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Antibiotic residues in milk from Moldavia, Romania

[1]Andrei C. Grădinaru, [2]Ovidiu Popescu, and [1]Gheorghe Solcan

[1]Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine of Iaşi, Romania.
[2]Veterinary School, Purdue University, West Lafayette, Indiana, USA.
Corresponding author: A. C. Grădinaru, andre_gradinaru@yahoo.com

Abstract. The present paper is a study based on the determination of antibiotic residues in milk samples collected from farms in the NE Romania (Moldavia). Contamination seasonality and the correlations with SCC and TBC values were investigated. Out of 2785 total milk samples, 124 were positive (+) (4.45%) and 130 samples were uncertain (±) (4.67%). The presence of antibiotics was confirmed in 109 positive (+) samples (87.9%) and 24 uncertain (±) samples (18.46%), the difference being false-positive reactions. Betalactams were identified in 27.90% of the samples, at an average concentration of 26.65 µg/kg. Gentamicin/neomycin were identified in 25% of samples, at an average concentration of 198.68 µg gentamicin/kg and 2048.53 µg neomycin/kg. Tetracyclines were identified in 24.42% of the samples, at an average concentration of 271.43 µg/kg. Gentamicin/streptomycin were identified in 15.11% of the samples, at an average concentrations of 198.68 µg gentamicin/kg and 280.61 µg streptomycin/kg. The macrolides were identified in 7.56% of the samples, at an average concentration of 97.87 µg tylosin/kg. The antibiotic contamination of milk was low in January (cs_i = 0.51) and July (cs_i = 0.59), and increased in April (cs_i = 1.77) and May (cs_i = 1.43). The milk contamination with antibiotics was associated with increased SCC and TBC values.
Key words: antibiotic residues, milk, statistical analysis.
Abbreviation key: ADI = acceptable daily intake; cfu = colony forming unit; cs_i = seasonality factor; MRL = maximum residue limits; SCC = somatic cell count; TBC = total bacteria count.

Introduction. In small quantities and long intake, antibiotics can have undesirable effects on humans and animals. The undesirable effects on consumer health can be direct and manifest as allergic or toxic reactions. The indirect effects are also important by the creating antibiotic-resistant organisms (Allison 1985; Bishop & White 1984; Popescu & Enache 1996). Some studies showed that milk polluted with antibiotics which was obtained from 20 cows delayed or stopped the fermentation processes of the milk collected from another 25,000 cows (Râpeanu 1975). Moreover, the samples with antibiotic residues are accompanied by an increase of the total number of somatic cells and of the total number of germ populations (Ruegg & Tabone 2000; Van Schaik et al 2002).

In this context, the control of the antibiotic presence in milk is an important issue, becoming mandatory in all milk processing units (Grădinaru 2010).

Materials and Methods. Investigations were conducted during 2006 - 2009 on 2785 milk samples harvested from some milk collecting centers and dairy farms located in NE Romania (Moldavia) (Fig. 1).

The sampling was done according to norms in force (Commision Directive 1987; SR EN 2000). Samples of 500 to 1000 mL of milk were collected in dry clean plastic containers; each sample was fitted with a label which specified the product name, date of collection, and any other useful information for further identification; the samples were collected in duplicates, of which one was sent to the laboratory for analysis and the other was freezed and stored in dark containers till the analyses were over.

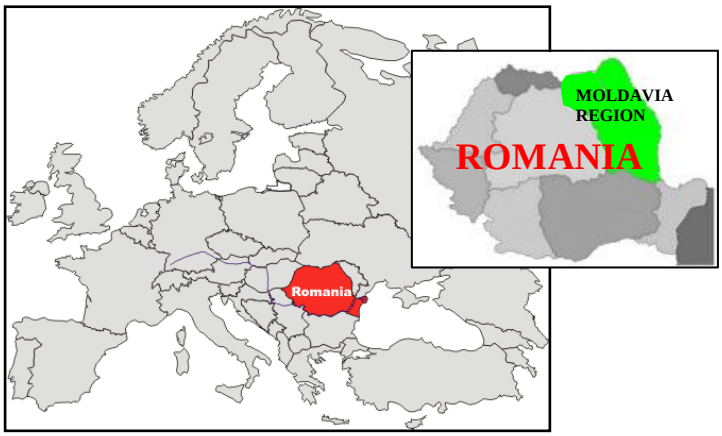

Figure 1. Geographical location of the study.

