Slov Vet Res 2006; 43 (4): 161-8
UDC 614:35-579.67:615.33:637.12

**Original Research Paper**

# MICROBIOLOGICAL 5-PLATE SCREENING METHOD FOR DETECTION OF TETRACYCLINES, AMINOGLYCOSIDES, CEPHALOSPORINS AND MACROLIDES IN MILK

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**Summary:** Within the field of food hygiene and food control, the antibiotic residues in food of animal origin are analysed because their presence may have undesirable consequences. These include for example, allergic reactions in people, spread of resistance to antibiotics among microorganisms and damages in the food industry. Methods available for the detection of antibiotic residues in food are microbial, chemical and immunoassays. Microbial methods are used as screening methods and are always the first choice for this purpose.

The aim of our study was to develop a microbial method for the detection of antibiotic residues from the macrolide, aminoglicoside, cephalosporine and tetracycline families. The study involved investigation of bacterial test strains and establishment of the limits of detection (LOD) of antibiotics.

For cephalosporines and macrolides, the most appropriate sensitive strain proved to be *Micrococcus luteus* ATCC 9341, for aminoglicosides *Bacillus subtilis* BGA and for tetracyclines *Bacillus cereus* ATCC 11778. A significant component in our experiment were the so-called confirmation solutions. Magnesium sulphate inactivates aminoglicosides and can be used to confirm their presence when more than one antibiotic group can produce inhibition zones on the same plate. Cephalosporinase inactivates cephalosporines and was used to distinguish this group of antibiotics from macrolides. The LOD was at or below the allowed maximum residue level (MRL) for all tested antibiotic groups.

**Key words:** food analysis - methods; antibiotics; drugs residues - analysis; microbial sensitivity tests - methods; milk

## Introduction

By definition, an antibiotic is either a natural product of a micro-organism, an identical synthetic product or a similar semi synthetic product, that inhibits the growth of other microorganisms (bacteriostatic effect) or destroys other microorganisms (bactericide effect). (1). The most common cause for the presence of antibiotic residues in food of animal origin is violation of withdrawal periods (2, 3, 4, 5). Other possible causes are overdosing of antibiotics and use of antibiotics banned for treatment of food producing animals (6, 7, 8). Also, antibiotic residues can be detected in bulk milk samples from a stable where individual animals are being treated for mastitis.

Received: 27 June 2006
Accepted for publication: 6 October 2006

In the field of food hygiene and food control we deal with analysis of antibiotic residues in food of animal origin due to the potential of unwanted consequences . Among them are sensitivity to antibiotics, allergic reactions and imbalance of intestinal microflora in people, spread of resistance to antibiotics in microorganisms and losses in the food industry where antibiotics can influence starter cultures used in the production of meat and milk products.

Microbial methods were the first choice of systematic detection of antibiotic residues in food in the past and are still mainstream screening methods. They allow determination of the presence of antibiotics in the sample and identification of specific antibiotic groups (9). Screening methods have acceptable false-positive result rates (10, 11) and allow detection of a wide spectrum of antibiotics (9, 12). Their other

advantages are the option to analyse a large number of samples simultaneously and the relatively short time needed for preparation of samples as no purification procedures are required (13, 14, 15, 16, 17, 18). As microbial methods can not be used to identify individual antibiotics a positive result should be confirmed with chemical or physical methods.

Tetracyclines are probably the most widely used therapeutic antibiotics in food producing animals because of their broad spectrum and cost effectiveness. In the United Kingdom and the Netherlands the amount of tetracyclines used for farm animals is nearly equal to the amount of all other antibiotics. Cephalosporines are used both for humans and animals. The first and second generation are approved worldwide strictly for treatment of mastitis infections in dairy cattle. A representative of the third generation, ceftiofur, and a representative of the fourth generation, cefquinome, have been developed strictly for veterinary use and approved in several countries for treatment of respiratory disease, foot rot mastitis in dairy cattle (9, 10, 15, 16, 19). Macrolides are used in veterinary medicine for the treatment of clinical and subclinical mastitis in lactating cows and for the treatment of chronic respiratory diseases (20). The aminoglycosides are broad-spectrum antibiotics also widely used for treatment of bacterial enteritis, mastitis and other infections. Aminoglycosides most commonly used as therapeutic agents are gentamicin, neomycin and streptomycin (21).

