

## Screening Methods Used for the Detection of Veterinary Drug Residues in Raw Cow Milk – A Review

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### Abstract

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At both national and international levels, increasing attention is paid to the evaluation of the risk of occurrence of veterinary drug residues in foodstuffs and foods of animal origin, and to the introduction of appropriate measures to reduce this risk. The design and strategy of antibiotics and sulphonamide detection in milk involve two different aspects: the ability to sell the milk depending on its quality (technological safety), and the health safety of the milk regulated by the recent legislative regulations (toxicological safety). Veterinary drug residues in milk represent a health risk for the consumer. This review describes the methods used for extensive monitoring of antimicrobial agents – microbial inhibitor methods and rapid specific assays.

**Keywords:** antibiotics; residues; methods

### INTRODUCTION

Veterinary drugs are pharmacologically and biologically active chemical agents especially designed for the treatment and prevention of animal diseases. At present, veterinary drugs are extensively used in animal production. This is related to the gigantic growth and intensification of animal production (BOTSOGLOU & FLETOURIS 2001). Antibiotics and other chemotherapeutics are administered in therapeutical quantities especially in the therapy and prevention of specific animal diseases. The most important and most frequently used group of veterinary drugs is that of antimicrobial agents (FISCHER *et al.* 2003). In subtherapeutical quantities, these agents have been also used as feed supplements. A

crucial change in the use of complementary agents designated as antibiotic growth stimulators has been brought by the Regulation of the EU, imposing a ban on further use of these agents for animal nutrition as from January 1<sup>st</sup>, 2006 (Regulation EU No. 1831/2003).

In lactating cows, antimicrobial agents are used mostly for the therapy of mastitis but also of other diseases (e.g. laminitis, respiratory diseases, metritis). Long-acting antimicrobials are commonly used in dry-cow therapy (HONKANEN-BUZALSKI & SUHREN 1999; BOTSOGLOU & FLETOURIS 2001). Antimicrobial agents administered to cows in the course of lactation can pass to milk in various levels. A frequent and prevailing source of the milk contamination is the intramammary (intracister-

nal) administration of a specific antibiotic. Other pathways for the milk contamination are cutaneous, intrauterine, subcutaneous, intramuscular, and intravenous drugs administrations (HEESCHEN & BLÜTHGEN 1991). In most countries, veterinary medicine is allowed to use only those agents that are officially registered and approved. In drugs which are registered for use with food-producing animals, protection periods are prescribed during which the quantity of residues in foodstuffs of animal origin (milk, meat, eggs) should be reduced to a level not threatening the consumer's health.

Countries worldwide rely on national regulatory agencies and international committees in evaluating the safety of all drugs used with food animals for potential human health risk as an integral part of the drug registration process. The Codex Alimentarius and Joint FAO/WHO programme have been developing the standards concerning the residues in foods since 1985. These standards are based upon scientific assessments performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) determining the acceptable daily intakes (ADIs) and giving recommendations for Maximum Residue Limits (MRLs) (HEESCHEN 1993; HERRMAN 1995; HONKANEN-BUZALSKI & REYBROECK 1997). Consequently, in the EEC countries the approval of an antibiotic or a sulfa drug for treatment of farm animals will require (HEESCHEN 1993): fixation of NOEL/ADI, development of a suitable detection method and fixation of the withholding period on the basis of the residues detected (ADI/MRL).

For the international registration of veterinary drugs in the EU, the Committee for Medicinal Products for Veterinary Use (CVMP) has been established. CVMP, based on the toxicological residue assessment, sets the MRL levels for the pharmacologically active chemical agents of the veterinary medicinal products occurring in foodstuffs. The establishing of the MRL level in the EU is regulated by the Council Regulation (EEC) 2377/90. All veterinary drugs at the European market specified for food animals must be toxicologically assessed and categorised into Annexes I–IV, depending on the MRL type. MRLs present the internationally acknowledged limits which specify maximum quantity of the drug residues that may be found in foodstuffs of animal origin. According to the Commission Regulation No. 1662/2006, food business operators must introduce procedures ensuring that raw milk will not be marketed if it

contains the residues of antibiotics in quantities exceeding the levels for any of the substances authorised in the Annexes I and III of the Regulation (EEC) No. 2377/90, or if the overall content of all antibiotic residues exceeds the maximum residue limits. At present, however, no method exists which can detect all of these agents at the levels of the established MRL values. In order to provide for the high technological quality of raw milk and, at the same time, the safety of the milk and milk products for the consumer, the IDF has developed a so called integrated system of checking veterinary drugs in milk and milk products. The system recommends the use of various methods for the antibiotic detection and specifies the responsibility for the health safety of milk and milk products of particular subjects in the whole of the technological process of producing and processing milk (HONKANEN-BUZALSKI & REYBROECK 1997; HONKANEN-BUZALSKI & SUHREN 1999).

