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## MACROSCOPIC PARASITIC LESIONS OF SHEEP MEAT AT TWO SLAUGHTERHOUSES IN THE NORTH OF ALGERIA

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### ABSTRACT

A total of 10,696 randomly selected sheep have been collected in two slaughterhouses in the north of Algeria (El Harrach and Boufarik) to determine the prevalence of muscular cysticercosis and macroscopic cysts of sarcosporidiosis, to find out the association between prevalence and potential risk factors, as well as to assess the distribution of these parasites in the surface's muscles of slaughtered sheep. All of the slaughtered sheep carcasses were visually and carefully inspected. Cysticercosis and sarcosporidiosis were found in 220 (2.06 %) and 76 (0.7 %) sheep, respectively. For both diseases, the prevalence was significantly higher in females than males. The prevalence of *Cysticercus ovis* increased with age, and the difference was statistically significant ( $P < 0.0001$ ), while all infected animals were old (over 5 years old) for sarcosporidiosis. For *C. ovis*, it didn't have significant difference between the seasons, however, all sheep were infected in the spring for sarcosporidiosis. All the detected cysts of *C. ovis* were non-viable, and were more frequently detected in the heart (51.82 %), followed by the diaphragm (30.77 %) and the oesophagus (17.41 %). This anatomical distribution of *C. ovis* cysts showed a significant variation

( $P < 0.001$ ) in different predilection sites. Whereas, for sarcosporidiosis, the species involved was identified as *S. gigantea* by histology and were distributed in the organs as follows: the skeletal muscles (38.15 %), the oesophagus (31.57 %) and the diaphragm (30.26 %); no significant difference were noted between the 3 sites. The present study has revealed that these parasites are present in Algeria. Appropriate control measures need to be introduced to eradicate these parasites in sheep.

**Key words:** muscular cysticercosis; sarcosporidiosis; sheep; slaughterhouses

### INTRODUCTION

The infection of meat with macroscopic parasitic cysts can involve both protozoan and helminth species. Cysticercosis and sarcosporidiosis constitute the major part.

Cysticercosis in livestock and wild animals is caused by the larval stages (metacestodes) of cestodes (taeniosis), the tape worm of humans, dogs and wild canids. Muscle cysticercoses in sheep are caused by *Taenia ovis* (the adult stage of *Cysticercus ovis*), of which adults develop in the intestines of dogs and wild canids [29]. Infections with the

larval stage of some species of *Taenia* are of veterinary importance because they cause economic losses. These losses are associated with total condemnation of infected offal or carcasses with generalized infestation and downgrading of carcasses which are subjected to refrigeration, in addition to the cost of refrigeration and transport [24]. Like beef and pork cysticercosis, ovine cysticercosis due to *Cysticercus ovis* is of special interest in meat inspection because it affects the musculature, that part of the animal which is at once the most valuable for food purposes and the most difficult to inspect thoroughly.

*Sarcocystis* is one of the most prevalent protozoan parasites in the striated muscles of livestock such as cattle, sheep and goat slaughtered for human consumption [5]. Sheep are intermediate hosts of five *Sarcocystis* species: *Sarcocystis tenella* (synonym *Sarcocystis ovicanis*), *Sarcocystis gigantea* (synonym *Sarcocystis ovifelis*), *Sarcocystis arieticanis* and *Sarcocystis medusiformis*. *S. gigantea* and *S. medusiformis* are transmitted by felids and are non-pathogenic. *S. tenella* and *S. arieticanis* are transmitted by canids and are pathogenic [13]. Another species, *Sarcocystis mihoensis* has been recently reported to occur in sheep [31]. The losses caused by discrimination of the carcasses with macroscopic cysts during meat inspection represented a serious economic problem. Despite it all, sarcosporidiosis in Algeria is underestimated, because the inspection of the carcasses for sarcosporidiosis is not obligatory.

In Algeria, unlike cattle, cysticercosis and sarcosporidiosis in sheep are not taken into consideration because they are not zoonotic and no legislation has been put in place concerning these diseases. Indeed, there are limited reports concerning these parasitic diseases in slaughtered animals. Considering the importance of these pathologies, and the lack of epidemiological information concerning these diseases in Algeria, the present work was conducted to determine the prevalence of muscular cysticercosis and macroscopic cysts of sarcosporidiosis from sheep in two slaughterhouses (El Harrach and Boufarik) in the north of Algeria.

## MATERIALS AND METHODS

### Climatic conditions in northern Algeria

Along the country's northern Mediterranean coast, the

climate is typically Mediterranean with hot, dry summers, but mild, rainy winters. Rainfall is between 600 mm and 800 mm. Most of the annual precipitation occurs between October and April. The temperatures are rather uniform: the average daily temperature (maximum and minimum) is around 11 °C–12 °C in January, the coldest month of the year, while the average daily temperature (maximum and minimum) is around 25 °C–26 °C in July–August, the hottest months of the year. Summers are long and sunny, with rather humid air on the coast, but also with sea breezes.

### Inspection of sheep carcasses for macroscopic cysts and sampling method

10,696 sheep carcasses were inspected over a two-year period, from January 2017 to December 2018 in two abattoirs in the north of Algeria (7,856 sheep in El Harrach and 2,840 in Boufarik) during our study for *Cysticercus* spp. and macroscopic species of *Sarcocystis* spp. The sheep were native breeds and they originated from different sub-districts within the municipality and their environs. The animals inspected, were of different sex and age. A few females were sampled only, because the females are kept for breeding purposes, age was estimated and the animals were divided into three age groups:  $\leq 1$  year, 1.5–3 years and 3–5 years. All of the slaughtered sheep carcasses were carefully inspected, with particular attention to the muscles (skeletal muscle, diaphragm, oesophagus, heart). The tongue and the masseters were not examined, because the head of the animals is separated from the carcass and delivered to trade without being examined. Macroscopic lesions namely translucent oval containing clear fluid, sometimes bloody, with a white point (proto-scolex) or degenerate cyst were considered to be *C. ovis*. While, macroscopic lesions of sarcosporidiosis in the form of whitish ovoid to fusiform cysts with a few millimetres long were packaged in an identified plastic bag, and taken to the Laboratory of Parasitology and Mycology in the Superior National Veterinary School, Algiers for confirmation of the species in question by direct examination and the histology technique.

### Identification of macroscopic sarcosporidiosis cysts

#### Direct examination

Identification of the contents of macroscopic *Sarcocystis* cysts by direct examination consists of spreading the contents

on a slide followed by MGG (May-Grünwald Giemsa) staining according to the method cited by Bussi ras and Chermette [8]. The slides were observed under an optical microscope 400  magnification to look for bradyzoites (RAINEY's corpuscle) of *Sarcocystis* spp.

### Histological technique

All samples were prepared in the Laboratory of Anatomy Pathological at the Superior National Veterinary School, Algiers. The fixed tissue samples were cut into 0.5 cm thick sections, dehydrated with serial dilutions of ethanol and xylene, processed into paraffin, sectioned to 4 to 5 microns, stained with haematoxylin and eosin (H&E), and examined with the light microscope at magnification (100 , 400 , 1000 ). The wall morphology of cysts in the muscle sections were examined by the light microscope with immersion oil (1000 ) for the identification of the species.

### Statistical analysis

For statistical analysis, we used the software program Microsoft Excel 2010. The comparison of the distribution of different populations were analysed by using Chi-Square test with the level of significance  $P < 0.05$ .

## RESULTS

Of the 10,696 carcasses examined, ovine cysticercosis was found in 220 (2.06%) carcasses, while 76 (0.7%) sheep were found to be infected with macroscopic cysts of *Sarcocystis* spp.

### Prevalence according to the predilection sites

The rate according to the predilection sites is represented in Table 1.

All muscular cysticercosis cysts found in sheep slaughtered were dry, in the form of hard whitish vesicles,

Table 1. Prevalence of cysticercosis and sarcosporidiosis according to the predilection sites

Organs	Organs infected with muscular cysticercosis of <i>C. ovis</i>		Organs	Organs infected with macrocysts of <i>Sarcocystis</i> spp.	
	N	%		N	%
Heart	128	51.82	Skeletal muscles	29	38.15
Diaphragm	76	30.77	Diaphragm	23	30.26
Oesophagus	43	17.41	Oesophagus	24	31.57
<b>Total</b>	<b>247</b>	<b>100</b>	<b>Total</b>	<b>76</b>	<b>100</b>

Table 2. Prevalence of cysticercosis and sarcosporidiosis according to risk factors

Factors	Category	Number of sheep examined	Sheep with muscular cysticercosis of <i>C. ovis</i>		Degree of significance and p value	Sheep with macrocysts of <i>Sarcocystis</i> spp.		Degree of significance and p value
			N	%		N	%	
<b>Gender</b>	Male	10641	200	1.88	$P < 0.0001$	26	0.24	$P < 0.01$
	Female	55	20	36.36		50	90.90	
<b>Age Year (s)</b>	$\leq 1$	2267	5	0.22	$P < 0.0001$	0	0	
	[1.5–3]	5269	79	1.50		0	0	
	[3–5]	3160	136	4.30		76	2.40	
<b>Season</b>	Winter	2280	56	2.46	$P > 0.05$	0	0	
	Spring	1439	45	3.13		76	5.28	
	Summer	3655	71	1.94		0	0	
	Autumn	3322	48	1.44		0	0	



pearlescent sometimes yellowish, about 1 cm in diameter on average, which could correspond to *C. ovis*. These cysts had a tendency to be located more in the heart (Fig. 3), followed by the diaphragm. However, a low percentage were found in the oesophagus and this difference between infections rate of heart and other organs was highly significant ( $P < 0.001$ ).

Sarcosporidiosis lesions were observed on the skeletal muscles (intercostal muscle and thigh), followed by the oesophagus (Fig. 4), then the diaphragm (Fig. 5). These organs were infested with several whitish fusiform to ovoid cysts 2 mm to 1.5 cm long. No significant differences were observed between the 3 preferred sites.

### Prevalence according to risk factors

The prevalence of ovine cysticercosis and sarcosporidiosis in relation to the season, the age and sex of sheep is shown in Table 2.

### Identification of macroscopic cysts of *Sarcocystis* spp.

Microscopic examination of the contents of the cysts (Fig. 1) allowed the observation of bradyzoites (banana-shaped structures) in all the samples examined.

Histological technique identified macrocysts with the thin wall and cauliflower-like protrusions surrounded by the secondary cyst wall corresponding to the description of *S. gigantea* (*S. ovifelis*), as shown in Fig. 2.

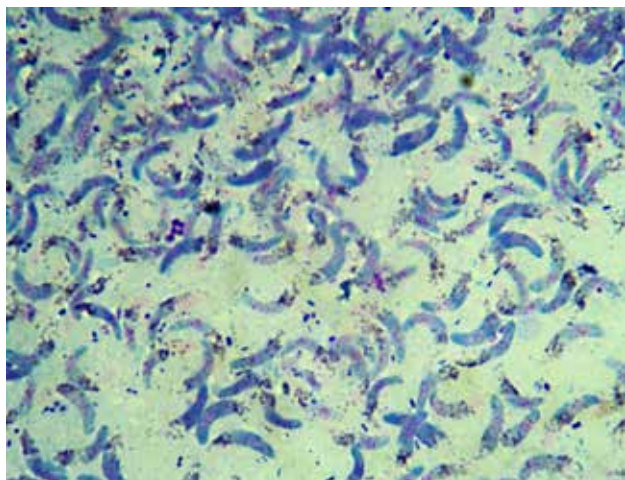


Fig. 1. *Sarcocystis* bradyzoite observed in samples of sheep oesophagus after MGG staining (400×)

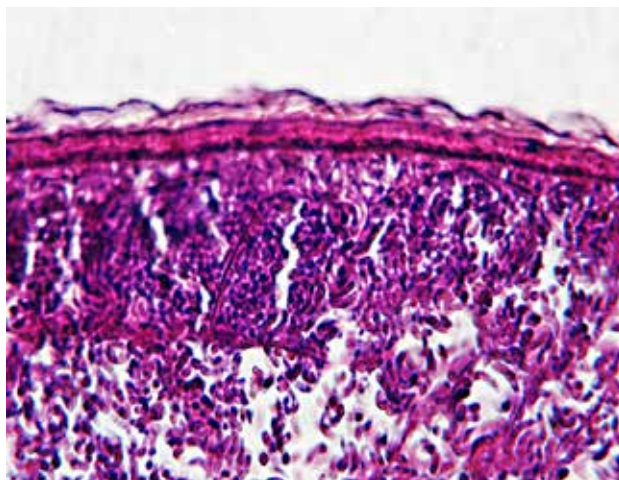


Fig. 2. *S. gigantea* in oesophagus with secondary cyst wall and cauliflower protrusions (H and E, 1000×)



Fig. 3. Degenerated *C. ovis* cysts in the heart of sheep



Fig. 4. Ovoid macroscopic cysts of *Sarcocystis* spp. in oesophagus of sheep





**Fig. 5. Fusiform macroscopic cysts of *Sarcocystis* spp. in diaphragm of sheep**

## DISCUSSION

### Overall prevalence

In our study the prevalence of muscular cysticercosis was found to be 2.06%. Similar findings have been reported in Benin (3.44%) [4], in Bulgaria (2.4 and 3.1%) in 1980 and 1981, respectively [18], in Southern Australia (2.39%) [35] and in Nigeria (1%) [10]. While higher prevalence compared to current results was reported from other countries: 7% in England [14] and 11.14% in the USA [21].

Christodoulou et al. [9] noted that the infection is influenced by several factors, such as, the improper disposal of dead animals, the access of farm dogs to the offal of slaughtered sheep, the carelessness of farmers to treat farm dogs with anthelmintics, and the grazing of flocks in fields where stray dogs have free access.

The disease is often associated with a lack of hygiene and poor farming practices. Samuel and Zewde [32] concluded that the prevalence is influenced by the type and stocking density and husbandry practices. The role of dogs is important in the transmission of pathology. Eichenberger et al. [14] showed that the frequent

use of fields by dog walkers represented a high risk for transmission of *T. ovis*.

In our study, all the cysts detected were non-viable. Broadbent [7] noted that 99.8% of the cysts detected were non-viable. Sheep probably have a good immune system that allows them to fight cysticercosis cysts. In effect, Euzéby [16] noted that this evolution of the lesions is due to the immune reaction of the host.

The reason for the low prevalence of muscular cysticercosis recorded in this study may be related to the low level of environmental contamination, also, our findings were based on surveys carried out on carcasses subjected to routine meat inspection procedures. This can possibly underestimate the incidences reported. According to Euzéby [15] in experienced meat inspectors could most likely miss out quite a number of viable cysticerci, which blend with the pinkish-red colour of the meat, or which are lodged in the intermuscular connective tissue, generally infiltrated with fat. Dorny et al. [11] reported that the sensitivity of visual inspection is considered low (<30%). However, it is believed that visual inspection of the meat can identify heavily infected carcasses, which also poses the greatest risk.

Macroscopic examination of sheep carcasses revealed the observation of macroscopic white cysts of *Sarcocystis* spp. with a prevalence of 0.7%. In Algeria, Nedjari [27] failed to confirm the presence of macroscopic cysts of sarcosporidiosis. In addition, from Australia, Hinaidy and Egger [20] revealed a prevalence of 2.4%, O'Donoghue and Ford [28] observed macroscopic cysts in 6.7% of the samples, but detected 2 types of species: *S. gigantea* (4.5 %) and *S. medusiformis* (3.1 %). Furthermore, other studies revealed a high prevalence of infections, in Turkey, the prevalence varies from one study to another: 7.89% was recorded by Aldemir et al. [3], 17.5% by Aldemir [2] and 24.5% by Beyazit et al. [5], while in Jordan a rate of 11.3% was noted by Aboshahada [1]. On the other hand, Fassi-Fehri et al. [17] in Morocco and Verduyck and Van Marck [34] in Senegal failed to observe macroscopic cysts.

In our study, the species identified was *S. gigantea* by histology. Differentiation between this species and other species of *Sarcocystis* has been made on the basis of cyst morphology and wall appearance [5, 12]. *S. gigantea* cysts are completely different. Protrusions are cauliflower-like,

reach a length of 4.5  $\mu\text{m}$ . In addition to these primary cyst wall differences, this species has a secondary cyst wall, which is always lacking in *S. tenella*, *S. arieticanis*, and *S. medusiformis* [12].

Sheep infested with the cysts probably lived close by the cats which are the definitive hosts, and were infected by ingesting the oocysts from the cat faeces.

### Prevalence according to the predilection sites

The present abattoir study revealed that among the organs accessible for detailed inspection, the heart, was the most affected by cysticercosis. This preferred predilection was similar with earlier findings reports by Bilan and Tassin [6], Jensen et al. [21], and Kebede [23].

Ezeby [16] noted that the elective muscular localizations were the heart, the masseter and pterygoid, the diaphragm and the tongue. From Kebede [23], the distribution of lesions on the various organs can be influenced by several factors such as muscle activity, age, and the geographic area.

For sarcosporidiosis, the skeletal muscles are the most infected with these cysts followed by the oesophagus and the diaphragm. In Iran, the species observed were *S. gigantea*, mainly in the oesophagus, *S. medusiformis*, mainly in the diaphragm [30]. In Turkey, the highest prevalence of *S. gigantea* was recorded in the oesophagus [5]. On the other hand, Meshkov and Kotominev [25] found that macroscopic cysts were present mainly on the exterior and interior parts of carcasses (abdomen and thorax).

No inflammatory lesions were observed in the evaluated sheep. The same results were observed by Beyazit et al. [5] and Tınak [33]. However, Jensen et al. [22] reported that all carcasses were discriminated for eosinophilic myositis indicating that opened sarcocysts killed the myocyte-host by causing the granuloma formation.

### Prevalence according to risk factors

Results from our study showed an increase in prevalence of ovine muscular cysticercosis with age confirming that a major source of infection for sheep is likely to be through the consumption of *Taenia* eggs from the environment. These results are similar to those of some previous investigations [4, 9, 35] indicating that age was an important factor for being positive as a measure of the cumulated life-time risk.

