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Original Research Paper

MICROBIOLOGICAL SCREENING METHOD FOR DETECTION OF AMINOGLYCOSIDES, β-LACTAMES, MACROLIDES, TETRACYCLINES AND QUINOLONES IN MEAT SAMPLES

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Summary: Antibiotics are natural products of a micro-organism, identical synthetic products or similar semi synthetic products that inhibit the growth of or destroy microorganisms. In veterinary medicine antibiotics are used for therapeutic, prophylactic, metaphylactic and nutritive purposes. The presence of antibiotics or their metabolites in food is potentially hazardous to health as it may cause allergic reactions in people and antibiotic resistance in pathogenic microorganisms. In addition antibiotics may influence starter cultures in food industry and lead to economic damage. The methods used to detect antibiotic residues in food of animal origin are microbiological, immuno-enzymatic and chemical. Microbiological methods are used as screening methods whose results direct the choice for more expensive and time consuming chemical confirmation methods. To serve this purpose the sensitivity of a microbiological method must be at or below the prescribed maximum residue level (MRL) for specific antibiotic group.

The purpose of our study was to develop a microbiological method for detection of macrolide, aminoglicoside, β-lactame, quinolone and tetracycline antibiotic families in meat by identifying the most appropriate set of bacterial test strains and to establish the limit of detection for each antibiotic group. *Micrococcus luteus* ATCC 9341 was selected as the most appropriate sensitive strain for detection of macrolides and β-lactams, *Bacillus subtilis* BGA for aminoglicosides, *Bacillus cereus* ATCC 11778 for tetracyclines and E. coli ATCC 10536 for quinolones. In addition magnesium sulphate was used to inactivate aminoglicosides β-lactamase to differentiate between macrolides and β-lactames. For all antibiotic groups the level of detection was below the MRL.

Key words: food analysis-methods; antibiotics; drug residues-analysis; microbiological sensitivity tests-methods; meat

Introduction

Antimicrobials are classified according to their chemical structure. Each class is characterized by a typical core structure and various members of the class are differentiated by the addition or removal of secondary chemical structures (1, 2). Antimicrobials can also be classified as broad or narrow spectrum, depending on the range of bacterial species against which they are active, or as bacteriostatic or bactericidal on the basis of their mechanism of action. The latter fall into four categories: inhibition of cell wall synthesis, damage to cell membrane function, inhibition of nucleic acid synthesis or function, and inhibition of protein synthesis. The aim of antimicrobiological therapy is to rapidly produce and then maintain an effective concentration of drug at the site of infection for sufficient time to allow host specific and nonspecific defenses to eradicate the pathogen (3).

The most commonly used antimicrobials in food-producing animals are β-lactams, tetracyclines, aminoglycosides, quinolones, macrolides and sulfonamides. Antimicrobials are administered to animals by injections (intravenously, intramuscularly, or subcutaneously), orally in food or water, topically on the skin and by intramammary and intrauterine

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infusions (4). Theoretically, all these routes may lead to residues appearing in foods of animal origin such as milk, meat and eggs (5).

Acquisition of resistance to antimicrobial agents by consuming food of animal origin has been receiving increasing attention in the literature, also raising awareness of the importance of minimizing exposure to antibiotic residues in food (6, 7). The most common causes for the presence of antibiotic residues in food of animal origin are violation of withdrawal periods, overdosing of antibiotics and use of antibiotics banned for treatment of economic animals (8, 9, 10, 11). The 2377/90 Council Regulation (12) determines a limited number of drugs allowed in veterinary medicine and defines MRL for each drug. This Regulation represented a milestone in food control because in addition to mere identification it also requested quantitative determination of antibiotic residues, the strategy of analyses and subsequently the methods therefore had to be adapted to the new legislation.

Microbiological, immuno-enzymatic and chemical methods are used for detection of antibiotic residues in food of animal origin and the protocol of control is usually based on two steps: screening for presence of different antibiotic groups and confirmation with identification of specific antibiotic in the sample and more accurate quantitative analysis. An ideal screening method would detect all licensed antibiotics at or below their Marls and should be robust, rapid, simple and cost effective (13). Chemical methods are too specific to be applied as a first choice screening method for the high number of monitored substances. In addition chemical methods require more expensive equipment. Microbiological methods are better suited for the first step screening, but unfortunately a single bacterial inhibition test for all antibacterial residues does not exist (14).