The reasons for monitoring veterinary drug residues in foodstuffs and foods of animal origin include the ethical ones (preventing undesired exposition of healthy consumers to therapeutic doses of drugs in food), hygienic (protection against possible harmful effects of the residues on the consumer's health), technological (preventing the disruption of the fermentation processes), and ecological (MÄYRÄ-MÄKINEN 1995; HONKANEN-BUZALSKI & REYBROECK 1997).

#### SCREENING METHODS FOR ESTABLISHING ANTIMICROBIAL AGENT RESIDUES IN MILK

The first test for establishing antimicrobial agent residues in milk (microbial inhibitor test) was developed as early as 1952 (MITCHELL *et al.* 1998). It was then known, too, that the presence of these agents could cause the inhibition of the starter cultures used in dairy industry, and for this reason the development of such methods was initiated so as to establish the inhibitor agent levels in milk. It was important that the methods be relatively cheap, simple to carry out, and capable of detecting a wide variety of antimicrobial agents. Of the methods used, microbial inhibitor methods suited best these requirements. A drawback which limits their use is a long incubation period. Therefore, rapid assays for antibiotic agent detection in milk have been developed which enable obtaining the results in the course of several minutes; they are

simple, sensitive, and specific. One of a plethora of rapid assays is the Penzyme test which was developed at the start of the 1980's. Later on, in 1988, Charm II. test for detecting 7 types of antimicrobial agents was introduced to the market, followed by other rapid assays, e.g. the LacTec test (1991), SNAP test (1994), Beta Star test, and Charm Safe Level test (MITCHELL *et al.* 1998).

International Dairy Federation (IDF) deals in its publications (bulletin) with the issues of establishing antimicrobial agents in raw milk. In 1987, the IDF issues a bulletin in which methods are described which can be used for the detection and confirmation of inhibitory agents in milk and milk products. In 1991, another bulletin was issued dealing with screening and confirmation methods for establishing antimicrobial agents and methods for specific analysis of these agents. IDF has set a conception and a control strategy concerning antibiotic and sulfonamid residues in milk and milk products – an integrated system. In a nutshell, the strategy of antibiotic and sulfonamid residues control is to be guided by the following rules (HEESCHEN 1993): microbiological screening inhibitory assays are to be used especially in primary food production. In order to establish agents which can not be detected by commonly used microbiological screening tests, other methods are to be used, e.g. microbiological receptor tests, immunological methods. The testing for antibiotics and sulfa drugs in the silo milk and in the heat-treated milk should use tests with the sensitivity required under toxicological aspects (microbial penicillin screening tests and in addition receptor and antibody tests).

In general, antimicrobial agent control system can be divided into two steps. The first of them is general monitoring of these agents during which tests are used making it possible to establish rapidly the presence of inhibitory agents. The second step is a specific analysis which makes use of the methods which enable the identification and quantification of inhibitory agents. For the general monitoring of antimicrobial agents in milk, common use is established of microbial inhibitor methods and rapid specific tests.

### Microbial growth inhibition methods

Microbial growth inhibition methods make use of a standard culture of the tested microorganism in a liquid or solid medium (HEESCHEN 1993). e.g.

*Geobacillus stearothermophilus* var. *calidolactis*, *Bacillus subtilis*, *Bacillus megaterium*, *Sarcina lutea*, *Escherichia coli*, *Bacillus cereus* var. *mycoides* or *Streptococcus thermophilus*. The analysed milk sample is applied on the agar surface either directly or with a paper disc (disc assay plate methods). In the course of incubation, the diffusion of the sample into the medium takes place (the agar diffusion principle), and if the sample contains inhibitor agents, reduction or total inhibition occurs of the tested microorganism growth. Depending on the method used, the presence of inhibitor agents in the tested sample is indicated by the formation of a clear zone of inhibition around the disc (disc assay plate methods) or a change in the medium colour (HUI 1993; MITCHELL *et al.* 1998; BOTSOGLOU & FLETOURIS 2001).