This study used the samples from abattoir where the majority of the slaughtered animals were male, and the only females less than 5 years old were slaughtered in accordance with Algerian regulations because females are preserved by breeders for reproduction. This may not reflect the reality of difference between gender in the occurrence of muscular cysticercosis.

In Benin, Attindehou and Salifou [4] noted that the infection was prevalent in all seasons with nevertheless high rates in the rainy seasons. In the USA, Jensen et al. [21] found that the prevalence was higher in the autumn than in the spring. The authors assumed that the lambs reached the infection from summer pastures contaminated by *T. ovis* eggs.

For sarcosporidiosis, the prevalence was significantly higher in females than males, while all infected animals were old (over 5 years old). The adult sheep are most often infected by the macrocysts [1]. For this reason, most infected sheep were old females over 5 years old, which is in accordance with the Algerian regulations stipulating slaughtering after that age. This may be due to the exposure time of sheep to the oocysts present in the faeces of the definitive host which is short for the young lambs. Or, to the prolonged development of these macroscopic cysts because species transmitted by felines require long periods of time to become infectious in intermediate hosts [19]. Munday [26] noted that of macroscopic sarcocysts in sheep was found in 0.6% of lambs, 8.8% in sheep (1 to 4 years old) and 66% in ( $\geq 4$  year olds). However, Beyazit et al. [5] found cysts of *S. gigantea* in 3 sheep under 1 year, and suggested that macrocysts might develop in less than 1 year.

## CONCLUSIONS

This study has revealed a non-negligible prevalence of ovine cysticercosis and sarcosporidiosis in the two slaughterhouses in the north of Algeria, suggesting a dispersion of *Sarcocystis* spp. oocysts and *T. ovis* eggs and parasite reservoir hosts in the environment. This study highlights the impact of these parasitic diseases in the study region, further studies are needed to determine the impact of these diseases in different regions in order to stimulate better efforts towards the control and possible eradication of these diseases.

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## THE HEAD AND NECK VASCULAR ANATOMICAL VARIABILITY IN THE LABORATORY RAT AND ITS SIGNIFICANCE TO MEDICAL SCIENCE

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### ABSTRACT

Laboratory rats are often used in experimental research of concern to human and veterinary medicine. There are several advantages of using rats as a scientific medium. In this study rats will be used as the scientific model as, previously discussed, they have proven their effectiveness in cardiovascular studies. The aim is to give a description of the cranial region, the head and neck of the rat as well as imaging of the vasculature of these regions to support the planning of surgical therapeutic methods to be applied to human and veterinary medical research. The research of the blood vessels morphology in anatomical studies is key to the prevention of ischemia during organ surgery. In recent times the laboratory rat has become one of the most popular models for experiments in medical research.

Corrosion casts were prepared on the cranial arteries of the body of 20 adult Wistar rats using Duracryl Dental® and PUR SP as the casting medium. We found the absence of the brachiocephalic trunk in some cases. Thyroid arteries originated independently or by the short common trunk from the right and left common carotid artery. The facial artery originated by the short trunk with the maxillary artery, or by the linguofacial trunk with the lingual artery from the common carotid artery. The results of this study revealed that, the functional anatomical relationship between the rat neck and head structures are important for the development of medical research of concern to human and veterinary experimental medicine.

**Key words:** arteries; blood supply; experimental medicine; laboratory animals

## INTRODUCTION

Laboratory animals (nonhuman primates, dogs, pigs, cats, sheep and laboratory rodents) have proved to be a suitable model for numerous scientific experiments such as: tumour, and cancer investigation, immunological, and metabolic studies, anatomy, and physiology, biochemistry, and experimental transplantation for example.

Laboratory animals are often used in experimental research relative to human and veterinary medicine interests. Many structures or organs of the body of laboratory animals were studied by many authors, for instance on the: mouse [8, 9], hamster [7, 27], rabbit [1, 14], or rat [3, 13, 21]; but some details have not yet been examined. The knowledge of anatomical variations through experimental study can be applied to surgical practice [30]. The study of the morphology of blood vessels in anatomical studies is key to the prevention of ischemia during organ surgery. In recent times, the laboratory rat has become one of the most popular models for experiments in medical research.

The laboratory rat takes its origins from the wild brown rat and it was an albino mutant that was first used in science as an experimental model in 1828 in a fasting study. After these numerous experiments using rats were performed, that led to the albino rat to become the first animal domesticated for purely scientific experimentation [17]. There are several advantages of using rats as a scientific medium. They have a short life span and a high reproductive rate with a large quantity of rats being able to be housed in a small area. In addition to this, they are easily handled and provide a low risk of injury if they attack. The rat has been chosen over the mouse as they more closely mimic human diseases, as well as, has having a better capacity for learning for use in behavioural studies. The rat has proved an excellent model for studies of the cardiovascular, reproductive, and nervous systems, as well as in studies for cancer and pharmaceuticals [12]. In this study, rats will be used as the scientific model as, previously discussed, they have proven their effectiveness in cardiovascular studies.

The aim of this paper is to give a description of the cranial region, the head and neck, of the rat as well as imaging of the vasculature of these regions to support the planning of surgical therapeutic methods to be applied to human and veterinary medical research interests [29, 30].

## MATERIALS AND METHODS

The experiment was performed on 20 one-year old laboratory rats (*Rattus norvegicus f. domestica*). Both sexes (10 females, 10 males) of Wistar rats were used in this experiment with body weights ranging from 350—520 g. The animals were obtained from standard breeding conditions from the Laboratory of Research Biomodels, University of P. J. Safarik in Kosice which is an accredited facility.

### Ethical statement

The experiment on rats was performed with approval of the Ethic Committee of the University of Veterinary Medicine and Pharmacy in Kosice and State Veterinary and Food Institute in Bratislava (No.SK P 12004) followed Slovakian protocols for ethical standards for the use of laboratory animals [15, 29, 30].

### Method of the preparing procedure of the corrosion casts specimens of the arterial system

The animals were euthanized by an intraperitoneal injection of sodium thiopental (50 mg.kg<sup>-1</sup>, Thiopental Valent, Valeant Czech Pharma, Praha, Czech Republic). Under total anaesthesia the left ventricle of the heart was dissected. A cannula was then introduced into the aorta through the left ventricle which was secured by a ligature. The right auricular appendage was opened to allow good distribution of the perfusion medium. First the vessels were perfused with 0.9% isotonic saline solution at a flow rate of about 10 ml/min for 30 s through the left cardiac ventricle. Then 0.05% NaOH solution (Mikrochem, Michalovce, Slovakia) was added to the perfusion medium to aid removal of blood clots from the vessels. A perfusion pressure of approximately 200—250 mm Hg (2.6—3.25 mm H<sub>2</sub>O) was maintained. The perfusion material used was injected in stoichiometric rates. The efficacy of the perfusion material was monitored by uniform colour fading of the tissues during the procedure. Corrosion casts were then prepared by using Duracryl Dental<sup>®</sup> resin (Spofa-Dental, Jičín, Czech Republic) and PUR SP resin (Institute of polymers, SAV, Bratislava, Slovakia). A suitable colour tone was achieved by the addition of 2—3 drops of red dye (oil-red paint 0). This was then applied after thorough mixing through the left ventricle of the heart. Once the resin formed a complete cast no manipulation of the casts or animals may be done for 30 minutes. After this time

the casts were submersed in 40—60 °C water for between 30 min and 24 hr to allow complete polymerization of the resin [18].

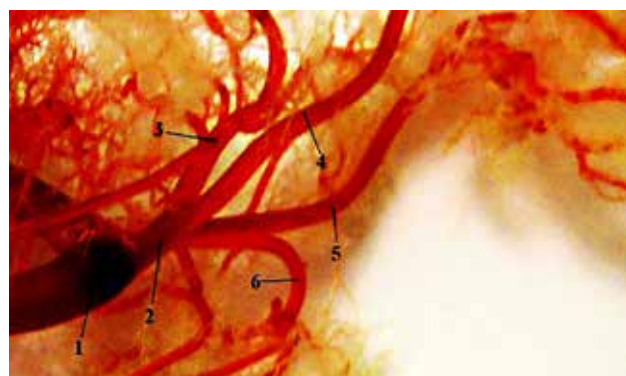
The soft tissue was then removed by chemical degradation in 2—4% KOH solution (Mikrochem, Michalovce, Slovakia) at 60—70 °C. The degradation of the tissue took approximately 2—3 days. The corroded specimens were then submerged in water prior to being dried at room temperature.

### Method of evaluation and interpretation of results

A stereo static Leica M 320 microscope (Leica Microsystems Schweiz AG, Heerbrugg, Switzerland) was used for the evaluation of the corrosion casts and anatomical dissection. Pictures were taken through a digital camera attached to the microscope. The results were then listed as percentages. The latest edition of the Veterinary Anatomic Nomenclature was consulted throughout this study [6].

## RESULTS

The common carotid artery (*a. carotis communis*) is the main artery of the head and neck. The left common carotid artery (*a. carotis communis sinistra*) originates from the aortic arch (*arcus aortae*), and the right common carotid artery (*a. carotis communis dextra*) is a branch of the brachiocephalic trunk (*truncus brachiocephalicus*) but without the bicarotic trunk (*truncus bicaroticus*) as it is absent in rats (Fig. 1). We detected in 15 cases (75%) that the aortic arch

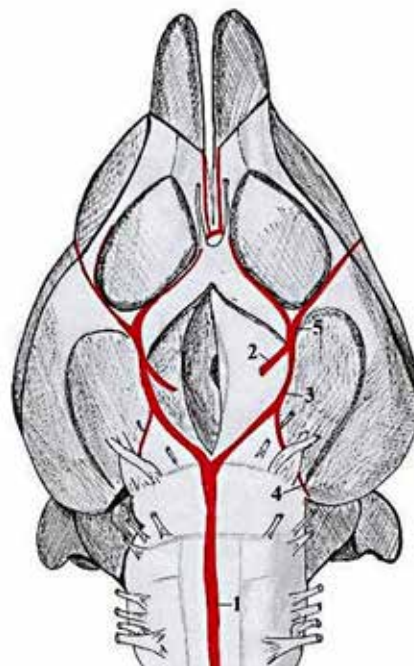


**Fig. 1. The division of arcus aortae (PUR SP)**

1—*arcus aortae*; 2—*truncus brachiocephalicus*; 3—*a. axillaris dextra*; 4—*a. carotis communis dextra*; 5—*a. carotis communis sinistra*; 6—*a. axillaris sinistra*

was divided into the brachiocephalic trunk, the left subclavian artery (*a. subclavia sinistra*) and the left common carotid artery. In only 5 cases (25%) the brachiocephalic trunk was absent (Table 2) and so instead the left and right common carotid artery, and left and right subclavian artery (*a. subclavia sinistra et dextra*) originated from the aortic arch. The *a. carotis communis* first runs ventrally along the trachea and then later along the dorsomedial surface of the trachea. At this level *a. carotis communis* is covered by the ventral muscles of the neck. The branches of the common carotid artery were described as the cranial and caudal thyroid artery (*a. thyroidea cranialis et caudalis*) and inconstant muscular branches (*rr. musculares*) which supply the thyroid gland and surrounding structures (larynx, pharynx). The cranial thyroid artery (Fig. 4) divides into the laryngeal branch (*r. laryngeus*) specific to the larynx and the pharyngeal branch (*r. pharyngeus*) specific to the pharynx. Our results detected that *a. thyroidea cranialis* and *caudalis* arose separately from the *a. carotis communis dextra et sinistra* in 17 cases (85%). In 3 cases (15%) these two arteries originated from the right and left common carotid artery by the short common trunk (Table 2).

Along the neck on both sides, many inconstant branches for the ventral muscles were found. In the region of the thyroid gland (*glandula thyroidea*) the common carotid ar-



**Fig. 2. The arteries of the brain**

1—*a. basilaris*; 2—*a. carotis interna*; 3—*a. communicans caudalis*; 4—*a. cerebri caudalis*; 5—*a. cerebri media*



Tab. 1. Blood supplying the area of the cerebral arteries

Cerebral artery	Part of the brain
<i>cerebri rostralis</i>	cerebral cortex
<i>cerebri caudalis</i>	dorsal surface of the mesencephalon
<i>communicans caudalis</i>	dorsal surface of the mesencephalon
<i>cerebri media</i>	lateral and dorsal surface of the hemispheres
<i>choroidea</i>	cerebral peduncles, hippocampus, lateral geniculate body, lateral ventricle of the brain
<i>basilaris</i>	caudal part of the brain

Tab. 2. The biggest variations are in the origin of these arteries

Vessel	Percentages
The absence of the brachiocephalic trunk	In 25 %
The short common origin of the both thyroid arteries from the left common carotid artery	In 15 %
The origin of the occipital artery from the common carotid artery	In 10 %
The short common trunk for the origin of the facial and maxillary artery from the common carotid artery	In 10 %
The presence of the linguofacial trunk	In 50 %

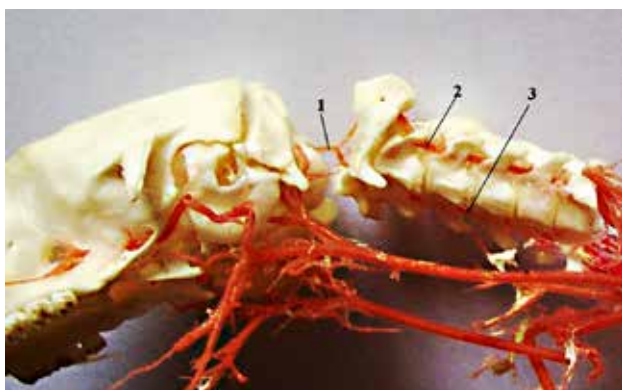
tery is divided into the internal and external carotid artery (*a. carotis interna et externa*).

The internal carotid artery is the main artery of the brain. It branches from the common carotid artery at the level of the thyroid gland. It proceeds directly to the base of the skull through the jugular foramen (*foramen jugulare*), to the tympanic bulla (*bulla tympanica*) by the name, of the stapedian artery (*a. stapedia*). The stapedian artery continues through three openings: the petrotympanic fissure (*fissura petrotympanica*), the caudal alar foramen (*foramen alare caudale*) and the rostral alar foramen (*foramen alare rostrale*) into the orbita. Here the stapedian artery divided into two branches: the supraorbital artery (*a. supraorbitalis*) and the infraorbital artery (*a. infraorbitalis*). This branching was found to occur on the right and left side in all cases. The supraorbital artery runs along the caudal border of the orbita. Its branch is the lateral superior palpebral artery (*a. palpebralis superior lateralis*) which supplies the lateral margin of the upper eyelid. This artery was also found to be on the right side and on the left side in all cases. The infraorbital artery is the second branch of the stapedian artery, which runs along the ventral margin of the eyelid, where it then runs through the infraorbital foramen (*foramen infraorbitale*). The pterygopalatine artery (*a. pterygopalatina*) originates from the internal carotid artery before the entrance to the cranial cavity. The *arteria pterygopalatina* then continues through the jugular foramen (*foramen jugulare*) into the skull as, the palatine artery (*a. palatina*). The palatine artery runs along the dorsal surface of the temporal bone through the petrotympanic fissure to the ventral surface of the skull, along the wing of the basisphenoidale bone and then into the hard palate

(*palatum durum*). The major palatine artery (*a. palatina major*) continues along the hard palate where it gives off the dental branches (*rr. dentales*) for molars. The palatine artery was found to be on the right side in 18 cases (90%) in 16 (80%) on the left side. The second branch of the pterygopalatine artery is the pharyngeal artery (*a. pharyngea*), which arises at the point of origin of the palatine artery. This artery was described in 18 cases (90%) on the right side and in 16 cases (80%) on the left side. A. pharyngea continues through the petrotympanic fissure (*fissura petrotympanica*) to the region of the pharynx. A. *pterygopalatina* was seen in all cases, on both sides.

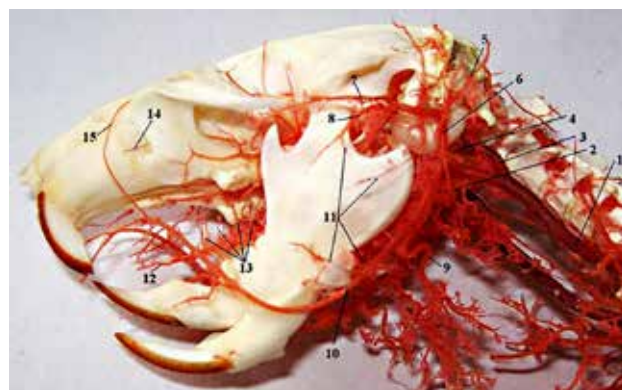
In this study it was observed that at the base of the brain were the following branches of the internal carotid artery: the caudal cerebral artery (*a. cerebri caudalis*), the caudal communicating artery (*a. communicans caudalis*), the middle cerebral artery (*a. cerebri media*), the rostral cerebral artery (*a. cerebri rostralis*) and the choroidal artery (*a. choroidea*) (Table 1).

