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EFFECTS OF TETRACYCLINE ON RUMINAL ACTIVITY AND BLOOD CALCIUM IN SHEEP

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ABSTRACT

The objective of this study was to assess the effects of tetracycline administration on the frequency of ruminal contractions and serum calcium concentrations. Rumen contractions were monitored by auscultation in 23 sheep prior to the administration of oxytetracycline and recorded every 12 hours for 84 hours after the intramuscular injection of the antibiotic. The blood for calcium analyses was collected by venipuncture of the jugular vein before and at 24, 48, 72, and 96 hours after the administration of oxytetracycline. The serum calcium concentrations were determined by atomic absorption spectrophotometry. The analysis of variance (ANOVA) was used to analyse the time effect of tetracycline treatment on the rumen contractions and serum calcium concentrations. There was a significant decrease ($P < 0.01$) in ruminal contractions following the application of oxytetracycline, with a maximum decrease at 24 hours following oxytetracycline application and a return to the initial rumen contraction frequency by 60—72 hours following the oxytetracycline application. The oxytetracycline administration resulted in a serum calcium decrease from 2.42 mmol.l^{-1} to 2.26 mmol.l^{-1} 24 hours after the administration ($P < 0.01$). In conclusion, the administration of tetracycline in sheep can be associated with a decline in ruminal motility potentially causing production losses, particularly in lactating ewes. Despite the resulting transient production decreases, oxytetracycline remains the antibiotic drug of choice for the treatment of bacterial infections in small ruminants, including foot rot especially.

Key words: calcium; rumen activity; sheep; tetracycline

INTRODUCTION

Belonging to broad-spectrum antibiotics, the group of tetracyclines is composed of: tetracycline, oxytetracycline, doxycycline and minocycline. They are actively transported into prokaryotic cells and inhibit protein synthesis by competing with tRNA for the A site of the ribosome. They

have a bacteriostatic effect. Tetracyclines chelate metal ions such as calcium, magnesium, iron and aluminium, forming non-absorbable complexes. Therefore if given orally, the presence of milk, antacids or iron preparations can decrease their absorption. The undesirable effects of the drug include gastrointestinal disturbances due to direct irritation and modification of the normal gut flora, and vitamin B complex deficiency can occur as a consequence. Since they chelate with Ca^{2+} ions, tetracyclines are deposited in bones and teeth, causing staining and sometimes dental hypoplasia and bone deformities [9]. Rumen and abomasal motilities are reduced in hypocalcaemia due to the general effects of a depression of levels of ionised calcium on smooth muscle contractility and on neuromuscular transmission [6, 7].

Tetracyclines are widely used in the treatment of the foot rot in ruminants. In a study of 209 sheep farmers, those which treated sheep with foot rot using parenteral antibiotics and foot sprays had a peak prevalence of 2 %, as opposed to a peak prevalence of 9 % in farmers who treated foot rot by hoof trimming and topical spraying [14]. In another study, the treatment of foot rot with parenteral oxytetracycline reduced clinical lameness in sheep significantly [10].

The aim of this study was to assess the effects of long acting tetracycline administration on the number of ruminal contractions and serum calcium concentrations.

MATERIALS AND METHODS

Rumen contractions were monitored in 23 adult merino female sheep (mean body weight 49 kg) prior to the administration of oxytetracycline and recorded every 12 hours for 84 hours after the intramuscular injection of the antibiotic. The sheep were admitted to the clinic for treatment of foot rot. After the diagnosis of foot rot, patients were treated with a single intramuscular injection of a long acting oxytetracycline (Tetradur LA 300, 30 $mg.kg^{-1}$). The drug was administered into the neck muscles (5 ml). The feeding of the experimental animals consisted of 0.2 kg of concentrates, and free access to hay and water. The ruminal contractions were counted by placing a stethoscope in the para-lumbar fossa on the left side of the animal and counting the number of ruminal contractions over a 5 minute period. In addition, the intensity of the rumen contractions were assessed by auscultation at the same time. The blood was collected by venipuncture of the jugular vein before and 24, 48, 72, and 96 hours after the administration of oxytetracycline. The serum calcium concentrations were determined by flame AAS method (Perkin Elmer AAnalyst 100).

The analysis of variance (ANOVA) with the post hoc Bonferroni test (IBM SPSS Statistics 23, 2015) was used to analyse the time effect of tetracycline treatment on the rumen contractions and serum calcium concentrations.

RESULTS

The ANOVA revealed a significant decrease ($P < 0.01$) in ruminal contractions following the application of oxytetracycline, with a maximum decrease at 24 hours following the oxytetracycline application and a return to the initial rumen contraction frequency by 60—72 hours following the oxytetracycline application (Table 1). As well as a reduction in the frequency of ruminal contractions, there was a decrease in the intensity of the contractions. In general, the contractions became quieter and sometimes difficult to distinguish effectively. Typically around 24 hours after the antibiotic administration, the intensity was at its lowest, coinciding with the greatest decrease in frequency. The strength of contractions gradually returned to normal over the same time period as the contraction frequency.

The oxytetracycline administration resulted in a serum calcium decrease from 2.42 $mmol.l^{-1}$ to 2.26 $mmol.l^{-1}$

Table 1. The rumen contractions and serum calcium after oxytetracycline administration (x ± s)

Collection time hours	Rumen contractions n/5 min	Serum calcium [$Mmol.l^{-1}$]
0	7.31 ± 1.55	2.42 ± 0.11
12	5.17 ± 1.32*	
24	4.09 ± 1.16*	2.26 ± 0.04*
36	5.00 ± 1.21*	
48	5.96 ± 1.19*	
60	7.17 ± 1.34	
72	7.65 ± 1.37	2.28 ± 0.09*
84	7.57 ± 1.34	
96		2.41 ± 0.05
ANOVA	P < 0.01	P < 0.01

* — values differ from 0 at P < 0.05 (Bonferroni test)

24 hours after the administration (Table 1). The serum calcium concentrations continued to decrease reaching the lowest values 48 hours after the drug injection (2.23 mmol.l^{-1}). The ANOVA showed a strong significant effect of the oxytetracycline administration on serum calcium ($P < 0.01$).

DISCUSSION

The rumen motility has a direct effect on the productivity levels of livestock, particularly on milk production. In sheep used for milking or ewes nursing lambs, a decreased rumen motility can cause a significant drop in milk quantity or decreased weight gains in lambs. The number of ruminal contractions is dependent upon: the type of feed (forage, concentrate), feed quality (herbage, rice straw), the form in which feed is ingested (hay, pellets), the amount of feed consumed and rumen wall stimulation [4]. The ruminal contraction frequency is highest during feeding (2.7 frequency.min^{-1}). The second highest frequency is recorded during rumination (2.3 frequency.min^{-1}), while the lowest frequency is recorded during rest periods (2.0 frequency.min^{-1}) [13]. In the present experiment in 23 sheep, the frequency of ruminal contractions decreased by 44 %, with the lowest number of contractions recorded 24 hours after application of long acting oxytetracycline. However, our study did not take into account other factors affecting rumen contractions, such as feeding regimen, composition of the dietary ration, underlying disease processes, and environmental temperature [2, 11].

Rumenal and abomasal motilities are reduced in hypocalcaemia due to the general effects of a depression of the levels of ionised calcium on smooth muscle contractility and on neuromuscular transmission [5]. In general, the tetracyclines can be divided into three groups based on their pharmacokinetic and antibacterial properties. The mostly used drugs are from the group 1. This group consists of the older agents which have reduced absorption and are less lipophilic than newer drugs and include tetracycline, oxytetracycline, chlortetracycline, demeclocycline (demethyl chlorotetracycline), lymecycline, methacycline, and rolitetracycline. All of these tetracyclines form insoluble complexes with calcium, magnesium, iron and aluminium, which can markedly reduce their absorption [1]. According to a pharmacodynamic study, intramuscular administration of oxytetracycline resulted in plateau-shaped concentration-time curves in serum and synovial fluid. Peak levels in serum (1.68 ± 0.47 μg.ml^{-1}) occurred at 3—8 hours post injection [3]. As oxytetracycline chelates Ca^{2+} ions [9], once the antibiotic reaches the bloodstream it will chelate with calcium in the serum, resulting in hypocalcaemia. One mole of oxytetracycline may form complexes with 1—2 moles of calcium, depending on the relative concentrations [8].

The calcium homeostatic mechanism operates very tightly to maintain extracellular calcium within physiological ranges. A change in physiological status, such as the initiation of lactation, creates a rapid disturbance in this mechanism. Plasma calcium exchanges with a large mass of calcium in soft tissues and bone surfaces, which may function to buffer the effect of such rapid changes in these pools. The sufficient calcium levels are necessary to decrease the incidence of periparturient hypocalcaemia (milk fever) in sheep [12]. The results presented in this study did reveal a significant serum calcium-lowering effect when each of the 23 animals were given oxytetracycline. The drop in blood calcium was not associated with any clinical sign, like neurological or locomotor disturbances. However, most of the experimental animals demonstrated a decrease in ruminal contractions.

In conclusion, the administration of tetracycline in sheep can be associated with a decline in ruminal motility, potentially causing the decline in ruminal motility which can cause production losses, particularly in lactating ewes. Despite the resulting transient production decreases, oxytetracycline remains the antibiotic drug of choice for the treatment of bacterial infections in small ruminants, especially foot rot.

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DEMOGRAPHIC FACTORS INFLUENCING THE RABIES ANTIBODY PREVALENCE OF DOGS IN FEDERAL CAPITAL TERRITORY, NIGERIA

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ABSTRACT

Canine rabies is enzootic in Nigeria occurring in all parts of the country. Rabies has been reported in Niger state neighbouring the Federal Capital Territory (FCT) and the movement of rabid dogs between the neighbour states is possible. Hence, a study to determine the immune status of dogs in Abuja to rabies was necessary. A cross sectional study was carried out to assess the rabies antibody titre of owned dogs and the rabies knowledge, attitude and practices of the dog owners. Serum samples from 276 dogs were collected and a structured questionnaire administered to each dog owner using a personal interview method. Associations between the demographic variables, protection titres and knowledge attitude and practice (KAP) were assessed using χ^2 analysis. Sera samples were analysed to measure for rabies antibodies using an indirect enzyme linked immunosorbent assay. Out of the 276 dogs sampled, 229 (83%) had a certified antirabies vaccination record. All vaccinated dogs had antibody titre against rabies greater than 0.6 EU.ml^{-1}. The dog owners had a mean knowledge score of 63.54 ± 23.82%, mean attitude score of 81.45 ± 20.37% and the mean practice score was 91.3 ± 21.39%. There was a significant association between the vaccination status of the dogs and categorized knowledge score ($P < 0.05$), attitude score ($P < 0.05$) and practice score ($P < 0.05$). A large proportion of the dogs (47.4%) owned by residents of satellite towns were not vaccinated against rabies. Hence mass vaccination of dogs in these suburban settlements is strongly recommended

Key words: antibody; dog owners; rabies

INTRODUCTION

Rabies is a zoonotic disease of high public health importance which has been in existence since 3000 BC [16, 21]. Dogs are universally accepted to be the most important source of the transmission of rabies to humans [8, 17, 20]. There have been few rabies cases where recovery has occurred, and they were predominantly caused by the bat variant rabies viruses [4, 5]. Most human rabies deaths

occur in the developing countries and although effective and economical control measures are available, their application in developing countries have been hampered by a range of economic, social and political factors [14, 21]. It is widely recognized that the number of deaths officially reported in most developing countries greatly underestimates the true incidence of the disease, with several factors contributing to this widespread under reporting [21]. The prevalence rate of rabies in Nigeria is between 15—20 % and the true picture cannot be easily determined because of under reporting [10]. The preventive vaccination against rabies virus is a highly effective method for preventing rabies in humans and other animals. In Nigeria where dog bites continue to be the main mode of transmission of the disease to humans, it remains a serious public health hazard [1, 12]. Documented reports have been published citing cases of rabies in a few vaccinated dogs [1, 13]. The World Health Organization stipulates a minimum of 0.5 IU.ml^{-1} serum levels of rabies antibody titre for the confirmation of immunity against rabies [21]. The serological evidence of rabies virus neutralizing antibodies in the serum of vaccinated and unvaccinated rabies occupational risk groups have been reported in Niger state bordering Federal Capital Territory (FCT) [9]. Rabies virus neutralizing antibodies can be present in roaming or hunting dogs that have been exposed to rabies virus (carriers) but the rabies virus antibodies are usually at suboptimal levels [15]. A knowledge attitude and practice (KAP) study is a social research method (survey) targeted at measuring changes in human knowledge, attitudes and practices in response to a specific project activity; usually involving education or outreach [6]. It is a representative study conducted on a specific population to collect information on what is known, believed and done in relation to any subject of interest [18]. Understanding the levels of knowledge, attitude and practice will enable a more efficient process of awareness creation as it will allow the program to be tailored more appropriately to the needs of the community [11]. Rabies knowledge, attitude and practice studies have been conducted in different parts of Nigeria on dog owners and dog meat processors with varied KAP scores [3, 7]. In the KAP study conducted on Abuja Municipal Area Council residents, 82 % of the respondents had a satisfactory knowledge of rabies, 165 (74 %) had a positive attitude and 91 (75 %) of dog owners had a satisfactory practice [7]. Rabies has remained endemic in Nigeria and has been reported in slaughtered dogs in Abuja Municipal Area of the Federal Capital Territory [7].

The aim of this study is to evaluate some demographic factors influencing the antibody response of dogs against rabies, the prevalence of rabies antibodies in owned dogs and determine the level of compliance of dog owners resident in the Federal Capital Territory of Nigeria to preventive canine rabies vaccination.

MATERIALS AND METHODS

Study area

This study was carried out in three phases and two major satellite towns (Kubwa and Gwagwalada) in the Fed-

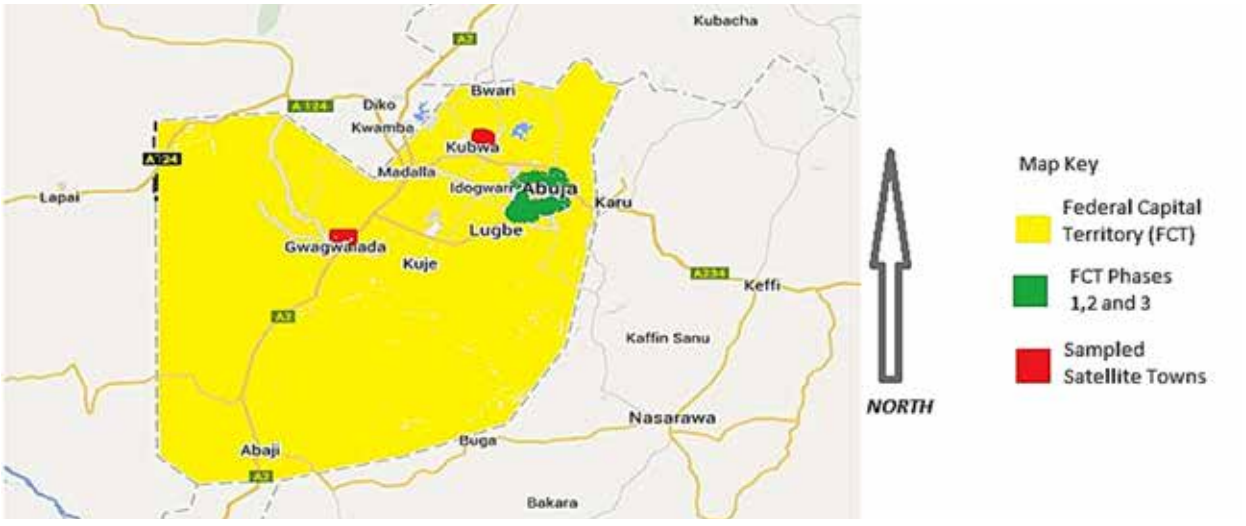

Fig. 1. Modified Google map of the Federal Capital Territory of Nigeria [19]

eral Capital Territory (FCT) of Nigeria. Abuja is the federal capital territory of Nigeria; it is a planned city with districts organized into three major phases (i.e. phases 1, 2 and 3). It is bordered by Kaduna state in the North, Nasarawa state in the South-East, Kogi state in the South-West and Niger state in the West. Geographically it lies between latitude 9˚4'0" North and longitude 7˚29'0" East with an area of 713 km^2 and had a population of 776,298 at the 2006 census. It has a few state-owned veterinary hospitals/clinics including the University of Abuja Veterinary Teaching Hospital and a host of private veterinary clinics dispersed across major commercial districts. English is the official language in Abuja and well-spoken by most residents. Abuja was formerly inhabited by the Gbagyi or Gbari (commonly known as Gwari) tribe who are predominantly farmers now resettled in bordering states, but a few pockets of locals are still found in areas such as Gwagwalada [19].

Sample size

A major limitation in this study was the fact that most dog owners in the Federal Capital Territory of Nigeria reside in estates with strict security setups therefore could not be easily accessed, this limited the sample size obtained within the specified period (December 2014 to February 2015). Two hundred and seventy-six samples were collected for this study due to accessibility and time (December 2014 to February 2015) constraints.

Survey methods

A total of 276 structured questionnaires were administered to certified dog owners following a pre-test survey conducted on 20 dog owners within the study area. A structured questionnaire was designed and pre-tested (employing personal interviews) on 20 dog owners within the study area. The pre-test questionnaires were then analysed and restructured for better effectiveness. A total of 276 restructured questionnaires were then administered to the dog owners (one questionnaire per dog) in 8 major cadastral zones within the 3 phases of Abuja metropolis (Wuse 2 and Asokoro in phase 1, Jabi in phase 2, Gwarinpa and Life Camp in phase 3) and two satellite towns (Gwagwalada and Kubwa) with the assistance of 8 private veterinary clinics located within these zones. The questionnaire administered comprised of the demographic information of the dog owners contained in section A, zoographic information and vaccination history of the owned dog was obtained in section B, whilst the knowledge, attitude and practice of the dog owners as it pertained to rabies was contained in sections C, D and E respectively which included questions on the mode of transmission, clinical symptoms and preventive measures.

The personal interviews were conducted by the researcher explaining the questionnaire to the dog owners and the responses were recorded. Responses for the Knowledge section was coded, "Yes", "No", and "Don't know"; the Attitude section was coded, "Agree", "Disagree", and "Indifferent"; the Practice section was coded, "Yes", or "No". A marking scheme of expected correct answers was prepared and used to grade the responses. "Don't know", "Indifferent", or undecided responses were considered wrong answers. Each correct response earned one point, whilst every wrong response was graded zero.

Sample collection

A total of 276 canine serum samples were collected from Wuse 2 and Asokoro in phase 1, Jabi in phase 2, Gwarinpa and Life Camp in phase 3. Canine serum samples were also collected from two satellite towns of Gwagwalada and Kubwa based on availability of dogs and the cooperation of the owners.

The dogs were restrained properly, the site for blood collection (cephalic vein) was well swabbed using a mild disinfectant and with the aid of sterile needles and syringes (22-gauge 5 ml syringes), 3—5 ml of blood was collected from each dog, into plain labelled sample bottles without anticoagulant and allowed to clot by placing them in a rack for 4—5 hours. The sera were then gently decanted into screw cap serum bottles and stored in the fridge (+4 °C) until they were analysed in the laboratory.

Laboratory analysis

The sera were stored at +4 °C (cumulatively for about 2 months, sera that were stored for more than 7 days were stored in the freezer compartment for stability) until tests were conducted using an ELISA technique (SERELISA® Rabies Ab Mono Indirect Kit). The optical density readings of the test wells were read using a monochromatic plate reader at 450 nm. The rabies antibody titre of sampled dogs was then deduced.

Statistical analysis

All data were collated and sorted out using Microsoft

EXCEL spreadsheet; analyses of the data were carried out using a Statistical Package for Social Sciences software, SPSS (version 17, SPSS Inc. Chicago IL USA). A regression analysis of the World Health Organisation standard serum optical readings was used to derive the mathematical model which was subsequently used to derive the equivalent unit per millilitre ($EU.ml^{-1}$) of antibody titre in each serum sample. A calculated titre greater than $0.6\,EU.ml^{-1}$ was considered protective, whilst a calculated titre of less than $0.6\ EU.ml^{-1}$ was considered nonprotective based on the kit's recommended standard. The association between the dependent variable and independent variables were assessed using a Chi-Square (χ^2) analysis at 95 % confidence interval; a P-value less than 0.05 were considered significant. A scatter plot was utilized to observe the relationship between the time lapse from vaccination to sample collection and the antibody titre of the sample sera. The mean knowledge, attitude and practice scores were computed, respondents with knowledge, attitude and practice scores equal or greater than the mean scores were considered good knowledge, attitude and practice, whilst those who had scores below the mean were categorized as having poor knowledge, attitude and practice [3]. The associations between demographic variables and categorized scores were assessed using χ^2 test of association at 95 % confidence intervals. P values less than 0.05 were considered significant in the χ^2 analysis. Binary regression analyses were carried out to assess the relationships between non-categorized scores.

RESULTS

The relevant demographic characteristics of dog owners and zoographic characteristics of dogs sampled in FCT, Nigeria, are presented in Tables 1 and 2.

Vaccination coverage of the dogs

A total of 200 (72.5 %) dogs were sampled in the City centre, i. e. phases 1, 2, and 3. The sum of 118 samples were collected in phase 1 out of which 109 (92.37 %) were vaccinated; in phase 2, 33 samples were collected out of which 32 (96.97 %) of the dogs had certificates of antirabies vaccination. The sum of 49 samples were collected in phase 3 out of which 48 (97.96 %) dogs had certificates of antirabies vaccination whilst out of the 76 dogs in the satellite towns, 40 (52.63 %) were certified vaccinated. A total of 276 dogs were sampled, 228 (82.6 %) were of exotic breed origin and 48 (17.4 %) were indigenous. Notably, 218 (95.6 %) of the exotic breed of dogs were vaccinated whilst only 11 (22.9 %) of the indigenous breed of dogs were vaccinated.

Rabies antibody profile (prevalence) of owned dogs

a) Prevalence of rabies antibodies in the dogs

A total of 276 serum samples were collected, processed and analysed during this study. Out of the 276 dogs, all vaccinated dogs i. e. 239 (86.6 %) had rabies antibody titre $> 0.6\,EU.ml^{-1}$ whilst all unvaccinated dogs, i. e. 37 (13.4 %) had less than $0.6\,EU.ml^{-1}$. There was a marked decline in rabies antibody titre with increase in time post vaccination

Table 1. Demographic characteristics of dog owners in FCT, Nigeria (December 2014 to February 2015)

Variables		Total number of respondents N = 276	Percentages [%]
Area of residence	City Centre (phases 1, 2, 3)	200	72.5
	Satellite towns/ Suburbs	76	27.5
Gender	Male	243	88.0
	Female	33	12.0
Occupation	Corporate institution personnel	153	55.4
	Traders	123	44.6
Education level	Tertiary	234	84.8
	Secondary/Primary	42	15.2

Table 2. Zoographic characteristics of dogs sampled in FCT, Nigeria (December 2014 to February 2015)

Variables	Response	Total number of respondents N = 276	Percentages [%]
Dog breed	Exotic breed	228	82.6
	Local breed	48	17.4
Dog's age	< 1year	17	6.2
	> 1year	259	93.8
Sex of dog	Male	158	57.2
	Female	118	42.8
Source of dog	Imported	53	19.2
	Breeder	223	80.8
Purpose for dog	Pet	86	31.2
	Breeding	25	9.1
	Security	165	59.8
Vaccination status	Certified Vaccinated	229	83.0
	Not-vaccinated	37	13.4
	Vaccinated but not certified	10	3.6

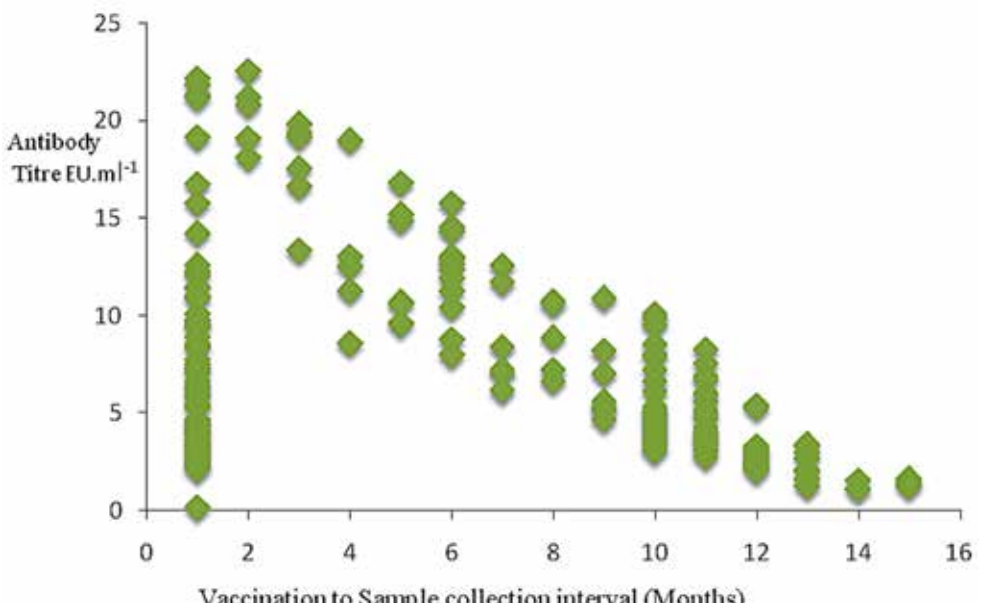

Fig. 2. Association between vaccination to sample collection interval and antibody titre of the dogs in FCT, Nigeria (December 2014 to February 2015)

as shown in Figure 2. For clarity, 0.5—1.4 months was categorized as 1 month, 1.5—2.4 months as 2 months, etc.

b) Association between demographic characteristics of dog owners and the rabies antibody titre of the dogs

The sum of 200 dogs was sampled in the City centre (phases 1, 2 and 3). Notably, all the dogs in FCT phases 1and 2 had their rabies antibody titre > 0.6 EU.ml^{-1}, only 1 (2 %) out of the 49 dogs in FCT phase 3 had rabies antibody titre less than 0.6 EU.ml^{-1}. However, 36 (47.4 %) out of the 76 dogs in the satellite towns/suburbs had rabies antibody titre less than 0.6 EU.ml^{-1}. When the gender of the dog owners was considered as a possible factor that can affect the vaccination of dogs against rabies and consequently rabies antibody titre, 36 (14.8 %) of the 243 dogs owned by male dog owners had rabies antibody titre less than 0.6 EU.ml^{-1} whilst only 1 (3 %) of 33 dogs owned by female dog owners had rabies antibody titre less than 0.6 EU.ml^{-1}. Following the consideration of the occupation of dog owners as a possible factor affecting the vaccination of dogs against rabies and consequently rabies antibody titre, it was observed that all 153 (100 %) of the dogs owned by civil servants, private sector workers and students (corporate institution personnel) had rabies antibody titre > 0.6 EU.ml^{-1} whilst 37 (30.3 %) of 123 dogs owned by business inclined persons (traders) had rabies antibody titre less than 0.6 EU.ml^{-1}. It was also observed that dog owners having 1—5 dogs were 184 (66.7 %) out of the 276 dogs but had only 5 (2.7 %) dogs with rabies antibody titre lesser than 0.6 EU.ml^{-1} in contrast to dog owners that owned more than 5 dogs with their frequency of occurrence as 92 (33.3 %) out of the 276 dogs but had 32 (34.8 %) of their dogs having rabies antibody titre less than 0.6 EU.ml^{-1}. Notably also out of the 234 dogs owned by tertiary school certificate holders, only 1 (0.4 %) had rabies antibody titre less than 0.6 EU.ml^{-1} whilst 36 (85.7 %) of the dogs owned by secondary and primary school leavers had rabies antibody titre less than 0.6 EU.ml^{-1}.