The performance criteria described by the Comision Decision 180/1991 and the Comision Decision 657/2002 were used for the study. The identification of antibiotic residues was based on a standardized method, with spores of *Bacillus stearothermophilus* calidolactis variety; the presence of antibiotics in the analyzed samples impaired/ halted the fermentation of the test microorganism. Positive (+) and uncertain (±) samples were heat treated at 82^0C for 10 minutes to eliminate false-positive results due to natural inhibitors (lysozyme, lactoferin, lactoperoxidase). Next, the confirmed positive samples were semiquantitatively analyzed to identify the families of antibiotics (the necessary equipment was provided by Charm Science Inc).

The determination of the antibiotic families was made through a competitive enzyme immunoassay method (ELISA), based on the principle of the antigen - antibody reaction. The determination of somatic cells was based on the microscopic procedure, computer equipment being used for staining and reading the microscope slides. The total number of germs was determined by a colorimetric method.

The interpretation of the investigation data involved the use of some basic statistical indicators, such as minimum, maximum, mean value, standard deviation, coefficient of variation, confidence interval of the mean value. For the seasonality calculation expressed as cs_i, the arithmetic method was used (Badea & Georgescu 2003; Jaba 2006). Seasonal factors were established using a series of monthly data for the four years of study. An average value was calculated for each month (Eq.1) which was compared with an overall average value of the period (Eq.2), achieving the value of seasonality factor (Eq.3).

$$\bar{X}_i = (\Sigma^n_{j=1} x_{ij}) \; / \; n \qquad (1)$$

$$\bar{X}_G = (\Sigma^m_{i=1} \bar{X}_i) \; / \; m \qquad (2)$$

$$cs_i = \bar{X}_i \; / \; \bar{X}_G \qquad (3)$$

where: cs_i - seasonality factor for the *i* month;

$\bar{X}_i$ - arithmetic mean of the *i* months of the four years of the study;

$\bar{X}_G$ - arithmetic average based on the $\bar{X}_i$ averages;
x_{ij} - values for the *i* months of the *j* year;
n - number of years;
m - number of months of the study period.

Whether $\bar{X}_i > \bar{X}_G$ it resulted a $cs_i > 1$ and the seasonal variation was considered positive.

For $\bar{X}_i < \bar{X}_G$ it resulted a $cs_i < 1$ and the seasonality was bad, the months average was smaller than the overall period average.

The consistency determination was made using the χ^2 test (chi-square). The observed frequencies of the antibiotic contaminations were compared with the expected ones (Eq.4).

$$\chi^2 = \Sigma^{l}_{i=1} \Sigma^{c}_{j=1}[(f^{o}_{ij} - f^{a}_{ij})^2 / f^{a}_{ij}] \quad (4)$$

where: f^{o}_{ij} = the observed frequencies for the *i* line and *j* column;
f^{a}_{ij} = the expected frequencies for the *i* line and *j* column;
l = the number of rows in the table (or the number of classes of the effect factor);
c = the number of columns in the table (or the number of classes of the active factor).

If there was no dependence trend, χ^2 value was 0, and if there was a dependence tendency the χ^2 value was greater as the dependence trend was stronger. To counter the trend of artificially increasing of the χ^2 coefficient value, the use of the Φ^2 pointer was imposed; this is obtained by dividing the χ^2 quotient to the cells number of Table (Eq.5).

$$\Phi^2 = \chi^2 / n \quad (5)$$

Results and Discussion. The results obtained using the microbial quality test to identify the antibiotic residues in the investigated samples are presented in Table 1.

Table 1

Qualitative microbial identification test for antibiotic residues

Year of study	*Analyzed samples*		*Results of the identification antibiotic residues*					
			(+) samples		(±) samples		(-) samples	
	no.	%	no.	%	no.	%	no.	%
2006	782	100	41	5.24	43	5.50	698	89.26
2007	734	100	32	4.36	34	4.63	668	91.01
2008	756	100	31	4.10	32	4.23	693	91.67
2009	513	100	20	3.89	21	4.09	472	92.02

Throughout the study period, 2785 milk samples were analyzed, of which 124 samples (4.45%) were contaminated with antibiotic residues (+), 130 samples (4.67%) had an uncertain reaction to the test (±) and 2531 samples (90.88%) were free of antibiotic residues (-).

