Detection of enrofloxacin residues in chicken meat by microbiological (growth inhibition test) and ELISA method after experimental prophylactic and therapeutic application

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Scientific paper

Summary

The paper presents detection results of enrofloxacin residues in muscle tissue and liver tissue of broiler chickens after the experimental application of prophylactic and therapeutic doses of the investigated drug. Two methods were used for the detection of enrofloxacin residues in muscle and liver tissue, and they are: microbiological method (growth inhibition test) and ELISA test. The aim of this research was to examine the reliability of the microbiological growth inhibition test (diffusion method) with the application of reference strain E. coli ATCC 10 536 as the microorganism- test for the detection of enrofloxacin residues in broiler meat and to compare the applied methods. By using phi correlation coefficient it was determined that there is a statistically very significant positive correlation ($p << 0.001$) between the data of the microbiological and ELISA method and in samples of muscle tissue and liver tissue. There was also determined that, in experimental conditions, both microbiological and ELISA method achieve equally positive results in the detection of the allowed quantities of enrofloxacin residues, although they are different measures (mm or ppb) of the same phenomenon.

Key words: *enrofloxacin, growth inhibition test, ELISA*

Introduction

The application of enrofloxacin, as one of the representatives of fluoroquinolones, has increased noticeably in the last ten years, equally in human and veterinary medicine. At the same time, the growth of resistance of pathogens has been noticed, especially of those which cause illnesses in people and which are transmitted by food, such as *Salmonella* spp. and *Campylobacter* spp. (Giguère et al., 2007).

Analysis of veterinary drug residues in products of animal origin has been developing intensively in the last years and it represents a new trend from the aspect of food safety. This development has been moving in the direction of finding quicker and more sensitive screening methods for the detection of residues in products of animal origin, and more sophisticated methods for the confirmation of results obtained by screening tests (De Brabander et al., 2009). Maximum residue limits of enrofloxacin in the EU are regulated in Table 1. of the Annex to Regulation 37/2010 (EC, 2010) and they are 100 µg/ kg for muscle tissue and 200 µg/ kg for liver tissue.

Enrofloxacin is a synthetic chemotherapeutic agent from the group of fluoroquinolones. Fluoroquinolones belong to the remaining three generations (II, III, and IV) of quinolones. When they appeared in clinical practice during the 80s of the last century, they were considered to be an almost ideal antimicrobial preparation because of their strength and width of antimicrobial spectrum of activity (Giguère et al., 2007). Enrofloxacin, together with ciprofloxacin, orbifloxacin, difloxacin, danofloxacin, marbofloxacin, sarafloxacin and norfloxacin belongs to the second generation of antimicrobial drugs. Enrofloxacin inhibits the function of two enzymes, topoisomerase II and topoisomerase IV. Topoisomerase II

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(DNA- gyrase) is responsible for DNA replication (secondary spiralization of linear double DNA helix) in the core of a bacterial cell (Bonagura, 2000; Boothe, 2001). It is a process which is necessary for grouping bacterial chromosomes within the cell (Dollery, 1999). In gram-negative causative agents, enrofloxacin inhibits its enzyme DNA- gyrase by binding to its subunit A, which also disables the synthesis of mRNK (Bonagura, 2000; Boothe, 2001). Enrofloxacin, the same as other fluoroquinolones, has a wide spectrum of activity which is shown in far lesser concentrations in comparison to traditional antibiotics, such as penicillins, cephalosporins, tetracyclines, macrolides, and even sulfonamides (Rivier and Papich, 2009).

Enrofloxacin is taken orally (with food, milk replacement, or in drinking water) and parenterally (s/c or i/m). After resorption, the same as other fluoroquinolones, it is distributed well and fast through all tissues of the organism because of its expressive lipophilicity and low ionization (Prescott, J. F., 2000). Enrofloxacin is metabolized to the active metabolite ciprofloxacin, which is also used in therapy (Anadon, 1995).

