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Bioluminescent bacteria assay of veterinary drugs in excreta of food-producing animals

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Abstract

The residues of pharmacological treatments on food-producing animals, present in the manure dispersed on agricultural land, can impact environmental and human health through toxic, genotoxic, and drug-resistance development effects. Biotoxicity assays can easily reveal the presence of noxious substances and those based on bioluminescent bacteria (BLB) are particularly simple and rapid. A BLB assay was developed as microplate format by using various strains of *Vibrio* sp. and was employed to evaluate their response to pure antibiotic solutions and to residues extracted from excreta of antibiotic treated pigs and turkeys. The residues were quantified by HPLC analysis. The BLB assay can be proposed as an easy-to-perform screening tool to assess the presence of residues due to undeclared current, or recently ended, pharmacological treatments, as well as to evaluate their permanence in manure.

Keywords: Antibiotics; Bioluminescent bacteria; Excreta; Food-producing animals; Biotoxicity

1. Introduction

The used amount of drugs for human or veterinary purposes is continuously growing and, in the same way, the amount of their residues in the environment. The residues of veterinary medicines used for treating food-producing animals are directly or indirectly released to the environment mainly through the application of manure to agricultural land [1]. From the soil they can be washed off to surface waters or can leach to groundwaters where they can impact human and environmental health, a problem that has already been recognised of prime importance [2,3].

The adverse effects of medical substances residues on the environment and the organisms include toxic and genotoxic effects as well as the development of drug resistant strains of bacteria [4]. Many drugs, such as antibiotics, are poorly absorbed in the gut and are excreted in the faeces. Literature data report that 30–90% of the administered dose of antibiotic to humans or animals is excreted with the urine as active substance [5].

The degradation processes can contribute to remove the parent drugs but at the same time can lead to the formation of several related products that may pose a further environmental risk. In 1996, the Committee for Veterinary Medicines has drawn up a document-guide, which describes an approach for environmental risk evaluation [6]. Of great importance for the development of any risk assessment protocol is a correct evaluation of the drugs content in treated animals excreta and manure at the moment of their application to the environment.

Apart from the direct determination by chromatographic methods, specific and sensitive but often expensive and requiring laborious procedures of sample treatment [7], the presence of compounds potentially noxious for living organisms can be revealed observing the appearance of toxic effects on sensitive organisms. Since a large number of veterinary drugs, namely the antibacterial agents and their derivatives, are by their very nature toxic to microorganisms, the presence of antibiotics is easily assessed using bacteria, and among them the bioluminescent strains offer several advantages [8]. Bioluminescence

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strictly depends on the vitality of bacterial cells, being directly coupled to respiration processes. Any compound able to damage the integrity of the bacterial metabolism or structure will modify the light emission and this effect can be evaluated in a very short time in comparison to that required for classical growth inhibition experiments, offering an average sensitivity close to that of other tests for organic and inorganic pollutants determination [9]. Several applications of the luminescent bacteria toxicity tests are reported in the literature. Among xenobiotics tested by these methods, antibiotics [10], polyaromatic hydrocarbons [11], analgesics [12], pesticides [13], heavy metals [14,15], anti-bacterial compounds such as triclosan [16], and non-ionic surfactants [17] are included.

Previously we developed, by using a *Vibrio* sp. strain, an assay on 96-well microplate to detect various toxic chemicals carried out at room temperature [18]. The advantages with respect to the methodology described in the European standard EN ISO 11348 [19] were the possibility to analyse a lot of samples at the same time, speeding up the screening analysis, and to use simpler instrumentations, since no cooling system was required. This can enlarge the possible applications out of the laboratory.

The aim of this work was to develop a similar bioluminescent assay, evaluating the response of different strains of bacteria to various classes of antibiotics, and to test its suitability to reveal in a quite direct and very simple way the presence of antibiotics in samples like excreta of treated animals. The results of such a bioassay were compared with the quantitative data obtained by HPLC measurements.

