Slov Vet Res 2004; 41 (3/4): 129-135
UDC 619:614.31:615.33:637.5

Original Research Paper

INTRODUCTION AND MODIFICATION OF A MICROBIOLOGICAL METHOD FOR IDENTIFYING FLUMEQUINE IN MEAT

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Summary: In this study a bacteriological method for identifying flumequine residues in the meat of slaughtered animals was modified. We introduced a quantitative evaluation of the results by adding a lyophilization step to the existing bacteriological method, which made the method more sensitive. Our results proved that *E. coli* ATCC 25922 is susceptible to the antibiotic fluoroquinolone, which was already evident according to available literature. Due to the current regulations that prescribe maximum residue levels for antibiotic residues in food, we needed to modify our existing bacteriological method as the results it produced were not sensitive enough to identify the maximum residue levels in cattle and pig meat. We added lyophilization to the process, which we believe is the first time this has been attempted as no evidence of this procedure being used in this manner was found in the literature available. This modified method can be used for the quantitative as well as for the qualitative identification of flumequine.

Key words: methods; antibiotics; flumequine; residues; bacteriological technique; meat

Introduction

In both veterinary and human medicine, antibiotics have therapeutic, prophylactic, metaphylactic and/or nutritive applications (1, 21, 22). Flumequine is member of the fluoroquinolones class of antibiotics and is one of the most commonly prescribed antibiotics in veterinary medicine (2, 7, 8). Fluoroquinolones display bactericidal activity by inhibiting bacterial enzyme deoxyribonucleic acid (DNA) gyrase, which is vital for bacterial DNA duplication (10, 21, 23).

Fluoroquinolones are bactericidal against Gram-negative bacteria – *E. coli*, *Proteus, Shigella spp., Campylobacter jejuni, Pseudomonas aeruginosa*, Gram-positive bacteria – *Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis*, anaerobes – *Clostridium spp., Bacteroides fragilis* and against *M. tuberculosis and Mycoplasma pneumoniae* (19, 7, 8).

Flumequine is registered in Slovenia for use in veterinary medicine and its residues in food can be identified with microbiological, chemical and immunoenzyme methods (6, 11). At the Institute for Food Hygiene, we have used a bacteriological method for identifying antibiotic residues in food of animal origin since 1968. The method relies on the fact that antibiotic activity on certain bacterial strains produces inhibitory growth zones. It was used at the Institute to identify flumequine residues in food of animal origin until 11.5.2000, when the old regulation (17) that encapsulated a zero-tolerance policy towards antibiotics in food was replaced by EU directives (4, 5) that prescribe maximum residue levels (MRL) for antibiotic residues in food.

Therefore, we needed to quantify the levels of fluoroquinolone residues in food of animal origin in order to identify whether they were within the prescribed MRL. Hence, we needed to modify our bacteriological method.

Received: October, 2004
Accepted for publication: November, 2004

Materials and methods

The zones of inhibition found on the plates used as media to grow the different bacterial strains were measured. Two different bacterial test strains were used, one fully susceptible and the other resistant to the antibiotic being tested for. The width of the inhibition zone was entered in a calibration curve and the appropriate concentration of antibiotic was read off. A negative result on the plate seeded with the resistant bacterial strain was used to confirm the presence of the antibiotic.

Most laboratories use *Escherichia coli* to detect fluoroquinolone residues in food (3, 14, 15, 16, 9). In this study, we used *E. coli* ATCC 25922, which had been imported from the United States of America.

Preparation of bacterial strains

Lyophilized bacterial strains were rehydrated by adding enriched bujona and 0.9 % NaCl, multiplied by a bacteriological loop before being transferred onto slant agar and then put in a thermostat for 24 hours at 37 °C. The purity of the strain was confirmed by Gram staining.

Bacterial strains were made viable for 10-14 days at 2-8 °C by using this method.

Preparation of media

An antibiotic seed agar A1 (code 1075 Biolife, Lot 1E5601), of pH 6.5, was used as the basic media for the test plates and was put into inclined tubes to obtain slant agars, which were then seeded with the bacterial strains and stored in a refrigerator at 2-8 °C.

Preparation of the plates

Ten millilitres of the basic media, heated to approximately 50 °C, was poured onto Petri dishes and left to cool and solidify. Then 5 ml of a sterile saline solution was transferred by pipette into the slant agar to obtain a suspension, 4 ml of which was then poured onto the basic media after it had cooled and solidified. The plates were then stored in a refrigerator at 2-8°C and were valid for five days.

Growth density

The growth density on the plates seeded with the *E. coli* 25922 tested strain was evaluated at the beginning of the experiment.

Different concentrations of the bacterial strains were transferred onto the media and kept at 37 °C for 24 hours. After optimal growth, the plates were evaluated with reference standard flumequine (Sigma; catalogue number F-7016).