Taking χ^2 as Chi square, df as the degree of freedom and P as the P-value, significant associations were observed between rabies antibody titre and the area of residence ($\chi^2 = 99.76$; df = 1; $P < 0.05$), occupation ($\chi^2 = 67.06$; df = 1; $P < 0.05$), number of dogs owned ($\chi^2 = 52.71$; df = 1; $P < 0.05$), the education of dog owners ($\chi^2 = 170.15$; df = 1; $P < 0.05$) and the gender of dog owners ($\chi^2 = 4.67$; df = 1;

Table 3. Association between demographic characteristics of dog owners and the rabies antibody titre of dogs in FCT, Nigeria (December 2014 to February 2015)

Categorical predictor variable	Protective titre frequency	Non-protective titre frequency	Likelihood ratio (LR)	Odds ratio	Confidence interval for OR (95 %)
Area of residence					
City centre (phases 1, 2, 3)	199 (99.5 %)	1 (0.5 %)	$\chi^2 = 99.76$ df = 1	0.006	0.001—0.042
Satellite towns/ Suburbs	40 (52.6 %)	36 (47.4 %)	P < 0.001	1	DV
Gender					
Male	207 (85.2 %)	36 (14.8 %)	$\chi^2 = 4.67$ df = 1	5.565	0.737—42.019
Female	32 (97 %)	1 (3 %)	P = 0.031	1	DV
Occupation					
Corporate institution personnel	153 (100)	0 (0)	$\chi^2 = 67.06$ df = 1	0.000	0.000—IND
Traders	86 (69.7)	37 (30.1)	P < 0.001	1	DV
Level of education Level of education					
Tertiary	233 (99.6)	1 (0.4)	$\chi^2 = 170.148$ df = 1	0.001	0.000—0.006
Primary/secondary	6 (14.3)	36 (85.7)	P < 0.001	1	DV

df — degree of freedom; IND — indeterminate (blank output); DV — dummy variable (cells are redundant)

P < 0.05) (Table 3). The Odds ratios showed that dog owners residing in the satellite towns, men, traders and those with only secondary or primary education had more dogs with non-protective rabies antibody titres (Table 3).

c) Association between the zoographic characteristics of sampled dogs in FCT, Nigeria and measured rabies antibody titre

Out of the 276 dogs, all 228 (100 %) of the exotic breed of dogs had rabies antibody titre > 0.6 EU.ml^{-1} whilst 37 (77.1 %) of the 48 indigenous breeds of dogs had less than 0.6 EU.ml^{-1} levels of rabies antibody titre. Age evaluation reflected that all 17(100 %) dogs lesser than 1year of age had > 0.6 EU.ml^{-1} rabies antibody titre whilst 37(14.3 %) of the 259 dogs that were > 1year of age had rabies antibody titre less than 0.6 EU.ml^{-1}. Following the evaluation of sex of the dogs as a possible factor affecting vaccination against rabies and consequently rabies antibody titre, 12 (7.6 %) of the 158 males and 25 (21.2 %) of the 118 female dogs had less than 0.6 EU.ml^{-1} rabies antibody titre. All 53 (100 %) of the dogs acquired by importation had rabies antibody titre > 0.6 EU.ml^{-1} whilst 37 (16.6 %) of the 223 dogs acquired from breeders (commercial breeders and friends whose dogs whelped) had less than 0.6 EU.ml^{-1} rabies antibody titre.

Significant associations were observed between breed ($\chi^2 = 165.83$; df = 1; P < 0.05), age ($\chi^2 = 5.06$; df = 1; P < 0.05), sex ($\chi^2 = 10.70$; df = 1; P < 0.05), source ($\chi^2 = 17.09$; df = 1; P < 0.05) and the rabies antibody prevalence of the dogs (Table 4). Also, the odds ratios showed that dogs of indigenous origin (local), acquired from friends and those raised for security purposes had more non-protective rabies antibody titres (Table 4).

Knowledge attitude and practices (KAP) of dog owners towards rabies

a) Knowledge of rabies

The mean knowledge score was 63.54 ± 23.82 % in

Table 4. Association between zoographic characteristics of dogs in FCT, Nigeria and their measured rabies antibody titre (December 2014 to February 2015)

Categorical predictor variable	Protective titre frequency	Non-protective titre frequency	Likelihood ratio (LR)	Odds ratio	Confidence interval for OR (95 %)
Breed					
Exotic	228 (100 %)	0 (0)	χ^2 = 165.83 df = 1	0.000	0.000—IND
Local	11 (22.9 %)	37 (77.1 %)	P < 0.001	1	DV
Age (years)					
< 1year	17 (100 %)	0 (0)	χ^2 = 5.064 df = 1	0.000	0.000—IND
>1year	222 (85.7 %)	37 (14.3 %)	P = 0.024	1	DV
Sex					
Male	146 (92.4 %)	12 (7.6 %)	χ^2 = 10.7 df = 1	0.306	0.146–0.638
Female	93 (78.8 %)	25 (21.2 %)	P = 0.001	1	DV
Source					
Imported	53 (100 %)	0 (0)	χ^2 = 17.09 df = 1	0.000	0.000—IND
Breeder (Commercial or friends with pups)	186 (83.4 %)	37 (16.6 %)	P < 0.001	1	DV
Purpose					
Pet	85 (98.8 %)	1 (1.2 %)	χ^2 = 33.49	0.042	0.006—0.313
Breeding	25 (100 %)	0 (0)	df = 2	0.000	0.000—IND
Security	129 (78.2 %)	36 (21.8 %)	P < 0.001	1	DV

df — degree of freedom; IND — indeterminate (blank output); DV — dummy variable (cells are redundant); P — P-value

a questionnaire of 15 items, 76 out of the 276 respondents (27.5 %) knew rabies is not curable; 146 (52.9 %) agreed rabies can affect humans and 221 (80.1 %) knew death to be the most likely the end result of rabies. One hundred and nine respondents (39.5 %) knew that rabies could be found in the saliva; all the respondents agreed that all dogs can be infected with and subsequently transmit rabies whilst 144 (52.2 %) knew that an unprovoked bite by a dog is a poten-

Table 5. Knowledge of dog owners in FCT, Nigeria, about rabies (December 2014 to February 2015)

Characteristics	Response	Total number of respondents N = 276	Percentages (%)
Rabies is curable	Yes	104	37.7
	No	76	27.5
	No idea	96	34.8
Rabies cannot affect humans	Yes	34	12.3
	No	146	52.9
	No idea	96	34.8
Death is rabies result	Yes	221	80.1
	No	0	.0
	No idea	55	19.9
Rabies is found in blood	Yes	109	39.5
	No	70	25.4
	No idea	97	35.1
All dogs can be infected/transmit rabies virus	Yes	276	100.0
	No	0	.0
	No idea	0	.0
Dogs are the major source of rabies	Yes	221	80.1
	No	0	.0
	No idea	55	19.9
Unprovoked bite is a rabies potential source	Yes	144	52.2
	No	69	25.0
	No idea	63	22.8
Rabies can never be transmitted from dog to humans	Yes	0	.0
	No	201	72.8
	No idea	75	27.2
Bite from infected animal cannot affect other animals	Yes	0	.0
	No	138	50.0
	No idea	138	50.0
Friendly dog suddenly turned aggressive may be rabid	Yes	213	77.2
	No	0	.0
	No idea	63	22.8
Excessive foamy salivation and biting tendency is not rabies sign	Yes	103	37.3
	No	35	12.7
	No idea	138	50.0
Contact with sick dog can endanger your health	Yes	276	100.0
	No	0	.0
	No idea	0	.0
Vaccination of dogs is the most effective rabies prevention method	Yes	213	77.2
	No	0	.0
	No idea	63	22.8
Dog registration and licensing help in controlling rabies	Yes	213	77.2
	No	0	.0
	No idea	63	22.8
Roaming increases the likelihood of dog rabies	Yes	221	80.1
	No	0	.0
	No idea	55	19.9

tial source of rabies. Two hundred and one of the 276 respondents (72.8 %) believe that rabies can be transmitted from dog to humans; 138 (50 %) knew that other animals can equally be infected by a bite from an infected animal.

Out of the 276 respondents, 213 (77.2 %) agreed that a friendly dog that suddenly turned aggressive may be rabid, whilst only 35 (12.7 %) knew that excessive foamy salivation and biting tendency were signs of rabies. All the respondents knew that contact with a sick dog can endanger their health; 213 (77.2 %) agreed that dog registration and licensing can help in controlling rabies and 221 (80.1 %) knew that allowing dogs to roam free increases the likelihood of dog rabies (Table 5).

b) Association between demographic variables and the categorized knowledge scores of dog owners in FCT, Nigeria

There was a significant association between categorized knowledge scores and the area of residence of dog owners ($\chi^2 = 25.59$; df = 1; $P < 0.05$), gender ($\chi^2 = 0.892$; df = 1; $P < 0.05$), occupation of dog owners ($\chi^2 = 11.66$; df = 1; $P < 0.05$) and owner's education level ($\chi^2 = 54.78$; df = 1; $P < 0.05$) (Table 6). The Odd ratios reflected the fact that dog owners in the satellite towns or suburban areas, males, traders, having not more than secondary or primary school leaving certificates had more respondents with poor knowledge about rabies.

c) Attitudes towards rabies

The mean attitude score was 81.45 ± 20.27 %. Two hundred and sixty nine out of the 276 respondents (97.5 %) agreed that bites by suspected animals should be reported immediately to the doctor and 208 (75.4 %) agreed rabies is a disease of great importance to humans. Out of the 276 respondents interviewed, 261 (94.6 %) agreed all dogs should be vaccinated against rabies; 227 (82.2 %) believe vaccinating dogs yearly is necessary to prevent rabies. Two hundred and thirty-five of the interviewed dog owners (85.1 %) agreed it is wrong to allow dogs to roam; 194 (70.3 %) agreed children must not play with stray animals and 178 (64.5 %) said washing bite wounds with soap and water before seeking medical help is necessary (Table 7).

d) Association of demographic variables to the categorized attitude scores of dog owners towards rabies

There was a significant association between catego-

Table 6. Association between demographic variables of dog owners with their categorized knowledge scores on rabies in FCT, Nigeria (December 2014 to February 2015)

Categorical predictor variable	Good knowledge	Poor knowledge	Likelihood ratio (LR)	Odds ratio	Confidence interval for OR (95 %)
Area of residence					
City centre (phases 1, 2, 3)	143 (71.5 %)	57 (28.5 %)	$\chi^2 = 25.59$ df = 1	0.246	0.141—0.429
Satellite towns/ Suburbs	29 (38.2 %)	47 (61.8 %)	P < 0.001	1	DV
Gender					
Male	149 (61.3 %)	94 (38.7 %)	$\chi^2 = 0.89$ df = 1	1.451	0.661—3.184
Female	23 (69.7 %)	10 (30.3 %)	P = 0.345	1	DV
Occupation					
Corporate institution personnel	109 (71.2 %)	44 (28.8 %)	$\chi^2 = 11.66$ df = 1	0.424	0.258—0.697
Traders	63 (51.2 %)	60 (48.8 %)	P < 0.001	1	DV
Level of education					
Tertiary	167 (71.4 %)	67 (28.6 %)	$\chi^2 = 54.78$ df = 1	0.054	0.020–0.14
Primary/secondary	5 (11.9 %)	37 (88.1 %)	P < 0.001	1	DV

df — degree of freedom; IND — indeterminate (blank output); DV — dummy variable (cells are redundant); P — P-value

Table 7. Attitude of dog owners in FCT, Nigeria, towards rabies (December 2014 to February 2015)

Variable N = 276		Frequency	Percentages [%]
Bite by suspected animal should be reported to the doctor	Agree	269	97.5
	Disagree	0	.0
	Indifferent	7	2.5
Rabies is of little importance to humans	Agree	34	12.3
	Disagree	208	75.4
	Indifferent	34	12.3
All dogs should be vaccinated against rabies	Agree	261	94.6
	Disagree	0	.0
	Indifferent	15	5.4
Vaccinating dogs yearly is not necessary	Agree	15	5.4
	Disagree	227	82.2
	Indifferent	34	12.3
It is wrong to allow dogs to roam	Agree	235	85.1
	Disagree	34	12.3
	Indifferent	7	2.5
Children must not play with stray animals	Agree	194	70.3
	Disagree	34	12.3
	Indifferent	48	17.4
Washing bite wounds with soap and water before medical help is necessary	Agree	178	64.5
	Disagree	41	14.9
	Indifferent	57	20.7

Table 8. Association of demographic variables to the categorized attitude towards rabies scores of dog owners in FCT, Nigeria (December 2014 to February 2015)

Categorical predictor variable	Positive attitude	Negative attitude	Likelihood ratio (LR)	Odds ratio	Confidence interval for OR (95 %)
Area of residence					
City centre (phases 1, 2, 3)	146 (73 %)	54 (27 %)	$\chi^2 = 20.54$ df = 1	0.284	0.164–0.492
Satellite towns/ Suburbs	33 (43.4 %)	43 (56.6 %)	P < 0.001	1	DV
Gender					
Male	154 (63.4 %)	89 (36.6 %)	$\chi^2 = 2.06$ df = 1	1.806	0.781–4.174
Female	25 (75.8 %)	8 (24.2 %)	P = 0.151	1	DV
Occupation					
Corporate institution personnel	112 (73.2 %)	41 (26.8 %)	χ^2 =10.50 df = 1	0.438	0.265–0.725
Traders	67 (54.5 %)	56 (45.5%)	P < 0.001	1	DV
Level of education					
Tertiary	168 (71.8 %)	66 (28.2 %)	$\chi^2 = 31.18$ df = 1	0.139	0.066–0.293
Primary/secondary	11 (26.2 %)	31 (73.8 %)	P < 0.001	1	DV

df — degree of freedom; DV — dummy variable (cells are redundant); P — P-value

rized attitude scores and area of residence of dog owners ($\chi^2 = 20.54$; df = 1; $P < 0.05$), occupation ($\chi^2 = 11.66$; df = 1; $P < 0.05$), and level of education ($\chi^2 = 54.78$; df = 1; $P < 0.05$) (Table 8). The odds ratios reflected the fact that dog owners in the satellite towns or suburban areas, males, traders, having not more than secondary or primary school leaving certificates had more respondents with negative attitude towards rabies prevention.

e) Practices of dog owners in FCT, Nigeria, towards rabies prevention

The mean practice score was 91.3 ± 21.39 % in a questionnaire with 6 items. Out of the 276 respondents, 213 (77.2 %) vaccinated their dogs against rabies, 254 (92 %) do take their dogs for clinical attention regularly and 254 (92 %) do not allow their dogs to roam. Two hundred and sixty-one (94.6 %) of the dog owners do not go for hunting with their dogs. All the respondents seek medical attention in the advent of a dog bite and 254 (92 %) keep a record of treatments administered to their dogs (Table 9).

f) Association of demographic variables of dog owners in FCT, Nigeria, to the categorized rabies prevention practice scores

There was a significant association between categorized practice scores and area of residence of dog owners ($\chi^2 = 26.43$; df = 1; $P < 0.05$), occupation ($\chi^2 = 16.20$; df = 1; $P < 0.05$), and level of education ($\chi^2 = 50.57$; df = 1; $P < 0.05$) (Table 10). The odd ratios reflected the fact that dog owners in the satellite towns or suburban areas, traders, having not more than secondary or primary school leaving certificates had more respondents with poor rabies preventive practices.

g) Association of rabies KAP scores of dog owners and the rabies antibody titre of dogs

Most, i. e. 171 (99.4 %) of the 172 dog owners with "Good" rabies knowledge scores had dogs with protective rabies antibody titres. Significant associations were observed between the rabies knowledge ($\chi^2 = 71.05$; df = 1; $P < 0.05$), attitude ($\chi^2 = 33.80$; df = 1; $P < 0.05$) and practice ($\chi^2 = 62.36$; df = 1; $P < 0.05$) scores (Table 11).

DISCUSSION

The zoographic information about the sampled dogs showed that a large proportion of the dogs were of exotic breed origin. This can be attributed to the aesthetic sentiment attached to owning foreign breeds of dogs and the relatively high cost of selling its offspring when compared to the indigenous breed of dogs. Most of the dogs were within 1—5 years of age which can be attributed to the agility and strength of the 1—5 years age group. Dogs acquired

Table 9. Rabies preventive practices of dog owners in FCT, Nigeria (December 2014 to February 2015)

Variable N = 276		Frequency	Percentages [%]
Do you vaccinate your dogs against rabies?	Yes	213	77.2
	No	63	22.8
Do you take your dog for clinical attention?	Yes	254	92.0
	No	22	8.0
Do you allow your dog roam?	Yes	22	8.0
	No	254	92.0
Do you take your dog for hunting?	Yes	15	5.4
	No	261	94.6
Do you seek medical attention in the advent of an animal bite?	Yes	276	100.0
	No	0	.0
Do you keep a record of treatments administered to your dog?	Yes	254	92.0
	No	22	8.0

Table 10. Association of demographic variables of dog owners in FCT, Nigeria, to categorized rabies preventive practice scores (December 2014 to February 2015)

Categorical predictor variable	Good practice	Bad practice	Likelihood ratio (LR)	Odds ratio	Confidence interval for OR (95 %)
Area of residence					
City centre (phases 1, 2, 3)	171 (85.5 %)	29 (14.5 %)	$\chi^2 = 26.43$ df = 1	0.209	0.115—0.382
Satellite towns/ Suburbs	42 (55.3 %)	34 (44.7 %)	P < 0.001	1	DV
Gender					
Male	187 (77 %)	56 (23 %)	$\chi^2 = 0.056$ df = 1	1.112	0.458—2.699
Female	26 (78.8 %)	7 (21.2 %)	P = 0.813	1	DV
Occupation					
Corporate institution personnel	132 (86.3 %)	21 (13.7 %)	$\chi^2 = 16.20$ df = 1	0.307	0.170—0.555
Traders	81 (65.9 %)	42 (34.1 %)	P < 0.001	1	DV
Level of education					
Tertiary	200 (85.5 %)	34 (14.5 %)	$\chi^2 = 50.57$ df = 1	0.076	0.036—0.16
Primary/secondary	13 (31 %)	29 (69 %)	P < 0.001	1	DV

df — degree of freedom; DV — dummy variable (cells are redundant); P — P-value

Table 11. Association of KAP scores and rabies antibody titre of dog owners in FCT, Nigeria (December 2014 to February 2015)

Categorical predictor variable	Protective titre frequency	Non-protective titre frequency	Likelihood ratio (LR)	Odds ratio	Confidence interval for OR (95 %)
Knowledge					
Good	171 (99.4)	1 (0.6)	$\chi^2 = 71.048$	0.011	0.001—0.082
Poor	68 (65.4)	36 (34.6)	P < 0.001	1	DV
Attitude					
Positive	171 (95.5)	8 (4.5)	$\chi^2 = 33.802$	0.110	0.048—0.252
Negative	68 (70.1)	29 (29.9)	P < 0.001	1	DV
Practice					
Good	205 (96.2)	8 (3.8)	$\chi^2 = 62.359$	0.046	0.019—0.108
Bad	34 (54)	29 (46)	P < 0.001	1	DV

df — degree of freedom; DV — dummy variable (cells are redundant); P — P-value

from friends or neighbours were predominant as they can be purchased at low prices from trusted sources. Most of the dogs were kept for security purposes because this is one of the chief reasons why people purchase dogs. Most of the dogs in phases 1, 2 and 3 had certified anti-rabies vaccination records whilst only 52.63 % of dogs in the satellite towns or suburban area had records of anti-rabies vaccination. The people most affected by rabid dog bites usually live in poor rural communities where medical resources are often sparse; these communities have problems that are often underscored by politicians and health authorities usually based in capital cities and are poorly informed about major public health issues affecting rural/suburban settlements [22]. This agrees with the study conducted in Abuja Municipal Area Council (city centre) where only 13.8 % of the dog owners had no evidence of anti-rabies vaccination of their dogs as documented by Edukugho [7]. Most of the exotic breed dogs had certified anti-rabies vaccination record whilst a meagre 22.9 % of the indigenous breed of dogs had anti-rabies vaccination record which is most likely since more care and attention is given to exotic breed of dogs by owners because of the high cost of purchasing them and the high returns to be generated from their litters.

There was an obvious decline in rabies antibody titre with increased time post anti-rabies vaccination with a marked decline after one year of vaccination (Figure 2). This can be attributed to the fact that rabies antibody titre wane with time, hence dog owners are advised to vaccinate their dogs against rabies yearly. The seroprevalence of rabies antibodies in FCT, Nigeria, was found to be 86.6 % with all exotic breeds having greater than 0.6 $EU.ml^{-1}$ whilst indigenous breed had the seroprevalence of only 22.9 %. This is at variance with the seroprevalence of rabies antibodies in Ilorin (Kwara State, Nigeria) where a seroprevalence of 49.1 % was observed in exotic breeds and 32.4 % was observed in the indigenous breed as documented by Aiyedun [2]. There was a significant association ($P < 0.05$) between the area of residence of dog owners in FCT, Nigeria and the antibody titre of the sampled dogs. This is probably since Abuja being the Federal Capital of Nigeria is a planned city with the upper social class residing mainly in the city centre (phases 1, 2, and 3), whilst the not so privileged reside in the satellite towns and suburban areas. The significant association between the occupation of dog owners in FCT, Nigeria, and the rabies antibody titre of dogs might be largely since the occupation is often a direct reflection of the level of education and traders are usually busy people having little or no time to vaccinate their dogs. There was a significant association ($P < 0.05$) between the number of dogs owned and the rabies antibody titre of dogs, as most of the dog owners with > 10 dogs were mainly commercial breeders, therefore they tried to keep their dogs as fit for sale as possible. The significant association between the education level and rabies antibody titre ($P < 0.05$) shown reflected the fact that the higher the level of education the more the likelihood of anti-rabies vaccination as education increases enlightenment about the dangers associated with not vaccinating dogs against rabies.

The association of dog breed to rabies antibody titre was significant, the cost of procuring exotic breed of dogs might be the major reason why dog owners pay more attention to their exotic breed of dogs and ensure their vaccination protocols are adhered to hence all the exotic breed of dogs had the required rabies antibody titre. This agrees with the seroprevalence of rabies antibodies in dogs in Ilorin (Kwara State, Nigeria) where there was significant association between the breed of the dogs and their rabies antibody titre with the exotic breeds having a higher seroprevalence than the indigenous breed as documented by Aiyedun [2]. A significant association between the age of dogs and their rabies antibody titre ($P < 0.05$) was observed, which might be due to the fact that more attention/care is usually given to newly acquired animals hence nearly all the dogs within 6 months to 5 years had the required rabies antibody titre (> 0.6 $EU.ml^{-1}$) and all the dogs older than 10 years of age also had their required rabies antibody titre as attachment to old dogs can also make dog owners pay more attention to their dogs. There was a significant association between the sex of dogs and their rabies antibody titre. This may probably be since male dogs are usually kept for security reasons and their vaccination protocol are thus adhered to. The association of the source of dogs and their rabies antibody titre was significant ($P < 0.05$), this might be largely since dogs acquired informally (from friends or neighbours) are less likely to be taken to clinics for proper veterinary attention than dogs acquired by importation or from commercial dog breeders. Worthy of note during this study was the fact that only one dog was certified vaccinated using the National Veterinary Research Institute (NVRI) vaccine and most of the veterinary clinics complained that this vaccine's availability is not commensurate to the demand for it in the market. The respondents in this study reflected

fair to good knowledge of rabies as it pertains to its mode of transmission and case fatality rate but their knowledge on the clinical signs of rabies was poor. This agrees with the study in Abuja Municipal Area Council where 82 % of the respondents had satisfactory knowledge about rabies but 54 % believed rabies could be cured after symptoms appear as documented by Edukugho [7]. Residents in the city centre were much more aware of good rabies knowledge, positive attitude and good rabies preventive practices than the residents in the suburban parts of FCT. Civil servants, private sector workers and students were also found to be more aware of better knowledge, attitude and practice than the traders. Residents with tertiary education were also more aware about rabies than respondents with only primary/secondary education.