Microbiological methods detect inhibitory substances diffusing from a piece of tissue (14, 15, 16) or from a paper disk soaked with tissue fluid (17) into an agar layer seeded with a susceptible bacterial strain. These methods are usually multiresidue screening tests able to detect several families of antimicrobial drugs and use one or more test plates which differ in bacterial strains, pH values of media and temperatures of incubation (15, 18, 19, 20, 21, 22, 23, 24). Ferrini et al (2006) reported a modification of such methods with the addition of neutralizing chemical substances that further help to narrow the spectrum of antibiotic families detected by a single test plate (24).

In our institution which is also the Slovenian National Reference Laboratory for detection of antibiotics in food of animal origin we have been using microbiological screening methods since 1968. These methods had to be adapted to the requirements of the above mentioned Council Regulation 2377/90. The aim of our study was to identify the appropriate combination of test strains for detection of aminoglycoside, macrolide, tetracycline, quinolone and beta-lactame antibiotic families and to determine the limit of detection (LOD) for each family in view of the prescribed MRLs for antibiotic residues. We also attempted to improve the specificity of different bacterial strains for different antibiotic groups by the addition of two neutralizing substances: β-lactamase and Mg SO_4.

Material and methods

The principle of method

Microbiological methods are based on 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. These principles are followed in the STAR (screening test for antibiotic residue) protocol (22) on which our method is based.

Bacterial strains

In our research we used the following strains: Bacillus cereus ATCC 11778 (Remel, Lenexa, USA), Micrococcus luteus ATCC 9341 (Remel), Escherichia coli ATCC 10536 (TCS Bioscience, Buckingham, UK), Staphylococcus epidermidis ATCC 12228 (Remel) and Bacillus subtilis BGA(Merck, Darmstadt, Germany). For confirmation solutions we used β-lactamase (BioChemika, Seelze, Germany) at the concentration of 5 mg/ml and $MgSO_4$ (Sigma&Aldrich, Taufkirchen, Germany)

Antibiotics

Antibiotics used in the study are described in Table 1. The procedure or preparing the standard and working solutions are described in the section Preparation of working solutions and meat samples.

Table 1: Antibiotic standards

ANTIBIOTIC	PRODUCER	PURITY (%)
Chlortetracycline hydrochloride	Sigma – Aldrich/ Taufkirchen/Germany	83.0
Streptomycin sulfate	Sigma - Aldrich	95.8
Tetracycline hydrochloride	Sigma - Aldrich	97.3
Neomycin	Calbiochem/Darmstadt/Germany	67.8
Penicillin G potassium salt	Sigma-Aldrich	99.8
Cephalexin	Sigma - Aldrich	99.7
Enrofloxacin	Sigma - Aldrich	98.1
Ciprofloxacin	Sigma - Aldrich	98.5
Amoxicilin	Sigma - Aldrich	99.9
Tylosin	Sigma - Aldrich	90.8
Erythromycin	Sigma - Aldrich	99.1

Culture media

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.

Preparation of test plates

Bacterial strains stored as cultures in original bacterial loops (Culti loop) were applied to a test tube containing 1ml Trypton soy broth (Oxoid, Hampshire, UK) 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 and 8 °C for up to one month. Before the composition of test plates a suspension of bacterial culture stored on blood agar was prepared and incubated at 37 °C for one hour. Density of the suspension was standardized with the Mc Farland method (Table 2).