Calibration curve

A calibration curve was developed from standardized solutions of the antibiotic, which were made by weighing and dissolving standard measures after considering their purity and chemical form.

The basic standardized solutions of flumequine were made using 0.1 M of NaOH as a solvent and the working solutions of flumequine were made by further mixing the standardized solutions with a buffer solution with a pH of 6.

Working concentrations for testing the *E. coli* susceptibility to flumequine ranged from 200 mg/l to 1200 mg/l to correspond with the limits set for flumequine in beef and pork (200 mg/l), poultry (400 mg/kg) and fish (600 mg/kg) (4, 5).

Evaluation of results

The results were evaluated in both a qualitative and quantitative manner. The quantitative assessments were made by using the calibration curve. Working solutions of five different decreasing concentrations – 1000, 800, 600, 400, 300 µg/kg (a down to e), were prepared. The 600-µg/kg (c) concentration was used as the reference. The different concentrations were made using a buffer solution with a pH of 6.

Each concentration was poured onto the 9-welled plates containing the bacterial culture. This was done nine times, except for the reference solution, which was poured 36 times.

The different antibiotic solutions, as well as the reference solution, were added to each plate three times and three parallels were made, which means that three plates were used for each concentration.

The plates were placed in an incubator set at 37 °C for 18-24 hours and then the width of the inhibition zone of the bacterial strain for each concentration was measured using an electronic

Table 1: The width of the inhibition zones in the presence of different concentrations of flumequine (in mm)

N	600µg/kg (c)	300µg/kg (e)
1	12.3	10.3
2	11.7	10.4
3	12.3	10.3
4	12.4	10.4
5	12.4	10.2
6	12.4	10.3
7	12.4	10.3
8	12.5	10.1
9	12.4	10.2
average	12.3	10.3

N	600µg/kg (c)	400µg/kg (d)
1	12.4	11.0
2	12.5	11.5
3	12.4	10.8
4	12.4	11.0
5	12.4	11.0
6	11.9	10.9
7	12.3	10.9
8	12.3	10.9
9	12.3	11.3
average	12.3	11.0

N	600µg/kg (c)	800µg/kg (b)
1	12.6	14.3
2	12.4	14.4
3	12.3	14.5
4	12.4	14.3
5	12.5	13.9
6	11.7	14.2
7	11.9	14.2
8	12.3	14.1
9	12.1	14.3
average	12.2	14.2

N	600µg/kg (c)	1000µg/kg (a)
1	12.3	15.2
2	12.6	15.6
3	12.2	15.1
4	11.9	15.1
5	12.1	15.1
6	12.4	15.2
7	12.3	15.2
8	11.9	15.1
9	12.1	15.2
average	12.2	15.2

N = number of measurements
average = average value

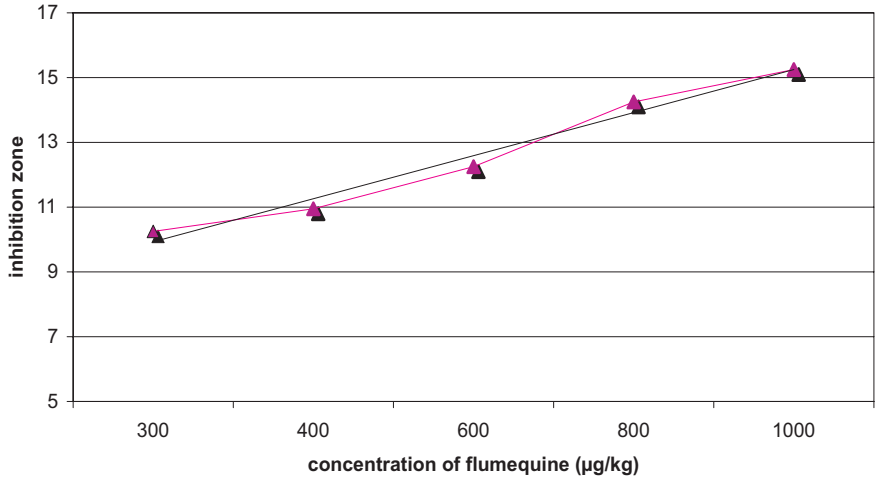

Figure 1: Calibration curve for flumequine
Axis x = concentration of flumequine (µg/kg)
Axis y = inhibition zone (mm)

movable tape (Stainless Hardened). The average values were calculated and the average value of the reference concentration was used to determine standard deviation. For each concentration of antibiotic, nine measurements were obtained and the average width of the zone was calculated for each concentration (Table 1).