The caudal communicating artery is very small and together with the caudal cerebral artery supplies the dorsal surface of the midbrain (*mesencephalon*) (Fig. 2). The middle cerebral artery is the strongest branch of the internal carotid artery and it reaches lateral and dorsal surfaces of the hemispheres (Fig. 2). The rostral cerebral arteries from both sides are fused and form the rostral cerebral trunk (*truncus cerebri rostralis*), which then supplies the cerebral cortex (*cortex cerebri*). The choroidal artery reaches the cerebral peduncle (*pedunculus cerebri*), the hippocampus, the lateral geniculate body (*corpus geniculatum laterale*) and the lateral ventricle (*ventriculus lateralis*). The caudal part of the rat brain is supplied by the



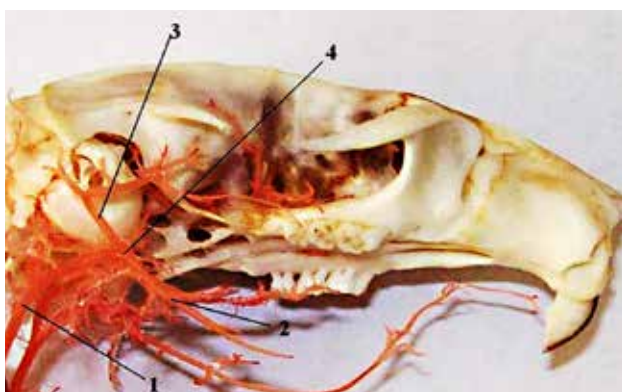
**Fig. 3. The presence of vertebral arteries and their fusion into *a. basilaris***

1—*a. basilaris*; 2—*a. vertebralis sinistra*;  
3—*a. vertebralis dextra*; Duracryl Dental®



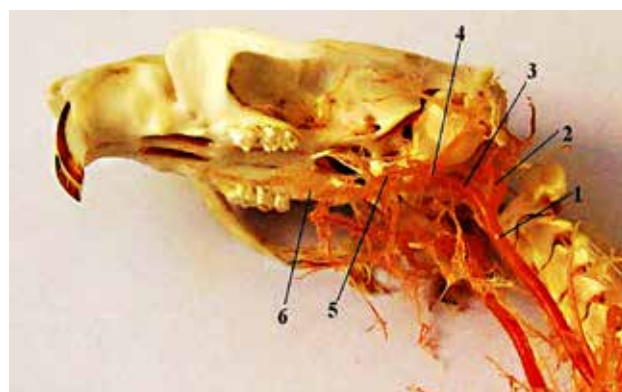
**Fig. 4. The division of the *a. carotis communis sinistra***

1—*a. carotis communis sinistra*; 2—*a. thyroidea cranialis*;  
3—*a. carotis interna*; 4—*a. pterygopalatine*; 5—*a. auricularis caudalis*;  
6—*a. maxillaris*; 7—*a. temporalis profunda*; 8—*a. transversa faciei*;  
9—*r. glandularis a. facialis*; 10—*a. facialis*; 11—*rr. masseterici*;  
12—*a. profunda linguae*; 13—*rr. dorsales linguae*; 14—*a. infraorbitalis*;  
15—*a. frontalis*; Duracryl Dental®



**Fig. 5. The common trunk of *a. maxillaris* and *a. facialis***

1—*a. carotis communis dextra*; 2—*a. facialis*; 3—*a. maxillaris*;  
4—The common trunk of *a. maxillaris* and *a. facialis*;  
Duracryl Dental®



**Fig. 6. Truncus linguofacialis**

1—*a. carotis communis sinistra*; 2—*a. carotis interna*; 3—*a. carotis externa*;  
4—truncus linguofacialis; 5—*a. facialis*; 6—*a. lingualis*; Duracryl Dental®

basilary artery (*a. basilaris*), which is a fusion of the right and left vertebral artery (*a. vertebralis dextra et sinistra*), (Fig. 3).

The external carotid artery forms the main continuation of the common carotid artery. It runs between the digastric muscle (*m. digastricus*) and the stylohyoid bone (*os stylohyoideum*). The external carotid artery has six branches, namely the occipital artery (*a. occipitalis*), the caudal auricular artery (*a. auricularis caudalis*), the superficial temporal artery (*a. temporalis superficialis*), the facial artery (*a. facialis*), the lingual artery (*a. lingualis*) and the maxillary artery (*a. maxillaris*). The occipital artery is the first branch that arises from *arteria carotis externa* immediately after the division of the common carotid artery. It runs along the jugular process (*processus jugularis*), where at this level, it then divides into a muscular branch

(*r. muscularis*) for the surrounding muscles and an auricular branch (*r. auricularis*) for the ear. Its main continuation is the condylar artery (*a. condylaris*). In 18 cases (90%) the occipital artery was found to be a branch of the external carotid artery and in the other 2 cases (10%) this artery was a branch of common carotid artery on both sides of the head (Table 2). The caudal auricular artery supplies the auricle by its branches. On the caudal margin of the auricle, the caudal auricular artery continues as the intermediate auricular artery (*a. auricularis intermedia*). The rostral part of the auricle is supplied by the rostral auricular artery (*a. auricularis rostralis*). In this investigation all cases proved that the caudal auricular artery was a branch of the external carotid artery. The intermediate auricular artery was stronger and longer than the rostral auricular artery. The caudal auricular artery was observed to divide into

two branches in 14 cases (70%) and in just 6 cases (30%) the rostral auricular artery was a branch of the intermediate auricular artery (Fig. 4). The superficial temporal artery is another branch of external carotid artery and it reaches the temporal region. This branch was found in all 20 rats. The strongest branch of the *a. carotis externa* is the facial artery. This artery runs along the medial pterygoid muscle (*m. pterygoideus medialis*) then ventrally through the notch for the facial vessels (*incisura vasorum facialis*) and along the lateral surface of the face, where it supplies muscles of the face. In this study variations in the origin of this artery were noted. In 10 cases (50%) facial artery had common origin with the maxillary artery on the right side and in 6 cases (30%) on the left side (Table 2; Fig. 5). Similarly, the linguofacial trunk (*truncus linguofacialis*) was observed to be on the right side in 10 cases (50%) and in 14 cases (70%) to be on the left side (Table 2; Fig. 6).

The facial artery has many branches. One such being the glandular branch (*r. glandularis*) which supplies the sublingual gland (*glandula sublingualis*). It is a very strong and marked branch and was found on both side of the face in all cases (Fig. 4). The other branch of the facial artery is the masseteric branch (*r. massetericus*) which supplies the masseter muscle (*m. masseter*). Masseteric branch arose from the right facial artery in 12 cases (60%) and from the left facial artery in 10 cases (50%). The inferior labial artery (*a. labialis inferior*) is the branch for the lower lip. In this experiment this branch was observed in 15 cases (75%) on the right side and in 2 cases (10%) on the left side. The mental branches (*rr. mentales*) are the branches for the chin and were observed in all cases on both sides. The three cases (15%) of the corrosion casts revealed the oral angular artery (*a. angularis oris*) which is the branch for the area of the angle of the mouth, to be on the right side, and then in 2 cases (10%) this artery was revealed to originate on the left side. In the region of the nose and orbita the other branch, the nasal lateral artery (*a. lateralis nasi*) was found in 5 cases (25%) to be on the right. Also, in the region of the frontal bone (*os frontale*), the frontal artery (*a. frontalis*) was found in 4 cases (20%) on the left side. The lingual artery, which enters the tongue near to the stylohyoid bone, is taken to arise from the *arteria carotis externa* close to the origin of the *arteria facialis*. However, the lingual artery did not originate from the external carotid artery in 10 cases (50%) where it was on the right side and 6 cases (30%) on the left side. Interestingly, in some

cases the short common trunk was found to be the origin of the lingual and facial arteries (Fig. 6). The deep lingual artery (*a. lingualis profunda*) arises from the lingual artery close to the root of the tongue. The dorsal lingual branches (*rr. dorsales linguae*) which originate from the deep lingual artery, go into the muscles of the tongue. The deep lingual artery and dorsal lingual branches were observed in all cases.

The maxillary artery runs along the medial surface of the mandible. The area which the maxillary artery supplies in other animals is supplied by the internal carotid artery in laboratory rats. The main continuation of the maxillary artery is the inferior alveolar artery which enters into the mandibular foramen (*foramen mandibulae*) through the mandibular canal (*canalis mandibulae*) and mental foramen (*foramen mentale*) and the mental artery (*a. mentalis*). It was observed that the buccal artery (*a. buccalis*), a branch of the maxillary artery in the rostral part of head, was on the right side in 5 cases (25%) and in 4 cases (20%) on the left side. Continuing dorsally from the maxillary artery to the temporal muscle (*m. temporalis*) is the deep temporal artery (*a. temporalis profunda*). The deep temporal artery was evident in all cases. Originating from the deep temporal artery is the transverse facial artery which is very important for blood supply to the face. The transverse facial artery (*a. transversa faciei*) runs in the rostral direction on the lateral surface of the zygomatic arch (*arcus zygomaticus*) into the facial region. The transverse facial artery was detected in 17 cases (85%) on the right side and in 15 cases (75%) on the left side. Many masseteric branches (*rr. masseterici*) were found supplying the masseter muscle, as were branches of transverse facial area.

## DISCUSSION

An exact description of the vasculature in the cranial parts of the rat body is fundamental for experimental surgical research in this model of laboratory animals. The knowledge of the anatomical variability of the vasculature of the head and neck would help to avoid critical complications when they perform different experimental surgery on these parts of the rat body. Many authors, previous publications and descriptions mentioned only partial results of rat morphology [18]. We have submitted well-knit knowledge about arterial vasculature of the cranial parts of the

rat body, which is more important for performing experiments in this model of laboratory animals, because venous drainage and alternative veins pathways of the rat brain were described by some authors [11, 15]. The morphological relationship between the head and neck of human and rat is still sufficiently undefined. The circulatory system of this part of the body in the rat is considered similar to human therefore the rat is the most often using laboratory animals [26, 32]. The origin, division and course of the cranial arteries of the aortic arch (Fig.1) and its branches are similar to human, but the area of supplying of single arteries is different. The experiment of the blood supply to the head and neck in rats showed the same scheme of the aortic arch division with some authors [11, 22, 24, 25]. Through this study it was found, in some cases, that the brachiocephalic trunk was absent whereas, in research carried out by [20] he found that the brachiocephalic trunk was absent in all of the rat fetuses he examined. In the cases in which the brachiocephalic trunk was absent from both the left and right common carotid and the left and right subclavian arteries it had originated directly from the aortic arch. The different anatomical nomenclature of the brachiocephalic trunk in rats is used by many authors [11, 22]. They named the brachiocephalic trunk in their study results as *arteria anonyma*. In this study, it was found that the cranial and caudal thyroid artery arose from the left and right common carotid artery (*a. carotis communis sinistra et dextra*) or, in some cases, it arose from the short common trunk. The cranial thyroid artery is divided into two branches, laryngeal and pharyngeal. The same division was described by N e j e d l ý [22] and P o p e s k o et al. [24]. The cranial thyroid artery (*a. thyroidea cranialis*), as named by P o p e s k o et al. [24], arises from the external carotid artery (*a. carotis externa*). In the study of Y a m a s a k i [31], the cranial thyroid artery formed a common trunk with the pharyngeal artery [15]. According to H e b e l and S t r o m b e r g [11] the caudal thyroid artery is a branch of the deep cervical artery (*a. cervicalis profunda*). Other authors describe the origin of the caudal thyroid artery as coming from the pericardiacophrenic artery (*a. pericardiacophrenica*) [31]. The internal carotid artery (*a. carotis interna*) is the main artery supplying the brain. It arises from the common carotid artery (*a. carotis communis*) at the thyroid gland. According to some authors the common carotid artery is at the level of the larynx [11, 22, 24], observed the division of this artery in the cau-

dal margin of the thyroid gland. As described by A l b i o n [2], the internal carotid artery then passes into the skull where it changes its name to the stapedia artery (*a. stapedia*). The stapedia artery has two branches: the supraorbital artery (*a. supraorbitalis*) and the infraorbital artery (*a. infraorbitalis*). In this study it was found that, in all cases, both of these arteries branched on both the left and right side. L e V e r e [19] drew attention to the supraorbital artery off which is a branch called the lateral superior palpebral artery (*a. palpebralis superior lateralis*). In our study it was found on both the left and right sides. The pterygopalatine artery (*a. pterygopalatina*), as described by H e b e l and S t r o m b e r g [11] branches from the internal carotid artery just before the skull cavity entrance. After it enters through the jugular foramen (*foramen jugulare*), as described by both C o x [5], H e b e l and S t r o m b e r g [11] the pterygopalatine artery (*a. pterygopalatina*) is the first branch of the internal carotid artery and passes through the tympanic bulla (*bullula tympanica*). The results of this study are concurrent with this. We found in all cases this branching on both sides. H e b e l and S t r o m b e r g [11] describes three branches of the internal carotid artery that supply the brain: the rostral cerebral artery (*a. cerebri rostralis*), the choroidal artery (*a. chorioidea*) and the caudal cerebral artery (*a. cerebri caudalis*). All three of these arteries were present in this study. Also found was the caudal communicating artery (*a. communicans caudalis*), and the middle cerebral artery (*a. cerebri media*). In agreement with H e b e l and S t r o m b e r g [11] we found the middle cerebral artery running dorsally to the cerebral hemisphere and ramifying both laterally and dorsally. The choroidal artery, in both our study and according to H e b e l and S t r o m b e r g [11] and H e et al. [10] runs to the cerebral peduncle (*pedunculus cerebri*), the lateral ventricle (*ventriculus lateralis*), the lateral geniculate body (*corpus geniculatum laterale*), and the hippocampus. According to T h o m a s [28] the brain is supplied by the common carotid artery and vertebral artery. O g a t a [23] on researching the ischemia of the rat brain, found that the main arteries for the brain are the common carotid artery, the vertebral artery and the basilar artery. Among these arteries are anastomoses. The external carotid artery (*a. carotis externa*), as described by H e b e l and S t r o m b e r g [11] and P o p e s k o et al. [24] is the largest continuation of the common carotid artery. The occipital artery (*a. occipitalis*) is the first branch that arises

from *a. carotis externa* immediately after the division of the common carotid artery. It runs along the jugular process (*processus jugularis*), where at this level it then divides into a muscular branch (*r. muscularis*) for the surrounding muscles and an auricular branch (*r. auricularis*) for the ear. Its main continuation is the condylar artery (*a. condylaris*). As described by Hebel and Stromberg [11] and Popesko et al. [24] the occipital artery runs dorsally along the jugular process and supplies the digastric muscle (*m. digastricus*) and the muscles of the neck. In line with Hebel and Stromberg [11] and Popesko et al. [24] this study found that, in many cases, the occipital artery branched from the external carotid artery, and in others, the occipital artery branched from the common carotid artery instead. The next branch of the external carotid artery is the caudal auricular artery (*a. auricularis caudalis*) which supplies the ear. The caudal auricular artery branches into the intermediate auricular artery (*a. auricularis intermedia*) and, as described by Popesko et al. [24] the rostral auricular artery (*a. auricularis rostralis*). It was found in all of the cases in this research that the caudal auricular artery branched directly from the external carotid artery. In many cases the caudal auricular artery was found to divide into two branches and, in the remaining cases, the rostral auricular artery divided from the intermediate auricular artery. The results of this study agree with research by Hebel and Stromberg [11]. Nejedlý [22] determined just one auricular branch from the external carotid artery and named it the *arteria auricularis magna*. Reaching the temporal region is another branch of the external carotid artery, the superficial temporal artery (*a. temporalis superficialis*). This branch was present in all cases of this study which agrees with research by Nejedlý [22] and Hebel and Stromberg [11]. According to Hebel and Stromberg [11] the superficial temporal artery gives off four branches: the *r. temporalis* and, *r. massetericus*, the transverse facial artery (*a. transversae faciei*), and the lateral palpebral artery (*a. palpebralis lateralis*). In this study in all the corrosion casts revealed that the superficial temporal artery was present. The facial artery (*a. facialis*) forms the largest branch of the external carotid artery. According to Hebel and Stromberg [11] and Popesko et al. [24] the facial artery branches from the external carotid artery running along the ventral margin of the mandible between the masseter and the digastric muscles. Nejedlý [22] found

the origin of this artery to be from the maxillary artery. Within this study it was found that the facial artery originated from the maxillary artery but in some cases the origin of the facial artery was from the short common trunk, the linguofacial trunk (*truncus linguofacialis*) with lingual artery. The linguofacial trunk (*truncus linguofacialis*) was observed by Popesko et al. [24]. The glandular branch (*r. glandularis*) is the first branch of the facial artery according to Hebel and Stromberg [11] and in this study this branch was present on both sides in all cases. Another branch of the facial artery is the inferior labial artery (*a. labialis inferior*) which supplies the lower lip in concurrence with Hebel and Stromberg [11]. The commissure of the mouth is supplied by the angular oris artery (*a. angularis oris*), a further branch of the facial artery according to Hebel and Stromberg [11]. However, in research done by Nejedlý [22] the angular oris artery is a branch of the inferior labial artery (*a. labialis inferior*). The lateral nasal artery (*a. lateralis nasi*) is the final branch of the facial artery. As described by Hebel and Stromberg [11] and Chagas [4], the lateral nasal artery originates at the level of the lateral part of the nose before it branches to the nostril and upper lip where they anastomose with the lateral nasal artery coming from the other side of the nose. Returning to the external carotid artery, another branch of it is the lingual artery (*a. lingualis*) which according to Popesko et al. [24] originates from the external carotid artery at the same level as the facial artery. These two arteries form the linguofacial trunk (*tr. linguofacialis*). Nejedlý [22] described the origin of the lingual artery as being from the maxillary artery but, according to Hebel and Stromberg [11] the lingual artery originates from the external carotid artery. Within this study it was found that in some cases the origin was from the linguofacial trunk. The lingual artery continues to the root of the tongue where it then becomes the deep lingual artery (*a. profunda linguae*) and this branch was found in all cases which concurs with research conducted by both Hebel and Stromberg [11] and Popesko et al. [24]. Also, in this research, the dorsal lingual branches (*rr. dorsales linguae*) were found in all cases which again agrees with research by Hebel and Stromberg [11] and Popesko et al. [24]. The maxillary artery (*a. maxillaris*), as described by Hebel and Stromberg [11] and Chagas [4] branches from the external carotid artery behind the mandibular joint before continuing along

the mandible and branching to form the alveolar mandibular artery (*a. alveolaris mandibularis*) and pterygoid artery (*a. pterygoidea*). Hebel and Stromberg [11] and Popeskó et al. [24] described that maxillary artery as the main continuation of the external carotid artery. Nejedlý [22] observed that the maxillary artery originates from external carotid artery and then it divides into two branches—the external and the internal maxillary arteries. From the maxillary artery originates the transverse facial artery, the buccal artery and the deep temporal artery. Other authors such as Hebel and Stromberg [11] and Popeskó et al. [24] described the same pattern.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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## BEHAVIOURAL PATTERNS AND GROWTH PERFORMANCE OF MALE WISTAR RATS EXPOSED TO CIGARETTE SMOKE: EFFECTS OF CURCUMIN AND HESPERIDIN

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### ABSTRACT

This study evaluated the behavioural responses of male Wistar rats to normal air or cigarette smoke (CS) and compared the effects of curcumin and hesperidin on growth performance. In experiment 1, male rats were randomised into two groups ( $n = 10$ ): control and CS-exposed groups. During exposure (four weeks), the dietary and behavioural patterns were monitored. In experiment 2, forty-eight rats were distributed across eight groups ( $n = 6$ ): normal control, CS control, CS + curcumin ( $10 \text{ mg.kg}^{-1}$ ), CS + curcumin ( $20 \text{ mg.kg}^{-1}$ ), CS + hesperidin ( $10 \text{ mg.kg}^{-1}$ ), CS + hesperidin ( $20 \text{ mg.kg}^{-1}$ ), curcumin ( $20 \text{ mg.kg}^{-1}$ ), and hesperidin ( $20 \text{ mg.kg}^{-1}$ ) for 6 weeks. Growth performance (feed intake, weight gain, and feed conversion ratio FCR) were assessed. In the first experiment, there was no significant difference ( $P > 0.05$ ) in the body weight of the CS-exposed group compared to the normal control, whereas feed intake was significantly ( $P > 0.05$ ) lower in the CS-group. The time to access feed and water was higher in the CS-group, while other behavioural responses (locomotion, stand upright, climbing, stand and stare, sniffing, sitting, and digging) were significantly reduced ( $P < 0.05$ )

compared with normal control, especially after two weeks. In the second experiment, weight gain, feed intake, and FCR were significantly lower in the CS-exposed group compared to the control group, whereas treatment with curcumin and hesperidin, especially at the higher dose ( $20 \text{ mg.kg}^{-1} \text{ b.wt.}$ ), significantly improved the growth performance of the CS-exposed groups. This study submits that CS exposure negatively impacts on the growth performance and behavioural patterns and demonstrates the potentials of curcumin and hesperidin in addressing these CS-provoked changes.