Material and methods

The research was conducted on the total of 210 broilers divided to five groups: the prophylactic group (P) and four test groups (G1, G2, G3 and G4). Each group consisted of 42 broilers of equal body weight. Chickens were put into five boxes prepared within the production facility of the farm. During the entire fattening period, chickens were fed *ad libitum* (feeders), whereas nipple drinking system was used for drinking. Fattening lasted for 42 days. During the experimental period, chickens were fed three kinds of feed. Starter feed (PT-1) was used from day 0 to 14, grover from day 15 to day 35, and finisher (PT-3) from day 36 to 42nd day of fattening. Immunoprophylactic procedure was conducted with all experimental groups, i.e. vaccination of broilers according to the determined program of immunoprophylaxis. Vetoflok® 10% powder ("Veterina" Ltd., Kalinovica, Croatia) was used for the experimental application of the drug, and dosage regime and preparation of the applied drug was made according to the producer's recommendation. The data on the applied enrofloxacin concentrations in food during the experiment are presented in Table 1.

Table 1. **Schedule of enrofloxacin application during the experiment**

Experimental group	Time of drug application	Drug concentration in ppm	Number of animals per group
Prophylactic group (P)	1) Giving animals a prophylactic dose in the period from day 1 to day 7 of fattening	60 ppm	42
Group 1 (G1)	1) Giving animals a prophylactic dose in the period from day 1 to day 7 of fattening 2) Giving animals a therapeutic dose in the period from day 25 to day 31 of fattening	100 ppm	42
Group 2 (G2)	1) Giving animals a prophylactic dose in the period from day 1 to day 7 of fattening 2)Giving animals a 2x higher dosage in relation to the therapeutic dose in the period from day 25 to day 31 of fattening	200 ppm	42
Group 3 (G3)	1) Giving animals a prophylactic dose in the period from day 1 to day 7 of fattening 2) Giving animals a therapeutic dose in the period from day 34 to day 41 of fattening	130 ppm	42
Group 4 (G4)	1) Giving animals a prophylactic dose in the period from day 1 to day 7 of fattening 2) Giving animals a 2x higher dosage in relation to the therapeutic dose in the period from day 34 to day 41 of fattening	260 ppm	42

In the last 8 days of fattening, i.e. from the 34th day, there were separated 4 broilers from each group and they were transported to the abattoir in separate and clearly marked boxes. After the slaughter, carcasses and livers were packed separately into plastic bags marked by the mark of the group, day of slaughter and number of the sample. Samples were delivered to the laboratory of the Department of Food Hygiene and Technology, Veterinary Faculty in Sarajevo, immediately after the slaughter. Carcasses and livers from three experimental animals were used for the analysis, and the carcass and liver from the fourth animal were stored as a spare sample. Broiler carcasses intended for the analysis were separated to three parts and stored at minimum temperature of -20°C until the beginning of analyses, i.e. until the end of slaughtering all animals.

Microbiological method (growth inhibition test – "one plate" method)

The method was performed according to Kirbiš (2007). As a microorganism test for the detection of

Table 2. **Results of the detection of enrofloxacin residues by the method of microbiological test of growth inhibition (growth inhibition zone diameter in mm) in broiler muscle tissue per days of slaughter. LOD of the microbiological test = 50 ppb. The size of growth inhibition zone of test microorganism (positive control) was 13 mm, which corresponds to the MRL value for poultry muscle tissue of 100 ppb.**

Mark of experimental group	Mark of sample	Day of slaughter							
		1	2	3	4	5	6	7	8
P	**1**	< 13	< 13	< 13	< 13	< 13	< 13	< 13	< 13
	2	< 13	< 13	< 13	< 13	< 13	< 13	< 13	< 13
	3	< 13	< 13	< 13	< 13	< 13	< 13	< 13	< 13
G1	**1**	< 13	< 13	< 13	< 13	< 13	< 13	< 13	< 13
	2	< 13	< 13	< 13	< 13	< 13	< 13	< 13	< 13
	3	< 13	< 13	< 13	< 13	< 13	< 13	< 13	< 13
G2	**1**	< 13	< 13	< 13	< 13	< 13	< 13	< 13	< 13
	2	< 13	< 13	< 13	< 13	< 13	< 13	< 13	< 13
	3	< 13	< 13	< 13	< 13	< 13	< 13	< 13	< 13
G3	**1**	28	35	32	30	28	30	29	28
	2	26	34	33	32	39	32	28	33
	3	30	33	34	32	30	30	30	30
G4	**1**	34	40	38	39	40	38	40	36
	2	34	39	37	35	38	35	34	40
	3	32	38	35	38	38	37	35	38