Microbial growth inhibition methods (wide spectral rapid tests) vary in the type of the testing organism, indicator, incubation period and temperature, spectrum and detection levels of the agents analysed. A series of these methods use as the testing microorganism *Geobacillus (Bacillus) stearothermophilus* var. *calidolactis*: BR-test/AS/Blue Star/6/7 (Enterotox Lab., Germany), Charm Blue Yellow Test (Charm Sciences Inc., USA), Delvo test SP-NT (Gist-brocades BV, The Netherlands), CMT – Copan milk test (Copan Italia, Italy), Eclipse 50 (Zeu-Inmunotec S.L., Spain). *Geobacillus stearothermophilus* is an outstanding testing microorganism for its properties from which the most important, according to KATZ and SIEWIERSKI (1995), are: the ability of rapid growth at higher temperatures (64°C) and a high sensitivity to the  $\beta$ -lactam antibiotics.

Commercially available microbial inhibitor tests play an important role in the integrated detection system. At present, many commercially produced microbial inhibitor tests are applied simultaneously with selective rapid tests for milk screening in primary production, in dairy industry, and in accredited laboratories (SUHREN 1995; HONKANEN-BUZALSKI & REYBROECK 1997; HONKANEN-BUZALSKI & SUHREN 1999; BOTSOGLOU & FLETOURIS 2001). The advantage of these methods is that they have a wide detection spectrum, they are simple to carry out, and they are not costly and can be used for the screening of a large number of samples (MITCHELL *et al.* 1998). These methods have their drawbacks, however, that limit their use: they do not enable specific antibiotic identification, they have limited detection

levels for a series of antibiotics, and they are only qualitative and require a long incubation period (2.5–3.5 h). They are highly sensitive to  $\beta$ -lactam antibiotics, mostly penicillin, but evidently less sensitive to other antimicrobial agents such as macrolides, sulfonamides, tetracyclines, or chloramfenicol (BOTSOGLOU & FLETOURIS 2001). Many studies proved that natural antimicrobial agents, if present in milk in higher concentrations, can bring about false – positive results (CARLSSON *et al.* 1989; ANDREW 2001; KANG & KONDO 2001; KANG *et al.* 2005).

Commercially produced microbial inhibitor tests are delivered in the form of ampoules (monotests) or in the form of microplates with a high number of testing cells. Apart from water bath or incubator, they do not require special laboratory equipment. To avoid subjective differences in the visual interpretation and to take the readings in an automated and more objective manner, some authors performing photometric measurements use the appropriate wavelength (590 nm) and another wavelength as reference (650 nm) in ELISA reader (ALTHAUS *et al.* 2003). When performing microbial inhibitor tests, it is necessary to meet the standards of good laboratory practice (protection against the contamination of the test), checking the pH value of the sample, observing carefully the correct temperature and the incubation period as specified by the producer's instructions and testing a positive as well as a negative control alongside with the sample. Out of microbial inhibitor screening methods, in frequent use are, for example: Eclipse test, Charm Cowside test, Charm AIM-96, Charm Farm test, VALIO T 101, Copan Milk test, and others.

#### Receptor binding and enzymatic colourimetric assays

An alternative method for establishing the  $\beta$ -lactam group antibiotics (active forms of the  $\beta$ -lactam structure) is the use of the receptor proteins.  $\beta$ -Lactam specific receptor proteins or penicillin-binding proteins (PBP) were successfully used in some methods and commercially produced tests (Biacore analysis, Penzym test, Beta Star test, SNAP test, Charm Safe Level test and DELVO-X-Press test and others) for establishing the  $\beta$ -lactam antibiotics residues (MITCHELL *et al.* 1998; GUSTAVSSON 2003).