Other studies also showed that in some milk markets the antibiotic residues may exist in approximately 8-15% of the processed milk (Baynes et al 1999; Shitandi & Sternesjo 2004). Chung et al (2009) identified 21 antibiotic contaminated samples out of 269 analysed milk samples, representing 7.8% of the total. Khaskheli et al (2008), analysing 137 milk samples and using the qualitative microbial method with *Bacillus subtilis* in plates for betalactams detection, identified 87 samples (63.5%) as negative and 50 samples (36.5%) were considered positive with mean inhibition zones of 8.91 ± 0.37 mm.

The milk samples with antibiotic residues (+) and those with uncertain results (±) were heated at 82^0C for 10 minutes. The samples with antibiotic residues (+) were confirmed at the following rates: 87.8% in 2006, 87.5% in 2007, 87.1% in 2008 and 90% in 2009. Among the samples with uncertain results (±) to the quality microbial test, 18.6% were confirmed as positive in 2006, 17.65% in 2007, 18.75% in 2008 and 19.05% in 2009. Considering the entire study period, 87.9% of the initially positive (+) samples and 18.46% of the initially uncertain (±) ones were confirmed by applying the heat treatment; the difference represented the false-positive reactions.

The application of the heat treatment to positive (+) and uncertain (±) samples resulted in a decrease of the positive (+) results by 10 - 12.9% and from 80.95 to

82.35% of uncertain (±) results, depending on the period of study; thus, this is one of the easiest ways to eliminate false-positive results. Thus, Tyler et al (1992) identified 45% of the false-positive milk samples collected from cows with mastitis experimentally induced by endotoxin; Van Eenennaam et al (1993) found that, in the naturally occurring mastitis cases, 37.7% false-positive responses were identified. The mixig of the milk in tank obviously lead to the dilution of the milk originated from cows with different forms of mastitis, which contain a number of natural inhibitors usually high (Kang et al 2005; Kosikowski & O´Leary 1963). Kosikowski & O´Leary (1963) stated that were able to neutralize all natural inhibitors in 11 milk samples by using the heat treatment, the subsequent application of the microbiological method in plates leading to negative results.

According to the objectives of the study, a statistical interpretation of results based on the seasonality factor calculation was performed, using an arithmetical method (Badea & Georgescu 2003; Jaba 2006). The average number of samples contaminated with antibiotics, according to the sample collecting season, and cs_i values are also presented in Table 2.

Table 2

The seasonal variation of the antibiotic positive (+) samples

Month	*Positive samples (+) number*				*Monthly average*	*Seasonality coefficient*
	2006	2007	2008	2009		
January	*2*	*2*	*2*	0	1.50	0.51
February	*3*	*2*	*1*	2	2.00	0.67
March	*3*	*3*	*3*	2	2.75	0.93
April	*6*	*5*	*6*	4	5.25	1.77
May	*5*	*4*	*3*	5	4.25	1.43
June	*4*	*3*	*4*	2	3.25	1.10
July	*2*	*1*	*2*	2	1.75	0.59
August	*3*	*2*	*3*	2	2.50	0.84
September	*4*	*3*	*2*	3	3.00	1.01
October	*5*	*4*	*2*	-	3.67	1.24
November	*3*	*3*	*3*	-	3.00	1.01
December	*4*	*2*	*2*	-	2.67	0.90
TOTAL	44	34	33	22		12.00
				General average	2.97	

An increasing number of samples contaminated with antibiotic residues was found in late winter - early spring with a maximum in April. By early summer there was a decrease in the number of contaminated samples. During the last months of summer - early autumn there was a further increase in the number of contaminated samples.

The seasonal variations recorded for antibiotic contamination of milk are considered an indirect consequence of seasonal calving system adopted in the North-Eastern Romania. Thus, due to the prolonged use of grazing, most births are scheduled for spring (February – March). Because of this, in spring occurs an immunity decrease on the general fund of post-partum stress associated with the scarcity of food. Also, the introduction of infusions during the rest breast period may contribute to the contamination of the milk samples collected in spring. The maintenance of the cows on pasture during the summer contribute to reducing the rate of breast disease through beneficial actions of ultraviolet light, open space and essential nutrients found in abundance in green fodder. In autumn and winter, the antibiotics are frequently used in the bovine pathology amid falling temperatures and changes in diet.