2. Experimental

Three strains of marine luminescent bacteria, all belonging to *Vibrio* sp., were employed to develop the bioluminescent assay. The strain named "*UCIBO*", isolated from the Mediterranean seawater, was cultivated at our laboratory as previously described [18]. A second strain, supplied in the lyophilised form, was purchased by the Biophysics Institute of the Russian Academy of Science (RAS), Siberian Branch, Novosibirsk, Russia. It was cultivated at our laboratory in the same conditions of *UCIBO* strain and was named "*Russian*". The third, *Vibrio fisheri*, was the strain employed in the official assay European standard EN ISO 11348, and it was supplied by the Institut Pasteur Laboratories, Paris, France.

All strains were cultivated both in liquid and on solid medium. Liquid medium: NaCl, 30 g; glycerol, 3 mL; peptone, 3 g; yeast extract, 3 g/L. Solid medium: 12 g of Agar per liter of liquid medium. All the reagents employed for bacteria cultivation and storage were from Sigma–Aldrich Co.

Cultures in broth were maintained, without stirring, at 21 °C under a continuous flow of filtered air (0.2 pore size) and were used both to prepare lyophilised stocks and to perform the analysis with fresh bacteria. On solid medium fresh bacteria can be cultivated for longer time and the colonies showing stronger light emission can be selected and isolated. The three strains were stored, as well as employed in part of the assays, in the lyophilised form. Lyophilised bacteria were prepared from fresh ones cultivated for 2 days in broth, collected by centrifugation, resuspended in lyophilisation media (12% lactose, 1% NaCl, 2% potato starch, in distilled water), divided into aliquots of 200 μL, refrigerated, and then lyophilised as previously described [18].

Pure samples of the antibiotics Amoxicillin, Flumequine, Chloramphenicol, Polimixin B, Amikacine, Ampicillin, Colistine, Gentamicine, and Piperacillin were supplied by Sigma–Aldrich Co. Cefodizime, Mezlocillin, and Sulphametazine were purchased as injectable solutions at local pharmacy. Stock solutions of the neat antibiotic compounds were prepared in distilled water, with the exception of Flumequine that was dissolved in ethanol. Working solutions were prepared by dilution in a saline solution (NaCl 2%). In case of Flumequine, the maximum allowed concentration of ethanol in the working solutions was 3%.

The food-producing animals selected to be treated specifically to develop this assay were turkeys and pigs. Treatments and sample collections were performed at the Istituto Zooprofilattico, Bologna. Two groups of 12 turkeys were placed into two water-cleaned, flooring boxes (Box 1 and Box 2) and were treated for 5 days with chloramphenicol, added to the drinking water at different doses:

Box 1: 20 mg/kg/die the first day; 40 mg/kg/die from day 2 to day 5.
Box 2: 20 mg/kg/die the first day; 60 mg/kg/die from day 2 to day 5.

Samples were collected the first, the third, and the last day of treatment (D1, D3, D5). Excreta of untreated animals were used as reference blank sample.

The pigs were divided into groups of four animals, each one, feeded with complete fodder for young animals (Agrizoo Center Romanini, Fiumana, Italy) that was added with an Amoxicillin/Flumequine mixture (Ascor Chimici s.r.l. Capocolle di Bertinoro, Italy), in case of group named "M" and with different amounts of Doxocicline in case of groups named "X_1", "X_2", and "X_5". The last group, named "X_0", was the control group (untreated animals). The medicated fodder containing the Amoxicillin–Flumequine mixture was administered to the "M" group for 5 days consecutively, with a daily intake of each antibiotic of 18.41 mg/kg b.w. Excreta, corresponding to the day before production, were collected in the morning before to feed the animals for 26 days.

The groups X_1, X_2, and X_5 were feeded for 6 days with the same fodder containing Doxocicline for a daily intake of 10, 20, and 50 mg/kg b.w./die, respectively. Samples of excreta corresponding to the last day of treatment and 2 days after this were collected following the same procedure as above. All samples were homogenised and immediately frozen.

2.1. Instruments

Quantitative measurements of the antibiotics content in the samples were performed by using a system of quaternary gradient pumps for Perkin-Elmer HPLC instrument mod. LC Pump Series 200, an autosampler mod. Advanced LC Sample Processor ISS200 equipped with Rheodyne injection valve mod.