PBP is found frequently in bacterial cell walls. Penicillin sensitive bacteria have various penicillin

binding proteins which, judging on their molecular weights, can be divided into two groups: proteins with low or high molecular weights (MASSOVA & MOBASHERY 1998). These proteins are further divided in subgroups by the amino acid sequence (GHUYSEN 1991). Various PBPs have different functions. They include transpeptidase, transglycosylase, and carboxypeptidase activities (MASSOVA & MOBASHERY 1998). The low molecular weight PBPs probably control the extent of cross-linking of the peptidoglycan in the cell wall by acting on D-alanyl-D-alanine-terminated peptides, but the high molecular weight PBPs lack this DD-peptidase activity (GRANIER *et al.* 1994). The greatest deal of attention was paid to soluble DD-carboxypeptidases *Streptomyces* R61 and *Actinomadura* R39 (GHUYSEN *et al.* 1973; FRÉRE *et al.* 1976). Bacteria excrete intracellular enzymes while in growth (e.g. R 61 and R 39), which are believed to be the soluble forms of membrane – bound transpeptidases participating in the bacteria cell wall synthesis. The enzymes can act as carboxypeptidases and transpeptidases (LEYH-BOUILLE *et al.* 1970). Natural substrates for these enzymes are peptides ended with D-alanyl-D-alanine (D-Ala-D-Ala). Penicillin is a structural analogue of the dipeptide D-Ala-D-Ala and therefore the enzymes react with the  $\beta$ -lactam structure.  $\beta$ -Lactam antibiotics create a covalent bond with carboxypeptidase giving rise to a very stable complex. Due to the formation of this complex, the enzyme activity is inhibited. The reaction is reversible if the enzyme is released from the complex – it has an identical affinity to the  $\beta$ -lactam antibiotic as that before the reaction.  $\beta$ -Lactam antibiotics are, however, degraded to phenylacetylglycine and N-formyl-D-penicillamine (GHUYSEN 1977; MASSOVA & MOBASHERY 1998).

#### Enzymatic colourimetric assays

**Penzym test** (UCB Bioproducts Belgium). Penzym test is a qualitative enzymatic colorimetric method for a rapid determination of  $\beta$ -lactam antibiotics in milk. The test principle is based on establishing the level of inactivation of the DD-carboxypeptidase enzyme by  $\beta$ -lactam antibiotics. These residues bind specifically with the enzyme and inactivate it, thus interfering with the bacterial cell wall formation. In the course of the Penzym test, lyophilised enzyme *Streptomyces* DD-carboxypeptidase is enclosed in an ampoule into which a sample of milk is introduced (50  $\mu$ l). Then the incubation is observed for 5 min at 47°C.

$\beta$ -Lactam antibiotics present in the sample create a stable complex with DD-carboxypeptidase in the sample. The degree of inactivation of the enzyme depends on the amount of antibiotics present in the sample. After a reagent tablet containing synthetic D-alanin oligopeptid and D-amino acid-oxidase is added, another incubation process follows for 8 min at 47°C. If the sample does not contain  $\beta$ -lactam antibiotics residues, after the specific substrate is added, the enzyme DD-carboxypeptidase hydrolyses the tripeptide (Ac-L-Lys-D-Ala-D-Ala) onto the dipeptide (Ac-L-Lys-D-Ala) with a simultaneous release of D-Alanin (D-Ala). The amount of the D-Ala released depends on the amount of the active DD-carboxypeptidase enzyme. Free D-Ala is further oxidised by D-amino-acid-oxidase onto pyruvic acid giving rise to hydrogen peroxide. The end products of the substrate and enzyme reaction (pyruvic acid and hydrogen peroxide) are measured using a redox colour indicator and the comparison of the final colour with the colour chart provided with the kit. Hydrogen peroxide is used to oxidise the organic redox indicator that will change into a pink-orange colour compound, indicating a negative result. If the colour is peach-coloured, the sample is close to the detection limit. If a yellow or yellow-orange colour is observed (test-positive outcome), the sample is suspected of containing an antibiotic residue (CULLOR 1993; MITCHELL *et al.* 1998; BOTSOGLOU & FLETOURIS 2001; GUSTAVSSON & STERNESJÖ 2004).

### Receptor binding assays

**Delvo-X-PRESS  $\beta$ L test** (Gist-brocades BV, The Netherlands). The Delvo-X PRESS  $\beta$ L test is a qualitative, competitive, receptor-enzyme assay. Although analogous in principle to immunochemical assays, the Delvo-X-PRESS test does not use antibodies to bind specifically  $\beta$ -lactams and therefore cannot be classified as an immunochemical test. The test involves a reagent substance (a tracer) containing a conjugate – a specific receptor protein isolated from the bacteria of *Bacillus stearothermophilus* species conjugated with an enzyme (horseradish peroxidase). One of the reaction compounds is non-specifically adsorbed onto the surface of a solid-the test tubes contain an active specific  $\beta$ -lactam layer.