Table 3. **Analysis results of detection of enrofloxacin residues by microbiological test (growth inhibition zone diameter in mm) in liver* of experimental animals per days of slaughter; LOD of the microbiological test = 50 ppb. The size of growth inhibition zone of test microorganism (positive control) was 22 mm, which corresponds to the value of MRL for poultry muscle tissue of 200 ppb.**

Mark of experimental group	Mark of sample	Days of slaughter							
		I	II	III	IV	V	VI	VII	VIII
P*	**1**	<22	<22	<22	<22	<22	<22	<22	<22
	2								
	3								
G1*	**1**	<22	<22	<22	<22	<22	<22	<22	<22
	2								
	3								
G2*	**1**	<22	<22	<22	<22	<22	<22	<22	<22
	2								
	3								
G3*	**1**	**30**	**32**	**31**	**32**	**32**	**30**	**31**	**38**
	2								
	3								
G4*	**1**	**42**	**43**	**45**	**45**	38	42	45	43
	2								
	3								

* value for collective sample of 3 broiler livers from the same experimental GROUP

enrofloxacin residues there was used a reference strain E. coli ATCC 10 536 which was previously enriched in ml Tryptone Soya Broth (Oxoid, UK), incubated at 37°C during the period of 1 hour and inoculated on blood agar and incubated for the next 16 hours at 37°C. Exclusively fresh and pure cultures of E. coli ATCC 10 536 prepared at the explained way were used for the test. Agar for the detection of antibiotic No. 2 (Merck, Germany) was used as a medium for the preparation of test plates. The agar prepared according to the producer's instructions (pH=8.0) was autoclaved at the temperature of 121°C during the period of 15 minutes and cooled at 40°C. E. coli ATCC 10 536 suspension was prepared in a physiological solution of 1 McFarland density which corresponds to 1.6 x 10^7 cfu/ml of agar. Out of that, 0.2 ml was added in 50 ml of previ-

Table 4. **Detection results of enrofloxacin residues by the ELISA method (ppb* ± SD) in muscle tissue of experimental animals per days of slaughter. LOD of the ELISA method = 3.5 ppb; bold values are higher than MRL for poultry muscle tissue (>100 ppb).**

Mark of experimental group	Mark of sample	Days of slaughter							
		1	2	3	4	5	6	7	8
P*	1 2 3	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5
G1*	1 2 3	24.26 ±1.0	20.15 ±3.13	8.22 ±1.08	6.5 ±0.95	3.39 ±0.37	<3.5	<3.5	<3.5
G2*	1 2 3	28.40 ±1.32	24.15 ±0.53	12.43 ±0.41	7.36 ±3.47	4.69 ±0.17	<3.5	<3.5	<3.5
G3*	1 2 3	258.27 ±23.93	259.47 ±26.74	309.13 ±40.85	290.72 ±20.05	249.79 ±11.93	282.28 ±22.42	328.76 ±21.5	390.43 ±7.25
G4*	1 2 3	486.95 ±75.51	592.30 ±71.13	493.99 ±39.80	551.85 ±27.34	663.62 ±31.84	457.91 ±77.72	578.33 ±29.95	709.75 ±50.46

* - mean value from 3 samples of muscle tissue

Table 5. **Detection results of enrofloxacin residues by the ELISA method (ppb) in liver of experimental animals per days of slaughter; LOD of the ELISA method = 3.5 ppb; bold values are higher than MRL for poultry liver (> 200 ppb).**