**Key words:** behavioural patterns; cigarette smoke; curcumin; growth performance; hesperidin; rats

### INTRODUCTION

Cigarette smoking—a major risk factor for the development of cancer, respiratory, cardiovascular and other systemic diseases—is still a common social habit globally [14]. Cigarette smoke contain several substances, such as nicotine and carbon monoxide, that can elicit chemo-mod-

ulatory effects in nerve endings, which can alter pulse rate, reaction time, awareness, etc. [24, 25]. Besides the addictive effect of nicotine, smokers tend to form a behavioural or psychosocial reliance on cigarette smoking, where they resort to smoking to deal with pressure. These behavioural patterns quickly become ingrained, coupled with negative reinforcement and the fear of withdrawal symptoms, continue to compel one to keep smoking [24]. It is therefore not surprising that a randomised clinical study revealed that only about 20 % of people that quit smoking sustained abstinence after 12 months [17].

Emerging evidence from experimental studies suggests that the impacts of cigarette exposure extends beyond physical health. In a murine study, Cardoso and colleagues revealed changes in anti-predatory response, following exposure to cigarette residue [5], while dependence behaviour has been reported in rats exposed to tobacco smoke [22]. Another study highlighted the adverse effects of aqueous extract of tobacco on feed intake and growth performance [1]. Other studies, both *ex vivo* [9] and *in vitro* [2], also indicated that cigarette smoke can inhibit growth.

Utilising natural products to combat disease conditions is now a common theme in research. The potentials of some phytochemicals against cigarette smoke-associated diseases have been reviewed elsewhere [28]. Curcumin, the major curcuminoid present in *Curcuma longa*, has been shown to demonstrate relevant pharmacological properties, including anti-inflammatory, antioxidant and neuroprotective activities [3, 7]. Hesperidin—a bioflavonoid present in citrus—demonstrates similar properties [3]. Interestingly, separate studies have demonstrated the roles of these phytochemicals against cigarette-induced pathologies [10, 26].

Most studies on cigarette smoke that utilised murine models focus mainly on the metabolic responses of the animals, making information on the effects of exploratory and consummatory behaviour, as well as on growth performance, of subjects exposed to cigarette smoke sparse in literature.

This study was designed with two objectives: 1) to elucidate some behavioural responses of male Wistar rats to normal air or cigarette smoke; 2) to compare any resulting effect of treatment with nutritional supplements (curcumin and hesperidin).

## MATERIALS AND METHODS

### Test substance

The cigarettes used in the experiments (Benson and Hedges) were products of Philip Morris British American Tobacco (Lausanne, Switzerland). Curcumin and hesperidin (both > 98 % purity) were purchased from Solarbio Life Science and Co. Ltd. (Tongzhou District, Beijing, China).

### Experimental animals

A total of fifty-eight adult male Wistar rats (average weight: 170—180 g) were used in this study and procured from the small animals' breeding unit of the College of Veterinary Medicine, Federal University of Agriculture, Abeokuta (FUNAAB). They were acclimatized for two weeks under ambient conditions (25 °C, 85 % relative humidity, 12 hours day-night cycles) in the animal house of the Department of Biochemistry, (FUNAAB).

### Ethical statement

The experiment was endorsed by the Ethical Committee of the Department, and assigned with ethical approval number FUNAABBCH-PG17/0226-01. All animals were handled according to the ARRIVE guidelines for animal care [15].

### Experimental design

For the first experiment, twenty animals were distributed, by simple randomisation, into two groups ( $n=10$ ), the control group and cigarette smoke (CS) exposed group. The animals were housed in separate propylene cages and observed physically when exposed to CS (CS-exposed group) and normal air (normal control). Whole-body exposure to cigarette smoke occurred for 90 minutes per day, every day, for a total of 4 weeks with a smoking chamber. The smoking chamber consisted of an animal containment system and a cigarette smoking control system [23]. The cigarette smoke was generated from double-filtered Benson and Hedges cigarettes (16 sticks daily). The exposure was monitored for particulate matter size, volatile organic compounds (VOC), and carbon monoxide levels using an aerosol gas analyser (Horiba PG-300; HePing, Shenzhen, China). The control animals were housed in their respective cages 150 metres away from the CS-exposed group. Feed was rationed to the animals in both experimental

groups, while drinking water was provided *ad libitum*. The feed intake and body weight data were monitored throughout the experimental period.

For the second experiment, forty-eight rats were distributed into eight groups (n=6): normal control, CS control, CS+curcumin (10mg.kg<sup>-1</sup> b.wt.), CS+ curcumin (20mg.kg<sup>-1</sup> b.wt.), CS + hesperidin (10mg.kg<sup>-1</sup> b.wt.), CS+hesperidin (20mg.kg<sup>-1</sup> b.wt.), curcumin (20mg.kg<sup>-1</sup> b.wt.), and hesperidin (20mg.kg<sup>-1</sup> b.wt.). Exposure to CS was the same as in the first experiment, but the duration was for 6 weeks. Olive oil, which was also given to the normal and CS-control groups, was used to dissolve curcumin and hesperidin. Treatments were administered via oral gavage.

### Data collection

For the first experiment, behavioural patterns of the animals (normal control and CS-exposed) were taken simultaneously daily, for four weeks at ten minutes intervals for the duration of exposure – ninety minutes – (Table 1). The first time to access drinking water and feed was taken ten minutes before exposure to CS, during the exposure period, and ten minutes post-exposure for the CS-exposed rats.

For the second experiment, growth performance parameters (feed intake, weight gain, and feed conversion ratio FCR) were assessed [16].

### Statistical analyses

Quantitative variables were expressed as mean±standard error mean (S.E.M) of ten rats. The normality of data distribution and homogeneity of variance was tested us-

ing the Shapiro-Wilk test and Levine's test, respectively. The significance of difference in particulate matter, body weight, feed intake, and FCR (parametric tests) were analysed with independent sample t-test, while other behavioural pattern evaluations were analysed with Kruskal Wallis non-parametric test. Data from experiment 2 were analysed using one-way analysis of variance and the Tukey test. All analyses were done with the Statistical Package for Social Sciences (IBM SPSS, version 20.0) and graphs were plotted using GraphPad Prism (version 8.02).

## RESULTS AND DISCUSSION

### Particulate matter evaluation

We begin our investigation by evaluating the composition of the air exposed to both the control and the CS-groups. The control groups were exposed to normal air, which contained significantly (P<0.001) lower levels of both PM 2.5 and PM 10 matter, compared to CS (Table 1). Airborne PM were often a mixture of solid and liquid particles and occurred in several sizes. It is a common practice to classify these PMs on the basis of their sizes. PM 2.5 and PM 10 are fine breathable particles of sizes ≤2.5 and ≤10µm, respectively [4]. The lesser the particulate size, the easier for them to infiltrate the respiratory system and the broader are the adverse outcomes [4, 29]. The PM levels vary within diverse cigarette products, as the contents of various additives, nicotine, and tar may contribute to the PM released. Nevertheless, the PM 2.5 and PM 10 levels we observed are similar to those reported by L o f f r e d o

**Table 1. Ethogram showing behavioural patterns monitored in the first experiment**

Behaviour	Description
Locomotion	When rat moves around their cages or smoking chamber
Climbing	When rat climbs the wall of the cages or smoking chamber
Sitting	When rat crouches in one corner of the cages or smoking chamber
Stand and stare	When rat stands still with the feet and forehead all on the floor of the cages or smoking chamber
Feeding	When rat takes food from the feeder and take it to the mouth to eat
Sniffing	When rat draws up air audibly through the nose to detect a smell
Stand-upright	When rat stands on their hind leg in the centre of the cage
Digging	When rat scratches or dig in the sawdust
Drinking	When rat licks the water from the drinker
Self-grooming	When rat uses paws to scratch their body, including mouth

**Table 2. Particulate matter (PM) evaluation**

Groups	PM2.5 [ $\mu\text{g.m}^{-3}$ ]	PM10 [ $\mu\text{g.m}^{-3}$ ]	CO [ppb]	VOC [ppb]
Normal air	35.67 $\pm$ 9.68	9.20 $\pm$ 15.93	92.67 $\pm$ 1.33	250.00 $\pm$ 28.87
Cigarette smoke	618.33 $\pm$ 35.24	855.00 $\pm$ 60.73	117 $\pm$ 2.19	5508.33 $\pm$ 92.61
P value	0.000	0.000	0.002	0.006

Data are means  $\pm$  SEM (n = 10); CO = carbon monoxide; VOC = volatile organic compounds; ppm = parts per million; ppb = parts per billion. Data were analysed with independent sample t-test

et al. [11] and Brun et al. [4], although these values for the normal air were lower than those reported by Lofredo et al. [11] for venues where tobacco smoking was banned (PM 2.5 levels of 72–81  $\mu\text{g.m}^{-3}$  compared to 35.67  $\mu\text{g.m}^{-3}$  reported here), probably owing to the controlled conditions of the animal house where the control groups were situated. Other gaseous and volatile components were also significantly higher in the CS than the normal air. Cigarettes contain several hydrocarbons that when partially combusted (as is the case during smoking) release CO and other volatile organic compounds. Inhaling high levels of CO have been said to increase heartbeat, induce headaches, nausea, and tiredness [19], which may in turn influence appetite and other habits.

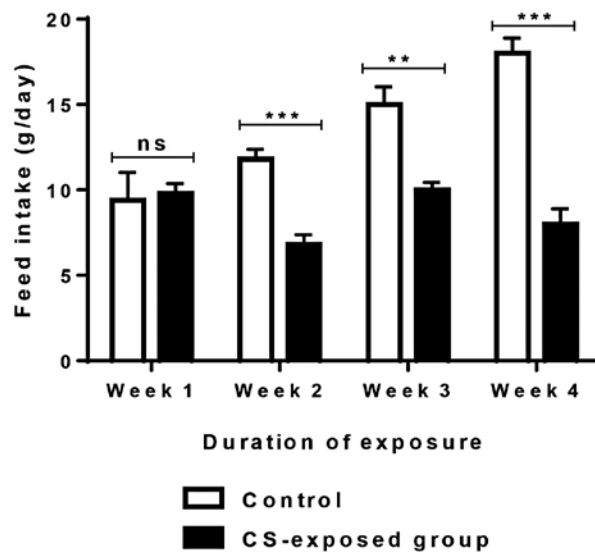
#### Exposure to cigarette smoke alters behavioural and dietary patterns in rats (Experiment 1)

The reductions in body weight of the CS-exposed rats did not attain statistical significance compared to the control group, after four weeks (Table 2). However, by the second week, the feed intake began to be significantly ( $P < 0.05$ ) lower in the CS-exposed rats, which was even more pronounced by the fourth week of CS exposure (Fig. 1). The feed intake of the CS-group was fairly consistent throughout the four weeks, whereas the control rats continued to consume more. Constituents present in CS, with nicotine being the chief suspect, are known to possess inhibitory effects on the hypothalamic-appetite regulating axis in the brain [8, 27]. By binding to the nicotinic acetylcholine receptors, nicotine stimulates the release of neurotransmitters, such as dopamine and serotonin, that decreases hunger and thereby, affecting the appetite [18]. Interestingly, we observed that the CS-exposed rats took longer time to access feed or drink, particularly after the CS-exposure, as early as the first week of exposure (Fig. 2 and 3). This delayed craving to drink or eat was sustained

**Table 2. Body weights of control and cigarette smoke (CS)-exposed groups**

Weeks	Control group	CS-exposed group
1	189.80 $\pm$ 9.17	209.20 $\pm$ 15.93ns
2	195.40 $\pm$ 9.22	209.60 $\pm$ 12.93ns
3	201.80 $\pm$ 8.97	185.20 $\pm$ 12.67ns
4	205.80 $\pm$ 8.28	193.60 $\pm$ 12.24ns

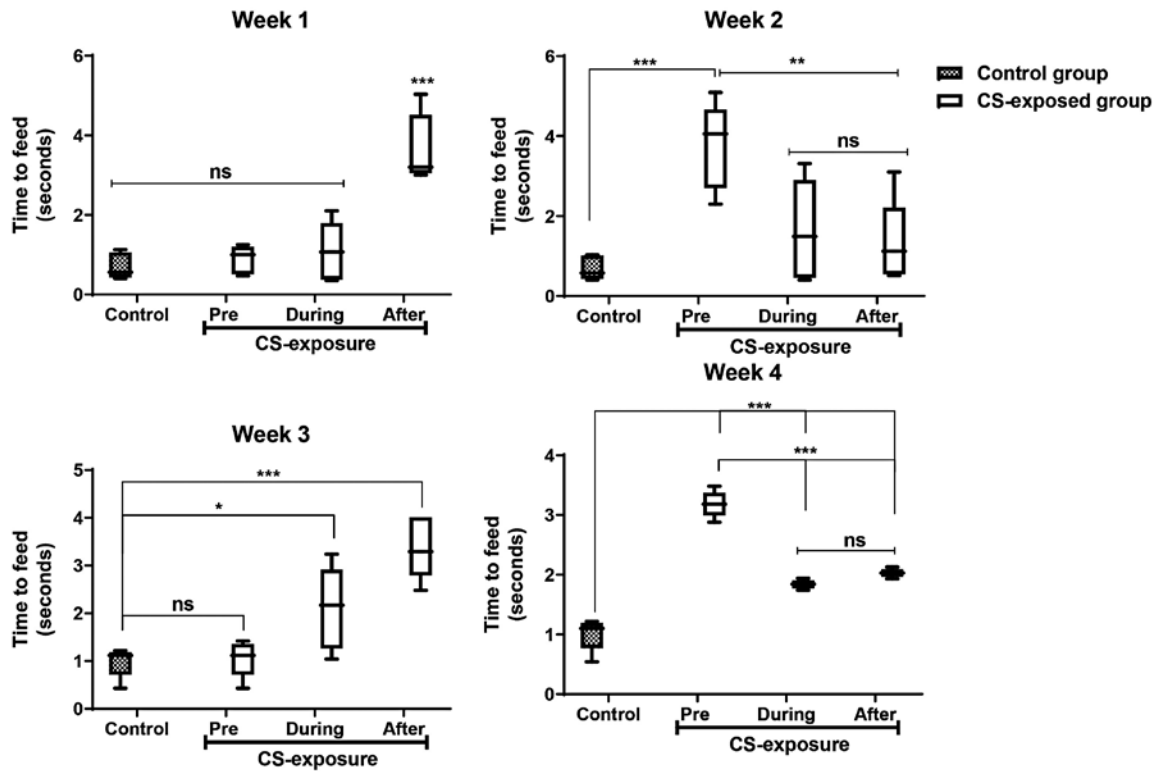
Data are means  $\pm$  SEM (n = 10); ns = not significantly different from the respective control, i.e.  $P > 0.05$ . Data were analysed with independent sample t-test

**Fig. 1. Weekly feed intake of control and cigarette smoke (CS)-exposed groups**

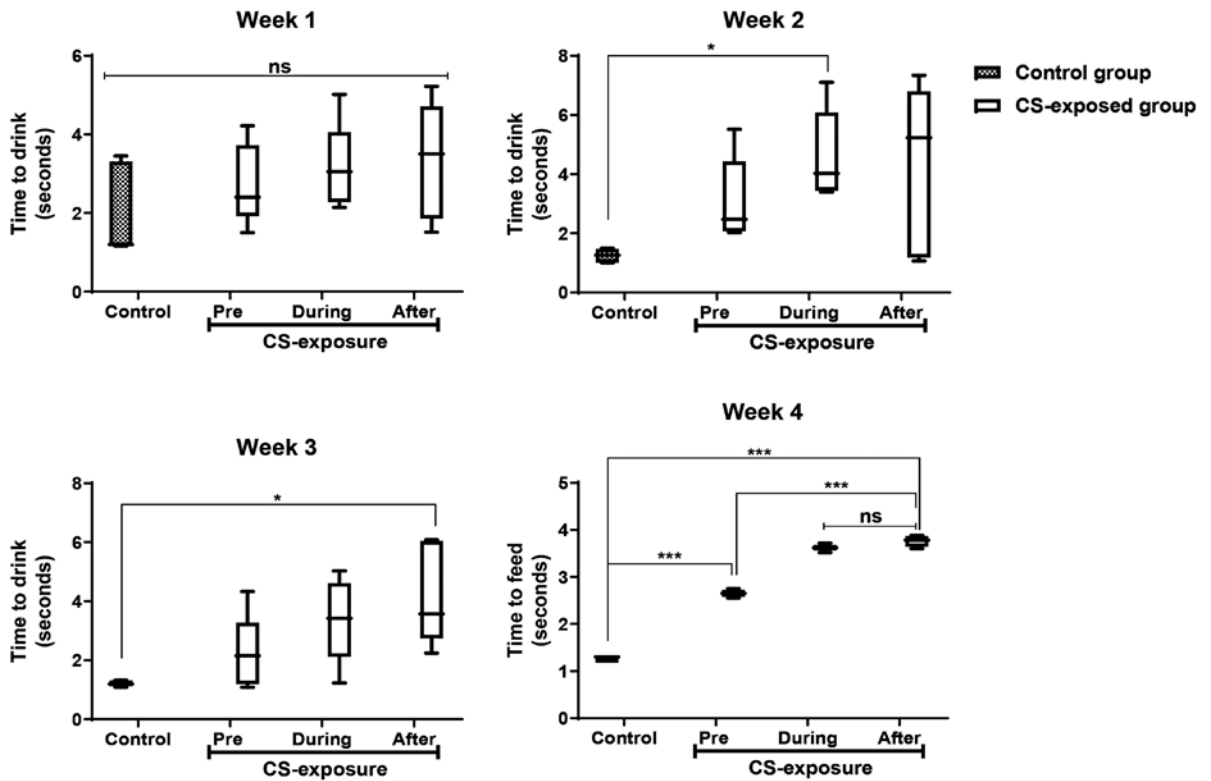
Bars are means  $\pm$  SEM of seven days. Significance: ns—not significant (i.e.,  $P > 0.05$ ); \*\*— $P < 0.01$ ; \*\*\*— $P < 0.001$ . Data were analysed with independent sample t-test

at the fourth weeks and further affirms that CS alters the dietary behaviour of exposed subjects.