The Delvo-X-PRESS kit uses a special workstation. The workstation integrates a heater, a manual,

a shaker and a reader unit. Via the display, the workstation gives the user step by step instructions. In the first step of the test, the measured volume of milk (0.2 ml) and the conjugate (enzyme bound onto the receptor protein), are mixed and incubated in a test tube (3 min at 64°C). The conjugate binds to the free  $\beta$ -lactam antibiotics which are present in the sample. Only the free conjugate (residual conjugate which did not bind to the  $\beta$ -lactam antibiotics present in the sample) is capable to bind onto the  $\beta$ -lactam layer of the test tube. The more free  $\beta$ -lactam antibiotics the reagent mixture contains, the fewerlabelled receptors bind to the immobilised  $\beta$ -lactam antibiotics adsorbed onto the surface of the test tube and *vice versa*. After the removal of the conjugate-free  $\beta$ -lactam antibiotics complex with repeated rinsing of the test tube, an enzymatic substrate (colour developer) is added. It serves for the detection of the residual conjugate binding onto the  $\beta$ -lactam layer. The addition of the enzymatic substrate results in the formation of blue colour which is inversely proportional to the concentration of the  $\beta$ -lactam antibiotics in the sample. The colour is measured photometrically at 660 nm. The same procedure is applied also in the test tube which contains, instead of the milk sample, 0.2 ml of penicillin G standard solution (concentration 5 ppb). The positivity of the sample is assessed photometrically by comparing optical densities (OD) of the sample and the standard. The samples yielding OD higher than 0 are classified as positive, while those yielding negative OD are assessed as negative (MITCHELL *et al.* 1998; ANGELIDIS *et al.* 1999; BOTSOGLOU & FLETOURIS 2001).

**IDEXX SNAP test** (Idexx Laboratories Inc., Westbrook, ME, USA). SNAP test is an enzyme-linked, receptor binding assay in which  $\beta$ -lactams are captured by a binding protein on a solid support adsorbent matrix housed in a moulded plastic unit. SNAP residues test consists of three components: SNAP device, pipette, sample tube. Using this test, penicillin can be detected in the amount of 4 ppb, ampicillin or amoxicillin in the amounts of 10 ppb, cephapirin 8 ppb, and ceftiofur 50 ppb.

The SNAP test utilises a  $\beta$ -lactam receptor protein conjugated to an enzyme. The assay procedure includes three simple steps with a total assay time of about 10 minutes for a sample. In the first step of test, calibrated amounts of milk and conjugate are mixed and incubated in a test tube, placed in a heating block (5 min, 45  $\pm$  5°C). The enzyme

conjugate binds with  $\beta$ -lactams present in the milk sample. The mixture is then transferred to the sample well of the SNAP device (plastic unit containing sample and control spots on filter paper strip) where the sample is allowed to migrate on a filter paper strip until it passes to the test spot. Test spots are coated with  $\beta$ -lactam antibiotic. Any free receptor will be captured at this spot, whereas the receptor protein that interacts with free  $\beta$ -lactams in the sample will not. The substrate is released and reacts with the enzyme attached to the captured receptor protein and a colour develops at the test spot. The results are read either visually or instrumentally (using reflectance) to provide the numerical interpretation of the visual result. The samples are declared positive or negative on the basis of the comparison of the intensity of the colour development between the sample and control spots on the SNAP test. If the colour of the test spot is weaker than that of the control spot, the result is interpreted as positive (BELL *et al.* 1995; NEAVES 1995; MITCHELL *et al.* 1998; GUSTAVSSON 2003).