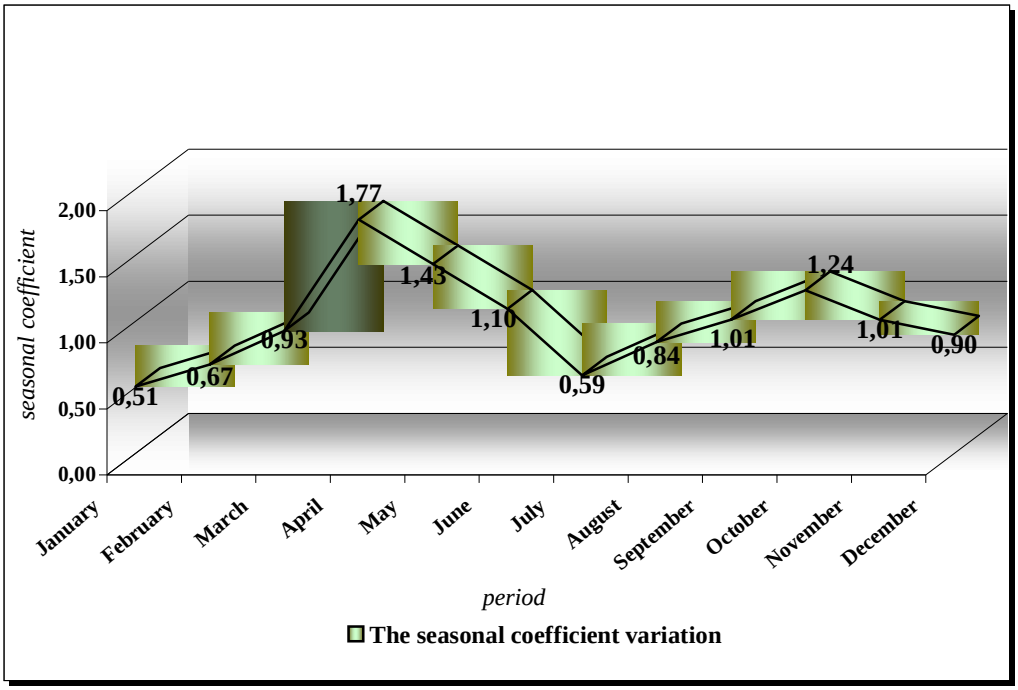

Figure 2. Season variation of the antibiotic positive (+) samples.

The differences between the observed and expected frequencies of the contaminations with antibiotic residues, in according to SCC and TBC classes, with χ^2, Φ^2 (χ^2 corrected) coefficient values and the χ^2 reference value at a $\alpha = 0.01$ probability, are presented in Tables 3 and 4.

Table 3

Interpretation of the match test between the antibiotic contamination and the somatic cell counts

SCC classes	Results of the identification antibiotic residues				Total	χ^2	Φ^2 (χ^2 corrected)	The distribution critical value χ^2_α ($\alpha = 0.01$)
	(+) samples	(±) samples	fals e (+) samples	(-) samples				
<200 000	*-48.82*	*-10.70*	*-59.90*	*119.42*	0.00			
200 000-400 000	*-31.04*	*-2.23*	*-44.35*	*77.62*	0.00	1927.56	120.47	21.67
400 000-600 000	*30.31*	*8.11*	*31.33*	*-69.75*	0.00			
>600 000	*49.54*	*4.81*	*72.93*	*-127.29*	0.00			
2006 – 2009 TOTAL	0.00	0.00	0.00	0.00	0.00			

The χ^2 and Φ^2 indicators are higher than the $\chi 2\alpha$ critical value of the theoretical distribution ($\alpha = 0.01$). This shows that among the investigated indicators (antibiotic residues vs. SCC and TBC values) is a strong correlation. Essentially, the antibiotic residue samples (+) had more than 600 000 cells/mL [$(f^o_{ij} - f^a_{ij}) = 49.54$] and more than 150,000 cfu/mL [$(f^o_{ij} - f^a_{ij}) = 50.25$]; the uncertain (±) samples contained between 400,000 and 600,000 cells/mL [$(f^o_{ij} - f^a_{ij}) = 8.11$] and 100 000 to 150 000 cfu/mL [$(f^o_{ij} - f^a_{ij}) = 5.83$]; the false-positive samples contained over 600 000 cells/ml [$(f^o_{ij} - f^a_{ij}) = 73.93$] and more than 150 000 cfu/mL [$(f^o_{ij} - f^a_{ij}) = 48.71$]; samples with no antibiotic residues (-) contained less than 200,000 cells/mL [$(f^o_{ij} - f^a_{ij}) = 119.42$] and 50,000 cfu/mL [$(f^o_{ij} - f^a_{ij}) = 108.69$].