The most frequently used microbial method is based on the principle of inhibition of growth of testing strains which is known as the STAR five-plate method (22). It is used for detection of antibiotics from the macrolide, aminoglicoside, tetracycline and cephalosporine families (19, 23, 24, 25). Detailed procedures of these tests vary among laboratories and tests are not standardised for minimal detectable antibiotic concentrations, therefore comparison of results is difficult (26, 27, 28). The aim of our study was to develop a microbial method for detection of antibiotic residues from the four above mentioned families and to determine LOD for each tested antibiotic according the EU Regulation 2377/90 which prescribed maximum residue limits (29).

## Material and methods

Microbiological methods are based on the measurement and evaluation of zones of inhibited bacterial growth on media. Two test strains are used to assess the presence of each antibiotic – one maximally sensitive and the other resistant to the tested substance. With the combination of different sensitive and resistant bacterial strains, specific antibiotic groups present in the sample can be identified. In our research we used the following strains with previously established sensitivity and resistancy profiles: *Bacillus cereus* ATCC 11778, *Micrococcus luteus* ATCC 2341, *Escherichia coli* ATCC 10536, *Staphylococcus epidermidis* ATCC 12228 and *E. coli* ATCC 10536 (manufactured by OXOID™).

### *Preparation of bacterial cultures and media*

Bacterial strains stored as cultures in original bacterial loops (Culti loop) were applied to a test tube containing 1ml Trypton soya broth (TSB) medium and incubated at 37 °C for one hour. The culture was then inoculated on blood agar and incubated for further 16 hours at the same temperature. Afterwards the purity of bacterial colonies was assessed with a light microscope and pure colonies were stored in a fridge at temperatures between 2 °C and 8 °C for up to one month. To compose test plates, bacterial culture was diluted with normal saline containing peptone water to produce a suspension which was then incubated at 37 °C for one hour and afterwards added to the agar medium specified below. The suspension density was standardised with the Mc Farland method.

Basic media for preparation of test plates were antibiotic agar No. 1 (Merck™) and antibiotic agar No. 2 (Merck™). Antibiotic agar No. 1 was prepared as follows: 1000 ml of distilled water was added to 30, 5 g of the medium, left for 15 min and then heated to boiling point so that the medium was completely dissolved. The medium was then autoclaved at 121 °C for 15 min. For antibiotic agar No. 2 1000 ml of distilled water was added to 15, 5 g of medium and then the same procedure was followed. After autoclaving, the pH of the media was set to desired values: pH 8 for Er, I BGA, Kin and AC plates and pH6 for E plates.

### *Preparation of test plates*

Test plates were marked according to the bacterial strain added to the medium: AC plate - Micrococcus luteus ATCC 2341, ER plate - Staphylococcus epidermidis ATCC 12228, I-BGA plate - Bacillus subtilis BGA, Kin plate - E. coli ATCC 10536 and E plate - Bacillus cereus ATCC 11778. The pH of the medium was maintained at 8.0 for AC, E and ER plates and at 6.0

for I-BGA and Kin plates. We defined the tolerance for the width of inhibition zone at (as) 8.5 mm – 0.5 mm wider than the width of the metal cylinder containing the sample. Inhibition zones between 8 mm and 8.5 mm wide were considered a non-specific reaction.

To prepare a test plate 0.45 ml of suspension of bacterial culture was added to 40 ml of basic medium and heated to 40 °C. Kin plate was an exception where 0.2 ml of suspension was added to 50 ml of medium. The mixture of medium and bacterial culture was poured into a petri dish (5 ml of mixture into each petri dish). At room temperature the petri dishes with silified medium were enveloped in a parafilm and stored in a fridge. The storage period of test plates was one week. Before application of samples to test plates, plates were warmed at room temperature for 20 to 30 min.

*Preparation of milk samples*

To test the sensitivity of our method, milk samples containing known concentrations of standard antibiotics were inoculated on test plates. Prior to the addition of antibiotics, milk was always tested for the presence of inhibitory substances.