Test plates were prepared as described in Table 2. To prepare each test plate 0,45 ml of suspension of bacterial culture was added to 40 ml of basic medium heated to 40 °C. Kin plate was an exception

Table 2: Assay plates

plate	bacterial strain	cfu/ml of agar	agar medium	pH	incub. temp. (°C)	McFarland standard
AC	Micrococcus luteus ATCC 9341	$5.6x10^6$	Antibiotic medium No 1	6.0	30	4
ER	Staphylococcus epidermidis ATCC 12228	$1.9x10^7$	Antibiotic medium No 1	8.0	37	0.5
I-BGA	Bacillus subtilis BGA	$1.9x10^7$	Antibiotic medium No 2	6.0	30	0.5
KIN	Escherichia coli ATTC 10536	$1.6x10^7$	Antibiotic medium No 2	8.0	37	1
E	Bacillus cereus ATCC 11778	$6.6x10^6$	Antibiotic medium No 2	6.0	30	0.5

where 0,2 ml of suspension was added to 50 ml of medium. The mixture of medium and bacterial culture was poured into a 90 mm diameter Petri dish (5 ml of mixture into each Petri dish) and after 15 min at room temperature the Petri dishes with solidified medium were enveloped in a parafilm and stored in a fridge. The storage period of test plates was seven days. Before application of samples to test plates, plates were warmed at room temperature for 20 to 30 min.

Confirmation solutions

To confirm the presence of antibiotic groups or their individual representatives we used confirmation solutions. They inhibit the action of certain antibiotics and can help to distinguish antibiotic groups which cause inhibition zones on the same test plates. Magnesium sulphate ($MgSO_4$) was used to neutralize the aminoglicosides and β-lactamase enzyme to neutralize the β-lactams.

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 β-lactamase was added to samples on AC and I-BGA plates to identify cephalosporins.

Preparation of working solutions and meat samples

First standard antibiotic solutions had to be prepared from reference standard antibiotics of known chemical composition and purity (Table 1). Standard antibiotics in powder were dissolved in appropriate solvents: tetracyclines in phosphate buffer (Merck) with pH value 4,5, β-lactames in phosphate buffer with pH value 6,0, aminoglicosides in phosphate buffer with pH value 8,0, quinolones in pH 8 and macrolides in methanol (J.T. Baker, Deventer, Netherlands). Standard solutions were then diluted to desired concentrations with 1 mg/ml to create working solutions which were then added to meat samples as follows. One milliliter of working solution was added to 9g of minced beef formed into a sphere and left to diffuse throughout the meat. After 1 hour the meat sample with the disposed working solution was transferred to a 10-ml test tube and heated to 80°C for 5 min to avoid later non-specific reaction on test plates due to antagonizing micro flora in meat. This procedure was shown not to affect the concentration of antibiotic (25). After heating, the samples were compressed to obtain a liquid meat extract; 100ml of the extract were transferred to test plates in 8 mm wide cylinders. Detection levels were obtained by placing 100 µl meat extract obtained from meat sample containing the working solution with known concentration of antibiotic into cylinders. The range of working solutions is shown in table 2. For the evaluation of reversible concentrations, 75 µl of standard solutions were applied to cylinders with addition of 25 µl of confirmatory solution. Each concentration of antibiotics was tested 10 times.

Test plates AC, E, I-BGA were incubated at 30 °C and plate ER and Kin at 37 °C for 18-24 hours (20, 24).

Evaluation of results

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

Results

We have identified sensitive and resistant bacterial strains for all antibiotic groups tested in our study. 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 the tetracyclines group. For 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 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 β-lactam group 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. For quinolones group *Escherichia coli* ATCC 10536 (Kin plate) was chosen as the sensitive strain.

Table 3 shows the limits of detection for meat samples containing standardized antibiotic solutions on selected test plates and also the limits of detection for pure standard solutions. The LOD was

Table 3: Detection levels, MRL, range of working solutions and diameters of inhibitions zones of antibiotics