The high point (HP) was calculated from the calibration curve (Equation 1):

Equation 1: $$HP \quad \frac{3a \quad 2b \quad c \quad e}{5}$$

In the same manner, the low point (LP) was also calculated from the calibration curve (Equation 2).

Equation 2: $$LP \quad \frac{3e \quad 2d \quad c \quad a}{5}$$

The calculations established the HP as being 15.25 mm and the LP as 9.95 mm.

The data was recorded and a trend curve was developed.

The trend curve is linear and the concentration of the tested antibiotic can be determined by the width of the inhibition zone.

Correction factor

A correction factor was used to modify the results. As microorganisms are living organisms their growth is never uniform; therefore, the size of the inhibition zones of the reference concentrations on each of the plates was never the same. With a correction factor we were able to get more accurate results taking into account the differences between each plate. The correction factor was determined using Equation 3:

Equation 3: $$X \quad \frac{C_{meas}}{C_{ref}} \quad 100$$

X = correction factor
C meas = measured antibiotic concentration
C ref = reference antibiotic concentration

The correction factor gives the percentage of the reference concentration that is obtained by testing each of the plates with the addition of the reference concentration of the same antibiotic. It is used when the results of food-sample analyses for the presence of antibiotic residues are interpreted in a qualitative manner.

The results can also be interpreted quantitatively, as follows:

The width of the inhibition zone is measured and the appropriate concentration of the antibiotic is read off the calibration curve. Before results are interpreted they must be massaged by the correction factor.

Example:

A correction factor of 80 means that when the reference concentration of a tested antibiotic is poured onto the media only 80 % of the poured antibiotic is identified.

$$Ca \quad \frac{C_{od} \quad 100}{X}$$

X = correction factor (for example 80)
C od = concentration read off the calibration curve
C a = antibiotic concentration in sample

Analysis of the samples

Meat from cattle, pigs, poultry and fish with known concentrations of the inoculated flumequine were analysed. Twenty grams of 0.5-cm chunks of meat were put into sterilized bags and then three times that amount of buffer, with a pH of 6, was added to assist in the flumequine identification.

A working solution of the antibiotic concentration was then added and mixed in a stomacher for 3 minutes. The mixture was then transferred into test tubes and heated for 5 minutes at 80 °C to inactivate any naturally occurring inhibitory substances and bacteria in the meat and exclude non-specific reactions on the plates. The mixture was then cooled and centrifuged for 10 minutes at 2500-3000 revolution per minute.

The upper layer was transferred into a clean test tube and a pipette was used to transfer 100 ml into an 8-mm cylinder. The plates were then put in an incubator for 18-24 hours at 37 °C.

Modifying the bacteriological method for identifying flumequine

The results we obtained using the bacteriological method to establish MRL values were not satisfactory, as our limit for identifying the flumequine residues was higher than the MRL.

As a result we modified the method with the addition of lyophilization. Samples with known concentrations of fluoroquinolone were lyophilized and rehydrated with a small amount of water. In this way higher concentrations of fluoroquinolone in the matrix were obtained.

Preparation of samples for lyophilization

Samples of muscle tissue were weighed and the prescribed concentrations of antibiotic were added. The samples were then homogenized for three minutes before being transferred into test tubes and centrifuged for 10 minutes at 2500-3000 rpm. Then approximately 2 ml of each sample was transferred into a rubber-sealed glass flask, closed and put into a lyophilizer.

Lyophilization

A DW6 freeze-dryer from Heto was used for the lyophilization, which took 24 hours and reduced

Table 2: Identification of predetermined concentrations of flumequine in beef and pork

	Concentration (µg/kg)							
N = 15	200	250	300	350	400	450	500	600
Beef Number of positive samples (%)	0 (0)	0 (0)	3 (20)	6 (40)	11 (73.3)	14 (93.3)	15 (100)	15 (100)
Pork Number of positive samples (%)	0 (0)	0 (0)	2 (13.3)	5 (33.3)	11 (73.3)	12 (80)	15 (100)	15 (100)

N = number of samples examined from each source

Table 3: Identification of predetermined concentrations of flumequine in poultry

	Concentration (µg/kg)							
N = 15	250	300	350	400	450	500	550	600
Number of positive samples (%)	0 (0)	6 (40)	8 (53.3)	12 (80)	14 (93.3)	15 (100)	15 (100)	15 (100)

N = number of samples examined

Table 4: Identification of predetermined concentrations of flumequine in fish

	Concentration (µg/kg)							
N = 15	400	450	500	550	600	650	700	800
Number of positive samples (%)	11 (73.3)	12 (80)	15 (100)	15 (100)	15 (100)	15 (100)	15 (100)	15 (100)

N = number of samples examined

the samples to 10-15 percent of their original mass on average.