As the initial step, standard antibiotic solutions were prepared using reference chemical composition and purity (Table 1). Standard antibiotics in powder were dissolved in appropriate solvents: tetracyclines in phosphate buffer with pH value 4.5, cephalosporines in phosphate buffer with pH value 6.0, aminoglicosides in phosphate buffer with pH value 8.0, and macrolides in methanol. Standard solutions were diluted to desired concentrations with UHT milk containing 1.6% fat (Ljubljanske mlekarne). These samples of milk with known concentrations of antibiotics were then poured into 10-ml test tubes and heated to 80 °C for 5 min to avoid later non-specific reaction on test plates. After heating, the samples were cooled and transferred to test plates in 8 mm wide cylinders. Test plates were incubated at 37 °C (I-BGA, AC, Er, Kin) or at 30 °C (E plate) for 18-24 hours.

For each antibiotic we used milk samples containing antibiotic concentrations equal to MRL and half the MRL for that substance. If at half the MRL the result was still positive, lower concentrations of antibiotic were applied until the minimal level of detection was reached.

*Confirmation solutions*

To confirm the presence of antibiotic groups or their individual representatives we used confirmation solutions. These solutions inhibit the action of certain antibiotics and can help to distinguish between antibiotic groups which cause inhibition zones on the same test plates. Magnesium sulphate ($MgSO_4$) was used to neutralise the aminoglicosides and cephalosporinase enzyme to neutralise the cephalosporines.

25 µl of 20% $MgSO_4$ solution in water was added to the sample on E, AC and I-BGA plates where inhibition zones are produced by aminoglicosides, macrolides or tetracyclines. 25 µl of cephalsporinase was added to samples on AC and I-BGA plates to identify cephalosporines.

**Table 1:** Antibiotic standards

| ANTIBIOTIC | TRADE MARK | CATALOGUE NUMBER |
|---|---|---|
| Streptomyicin | Sigma - Aldrich | 46754 |
| sGentamicin | Sigma - Aldrich | 46305 |
| Neomycin | Calbiochem | 4801 |
| Cephalexine | Sigma - Aldrich | 33989 |
| Cephazoline | Sigma - Aldrich | 22127 |
| Cefoperazone | Sigma - Aldrich | 22129 |
| Chlortetracycline | Sigma - Aldrich | 46133 |
| Tetracycline | Sigma - Aldrich | 46935 |
| Erythromycin | Sigma - Aldrich | 46256 |
| Tylosin | Sigma - Aldrich | 46992 |

*Evaluation of results*

Results of microbial methods can be evaluated both qualitatively and quantitatively. Qualitative results are obtained by analysing the effect of antibiotics on a combination of sensitive and resistant bacterial strains. When required, neutralising substances (confirmation solutions) can help to differentiate between antibiotics with similar action on test bacterial strains.

Quantitatively the concentration of antibiotic can be assessed with microbial methods if the sample contains a known antibiotic or an antibiotic that has previously been identified qualitatively. In each case a calibration curve is required.

## Results

We have confirmed sensitive and resistant bacterial strains for all antibiotic groups tested in our study (Table 2). Based on our results we chose to use *Bacillus cereus* ATCC 11778 (E plate) as the sensitive and *Micrococcus luteus* ATCC 9341 (AC plate) as the resistant strain for tetracycline and chlortetracycline from the tetracyclines group. For tylosine and erythromycine from the macrolides group *Micrococcus luteus* ATCC 9341 (AC plate) was chosen as the sensitive and *Escherichia coli* ATCC 10536 (Kin plate) as the resistant strain. For gentamycine, sterptomycine and neomycine from the aminoglicosides group *Bacillus subtilis* BGA (I-BGA plate) was chosen as the susceptible and *Staphylococcus epidermidis* ATCC 12228 (ER plate) the resistant strain. For cephalexine, cephoperasone and cephasoline from the sensitive group *Micrococcus luteus* ATCC 9341 (AC plate) was chosen as the susceptible and Staphylococcus epidermidis ATCC 12228 (ER plate) as the resistant strain.

We differentiated between antibiotic groups using a combination of five test plates (Table 3). To discriminate between aminoglicosides and macrolides we had to utilise used magnesium sulphate which inactivates the aminoglicosides. To discriminate between cephalosporines and macrolides we used the cephalosporinase enzyme.

Table 4 shows the limit of detection for milk samples containing standardised antibiotic solutions on selected test plates. The level of detection was at or below the MRL in all tested antibiotics.