4293, a fluorescence detector mod. LC240, an interface mod. 600 Series Link and mod. 900 Series, and data processing software Turbochrom 6.1.1, all from Perkin-Elmer (Wellesley, MA, USA).

The bacterial light emission was measured in a Multilabel Counter Mod. *Victor* 1420 (Wallac, Sweden), employing 96-well microplate (ThermoLabsystem, Vantaa, Finland). The luminometer was equipped with a computerised collection of data, elaborated on Microsoft Excel software.

2.2. HPLC measurements

2.2.1. Chloramphenicol

The analytical procedure was that employed to detect the presence of this antibiotic in tissues [20–23], however, due to the high content of residues, a lower amount of sample with respect to tissues and dilutions of the extracts are often required. Shortly, 10 g of sample were added to 50 mL of ethanol, homogenised and gently shaked for 1 h, then centrifuged at $3660 \times g$ for 20 min. Ten milliliters of the supernatant were again centrifuged and the surnatant directly injected in the HPLC column (Supelco LC18, 150 mm × 3.2 mm, 3 μm particles size (Sigma–Aldrich Co.).

2.2.2. Flumequine and Amoxicillin

The pig excreta were analysed according to the validated procedures for the assay of these antibiotics in tissues and biological fluids [20,24–26]. The extracts were injected on a Sphereclone 150 mm × 3.2 mm (5 μm particles size, cod 290805) column (Phenomenex, Torrance, USA) to measure the Amoxicillin content and on Waters Novapack C18 3.9 nm × 300 nm, 5 μm particles size column (Waters Corporation, Milford, MA, USA) to assess the Flumequine content.

2.3. Bioluminescent assay

All samples, both treated and blank, underwent the same extraction procedure: 1 g of a thinly grinded sample was added to 5 mL of ethanol and stirred for 1 h. The suspension was passed through a paper filter and the filtered solution centrifuged 15 min at $2056 \times g$. The surnatant was diluted in 2% NaCl to obtain final solutions containing different amounts of ethanolic extract.

The long-term (18 h) toxic effects of pure antibiotics and sample extracts were evaluated both on lyophilised, reconstituted, bacteria as well as on bacteria cultivated in liquid medium. Lyophilised bacteria were reconstituted with 200 μL of 2% NaCl, added to 10 mL of culture medium and left at room temperature for 30 min before to distribute them, 100 μL per well, on the microplate. When fresh bacteria were used, 100 μL of the liquid culture were added to 10 mL of fresh liquid medium, then distributed in the microplate. One hundred microliters of standard solutions or sample extracts was added to each well and the light emission collected each 10 min during a period of 18 h. The light emission intensity of the just reconstituted bacteria was measured before the addition of the samples, to check their vitality. The toxicity values were expressed as percent of inhibition of the light emitted by the reference blank:

$$\frac{B_e - S_e}{B_e} \times 100$$

where B_e is the emission of the blank and S_e that of the sample at the same time which was fixed at 960 min.

Table 1
HPLC determination of the content of antibiotic residues in pigs and turkey samples

Sample (day of treatment)	Amoxicillin (ppm)	Flumequine (ppm)	Chloramphenicol (ppm)	
			Box 1	Box 2
D1	183.9	116.0	8.9	29.7
D2	128.2	218.2	–	–
D3	394.8	441.5	4.3	14.4
D4	272.0	359.1	–	–
D5	273.1	313.8	1.1	11.0
D6	30.4	194.5		–
D7	2.4	3.1		–
D8	0.9	2.2		–
D9	0	3.8		–
D10	0	2.5		–

3. Results and discussion

3.1. HPLC measurements

The specificity of the employed methods to determine the respective analytes in this particular matrix was checked testing a group ($n = 20$) of samples surely analyte-free. The analysis demonstrated the absence, for all three assays, of significant interferences produced by the matrix.