Mark of experimental group	Mark of sample	Days of slaughter							
		1	2	3	4	5	6	7	8
P*	1 2 3	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5	<3.5
G1*	1 2 3	72.53	52.43	45.09	31.84	28.78	25.76	17.68	13.79
G2*	1 2 3	240.40	109.47	81.77	64.50	29.18	21.09	19.52	15.88
G3*	1 2 3	700.78	888.41	881.18	845.87	648.67	567.98	683.83	773.09
G4*	1 2 3	988.07	1023.72	1060.65	1009.86	745.85	820.34	940.76	993.50

* value for collective sample of 3 broiler livers from the same experimental group

ously prepared and cooled agar for the detection of antibiotic No. 2. After that, 10 ml of the prepared examining medium was put in Petri dishes of 90 mm diameter. Mean value of growth inhibition zone for the concentration of 100 ppb was 13 mm, and 22 mm for the concentration of 200 ppb. 100 µl of examining medium, i.e. extracted fluid of meat was applied to test plates with previously made concavities. The plates were incubated at 37°C for 18 to 24 hours. A positive control with enrofloxacin standards (Sigma Aldrich, Germany) was made in each test plate in a concentration of 100 ppb for muscle tissue and 200 ppb for liver. Growth inhibition zones (in mm) were measured in positive control and examining samples after incubation.

ELISA test for enrofloxacin detection

For the detection of enrofloxacin residues in chicken muscle and liver by the ELISA method there were used commercial ELISA kits (MaxSignal®-Enrofloxacin ELISA Test Kit, Bioo Scientific, USA) according to the producer's instruction. ELISA reader (IDEXX, USA) was used for reading at 450 nm wavelength. The interpretation of results was done by using BIOO MaxSignal® ELISA Analysis Program (Bioo Scientific, USA) identification software.

Statistical analysis of sample analysis results was performed by applying SPSS 17 Statistical Package for Social Sciences. A calculation of phi correlation coefficient (j or r_j) was used for determining the degree of data association obtained by one, that is, the other method.

Results and Discussion

Correlation of results on researching enrofloxacin residues in muscle tissue analyzed by the method of microbiological test are presented in Table 2. The analyzed samples from the prophylactic group (P) and groups G1 and G2 were negative during all eight days of slaughter, whereas growth inhibition zones in all of the analyzed samples from group G3 were higher than 13 mm, so they were considered to be positive samples. Growth inhibition zones in samples from group G3, where the animals were given therapeutic enrofloxacin dose, were in the range from 26 to 39 mm, whereas in group G4, where the animals were given a 2x higher dose in relation to the therapeutic dose, growth inhibition zones were even more expressed and were in the range of 32 to 40 mm.

Table 3. presents the readings of growth inhibition zones in the analyzed liver samples of experimental animals. These results show a similar

trend as in the samples of muscle tissue where all the analyzed samples from the prophylactic group (P) and groups G1 and G2 were negative, i.e. concentrations of enrofloxacin residues were below MRL. Growth inhibition zones for all the analyzed liver samples from groups G3 and G4 during all days of slaughter were over 22 mm and were in the range from 30 to 38 mm for the group G3 and 38 to 45 mm for the group G4.

Detection results of enrofloxacin residues by the ELISA method in muscle tissue (Table 4.) show that all the samples from the prophylactic group (P) were negative during all days of slaughter and that concentrations of enrofloxacin residues were below detection level (< 3.5 ppb). Concentration of enrofloxacin residues in samples of muscle tissue taken on the first day of slaughter from the experimental group G1 were below MRL and were in the range from 24.26±1.0 ppb, and in samples taken from the first to the eight day of slaughter a trend of a constant decrease of enrofloxacin concentration can be noticed. In the same experimental group there has been noticed a sudden decrease in enrofloxacin concentrations in samples of liver taken on the third day of slaughter, whereas these concentrations in samples taken from the sixth to the eight day of slaughter were below detection limit (Graph 3.). Detection levels of residues in samples from group G2 on the first day of slaughter were somewhat higher in relation to the same samples from the group G1, but they were still lower in relation to MRL and they were in the range from 28.40±1.32 ppb. In this experimental group too there has been noticed a trend of constant decrease in concentrations of enrofloxacin residues during the following days of slaughter and, similar as with the experimental group G1, there has been noticed a sudden decrease in concentrations on the third day of slaughter. From the sixth to the eight day of slaughter there were recorded enrofloxacin concentrations below the detection limit (Graph 1.). Results of enrofloxacin detection for the experimental groups G3 and G4 show that the analyzed samples from both groups were positive on the first day of slaughter already. i.e. that enrofloxacin concentrations were far over the maximum allowed limit. Residue concentrations of the examining antibiotic in samples of the experimental group G3 on the first day of slaughter were from 258.27±23.93 ppb. During the following seven days of slaughter the level of enrofloxacin residues in samples from the experimental group G3 was constant with somewhat slighter oscillations of the marked concentrations (Graph 2.). From the second to the third day of slaughter, a slight increase in concentrations is noticed, following by a slight decrease until the fifth day of slaughter, and then a new increase until the last day of slaughter where the highest concentrations were marked: 390.43±7.25 ppb.