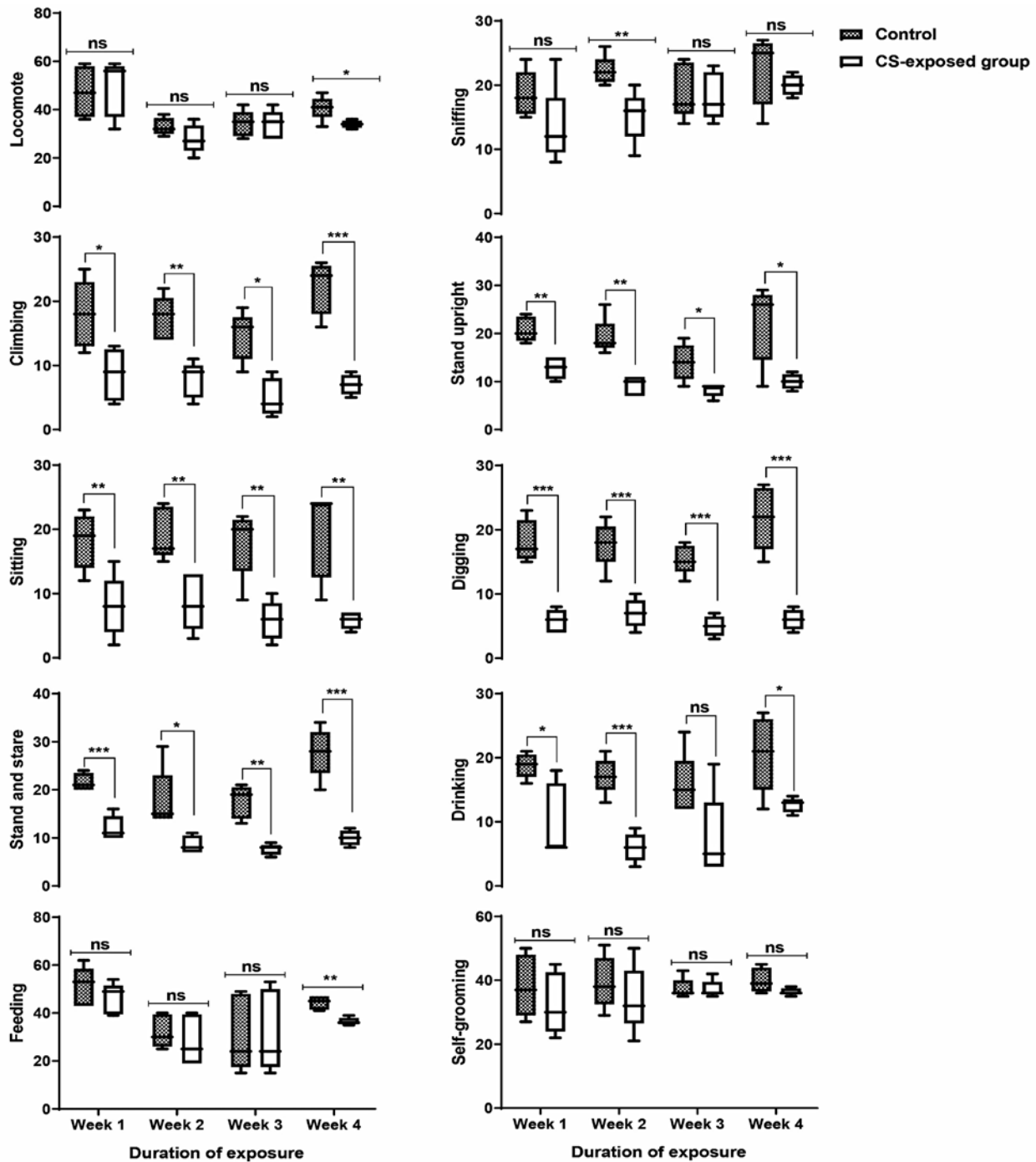
The behavioural patterns examined during exposure to CS, in the first experiment, were locomotion, climbing, sitting, stand and stare, feeding, sniffing, stand upright, dig-



**Fig. 2.** Box and whisker plots of time to feed (seconds) in control and cigarette smoke (CS)-exposed groups  
Data were collected for the seven days of each week. Significance: ns—not significant (i. e.,  $P > 0.05$ ); \*— $P < 0.05$ ;  
\*\*— $P < 0.01$ ; \*\*\*— $P < 0.001$ . Data were analysed with Kruskal Wallis non-parametric test



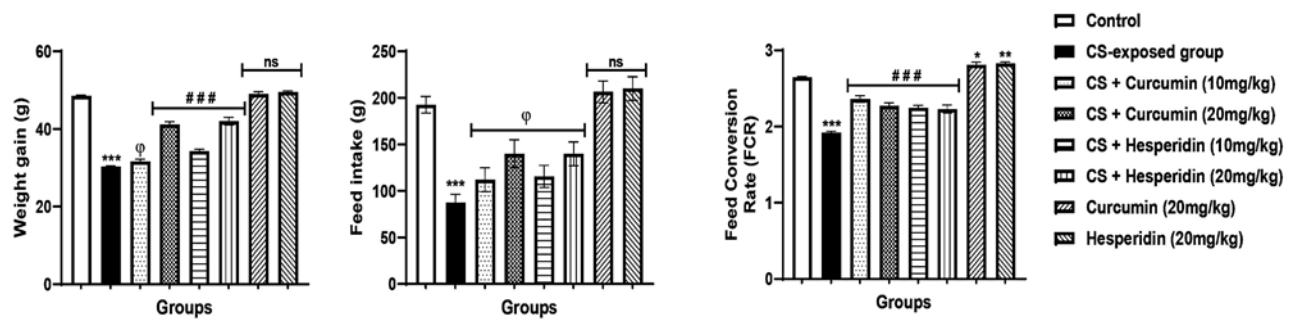
**Fig. 3.** Box and whisker plots of time to drink (seconds) in control and cigarette smoke (CS)-exposed groups  
Data were collected for the seven days of each week. Significance: ns—not significant (i. e.,  $P > 0.05$ );  
\*— $P < 0.05$ ; \*\*— $P < 0.01$ ; \*\*\*— $P < 0.001$ . Data were analysed with Kruskal Wallis non-parametric test



**Fig. 4. Box and whisker plots of behavioural patterns in the control and cigarette smoke (CS)-exposed groups**  
 Data were collected for the seven days of each week. Significance: ns—not significant (i.e.,  $P > 0.05$ );  
 \*— $P < 0.05$ ; \*\*— $P < 0.01$ ; \*\*\*— $P < 0.001$ . Data were analysed with Kruskal Wallis non-parametric test

ging, drinking, and self-grooming (Fig. 4). We report that CS distorted the behavioural patterns in exposed rats, characterized by reduced willingness to locomote or explore their environment (by climbing, stand and stare, digging). This may suggest reduced locomotor activity or anxiety (due to unwillingness to explore). Besides, reduced activity, anxiety, and panic-related behaviours have been

reported in rats exposed to tobacco-free CS [6]. Smokers often report a sense of calm during and immediately after smoking, though transient [24]. This false sense of calm, attributed to satisfying one's nicotine craving, may be involved in the reduced behavioural activity, as observed in the CS-exposed rats.



**Fig. 5. Growth performance parameters of control and cigarette smoke (CS)-exposed groups treated with(out) curcumin and hesperidin** (A) Weight gain (g); (B) Feed intake (g); and (C) Feed conversion ratio (FCR). Bars are means  $\pm$  SEM of six rats. Significance: ns—not significant compared to control; \*, \*\*, \*\*\*—significant compared to control at  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively;  $\phi$ —not significant compared to the CS-exposed group; ###—significant compared to control at  $P < 0.05$ . Data from experiment 2 were analysed using one-way analysis of variance and Tukey test

### ExCurcumin and hesperidin improves growth performance in CS-exposed rats (Experiment 2)

In the second experiment, we compared the effects of some nutritional supplements (curcumin and hesperidin) on growth performance parameters in the control and CS-exposed rats, while using a longer duration (6 weeks) than the first experiment. After 6 weeks of exposure, weight gain, feed intake, and FCR were significantly lower in the CS-exposed group compared to the control group (Fig. 5), which is in line with the works of Andong et al [1] and Ypsilantis et al [27]. Nicotine, in the short term at least, upregulates energy expenditure, which impairing appetite, leading to reduced body weight gain [8], and may explain the reduced growth performance in rats exposed to CS. Treatment with curcumin and hesperidin, especially at the higher dose ( $20\text{mg}\cdot\text{kg}^{-1}$  b.wt.), significantly improved the weight gain and feed intake of the CS-exposed groups. Separate studies have reported similar beneficial effects on weight gain, feed intake, and FCR, in different animal models and under different conditions [12, 13, 20, 21]. Also, although the weight gain and feed intake in the normal rats treated with curcumin and hesperidin (at the dose of  $20\text{mg}\cdot\text{kg}^{-1}$  b.wt.) were not significantly different ( $P > 0.05$ ) compared to the control group, the FCR was significantly higher in both treatments compared to the control. This further corroborate the benefits of these nutritional supplements.

### CONCLUSIONS

Exposure to cigarette smoke impairs exploratory and consummatory behaviour in rats, characterized by reduced

feed and water intake as the duration of exposure is prolonged. Furthermore, behaviour patterns, such as standing, climbing, and locomotion, were impaired, suggesting that CS smoke might cause a reduction in the physical fitness and willingness to explore in the animals. More so, attributes such as digging, and sitting which might be taken as signs of comfort and wellbeing decreased significantly, suggesting an inherent discomfort to the animals due to CS exposure. Treatments of CS-exposed rats with curcumin and hesperidin demonstrated improvements in the growth performance, particularly at the higher dose ( $20\text{mg}\cdot\text{kg}^{-1}$  b.wt.).

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## EFFICACY OF DISINFECTANTS USAGE AT DAILY LIVE BIRD MARKETS IN FOUR NORTH-WESTERN STATES OF NIGERIA

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### ABSTRACT

Maintaining strict biosecurity measures are essential in preventing disease spread from live bird markets (LBMs), which serve as a major intermingling area for poultry from different sources. This study evaluated the efficacy of disinfectants used in daily live bird markets of four north-western states in Nigeria. Seven different disinfectants were identified as commonly used in the LBMs. They were analysed by suspension and surface disinfection tests against standard strains of *Escherichia coli*, *Salmonella Enteritidis*, and *Staphylococcus aureus*. Isolates from swab samples of birds' cages in the LBMs were initially subjected to biochemical tests and, subsequently, susceptibility tests against commercial disinfectants. All of the 7 (100 %) disinfectants used in the LBMs killed/inhibited the growth of *E. coli*, *S. Enteritidis*, and *S. aureus* with the suspension test, while following the surface disinfection test, all 7 (100 %) killed/inhibited the growth of *E. coli* and *S. Enteritidis* but only 4 (57 %) killed/inhibited the growth of *S. aureus*. Seven (0.02 %) samples out of the 400 swabs were positive for *E. coli* comprising 1 (14 %), 2 (29 %), and 4 (57 %) from LBMs in Katsina, Kaduna, and Kano, respectively. There were varying growths of

*E. coli* at different concentrations and exposure times. Six (17 %) of the LBMs sampled had and used disinfectants. *E. coli* was isolated from 1 (17 %) out of the 6 LBMs that had and used disinfectants and 5 (17 %) out of the 29 LBMs that did not have or use disinfectants. The standard organisms were most susceptible to orthobenzyl chlorophenol-based disinfectants and least susceptible to chlorophenol-based disinfectants. This study has shown the importance of the use of disinfectants in LBMs. There should be enforcement of disinfectants usage in LBMs for public safety.

**Key words:** biosecurity; efficacy; disinfectants; live bird markets; North-western Nigeria

### INTRODUCTION

The transmission of diseases through surfaces plays an essential role in spreading infection among humans and other animals. The survival of pathogens such as viruses, bacteria, fungi, etc. on surfaces can have a direct impact on preventive measures, including hygiene guidelines and disinfection strategies [29]. Disinfection is the killing/inhibition of microorganisms, not necessarily destruction but

reducing to a lower acceptable level of microbial activity. Hence, a termination of the chain of bugs transmission to avoid infections [17]. Disinfection is an integral part of preventive medicine, which cuts off infection routes. However, the effectiveness of disinfection depends on selecting disinfectants according to the situation and environment, which usually requires expertise and experience. Several properties of an ideal disinfectant and five critical considerations for selecting the optimal disinfectant have been highlighted. Fundamental properties of a good disinfectant have been described by R u t a l a and W e b e r [24]. It is essential to make use of a disinfectant that has the majority of these important properties, and it is advisable to select a disinfectant with a longer contact time to kill a broader spectrum of microorganisms. The growth rate of disinfectant-resistant bacteria is shocking, which will greatly reduce the killing efficiency of disinfectants [31]. Disinfectant resistance is related to the bacterial characteristics, concentration and physical state of disinfectants and environmental conditions [28]. High-level disinfection will inactivate all microorganisms except large numbers of bacterial spores [23]. Intermediate-level disinfection will inactivate vegetative microorganisms and possibly low numbers of bacterial spores. Low-level disinfection inactivates most vegetative bacteria and some fungi and viruses, but it does not inactivate bacterial spores. Alcohol is an excellent example of a low-level disinfectant, with a relatively broad spectrum for microorganisms but is slow to act against non-enveloped viruses. Additionally, alcohol is not a good choice to achieve coverage of the necessary contact period with microorganisms because of its volatility, and it is not recommended for use on large surfaces such as cages of live bird markets because of its flammability. Generally, the contact period should be longer than or equal to the kill time. Several disinfectants that contain different classes of chemical compounds, including acids, alcohols, aldehydes, alkalis, biguanides, halogens, oxidizing agents, phenols, and quaternary ammonium compounds as active substances have been developed and proposed for practical use, and they are recommended for use in different branches of industry [22].

Live bird markets (LBMs) are also known as wet markets and are common in many parts of the World [10], including Nigeria. There were no dedicated live bird markets (LBMs) and poultry processing facilities in Nigeria until recently. The majority of the processing facilities are also

devoid of formal dedicated Veterinary Inspectors, thus lacking expertise in dilution/preparation of disinfectants. Hence, there is a need to evaluate overall live bird market operations to assess the country's public health problems related to poultry processing [19]. In some selected urban LBMs, the marketers have been trained and educated on the dangers of highly pathogenic avian influenza (HPAI) to poultry and humans [3] and were taught the disinfection procedures, but the impact of these measures on the safety of poultry products and the control of other zoonoses were never evaluated. In Nigeria, there is also a problem with the quality of drugs and chemicals, where producers may use a lesser amount of the active ingredient required for it to be effective. In addition, the live bird marketers are generally uneducated, which makes it difficult for them to follow manufacturers' instructions strictly, leading to improper use of these chemicals. These will consequently promote resistance and the failure of disinfection.

Infected poultry can contaminate the LBM environment, such as swabs from floors, cages, drains, walls, scales, and microorganisms may persist for days or weeks depending on factors such as humidity, temperature, and the presence of several host ranges [12]. There is a dearth of information on disinfectants' efficacy in Nigeria's LBMs. This study will generate information on the efficacy of disinfectants that can guide LBM operators (and other relevant authorities) on the correct use of disinfectants in LBMs. It will, in turn, reduce the likelihood of the development of resistance and the spread of pathogens during poultry handling and processing and hence reduce human exposure to poultry zoonoses. It will reduce the cost of treatment in both poultry and humans, which will positively impact the economy.