There was a statistically significant association between the areas of residence, occupation, number of dogs owned, level of education and the knowledge of dog owners about rabies. This can be attributed to the structure of Abuja as a planned territory with most of the socially privileged and enlightened individuals residing in the city centre (FCT phases 1, 2, and 3) whilst the less educated or financially limited persons reside in satellite towns or suburban settlements. There was no statistically significant association between the gender of dog owners and their knowledge about rabies. This agrees with the study in Abuja Municipal Area Council as documented by Edukugho [7]. The respondents' attitude of promptly washing dog bite wound, reporting dog bite incidents to the doctor, vaccinating their dogs yearly, not permitting roaming of dogs, discouraging children from playing with stray dogs reflects their positive attitude towards rabies prevention. This agrees with the study documented by Edukugho [7] in Abuja Municipal Area Council where 74 % of the respondents had a positive attitude and 24 % had a fair attitude towards rabies. Good practices including vaccination of dogs, regular clinical evaluation, keeping record of treatments administered, show the importance the dog owners attach to rabies prevention. This is also in agreement with the result of the study documented by Edukugho [7] in Abuja Municipal Area Council where he documented that 75 % of the respondents had satisfactory practice and 25 % had fair practice towards rabies prevention. The analysis reflected the significant statistical association of knowledge, attitude and practice scores with anti-rabies vaccination status and this is largely due to the fact that educational enlightenment moves dog owners to seek rabies prevention by vaccination. This study reflects the need for mass enlightenment campaigns against rabies in Abuja, Nigeria placing emphasis on educating the residents on the clinical manifestations of the disease. Following this cross-sectional study conducted in FCT, Nigeria, within the period December, 2014 and February, 2015 to ascertain the antibody prevalence of dogs in Abuja against rabies it was observed that a majority (83 %) of the sampled dogs were vaccinated against rabies, all vaccinated dogs had rabies antibody titre $> 0.6\,\mathrm{EU.ml^{-1}}$, the rabies vaccination status of dogs in FCT satellite towns were unsatisfactory, hence mass vaccination of dogs in the satellite towns is recommended.

CONCLUSIONS

The Federal Capital Territory of Nigeria have enlightened residents with good knowledge as it pertains to rabies and its preventive measures. Their antirabies dog vaccination culture is commendable with the canine antirabies antibody titres largely protective. However, the suburban settlement pockets in the territory still contains large populations of unvaccinated dogs owned by poorly enlightened residents. These suburban settlements thus require prompt massive rabies enlightenment campaigns and vaccinations to lessen the likelihood of these areas serving as rabies nidus and reservoirs soon.

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RELATIONSHIP BETWEEN CANINE LYMPHOCYTE AgNOR COUNTS AND HAEMATOLOGICAL INDICES OF HEALTH

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ABSTRACT

A modified agyrophil technique was applied to peripheral blood smears to determine the mean AgNOR counts (MAC) of lymphocytes and ultimately assess the state of the lymphoid system in various clinical conditions of dogs. Fifty dogs, from clinically normal to pets with leukaemia, presented to the Veterinary Teaching Hospital, were recruited. Blood smears from each dog were stained with routine Romanowsky and modified agyrophil stains. Signalment, clinical diagnoses and hematologic parameters of the dogs were related to the MAC. An AgNOR proliferative index (AgPI) — percentage of lymphocytes with 3 or more AgNORs, was determined, and correlated with MAC. The statistical significance was determined at $P < 0.05$. MAC ranged from 1.17 in clinically healthy patients to 6.00 in leukaemic patients. The MAC was 2.00 in patients (n = 26) with lymphocyte counts within reference intervals (900—2400 per microliter); 2.23 in patients (n = 4) with lymphopenia; 2.18 in patients with lymphocytosis (n = 18) and 4.73 in patients (n = 4) with lymphocytic leukemia. Also, the MAC was 2.00 in non-anemic dogs while it was 2.47, 2.49 and 3.06 in patients with mild, moderate and severe anaemia, respectively. The MAC correlated strongly with AgPI (r = 0.91). The ancillary AgNOR technique provides a cheaper, more rapid and sensitive tool than routine lymphocyte counts in assessing the state of lymphoid proliferation in a variety of conditions in the dog.

Key words: AgNOR; canine; haematology; lymphocyte

INTRODUCTION

Nucleolar organizer regions (NORs) are loops of chromosomal DNA which contain clusters of ribosomal RNA genes and are transcribed by RNA polymerase I. NORs ultimately direct protein synthesis. NORs vary in size and number according to the degree of nucleolar transcription, and are intimately related to the cell cycle and degree of cellular proliferation [3]. Transcriptionally active NORs are selectively stained by a silver colloid technique and can be identified as black dots (AgNORs) under a microscope [16]. Several modifications to the original technique by Ploton et al. [16] have been made [6, 10, 14, 21].

Ever since the agyrophilic technique was introduced to detect NORs in human chromosomes [5], it has found extensive use in human histopathology [1], human haematology [7, 24] as well as veterinary histopathology [8, 22]. Detection of AgNORs has been seen to correlate with other markers of proliferation such as Ki67, PCNA BrDU, DNA flow cytometry and immunohistochemistry [3], and the mitotic index [9]. Enumerating AgNORs via counts [24], morphometry [2] or a combination of both methods of enumeration [4, 11] has led to the indication of AgNOR as a possible prognostic tool [1, 12, 13, 19]. Consequently, there has been discussion about the use of the AgNOR proliferative index, a derivative of the mean AgNOR counts, as a more sensitive tool to delineate among normal, hyperplastic and benign lesions or even to predict survival times [8, 12, 15, 18, 19].

Lymphocytes are unique among peripherally-circulating leukocytes in their ability to recirculate and undergo mitosis. The haemograms of canine patients presenting with diverse clinical conditions are varied and may be at times unspecific, non-diagnostic and inconclusive.

In this study, a modified agyrophil technique was applied to peripheral blood smears to determine the mean AgNOR counts (MAC) of lymphocytes and ultimately assess the state of the lymphoid system in various clinical conditions of dogs (*Canis familiaris*).

MATERIALS AND METHODS

Study population

Fifty dogs, whose health status ranged from apparently clinically-healthy to obviously clinically-ill, presented to the Veterinary Teaching Hospital, were included in the study. The pets comprised of 30 males and 20 females, aged between 3 months to 6 years.

Collection of blood and haematology

Two millilitres of blood obtained from the cephalic vein was collected into EDTA-containing vials. The complete blood counts and differential leukocyte counts were carried out manually. The blood smears from each dog were stained with routine Romanowsky and an adapted agyrophilic staining technique as described by Ogunsola and Antia [14]. We defined lymphopaenia as less than 700 per µl, and thrombocytopaenia as less than 200,000 per µl. The pets were grouped based on the erythrocytic, leukocytic, lymphocytic and thrombocytic values (Table 1).

Detection of AgNORs

After staining with the modified agyrophilic technique, the blood smears were observed under a microscope using the oil-immersion objective. AgNORs in lymphocytes were generally detected as brown dots in a golden yellow nucleus. Specifically, only AgNORs in lymphocytes were enumerated.

Table 1. Lymphocyte mean AgNOR counts (MAC) and patients' clinical and haematologic characteristics

	N	MAC ± SD
Total white cell counts		
Leukopaenia	2	2.63 ± 0.29*
Within reference rangea	23	1.94 ± 0.43
Leukocytosis	25	2.47 ± 1.18*
Lymphocyte counts		
Lymphopaenia	4	2.23 ± 0.51*
Within reference rangeb	25	2.00 ± 0.51
Lymphocytosis	18	2.13 ± 0.51*
Lymphocytic leukaemia	3	4.73 ± 2.19**
Packed cell volume (%)c		
Non-anaemic (35–57)	32	2.00 ± 0.49
Mild anaemia (30–34)	7	2.47 ±1.57
Moderate anaemia (20–29)	6	2.49 ± 0.28**
Severe anaemia (< 20)	4	3.06 ± 2.04*
Platelet counts		
Thrombocytopaenia	10	2.24 ± 0.46
Within reference ranged	34	2.02 ± 0.49
Thrombocytosis	6	3.54 ± 1.93**
Clinical health status		
Clinically healthy	5	1.36 ± 0.16
Clinically sick	45	2.33 ± 0.91**
Breed		
Alsatian	23	2.01 ± 0.54
Boerboel	4	2.31 ± 0.17
Rottweiler	4	2.21 ± 0.48
Others	19	2.48 ± 1.32
Sex		
Male	30	2.33 ± 1.09
Female	20	2.07 ± 0.54
Age		
< 1year	10	2.29 ± 0.52
1–3 years	23	2.01 ± 0.52
3 years	17	2.48 ± 1.38

N — number of patients; SD — standard deviation; * — $P < 0.05$
** — $P < 0.01$
[a] — 7–14 ($\times 10^3.\mu l^{-1}$); [b] — 0.7–2.9 ($\times 10^3.\mu l^{-1}$); [c] — N = 49
[1] — patient had PCV = 62 %, MAC = 3.07, AgPI = 59 %
[d] — 200–600 ($\times 10^3.\mu l^{-1}$)
[e] — Breeds include Pitbull, Caucasian, Mixed, Nigerian indigenous

Calculation of mean AgNOR counts (MAC) and AgNOR proliferative index (AgPI)

The MAC is defined as the total number of AgNORs detected in 100 lymphocytes divided by 100. AgPI was calculated as the percentage of lymphocytes that contained 3 or more AgNORs.

Statistical analysis

Mean AgNOR counts, calculated for each sub-type of haematologic diagnosis, were compared using the one-way ANOVA test. Following the ANOVA test, post hoc analysis was also carried out where necessary. The MAC was correlated with the AgPI. Statistical significance was determined at $P < 0.05$ using the SPSS software v.20.

Ethical consideration

The study was carried out according to set ethical guidelines approved by the University of Ibadan ACUREC. The informed consent of all clients who owned the pets employed was obtained.

RESULTS

Lymphocyte mean AgNOR counts (MAC)

Generally, MAC ranged from 1.17 in a clinically-normal patient to 6.00 in a leukaemic patient. Clinically healthy patients (n = 5) had MAC of 1.36 while clinically-sick animals (n = 45), irrespective of the presenting complaints and clinical diagnoses) had MAC of 2.33. When leukaemic patients with MAC of 4.73 are excluded from the list of clinically sick patients, the MAC of clinically-sick patients drops to 2.16.

The MAC of lymphocytes varied with the degree of anaemia. The counts were consistently higher in anaemic patients than in non-anaemic patients. MAC was 2.00 in non-anaemic dogs while it was 2.47, 2.49 and 3.06 in patients with mild, moderate and severe anaemia respectively. Also, the MAC of lymphocytes was higher in dogs with total white cell counts outside the reference intervals (leukopaenia = 2.63; leukocytosis = 2.46) than those with total white cell counts within the reference intervals (1.94). Specifically, MAC was 2.00 in patients with lymphocyte counts within reference intervals (700—2400 μl^{-1}); 2.23 in patients showing lymphopaenia; 2.18 in patients with lymphocytosis, and 4.73 in patients with lymphocytic leukaemia. Similarly, the MAC of lymphocytes was higher in dogs that had thrombocyte values outside the reference interval (200,000—600,000 μl^{-1}). Patients showing thrombocyte values within the reference interval had MAC of 2.02 while patients with thrombocytopaenia and thrombocytosis had MAC values of 2.24 and 3.54 respectively. Details are presented in Table 1.

AgNOR proliferative index (AgPI)

The AgPI of lymphocytes ranged from 0 % in patient with MAC of 1.20 to 100 % in patients with MAC of 6.00. AgPI greater than 29 % was set as a benchmark for increased lymphocyte proliferation. All lymphopaenic patients and 70 % of patients presenting with lymphocytosis had AgPI greater than 29 %.

Correlation between total white cell counts, lymphocyte counts, MAC and AgPI of lymphocytes

There was a positive correlation between the MAC and lymphocyte counts (r = 0.71) as well as between MAC and AgPI of lymphocytes (r = 0.91). Details are presented in Table 2.

Table 2. Correlations between lymphocyte mean AgNOR counts (MAC), proliferative index (AgPI) and total white cell (TNCC) and lymphocyte (Lymphs) counts

	MAC	AgPI	TNCC	Lymphs
MAC	1	0.912**	0.586**	0.710**
AgPI	0.912**	1	0.485**	0.472**
TNCC	0.586**	0.485**	1	0.738**
Lymphs	0.710**	0.472**	0.738**	1

*P < 0.05; ** P < 0.01

DISCUSSION

Increasing lymphocyte counts were observed to correlate strongly with increasing MAC of lymphocytes as patients with lymphocytic leukaemia had the highest MAC of 6.00. This probably indicates that most causes of lymphocytosis were due to increased cellular proliferation of lymphocytes, and migration of this proliferating pool of lymphocytes into the peripheral vascular pool. It is noteworthy that 70 % of cases that presented with lymphocytosis had AgPI greater than 29 %.

However, there were a few cases where patients with lymphocytosis did not show a corresponding increase in AgPI. This finding was mostly observed in cases that presented with "leukaemoid" neutrophilic counts. This finding may be due to the fact that the absolute specific white cell counts are obtained from the product of differential counts and total white cell counts. The product will increase the lymphocyte counts without necessarily indicating a state of increased lymphocyte proliferation.

Also, patients with lymphopaenia had higher MAC than patients with lymphocyte counts within reference intervals. It is plausible that there is increased egress of proliferating/stimulated lymphocytes from the vascular pool into tissues or sites of injury. This is supported by the finding that all the patients that presented with lymphopaenia had AgPI greater than 29 %.

A rather puzzling but novel finding was that MAC of lymphocytes increased with increasing severity of anaemia. It is safe to assume that most of the causes of canine anaemia in the tropics are infectious agents. Indeed, about 80 % of the patients in this study had clinical diagnoses of some form of haemoparasitic infection. Commonly encountered infectious agents, such as *Babesia canis*, *Leptospira icterohaemorrhagiae*, *Ehrlichia canis*, canine parvovirus are antigenic and as such, they stimulate the immune system and ultimately induce lymphoid proliferation. It might also not be out of place to consider anaemia in itself, regardless of cause, as a stimulus for increased cellular proliferation of lymphocytes. In a previous study by VanBelle and Cocquyt [23], anaemia in itself has been considered as a prognostic and predictive factor in a number of neoplastic conditions. According to Yasunaga et al. [25], anaemia and AgNOR counts are the significant clinicopathologic prognosticators of survival in renal cell carcinoma. AgNOR counts have also been reported to increase with increasing severity of aplastic anaemia [17]. However, a decrease in AgNOR sites (compared with healthy controls) in haemopoietic cellular series in myelodysplastic syndrome was attributed to a decrease in the proliferative potential with disease progression [11].

The changes in platelet values and corresponding changes in MAC of lymphocytes in this study cannot be explained from the findings in this study. Breed, sex and age of the patients did not seem to significantly influence MAC of lymphocytes. This finding is in tandem with that reported by Sur et al. [20].

The strong positive relationship ($r = 0.91$) between MAC and AgPI of lymphocytes indicates that both indices may be used interchangeably. From the viewpoint of cellular kinetics, the AgPI may be a more sensitive index than the MAC. This is supported by the finding of a stronger correlation ($r = 0.71$) between lymphocyte absolute counts and AgPI, than the correlation ($r = 0.59$) between lymphocyte absolute counts and MAC. This corroborates the findings of Bukhari et al. [1] and Salehinejad et al. [19].

CONCLUSIONS

The agyrophilic technique is a relatively cheap and easy method of determining cellular proliferation, when compared with other cellular markers. Increasing lymphocytes' MAC correspond to increasing cellular proliferation of lymphocytes, and is associated with increasing severity of anaemia. The AgPI is an easier-to-estimate, more sensitive alternative to the MAC in determining the proliferative activity of lymphocytes in the dog.

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IMMUNOHISTOCHEMICAL STUDY OF THE STROMAL CELLS IN THE LACTATING BOVINE MAMMARY GLAND

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ABSTRACT

The bovine mammary gland is a special gland characterized by high secretory activity. During lactation the cellular and fibrous components of the interstitial tissue septa are exposed to store accumulated secretory products. The aim of this study was to find and study the cells in the stroma of the bovine lactating mammary gland. For this purpose, the immunohistochemical methods and antibodies against the smooth muscle actin, vimentin, and desmin were used. The myoepithelial cells (MEC) which stained with smooth muscle actin (SMA), were found supporting the secretory units and the intralobular ducts. Coexpression of the SMA and desmin were found in the smooth muscle cells of the blood vessels. The fibroblasts (myofibroblasts) and free cells positive to vimentin were located in the connective tissue septa. The results of this study on the mammary glands indicated that smooth muscle cells (SMC) were altered in the lactating mammary gland, with additional cells such as fibroblasts (myofibroblasts) participated in the storage and after milk let-down they allowed the mammary glands to return to their original state.

Key words: bovine; immunohistochemistry; mammary gland; stromal cells;

INTRODUCTION

The cells of the mammary gland are in intimate contact with other cells and with the extracellular matrix, both of which provide not only a biochemical context, but a mechanical context as well. Cells within the mammary gland respond to changes in the stiffness of their environmental structures, serving as an important role for cellular force and mechanosignaling events in the normal development and differentiation of the gland at puberty, pregnancy, lactation, and involution [23].

Studies carried out with anti-smooth muscle actin (SMA), anti-desmin and anti-vimentin antibodies in several glandular organs confirmed the heterogeneity of stromal

cells [10]. The heterogeneity of stromal cells was confirmed after using anti-alpha SMA, anti-desmin and anti-vimentin antibodies in salivary, sweat and mammary glands [5, 11]. Antibodies to keratin and smooth muscle actin have been described in myoepithelial cells but they are not specifically stained by antibodies to vimentin nor desmin [21, 24]. The smooth muscle-specific proteins and vimentin were found in the myoepithelium and stromal myofibroblasts of normal and malignant mammary glands [10, 15]. Smooth muscle actin was observed in the stroma of neoplastic breast tissue to identify myofibroblasts [3]. In carcinoma cases examined, numerous brightly stained elongated stromal cells were positive for SMA whereas no immunoreactivity was detected in fibroblasts of normal human breast tissue [10].

The loose fibrovascular stroma of the mammary gland was positive for vimentin and basal cells covering the ductal epithelium for alpha-smooth-muscle actin suggesting myoepithelial cells [7]. These cells are characteristized by their high metabolic activity and have an important function. They play the central role in milk ejection during lactation, and actively participate in mammary morphogenesis and are assumed to influence the proliferation, survival and differentiation of luminal cells [4, 18]. Myoepithelial cells, which tightly surround ductal epithelium and loosely encase the luminal epithelial cells, are specifically designed for contractility, and likely create mechanical events within the gland that differ between ductal and alveolar cells and with hormone status [23].

The interaction between stromal cells and tumor cells is known to play a major role in cancer growth and progression. The mammary stromal fibroblasts express and produce cytokines which plays roles in mammary cancer [9]. Studies relative to a stromal components of the mammary gland, mainly those involved in the ejection of milk and the recoil of the gland, are scarce. The objective of this study was to localize supporting and contractile cells in the stroma of the bovine lactating mammary gland and to appraise their role in the process of milk ejection.

MATERIALS AND METHODS

Mammary gland tissue samples were obtained from five lactating cows (Holstein) at the slaughter house. The samples were placed in 0,1 mol phosphate buffered 10 % formalin for 24 hours at room temperature, dehydrated and embedded in paraffin. The sections of thickness 5 mm were deparaffinised and rehydrated. For immunohistochemical study sections were immunostained for their reactivity with monoclonal antibodies. For this purpose, the histological sections were pre-treated with 3 % H_2O_2 in methanol for 30 min to reduce endogenous activity and preincubated with 2 % goat serum to mask nonspecific binding sites. Afterwards the sections were incubated at 4 °C overnight with the primary antibodies (Table 1). Sections were then incubated with biotinylated secondary antibody for 45 min. Tissue samples were then incubated with the streptavidin-biotin peroxidase complex method. The peroxidase activity was visualized with 0.05 % 3´.3´- diaminobenzidine (DAB) and 0.03 % v/v H_2O_2. Some sections were counter-stained with Mayer's hematoxylin. Negative controls were performed by omitting the primary antibody.

RESULTS

Under a light microscope the bovine mammary gland demonstrated itself as a highly modified tubulo-alveolar apocrine gland. The branching ducts and alveoli were lined by an inner layer of the secretory luminal epithelial cells that produced milk and were surrounded by the contractile myoepithelial cells and the basement membrane. The secretory units drained into intralobular ducts, which left the lobule and opened into the interlobular ducts. Groups of tubuloalveolar secretory units forming lobules are separated by different mounts of connective tissue septa. The interalveolar connective tissue within the lobule was loose and rich in the capillary network. Interlobular connective

Table 1. Primary antibodies

Antibodies used	Donor	Code	Isotype	Dilution	Source
Smooth muscle actin	Mouse	M 851	IgG2a	1:200	Dako
Vimentin	Mouse	1074	IgG1	1:50	Imunotech
Desmin	Rabbit	PS 031	OgG1	1:50	Imunotech

tissue was usually dense and fibrous and contained small blood vessels and nerves.

Smooth muscle actin

The myoepithelial cells and smooth muscle cells were strongly stained with alfa-SMA whereas the secretory cells gave no positive staining for SMA (Fig. 1). The myoepithelial cells were seen to cover stromal surface of the epithelium of the secretory alveoli and the intralobular ducts. The typical location of the myoepithelial cells around the alveolar epithelium was well seen in cross sections through alveoli where the myoepithelial cells formed a continuous layer. In the intralobular ducts the myoepithelial cells formed an incomplete layer. Smooth muscle cells of blood vessels and pericytes of the capillaries revealed a strong reaction to SMA (Fig. 1). Numerous blood capillaries in the interalveolar space demonstrated a rich blood supply of the lactating mammary gland.

Vimentin

The vimentin was observed in fibroblasts (myofibroblasts) disposed in the connective tissue of the interalveolar and interlobular septa (Fig. 2). Vimentin was also observed in some cells of the external epithelial surfaces of the secretory alveoli and interlobular outlets. Some free cells, such as lymphocytes and macrophages were present in the connective tissue septa, and they also expressed different intensities of the positive reaction for vimentin (Fig. 2). In the intralobular ducts, the positive reaction for vimentin was seen in myoepithelial cells (MEC) forming a layer in the subepithelial space. The endothelial cells of blood vessels were strongly stained for vimentin.

Desmin

Desmin-positive smooth muscle cells were seen in the wall of the blood vessels located in the connective tissue of the inter-lobular space (Fig. 3). No positive reaction was observed in the myoepithelial cells. A few desmin-positive smooth muscle cells were observed closely associated with capillaries. These cells had the morphological features of pericytes. Coexpression of SMA and desmin was observed in the smooth muscle cells of larger blood vessels.