Antibiotic	Bacterial strain/plate	LOD st.s. (µg/kg)	LOD meat samples (µg/kg)	MRL bovine meat (µg/kg)	Range of working solutions (µg/kg)	Diameters of inhibitions zones (mm)
Cephalexin	M.l.1/ AC	50	50	200	50-250	11.87-19.75
Tetracycline	B.c/ E	30	50	100	50-150	17.23-22.0
Chlortetracycline	B.c/ E	40	50	100	50-150	18.65-22.5
Erythromycin	M.l.1/ AC	50	50	200	50-400	10.45->25
Tylosin	M.l.1/ AC	30	100	100	100-200	11.14-13.5
Neomycin	B.s.BGA/ IBGA	50	100	500	100-1000	9.91->25
Streptomyicin	B.s.BGA/ IBGA	80	100	500	100-1000	11.37->25
Penicillin	M.l.1/ AC	4	20	50	20-100	10.87-18
Amoxicilin	M.l.1/ AC	4	20	50	20-100	11.31-19.3
Enrofloxacin	E.c./KIN	20	50	100	50-200	10.17->25
Ciprofloxacin	E.c./KIN	20	30	100	30-200	11.35->25

LOD st.s limit of detection of standard solution
LOD meat limit of detection in meat samples
MRL meat maximum residue level in meat samples
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 4: Mean diameter of inhibition zone (mm), recovery and standard deviation (SD) for each inoculated antibiotic

	number of samples	conc. (µg/kg)	mean inhibition zone at the limit of detection (mm)	recovery (%)	SD of inhibition zone (mm)
Cephalexin	10	50	11.87	94	0.17
Tetracycline	10	50	17.23	76	0.40
Chlortetracycline	10	50	18.65	98	0.23
Erythromycin	10	50	10.45	82	0.31
Tylosin	10	100	11.14	84	0.14
Neomycin	10	100	9.91	85	0.36
Streptomycin	10	100	11.37	96	0.22
Penicillin	10	20	10.87	90	0.59
Amoxicilin	10	20	11.31	90	0.43
Enrofloxacin	10	50	10.17	92	0.80
Ciprofloxacin	10	30	11.35	87	0.67

at or below half the MRL for all tested antibiotics, both for meat samples and for standard solutions. Determination of LOD in standard solutions is essential to assess the influence of matrix (in our case meat) on the sensitivity of the method. Knowing the LOD both in standard solutions and in meat samples allows calculation of the recovery which is one of the measures of the reliability of the method.

Table 4 shows the recovery and standard deviation of the inhibition zones at the LOD for each tested antibiotic. For all antibiotics the recovery was above 80% which is the limit set by the Directive EEC657/2002 (26). The only exception where the recovery was 76% was tetracycline.

Discussion

Microbiological methods for detection of antibiotic residues in food are used in practically every laboratory in Europe involved in controlling food of animal origin. Although the method is widely know as the "four-plate method", many variations are used and most laboratories apply a specific approach with a different number and types of bacterial strains and therefore a different number of test plates (19, 21, 24). Methods using between one and eighteen 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, and, most importantly, differences in detection levels (27, 28, 29, 30).

Microbiological methods for detection of antibiotic residues in food are screening methods able to detect and differentiate only between antibiotic groups. Their results are used to minimize the number of chemical and immuno-enzymatic methods that are required to confirm the presence of antibiotics and identify specific substance within the antibiotic group (31, 32).

The sensitivity of a microbiological method must be high enough to allow detection of antibiotic residues level below the MRL prescribed in the current legislation (33). In some of the already published studies only working solutions were used instead of the real matrix investigated in routine analyses. This may lead to falsely low levels of detection. According to our experience the influence of matrix on the results should not be neglected, and this is especially true in the case of meat as a solid matrix whose preparation is especially troublesome. For this reason the recovery should always be calculated. In our method the values were within the values demanded by the Directive EEC 657/2002 (26) which confirms the reliability of our method. The standard deviations for inhibition zones are comparable with data reported by Ferrini et al(2006) and Myllyniemi et al(2001). Low values of standard deviations show the high accuracy of our method.

In several Scandinavian countries kidneys are used as the matrix from which the level of antibiotics in meat is assessed, because the concentration of antibiotics and therefore the MRL are higher in kidney tissue compared to meat. Using this approach a method that would otherwise have failed to reach MRL in muscle tissue was proved to be sensitive enough to detect antibiotic residues at half the MRL in kidney matrix and thus met the requirements of EEC 657/2002 (26). One of the problems of this approach is the rate of false positive results caused by natural inhibitors of bacterial growth such as lysozyme which are often present in kidneys. Ferrini et al (2006) managed to avoid their influence by placing a dialysis membrane between the growth media and the analyzed matrix (24). Despite some known advantages of analyses of kidney tissue, in Slovenia we opted for the use of meat in routine investigations.