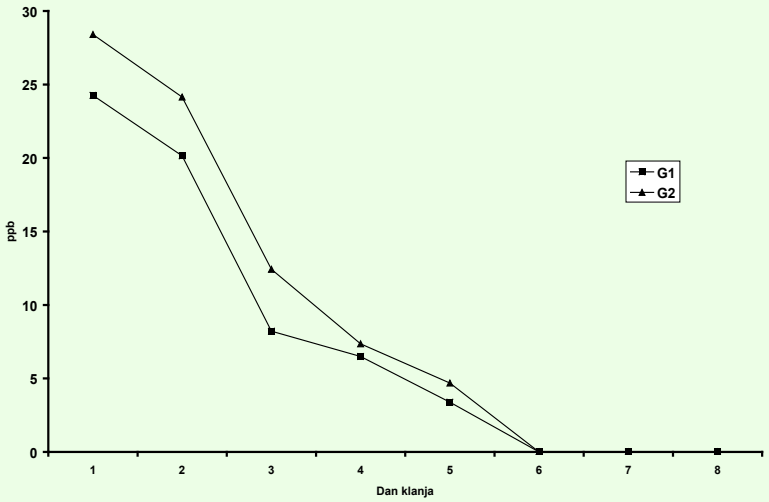
Enrofloxacin residues were also detected in samples from the experimental group G4 during all days of slaughter in significantly higher concentrations in relation to other experimental groups (Graph 2.). On the first day already there were marked concentrations from 486.95±75.51 ppb, and starting from the third to the fifth day there can be noticed a slight trend of increase in concentrations. The lowest marked value of the read enrofloxacin concentrations was measured on the sixth day and it was 457.91±77.72 ppb and then followed a growth until the eight day when at the same time there was marked the highest concentration of enrofloxacin residues in relation to all the experimental groups and all days of slaughter (709.75±50.46). Detection results of enrofloxacin residues by the ELISA method in liver samples are shown in Table 5. and Graph 3. Liver samples from the prophylactic group (P) during all days of slaughter were negative, i.e. levels of detected residues were below detection limit and MRL. Group liver sample from three broilers from the same experimental group (G1) on the first day of slaughter was negative, i.e. concentrations of enrofloxacin residues were below MRL (200 ppb) and they were 72.529 ppb. During the following days of slaughter, a slight decrease in concentrations of detected residues can be noticed, and on the last day of slaughter (8th day) residue concentration was 13.79 ppb. By analyzing results of liver samples from the first group G2, we can notice that only the sample from this group on the first day of slaughter was positive with the read value of 240.40 ppb, and from the second to the eight day of slaughter a constant decrease in concentrations with values below MRL can be noticed. Concentrations of enrofloxacin residues in liver samples from the experimental group G3 were expressedly high and even from the first day of slaughter it was 700.78 ppb and the highest concentration was marked on the second say of slaughter and it was 888.41 ppb. During the following days of slaughter there are noticed slight oscillations in found concentrations in the form of a decrease in concentrations from the third and sixth day of slaughter, and a slight increase until the last day of slaughter when the concentration of enrofloxacin residue was 773.08 ppb on the eight day. Concentrations of residues of the experimental group G4 are higher in relation to the group G3 with the initial concentration of 988.07 ppb on the first day of slaughter, then follows a slight increase of the concentrations on the second, third and fourth day of slaughter when there were determined the highest concentrations in this group of 1023.72, 1060.65 and 1009.86 ppb. Then fol-

lowed a decrease in the concentration on the fifth day, which is also the lowest concentration of this group (745.85 ppb). On the sixth, seventh and eight day there followed an increase where the concentrations of 820.34, 940.76 and 993.50 ppb were read.