This study aimed to identify the type(s) of disinfectant(s) used in the LBMs and determine their efficacy against reference strains of *Escherichia coli*, *Salmonella Enteritidis*, and *Staphylococcus aureus*. Also, to determine the *in vitro* antibacterial activity of the identified disinfectants.

## MATERIALS AND METHODS

### Study area

The study was conducted at the daily live bird markets in some North-western states (Kaduna, Kano, Katsina, and Zamfara) of Nigeria. Northwest is one of the six geopolit-



**Fig. 1. Map of North-western Nigeria**  
(Source; Google images)

ical zones in Nigeria and consists of seven (7) states: Jigawa, Kaduna, Kano, Katsina, Kebbi, Sokoto, and Zamfara states (Fig. 1).

### Study design

The study design was experimental, and it was divided into two parts. The first part was conducted to test the efficacy of routinely used disinfectants in daily LBMs of the states against standard control organisms. *Escherichia coli* isolated from swab samples taken from the LBMs were subjected to a susceptibility test using some commercial disinfectants for the second experiment.

### Sampling techniques

Disinfectant samples from managers were collected based on availability. The swab sample size collected from Marketers was determined using the formula by *Thrusfield* [27]. The prevalence rate of 56% [22] was used to obtain the sample size was 379. In the present study, 5.5% of the calculated sample size was added to minimize loss due to sampling error. Hence, 400 swab samples were collected from the bird's cages of the marketers.

Live bird markets within the metropolitan area of these states were identified. Disinfectant (10ml each) and swab samples were collected from managers based on convenience and availability. The number of swab samples taken from each state was calculated by proportionate sampling from which Kaduna (with 12 LBMs)=137, Kano(15)=171, Katsina(4)=46, and Zamfara(4)=46. Swab samples of birds' cages (a single swab from each marketer) of about 10 cm<sup>2</sup> of the innermost area of the floor of the birds'

cages were collected using a sterile swab stick, and this was transferred into transport media and taken to bacteria laboratory for isolation.

### Testing of disinfectant samples

It involved a standard laboratory protocol according to the European Committee for Standardization guideline, where the basic principle for testing the efficacy of a disinfectant is in three phases; first, testing for its antimicrobial activity; second, testing the condition and in-use dilution at which it is active; and lastly an in situ test [21]. The first two phases were carried out in this study.

### Suspension test

Standard strains of reference *Escherichia coli* (ATCC 10536; NCTC 10418), *Salmonella Enteritidis* (ATCC 13076; NCTC 12694), and *Staphylococcus aureus* (ATCC 6538; NCTC10788), were grown on media. Nutrient broth cultures of these reference bacteria were prepared in a test tube to make a 10<sup>4</sup> dilution of the bacteria.

A loopful (10µl) of the suspension was taken and brought into contact with 2ml of the disinfectant in a second test tube. A loopful (10µl) of the resultant mixture was inoculated onto a culture medium. Eosin methylene blue (EMB) was used for *Escherichia coli*; eoxycholate citrate agar (DCA)/*Salmonella Shigella* agar (SSA) for *Salmonella Enteritidis* and Mannitol salt agar (MSA) for *Staphylococcus aureus*. A control was included for each category. These were incubated at 37°C for 24h after which they were observed for growth. Growth was recorded as positive (+), whereas its absence was negative (–).

### Surface disinfection test

The disinfectant samples collected from the LBMs were prepared by diluting according to the working dilution of the LBMs. There were prepared 2-fold and 4-fold dilutions to achieve three different concentrations. A microscopic glass slide (serving as the test surface) was contaminated with 0.2ml of a 10<sup>4</sup> dilution of a nutrient broth culture of the test bacteria. The slides were dried and placed on a rack. One ml each of the disinfectant was dropped on the slides using a pipette and distributed evenly. A control was included with the disinfectant substituted with sterile distilled water. These were left to stand for different intervals (5, 15, and 30 minutes), after which the slides were rinsed with 50 ml of distilled water. The rinsing

fluids were inoculated on a culture medium and incubated at 37°C for 24–48 h.

### Isolation of *E. coli*

One ml of the swabbed sample was taken and added to 9 ml of tryptone soy broth as enrichment and incubated for 24 hours. This was then plated on a selective media (EMB) and incubated for 24 h as first described by Levine [16]. Suspected positive colonies (with characteristic greenish metallic sheen on *E. coli*) were transferred into nutrient broth in sterile sample bottles and afterward subjected to conventional biochemical and kit (Microbact™) tests.

### Susceptibility test

The isolated *Escherichia coli* from LBMs were subjected to susceptibility tests against commercially available disinfectants. Four commercially available disinfectants were used, referred to here as A, B, C, and D. A contains ortho-benzyl chlorophenol, B contains chloroxylenol, C contains chlorophenol, and D contains sodium hypochlorite as active ingredients. The maximum concentration was the available commercial concentration from the manufacturers. These concentrations were diluted with sterile distilled water to obtain 1.28 %, 0.32 %, 0.08 %, and 0.02 % for the respective disinfectant.

A: 0.02, 0.08, 0.32, 1.28 and 5.12 %

B: 0.02, 0.08, 0.32, 1.28 and 4.80 %

C: 0.02, 0.08, 0.32, 1.28 and 5.12 %

D: 0.02, 0.08, 0.32, 1.28 and 3.50 %

The isolates were standardized to 1 % McFarland in normal saline, inoculated into the nutrient broth, and incubated at 37°C for 24 h.

A half ml of the 24 h culture was added to 5 ml of the different concentrations of disinfectant solutions, placed in a water bath (at 19°C), and shaken gently. These were removed at different time intervals (5, 10, 15, 30, and 60 min). One loopful of the mixture was then sub-cultured into 5 ml of nutrient broth and incubated at 37°C for 48–72 hrs.

The results were recorded as positive when there was an increase in turbidity or negative when there was none when compared to the controls. These were quantified and also presented as percentages.

## RESULTS

Results of the suspension, surface disinfection, and susceptibility tests are presented in tables.

### Types of disinfectants used in the LBMs and their efficacy

Table 1 shows LBMs that had disinfectant samples, the name of the disinfectant, form, chemical composition, and the dilution factor used in the LBM.

The characteristic growth of the organisms was obtained when inoculated onto individual selective media. *E. coli* on eosin-methylene blue (EMB) appeared as dark,

**Table 1. Disinfectants used in some LBMs of north-western Nigeria**

	State	LBM	Disinfectant	Form	Chemical composition
1	Katsina	Sabuwar kofa	Diskol I	Liquid	Glutaraldehyde, ammonium chloride, formaldehyde
2	Katsina	Central market	Lysol	Liquid	Methylphenol
3	Kano	Sharada	Virkon-S I	Powder	Potassium peroxymonosulphate, sodium dodecylbenzene sulfonate, sulphamic acid
4	Kano	Sharada	Diskol II	Liquid	Glutaraldehyde, ammonium chloride, formaldehyde
5	Kano	Sheka	Hypo	Liquid	Sodium hypochlorite
6	Kano	Sabon titi	Vinkokill	Liquid	Chlorophenol
7	Kaduna	Sokoto road	Virkon-S II	Powder	Peroxymonosulphate

S/N—Serial Number

**Table 2. The activity of some disinfectants used in live bird markets in north-western Nigeria on *Staphylococcus aureus***

Time	Dilution	C	Diskol I	Disinfectants			Hypo	Vinkokill	Virkon-S II
				Lysol	Virkon-S I	Diskol II			
5 min	Market	+	–	–	–	–	–	–	–
	2-fold	+	+1	–	–	–	+4	–	+1
	4-fold	+	+3	–	–	+1	+5	–	+7
15 min	Market	+	–	–	–	–	–	–	–
	2-fold	+	–	–	–	–	–	–	–
	4-fold	+	–	–	–	–	–	–	–
30 min	Market	+	–	–	–	–	–	–	–
	2-fold	+	–	–	–	–	–	–	–
	4-fold	+	–	–	–	–	–	–	–

blue-black colonies with a greenish metallic sheen. *Salmonella Enteritidis* on desoxycholate agar (DCA) as pale/colourless colonies with dark centres due to hydrogen sulphide production. *Staphylococcus aureus* on mannitol salt agar (MSA) as yellow colonies with yellow zones.

Following the suspension test, no growth was observed in all inoculates except controls.

All the disinfectants inhibited the growth of *Escherichia coli* at all three dilutions and the different periods of exposure. Growths were only seen in the controls. There was no growth at the three dilutions and at different exposure times when *Salmonella Enteritidis* was exposed to disinfectants. Growth was also seen in the controls. Table 2 shows the action of the disinfectants on *Staphylococcus aureus* at different time intervals (of exposure) and different concentrations following the surface disinfection test. For *Staphylococcus aureus*, there was growth in all of the controls. Growth was recorded after exposure to disinfectants 1, 4, 5, and 7. The growth was seen at 5 min exposure time and at second and third dilution except for disinfectant 4 where growth was only seen at the third dilution. The number of colonies increased with increasing dilution.

#### **In vitro antibacterial activity of some commercial disinfectants**

Out of the total of 400 swab samples collected, 121 produced the characteristic “blue-black” colonies with greenish metallic sheen on eosin-methylene blue following the culture and isolation for *E. coli*. These were the suspect positives.

Following the MR/VP, citrate, urease, SIM, and TSI tests, and the kit (Microbact™) tests on the 121 suspect positive samples, 7 samples were positive for *E. coli*. These were urease negative, citrate negative, indole positive, motile, hydrogen sulphide negative, methylene red positive, and Vvoges Proskauer negative, and showed percentage probability (of being *E. coli*) from the ID system of 97.11, 91.92, 84.29, 97.11, 97.11, 92.97 and 92.11 % comprising of 1 from an LBM in Katsina, 2 from Kaduna and 4 from Kano (KT 01 13, KN 05 01, KN 06 03, KN 09 03, KN 13 03, KD 06 03 and KD 11 01. Where the first number represent the LBM and the second number represent the sample number).

The action of each disinfectant on individual isolates at varying concentrations and at different exposure times was observed as the presence of growth (+) or no growth (–) and presented as tables of concentration (horizontal axis) against periods of exposure (vertical axis) (Tables 3, 4, 5, and 6).

#### **The activity of orthobenzyl chlorophenol on *Escherichia coli***

Two isolates, one from Kano (KN 06 03) and the second from Kaduna (KD 06 03), were most susceptible as growth was observed only at the least concentration of 0.02 % and an exposure time of 5 minutes. Another from Kano was a bit more resistant, with growth at the next concentration of 0.08 %. The least susceptible was an isolate from Kaduna (KD 11 01), with growth observed at a concentration of 1.28 %, and an isolate from Kano (KN 13 03),



Table 3. The activity of orthobenzyl chlorophenol on *Escherichia coli*

<i>E. coli</i> isolate	Conc. [%]	Duration of exposure [min]					<i>E. coli</i> isolate	Conc. [%]	Duration of exposure [min]				
		5	10	15	30	60			5	10	15	30	60
KT 01 13	5.12	–	–	–	–	–	KN 05 01	5.12	–	–	–	–	–
	1.28	–	–	–	–	–		1.28	–	–	–	–	–
	0.32	–	–	–	–	–		0.32	–	–	–	–	–
	0.08	+	+	–	–	–		0.08	+	–	–	–	–
	0.02	+	+	–	–	–		0.02	+	–	–	–	–
KN 06 03	5.12	–	–	–	–	–	KN 09 03	5.12	–	–	–	–	–
	1.28	–	–	–	–	–		1.28	–	–	–	–	–
	0.32	–	–	–	–	–		0.32	+	–	–	–	–
	0.08	–	–	–	–	–		0.08	+	–	–	–	–
	0.02	+	–	–	–	–		0.02	+	+	–	–	–
KN 13 03	5.12	–	–	–	–	–	KD 06 03	5.12	–	–	–	–	–
	1.28	–	–	–	–	–		1.28	–	–	–	–	–
	0.32	+	–	–	–	–		0.32	–	–	–	–	–
	0.08	+	+	–	–	–		0.08	–	–	–	–	–
	0.02	+	+	+	–	–		0.02	+	–	–	–	–
KD 11 01	5.12	–	–	–	–	–		5.12	–	–	–	–	–
	1.28	+	–	–	–	–		1.28	–	–	–	–	–
	0.32	+	–	–	–	–		0.32	–	–	–	–	–
	0.08	+	–	–	–	–		0.08	–	–	–	–	–
	0.02	+	+	–	–	–		0.02	+	–	–	–	–

with growth observed at an exposure time of 15 minutes (Table 3). The percentage growth was recorded as 16, 8, 4, 16, 24, 4 and 20 % for *E. coli* isolates KT 01 13, KN 05 01, KN 06 03, KN 09 03, KN 13 03, KD 06 03 and KD 11 01, respectively.

#### The activity of chloroxylenol on *Escherichia coli*

The most susceptible was an isolate from Kano (KN 09 03), with growth observed only at a concentration of 0.02 % with a 5 min exposure time. The least susceptibility was observed in an isolate from Kano (KN 13 03), with growth even at concentrations of 1.28 %, 0.32 % at 10 min, and with an exposure time of 15 min (Table 4). The percentage growth was recorded as 28, 32, 12, 4, 32, 20 and 20 % for *E. coli* isolates KT 01 13, KN 05 01, KN 06 03, KN 09 03, KN 13 03, KD 06 03 and KD 11 01, respectively.

#### The activity of chlorophenol on *Escherichia coli*

An isolate from Kano (KN 05 01) and two from Kaduna (KD 06 03 and KD 11 01) were least susceptible to the disinfectant even at the highest concentration of 5.12 %. The second from Kaduna (KD 11 01) was resistant to the disinfectant at the highest exposure time of 60 minutes. The highest susceptibility was observed with an isolate from Kano (KN 06 03) with inhibition of growth from the concentration of 0.32 % (Table 5). The percentage growth was recorded as 60, 36, 28, 28, 56, 56 and 52 % for *E. coli* isolates KT 01 13, KN 05 01, KN 06 03, KN 09 03, KN 13 03, KD 06 03 and KD 11 01, respectively.

#### The activity of sodium hypochlorite on *Escherichia coli*

The least susceptibility was observed with the isolate from Katsina (KT 01 13), with observed growth at

Table 4. The activity of chloroxylenol on *Escherichia coli*

<i>E. coli</i> isolate		Duration of exposure [min]					<i>E. coli</i> isolate	Conc. [%]	Duration of exposure [min]				
		5	10	15	30	60			5	10	15	30	60
KT 01 13	4.80	-	-	-	-	-	KN 05 01	4.80	-	-	-	-	-
	1.28	-	-	-	-	-		1.28	-	-	-	-	-
	0.32	+	+	-	-	-		0.32	+	+	-	-	-
	0.08	+	+	-	-	-		0.08	+	+	-	-	-
	0.02	+	+	+	-	-		0.02	+	+	+	+	-
KN 06 03	4.80	-	-	-	-	-	KN 09 03	4.80	-	-	-	-	-
	1.28	-	-	-	-	-		1.28	-	-	-	-	-
	0.32	-	-	-	-	-		0.32	-	-	-	-	-
	0.08	+	-	-	-	-		0.08	-	-	-	-	-
	0.02	+	+	-	-	-		0.02	+	-	-	-	-
KN 13 03	4.80	-	-	-	-	-	KD 06 03	4.80	-	-	-	-	-
	1.28	+	-	-	-	-		1.28	-	-	-	-	-
	0.32	+	+	-	-	-		0.32	+	-	-	-	-
	0.08	+	+	-	-	-		0.08	+	-	-	-	-
	0.02	+	+	+	-	-		0.02	+	+	+	-	-
KD 11 01	4.80	-	-	-	-	-							
	1.28	-	-	-	-	-							
	0.32	+	-	-	-	-							
	0.08	+	-	-	-	-							
	0.02	+	+	+	-	-							

a concentration of 3.5% at both exposure times of 5 and 10 minutes. The highest susceptibility was observed with an isolate from Kano (KN 09 03), with growth observed at a concentration of 0.32% (Table 6). The percentage growth was recorded as 48, 24, 40, 12, 12, 44 and 32% for *E. coli* isolates KT 01 13, KN 05 01, KN 06 03, KN 09 03, KN 13 03, KD 06 03 and KD 11 01, respectively.

## DISCUSSION

From our study, the calculated prevalence as regards to the use of disinfectants in LBMs was 17% which was close to the 23% reported in Bangladesh [7]. It is far lower than the 56% prevalence of FAO in 2008 [9] but higher than the 4.5% disinfectant usage reported in Malaysia

[16]. The high prevalence reported by FAO could be attributed to the rigorous control campaign with the call for the use of disinfectants in LBMs following the avian influenza outbreaks between 2006 and 2009. The stakeholders have since been growing more complacent about disinfection in LBMs, which has led to the decline.

Following the suspension test, the absence of growth in all reactions showed that all of the disinfectants could kill/inhibit the growth of the bacteria they were tested against. However, the suspension test alone cannot be used to justify the efficacy claim of the disinfectants or the susceptibility of the bacteria because it does not represent the effectiveness of the disinfectant in the actual environment where the bacteria are attached to a specific material.