**Beta-Star test** (UCB Bioproducts, Belgium). The test involves a specific  $\beta$ -lactam receptor linked to gold particles. It is a dipstick test that detects penicillins and cephalosporins. The milk sample (0.2 ml) is added to a vial containing the test reagents (receptor protein linked to gold particles), mixed and incubated at 47.5°C in the incubator for 3 minutes. During incubation, the receptor will react with the free  $\beta$ -lactams contained in the sample. After 3 min of incubation, the dipstick is added and incubation is continued (2 min at 47.5°C). The mixture is transferred to a strip of immuno-chromatography paper where it migrates towards the test field. With milk samples free of  $\beta$ -lactam residues, the receptor protein will be captured by a biomolecule immobilised at the test field of the chromatography paper. Since the receptor protein is linked to gold particles, the captured protein-gold complex will appear as a pink-coloured band. With the sample where the receptor protein has interacted with free  $\beta$ -lactam molecules, the receptor protein will not be captured at the test field and no band will occur. The colour intensity of the test band is visually compared with that of the reference band: if the colour intensity of the test band is weaker than that of the reference band, the sample is classified as positive (GUSTAVSSON 2003; GUSTAVSSON and STERNESJÖ 2004).

### *Microbial receptor assays*

**CHARM I and II tests** (Charm Sciences, Inc., USA). CHARM I and II tests are qualitative microbial receptor assays. The CHARM I test developed for  $\beta$ -lactams in milk was the first rapid test recognised by the AOAC (Association of Official Analytical Chemists) with a test time of 15 minutes. In 1984–1985, the CHARM I test was further developed to a test for antibiotics including, apart from  $\beta$ -lactams, tetracyclines, sulfonamides, aminoglycosides, chloramphenicol, novobiocin, and macrolides. The extended version the CHARM I test has been referred to as CHARM II test (BOTSOGLOU & FLETOURIS 2001).

CHARM II test is based on the irreversible binding reaction between the functional groups of antibacterials and receptor sites on or within the cells of the added microorganisms. Two types of bacterial cells (*Bacillus stearothermophilus*) are used to provide the binding sites for seven drug families. In tetracycline and chloramphenicol test kits, an antibody coating is used. The test employs  $^{14}\text{C}$ - or  $^3\text{H}$ -radiolabelled antibacterials (tracer reagent) to compete for the binding sites. This competition for the receptor sites prevents the radiolabelled antibacterial from binding. Thus, the more radiolabelled compound binds, the less analyte is in the sample.

The procedure is relatively fast and simple. Milk is added to a freeze-dried pellet of the binding reagent in a test tube and the resulting sample is mixed and incubated. During incubation, any antibiotic present in the milk will bind to its specific natural receptor site on the bacterial cell. The tracer reagent is then added to the mixture and the sample is mixed and incubated. At this time, any free receptor sites on the bacterial cell will bind with radiolabelled antibiotics. The sample is then centrifuged to collect the bacterial cells at the bottom of the test tube. The supernatant is discarded, the precipitate is resuspended in water to be further mixed with the scintillation fluid. The rate of binding is measured with a scintillation counter and compared to the positive and negative controls. The higher the amount of the antibiotic present in the sample, the lower the counts detected by the equipment (CULLOR 1993; HUI 1993; MITCHELL *et al.* 1998; BOTSOGLOU and FLETOURIS 2001).

### Immunoassays

Immunochemical methods are based on the reaction of an antigen with an antibody – a reaction of antigenic determinants with the antibody linking site. Chemically, antigens are polymers-proteins, polypeptides, polysaccharides, or nucleoproteins. They have two fundamental properties – they incite a specific immune response, and they react specifically with the products of this response (with antibodies and immunocomplementary cells). Both properties can be found in the complete antigen-an immunogen which is made of a macromolecular carrier and antigenic determinants-epitopes. A low molecular substance which itself can not incite the antibody production but which reacts specifically with the products of the immune response is termed a hapten. Antibodies are proteins, specifically immunoglobulins, formed by the host animal in response to the invasion by antigens. An immunoglobulin molecule contains two identical light (L) and two identical heavy (H) polypeptidic chains mutually linked with disulphide bonds. One of the Ig molecules always contains one light and one heavy chain type. The light chain determine the immunoglobulin molecule type. The heavy chains are decisive in classifying into the immunoglobulin class. C-ending sections of the chain make up their constant area, N-endings of chain are denoted as the variable sections and they represent the section of the molecule binding to the antigen – a paratope. Immunochemical methods make use of monoclonal or polyclonal antibodies. Modern immunoanalytical methods accomplish an increase in the sensitivity by labelling one of the reagents – the antigen or the antibody. The label can be a radio-isotope, enzyme, fluorescence or chemical scintillation agent (MITCHELL *et al.* 1998; ROEDER & ROEDER 2000; STEPANIAK *et al.* 2003)