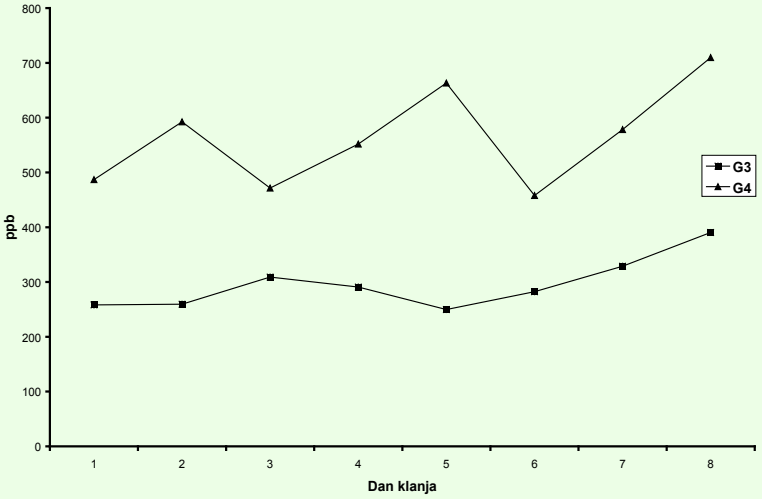
Anadon et al. (1995) also conducted a similar experiment by treating chickens with 10/mg/kg/day during four days. One day after the last application of the drug, they determined 540 ng/g of enrofloxacin and 650 ng/g of ciprofloxacin in muscle tissue, and 990 ng/g of enrofloxacin and 960 ng/g of ciprofloxacin in liver.

Similar researches were conducted by Petrović et al. (2006) with the aim of determining target tissue of enrofloxacin residues and its basic metabolite ciprofloxacin, so they treated chickens per orally with enrofloxacin dose of 10/mg/kg/day during the period of five days. Samples of muscle tissue and liver of the treated animals during five days of the therapeutic period were positive, i.e. they achieved values higher than MRL and concentrations of enrofloxacin residues in liver were up to 3.8 times higher in relation to the samples of muscle tissue. On the forth day since the last therapy there weren't determined drug concentrations in examining samples, which is in accordance with our results.

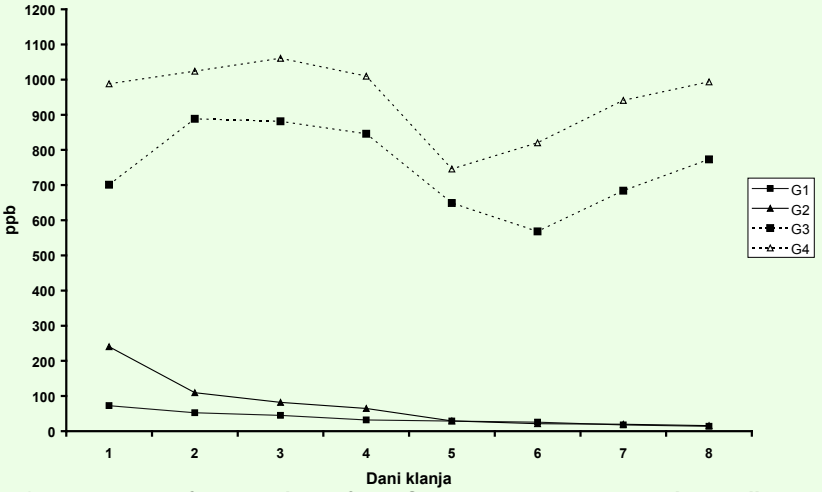
By comparing total results of the applied screening methods of detection of enrofloxacin residues in samples of muscle tissue and liver from the prophylactic group (P) it is noticeable that all the samples were negative, i.e. the found concentrations were lower than MRL. In the report of European Medicines Agency (Anonymous, 1998), similar data can be found on the results of analyses from a similar experiment where it is stated that concentrations of enrofloxacin residues on the 15th day after