Despite resistant *E. coli* and *Salmonella* spp. have been reported to be prevalent and associated with older farms

Table 5. The activity of chlorophenol on *Escherichia coli*

<i>E. coli</i> isolate	Conc. [%]	Duration of exposure [min]					<i>E. coli</i> isolate	Conc. [%]	Duration of exposure [min]				
		5	10	15	30	60			5	10	15	30	60
KT 01 13	5.12	–	–	–	–	–	KN 05 01	5.12	+	–	–	–	–
	1.28	+	+	+	–	–		1.28	+	–	–	–	–
	0.32	+	+	+	–	–		0.32	+	–	–	–	–
	0.08	+	+	+	+	–		0.08	+	+	–	–	–
	0.02	+	+	+	+	+		0.02	+	+	+	+	+
KN 06 03	5.12	–	–	–	–	–	KN 09 03	5.12	–	–	–	–	–
	1.28	–	–	–	–	–		1.28	–	–	–	–	–
	0.32	–	–	–	–	–		0.32	+	+	–	–	–
	0.08	+	+	+	–	–		0.08	+	+	–	–	–
	0.02	+	+	+	+	+		0.02	+	+	+	–	–
KN 13 03	5.12	–	–	–	–	–	KD 06 03	5.12	+	+	–	–	–
	1.28	+	+	–	–	–		1.28	+	+	+	–	–
	0.32	+	+	–	–	–		0.32	+	+	+	–	–
	0.08	+	+	+	+	+		0.08	+	+	+	–	–
	0.02	+	+	+	+	+		0.02	+	+	+	–	–
KD 11 01	5.12	+	–	–	–	–							
	1.28	+	–	–	–	–							
	0.32	+	+	–	–	–							
	0.08	+	+	+	+	–							
	0.02	+	+	+	+	+							

and live bird markets [2, 4], the surface disinfection test in our study revealed that all of the disinfectants were active against *E. coli* and *S. Enteritidis*, even at the lowest concentration and least exposure time. A previous study reported a similar result in a response of *Escherichia coli* and *Salmonella Enteritidis* to disinfection, and it proposed the use of *E. coli* as an indicator for possible *Salmonella* presence after cleaning and disinfection [8]. In the case of *S. aureus*, growth was observed under only five minutes of exposure time for the various disinfectant with the exception of Lysol and Vinkokill. This may be due to the variation in time required for the mechanism of action of the respective disinfectants to manifest. Although, it has been demonstrated that complete inhibition can be obtained with a shorter exposure duration using a certain concentration of the disinfectant [6]. The differences in

growth activity from *S. aureus* between similar disinfectants (Diskol I and II, Virkons I and II) may be due to the fact that they were sourced from different LBMs where they were subjected to different storage conditions and periods. Disinfects not stored at cool, dark, and appropriate covering usually result in instability and inactivation of the solution, rendering it less or ineffective [25].

*Escherichia coli* was isolated from the swab samples collected, comprising one from Katsina, two from Kaduna, and four from Kano. The results from the susceptibility testing showed varying reactions. From our study, the activity of orthobenzyl chlorophenol on the isolates shows that KN 13 03 and KD 11 01 had a higher percentage growth (24% and 20%, respectively) than the other isolates. This may be due to these LBMs being larger, more populated by humans and birds, and hence more activities.

Table 6. The activity of sodium hypochlorite on *Escherichia coli*

<i>E. coli</i> isolate	Conc. [%]	Duration of exposure [min]					<i>E. coli</i> isolate	Conc. [%]	Duration of exposure [min]				
		5	10	15	30	60			5	10	15	30	60
KT 01 13	3.50	+	+	–	–	–	KN 05 01	3.50	–	–	–	–	–
	1.28	+	+	–	–	–		1.28	–	–	–	–	–
	0.32	+	+	–	–	–		0.32	+	–	–	–	–
	0.08	+	+	+	–	–		0.08	+	+	–	–	–
	0.02	+	+	+	–	–		0.02	+	+	+	–	–
KN 06 03	3.50	–	–	–	–	–	KN 09 03	3.50	–	–	–	–	–
	1.28	–	–	–	–	–		1.28	–	–	–	–	–
	0.32	+	+	–	–	–		0.32	–	–	–	–	–
	0.08	+	+	+	–	–		0.08	+	–	–	–	–
	0.02	+	+	+	+	+		0.02	+	+	–	–	–
KN 13 03	3.50	–	–	–	–	–	KD 06 03	3.50	–	–	–	–	–
	1.28	–	–	–	–	–		1.28	+	–	–	–	–
	0.32	+	–	–	–	–		0.32	+	+	+	–	–
	0.08	+	–	–	–	–		0.08	+	+	+	–	–
	0.02	+	–	–	–	–		0.02	+	+	+	+	–
KD 11 01	3.50	–	–	–	–	–							
	1.28	–	–	–	–	–							
	0.32	+	–	–	–	–							
	0.08	+	+	–	–	–							
	0.02	+	+	+	+	+							

These huge activities will require the need to disinfect frequently, but the workers and managers do dilute the disinfectant below recommended dilution so as to cut costs. It has been reported that places with more human activity tend to have more antimicrobial-resistant *E. coli* [30]. Also, the level of contamination and spread of disease and resistant bacteria in LBM may be associated with the volume of trade, environment, and type of poultry at the market [15]. Our study revealed that orthobenzyl chlorophenol inhibits/kills the isolated *E. coli* completely from 15 minutes upward. Hence, LBM workers and managers need to use this disinfectant for an exposure duration greater than 15 minutes during cleaning and disinfection.

The least susceptible isolate to chloroxylonol was from KN 13 03, where growth was recorded at 15 minutes exposure time and at 1.28% concentration. Although chlorox-

ylonol has been established to kill bacteria and enveloped viruses by compromising the membrane proteins responsible for transport and signalling. However, it shows some level of discrimination against bacteria, possibly due to the magnitude of changes in the bacteria membrane environment [20].

Virtually all of the isolates showed growth when exposed to chlorophenol at a lower concentration irrespective of the duration of the exposure. Hence, this indicates disinfection cannot be achieved with chlorophenol at a lower concentration even when exposed for a long period of time. This may be due to chlorophenols being primarily formulated in soap solutions to increase their invasive potential and are considered bactericidal at 5% concentration [13]. Chlorophenols do have a cloudy or milky appearance when added to water, and this factor may encourage users

to over dilute it and thus expose the bacteria to a lesser dose leading to the development of resistance [5].

The isolate from Katsina (KT 01 13) had the highest percentage growth and it was least susceptible at a lesser exposure time. Thus, there is a need to advocate for prolonged exposure period during disinfection with sodium hypochlorite. A previous study reported sodium hypochlorite to have rapid bactericidal and sporicidal activity only at a high concentration which can be corrosive and not safe to use. However, at low concentrations, the vegetative bacteria can be inhibited. Sodium hypochlorite has been reported to be sensitive to light and certain materials which can easily and rapidly render the disinfectant property inactive [14].

Overall, the *E. coli* isolates were most susceptible to orthobenzyl chlorophenol-based disinfectant (Izal®) and least susceptible to chlorophenol-based disinfectant (Virkon-S®). A previous study by Okore et al. [18] showed Dettol®, Z-Germicide®, and Jik® being more effective compared to Izal® on some bacteria isolates (including *E. coli*). Another study by Agunwamba et al. [1] showed Izal® to be more effective in aerobic sewage degradation (which includes its effect on faecal coliforms) compared to Dettol®. It could mean that the susceptibility of *E. coli* to disinfection differs between strains. There is also a possibility of disinfectant resistance and biofilm formation capacity of *E. coli* in live bird markets [26]. It could also be due to the presence of the *qac* resistance gene and antibiotic resistance gene in *E. coli* which are linked to their resistance to disinfectants [11].

## CONCLUSIONS

In conclusion, only 17% of the Live Bird Markets sampled had and used disinfectants (17% prevalence). From the LBMs that had disinfectants, the dilution in use was sufficient to kill/inhibit the growth of *Escherichia coli*, *Salmonella Enteritidis*, and *Staphylococcus aureus* at minimal exposure time, but a lower concentration will not be active against *Staphylococcus aureus*. *Escherichia coli* was isolated from 6 LBMs sampled. *E. coli* was not isolated from any LBM that used disinfectant samples except one. One particular isolate was most susceptible to the disinfectants used. The results of the susceptibility test showed that the isolates were least susceptible to

chlorophenol-based disinfectant and most susceptible to orthobenzyl chlorophenol-based disinfectant.

It is therefore recommended that live bird marketers should be more proactive towards disinfection practices by ensuring that they disinfect their premises, cleaning before disinfection, and properly use disinfectants. Also, there should be a routine evaluation of the efficacy of disinfectants by the relevant authorities and encourage further research in LBMs to understand the dynamics of diseases in the LBMs.

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## MOLECULAR DETECTION AND CHARACTERIZATION OF TRYPANOSOMES INFECTING TRADITIONALLY MANAGED CATTLE IN THE TROPIC WARM SUB-HUMID ZONE OF NIGERIA

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### ABSTRACT

Traditionally managed cattle constitutes the main source of animal protein to humans in Nigeria. However, seasonal migration in search of pasture exposes them to several vector-borne infections such as the African Animal Trypanosomosis (AAT), which limits their productivity. In this study, blood samples from 130 cattle in Plateau and Nasarawa states collected from May to June, 2021 were examined by the Polymerase Chain Reaction (PCR) and sequencing methods to determine the prevalence of pathogenic trypanosomes. Overall, the DNA of *T. vivax* was detected in 19 out of the 130 (14.6%) samples examined by the PCR. However, using the micro-hematocrit centrifugation technique, motile haemoparasites were detected in only six (4.6%, confidence interval [CI] 0.5—6.9%) of the samples. The higher prevalence of *T. vivax* was recorded in samples sourced from the abattoir than in samples submitted from the field in Plateau state (16.7% versus 11.5%). However, the reverse was the case in Nasarawa state (2.9% versus 37.5%). The difference in prevalence of *T. vivax* between the abattoir and field samples was significant ( $P=0.009$ ) in Nasarawa state, but not in Plateau state ( $P=0.55$ ). The mean PCV (Packed

Cell Volume) of the trypanosome infected animals was lower than that of the non-infected animals, but the difference was not significant ( $P=0.29$ ). The internal transcribe spacer region (ITS) nucleotide sequences of *T. vivax* generated in this study were 100% identical to each other and formed a monophyletic cluster with the sequences of *T. vivax* from different countries in the GenBank. AAT remains a major constraint to profitable cattle production and food security in Nigeria and deserves more attention.

**Key words:** cattle; ITS; mHCT; Nigeria; PCR; Trypanosomes

### INTRODUCTION

Pastoral cattle production in the form of semi-sedentary, agro-pastoralists and transhumance are the dominant cattle management systems in most parts of Nigeria [3]. These practices do not only exert stress on the animals, but also expose them to disease vectors during the course of migration across different ecological zones [21]. One of such diseases is the African Animal Trypanosomosis (AAT). There are 11 different pathogenic trypanosomes



known to exist in Africa [15, 22]. AAT has a significant impact that can lead to reduced animal productivity as much as 38% in high risk areas [28]. The condition is characterized by: fever, hair loss, stupor, oedema, anaemia, bilateral lacrimation, keratitis, weight loss, abortion and eventually death. The name Nagana is derived from the Zulu word “N’gana”, meaning “useless”, because of the progressive, wasting nature of the disease [25]. In addition, AAT can also cause immunosuppression, making the host more susceptible to coinfections [27]. The common cause of AAT in cattle in sub-Saharan Africa are: *Trypanosoma congolense*, *Trypanosoma brucei*, and *Trypanosoma vivax* leading to a chronic wasting disease [22, 28]. Trypanosomes are mainly transmitted biologically between mammalian hosts by the bite of an infected *Glossina* species [8]. However, some *Trypanosoma* spp. such as *T. vivax* and *T. evansi* can be mechanically transmitted by hematophagous Diptera belonging to the Tabanidae, Stomoxyinae, and Hippoboscidae [13]. Therefore, *T. vivax* and *T. evansi* are found in areas beyond the range of the tsetse flies [6]. Taken together, the epidemiology of trypanosomosis is not dependent on the availability of the biological vectors only, but also on the cattle management system, trade in livestock, and wildlife, socio-cultural, and political factors [21]. The Standard Trypanosomes Detection Methods (STDMS); wet film, thick and thin smear, microhaematocrit centrifugation technique and animal inoculation are the commonly used methods for the diagnosis of AAT in Nigeria. However, these techniques have low sensitivity and specificity and lack the ability to accurately differentiate between the various trypanosomes [15]. The polymerase chain reaction (PCR) has been employed for the diagnosis of trypanosomes with high efficiency, although, technical requirement and cost have precluded its widespread use [5, 22].

So far, there are a few studies on AAT in Nigeria using the PCR. The prevalence of 77%, 46% and 37% of AAT have been reported in cattle in Ogun, Plateau and Kaduna states, respectively, using the PCR [17, 29], suggesting widespread AAT across the ecological zones of Nigeria. Thus, the aim of this study was to apply PCR and sequencing approach to determine the prevalence and characterize the *Trypanosoma* spp. infecting cattle in two contiguous states; Plateau and Nasarawa located in tropic sub-humid zone of Nigeria.

## MATERIALS AND METHODS

### Ethical approval

Approval for this study was granted by the Institutional Animal Care and Use Committee (IACUC), National Veterinary Research Institute (NVRI), Vom, Nigeria, approval number: AEC/03/108/21. Oral consent was obtained from the management of the abattoirs and cattle owners before the animals were sampled.

### Study Area

The study was conducted in Plateau and Nasarawa states located in the tropic sub-humid zone of Nigeria (Fig. 1) [11]. The climate over this region is the tropical savanna climate and exhibits a well-marked rainy season and a dry season. The single dry season experienced in the study areas begins from October to May and the rainy season lasts from June to September [3, 21]. The temperatures are above 18°C, and the average annual precipitations ranges from 750mm to 1,100mm. The vegetation cover is predominantly made up of plains of tall grass which are interrupted by trees, that provide a suitable land for crop and animal production. Furthermore, the near temperate climate experienced in this region, coupled with the availability of good vegetation cover and crop residues as a source of animal feed makes this zone an ideal choice for livestock production [16].

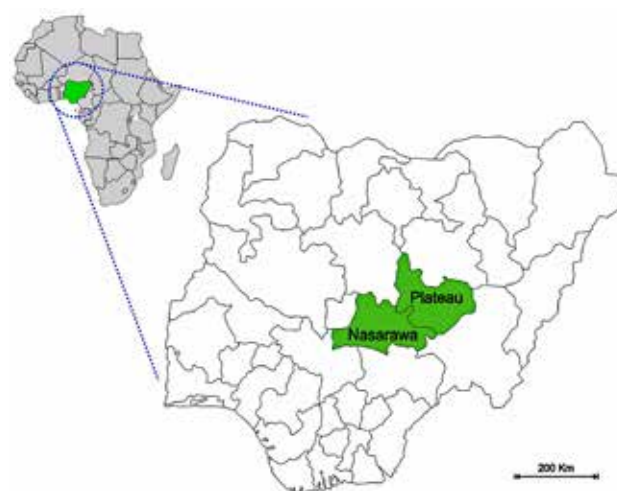


Fig. 1. Map of Nigeria showing the study areas (shaded)

### Study design and blood sample collection

Samples analysed in this study were obtained from two sources. The first group consisted of anticoagulated blood samples from cattle in Plateau and Nasarawa states, submitted to the Parasitology Laboratory, NVRI Vom, for parasitological analyses. The second group consisted of blood samples collected from cattle slaughtered in the main abattoirs in Jos and Lafia; the capital cities of Plateau and Nasarawa states, respectively. In the abattoirs, the samples were collected at random from the jugular vein before slaughter over a period of two months (May–June, 2021). Approximately 3 ml of blood samples were transferred into ethylene diamine tetraacetic acid (EDTA) tubes, labelled and kept on ice before they were transported to the Parasitology laboratory NVRI Vom and processed accordingly.

The sex, age and breed of the animals were recorded before the collection of blood samples.

### Determination of the packed cell volume (PCV) and microhaematocrit centrifugation technique (mHCT)

The presence of motile parasites in the blood was determined using the mHCT according to standard procedures [32]. Briefly, for every blood sample, a microhaematocrit capillary tube was filled to 75 % and stoppered with a sealant. After centrifugation at 12,000 rpm for 5 min, the PCV was recorded. The tubes were placed on a grease free microscope slide and examined under the microscope at 100× magnification with low light intensity for the presence of motile parasites at the level of the buffy coat.

### DNA extraction

DNA extraction was achieved using a commercially available kit; Quick-DNATM MiniPrep (ZymoResearch). Approximately 150 µl blood was subjected to genomic DNA extraction according to the manufacturer's instructions. DNA was eluted in 50 µl and stored at –20 °C until use.

### Amplification of *Trypanosoma* species DNA using conventional PCR

All the DNA samples were initially screened for the presence of the DNA of pathogenic trypanosomes. The initial PCR was conducted using the internal transcribed spacer one (ITS1) ribosomal DNA (rDNA) based primers reported to efficiently detect the DNA of all pathogenic trypanosomes at different base pairs in a single reaction. Amplification was performed in 25 µl reaction volumes, containing 12.5 µl of 2X PCR Mastermix with standard buffer (New England Biolabs Inc), 0.5 µl of each primer (10 mM), 5 µl of template DNA and 6.5 µl of nuclease-free water (BioConcept, Switzerland). Amplification involved 30 s at 94 °C initial denaturation followed by 40 cycles of 1 min at 94 °C, 1 min at 60 °C, and 45 s at 68 °C followed by a final elongation at 68 °C for 7 min. The conventional PCR was conducted on a GenAMP 7400 (Applied Biosystems, Foster City, CA). Positive samples were further amplified using species-specific primers to differentiate the presence of different trypanosomes. The various primers and reaction conditions used in the study are listed in Table 1.

**Table 1. Primers and reaction conditions for the detection of *Trypanosoma* spp. in cattle in north-central Nigeria**

Code	Primer sequence (5'-3')	Annealing Temperature [°C]	Amplicon length [bp]	Reference
TITS1F	CCGGAAGTTCACCGATATTG	60	250–710	[24]
TITS1R	TTGCTGCGTTCTTCAACGAA			
TCFF	GGACACGCCAGAAGGTACTT	55	350	[18]
TCFR	GTTCTCGCACCAATCCAAC			
TCSF	CGAGCGAGAACGGGCAC	55	316	[18]
TCSR	GGGACAAACAATCCCGC			
TCKF	GTGCCCAATTGAAGTGAT	55	294	[19]
TCKR	ACTCAAAATCGTGACCTCG			

Bp—base pairs.

The PCR products were electrophoresed in a 1.5% agarose gel stained with SafeView™ Classic (Applied Biological Materials, Richmond, BC, Canada) and were visualized under a Blue light Transilluminator (Cleaver Scientific, UK).