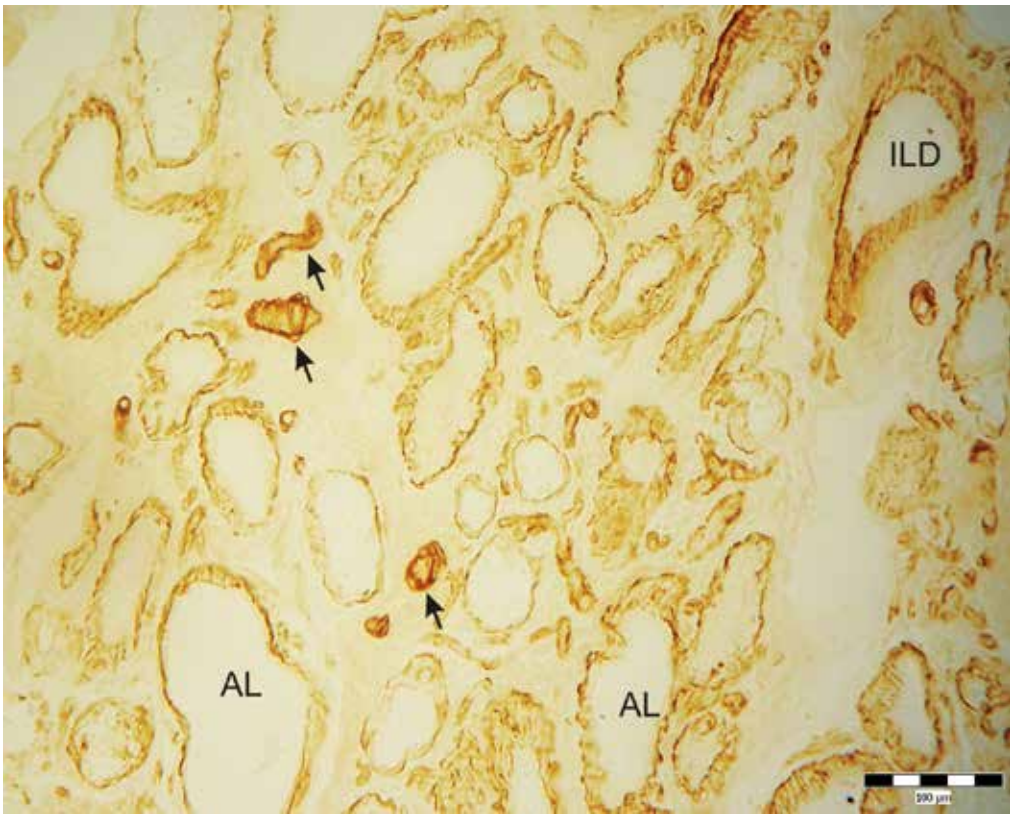

Fig. 1. Immunostaining for SMA. The myoepithelial cells form a complete layer on the periphery of alveoli (AL) and intralobular ducts (ILD). Positive reaction for SMA was strong also in SMC of capillaries and arterioles (arrows)

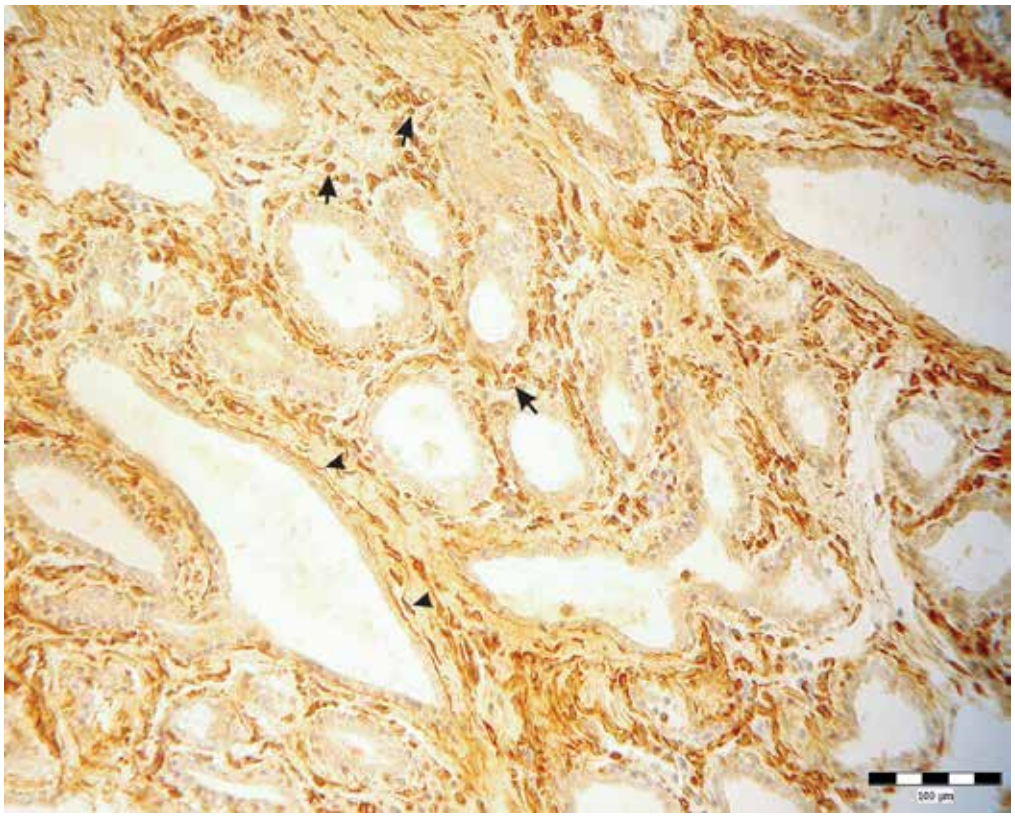

Fig. 2. Immunostaining for vimentin. Strong positive reaction was seen in the fibroblasts (arrowheads). Numerous free cells express positive reaction for vimentin (arrows)

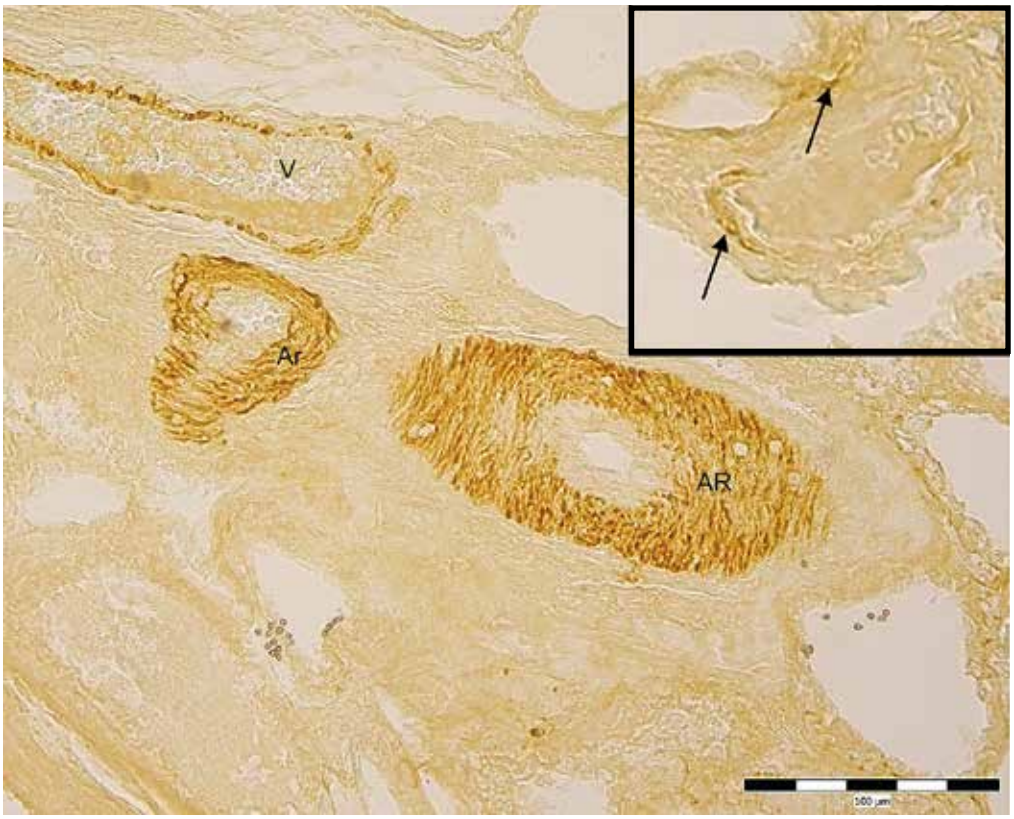

Fig. 3. Immunostaining for desmin. Positive reaction is seen in smooth muscle cells of the blood vessels: arterioles (Ar), arteries (AR) and veins (V). Top right: A few desmin-positive smooth muscle cells were observed closely associated with capillaries

DISCUSSION

Immunohistochemical studies carried out with SMA, desmin and vimentin antibodies in several glandular organs confirmed the heterogeneity of stromal cells. The smooth muscle-specific proteins and vimentin were found in the myoepithelium and stromal myofibroblasts of normal and malignant mammary glands [10]. Although SMA have been described in myoepithelial cells they are not specifically stained by antibodies to vimentin nor desmin [21, 24]. The myoepithelial cells and some stromal cells corresponding to myofibroblasts were stained with SMA thus hampering the identification of MEC [2, 5]. The MEC and stromal fibroblasts have epithelial and mesenchymal cells origin, which coordinate their expression of a set of smooth muscle markers while maintaining their specific original features. The dual nature of MEC and the phenotypic transition of fibroblasts to myofibroblasts are the examples of the plasticity of the differentiated cell phenotypes [10].

Vimentin was expressed in a number of cells of loose fibrovascular stroma of the ovine mammary gland [7]. In the mammary gland of pregnant rats, Warburton et al. [25] observed a much weaker reaction for vimentin in the MEC of developing alveolar buds than in the main ducts. Gould et al. [6] found in humans, vimentin-positive cells in 8 of 12 normal breasts and in 12 of 20 of fibrocystic disease. According to the authors, these cells in most cases appeared to be myoepithelial. In the bovine mammary gland vimentin was found in numerous cells located in the connective tissue septa. Though the main cells positive for vimentin were fibroblasts and/or myofibroblasts, some cells located covering secretory epithelium showed also positivity for vimentin. According to their location and shape, these cells correspond to myoepithelial cells. Michalczyk et al. [12] observed in the human mammary gland an intensive reaction for vimentin in MEC. There was a four-fold increase in vimentin protein levels in lactating tissue relative to resting tissue, and this may be related to the increased cellular activity of the myoepithelial cells which surround secretory alveoli [12]. A positive reaction for vimentin in these cells have been described in both the resting and lactating gland. Positive cells for both vimentin and SMA were found also in the connective tissue cells in canine mammary tumors [3] and in breast carcinomas [15].

Myofibroblasts are a unique group of smooth-muscle-like fibroblasts that have a similar appearance and function regardless of tissue of residence. In the canine connective tissue of mammary tumors Destexhe et al. [3] described myofibroblasts positive for both vimentin and alfa-actin. In normal canine mammary tissues, mixed tumors or complex adenomas have been observed by some authors with immunopositive reactions to vimentin in every type of myoepithelial cells and cells of mesenchymal tissues [3, 19].

Structural changes in the mammary gland during milk production leads to an increase in the activity of vimentin positive MEC and the appearance of a number of free cells, including macrophages (V-type) which under certain conditions are immuno-positive to vimentin [18]. Vimentin was also observed on the cell surfaces of activated macrophages, but also in apoptotic T lymphocytes, aged neutrophils and platelets [13]. Secreted vimentin could play a role in mediating the movement of circulating blood cells across the endothelium, a process in which activated macrophages and activated platelets participate [26]. The detailed functions of macrophages are extensive, as their precursor cells can respond to a variety of physiological situations and mature along a spectrum of phenotypes [17]. Mor–Vaknin et al. [14] demonstrated that activated human macrophages secrete vimentin into the extracellular space. The studies of Ingman et al. [8] revealed a role of macrophages in collagen fibrillogenesis and in the organization of the structure of terminal end buds. Besides macrophages, other types of cells may be present in the stroma of mammary gland. Radu et al. [20] reported on the *in vitro* isolated Cajal-like interstitial cells from human inactive mammary-gland stroma which expressed c-kit/CD117 and vimentin.

Smooth muscle actin detected in smooth muscle cells (SMC) of the bovine mammary gland was found irregularly scattered in the connective tissue septa between the alveoli or lobules and along the intralobular ducts. SMA positively stained myoepithelial cells were found to be a stable cellular supporting and contractile component of secretory units of the bovine mammary gland. The position of SMC in the bovine mammary gland was same as in other species [1, 4, 22].

The positive reaction for desmin in the bovine mammary gland, was found in SMC of blood vessels located in thick interlobular connective tissue septa and around the blood capillaries. The desmin was found in a similar position also in a mammary fibroadenoma in a lamb [7]. Few positive spindle cells in the interalveolar space may present pericytes of blood capillaries. In the normal condition, the

desmin-positive pericytes are located around the endothelial cells of the capillary plexus and of larger vessels in the intermediate mesenchyme [16]. In this position pericytes stabilize vessel wall, participate in the regulation of blood flow microcirculation and influence endothelial proliferation, survival, migration and maturation.

CONCLUSIONS

The results of this study showed that the stroma of the bovine lactating mammary gland contains a population of various types of cells. Immunohistochemical studies carried out with anti-alpha SMA, anti-desmin, anti-vimentin antibodies revealed a heterogeneity of stromal cells located in the supporting connective tissue. It has been shown that the cellular components play a significant part of the lactating mammary glands of cattle. Supporting and contractile components are involved in the ejection of milk and recoil of the mammary gland.

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IN VITRO EVALUATION OF BIOLOGICAL EFFECTS OF DANDELION (*TARAXACUM OFFICINALE*) EXTRACTS

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ABSTRACT

Dandelion (*Taraxacum officinale*) of the *Asteraceae* family is known for its pharmacological effects and has been used in therapy for centuries. Currently extracts of all parts of this plant are used — root, leaves and flowers. The extracts are prepared using various extraction agents that may significantly affect the effectiveness and therapeutic spectrum of the extracts. The aim of this study was to use three different solvents for the preparation of the extracts from dandelion (*Taraxacum officinale*) leaves and flowers, namely triton X-100 (2 %), nonidet P-40 (2 %) and acetone (30 %). After extraction, the extractants were evaporated and the dried extracts were dissolved in water to obtain a series of solutions of the concentrations: 125, 250, 500 and 1000 µg.ml^{-1}. The biological effects of the extracts were investigated by means of the MTT test of cell viability. Rabbit kidney epithelial cells (RK13) exposed to the extracts for 24 and 48 hours were used as a model cell line. We observed that the acetone extract of dandelion leaves and flowers at lower concentrations caused an increase in the viability of the treated cells in comparison with the control cells which were not exposed to the extracts ($P < 0.05$). At the same time, we observed a significant effect of the solvent used for the preparation of the dry extracts on the viability of the cells. The residues of the extractants caused a decrease in the cell viability almost to zero, which in fact means the death of the cells. The selection of the correct extractant for the preparation of the extracts is essential regarding the use of extracts in the pharmaceutical or cosmetic industries.

Key words: cell culture; MTT; solvent; viability

INTRODUCTION

The pharmaceutical industry constantly maintains an interest in the herbaceous plants owing to the fact that phytotherapy is perceived as a part of modern medicine. Officinal plants play an important role in the treatment and prevention of various diseases and disorders. Phytotherapy is based on the use of pharmacologically effective ingredients

found in plants in order to achieve the required therapeutic effect on target molecules, enzymes and receptors [3, 12].

Dandelion (*Taraxacum officinale*) of the *Asteraceae* family belongs among plant species commonly grown and widely-spread throughout Slovakia, containing many effective ingredients. Owing to their broad spectrum of pharmacological profile and safety of applications, they are widely used in the therapy and prevention of various diseases and organism disorders, such as: digestive and urinary tract disorders, inflammatory processes, pro-thrombotic states and oxidation stress. The whole plant — leaves, flowers, stems and roots – have curative use. Many pharmaceutical products are prepared on the plant basis and a great number of herbs are used for the preparation of various tea blends. Medicinal preparations of the current modern phytotherapy include not only infusions, decoctions or tinctures, but also dry standardized extracts or single individual fractions of such extracts. Extracts are the raw material of the pharmaceutical industry that suit to the current requirements of processing, analysis and control as well as the synthetic medicines [8, 12]. The preparation of these dry extracts involves the use of various extraction reagents. Solvents are widely used in the chemical industry in many applications, for example: in the processing (synthesis, separation, purification of active ingredient), medicinal formulations and during cleaning and washing, and exhibit different properties [4]. It is assumed that drying involves evaporation of the solvents. The effect of extractants on the live organism is frequently unknown and can even be harmful.

The aim of our study was to validate the effect of extracts of the herbaceous plant dandelion (*Taraxacum officinale*), prepared by means of three different extractants, acetone, nonidet P-40 and triton X-100, on a live cell line — model line of rabbit kidney epithelial cells RK13 — and by means of the observation of the viability of these cells to compare the influence of the solvents used in the preparation of dry extracts.

MATERIALS AND METHODS

Preparation of extracts

In the experiment we investigated the action of the extracts of dandelion (*Taraxacum officinale*, *Asteraceae*) leaves and flowers. The plants originated from the Subcarpathian region (Rzeszow, Poland). About 10—15 dandelion plants were collected from one location (a meadow outside of the town) in May, 2017, and were air dried. One gram of dried, pulverized dandelion leaves or flowers was extracted with 20 ml of proper solvent in an ultrasonic bath (Sonorex RK 30, Bandelin, Germany) for 30 min. The following solvents were used: aqueous acetone 30 % v/v; aqueous solution of nonidet P-40, 2 % v/v; aqueous solution of triton X-100, 2 % v/v. Nonidet and triton are non-ionic surface active substances — viscous liquids easily soluble in water (Sigma Aldrich, Germany).

The extracts were then centrifuged (10 min, 6500 × g) and the supernatants were collected. After removal of acetone by vacuum evaporation, all supernatants were lyophilised (24 h, lyophylisator Alpha, Christ GmBH, Osterode am Harz, Germany) to obtain dry extracts. Immediately before the experiments, we prepared a series of fresh water solutions from the dried extracts of concentrations: 125, 250, 500 and 1000 $\mu g.ml^{-1}$.

Cell cultivation

Rabbit kidney epithelial cells RK13 (ATCC® CCL-37™) were selected as a model cell line for evaluation of the biological action of the prepared extracts. The cells were grown in a complete culture medium EMEM (Earl's Minimal Essential Medium; Lonza, Belgium) with 10 % foetal bovine serum, 1 % L-glutamine, 0.1 % gentamycin, 0.5 % Fungizone (amphotericin B) and 1 % antibiotic (combination of penicillin and streptomycin) at 37 °C in an atmosphere containing 5 % CO_2 until they reached approximately a 90 % confluent monolayer. During cultivation, the cells were regularly checked for the absence of mycoplasma contamination [15].

MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay

The cell suspension (100 µl) of density 22,000 cells per well area was cultivated in 96-flat bottom well plates (Greiner Bio One, Greiner, Germany). After 24-hour cultivation, the cells in the growth phase were exposed to extracts of dandelion leaves and flowers for 48 hours. Wells with the cells not exposed to the action of extracts served as a control. After 24 and 48 hours of exposure, the medium was removed from wells and the adhered cells were washed with PBS (100 µl). PBS was then removed and 90 µl of cultivation medium and 10 µl (concentration 5 $mg.ml^{-1}$) of yellow tetrazolium stain MTT 3-(4,5-dimethylthiazol-

2-yl)-2,5-diphenyltetrazolium bromide) was added to each well. In metabolically active, i.e. live cells, this stain turns to a purple formazan. After 4-hour incubation in a CO_2 cell incubator, the MTT medium was replaced by concentrated DMSO (100 µl) solution to dissolve formazan crystals. Immediately after addition of Sorensen glycine buffer (12.5 µl per well), the absorbance at 570 nm was read (spectrophotometer Synergy HT, Biotek, Winooski, VT, USA).

Statistical processing of results

The spectrophotometrically determined absorbance values were converted to % of cell viability by the following formula:

% = absorbance of sample × 100/absorbance of control

The cells not exposed to extracts served as a control (viability 100 %). The results were presented as means (n = 3) with standard deviation (± sd). The results were evaluated statistically using the software Graf Pad Prism 3.00 (GraphPad Software, San Diego, CA, USA) and Dunnett's comparison test. The significance level was set to $P < 0{,}05$.

RESULTS

Changes in the viability of cells following their exposure to the extracts of dandelion leaves are summarised in Fig. 1. An increase in the metabolic activity above 100 % was observed after 24-h exposure to acetone extracts of concentrations 125 and 250 $\mu g.ml^{-1}$ and after 48-h exposure to extract of concentration 500 $\mu g.ml^{-1}$.

In the case of extracts prepared with nonidet and triton, almost all values were low, close to zero ($P < 0.05$) which indicated the death of cells. Viability of all cells exposed to extracts differed significantly ($P < 0.05$) from the viability of untreated cells with the exception of acetone extracts of concentrations 250 and 500 $\mu g.ml^{-1}$ after 24-h exposure ($P > 0.05$).

The investigation of the metabolic activity of the model cell line treated with extracts of dandelion flowers showed a marked increase in the activity of cells already after 24-h exposure (Fig. 2). Similar to the action of dandelion leaf extracts, the acetone extracts affected positively the viability of cells. In the case of concentrations 125 and 250 $\mu g.ml^{-1}$, an increase in viability of the treated cells above 100 % was observed after 24-h of the action of extracts ($P < 0.05$). After exposure to concentrations 500 and 1,000 $\mu g.ml^{-1}$, the metabolic activity of the cells decreased to 80 %. On the contrary, the viability of the cells exposed to the extracts of dandelion flowers prepared with nonidet and triton, was very low, almost zero, resembling the action of leaf extracts. All differences in the activity in comparison with the control were significant ($P < 0.05$).

DISCUSSION

Dandelion (*Taraxacum officinale*) exhibits a broad pharmacological profile and with regard to the occurrence of undesirable effects, it is classified as safe. A number of studies have confirmed its pharmacological effects — diuretic, choleretic, anti-inflammatory, antioxidant, anticancerigenic, antihyperglycaemic, anticoagulant and prebiotic [14].

In our investigations of the biological activity of dandelions we observed the effects of different extracts of dandelion leaves and flowers on the viability of a model cell line RK13. We detected a significant influence of the solvent used for the preparation of dry extracts. The common methods of selection of the most suitable solvent uses the available powerful tools that help to reduce the effort by focusing on a pre-selected set of solvents suitable for the relevant processing step [4]. Acetone dissolves many hydrophilic and lipophilic plant components, is water-miscible, volatile and its toxicity for the biological test is low. Although water is used as a general solvent, plant extracts prepared with organic solvents exhibit higher antimicrobial activity in comparison to water extracts. [11]. Our study showed an increased metabolic activity of cells exposed to acetone extracts from dandelion leaves and flowers. One can assume that this was related to the influence of some specific components as dandelions contain a large amounts of polyphenols and flavonoids (19 %) [7]. Triton X-100 and nonidet P-40 belong to the most used non-ionic surface active substances utilized for lysis of cells at extraction of proteins and other cell organelles and also as a reagents that induce the permeabilisation of membranes [2]. Surfactants are also used as modifiers of aqueous extraction in the so-called micelle mediated extraction (MME). Formation of surfactant micelles in a solution is a factor improving solubilization of various analytes. The MME method is widely used in sample preparation processes, also for extraction of biologically active plant metabolites, e. g. terpenes [10, 13], alkaloids [9] or polyphenols [1, 5, 6]. The removal of

surfactant from the extract is problematic, therefore even after lyophilisation some residues of this compound remain in the sample. Despite complete evaporation of the solvent during preparation of dried powdered extracts, comparative studies proved the influence of solvent residues on metabolic activity or the viability of the cells. Their residues most likely caused a decrease in the metabolic activity of the treated cells down to the zero level, i. e. to cell death. The cytotoxic effects of surfactants has been confirmed when various substances of this type were tested on cells. Triton X-100, used in our research, is classified as one of the most cytotoxic surfactants [1]. Nonidet P-40 is structurally similar, so it can be concluded, that its effects may be comparable. Our study showed that the selection of suitable solvent for obtaining plant extracts is very important as it can have adverse effects on live organisms. Thus, despite the positive effects of the relevant plant, the final impact on health can be negative.

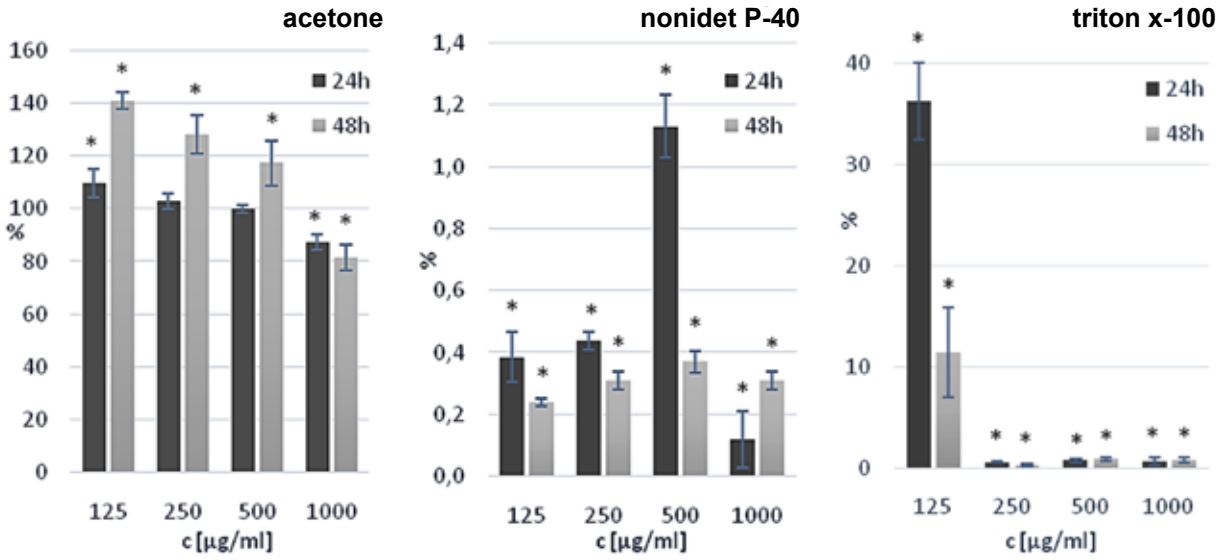

Fig. 1. Changes in the viability of cells (%) in comparison with the control (100%)after exposure to extracts prepared with acetone, nonidet and triton from dandelion leaves in relation to extract concentrations (µg.ml^{-1})

*the differences in comparison with the control were significant at the level of P < 0.05

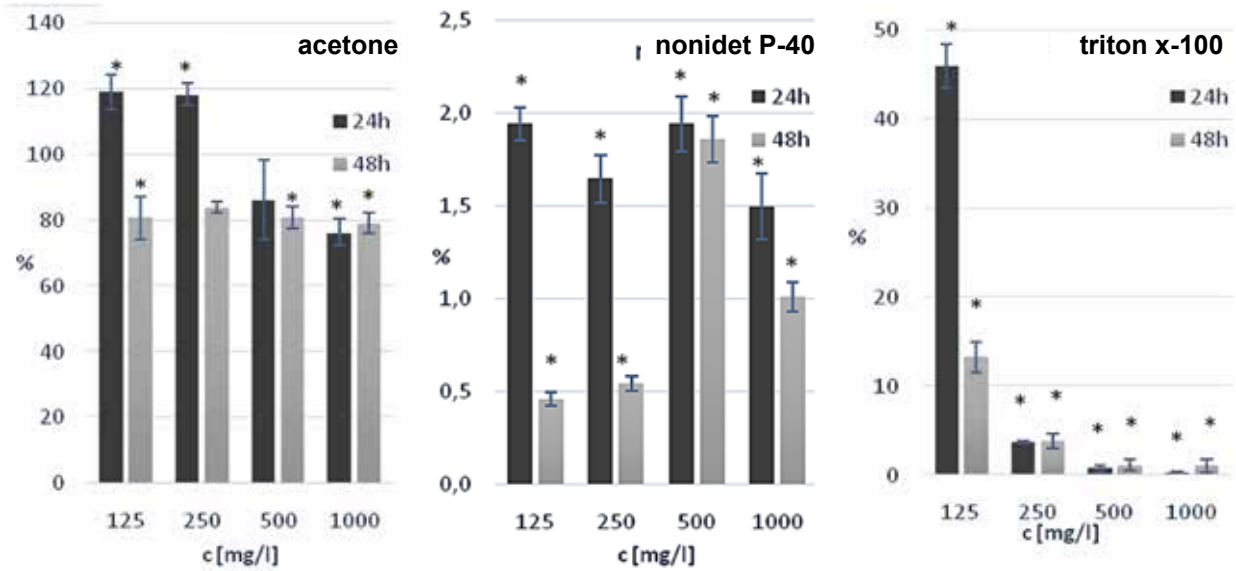

Fig. 2. Metabolic activity of cells (%) in comparison with the control (100 %) after exposure to extracts prepared with acetone, nonidet P-40 and triton x-100 from dandelion flowers in relation to extract concentrations (µg.ml^{-1})

*the differences in comparison with the control were significant at the level of P < 0.05

CONCLUSIONS

Extracts of medicinal plants are frequently used in phytotherapy. The selection of solvents used for their preparation is very important as it affects their biological action. Acetone appears a suitable solvent for preparation of extracts from dandelions with regard to the complex of components present in this plant. Exposure of model cells to acetone extracts caused a significant increase in their metabolic activity above 100 % which can be considered a supportive effect. On the contrary, the use of surface active substances, ionic detergents, affected negatively metabolic activity and viability of cells, in fact it caused their death. The residues of extractants can markedly affect the action of dry plant extracts and eventually have a direct negative effect on live organisms. This stresses the importance of the selection of the correct extractants in order to ensure the optimum use of beneficial components of medicinal plants and also the way of application of the extract in cosmetic or pharmaceutical industries.