The method developed in our study allows identification of b-lactame, aminoglicoside, macrolide, tetracycline and qionolone antibiotic groups at or below the MRL prescribed for meat. To improve the ability for differentiation between the listed antibiotic groups we used $MgSO_4$ which inhibits the action of tetracyclines, aminoglicosides and quinolones and enzyme b-lactamase which inactivates b-lactames but not macrolides which otherwise cause inhibition of growth on identical set of test plates. The drawback of b-lactamase is resistance of some synthetic b-lactames, for example newer generations of cephalosporins. A substance that would inactivate these groups of antibiotics would be an important step forward in the development of antimicrobiological screening methods.

Conclusion

The microbiological method described in this paper allows differentiation between five antibiotic groups and detection of antibiotic residues at or below the MRL prescribed for each group. The time required to perform the analysis is short (be-

tween 18 and 24 hours) and no expensive equipment is needed therefore the cost of investigation is relatively low. The drawbacks of the method are that any positive result must be confirmed by chemical methods and that the results are difficult to interpret quantitatively because a separate calibration curve would have to be constructed for each antibiotic. Altogether we consider this method as an appropriate and highly efficient screening method for detection of antibiotic residues in meat, especially for monitoring purposes where a high number of samples must be investigated for a high number of different antibiotics.

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MIKROBIOLOŠKA PRESEJALNA METODA ZA UGOTAVLJANJE AMINOGLIKOZIDNIH, β-LAKTAMSKIH, MAKROLIDNIH ANTIBIOTIKOV TER TETRACIKLINOV IN KINOLONOV V MESU

A. Kirbiš

Povzetek: Antibiotik je po definiciji naravni produkt mikroorganizmov ali naravnemu produktu enaka sintetična ali podobna polsintetična spojina, ki zavira razmnoževanje drugih mikroorganizmov in deluje bakteriostatično ali pa bakterije ubija, kar imenujemo baktericidni učinek. V veterinarski medicini se antibiotiki uporabljajo v terapevtske, profilaktične, metafilaktične in nutritivne namene. Ostanki antibiotikov in njihovi metaboliti v živilih so lahko nevarni za zdravje ljudi, saj lahko povzročajo alergijske reakcije oziroma vplivajo na nastanek odpornosti pri mikroorganizmih, povzročajo pa tudi gospodarsko škodo, saj delujejo zaviralno na štarterske kulture. 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. Meja detekcije metode za posamezne skupine antibiotikov mora biti vsaj na meji MRL vrednosti oziroma pod njo. Rezultati, ki jih dobimo z zanesljivo presejalno metodo, so zelo dobra orientacija za potrjevalne kemijske metode, ki so zamudne in zelo drage.

Namen raziskave je bil uvedba mikrobiološke metode za ugotavljanje antibiotikov s testiranjem in uvedbo testnih sevov bakterij in ugotoviti minimalno količino antibiotikov, ki jih je s posamezno metodo mogoče ugotoviti. Določili smo občutljive in odporne bakterijske seve za skupine makrolidnih, aminoglikozidnih in β-laktamskih antibiotikov kakor tudi kinolonov in tetraciklinov v mesu. Za ugotavljanje β-laktamskih in makrolidnih antibiotikov uporabljamo bakterijski sev *Micrococcus luteus* ATCC 9341 kot občutljivi sev, za aminoglikozidne antibiotike bakterijski sev *Bacillus subtilis* BGA , za tetracikline *Bacillus cereus* ATCC 11778 ter za kinolone *E.coli* ATCC 10536. Pri poskusu smo uporabili potrditvene spojine, in sicer magnezijev sulfat, ki inaktivira aminoglikozidne antibiotike, ter predstavnike kinolonov in tetraciklinov, pa tudi encim β-laktamaza, ki inaktivira delovanje β-laktamskih antibiotikov. Rezultati, ki smo jih dobili, so pod MRL vrednostmi za posamezne predstavnike antibiotikov.

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