Samples from pigs treated with the Amoxicillin/Flumequine mixture and from turkeys were analysed to quantify exactly the residues amount and the mean values are reported in Table 1. All the 26 samples of the "M" group were measured, in order to evaluate how long after treatment end the residues can be detected in excreta. Samples from D11 to D26, not reported in the table, showed no residues of Amoxicillin, and a variable amount of Flumequine in the range 1.1–0.5 ppm.

3.2. Bioluminescent assays

The three strains were tested at the same time on the same compounds to compare their performances in the chosen experimental conditions. Especially the "*UCIBO*" strain was of particular interest having demonstrated any problem working at room temperature. On the contrary, the optimal working temperature of *V. fisheri* is 15 °C and this entails the need for a cooling device in the measuring instruments, expensive and difficult to apply to a microplate reader.

The various strains of luminescent bacteria showed a slightly different behaviour in the light emission kinetics after reconstitution of the lyophilised aliquots. *UCIBO* bacteria showed a maximum after 1 h from reconstitution, then a decrease of the intensity and a new maximum after 18–20 h. The *Russian* bacteria displayed a low emission immediately after reconstitution

Table 2
EC_{50} values of the tested antibiotics, expressed as ppm of the active compound with the only exception of Polimixin B, expressed as units

Strain	Compound	Lyophilised	Fresh
V. fisheri	Amoxicillin	18.37	34.45
Russian	Chloramphenicol	0.04	0.02
UCIBO	Chloramphenicol	0.04	0.02
V. fisheri	Chloramphenicol	0.10	0.01
Russian	Flumequine	0.03	0.05
UCIBO	Flumequine	0.06	0.04
V fisheri	Flumequine	0.06	0.02
Russian	Polimixin B	0.000032	0.000026
UCIBO	Polimixin B	0.000048	0.000038
V. fisheri	Polimixin B	0.000028	0.000031
Russian	Amikacine	0.30	0.29
UCIBO	Amikacine	0.35	0.31
V. fisheri	Amikacine	0.30	0.28
Russian	Ampicillin	11.94	10.20
UCIBO	Ampicillin	10.32	8.92
V. fisheri	Ampicillin	8.72	6.31
Russian	Cefodizime	0.05	0.04
UCIBO	Cefodizime	0.05	0.06
V fisheri	Cefodizime	0.07	0.05
Russian	Colistin	0.52	0.32
UCIBO	Colistin	0.74	0.58
V. fisheri	Colistin	0.38	0.47
Russian	Gentamicine	2.10	1.34
UCIBO	Gentamicine	1.43	1.51
V. fisheri	Gentamicine	0.76	0.40
Russian	Mezlocillin	35.12	34.62
UCIBO	Mezlocillin	20.77	15.73
Russian	Piperacillin	2.58	2.32
UCIBO	Piperacillin	0.75	0.54
Russian	Sulphametazine	4.52	3.85
UCIBO	Sulphametazine	4.57	4.22
V. fisheri	Sulphametazine	3.68	4.15

and a maximum after 8–9 h. The *V. fisheri* strain showed the slowest emission kinetics: the initial emission was close to zero and the maximum was reached after 20 h, i.e. after the end of the assay time the emission was still growing.

When fresh bacteria were used the light emission kinetics of the three strains were different from those of the lyophilised ones but very similar among them: the initial emission was higher then in case of lyophilised bacteria and it decreased very quickly, then the maximum was reached about at the end of the assay time (18 h).

Pure antibiotic solutions were prepared to obtain a wide range of concentrations. In case of Flumequine it was not possible to obtain solutions at higher concentration than 10 ppm because of the low solubility in water of this molecule and of the low amount of ethanol (not higher than 3%) that can be present in the solutions to put in contact with bacteria.

The various concentrations were tested on all strains, both on lyophilised and fresh bacteria and the EC_{50} values, i.e. the concentrations that reduced the light emission intensity to the 50%, were calculated. These data are reported in Table 2. The coefficients of variation were between 5 and 14%.

Amoxicillin showed very particular effects: on "*UCIBO*" and "*Russian*" bacteria, concentrations lower than 500 ppm stimulated the light emission, that was higher than in the respective blanks. Only concentrations above 500 ppm produced inhibition of the light emission, as shown in Fig. 1. Being these concentrations abnormally high, it was not possible to obtain a useful EC_{50} value.