Rehydration

The freeze-dried samples were then weighed and the weight difference was used to calculate the amount of water that needed to be added to obtain higher concentrations of the antibiotic.

Results

Examination of the samples

The samples of cattle, pig, poultry and fish meat with known concentrations of flumequine were analysed and the influence of the matrix on the sensitivity of the method was also examined.

We tested for the presence of flumequine residues that ranged from 1 to 3 times the MRL for beef and pork (200-600 µg/kg), from 0.625 to 1.5 times the MRL for poultry (250-600 µg/kg) and from 0.66 to 1.33 times the MRL for fish (400-800 µg/kg).

All the samples examined were positive (100 %) at concentrations of 500 µg/kg (Tables 2, 3 and 4), which is too high as the MRL for flumequine in beef and pork is only 200 µg/kg and 400 µg/kg in poultry. However, it is satisfactory for identifying flumequine residues in fish as the MRL for flumequine in fish is 600 µg/kg.

Modification of the microbiological method

The microbiological method was modified by lyophilizing the samples, which were then rehydrated to the appropriate concentration levels. This allowed the actual concentration of flumequine to be multiplied as many times as was needed to get the concentration of 800 µg/kg. Samples were then transferred onto the plates.

Table 5: Susceptibility of the bacterial strains after lyophilization

Lyophilized to 800 µg/kg	Concentration (µg/kg) in samples				
N = 10	100	150	200	250	300
Beef Number of positive samples (%)	8 (80)	10 (100)	10 (100)	10 (100)	10 (100)
Pork Number of positive samples (%)	8 (80)	10 (100)	10 (100)	10 (100)	10 (100)
Poultry (muscle tissue) Number of positive samples (%)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)

N = number of samples examined

The procedures used to prepare, lyophilize, rehydrate and transfer the samples to the plates are described in the Materials and methods section.

Discussion

In this study, we used the reference strain *E. coli* ATCC 25922 and observed its growth under different concentrations of flumequine in media. When an appropriate dilution was found we continued with the procedure. Using the same antibiotic seed agar medium as was used in other studies (3, 12, 15, 16), we identified 10^7 colonies per plate, which is comparable to results already described in literature (3, 12, 15). The method used to prepare the plates, the pH of the medium and the incubation procedures were the same as those described by Okerman (16).

We had some difficulties obtaining the MRL values for flumequine residues in pork and beef (200 µg/kg) and also, to some extent, with poultry (400 µg/kg). Even though we were able to determine the MRL values for flumequine residues in poultry, we were only partially successful (80 %). However, we identified all (100 %) of the flumequine residues in fish where the MRL value is 600 µg/kg.

The calibration curve for flumequine was developed using the same method as is used for other antibiotics. The concentrations of flumequine residues in the matrix were calculated from the calibration curve, which included a correction factor, and the growth-inhibition zones of the bacterial strains.

As we were unable to obtain the MRL values for flumequine residues in pork and beef, we modified the microbiological method by adding a lyophilization step. We were unable to find any reference to lyophilization as a step in the identification methods for lower concentrations of antibiotics in any available literature.

Our results were within MRL limits; therefore, our lyophilization-enhanced microbiological method is suitable for use in everyday practice.

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UVEDBA IN MODIFIKACIJA MIKROBIOLOŠKE METODE ZA UGOTAVLJANJE FLUMEKVINA V MESU

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Povzetek: Z raziskavo smo želeli razširiti analitiko ostankov antibiotikov v mišičnem tkivu klavnih živali. V ta namen smo uvedli in modificirali bakteriološko metodo za ugotavljanje flumekvina.Metodo smo modificirali na ta način, da smo kvalitativnemu vrednotenju dodali še kvantitativno vrednotenje rezultatov, in sicer liofilizacijo vzorcev. Na ta način smo bistveno izboljšali občutljivost metode.Rezultati so potrdili dejstvo, ki smo ga povzeli po literaturi, da je bakterijski sev E. coli ATCC 25922 dejansko občutljiv za predstavnike fluorokinolonov. Ta občutljivost pa glede na to, da obstajajo najvišje dopustne meje vsebnosti ostankov antibiotikov v živilih (MRL, maximum residue level), ni bila povsod zadostna. Naleteli smo na težave pri doseganju MRL v mišičnih tkivih prašičev in goveda. To je tudi bil razlog, da smo metodo modificirali. Modifikacijo metode z uvedbo liofilizacije ekstraktov vzorcev prištevamo k originalnosti raziskave, saj v literaturi nismo zasledili podobnih prijemov za izboljšanje občutljivosti metode. Zato tako modificirano metodo lahko uporabljamo tako za kvali- kakor tudi za kvantitativno ugotavljanje flumekvina.

Ključne besede: metode; antibiotiki; flumekvin; zdravila; bakteriološka tehnika; meso