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ANALYSIS OF SISTER CHROMATID EXCHANGES AND PROLIFERATION OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES EXPOSED TO EPOXICONAZOLE

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ABSTRACT

The potential genotoxic/cytotoxic effect of epoxiconazole was evaluated by means of sister chromatid exchanges (SCE) following the 24 and 48 h *in vitro* exposure of human peripheral blood lymphocytes to epoxiconazole at concentrations of: 5, 10, 25, 50 and 100 µg.ml^{-1}. Dimethyl sulphoxide (DMSO), used as an epoxiconazole solvent, was used as a negative control and mitomycine (MMC) as a positive control. After the 24-hour exposure, we failed to observe a significant increase in SCE frequencies in comparison with the negative control, however, the concentrations of 10—100 µg.ml^{-1} caused a significant decrease in the proliferation index (PI; $P < 0.001$). Also, the 48-hour exposure produced no significant alterations in the SCE frequencies in comparison with the control. At epoxiconazole concentrations ranging from 10 to 50 µg.ml^{-1} we recorded a moderate to strong, dose-dependent inhibition of PI ($P < 0.05$; $P < 0.01$; $P < 0.001$), while at the highest dose (100 µg.ml^{-1}) the reduction in PI compared to the control was less pronounced ($P < 0.05$). The reduction in PI at the concentration range of 10—100 µg.ml^{-1} depended on the number of cells in the M_1, M_2 and M_3 phases of the cell cycle per total number of 100 evaluated metaphases. Our results indicated a significant cytotoxic or cytostatic effect on human peripheral blood lymphocytes.

Key words: epoxiconazole; proliferation index; sister chromatid exchanges

INTRODUCTION

Azole antimycotics are widely used not only in agriculture but also in human and veterinary medicine against a broad spectrum of fungal infections [6]. The mechanism of effect of azole compounds is based on an inhibition of the enzyme lanosterole 14α-demetylase (CYP51), responsible for conversion of lanosterole. Because of this inhibition, ergosterole, the essential component of membranes of yeasts and fungi, is not produced [3]. In agriculture, epoxiconazole has appeared effective in the prevention and treatment of fungal infections of plants caused by an as-

comycete fungus *Mycosphaerella fijiensisa* and *M. musicola* (black and yellow sigatoka) from the family *Mycosphaerellaceae*, occurring on banana leaves, and rust forming fungus *Hamileia vastatrix* (*Pucciniaceae*) parasitizing leaves of plants from the genus *Coffea* [2]. A characteristic feature of epoxiconzole is its low biodegradability in the environment that may result in its accumulation in the soil and, subsequently, contamination of water [7]. Water fauna is therefore considerably used as suitable objects in the evaluation of toxic and teratogenic effects of various azole compounds. For example, Zhu et al. [15] exposed *Gobiocypris rarus* embryos to five triazole compounds (myclobutanil, fluconazole, flusilazole, triflumizole and epoxiconazole) at various concentrations and confirmed their teratogenic effects. Because azole compounds are capable of inhibiting biosynthesis of androgens and oestrogens, one may assume that chronic exposure to these compounds could result in a number of disturbances in humans and other animals, for example reproductive disorders, foetal malformations, feminisation, neuro-behavioural and tumorous diseases [12, 16].

With regard to the potential exposure of humans and animals to azole compounds we focused on the investigation of the potential *in vitro* genotoxic and/or cytotoxic effects of epoxiconazole by means of analysis of SCE and PI in human peripheral blood lymphocytes.

MATERIALS AND METHODS

Materials and the tested compounds

Epoxiconazole (CAS registration number 133855-98-8, purity 99 %, Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulphoxide (DMSO, Sigma, St. Louis, MO, USA) and added to culture media at concentrations of: 5, 10, 25, 50 and 100 $\mu g.ml^{-1}$. DMSO served as a negative control and its final concentration in the experimental and control media was 0.1 %. Mitomycine (MMC) (Sigma, St. Louis, MO, USA, 0.4 $\mu g.ml^{-1}$) was used as a positive control.

In the experiment we used lymphocytes from the peripheral blood of a healthy, 20 years old woman.

Cultivation of lymphocytes

The culture medium contained 4 ml RPMI 1640 (Sigma Chemical Co., St. Louis), L-glutamine and 15 µmol HEPES (GE Healthcare Hycone Lab, Utah, USA), 1 ml BoFeS (bovine foetal serum; Sigma Chemical Co., St. Louis, MO, USA), phytohaemagglutinine (PHA; 20 $\mu g.ml^{-1}$, Welcome, Dartford, England), mixture of antibiotics and antimycotics (100 U. ml^{-1} penicillin, 0,1 $mg.ml^{-1}$ streptomycin a 0,25 $mg.ml^{-1}$ amphotericin, Sigma Chemical Co., St. Louis, MO, USA) and 0.2 ml of blood. The cultivation took place in test tubes in a thermostat set to 37 ± 1 °C and lasted 72 hours. Fifty µl aliquots of epoxiconazole solution in DMSO of concentrations specified above were added to the cultures 24 and 48 hours before termination of cultivation. After 24 hours of cultivation, we added bromdeoxyuridine (BrdU; 8.0 $\mu g.ml^{-1}$, Sigma Chemical Co., St. Louis, MO, USA) to all culture flasks (control and experimental) in order to detect SCE and differentiate the cell cycles. For detection of SCE and differentiation of cell cycles in metaphases we used the FPG (Fluorescence Plus Giemsa) method.

Evaluation of results and statistical methods

For each concentration and time of exposure we counted SCE in 30 metaphases selected at random (M_2 cells, i. e. cells in the second cell cycle). At the same time, we calculated and recorded the numbers of cells in the first, second and third cycle from among the total number of 100 evaluated cells. PI was calculated by the following formula:

$$PI = (M_1 + 2M_2 + 3M_3)/N$$

Where: M_1 is the number of metaphases in the first (uniformly stained chromosomes) cell cycle; M_2 in the second (the so-called harlequin chromosomes) and M_3 in the third cycle (Fig. 1) and N is the total number of metaphases, altogether in 100 cells.

The statistical evaluation of the occurrence of SCE in the experimental groups in comparison to the control was carried out by ANOVA and subsequently by the Student's t-test; the proliferation rate indices were evaluated by the χ^2 test. The significance level was set to $P < 0.05$.

RESULTS

Detection of SCE in human lymphocytes exposed to epoxiconazole

The results of the detection of the sister chromatid exchanges following the exposure to epoxiconazole are shown in Fig. 2 and the relationship between the PI and epoxiconazole concentration is depicted in Fig. 3.

After 24-hour exposure, we failed to observe a signifi-

Fig. 1. M_1, M_2 and M_3 cell cycle phases

On the left: M_1 phase, uniformly stained, dark chromosomes (46, XX). In the centre: M_2 phase with BrdU incorporated in both DNA strands of one chromatid — during two cell cycles (light chromatid), second chromatid coloured dark — BrdU is incorporated in one of two DNA strands, the so-called harlequin chromosomes that enable evaluation of SCE. On the right: M_3 phase, chromosomes are light (with incorporated BrdU in both DNA strands of both chromatids) or of harlequin type

cant increase in SCE frequency in comparison with the negative control (DMSO), however, starting with the concentration of 10 μg.ml^{-1}, up to the concentration of 100 μg.ml^{-1}, we recorded a significant decrease in the proliferation rate index (PI; $P < 0.001$; χ^2 test).

Similarly, after the 48-hour exposure, there were no significant changes in the SCE frequency in comparison with the control. Starting from the epoxiconazole concentration of 10 μg.ml^{-1} up to 50 μg.ml^{-1} we recorded a dose-dependent inhibition of PI ($P < 0.05$; $P < 0.01$; $P < 0.001$; χ^2 test), but at the highest dose (100 μg.ml^{-1}) the PI reduction in comparison with the control was less pronounced ($P < 0.05$; χ^2 test).

The differences in the number of the observed M_1, M_2 and M_3 metaphases produced by individual concentrations are illustrated in Fig. 4 and Fig. 5. Fig. 6 shows metaphase M_2 with sister chromatid exchanges marked with arrows.

The curves show proportions of metaphases in the first, second and third cell cycles in dependence on concentration of epoxiconazole. DMSO served as a negative control and MMC as a positive control. We evaluated 100 mitoses for each concentration.

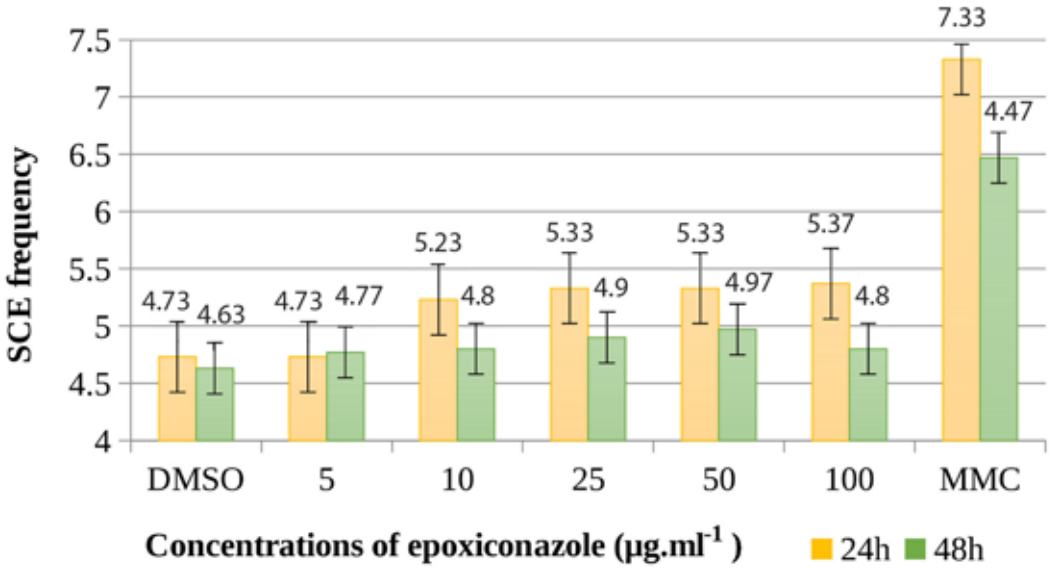

Fig. 2. Relationship between SCE frequency and concentration of epoxiconazole after 24h and 48h exposure of human peripheral blood lymphocytes

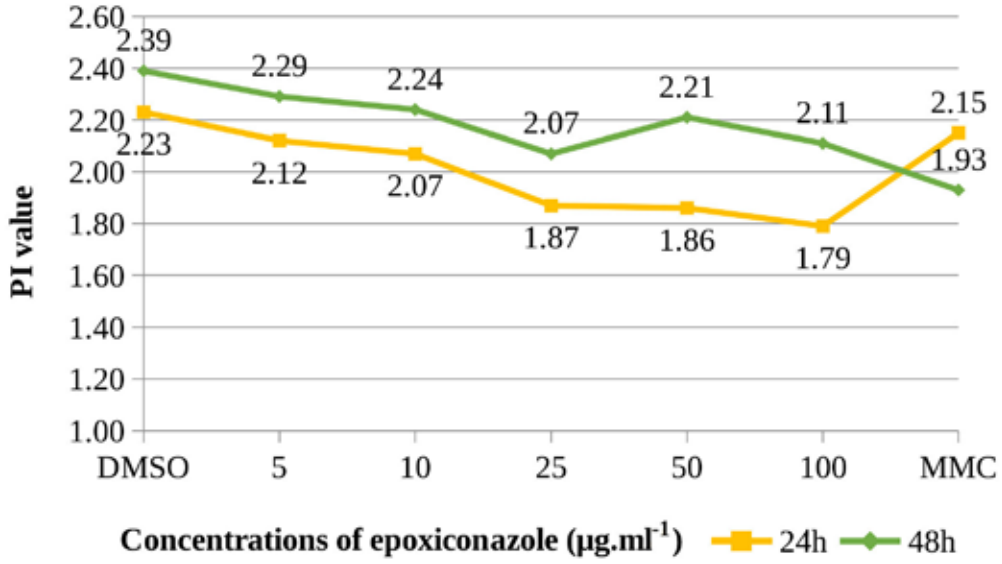

Fig. 3. Relationship between PI value and concentration of epoxiconazole after 24h and 48h exposure of human peripheral blood lymphocytes

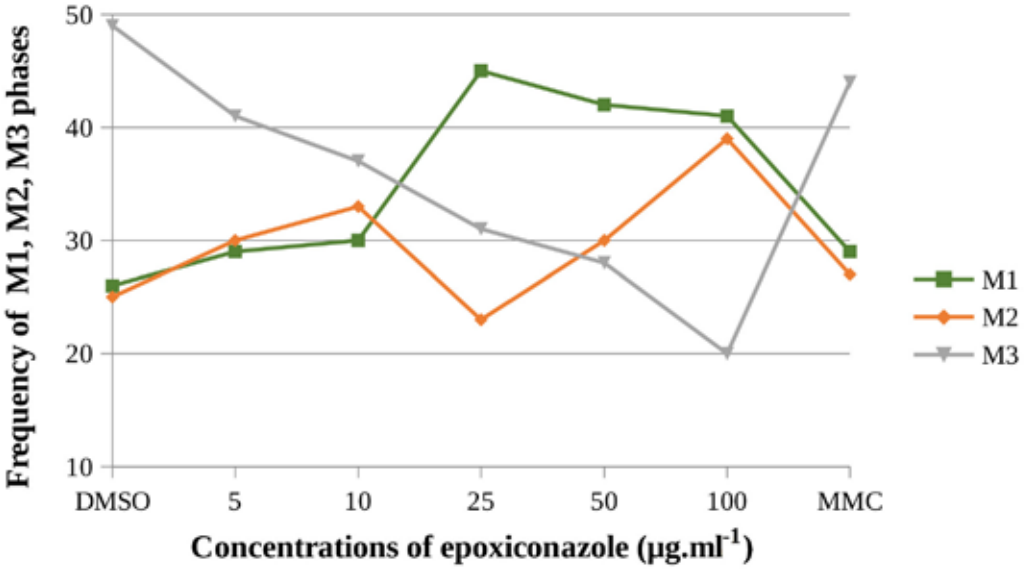

Fig. 4. Relationship between frequency of M_1, M_2 and M_3 phases of the cell cycle and the concentration of epoxiconazole after 24 h exposure

DISCUSSION

The evaluation of sister chromatid exchanges (SCE) in the human peripheral blood lymphocytes is one of the complex of cytogenetic methods required by Organisation for Economic Cooperation and Development (OECD) for the evaluation of the potential mutagenic/carcinogenic effects of chemicals. Despite its advantages, there are two main practical issues associated with its use. First, after the exposure to genotoxic chemicals, the SCE frequencies are evaluated only in cells that have reached the metaphase stage. The cells are able to reach this stage after exposure to non-cytotoxic doses of chemicals. The cells with the cell cycle arrested in the G_2 phase are not included in the analysis. Because the undamaged cells, the cell cycle of which was not affected, pass through metaphase, the conventional SCE analysis cannot sufficiently detect the genotoxic/mutagenic potential of various chemical

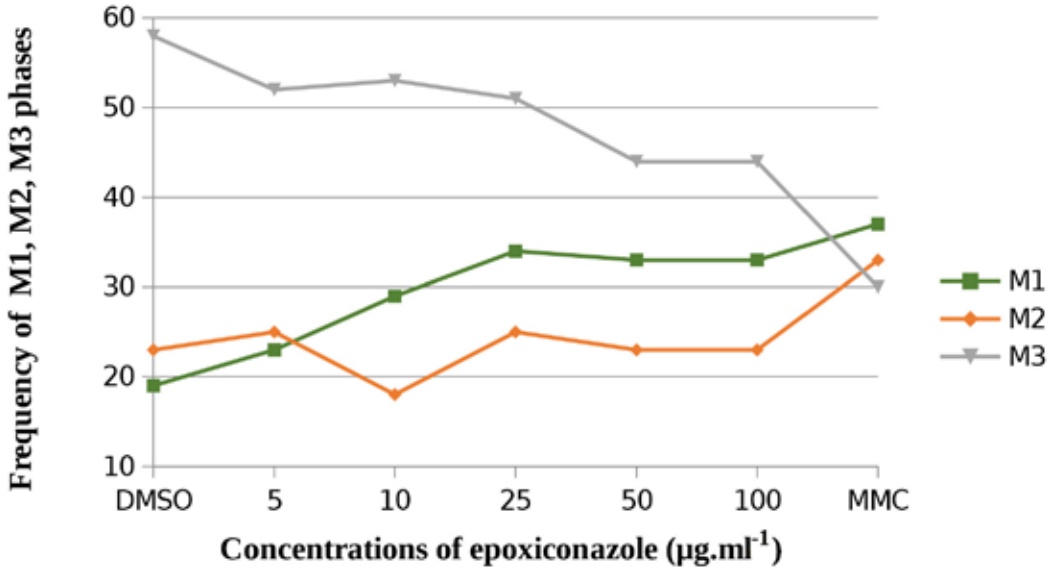

Fig. 5. Relationship between frequency of M_1, M_2 and M_3 phases of the cell cycle and the concentration of epoxiconazole after 48 h exposure

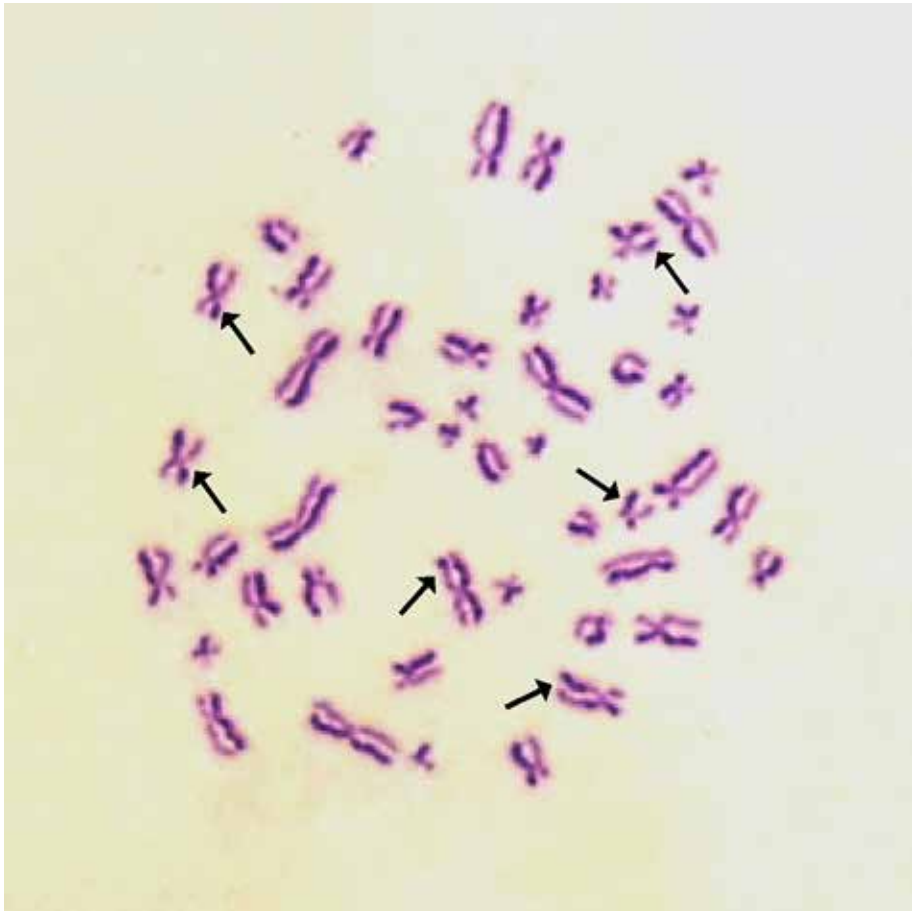

Fig. 6. M_2 phase of the cell cycle
Human metaphase — woman (46, XX); second cell cycle (M_2 phase) — harlequin chromosomes; sister chromatid exchanges are marked with arrows

compounds. Second, the influence of the concentration of BrdU incorporated in DNA can be sometimes higher than the effect associated with the exposure to genotoxic carcinogens [13].

Our experiment, that involved analysis of SCE after 24 and 48 h exposure to epoxiconazole, showed no genotoxic effect of pure azole antimycotic on human peripheral lymphocytes (Fig. 2).

Amara et al. [1] described a synergistic effect of epoxiconazole and pyrethroid insecticides and herbicides on aquatic crustaceans and phytoplankton and their studies allowed them to present an assumption that azole antimycotics are toxic to aquatic fauna — crustaceans, fish and some algae. Similarly, biomonitoring studies that involved farmers professionally exposed to pesticides (cyproconazole, epoxiconazole, tebuconazole, hexaconazole and other) conducted by Lebailly et al. [5] documented a significant damage to DNA detected by alkaline single-cell gel electrophoresis (SCGE — single-strand breaks, alkaline labile DNA sites and double-strand breaks). Ross et al. [8, 9] conducted a study on murine liver cells and observed that triadimefon at a dose of 1800 ppm and propiconazole at a dose of 2500 ppm induced increased frequencies of mutations during a 4-day exposure. Additional authors, for example, Zhu et al. [15] demonstrated teratogenic effects on embryos of *Gobiocypris rarus* fish.

In agreement with our results obtained in the study on human lymphocytes, no genotoxic and/or clastogenic effect of epoxiconazole was confirmed in cultivated bovine peripheral lymphocytes [11]. However, the proliferation rate was affected significantly after 24 and 48 h of exposure to epoxiconazole with cytotoxic or cytostatic effects. In a similar experiment conducted by Schwarzbacherová et al. [10], the authors obtained negative results by evaluation of clastogenic and aneugenic effects of commercial triazole fungicide containing tebuconazole and protioconazole on bovine peripheral lymphocytes *in vitro*. In their evaluation they used a micronuclei and comet assays. The observed inhibition of cytokinesis-block proliferation index (CBPI) indicated a significant cytostatic/cytotoxic effect of the investigated fungicide.

Our experiments showed a pronounced effect on proliferation at both exposures of human lymphocytes to epoxiconazole (basic and prolonged). In dependence on concentration, we recorded a reduction in PI in the concentration range of 10—100 $\mu g.ml^{-1}$ after 24 h exposure and in the range of 10—50 $\mu g.ml^{-1}$ after 48 h exposure to epoxiconazole. On the basis of the reduced number of M_1 metaphases and persistence of cells in M_2 and M_3 cell cycles (Fig. 5) we assumed that after 48 h exposure to epoxiconazole, the cell division was arrested in the G_1/S phase of the cell cycle, therefore the cells could not proceed to the replication phase. This indicates the cytotoxic/cytostatic effect of epoxiconazole on the peripheral lymphocytes (Fig. 5).