Table 4

Interpretation of the match test between the antibiotic contamination and the total bacterial count

TBC classes	Results of the identification antibiotic residues				Total	χ^2	Φ^2 (χ^2 corrected)	The distribution critical value χ^2_α ($\alpha = 0.01$)
	(+) samples	(±) samples	false (+) samples	(-) samples				
<50 000	*-49.36*	*-9.82*	*-49.51*	*108.69*	0.00			
50 000-100 000	*-31.91*	*-0.98*	*-33.10*	*65.98*	0.00			
100 000 – 150 000	*31.02*	*5.83*	*33.90*	*-70.75*	0.00	1488.29	93.02	21.67
>150 000	*50.25*	*4.97*	*48.71*	*-103.92*	0.00			
2006 – 2009 TOTAL	0.00	0.00	0.00	0.00	0.00			

Results similar to those obtained in this study on the correlation between the contamination of milk with antibiotics and other risk factors were reported by Rüegg & Tabone (2000) who observed an increase of the somatic cell populations in samples contaminated with antibiotic residues. In that research, the milk from A class with 700,000 cells/mL presented a risk of contamination with antibiotic residues approximately seven times higher than that with less than 250,000 cells/mL. Also, the residual outruns risk was about 2.5 times higher for the milk with less than 700,000 somatic cells/mL compared to that with 551,000 to 700,000 cells/mL. For the milk from B class, the antibiotic contamination rate was about 5.5 times higher in samples with more than 700,000 cells/mL compared to those with less than 250,000 cells/mL. In contrast, the outruns risk of antibiotic residues in the milk with more than 700,000 cells/mL was approximately 1.2 times greater than in that which contains between 551,000 and 700,000 cells/mL.

After applying the Charm II test the following antibiotic families were identified (see Table 5).

Table 5

Families of antibiotics identified in the samples confirmed

Year of study	betalactams		gentamicin/ neomycin		gentamicin/ streptomycin		macrolides		tetracyclines		TOTAL	
	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%
2006	17	29.31	12	20.69	10	17.24	5	8.62	14	24.14	58	100
2007	13	29.54	12	27.27	5	11.36	3	6.82	11	25.00	44	100
2008	9	20.93	11	25.58	8	18.60	3	6.97	12	27.91	43	100
2009	9	33.33	8	29.63	3	11.11	2	7.41	5	18.52	27	100

During the four years of study, the betalactams have been identified in 27.90% of samples (n = 48), the gentamicin/neomycin aminoglycosides in 25.00% of samples (n = 43), the tetracyclines in 24.42% of the samples (n = 42), the gentamicin/streptomycin in 15.11% of samples (n = 26) and the macrolides in 7.56% of samples (n = 13). The results obtained applying the Charm II test to identify the antibiotic families confirmed some treatment practices used in the cattle farms. Thus, the betalactams are the most used antibiotics to treat infectious diseases; due to lower costs, these are included in a significant number of drug combinations. These molecules were also identified in other investigations (Ghidini et al 2002; Heeschen & Suhren 1996; McEwen et al 1991). The gentamicin/neomycin and tetracyclines were also identified in a significant number of samples; this is closely related to their use in the treatment of the mastitis.

The results of quantitative determinations of the antibiotic residues (betalactams, gentamicin, neomycin, streptomycin/dihydrostreptomycin, tylosin and tetracyclines) are presented in Table 6.