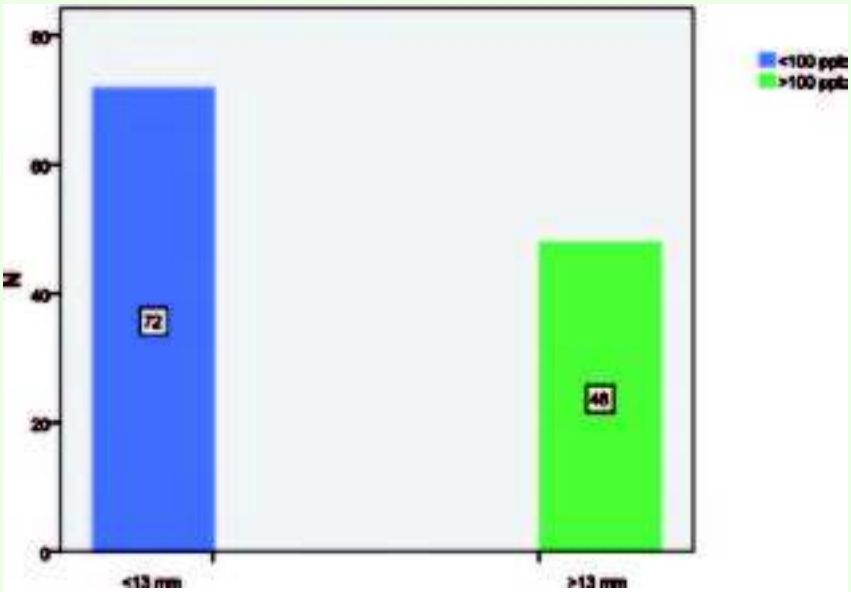
Graph 1. **Dynamics of mean values of enrofloxacin concentration (ppb) in samples of broiler muscle tissue (n = 3) for experimental groups G1 and G2 per days of slaughter by using the ELISA method**



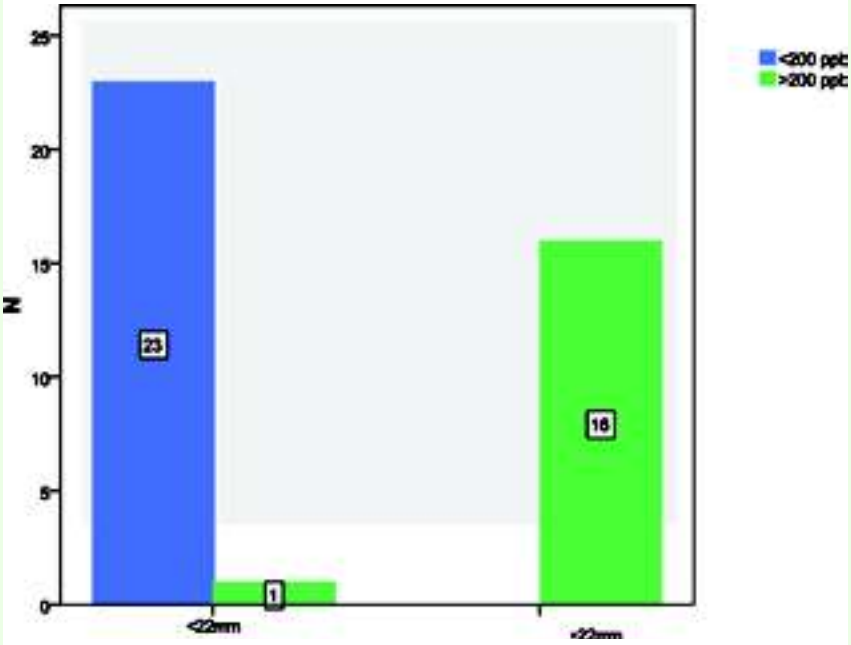
Graph 2. **Dynamics of mean values of enrofloxacin concentration (ppb) in samples of broiler muscle tissue (n = 3) for experimental groups G3 and G4 per days of slaughter by using the ELISA method**