## Discussion

Microbial methods for detection of antibiotic residues in food of animal origin are used as a screening method in the majority of laboratories in Europe that deal with analyses of drug residues in food

**Table 2:** Sensitivity of bacterial strains:

| ANTIBIOTIC | B.c/ E | M.l.1/ AC | B.s.BGA/ IBGA | S.e./ ER | E.c./ KIN |
|---|---|---|---|---|---|
| Cephalexine | - | + | + | - | - |
| Cephasoline | - | + | + | - | - |
| Cefoperazone | - | + | + | - | - |
| Gentamicin | + | - | + | - | + |
| Neomycin | + | - | + | - | + |
| Streptomycin | + | - | + | - | + |
| Erythromycin | - | + | + | - | - |
| Tylosin | - | + | + | - | - |
| Tetracycline | + | - | + | - | - |
| Chlortetracycline | + | - | + | - | - |

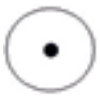
| | |
|---|---|
| + | sensitive strain |
| - | resistant strain |
| B.c/E | Bacillus cereus ATCC 11778/ plate E |
| M.l.1/AC | Micrococcus luteus ATCC 9341/ plate AC |
| B.s.bga/IBGA | Bacillus subtilis BGA/ plate IBGA |
| S.e./ER | Staphylococcus epidermidis ATCC 12228/ plate ER |
| E.c./KIN | Escherichia coli ATTC 10536/ plate KIN |

**Table 3:** Interpretation of results of 5-plate method:

| Plate E | Plate Ac | Plate IBGA | Plate Er | Plate Kin | Antibiotics | Antimicrobial family |
|---|---|---|---|---|---|---|
|  |  |  |  |  | Cephazoline<br>Cephalexine<br>Cefoperazone | Cephalosporins |
|  |  |  |  |  | Gentamicin | Aminoglycosides |
|  |  |  |  |  | Neomycin | |
|  |  |  |  |  | Streptomyicin | |
|  |  |  |  |  | Erythromycin | Macrolides |
|  |  |  |  |  | Tylosin | |
|  |  |  |  |  | Tetracycline<br>Chlortetracycline | Tetracyclines |

| | |
|---|---|
| plate E | Bacillus cereus ATCC 11778 |
| plate Ac | Micrococcus luteus ATCC 2341 |
| plate IBGA | Bacillus subtilis BGA |
| plate Er | Staphylococcus epidermidis ATCC 12228 |
| plate Kin | E. coli ATCC 10536 |
| Sa + ceph | sample + confirmatory solution - cephalosporinase |
| Sa + MgSO4 | sample + confirmatory solution – magnesium sulphate |

sample with inhibition zone after incubation



sample withouth inhibition zone after incubation.

(30, 31, 32, 33). They are always the method of choice for screening purposes as they allow qualitative detection of antibiotics in the sample and identification of antibiotic groups. This facilitates subsequent confirmation of specific antibiotic residues with chemical methods. Microbial methods are relatively inexpensive, easy to use, do not require expensive equipment and can be efficiently adopted by laboratory staff. Although minimal expenditure is a significant factor of analyses, no test is valuable if it does not give reliable results (34, 35). We succeeded in developing a microbial method which is sensitive and meets the legislative requirements – to detect concentrations of antibiotics below the MRL. For some antibiotics the level of detection was at half the MRL or lower.

Microbial methods are semi quantitative, therefore any positive or suspicious result should be confirmed by chemical methods (36). In accordance with the EC 2002/657/EC regulation results of microbial methods are not reported as negative and positive, but as satisfactory or suspect when the MRL is exceeded.