Also *V. fisheri* showed quite high resistance to this antibiotic in comparison to the other two tested compounds, but with this strain it was possible to calculate the EC_{50}, testing Amoxicillin in the range 1–400 ppm.

This stimulating effect of Amoxicillin was not a problem during measurements of real samples, because it was negligible at the Amoxicillin concentrations present in the diluted samples analysed. In fact, in the wells was present not more than 1.5% of the actual amount in the sample.

Concerning the toxicity of the other tested antibiotics quite in all cases the three strains showed very similar behaviour, both as lyophilised and fresh cultures.

To evaluate the response of luminescent bacteria to the antibiotics, the residues had to be extracted from the samples. Various solvents were tested, since some of the antibiotics were not water-soluble. Among dimethylsulfoxide, methanol, and ethanol only the last one showed no one or negligible effects on light emission when put into contact with the bacteria at concentrations till 5% in saline solutions. To a higher concentration of ethanolic extract corresponded a higher percentage of inhibition of the light emission, and since amounts of ethanol in saline solution till the 3% produced no effect on the blank emission, all samples were tested by using the dilution containing 3% of ethanolic extract.

The *V. fisheri* strain was not employed to test the real samples since the effects of the antibiotics, with the exception of Amoxicillin, were very similar on the three strains and the *UCIBO* and *Russian* ones reached the maximum of light emission within the measurement time, showing a more reproducible emission behaviour. Moreover, bacteria were used in the lyophilised form since, as mentioned above, no significant differences were noted between the data obtained treating fresh and lyophilised bacteria with pure antibiotics and both for laboratory as well as on-field analysis it is easier to handle them in this form.

Concerning the turkey samples the highest percentage of inhibition was provoked, on both bacterial strains, by the addition of the samples collected at the beginning of the treatment (first day), and the effect decreased in the middle and end of treatment (Fig. 2), in parallel with the decreasing of antibiotic concentration in the excreta, as determined by HPLC measurements. The correlation between these two parameters was expressed by the linear regression equations as it follows: Box 1, "*Russian*": $y = 4.66x + 164.16$; $R^2 = 0.870$. "*UCIBO*": $y = 2.19x + 38.41$; $R^2 = 0.927$. Box 2, "*Russian*": $y = 0.15x + 95.96$; $R^2 = 0.439$. "*UCIBO*": $y = 2.82x + 13.27$; $R^2 = 0.740$.

The 26 samples of pig excreta from group "M" were all tested on luminescent bacteria, even the HPLC measurements had shown a drastic drop in residues concentration few days after the end of treatment. The aim was to assess if antibiotic metabolites, not detected in the HPLC assay, could display toxic effects.

Actually, it was possible to observe inhibitory effects only with the samples from no. 1 to no. 6, i.e. during the 5 days of treat-