A similar effect of azole compounds on cell proliferation was documented also by other authors. For example, Yilmaz et al. [14] described a genotoxic effect of a preparation Conan 5FL, containing hexaconazole (50 $g.l^{-1}$), on murine bone marrow cells *in vivo* and human lymphocytes *in vitro* and a significant, dose-dependent decrease in mitotic index (17.5, 30 and 70 $\mu g.ml^{-1}$).

Our results agree also with those obtained by Herrera–Martínez et al. [4], who evaluated the effects of ketoconazole (imidazole antimycotic) on cell lines BON-1 (human serotonin-producing neuroendocrine pancreas tumour, derived from lymph node metastases) and DMS-79 (small-cell lung carcinoma). A significant suppression of cell growth by ketoconazole in BON-1 cells was dose and time dependent. The authors recorded a maximum inhibitory effect (41—95 %) at the concentration of 10 µM after 3 to 7 days ($P < 0.0001$). Ketoconazole also induced stopping of the cell cycle in the G_1 phase, accompanied by reduction of S and G_2 phases and significantly induced apoptosis ($P < 0.001$). In the less susceptible DMS-79 cells, the authors observed the highest inhibitory effect (44—94 %) at concentration of 50 µM after 3 to 7 days. Ketoconazole at the concentration of 10 µM suppressed the secretion of adrenocorticotropic hormone.

The results of our experiments allowed us to conclude that the concentrations used of epoxiconazole did not induce damage to the DNA or chromosomes but significantly affected the proliferation as manifested by the cytotoxic and cytostatic effects.

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DETECTION OF RESIDUES OF ANTIMICROBIAL COMPOUNDS IN EGGS BY THE RAPID SCREENING METHODS

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ABSTRACT

Eggs belong to the most frequently consumed products of animal origin worldwide, and therefore the safety of eggs is a substantiated issue. Conventional poultry rearing involves the use of antimicrobials added to their feed or potable water particularly for disease treatment, however, in some countries also for the prevention of diseases, promotion of growth and better utilisation of the feed. Thus, effective control of residues of such substances in eggs is very important for the protection of the public health. The aim of this study was to detect the potential presence of antimicrobial residues in fresh hen eggs using commercially available rapid screening methods (Premi®Test and EXP Ampulle test) and compare the results of both of these tests. We examined 22 samples randomly selected from among 66 samples purchased in 11 European countries. We respected the procedures as supplied by the manufacturers of the tests together with their respective test kits. The examination of eggs by the Premi®Test did not detect the presence of antimicrobial residues in the samples, while the EXP Ampulle test provided 8 positive and 6 dubious results. Our results allowed us to conclude that the EXP Ampulle appears to be more sensitive and allows one to carry out more effective control of the presence of antimicrobial residues in hen eggs intended for human consumption.

Key words: antibiotics; detection; eggs; residues

INTRODUCTION

The essential role of egg production is to ensure the reproduction of the respective species. The development of the progeny takes place outside of the maternal body, therefore eggs must contain all of the important nutrients necessary for the embryonal development. Eggs contain high levels of full-value proteins that are a rich source of essential amino acids. Other important components are fats, vitamins and minerals. Eggs are an inevitable product for human nutrition. Besides their direct consumption, eggs are used also as a raw material in many branches of the food industry but also in other industries. They are important

also for their use in human and veterinary medicine, e.g. in vaccine production or as an insemination diluent [7].

In poultry production, antibiotics and antiparasitics are used to prevent and treat infectious diseases. Although in some countries antimicrobials are still used as growth promoter, in the European Union the use of antibiotics for this purpose was banned in 2006 in all species of food producing animals due to the increasing risk of the development of antimicrobial resistance [9]. The exception are coccidiostats that are administered preventively to poultry as feed supplements intended for killing or inhibition of the growth of pathogenic protozoa. Other antimicrobials most frequently used in poultry include polypeptides, tetracyclines, penicillins and sulphonamides, either separately or in combination with trimethoprim and chinolones [8].

In order to ensure good functioning of the internal market involving food of animal origin and to protect the public health, the EU accepted Commission Regulation (EU) No. 37/2010 as of 22 December 2009 on pharmacologically active substances and their classification regarding the maximum residue limits (MRL) in foodstuffs of animal origin. These MRL, accepted in the respective food matrix of the respective animal species, guarantee its safety to consumers [10]. The presence and the level of residues in products of animal origin are affected by a number of factors. The most important include: the dose of the medicine and the way and length of its administration, combination of medicines, physico-chemical properties and metabolism, withdrawal period, contamination of feed and water, age and physical condition of the animal and the composition of the food matrix itself [1, 2].

Egg consists of the egg white and the egg yolk. After administration of antimicrobials to layer hens, their residues occur in one or both egg components. Absorption of drugs in the intestine and their transport by the blood circulation to the left ovary, the protein-producing part of the oviduct (magnum) and uterus results in the deposition of antimicrobial residues in the egg yolk and thick and thin egg white during egg formation. The amount of accumulated residues depends first of all on the physico-chemical properties of the drug, its polarity, ability to bind to plasma proteins and the duration of relevant phases of formation of individual egg components [6].

The presence of residues in eggs for consumption is subject to compulsory testing. The EU has established a unified effective system for the determination of the presence of antimicrobial residues in products of animal origin in agreement with the Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC [14]. This effective system of residue monitoring consist of two linked-up steps, the screening and confirmation. The Directive 96/23/EC stipulates that the screening of residues can be carried out only by those analytical methods for which it can be demonstrated in a documented traceable manner that they are validated, and at the level of interest (concentration of the analyzed substance in the sample important for determination of its compliance with legislative regulations – MRL) have a false compliant rate of < 5 % (β-error). In the case of a suspected non-compliant result, this result shall be confirmed by a confirmatory method.

The screening methods that comply with the Directive 96/23/EC include also Premi®Test and EXP Ampulle test. Both these tests are broad-spectrum qualitative commercial testing systems combining the principle of agar diffusion and indicator colour change as a result of the active metabolism of the test strain *Bacillus stearothermophilus* var. *calidolactis* with the absence of an inhibitor. If the sample contains substances inhibiting the growth of the test strain, the purple colour of the indicator remains unchanged.

As the Premi®Test is currently the official method for laboratory diagnosis approved by the relevant authority of the Slovak Republic for the detection of antibiotic residues in products of animal origin including eggs [11], and EXP Ampulle test is one of the most recent available commercial tests, also recommended for detection of antibiotic residues in products of animal origin including eggs, the aim of our study was to detect the potential presence of antimicrobial residues in fresh hen eggs available on the market of 11 European countries by both above mentioned commercial rapid screening methods and compare the results of both tests.

MATERIALS AND METHODS

In the period of 2017—2018, we tested 22 samples randomly selected from among 66 samples of eggs purchased in chain stores in 11 European countries (Tab. 1) using Premi®Test (R-Biopharm AG, Germany) and EXP Ampulle

test (Packhaus Rockmann GmbH, Germany). In the case of a positive result, we tested separately the egg yolk and the egg white by the test that provided the initial positive result. Both tests are based on the inhibition of growth of the test strain *Bacillus (Goebacillus) stearothermophilus*, susceptible to a broad spectrum of antimicrobial substances used in veterinary medicine (β-lactams, cephalosporins, tetracyclines, macrolides, sulphonamides, and aminoglycosides) at their MRL level. The preparation and analysis of the samples complied with the procedures recommended by the manufacturers of Premi®test and EXP Ampulle test.

Table 1. Labeling of the samples and country of their origin/purchase

Sample No.	Country of origin
1, 2	Italy
3, 4	Spain
5, 6	France
7, 8	Rumania
9, 10	Bulgaria
11, 12	Ukraine
13, 14	Hungary
15, 16	Czechia
17, 18	Austria
19, 20	Poland
21, 22	Slovakia

Preparation of samples

The contents of the eggs used for the analysis of residues (whole egg content; egg yolk; egg white) were transferred to a sample container and homogenised thoroughly.

Procedure

Premi®Test: using a micropipette of a defined volume we transferred 100 µl of the homogenised sample to a Premi®Test ampoule (containing an agar medium, pH indicator and spores of *Bacillus stearothermophilus* and covered the ampoule with an adhesive foil supplied by the test manufacturer. The ampoule was then labelled and inserted into a thermoblock (Acublock Digital Dry Bath D 1200, Labnet, USA) where it was first pre-incubated at 80 °C for 10 min and then incubated for approximately 3—3.5 hours at 64 ± 0.5 °C. The incubation was terminated when the colour of the agar medium in the negative control sample turned from purple to yellow.

EXP Ampulle: 10 ml of sterile demineralised water was added to 10 ml of the homogenized egg contents. After thorough mixing, the diluted sample was warmed up for 3 min in a water bath at 100 °C with occasional mixing by a glass rod to prevent coagulation. Then, using a micropipette of a defined volume, 100 µl of the diluted homogenised sample were transferred to an ampoule supplied with the EXP Ampulle kit, covered the ampoule with a provided adhesive foil and inserted it into a thermoblock where it was incubated at 65 °C ± 1 °C for approximately 3 hours (2 hours and 30 min — 3 hours and 15 min), until the agar medium of the negative control samples turned from purple to yellow.

Evaluation of results

The colour of the agar medium in the sample ampoule was compared with that of the negative control. The yellow colour of the agar medium indicated the absence of antibiotics, therefore a negative result. The purple colour of the agar medium indicated the presence of antibiotics and the sample was evaluated as positive. The yellow-purple colour of the agar medium is indicative of the presence of antibiotics at the level equal to the detectability of the test. Such results are considered as dubious, because any colour differing from the yellow colour of the agar medium of the negative control indicates the presence of a residue. In the case of a dubious result, the analysis should be repeated again.

RESULTS AND DISCUSSION

The results of both tests were evaluated by comparing the colour of the agar medium with the colour chart which is part of the commercial test kit (Fig. 1 and 2). On the basis of this comparison we could determine whether the relevant sample was positive, negative or dubious.

The samples for the EXP Ampulle test were prepared according to the manufacturer's instructions. However, during the primary pre-incubation of the samples at 100 °C for 3 min, the samples coagulated despite their previous dilution with a sterile demineralized water (1 : 1 v/v). Because the pre-incubation of eggs is important for the inactivation of the enzyme lysozyme present in the egg white, which has an antimicrobial effect and thus can lead to false positive

Fig. 1. Evaluation of the change of colour of the agar medium bythe Premi®test

Fig. 2. Evaluation of the change of colour of the agar medium by the EXP Ampulle test

Table 2. Results of the examination of the whole egg content obtained by the Premi®testom and EXP Ampulle tests

Test	Sample																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Premi®test	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EXP Ampulle	+	–	–	–	+	–	–	–	+	–	+	+	–	+	–	–	–	–	+	+	–	–

+ — positive sample; ± — dubious sample; – — negative sample

Table 3. Results of the examination of individual components of the positive eggs by the EXP Ampulle test

	Sample							
	1	5	9	11	12	14	19	20
Egg yolk	–	–	±	±	±	±	±	±
Egg white	+	+	+	+	+	+	+	+

+ — positive sample; ± — dubious sample

results, we respected the requirement of sample dilution but pre-incubated the samples using the thermal regimen of the Premi®test (pre-incubation at 80 °C for 10 min).

The testing of all 22 samples (whole egg content) by the Premi®test provided no positive results while with the EXP Ampulle test 8 samples tested positively. The results of the determination of residues of antimicrobial compounds in whole egg content obtained by Premi®test and EXP Ampulle test are presented in Table 2 and Figures 3a, 3b, 4a, 4b.

Eight positive results (samples 1, 5, 9, 11, 12, 14, 19, 20) obtained by the EXP Ampulle test when testing the whole egg content inspired us to test the individual components of the eggs, i.e. the egg yolk and the egg white separately. When examining the egg yolks, 6 samples tested dubious, but all 8 egg whites tested positive. The results of the determination of antimicrobial residues in egg whites and egg yolks of the positive samples by EXP Ampulle test are presented in Table 3 and Figures 5a, 5b and 5c.

The national plans of monitoring the residues of antimicrobial compounds in the food of animal origin are implemented by the EU member states. The residue control plan is aimed at surveying and revealing the reasons for residue hazards in foods of animal origin on farms, slaughterhouses, dairies, fish processing plants, and egg collecting and packing stations. The plan of residue control must agree with respect to the extent and frequency of sampling with the requirements set by the Directive 96/23/EC and the national legislative regulations.

The levels and frequencies of sample collection for the monitoring of some compounds and their residues in eggs are stipulated in the Annex of the Commission Decision 97/747/EC, which supplements the levels and frequencies

Fig.3a (left) and 3b (right). Determination of the antimicrobial residues in whole egg contents by the Premi°test

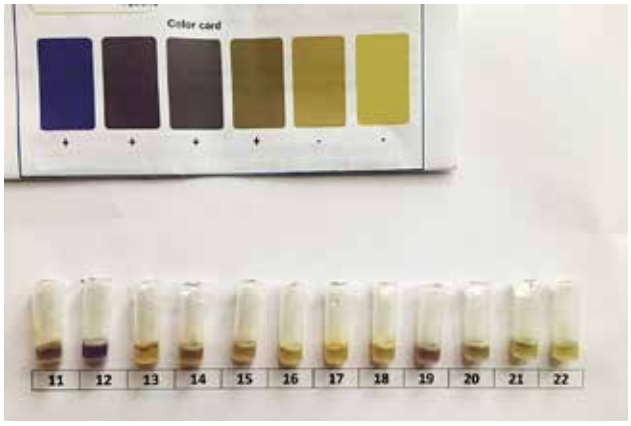

Fig. 4a (left) and 4b (right). Determination of the antimicrobial residues in whole egg contents bythe EXP Ampulle test

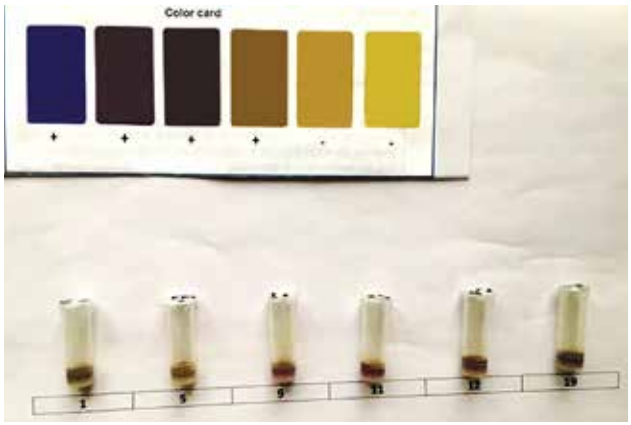

Fig 5a. Results of the examination of egg white of the positive eggs (1, 5, 9, 11, 12, 19) usingthe EXP Ampulle test

Fig 5b. Results of the examination of egg yolks of the positive eggs (1, 5, 9, 11, 12, 19) using the EXP Ampulle test

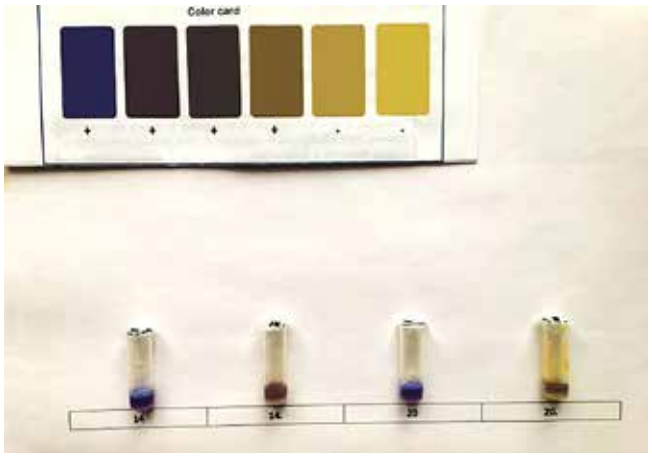

Fig 5c. Results of the examination of the egg white and egg yolks of the positive eggs (14/white/, 14/yolk/, 20/white/, 20/yolk) usingthe EXP Ampulle test

of sampling described in the Annex IV of the Directive 96/23/EC [13]. In the case of hen eggs, each official sample must be collected by the authorised veterinary administration body in a manner that allows one to identify retroactively the farm from which the eggs originated. The samples can be collected either at the farm level or grading or packing stations. The size of the sample is 12 or more eggs, according to the analytical methods used. The number of samples collected per year must be equal to at least one sample per 1,000 tonnes of annual production of eggs intended for consumption; the minimum being 200 samples.

Each member state of the EU can decide about the structure of sample collection depending on the structure of the egg industry, particularly with regard to its integration level. At least 30 % of the samples must be collected from egg grading and packing stations that represent the most significant proportion of eggs supplied for human consumption. From the point of view of the monitored compounds, 70 % of the samples must be tested for at least one compound from group A 6 (unauthorised substances), B 1 (antibacterial compounds /beta-lactams, tetracyclines, macrolides, aminoglycosides/including sulphonamides and chinolones) and B 2, letter b) (anticoccidials including nitroimidazoles) listed in the Annex II of the Directive 96/23/EC, and 30 % of samples must be collected according to the situation in the respective country, however, this must include some analysis of compounds from group B 3, letter a) (organochlorine compounds including chlorinated biphenols).

In the case of eggs from other poultry species, the proportions of samples from these species are determined individually by each member state according to the level of production and the detected problems. The eggs from other poultry species must be included in the plan of sample collection in the form of supplementary samples to samples of hen eggs.

The detection of antibacterial compounds by many screening methods based on microbiological analyses involves the testing of samples or sample extracts by means of the inhibition of growth of the test bacterial strains. The inhibition of the growth of the respective bacterial strain after some period of incubation indicates the presence of an antibacterial compound in the sample. Some EU member states use microbiological methods as a part of their specific control programmes intended for the control of antimicrobial residues. In some cases, the results of such tests are sufficient to decide about the presence of residues in the samples and no additional physical or chemical analysis are required for confirmation or identification of the relevant compounds. In the cases that require additional confirmatory tests, the positive result of the screening test is confirmed by immunochemical or physico-chemical tests capable of identifying the relevant compounds and determining whether its concentration is lower or higher than the respective MRL [12].

The three latest reports on the results of the monitoring of the residues of veterinary medicines and other substances in live animals and animal products in the years 2014,

2015 and 2016, published by the European Food Safety Authority (EFSA), presented the following results for eggs: in 2014 the examination of 4,674 samples of eggs for the presence of antimicrobials showed that 4 (0.09 %) of the samples were positive for doxycycline, enrofloxacin, flumechin and sulfadiazine, and the examination of 4,367 samples of eggs for the residues of coccidiostats revealed that 18 (0.41 %) of the samples were positive for diclazuril, lasalocid, monensin, narasin, nicarbazin, robenidin, salinomycin and toltrazuril sulfone [3]; in the 2015 examination of 4,454 samples of eggs for the presence of antimicrobials showed that 7 (0.16 %) of the samples were positive for doxycycline, enrofloxacin, sulfadimetoxine in combination with trimethoprim and sulfadiazine, while of 4,823 samples of eggs examined for anticoccidials and coccidiostat residues 23 (0.54 %) of the samples were positive for diclazuril, lasalocid, maduramycin, monensin, narazin, nicarbazin, robenidin, salinomycin and toltrazuril sulfone [4]; the examination of 4,476 samples of eggs for antimicrobial residues in 2016 revealed positivity of 8 (0.18 %) samples for doxycycline, enrofloxacin, flumechin and sulfadiazine, and tests for anticoccidial and coccidiostat residues conducted in the same year on 3,933 egg samples detected positivity of 32 (0.81 %) samples for lasalocid, monensin, narasin, salinomycin, diclazuril, dinitrokarbanyl, toltrazuril, toltrazuril sulfone and robenidin [5].

The results above clearly indicate that the monitoring and control of residues are essential to the protection of the public health and to validation the safety of products of animal origin. The results presented in our study clearly indicated the presence of antimicrobial residues in fresh hen eggs intended for human consumption that were randomly selected for screening tests. Hen eggs are an important food matrix and their safety in the first link of the food chain, i. e. by egg producers, should be guaranteed.

CONCLUSIONS

The aim of this study was to use and compare the current methods available for screening of residues (Premi®Test and EXP Ampulle tests) for the determination of the potential presence of antimicrobial residues in fresh hen eggs available on the market in 11 European countries. These two commercial tests were developed for the detection of a broad spectrum of antimicrobial residues in various food matrices including eggs. The higher number of positive and dubious results detected by EXP Ampulle test indicates the relevance of the residue control and use of microbiological methods for simple, rapid and economic detection of potentially non-compliant results. However, the confirmation of this potentially non-compliant results and necessity of taking relevant corrective measures requires identification and quantification of the respective antimicrobial compound.

ACKNOWLEDGEMENT

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MASTITIS PATHOGENS ISOLATED FROM RAW MILK SAMPLES ON SHEEP FARMS SITUATED IN MARGINAL PARTS OF SLOVAKIA

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ABSTRACT

Relatively large parts of the Slovak territory are marginal regions, which in terms of the economy of ruminant rearing can effectively produce animal commodities only occasionally. The aim of this study was to evaluate the occurrence and aetiology of mastitis during one milking season in two sheep dairy herds of 224 (A) and 270 (B) ewes situated in the marginal parts of Slovakia. A complex examination of health status of ewes based on: the clinical examination of the udder, macroscopic evaluation of milk with the California mastitis test (CMT) and the bacteriological analysis of raw milk samples from individual halves were carried out at the beginning (April) and at the end (September) of the milking season. The prevalence of intramammary infection (IMI) in the herds of ewes (A and B) were 19.1 % and 14.3 %, respectively. In both herds we confirmed predominantly the subclinical forms of IMI. The highest percentages of aetiological agents were determined for coagulase negative staphylococci (CoNS), such as *Staphylococcus chromogenes*, *Staphylococcus schleiferi*, *Staphylococcus epidermidis*, *Staphylococcus xylosus*, *Staphylococcus piscifermentans* and *Staphylococcus intermedius*, the occurrences of which were determined in 41 (48.2 %) and 37 (47.9 %) positive milk samples, respectively.

Key words: coagulase negative staphylococci; ewes; marginal parts; mastitis; prevalence

INTRODUCTION

A problem involving the agricultural production in marginal areas exists not only in highly developed countries but is typical also for countries in the transient period of economy development. Slovakia is one of these countries, with a relatively high share of marginal agricultural areas [15]. Products from dairy ruminants are unique, especially in the field of rational nutrition of consumers. Many of these products and specialties can be included among the functional foods [20].

The production of sheep milk is currently the main aim of many agricultural farms localized in marginal regions.

Milk plays a crucial role in the economy of cooperatives and farms. The price of milk is affected by sheep milk market, but also by farmers themselves. On the farmer's side, there are legislative limits for the total number of microorganisms in the milk delivered, which cannot be exceeded. An equally important contribution of breeders is also the hygienic safety of the milk for the consumers, especially in the marketing of milk and milk products directly sold on the farm [7, 17].

Mastitis is an important disease of ewes occurring in all countries of the world where sheep are kept. This inflammation of the mammary gland is known to be a complex and costly disease [1, 16]. The disease is associated with a decrease in milk production, an increase of veterinary services, treatment, labour costs and culling [3, 14].

Sheep mastitis is predisposed by several epidemiological risk factors that play a significant role in causing mammary incompetence to protect it from the invasion of infectious agents. Many bacterial agents, such as *Staphylococcus aureus, Streptococcus agalactiae, E. coli* or coagulase negative staphylococci (CoNS) have been found to be associated with clinical (Fig. 1) or subclinical mastitis in ewes [2, 7].

The aim of this study was to evaluate the occurrence and aetiology of mastitis at the beginning (April) and the end (September) of the milking season in two dairy sheep herds situated in marginal parts of Slovakia.

MATERIALS AND METHODS

Animals and milking

The practical part of the study was performed on two sheep herds situated in marginal parts of eastern Slovakia (Gelnica, Trebišov), kept under standard animal husbandry and hygiene conditions. The herd A (224 sheep) consisted of the breeds, Improved Valaska and Lacaune. The herd B (270 sheep) consisted of the breeds, Improved Valaska and Tsigaia. The ewes were milked twice a day after weaning of their lambs at the beginning of April. In herd A, machine milking was performed using two-line milk parlour 2 × 14 Miele Melktechnik, (Hochreiter Landtechnik, Germany) and in herd B sheep were milked in two-line milk parlour 2 × 15 Alfa Laval Agri (Alfa Laval, Sweden).

Collection of samples and laboratory analyses

The complex examination of the health status of the udders in ewes was carried out at the beginning (April) and the end (September) of the milking season. The sheep were examined clinically according to Hariharan et al. [5], e. g. for swelling, presence of lesions or anatomical malformations, and milk from individual halves were evaluated by the California mastitis test (CMT). The CMT scores were 0, +, ++, and +++ for: "negative", "weak positive", "positive", and "strong positive", respectively [3]. The emphasis was put on aseptic sampling and transport of the milk samples from individual halves intended for bacteriological examination.