Although the STAR five-plate test is the official method approved by the Community Reference Laboratory, many variations of microbial methods are

**Table 4:** Limit of detection and maximum residue levels (MRL) of antibiotics in milk

| Antibiotic | Bacterial strain/ plate | LOD st.s. (µg/kg) | LOD milk (µg/kg) | MRL milk (µg/kg) |
|---|---|---|---|---|
| Cefalexin | M.l.1/ AC | 50 | 50 | 100 |
| Cefazolin | M.l.1/ AC | 20 | 25 | 50 |
| Cefoperazon | M.l.1/ AC | 50 | 50 | 50 |
| Tetracycline | B.c/ E | 5 | 20 | 100 |
| Chlortetracycline | B.c/ E | 10 | 20 | 100 |
| Erythromycin | M.l.1/ AC | 20 | 20 | 40 |
| Tylosin | M.l.1/ AC | 20 | 10 | 50 |
| Gentamicin | B.s.BGA/ IBGA | 20 | 30 | 100 |
| Neomycin | B.s.BGA/ IBGA | 50 | 80 | 1500 |
| Streptomyicin | B.s.BGA/ IBGA | 80 | 100 | 200 |

LOD st.s limit of detection of standard solution
LOD milk limit of detection in milk
MRL milk maximum residue level in milk
B.c/ E Bacillus cereus ATCC 11778/ plate E
M.l.1/AC Micrococcus luteus ATCC 9341/ plate AC
B.s.bga/IBGA Bacillus subtilis BGA/ plate IBGA
S.e./ER Staphylococcus epidermidis ATCC 12228/ plate ER
E.c./KIN Escherichia coli ATTC 10536/ plate KIN

used across the world and most laboratories apply a specific approach with a different number and types of bacterial strains and therefore a different number of test plates (31, 37, 38). Methods using between one and 18 plates have been described in the literature. There are also differences in incubation periods, pH values of media and the quantity of media on which the bacteria are cultured. Considering the length of time since the development of microbial methods it is perhaps surprising that relatively few studies have been published on this topic (33, 38). In this paper we have presented a method based on the STAR test but with additional use of two confirmation solutions and LOD for aminoglycosides below the MRL.

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# MIKROBIOLOŠKA METODA PETIH PLOŠČ ZA UGOTAVLJANJE TETRACIKLINOV, CEFALOSPORINOV TER MAKROLIDNIH IN AMINOGLIKOZIDNIH ANTIBIOTIKOV V MLEKU

A. Kirbiš

**Povzetek:** Na področju higiene in nadzora živil se ukvarjamo z analitiko ugotavljanja ostankov antibiotikov v živilih živalskega izvora zaradi težav, ki jih lahko le-ti povzročijo. To so senzibilizacija in alergijske reakcije, širjenje rezistence na antibiotike med mikroorganizmi in ne nazadnje škoda, ki jo lahko povzročijo v živilski industriji, kjer lahko vplivajo na starterske kulture, ki se uporabljajo za proizvodnjo mlečnih in mesnih izdelkov.

Metode, ki se uporabljajo za ugotavljanje ostankov antibiotikov v živilih živalskega izvora, so mikrobiološke, imunoencimske in kemijske. Mikrobiološke metode se uporabljajo kot presejalne oziroma screenig metode in so vedno prvi izbor pri tovrstni analitiki.

Namen raziskave je bil uvedba mikrobiološke metode za ugotavljanje antibiotikov s testiranjem in uvedbo testnih sevov bakterij in ugotavljanje minimalne količine antibiotikov, ki jih je s posamezno metodo mogoče ugotoviti. Določili smo občutljive in odporne bakterijske seve za skupine makrolidnih, aminoglikozidnih antibiotikov, cefalosporinov in tetraciklinov v mleku. Za ugotavljanje cefalosporinov in makrolidnih antibiotikov uporabljamo bakterijski sev Micrococcus luteus ATCC 9341 kot občutljivi sev, za aminoglikozidne antibiotike bakterijski sev Bacillus subtilis BGA ter za tetracikline Bacillus cereus ATCC 11778.

V veliko pomoč pri poskusu pa so bile t. i. potrditvene spojine. Magnezijev sulfat inaktivira aminoglikozidne antibiotike. Uporabimo ga lahko pri sumljivih vzorcih za potrditev prisotnosti le-teh.

Druga potrditvena snov je bil encim cefalosporinaza, ki inaktivira cefalosporine. Uporabljamo jo, kadar imamo na plošči AC pozitiven rezultat, saj bi sicer lahko prišlo do zamenjave z makrolidnimi antibiotiki, ki jih cefalosporinaza ne inaktivira.

**Ključne besede:** hrana, analize - metode; antibiotiki; zdravila, ostanki - analize; mikrobni občutljivostni testi - metode; mleko