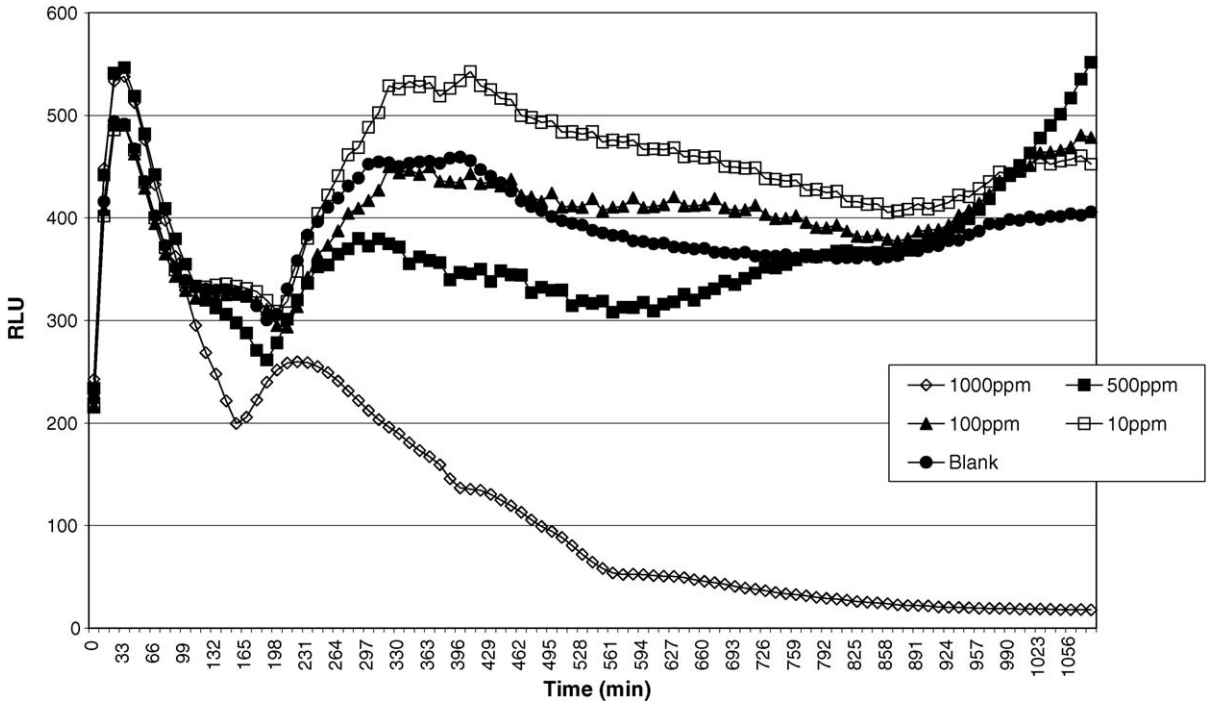

Fig. 1. Effect of various concentrations of Amoxicillin on light emission of "*Russian*" bacteria. Concentrations under 400 ppm and above 1 ppm produced a stimulation on light emission, higher concentration an inhibition. At concentrations lower or equal to 1 ppm no stimulating effect was observed.

ment to one after its end. The percentage of inhibition were in the range 99–78% for samples containing 3% of ethanolic extracts, decreasing in parallel with the decrease of the antibiotics content. The amount of antibiotics contained in the D7–D26 samples was not enough to produce an inhibition of the light emission that was, on the contrary, higher than the reference blank (samples from X_0 group), displaying a phenomenon of stimulation by low levels of toxicants (hormesis) and/or matrix components widely described in literature [15,27].

With the aim to obtain better differentiation among the samples the percentage of extracts in the final solutions was reduced to obtain from the 20 till the 80% of the amount usually