The bacteriological examinations were performed according to the commonly accepted rules [11]. Milk samples (0.05 ml) were inoculated onto blood agar (Oxoid, UK) and cultivated at 37 °C for 24 hours. Based on the colony morphology and Gram staining, bacteria *Staphylococcus* spp. were selected for the tube coagulase test (Staphylo PK, ImunaPharm, Slovakia). The suspected colonies of *Staphylococ-*

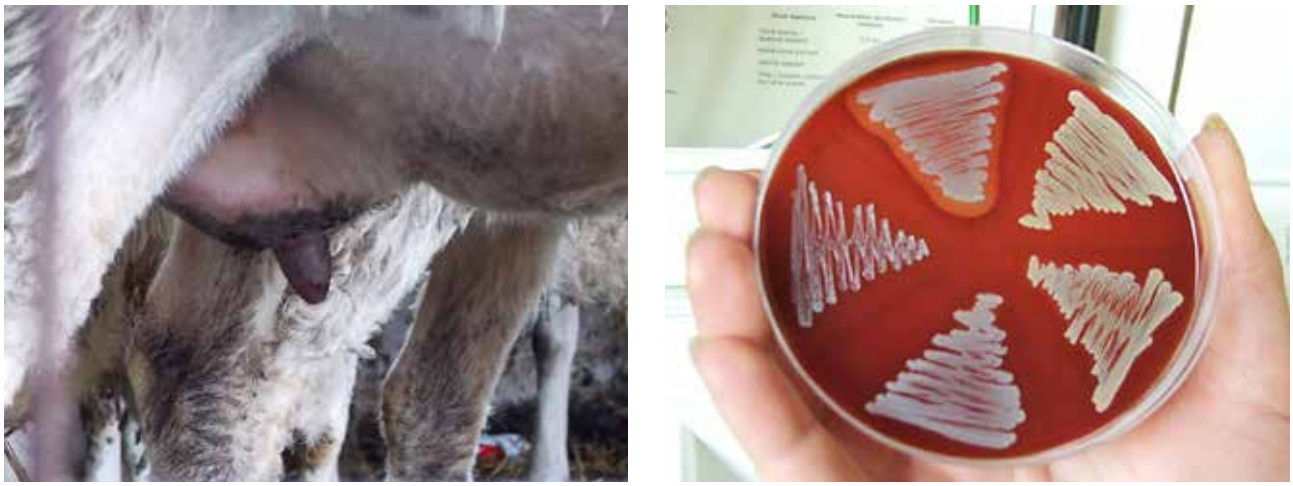

Fig. 1. Peracute mastitis caused by *Staphylococcus aureus*

cus spp., *Streptococcus* spp. and *Enterobacteriacae* spp. were isolated on blood agar, cultivated at 37 °C for 24 hours and identified biochemically using the STAPHY-test, STREPTO-test, or ENTERO-test and identified by software TNW Pro 7.0 (Erba-Lachema, Czechia).

The health of the udder and individual forms of mastitis (subclinical and clinical) based on clinical signs, abnormal udder secretions, CMT scores and bacteriological examination with positive culture result were classified according to Fthenakis [3, 4].

Statistical analysis

The results were analysed statistically using the GraphPad Prism 6.0 (GraphPad Software Inc., USA). The differences in the prevalence of mastitis among herds were statistically analysed using the Chi-square test. The level of significance was set to $P < 0.05$.

RESULTS

Table 1 shows the prevalence of intramammary infection (IMI) in dairy sheep herds during the milking season. The evaluation of the CMT showed that 23.2 % of the samples in herd A and 16.6 % of the samples in herd B were scored as either weak positive, positive or strong positive. In herd B, we observed a decreased prevalence of positive halves as well as a higher number of healthy halves. The prevalence of IMI with the positive CMT and bacteriological cultivation of individual raw milk samples in the monitored herds A and B of ewes was 19.1 % to 14.3 %, respectively.

The prevalence of subclinical mastitis among herds A and B varied from 13.9 % to 6.5 %, respectively. The differences in the prevalence of subclinical forms in the examined halves among herds were significant at the beginning of milking season ($P < 0.05$) (Fig. 2). The prevalence of clinical mastitis ranged from 1.8 % to 2.2 %.

The bacteria isolated from the infected halves of the herds are presented in Tables 2 and 3. Pathogenic bacteria were isolated from 16.5 % (162) of all of the 981 investigated halves. The most frequent aetiological agents of mastitis were CoNS, *Staphylococcus aureus, Streptococcus sanguinis, Streptococcus uberis* and *Enterococcus* spp. Staphylococci were the main aetiological IMI agents in the dairy sheep herds. In both herds CoNS occurred in the highest proportion (A: 41, [48.2 %]; B 37, [47.9 %]) in the positive cases. The bacteria of the genus *Staphylococcus* were the most numerous and besides *Staphylococcus aureus* there were isolated also *Staphylococcus chromogenes, Staphylococcus schleiferi, Staphylococcus piscifermentans, Staphylococcus epidermidis, Staphylococcus intermedius,* and *Staphylococcus xylosus*. Of these, CoNS, *Staphylococcus chromogenes* was isolated regularly throughout the milking season in the breeds with clinical IMI in two cases. S*taphylococcus aureus* with *Streptococcus uberis* and *Streptococcus sanguinis* were the most frequent isolates from the clinical mastitis cases.

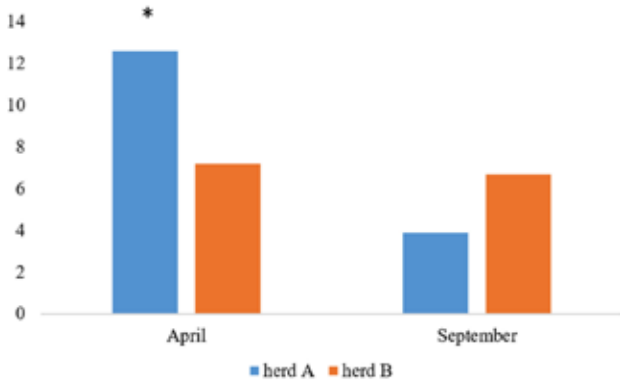

Fig. 2. Comparison of subclinical forms of mastitis [%] during the milking season
*P < 0.05 — significant differences in the incidence of subclinical mastitis among herds

Table 1. Prevalence of mastitis in the examined halves of dairy sheep herds

Herd	No. of examined sheep	No. of examined halves	Healthy halves		Rejected halves	Positive halves*		Infected halves	
			n	%		n	%	n	%
A	224	444	341	76.8[a]	4	103	23.2[a]	85	19.1
B	270	537	448	83.4[b]	3	89	16.6[b]	77	14.3
Total	**494**	**981**	**789**	**80.4**	**7**	**192**	**19.6**	**162**	**16.5**

* — CMT with score +, ++ and +++; [a, b] — values within the same column with different superscript letters differ significantly at $P < 0.05$

Table 2. Bacteria isolated from positive halves of herd A

Bacteria			Subclinical forms				Clinical forms			
			April		September		April		September	
	n	%	n	%	n	%	n	%	n	%
S. aureus	9	10.6	4	4.6	1	1.1	3	3.4	1	1.1
S. chromogenes	15	17.2	11	12.6	3	3.4	–	–	1	1.1
S. piscifermentas	11	12.6	9	10.6	2	2.3	–	–	–	–
S. schleiferi	10	11.5	7	8.0	3	3.4	–	–	–	–
S. xylosus	5	5.7	5	5.7	–	–	–	–	–	–
Str. sanguinis	8	9.2	4	4.6	2	2.3	1	1.1	1	1.1
Ent. faecalis	14	16.1	10	11.5	3	3.4	1	1.1	–	–
E. coli	7	8.0	2	2.3	5	5.7	–	–	–	–
Other*	6	6.9	4	4.6	2	2.3	–	–	–	–
Total	**85**	**100**	**56**	**64.3**	**21**	**24.1**	**5**	**5.7**	**3**	**3.4**

n — number of isolated bacteria; other* — *Proteus* spp., *Aerococcus* spp.

Table 2. Bacteria isolated from positive halves of herd B

Bacteria			Subclinical forms				Clinical forms			
			April		September		April		September	
	n	%	n	%	n	%	n	%	n	%
S. aureus	17	22.1	6	7.8	7	9.1	1	1.3	3	3.9
S. intermedius	8	10.4	1	1.3	7	9.1	–	–	–	–
S. epidermidis	11	14.3	4	5.2	7	9.1	–	–	–	–
S. chromogenes	11	14.3	3	3.9	6	7.8	1	1.3	1	1.3
S. schleiferi	8	10.4	5	6.5	3	3.9	–	–	–	–
Str. uberis	7	9.1	2	2.6	2	2.6	1	1.3	2	2.6
Str. sanguinis	2	2.6	1	1.3	–	–	1	1.3	1	1.3
Ent. faecalis	2	2.6	2	2.6	–	–	–	–	–	–
Ent. gallinarum	2	2.6	2	2.6	–	–	–	–	–	–
E. coli	4	5.2	4	5.2	–	–	–	–	–	–
Other*	5	6.5	2	2.6	3	3.9	–	–	–	–
Total	**77**	**100**	**32**	**41.6**	**35**	**45.8**	**4**	**5.2**	**7**	**8.8**

n — number of isolated bacteria; other* — *Proteus* spp., *Bacillus* spp.

DISCUSSION

The prevalence rate of mastitis in sheep flocks usually varies from 1 to 30 %. In our study the prevalence of mastitis in the A and B herds of dairy ewes was 19.1 % and 14.3 %, respectively. In the study by Contreras et al. [2], in only 10 % of the flocks had a prevalence that exceeded 10 %. In fact, within a flock the prevalence risk of mastitis exceeding 20 % is very rare; as is bilateral mastitis caused by common mammary pathogens.

Subclinical mastitis is financially the most crippling form of the disease [19] because it has detrimental effects on the milk yield of ewes and on the growth of their lambs [8]. At the start and end of the lactation season we confirmed predominantly subclinical forms in both herds. The economic losses are more associated with subclinical mastitis which is 40 % more prevalent than clinical mastitis [6]. The clinical cases were most frequently caused by *Staphylococcus aureus*, *Str. uberis* and *Str. sanguinis*.

Many microbial species that are common causes of ruminant mastitis, such as *Streptococcus agalactiae, Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae*, also occur as commensals or pathogens of humans whereas other causative species, such as *Streptococcus uberis, Streptococcus dysgalactiae* subsp. dysgalactiae or *Staphylococcus chromogenes*, are almost exclusively found in animals [9, 21].

Although a wide range of microorganism species may cause sheep mastitis, most cases are reported to be due to staphylococci [12].

Lafi and Hailat [10] reported that *S. aureus* was the predominant bacterial strain (50 %) in milk from ewes, followed by *E. coli* (27 %) and *Pseudomonas aeruginosa* (7 %). Narenji et al. [13] reported that *Staphylococcus aureus* (72.2 %) and CoNS (66.6 %) were the most common isolates from dairy ewes' subclinical mastitis. The main *Staphylococcus aureus r*eservoirs in sheep are suggested to be the infected mammary glands and teat lesions. However, *Staphylococcus aureus* can also be cultured from intact teat skin and other body sites.

The importance of coagulase negative staphylococci in the aetiology of sheep mastitis was also confirmed in our study when six species isolated from subclinical mastitis were recorded in the 79 individual samples from the two sheep breeds. Only *Staphylococcus chromogenes* together with the coagulase positive *S. aureus* caused the clinical forms of mastitis.

Thorberg et al. [18] in their study isolated *Staphylococcus epidermidis* and *Staphylococcus chromogenes* from the raw milk of dairy ruminants. The same bacteria were also isolated from skin of the people that milked the dairy animals, because isolation of *Staphylococcus epidermidis* from human skin is more common than isolation from skin of the udder. The authors concluded that humans are probably the main source of infection with coagulase negative species during milking.

CONCLUSIONS

In conclusion, the results of this study indicated that the prevalence of mastitis in the herds A and B reached 19.1 % and 14.3 %, respectively. It was also observed that CoNS are the most common cause of subclinical mastitis in dairy sheep herds situated in the marginal parts of Slovakia.

A successful mastitis control program should focus on good management that can ensure: a clean and comfortable environment, proper feeding and adequate supplementation of the diet with vitamins and trace elements. The prevention of mastitis consists in a reduction of the exposure of the teat ends of the mammary gland to the environmental pathogenic bacteria capable of surviving in the external environment outside the udder.

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PYGOMELIA AND TRUE HERMAPHRODITISM IN A NINE WEEK OLD LARGE WHITE PIGLET CASE REPORT

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ABSTRACT

A nine weeks old female Large White piglet which was presented to the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta, with a complaint of extra limbs was diagnosed with pygomelia and concurrent true hermaphroditism based on gross morphologic features, radiography, exploratory laparotomy and histopathology of the malformed organs. The piglet had two well-developed extra hind limbs consisting of the femur, tibia, fibula and the phalanges. Radiographically, the accessory limbs were attached to the ischium through a rudimentary pelvic bone. The supernumerary limbs were smaller than the normal appendages, but contained equal digits. The anal orifice was observed cranial to the right supernumerary limb. Caudal to the left supernumerary limb a rudimentary penis was observed. Two oval shaped fibrous masses were palpated in the inguinal canal of the piglet. In addition, there was a transparent tubular tract measuring 24 cm in length which contained serous fluid. The right kidney was rudimentary measuring 2.10 cm, while the left kidney appeared hypertrophied measuring 6.10 cm. The histology of the left kidney showed dysplastic areas of undifferentiated mesenchymal stroma in the cortex and medulla with the presence of groups of immature glomeruli in the cortex. The tubules in the medulla were scanty in number and had atypical epithelium. The adrenal glands had normal architecture with ectopic adrenal tissue in the adrenal capsule, while the ovaries and uterus were normal. It was concluded that the complex anomalies in the piglet might be as a result of a complex mode of inheritance.

Key words: hermaphroditism; intersex; pigs; pygomelia

INTRODUCTION

Developmental malformations occur relatively frequently in swine in comparison to other domestic species. Frequently reported disorders include myofibrillar dysplasia, umbilical and inguinal hernias, cryptorchidism, intersexes, and anal atresia [3]. The overall incidence of develop-

mental defects in piglets was 2.07 % [14]. The frequency of developmental anomalies in pigs depends upon the breed and population, and has been reported to be decreasing in some countries [2].

Congenital anomalies may be caused by genetic factors (transgenes, chromosomes), environmental agents (infections, teratogens, toxins, fertilization techniques, management) or a combination of factors [7]. There have also been reports indicating that chromosomal aberrations are associated with congenital limb malformations [9]. A polygenic mode of inheritance has been suggested in most of the cases, however, not all congenital defects are heritable.

The congenital anomalies of the musculoskeletal system are considered to be one of the most frequent anomalies found in humans and animals [8]. Supernumerary ectopic limbs or polymelia is a congenital anomaly in which accessory limbs are attached to the various body regions [4]. These accessory limbs are smaller than normal limbs, and are associated with stiff joints and sparse muscles without innervations. The condition has been reported in calves, ewes, goats, poultry and in humans, but they are extremely rare in swine [12, 13]. Only one case of polymelia has been described in swine and it was associated with features such as *duplicitas coli partialis et recti*, *atresia ani* with *recto-genital fistula*, *duplicitas corpori uteri*, *cervicis*, v*aginae et vulvae*, and *duplicitas vesicae*, *urethrae et renalis* [11].

Pygomelia is a variant of polymelia in which the accessory limbs are attached to the pelvis through rudimentary *os coxae*. It usually results from a caudal bifurcation of the long axis of the body. It has been reported in cross breed calves [6, 10]. However, no record of pygomelia in pigs could be found in the literature. This paper described a rare case of pygomelia in a nine weeks old female large white piglet. The congenital defect was also associated with a true hermaphroditism.

CASE PRESENTATION

A nine weeks old female large white piglet was presented with a complaint of extra limbs to the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta; from a farm of 40 adult pigs located at Ifo local government area of Ogun State in Nigeria. It was one of a litter of six piglets farrowed by a primiparous 16 months old sow mated to a 14 months old boar. The pigs on the farm were fed blood meal, palm kernel cake, cassava peels and water leaves. An outbreak of African swine fever was reported on the farm in 2009, although the current pigs on the farm were from a new stock. There were no records of umbilical hernia or any other congenital anomaly from the farm except for the current case. The anomaly was detected at birth and did not affect the parturition in the sow.

The piglet weighed 3 kg at presentation, while the posture and conformation of the piglet reflected a healthy appearance. The skin was smooth with no abnormality. All the physiological parameters were essentially normal. An examination revealed two well-developed extra hind limbs (Fig. 1), consisting of the femur, tibia, fibula and the phalanges, which were attached around the mid pelvis caudal to the tail of the piglet. The supernumerary limbs were smaller than the normal appendages, but contained equal number of digits and the appendages were non-functional. The anal orifice was observed cranial to the right supernumerary limb, and caudal to it was a vagina, while a penis was observed caudal to the left supernumerary limb. Two oval shaped fibrous masses thought to be the testes were palpated in the inguinal canal of the piglet. Other external features of the piglet were essentially normal.

MANAGEMENT AND OUTCOME

Antero-posterior and lateral radiographs of the abdomen and hip of the piglet were obtained using a mobile

Fig. 1. Picture of a neight weeks old Large White piglet with supernumerary limb (SL)

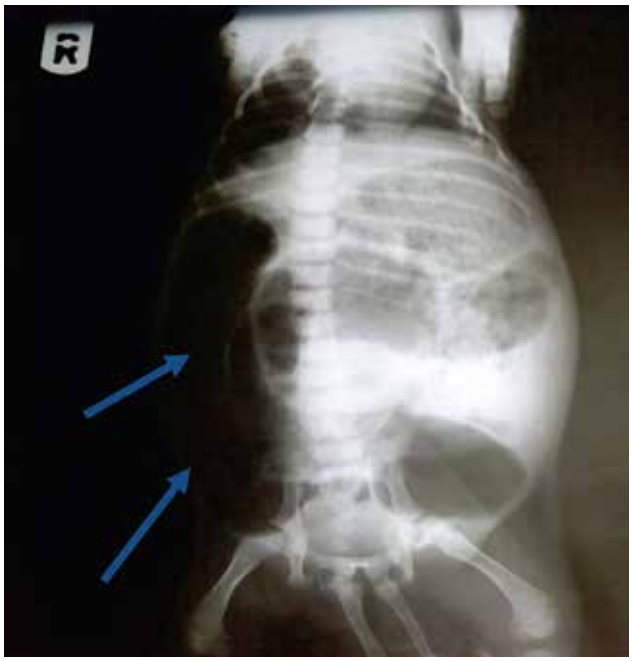

Fig. 2.Ventro-dorsal radiograph of the abdomen of an eight weeks old Large White piglet showing the gas filled caecum and colon

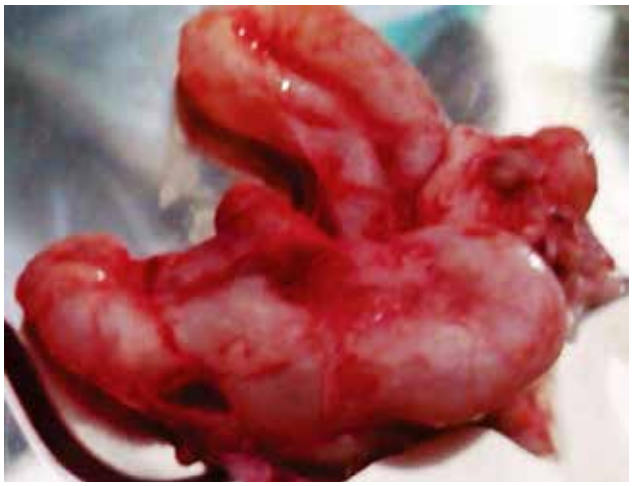

Fig. 4. Caudo-dorsally located transparent tubular structure from the abdomen of an eight weeks old Large White piglet

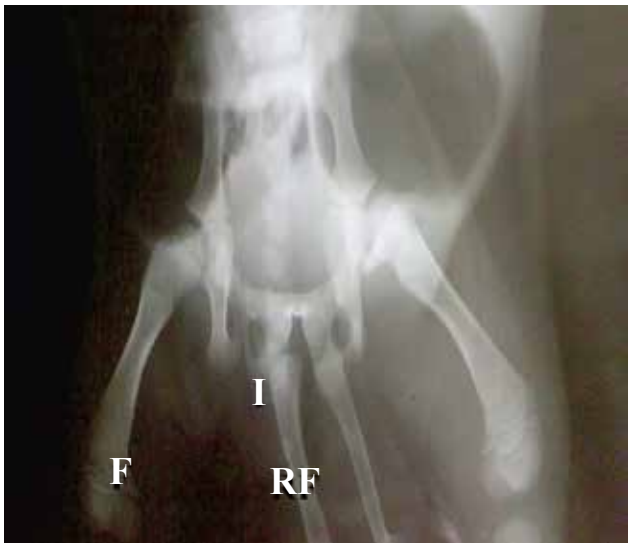

Fig. 3.Antero-posterior radiograph of eight weeks old piglet showing a rudimentary limb (RF) attached to the ischium (I) of the piglet. The main femur (F) is parallel to the rudimentary femur

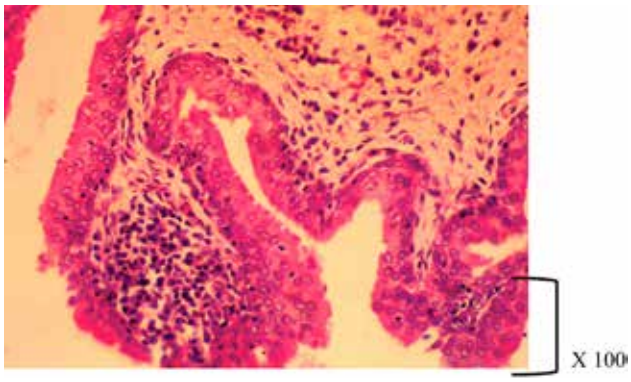

Fig. 5. Photomicrograph of a transparent caudo-dorsal abdominal tubular structure showing segmental hyperplasia of transitional epithelial mucosa and hyperplasia of associated lymphoid tissue. Magn. ×100)

x-ray machine (PLH Medicals, Herts, UK). Thereafter, the piglet was anaesthetized using intramuscular injections of 0.04 mg.kg^{-1} of atropine sulphate (Atocan®, Sishui Xierkang Pharma, China), 0.5 mg.kg^{-1} of 2 % xylazine (Xylazine 20 Inj®, Kepro, Holland), 0.5 mg.kg^{-1} diazepam (Calmpose®, Ranbaxy, Dewas, India) and 10 mg.kg^{-1} of 5 % ketamine hydrochloride (Rotexmedica, Trittau, Germany). Two percent lignocaine was also infiltrated around the ventral midline to obtain good somatic analgesia. Thereafter, an exploratory laparotomy was done to check if there were other anomalies internally. The structurally deformed abdominal organs were excised and fixed in 10 % formaldehyde for histopathology.

The abdominal radiograph of the piglet revealed radiolucent gas filled caecum and colon and increased soft tissue opacity around the middle and caudal abdominal cavity (Fig. 2). There was a completely formed but rudimentary pelvic bone which attached the accessory limb to the ischium of the piglet (Fig. 3). The colon and caecum were gas distended and occupied most of the part of the abdominal cavity. A transparent tubular tract measuring about 24 cm in length and containing serous fluid was observed craniodorsal to the bladder (Fig. 4). The histology of the structure

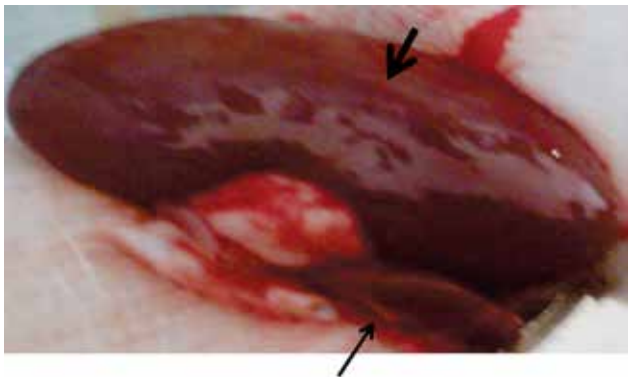

Fig. 6. Kidneys of an eight weeks old Large White piglet showing a normal kidney (Big arrow) and a rudimentary kidney (Small arrow)

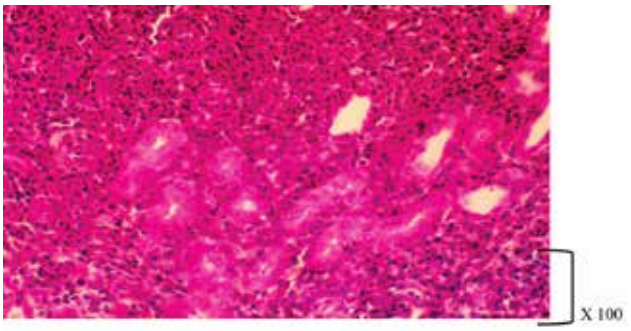

Fig. 7. Photomicrograph of the rudimentary kidney of the piglet showing dysplastic areas of undifferentiated mesenchyme in the cortex and medulla with the presence of immature blind ending tubules. Magn. ×100)

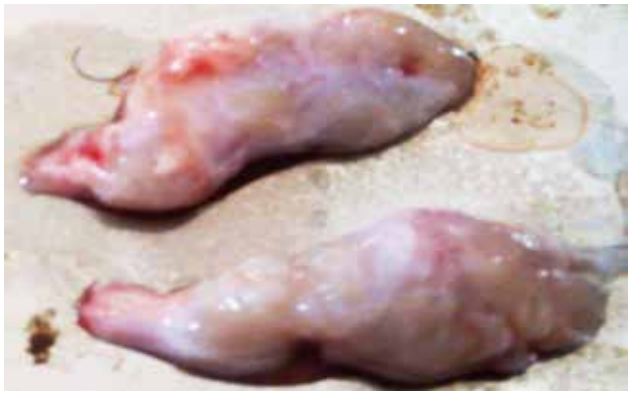

Fig. 8. Paired rudimentary oval shaped structures measuring about 2.4 cm in length from the inguinal canal of the piglet

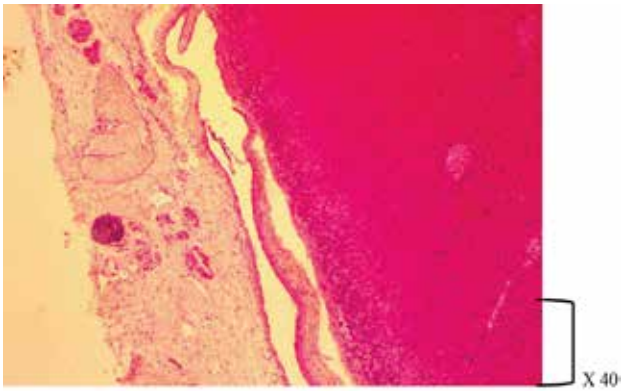

Fig. 9. Photomicrograph of the inguinal structure showing a normal architecture of the adrenal gland with ectopic medullary tissue in the cortex. Magn. ×40

revealed segmental hyperplasia of the transitional epithelia mucosa and the associated lymphoid tissue (Fig. 5). The ovaries and uterus were normal. The right kidney was rudimentary measuring 2.10 cm, while the left kidney appeared hypertrophied measuring 6.10 cm (Fig. 6). The histology of the left kidney showed dysplastic areas of undifferentiated mesenchymal stroma cells in the cortex and medulla with the presence of groups of immature glomeruli in the cortex (Fig. 7). The tubules in the medulla were scanty in number and had atypical epithelium. The spleen, liver and stomach of the piglet appeared essentially normal. An incision into the inguinal canal revealed two rudimentary oval shaped structures measuring about 2.4 cm in length (Fig. 8). The histology of the adrenal gland showed normal architecture with foci of ectopic parenchymal tissue in the thickened adrenal capsule (Fig. 9).

