest P with the Delvotest SP, though others are still deliberating. At least one quality testing laboratory has been using Delvotest SP for farmer quality payment purposes for over 2 years whilst another uses different tests for different customers. The third laboratory still requires a milk sample to fail the Delvotest P before it penalises a farmer. The situation should soon be resolved, however, because Gist-brocades has announced its intention to withdraw the Delvotest P from sale in the UK as from October or November 1999.

As far as immune receptor tests are concerned, the current UK situation could be described as a 'free-for-all'. The choice of an immune receptor test depends on several factors outside the realms of science and technology, cost, ease of use, the need for capital equipment or a maintenance contract and other factors all confounding the difficult decision. Almost all of the immune receptor tests are used in the UK, though their market shares vary considerably.

Following the publication of the Hannah survey of antibiotic test methods (Muir, 1999), the Milk Quality Forum has to decide which tests shall be included in a revised Blue Book. The original intention was that one or a limited number of tests would be included but it has become very clear that for technical, commercial and even political reasons this is unlikely to be the case. Many dairies have avoided choosing a new test until this occurs. We await the big decision!

## REFERENCES

1. Anon. (1998) ISO/CD 13969 *Milk and Milk Products - Guidance for the Standardized Description of Microbial Inhibitor Tests*, International Organisation for Standardisation
2. D.D. Muir (1999). *Report of a survey of antibiotic test methods for raw milk*, commissioned by the Dairy Industry Federation, Hannah Research Institute: Ayr.
3. W.H. Heeschen & G. Suhren (1995). *Proceedings of a Symposium on Residues of Antimicrobial Drugs and Other Inhibitors in Milk*, International Dairy Federation: Brussels.

**Table 1. Manufacturers' claims for sensitivity and specificity of some antibiotics tests**

Test	Antimicrobial										
	Penicillin G	Amoxicilli n	Ampicilli n	Cloxacilli n	Dicloxacilli n	Oxacilli n	Nafcillin	Cephapir n	Cephaloni m	Ceftiofu r	Sulphameth ne
<b>Microbial inhibitor tests</b>											
Delvotest P (2½ h)	3†	5-6	5	25	20	10-15	10	8-10	15-20		10000+
Delvotest SP (2¾h)	2.5	3-4	3-4	15-25	10-15	10	5-8	5-8	10-20		50-100
Delvotest MCS	2-4										
AIM-96	3-5										10-50
Charm Farm V/MV	3-4	4	4					10		50	50-100
<b>Immune receptor tests</b>											
LacTek β-L	≤ 4.8			≤ 7				NS	NS	NS	NS
Delvo X-Press	2-4	4-8	4-8	30-40				4-8		4-8	NS
SNAP β-L	≤ 4										NS
Beta Screen	1	10	3	20	30	20	10	NS	NS	NS	NS
Charm II β-L*	2	5	4	30	20	30	30	3		40	NS
Beta STAR**	2-4	2-4	2-5	5-10	5-10	5-10	8-20	8-16	7.5-15	75- 150	NS
Charm MRL	3	4	4	30		50		10		100	NS
Penzym	4-6	4-6	4-7	60-100		30-50		5-7	10-15	40-70	NS

All values are µg/Kg

\* MRL version

\*\* 25 and 100 test kits

† 3 µg/Kg = 0.005 IU/ml

‡ 2.5 µg/Kg = 0.004 IU/ml

NS = Not sensitive