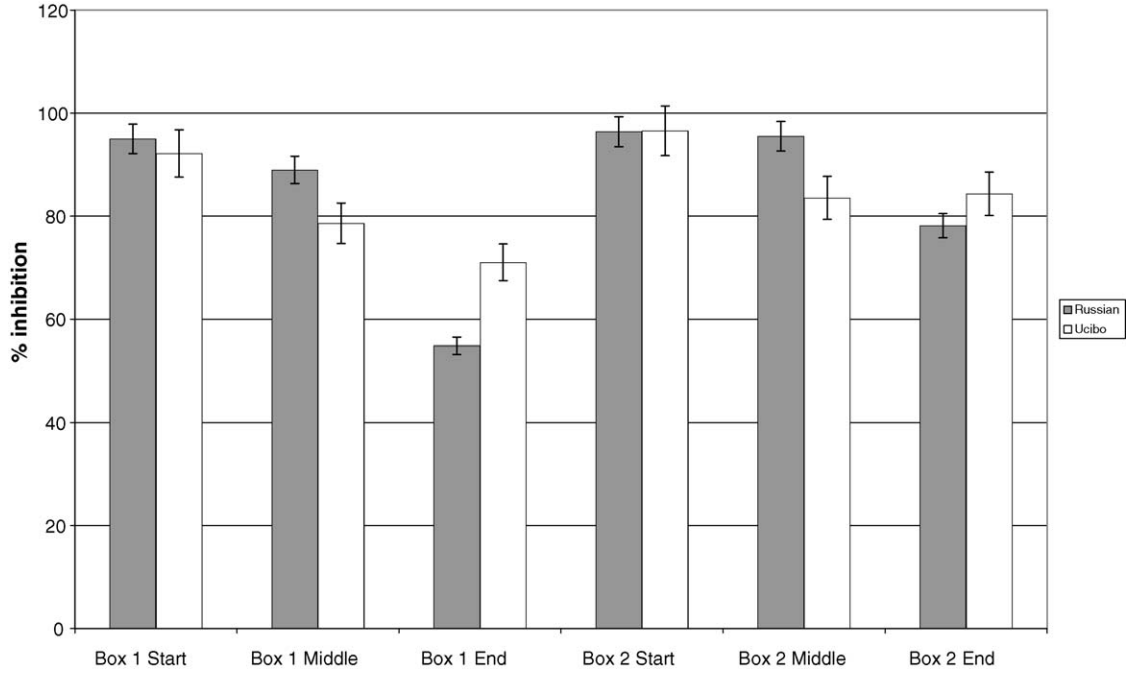

Fig. 2. Inhibitory effects of the extracts of turkeys excreta on light emission of the "*Russian*" and "*UCIBO*" strains (3% of ethanolic extract in NaCl).

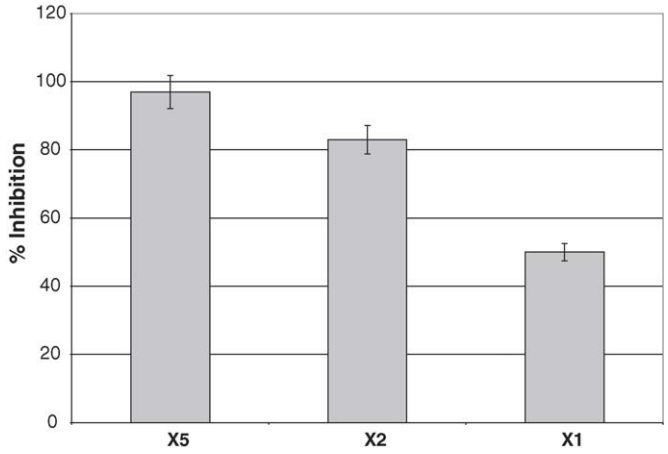

Fig. 3. Percent of inhibition of the light emission of "*UCIBO*" bacteria produced by the extracts (2.4% in NaCl) of pig samples treated with Doxocicline and collected 2 days after the end of the treatment.

employed. The decrease of the percentage of inhibition was proportional to the dilution of the extracts, indicating that the response of the bacteria was directly correlated to the content of toxicants, but the reciprocal differences among the samples resulted very similar.

Results obtained measuring the samples from groups X_1, X_2, and X_5 were analogous, with an important difference in the sensitivity of bacteria to the Doxocicline. In fact, samples collected 2 days after the end of treatment still produced strong inhibition, as shown in Fig. 3 for solutions containing only the 2.4% of ethanolic extract.

4. Conclusions

A bioluminescent bacteria assay was optimised to test the toxicity of antibiotics in standard solutions and in excreta of food-producing animals. The developed assay could be considered a useful preliminary tool to assess going-on or just-ended veterinary treatments, possibly undeclared, or to evaluate the presence of antibiotics, especially resistant ones to degradation, prior to the application of manure to agricultural land.

The BLB assay proved to be rapid and versatile, allowing to work at room temperature when appropriate bacterial strains are chosen. The use of 96-well microplates allowed the simultaneous analysis of several samples, reducing the analysis time and the cost per assay. The exact quantification of the positive samples, when required, or the identification of the toxic compound, can be obtained by chromatographic methods. The bacteria could be easily stored and transported after lyophilisation. A linear relationship between luminescence inhibition and the amount of antibiotics in the samples was generally demonstrated, in spite of the complexity of the analysed matrix and of its overall effect on the bacterial metabolism. It is important to point out that to minimise these effects any result must be evaluated by comparison with a proper blank sample of the analysed matrix.

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