DISCUSSION

Congenital defects like umbilical and inguinal hernias, cryptorchidism, intersexes, and anal atresia occur relatively frequently in swine [3]. However, congenital defects of the limbs are very rare in pigs. Polymelia with dactylogryposis have been reported in a five months old female Landrace and Pietrain piglet cross [11]. There appears to be no report of congenital limb anomaly in the Large White pigs. This report describes a complex malformation in a Large White piglet characterized by accessory limbs, rudimentary left kidney, an accessory and rudimentary urinary system, and ectopic adrenal tissues located within the thickened adrenal capsule. In addition, the piglet was an intersex with an external penis, inguinally located testes with bulbourethral gland and well-formed ovary, uterus and vagina. This report appears to be the first case of pygomelia in pigs with concurrent hermaphroditism.

The common causes of genetic abnormality in pigs include: folic acid deficiency, exposure to radiation and chemicals, toxins such as mycotoxin and viral diseases. In only 13 % of congenital defects in pigs, are the causes known or believed to be heritable, or a known environmental or teratogenic agent identified [11]. The exact cause of the anomaly in this piglet is unknown. The history of the stock did not contain any prior congenital anomalies and also precluded the possibility of exposure to poisonous plants, radiation or chemicals. Although there was an outbreak of African swine fever on the farm, the farm had been rested for five years prior to re-stocking. Also, the present stocks were reported to be obtained from a new source.

Intersexuality is a frequently occurring defect in pigs with reported frequencies ranging from 0.1 percent 1.4 percent [3]. In general, intersexes may be either true hermaphrodites, which have gonads of both sexes, or pseudohermaphrodites having either female or male gonads. The greater majority of pig intersexes have been reported to belong to be pseudohermaphrodites and have been shown to possess the normal female XX chromosome constitution. This piglet can be regarded as a true hermaphrodite owing to the presence of: a penis, inguinally located testes, bulbourethral gland, a pair of ovaries and a well-formed uterus with an externally located vagina. The probable cause of the intersex in the piglet may be a recessive autosomal gene with sex-limited expression either with polygenic inheritance or more likely due to recessive genes at very few autosomal loci.

The most common abnormality in the male reproductive system is cryptorchidism. It is hypothesized to be due to complete penetrant recessive genes at two autosomal loci [1]. Most cases of cryptorchidism in piglets are also associated with other abnormalities. Although the piglet can be regarded as a true hermaphrodite, the presence of inguinally located testes is suggestive that the genetic cause for the intersex might also be responsible for this abnormality.

The inheritance of polydactylism is much less clear, owing to the irregular expression of the defect. A case of autosomal dominant complete polydactyly has been reported in Papua New Guinea village pigs [5]. Although cytogenetic analysis was not done for this piglet, it is logical to presume that the skeletal anomaly was inherited and the mode of inheritance was probably complex and related to the factors responsible for the intersex in the piglet.

Finally, the presence of a unilateral renal dysplasia might have compromised the urinary functions of the piglet which probably led to the hypertrophy of the second kidney. This might explain why the piglet did not recover from a single injection of ketamine hydrochloride since the anaesthetic agent is excreted through the kidney unchanged. The vesicular structure in the abdomen will appear to be a rudimentary accessory urogenital tract or an accessory rudimentary bladder because of the predominant transitional epithelium of its mucosa later seen in the histology.

In conclusion, this report presents probably the first case of a complex malformation of true hermaphroditism and pygomelia in a pure bred Large White piglet.

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ANTIOXIDANT ACTIVITY OF THE FUNGUS *CORDYCEPS SINENSIS* GROWN ON TWO DIFFERENT MEDIA

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ABSTRACT

***Cordyceps sinensis*, a species of the genus *Ascomycetes*, is recognised as the most famous tonic herb and natural remedy in traditional Chinese medicine for centuries. Various pharmacological actions of the chemical constituents of *C. sinensis* have been reported, including: antitumour effects, hepatoprotective and anti-inflammatory effects, and antioxidant, nephroprotective and anti-apoptotic properties. In this study we tested the antioxidant activity of extracts of the fungus *C. sinensis* grown on two subspecies of rice, *Oryza sativa* var. *Indica* and *Oryza sativa* var. *Japonica*. The extracts were prepared with methanol by two different extraction procedures (reflux and ultrasound). The antioxidant activity of the extracts was determined by the DPPH assay. Our investigations showed that the sample 1 (grown on *Oryza sativa* var. *Japonica*) exhibited higher antioxidant activity than the sample 2 (grown on *Oryza sativa* var. *Indica*). The higher antioxidant activity of the sample 1 was observed with both extraction procedures.**

Key words: antioxidant activity; *Cordyceps sinensis*; DDPH radical; infrared spectroscopy; *Oryza sativa* var. *Indica*; *Oryza sativa* var. *Japonica*

INTRODUCTION

Medicinal mushrooms or mushroom extracts have been traditionally used for centuries in China and Japan as herbal medicines because they possess large amounts of essential amino acids, nucleotides, important minerals, vitamins and enzymes [10]. *Cordyceps sinensis* is one of such medicinal fungi, which belongs to the *Clavipitaceae* family and the genus *Ascomycetes* [18].

Up to the present, more than 680 *Cordyceps* species, many with various curative effects have been documented, for example: *Cordyceps sinensis*, *Cordyceps militaris*, *Cordyceps sobolifera*, *Cordyceps subssesilus* and *Cordyceps ophioglossoides* [7, 12].

The first written mention of *C. sinensis* in China appeared in 620. It described *Cordyceps* as a "creature" which changes from animal to plant in the summer and from plant

to animal in the winter. This "medicinal animal/plant" has been discussed by Tibetan scholars since the 15th century. The first scientific description of this fungus was presented by Ben Cao by Wu-Yiluo in 1757 [5].

C. sinensis is an entomogenous fungus that parasitizes the larva of the Tibetan ghost moth (some authors use incorrectly the term, "bat moth" of the family *Hepialus Armoricanus*) [6] and this entire fungus and larva combination results in an unique profile of secondary metabolites which is used for medicinal purposes [18]. Its normal harvesting period stretches from April to August. It grows only in high-altitude regions of about 3800 m above sea level, in cold grassy alpine meadows of the Himalayan Mountains.

C. sinensis and other *Cordyceps*, although they are not normally considered as food (mostly due to its small size, endemic character and tough structure) they contain a broad range of nutritional compounds. This includes all basic amino acids, vitamins E and K and water soluble vitamins B_1, B_2 and B_{12}. In addition, they contain many sugars including mono-, di- and oligosaccharides and many complex polysaccharides, proteins, sterols, nucleosides and trace elements [1].

Chen et al. [2] characterized cordycepin in *Cordyceps sinensis* extract by means of nuclear magnetic resonance (NMR) and infrared spectroscopy. They identified additional components such as various saccharides and polysaccharides, including cyclofurans and many other nucleosides such as: uridine, adenosine, guanidine, deoxyguanidine and deoxy-nucleotides, not found anywhere in nature. Particularly important are immunosuppressive compounds such as cyclosporine [2] and cordycepic acid [16, 17]. Investigations have demonstrated that polysaccharides are effective in the regulation of blood sugar levels [8] and have also antimetastatic and antitumour effects [15].

Fungi of the genus *Cordyceps* are currently among the most expensive fungi/mushrooms. In the past they were used only by members of the Chinese imperial court or noblemen. The increased interest in their beneficial effects have resulted in a considerable increase in gathering the natural *Cordyceps* and, consequently, its natural market shortage. This has stimulated interest in its artificial culturing. Modern technological progress in artificial culturing has led to an increased availability of *Cordyceps* to the public and, at the same time, enabled testing of its medicinal activity. The high cost of both natural and cultured *Cordyceps* eventually resulted in the intentional replacement of high quality products by low-grade, impure ones.

The difference between cultured *Cordyceps* and wild growing fungi led to an assumption that the wild varieties are more effective. However, it was revealed that such an assumption is ungrounded [4]. The quality of *Cordyceps* grown in an artificial environment where the natural conditions of the Himalayan plateau are strictly simulated (clean air, low temperature, low atmospheric pressure) is equal to the natural product. The current modern culturing methods are capable of approximating the climate conditions in which the natural fungus grows and thus produce sufficient quantities of the cultured *Cordyceps*. Owing to new procedures, *Cordyceps sinensis* can be grown on other nutrient media such as rice, millet, corn and others.

The quality of cultured *Cordyceps* is assessed according to the content of mannitol and polysaccharides. Therefore, the need for information about these components are inevitable. The proportion of polysaccharides should exceed 40 % and the content of mannitol should reach minimally 15 %.

Oxidative stress is associated with an increased production of oxidising species or a significant decrease of antioxidants and is involved in various human diseases such as in: cellular necrosis, cardiovascular disease, cancer, neurological disorder and even ageing [3]. Non-toxic antioxidants from natural sources, especially medicinal plants are known to prevent oxidative damage caused by free radicals and are rich in polyphenolics and bioactive compounds [11].

The aim of our study was to contribute to the knowledge about antioxidant properties of methanol extracts of *Cordyceps sinensis* grown on two different media.

MATERIALS AND METHODS

Investigated samples

Samples of *C. sinensis* in a powdered form were obtained from the Technical University in Zvolen, Slovakia. Both samples were grown under the same local environmental conditions (incubation at 22 °C for 30 days, alternating 12 hours of light and darkness; drying at 40 °C in an APT Line Dryer, Binder GmBH, Tuttlingen, Germany; ground in a SM-100 mill (Retch Co., Haan, Germany).

In this study, which was part of a more extensive study, we tested the antioxidant activity of *Cordyceps sinensis*

grown on two substrates (subspecies of rice):

Sample 1: grown on *Oryza sativa* var. *Indica*

Sample 2: grown on *Oryza sativa* var. *Japonica*.

Preparation of extracts

The extracts from *C. sinensis* for testing of the antioxidant activity were prepared with 99 % methanol p. a (Sigma Aldrich). The selection of this extractant was based on our preliminary testing of various extraction agents (not published). Methanol extracts appeared suitable for the prospective pharmacological purposes; it provided a good yield and demonstrated better stability than water extracts.

Two different procedures were used for the preparation of extracts for each sample:

Ten grams of the sample were macerated with 100 ml of methanol for 24 hours in the dark, at room temperature. The macerate was refluxed for 4 hours, then filtered and concentrated to dryness by means of a rotary vacuum evaporator.

Ten grams of the sample were macerated with 100 ml of methanol for 24 hours in the dark, then extracted in an ultrasonic bath (Bandelin Sonorex Digitec s frekvenciou 35 kHz, P 140/560 W and DT 103H, Germany) for 1 hour at 60 °C, filtered and concentrated to dryness by means of a rotary vacuum evaporator.

Determination of antioxidant activity

The antioxidant activity of the samples were determined by DPPH (1, 1-difenyl-2-pikrylhydrazyl, p. a, Sigma Aldrich) assay based on the determination of the percentage reduction of the activity of a DPPH radical solution with measurements of the absorbance at $\lambda_{max} = 517$ nm [13] at room temperature, employing a UV-VIS spectrophotometer Libra S12 Biochrom Ltd., Cambridge CB4, OFJ, England). The measurements were carried out on a series of solutions with different concentrations of *C. sinensis*, diluted 1 : 1. As a blank we used 99 % methanol. The mass concentrations of *C. sinensis* in the solutions are presented in Figures 1—3.

The antioxidant activity of each solution was calculated as follows:

$$I\ (\%) = (AB - AS/AB) \times 100$$

Where I is the inhibition of DPPH radicals in per cent, AB is the absorbance of the blank DPPH solution, and As is the absorbance of the sample after 30 min of incubation with the DPPH solution in the dark at room temperature.

Determination of infrared spectra

The infrared spectra of the extracts were measured at the Faculty of Natural Science of the Pavol Jozef Šafárik University in Košice using the Alternate Total Reflection method (ATR). A spectrometer Thermo Scientific Nicolet 6700 FTIR was used.

RESULTS AND DISCUSSION

The measurements of the antioxidant activity of both samples were carried out in a series of solutions of *C. sinensis* extracts in methanol with increasing mass concentration of *C. sinensis*, diluted 1 : 1 (Figures 1—3).

Our measurements of the antioxidant activity of ex-

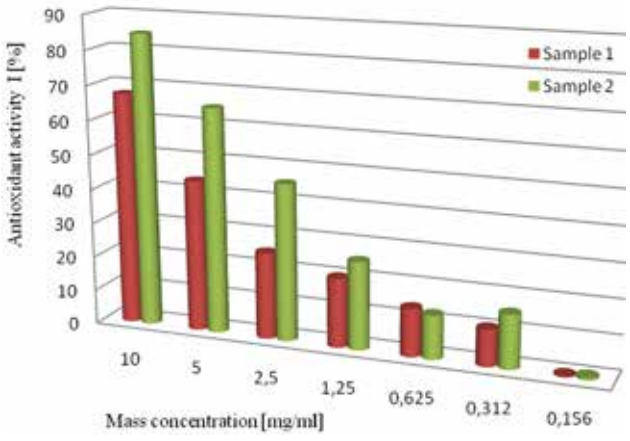

Fig.1. Comparison of the antioxidant activity of extracts of sample 1 (red) and sample 2 (green) prepared by theultrasound procedure

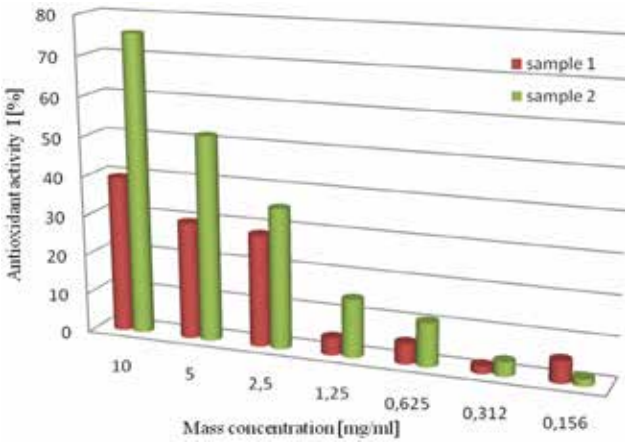

Fig. 2. Comparison of the antioxidant activity of extracts of sample 1 (red) and sample 2 (green) prepared by the reflux procedure

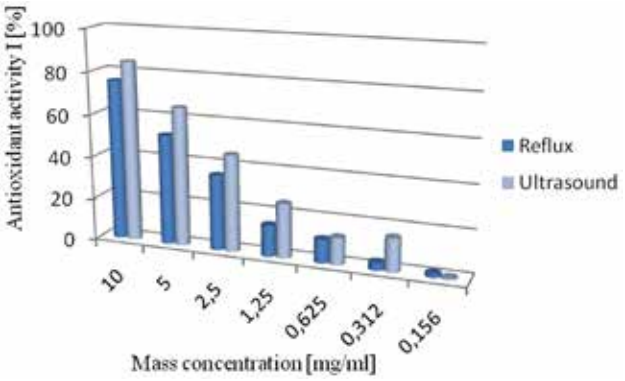

Fig. 3.Comparison of antioxidant activity of extracts of sample 2 obtained by reflux and ultrasound procedures

tracts of sample 1 demonstrated that the ultrasound extraction procedure resulted in a considerably lower (67.5 %) antioxidant activity in comparison with that of the sample 2 (Fig. 1) and, at the same time, in comparison with the reflux extraction (39.6 %) (Fig. 2). The inhibition percentages refer to the highest mass concentration of *C. sinensis* in $mg.ml^{-1}$.

The results indicated that the highest antioxidant activity (84.8 %) was determined for sample 2 in the extract obtained by the ultrasound procedure. This sample was grown on *Oryza sativa* var. *Japonica*. The antioxidant activity of extracts of this sample obtained by reflux was lower (75.7 %) (Fig. 3).

The results presented above indicated that sample 2 grown on *Oryza sativa* var. *Japonica* exhibited higher antioxidant activity than sample 1 grown on *Oryza sativa* var. *Indica*. The higher activity was observed with both extraction procedures, i.e. when using reflux and ultrasound. We assume that this difference was caused by the presence of different components in the medium that may have affected the production of *C. sinensis* constituents with antioxidant activity.

Liu et al. [9] published a review article on the chemical constituents and pharmacological actions of *Cordyceps sinensis*. According to references cited in this article, chemical constituents with a particular antioxidant activity included exopolysaccharides, CPS-1 — a water-soluble polysaccharide, and CME-1 — a water-soluble polysaccharide fraction with a molecular mass of 27.6 kD. This review article concluded that as investigations of this fungus continues, more bioactive constituents with potential therapeutic value will be isolated. However, new methods and technologies need to be adopted to extract and analyse the components, requiring evaluation along the modern scientific line. Overall, so far, we know only a little of the wonders of this medicinal fungus and it still has many secrets for us to discover.

Sample preparation is the first and very important step, which can greatly influence the accuracy of the analysis. *Cordyceps* extracts have been commonly prepared with water of varying temperature and ethanol (100 % or diluted with water). Yang and Li [19] used three extraction methods, i. e. organic solvent pressurized liquid extraction, boiling water extraction and ambient temperature water extraction for a quantitative determination of five nucleosides in natural and cultured *Cordyceps*, including: adenosine, guanosine, inosine, uridine and cordycepin. Similar results were obtained when organic solvent pressurized liquid extraction and boiling water extraction were applied. However, the amounts of nucleosides in the natural *C. sinensis* extracted with ambient temperature water was greatly increased except those of adenosine. The amount of the investigated nucleosides in cultured *C. sinensis* had no obvious variation among the three extraction methods.

In our study we used methanol for extraction of the cultured *C. sinensis*. Selection of this extractant was based on our preliminary testing of a range of various extraction agents. Methanol extracts appeared suitable for prospective pharmacological purposes and showed better stability than the other tested extracts.

It should be mentioned that there are other aspects of sample preparation that can affect the final results besides the extractant itself or the extraction procedure, such as the time of extraction. Thus, a number of various combinations of relevant factors should be tested to provide a more reliable answer.

The determination of the infrared spectra (Figs. 4—7) also confirmed differences in all four types of extracts indicated by the essential infrared absorption peak positions. In these spectra one can observe valence vibrations of carboxyl groups ν(C = O). Asymmetric vibrations were observed in the wavenumber range 1550–1610 cm^{-1} while symmetric vibrations were seen in the range 1335—1420 cm^{-1} [14]. These bands were present in all extracts. In our study of the extracts obtained by reflux procedure an asymmetric vibration appeared at 1589 cm^{-1} in sample 1 and at 1573 cm^{-1} in sample 2. Symmetric vibrations were present at 1406 cm^{-1} (sample 1) and 1386 cm^{-1} (sample 2). An asymmetric vibra-

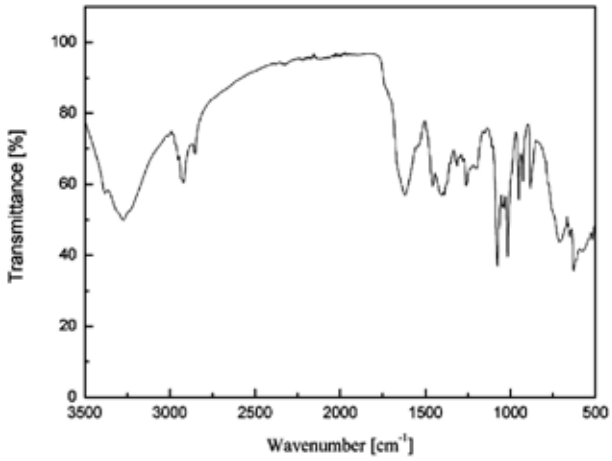

Fig. 4. IR spectrum of sample 1 (ultrasound extraction)

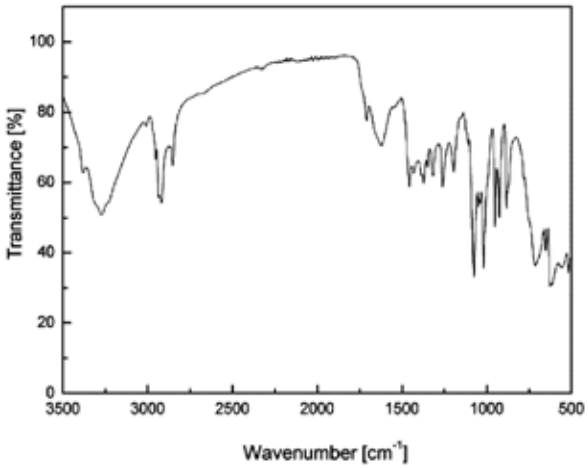

Fig. 5. IR spectrum of sample 1 (ultrasound extraction)

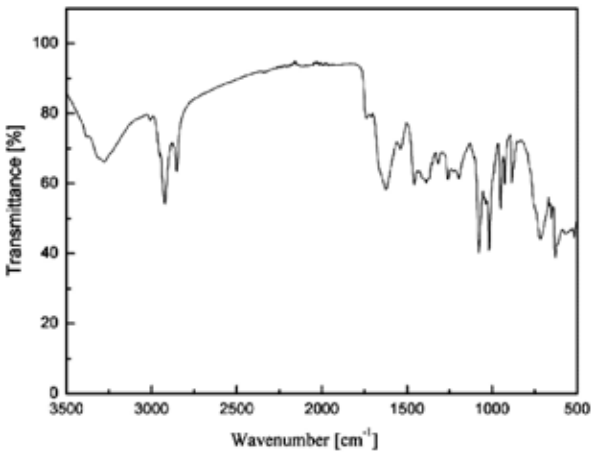

Fig. 6. IR spectrum of sample 1 (reflux)

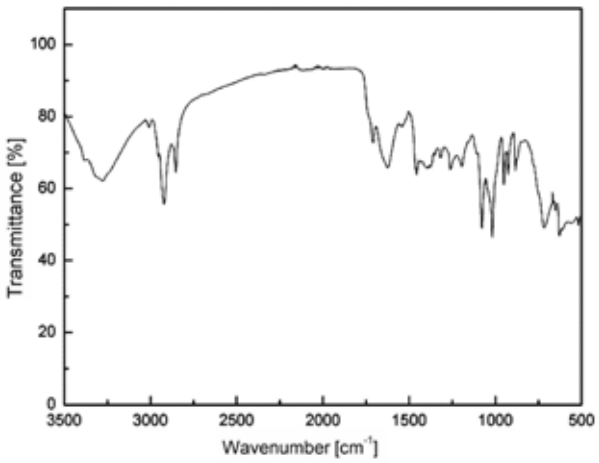

Fig. 7. IR spectrum of sample 1 (reflux)

tion in ultrasound extracts was located at 1617 cm^{-1} and the symmetric one at 1400 cm^{-1} (sample 1). In the IR spectrum of sample 2, the corresponding vibrations were observed at 1371 cm^{-1} and 1623 cm^{-1}. In all IR spectra there were bands indicating valence vibration ν(N-H) of the amino groups in the range of 3300—3500 cm^{-1}. There were also bands in the range of 3300 cm^{-1} to 2500 cm^{-1}, appertaining to valence vibrations of the –OH groups.

CONCLUSIONS

The determination of the antioxidant activity of extracts of two samples of cultured *Cordyceps sinensis* grown on different media, prepared by two different extraction procedures demonstrated that sample 2 grown on *Oryza sativa* var. *Japonica* exhibited higher antioxidant activity than sample 1 grown on *Oryza sativa* var. *Indica*. The higher activity of this sample was observed with both extraction procedures. This difference could be caused by the presence of different components in the medium that could affect the production of *C. sinensis* constituents with antioxidant activity. This assumption is supported also by the differences in the IR spectra of all four investigated extracts. However, the influence of the media used for growth of this fungus on the antioxidant activity of the extracts is a very complex issue and more research is needed in this regard.

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