Table 6

The quantitative determinations of antibiotic residues in milk (µg/kg)

Antibiotic/ Antibiotic class	*Year*	*Samples no.*	*X*	*minimum*	*maximum*	σ_x	*V %*	*C.I.(α=0,01)*
	2006	17	29.18	14.02	37.01	6.27	21.5	29.18±3.92
betalactams	2007	13	27.63	15.07	38.73	7.06	25.56	27.63±5.05
	2008	9	25.85	13.92	34.27	6.16	23.84	25.85±5.29
	2009	9	23.94	14.75	32.57	5.82	24.33	23.94±4.33
	2006	22	210.05	98.97	310.02	66.71	31.76	210.05±38.43
gentamicin	2007	17	211.41	96.21	308.22	69.60	32.92	211.41±46.29
	2008	19	194.05	91.56	290.15	58.89	30.35	194.05±39.17
	2009	11	179.20	95.24	268.59	53.18	29.68	179.20±39.54
	2006	12	2222.79	1495.26	2870.23	440.05	19.80	2222.79±327.21
neomycin	2007	12	2082.38	1412.36	2893.27	415.84	19.97	2082.38±309.21
	2008	11	1959.75	1398.52	2672.14	352.20	17.97	1959.75±273.53
	2009	8	1929.21	1403.44	2467.21	309.06	16.02	1929.21±265.36
streptomycin	2006	10	278.85	196.77	397.77	72.89	26.14	278.85±59.37
dihydro-	2007	5	311.93	251.65	395.34	-	-	-
streptomycin	2008	8	295.39	173.20	394.57	83.65	28.32	295.39±76.18
	2009	3	236.29	198.64	275.96	-	-	-
tylosin	2006	5	92.51	62.56	110.17	-	-	-
	2007	3	103.43	75.15	129.51	-	-	-
	2008	3	89.80	69.78	103.29	-	-	-
	2009	2	105.75	87.54	123.95	-	-	-
tetracyclines	2006	14	261.66	143.16	398.24	87.49	33.44	261.66±60.23
	2007	11	324.24	167.49	458.72	101.31	31.25	324.24±78.68
	2008	12	250.91	159.45	374.58	90.22	35.96	250.91±67.08
	2009	5	248.91	172.62	424.59	-	-	-

The betalactams were determined at average concentrations of 23.94 - 29.18 µg/kg. Gentamicin was determined at average concentrations of 179.20 - 211.41 µg/kg. Neomycin was determined at average concentrations ranging between 1929.21 and 2222.79 µg/kg. Streptomycin/dihydrostreptomycin residues were determined at average concentrations of 236.29-311.93 µg/kg. Tylosin was determined at average concentrations of 89.80-105.75 µg/kg. Tetracyclines were found at average concentrations of 248.91-324.24 µg/kg.

The most concentrations of antibiotics did not exceed the acceptable daily intakes or the maximum limits to be taken with foods by an adult, except penicillins, for which was not established an acceptable daily intake (Table 7). However, the samples with antibiotic residues after the microbial quality test application are not directed for human consumption. Moreover, the introduction of milk contaminated with antibiotics in the technological flow processing would be contrary to the interests of producers to obtain appropriate products, knowing that these drugs interfere with the coagulation processes based on lactic bacteria activity.

Table 7

Limits of the antibiotic residues in milk for human consumption

Antibiotic residues	*Limits*			
	MRL in milk µg/kg	Norm in force	ADI µg/kg bodyweight	Norm in force
Penicillin G, amoxicillin, ampicillin.	4	Council Regulation, 1990	-	-
Cloxacillin, dicloxacillin, nafcillin, oxacillin.	30		-	-
Gentamicin	100	NSVFSA Ord., 2005	0 - 4	WHO, 1995
Neomycin	1500		60	WHO, 2003
Streptomycin / dihydrostreptomycin	200		50	WHO, 2000
Tylosin	50		6	WHO, 2008
Tetracyclines	100		25	FDA

The results obtained from the analysis of milk from the NE Romania (Moldavia) are considered satisfactory, given the interest of farmers and processors to obtain the least contaminated products and even organic ones is more and more obvious.

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Authors:
Andrei Cristian Grădinaru, University of Agricultural Sciences and Veterinary Medicine of Iasi, Faculty of Veterinary Medicine, 8 Sadoveanu Alley, 700489, Iasi, Romania; andre_gradinaru@yahoo.com
Ovidiu Popescu, Purdue University, Veterinary School, 625 Harrison Street, West Lafayette, IN 47907 USA (765) 494-7607.
Gheorghe Solcan, University of Agricultural Sciences and Veterinary Medicine of Iasi, Faculty of Veterinary Medicine, 8 Sadoveanu Alley, 700489, Iasi, Romania.
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