Nonisotopic immunoassays such as ELISA (enzyme linked immunosorbent assay), FPIA (fluorescence polarisation immunoassay), PCIA (particle-concentration immunoassay), PCFIA (particle-concentration fluorescence immunoassay), and monoclonal-based immunoassays will, in all likelihood, play an increasingly important role in antibiotics screening immunoassay determinations (ROEDER & ROEDER 2000). The most frequently used immunochemical method for rapid diagnostics of veterinary drug residues is enzyme immunoanalysis (EIA). As an enzyme label, horse radish peroxidase, alkaline phosphatase, glucose

oxidase, pyruvate dehydrogenase and recombinant  $\beta$ -galactosidase are used. These enzymes catalyse the reactions that cause the substrates degradation and form coloured products that can be read spectrophotometrically or visually (BOTSOGLOU & FLETOURIS 2001).

LacTec test ( $\beta$ -lactam) is based on the immunobinding principles of the enzyme-linked immunosorbent assay system (CULLOR *et al.* 1992; CULLOR 1993; BOTSOGLOU & FLETOURIS 2001). Among other methods is used e.g. the Fluorophos Beta Screen E.U. test – qualitative enzyme immunoassay based on fluorescence detection for the determination of six  $\beta$ -lactam antibiotics – amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin, penicillin G in raw milk (STERNESJÖ & JOHNSON 1998). Parallax (IDEXX Laboratories Inc., USA) can be used to detect different antimicrobial agents. The method is based on competitive fluorescent immunoassays in glass capillary tubes (GUSTAVSSON & STERNESJÖ 2004).

Most of immunoassays are normally performed in a laboratory and therefore it takes quite a long time before the outcome of the test is known. For this reason, there is a growing need for on-site screening at the beginning of the food chain using fast and easy to perform test methods. A number of immunochemical tests are commercially available in a kit format for many drugs (card format, one step strip test). A promising approach in the rapid antibiotics detection is the use of a dipstick format (lateral flow devices).

A lateral flow test comprises five different basic components: sample filter (a paper like material with two functions-filtering out a solid material and buffering the sample after extraction/pretreatment); conjugate pad (gold pad) is a fibreglass-like pad which can either be sprayed by, or bathed in gold-, latex- or carbon-conjugated solutions; membrane (membrane is composed of nylon or nitrocellulose and glued upon a backing material) are made to display a maze structure with various pore sizes; reservoir – absorption pad (has one purpose – to adsorb the liquid at the end of the strip); test line and control lines (lines are sprayed onto the nitrocellulose/nylon membrane). The sample is applied to the sample filter, housed inside the device casing. The sample runs through the sample filter and conjugate pad. This conjugate pad contains a labelled antibody, specific for the analysis under test. Antibiotics, if present, will form a complex with the conjugate and migrate further slowly to the

membrane in the test zone. This zone contains an immobilised antibody, specific for the analysis, but preferably not competing with the conjugated antibody for the same or adjacent epitopes. The test line will thus capture the migrating analyse-conjugate complex. The intensity of the test line correlates well with the amount of analyte in the sample (VAN HERWIJNEN 2006). Immunoassay can be sensitive, class specific, accurate, and can provide a means for a rapid screening of samples for antibiotics.

## CONCLUSIONS

In general monitoring of antimicrobial agent residues, microbial growth inhibition methods and rapid tests are used. Microbial inhibitor screening methods are easy to perform and enable the detection of a wide spectrum of agents. The microbial inhibitor screening methods do not attain with some antibiotics the sensitivity at the levels specified by the MRLs. For the determination of these agents, other methods should be used (immunochemical, receptor). Another disadvantage is a long period needed to perform the test and the occurrence of falsely-positive results if the tests are used for the analysis of individual samples containing higher levels of naturally occurring antimicrobial agents. Rapid tests enable to obtain the result of a test in the course of several minutes and they are highly specific. A majority of the rapid assays were aimed above all, at the detection of  $\beta$ -lactam antibiotics. Even in these tests, falsely-positive results can occur.

In general monitoring, the methods should be combined so that raw milk health safety is assured. A new trend is represented in the development of rapid tests for the detection of more antibiotic groups, e.g. Twinsensor (Unisensor S.A., Belgium) which enables to detect  $\beta$ -lactam and tetracycline antibiotics as well.

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