Graph 3. **Dynamics of mean values of enrofloxacin concentration (ppb) in collective liver samples of 3 broilers for experimental groups G1, G2, G3 and G4 by using the ELISA method.**



Graph 4. **Results correlation of researching enrofloxacin residues in muscle tissue samples (n = 120) obtained by methods of microbiological test and ELISA. Correlation coefficient (j) = 1.00; p << 0.001.**



Graph 5. **Results correlation of researching enrofloxacin residues in liver samples (n = 40) obtained by methods of microbiological test and ELISA. Correlation coefficient (j) = 0.95; p << 0.001.**

the last application were extremely low, which justifies our findings that 35 days after the last application of therapeutic dose of enrofloxacin, its concentration was lower than MRL.

By using phi correlation coefficient it was determined that there is a statistically very significant positive correlation (p <<0.001) between the data of the microbiological and ELISA method (with the level of significance lower than 5%) in the direction: if ELISA > 100 ppb, then microbiological test > 13 mm and vice versa, regardless on the time of taking a sample (day of slaughter), that is, the quantity of the applied enrofloxacin (Graph 4.). In other words, in experimental conditions, both microbiological and ELISA method give equally reliable results of detection of allowed quantities of enrofloxacin residues, although they are different measures (mm or ppb) of the same phenomenon. Out of 72 samples of muscle tissue collected during eight days of slaughter in different experimental groups of broilers, which showed a negative result in the ELISA test (residue quantity lower than 100 ppb which is the value of MRL for muscle tissue), all 72 samples resulted in a negative microbiological test (MRL > 13 mm). On the other hand, the rest 48 samples which were positive in the ELISA method (MRL > 100 ppb) were also positive in the microbiological test (MRL > 13 mm). No case was recorded as a case deviating from the above listed findings, which is the reason for these two diagnostic methods to show a 100% positive correlation (j = 1,00).

Except on muscle tissue, these two methods for the detection of enrofloxacin residues were applied on liver too. The allowed MRL for liver is 200 ppb, so this value is used as a criterion of dichotomy of data (positive- negative) obtained by the ELISA method, whereas in the microbiological method that yield criterion was 22 mm diameter of growth inhibition zone of test microorganism.

By using phi correlation coefficient it was determined that there is a statistically significant positive correlation between the data obtained by the microbiological and ELISA method (with the level of significance lower than 5%) in the direction: if ELISA > 200 ppb, then microbiological test > 22 mm and vice versa, regardless on the time of taking a sample (day of slaughter), that is, the quantity of the applied enrofloxacin (Graph 5.) Out of 23 liver samples collected during 8 days of slaughter in different experimental groups of broilers which resulted in the residue quantity less than 200

ppb (a negative result) in the ELISA test, it also resulted in a negative microbiological result (inhibition zone diameter < 22 mm). On the other hand, out of the remaining 17 cases which resulted in a positive finding (> 200 ppb) in the ELISA test, there was marked only one case of a microbiological testing which resulted in inhibition zone diameter larger than 22 mm. Of course, that one case is not an aberration large enough to affect the significance of correlation ((j) = 0.95; p << 0.001) between the microbiological test and ELISA test and when it comes to liver too, not only muscle tissue.

Conclusions

1. The method of microbiological test of growth inhibition (diffusion method) with the use of reference strain E. coli ATCC 10 536 as the microorganism- test, it can be considered a method reliable enough for the detection of enrofloxacin residues in broiler meat in relation to the values of MRL;
2. In experimental conditions, both microbiological and ELISA method give equally reliable results of detection of enrofloxacin residues in broiler meat in relation to the values of MRL;
3. By using methods of the microbiological test of growth inhibition with the use of reference strain E. coli ATCC 10 536 and the ELISA test, enrofloxacin residues in muscle tissue and liver of broilers can be detected successfully as soon as 24 hours from the beginning of a therapeutic application.

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