Positive amplicons were sequenced at a commercial facility (Inqaba Biotech West Africa Ltd, Ibadan, Nigeria) using the PCR primers. Sequences were edited manually using the Bioedit Software [10]. The nucleotide sequences were checked using a BLASTn search hosted by the National Centre for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for the comparison with other known nucleotide sequences in the GenBank. Sequences generated in this study were deposited in the GenBank under the accession numbers ON139612–ON139618.

### Phylogenetic Analysis

The phylogenetic tree was inferred using the Neighbor-Joining [26], and the p-distance method [23]. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [7]. The evolutionary analyses were conducted in MEGA11 [30].

### Data analysis

The data generated in this study were recorded in Microsoft excel and analyzed using descriptive statistics. Furthermore, the Odds ratio was computed to determine the association between the prevalence of trypanosomes

and samples collected from abattoirs and those submitted from farms using the MedCalc® Statistical Software version 20.015 [20]. The effects of trypanosomes on the PCV was determined using the student t-test. P-values <0.05 were considered significant.

## RESULTS

A total of 130 cattle were examined in this study. This included 80 (61.5%, CI 50.8–68.7%) from Plateau state and 50 (38.5%, CI 31.3–49.1%) from Nasarawa state. Seventy-eight (67.7%, CI 61.6–78.2%) of these samples were sourced from abattoirs while 42 (32.3%, CI 21.8–38.4%) were samples submitted to our laboratory from cattle herds for parasitological analyses (Table 2). In Plateau state, a higher prevalence of *T. vivax* was recorded in samples sourced from the abattoir than in samples submitted from the field (16.7% versus 11.5%). However, the reverse was the case in Nasarawa state (2.9% vs. 37.5%). The difference in prevalence of *T. vivax* between the abattoir and field samples was significant (P=0.009) in Nasarawa state, but not in Plateau state (P=0.55). Near equal prevalence (15.0% vs. 14.0%) of *T. vivax* DNA was recorded in samples from Plateau and Nasarawa states (Table 2). Overall, the DNA of *T. vivax* was detected in 19 out of the 130 (14.6%) of samples examined by PCR. However, using the mHCT, motile haemoparasites were detected in only six (4.6%, CI 0.5–6.9%) of the samples.

**Table 2. Prevalence of *Trypanosoma vivax* in cattle in north-central Nigeria**

Sample location	Source of sample	Number positive/ No. tested [%]	OR	95 % CI	Z	P
Plateau	Abattoir	9/54 (16.7)	1.53	0.38–6.22	0.60	0.55
	Farms	3/26 (11.5)				
Total		12/80 (15.0)				
Nasarawa	Abattoir	1/34 (2.9)	0.05	0.005–0.47	2.62	0.009
	Farms	6/16 (37.5)				
Total		7/50 (14.0)				
Overall	Abattoir	10/88 (11.4)	0.47	0.18–1.26	1.50	0.13
	Farms	9/42 (21.4)				

OR—Odds ratio; Z—Z-statistics; CI—confidence interval; P—Significance level

Table 3. Detection of trypanosomes by mHCT and PCR in cattle in Nigeria

		PCR		
		Positive	Negative	Total
mHCT	Positive	5	1	6
	Negative	14	110	124
	Total	19	111	130

Table 4. Effects of *Trypanosoma* spp. infection on PCV of cattle in Nigeria

Sample location	Infection status	Mean PCV (%) $\pm$ SD (Normal value = 25–46)	95 % CI	T	P
Plateau	Non-infected	33.1 $\pm$ 6.6	0.07–0.23	0.2	0.42
	Infected	32.6 $\pm$ 7.5	0.77–0.93		
Nasarawa	Non-infected	30.2 $\pm$ 10.9	0.73–0.94	0.35	0.36
	Infected	29.0 $\pm$ 5	0.06–0.27		
Overall	Non-infected	31.65 $\pm$ 9.0	0.79–0.91	0.56	0.29
	Infected	31.41 $\pm$ 6.2	0.08–0.21		

T—T-test; P—significance level; PCV—packed cell volume; SD—standard deviation

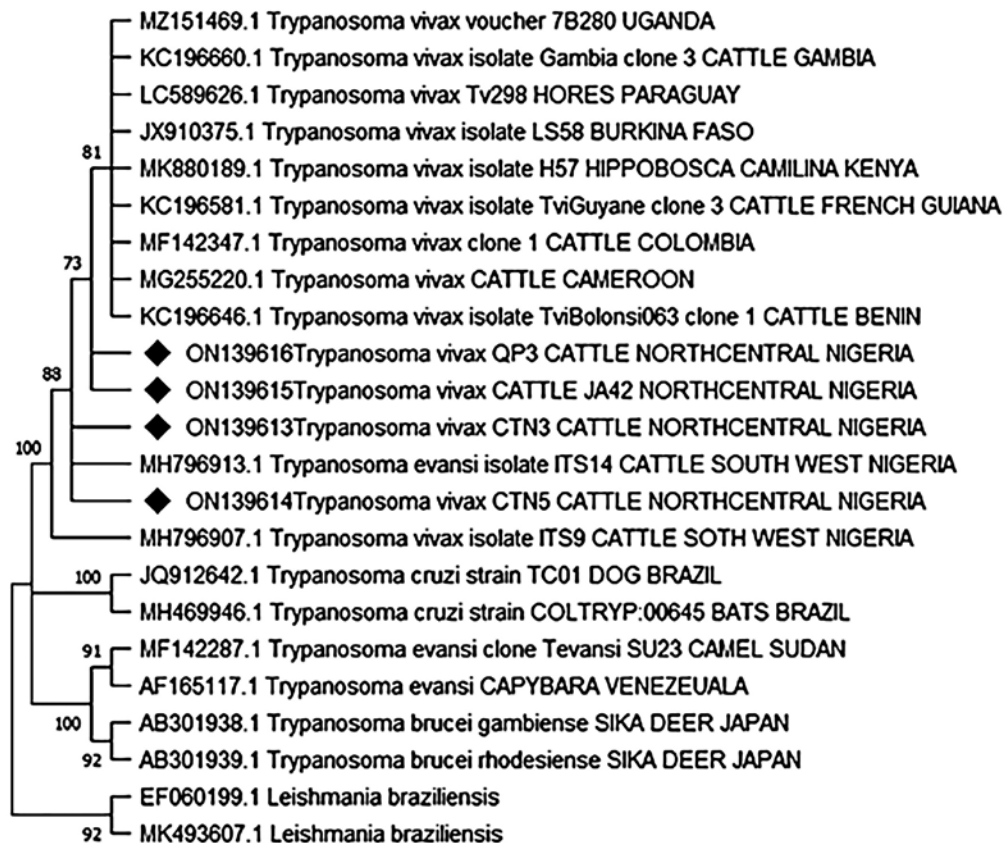


Fig. 2. Neighbor-Joining phylogenetic tree showing the relationship of sequences of *T. vivax* obtained in the study (black rhombus) and other sequences in the GenBank. Countries and host species where sequences were obtained are indicated. Percentage of 1000 replicate are shown next to the branches. *Leishmania braziliensis* (EF060199 and MK493607) were used as outgroups

All the samples positive in the mHCT except one were positive for the DNA of *T. vivax* in the PCR test (Table 3). Similarly, all the mHCT positive samples were from those submitted from farms for the parasitological analyses. Generally, the mean PCV of trypanosome infected animals was lower than that of non-infected animals, but the difference was not significant ( $T=0.56$ ;  $P=0.29$ ) (Table 4). None of the positive samples were amplified using any of the *Trypanosoma congolense* species-specific primers used in this study.

The nucleotide sequences generated in this study were 100% identical to each other and 98.5–100% identical to *T. vivax* sequences in the GenBank. The *T. vivax* sequences from this study and formed a monophyletic cluster with sequences of *T. vivax* from different countries in the GenBank (Fig 2).

## DISCUSSION

The primer pair ITS1 F and ITS1 R used in this study is reputed to amplify the ITS1 region of all pathogenic trypanosome species, producing PCR products with species-specific sizes and thus enabling the multiplex detection of different parasite species [24]. Amplification of an approximately 250bp was achieved in 19 out of the 130 samples (14.6%) screened in this study, suggesting that the amplified DNA belonged to *T. vivax*. The analysis of the ITS1 nucleotide sequences obtained in this study showed 99–100% identity to sequences of *T. vivax* in the GenBank. Thus, confirming *T. vivax* as the trypanosome species infecting the cattle examined in this study. This is not surprising as *T. vivax* can be transmitted both biologically and mechanically and is reputed as the most widespread species infecting cattle in Nigeria.

The prevalence of 14.6% *Trypanosoma* spp. DNA in samples from the Plateau state in this study by PCR is higher than the earlier reports of 7.8% and 5.1% by classical methods [4, 14], but lower than the 46.8% by the PCR [17]. However, the prevalence of 14.0% recorded for samples from Nasarawa state in this study was lower than the 53.3% previously reported for abattoirs samples from the same location [12]. These differences may be attributed to the variation in the study design, sample size, and importantly, the diagnostic methods employed in each of the studies. It is evident even from our report that the PCR was three times

better at detecting trypanosomes than the mHCT. Another factor, that may account for differences in the results is that the method of preservation and the duration before testing. The integrity of samples may be compromised with poor preservation over prolonged periods. We had higher detection rates in samples submitted from the field than samples from abattoirs, being that samples from the field were collected and transported to the laboratory and analysed within a short time period (within 24 hours). This was not the case with most of the samples collected in the abattoirs, especially those from Nasarawa state, where they were preserved for 48–72 hours before they were transported to the laboratory for analysis. Our finding of the DNA of *T. vivax* only in cattle in this study is at variance with previous reports where in addition to *T. vivax*, *T. congolense*, and *T. brucei* were also reported in the study area [4, 12, 14, 17]. Although, most of the results were based on morphological identification of the trypanosomes, which is highly subjective, one of the studies was based on PCR and sequencing [17]. Although, the presence of *T. congolense* and *T. brucei* infection in cattle in Plateau state had been earlier reported, the relatively small sample size and the short sampling period (3 months) in this study might have limited our scope of trypanosome species detection. Seasonal variation in trypanosomes prevalence, with the lowest prevalence in the early wet season which coincided with our sampling period have been reported in Plateau state [17]. Drivers of livestock migration on the Jos Plateau have been identified to include: lack of water, pasture, lack of access to grazing land or water and avoidance of tsetse flies [16]. It should be noted, however, that seasonal variation in trypanosome prevalence does not always follow a clear cut pattern. It would appear that multiple factors, such as: management practice, veterinary care, nutrition and inter-current diseases superimpose and alters known epidemiological factors [21]. Another worrisome dimension is the prevailing unsupervised veterinary practice in Nigeria. It has been observed that herdsmen administer drugs to their animals without adequate supervision, therefore, the total curative effects may not be achieved. Hence, a chronic carrier state may be the norm rather than the exception. This may explain the deviation from the predictive seasonal prevalence of trypanosomes [1, 31]. Therefore, it will be interesting to also obtain information on the use of trypanocide among herdsmen during survey studies to evaluate perception, handling, administration and efficacy of the treatments.

We observed that there was no significant association between trypanosome infection and PCV of infected cattle compared to those that were not infected. This is in accord with the report of Birhanu et al. [2] in Ethiopia but at variance with the reports from Nigeria [17, 29]. Pastoralist cattle in Nigeria are usually infected with several haemo-parasites in a state of endemic stability and therefore may not manifest severe haemolytic signs as in naïve or exotic breeds [9]. The majority of the samples examined in this study were obtained from animals in the abattoir. Under the traditional cattle production system in Nigeria, cattle ownership is a family prestige and status symbol, hence, sales of animals are highly restricted. Whenever it becomes necessary, aged cows or those in poor health condition are the prime candidates for sale. This may explain the low PCV value in most of the animals examined in this study regardless of their trypanosome infection status. The *T. vivax* nucleotide sequences generated in this study were 100% identical to each other and 98.5–100% identical to sequences in the GenBank suggesting as evolution of this species.

## CONCLUSIONS

In conclusion, only *T. vivax* DNA was found in blood samples of cattle from Plateau and Nasarawa states in Nigeria. The persistence of this parasite in areas at the fringe or outside the known tsetse fly distribution zone is attributed to its ability to be transmitted by mechanical and biological routes. Recent events in Nigeria such as insurgency and conflicts between farmers and herders over grazing land have led to displacement of many livestock farmers consequently altering the epidemiology of AAT. More studies are needed to determine the nationwide prevalence, species distribution and vector and reservoirs of AAT in Nigeria. This will form an essential first step in the design of cost effective control measures for the disease.

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## CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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## HEREDITARY EYE DISEASES IN GERMAN SHEPHERD DOG

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### ABSTRACT

**Hereditary eye diseases occur to varying degrees in all dog breeds. Individual purebred breeds have specific predispositions to various eye disorders. The German Shepherd is diagnosed mainly with chronic superficial keratitis/pannus, but also with: distichiasis, plasmoma/atypical pannus, corneal dystrophy, persistent pupillary membranes, cataract, cone degeneration, retinal dysplasia, optic nerve hypoplasia/micropapilla, and limbal melanoma. Individual ocular abnormalities are manifested by characteristic clinical manifestations and ophthalmological findings. Some eye diseases can lead to blindness, others affect the comfort of life or work use of the dog to varying degrees. A thorough knowledge of individual ocular pathologies in a particular breed leads not only to the identification of the diagnosis but also to the correct assessment of the dog's breeding usability.**

**Key words: eye diseases; German Shepherd dog; hereditary**

### INTRODUCTION

The German Shepherd is a working dog that was initially developed for guarding and herding sheep [29]. It

is classed under the number 166 in the FCI (Federation Cynologique Internationale) Group 1—Sheepdogs and Cattle Dogs [13]. The German Shepherd Dog is a relatively new breed of purebred dogs that only dates back to 1899. It is the product of Captain Max vom Stephanitz's (1864—1936) vision of creating the perfect working dog that would possess the following essential qualities: intelligence, ability, a weatherproof coat and beauty [29].

Not only nature but also health is important for its usability. The breed is predisposed to several diseases, including eye diseases. Visual disturbances can be caused by a variety of causes. A specific group consists of hereditary eye diseases (HED).

The inherited forms of eye diseases are arguably the best described and best characterized of all inherited diseases in the dog. A principal reason is that the eye is very accessible, and much of it can be examined in detail using non-invasive techniques, making it relatively easy to detect abnormalities, even if they do not impair vision significantly [25]. The HED group involves disorders present at birth (e.g., microphthalmia), but also those appearing in the adult age (e.g., hereditary cataracts, and late-onset progressive retinal atrophy). In addition, these HEDs include sluggish diseases with minimum influence on vision (e.g., a mild form of a persistent pupillary membrane, and multifocal retinal dysplasia); progressive ones seriously impairing vision (e.g., corneal endothelium dystrophy,



and progressive retinal atrophy); polygenic diseases (e. g., entropion, and ectropion); and those transmissible according to simple Mendelian rules (with autosomal recessive/dominant trait) inherited by siblings at high frequency (e. g., prcd (Progressive Rod-Cone Degeneration) form of progressive retinal atrophy) [1, 27, 33, 35, 36, 37].

The Genetics Committee of the American College of Veterinary Ophthalmologists maintains a database of eye diseases in dogs. It provides an up-to-date overview of the prevalence of individual eye abnormalities in specific purebred breeds. According to the database, the most common eye diseases found in German Shepherd Dogs are: distichiasis, plasmoma/atypical pannus, corneal dystrophy, chronic superficial keratitis/pannus, persistent pupillary membranes, cataract, cone degeneration, retinal dysplasia, optic nerve hypoplasia/micropapilla and limbal melanoma [2]. There is also a reference that German Shepherds could be prone to lens luxation when the incidence is found in family lines [21, 26].

### **Distichiasis**

Distichiasis refers to single or multiple hairs arising from the free lid margin. They usually arise singly or with two or more hairs from the Meibomian duct openings. Distichiasis is considered to be inherited, but the exact mode of transmission is unknown [34]. However, the use of the dog in breeding is possible [2].

The clinical signs of cilia disorders are: blepharospasm, epiphora, conjunctival hyperaemia, and corneal ulceration [3]. The treatment for canine distichiasis consists of either temporary removal of the offending distichia by manual epilation or the permanent destruction of the distichia follicle by electroepilation, cryoepilation, or other various surgical procedures [14].

### **Plasmoma/atypical pannus**

Plasma cell infiltration of the nictitating membrane or plasmoma can cause thickening, depigmentation, and follicle formation. German shepherds appear to be predisposed [19]. Patchy depigmentation and nodular thickening of the anterior surface of the third eyelid, usually near the margin, is commonly seen in association with the keratitis or rarely occurs without concurrent corneal lesions. In the latter instance, this syndrome has been called plasmoma, but it is important to realize that this is not a neoplastic condition [24]. German Shepherds appear to be predis-

posed to this condition [19]. The inheritance of plasmoma is not defined, and the use of a dog with plasmoma is not recommended in breeding [2]. Treatment generally consists of topical ophthalmic dexamethasone four times daily, initially, or subconjunctival or systemic corticosteroids. Topical ophthalmic cyclosporine ointment, tacrolimus drops or 1 % pimecrolimus administered 2—3 times daily are also effective [19].

### **Corneal dystrophy**

Corneal dystrophy is any primary, bilateral, inherited disorder of the cornea not accompanied by corneal inflammation or systemic disease. Most corneal dystrophies in the dog appear clinically as grey-white or silver, crystalline or metallic opacities in the central or paracentral cornea. The condition is bilateral and often appears as nearly symmetric lesions. Corneal dystrophy may affect the corneal epithelium, stroma or endothelium [4, 5, 6, 7, 38].

In the years 1991—2015, corneal dystrophy capture rate in the German Shepherds was 4.6% and in the years 2015—2020 it was up to 4.5%. The mode of inheritance is not defined; however, the breeding usability of a dog with this diagnosis is possible [2]. In general, corneal dystrophies do not respond to medical treatment. The corneal lesions can be removed by keratectomy if the opacity is obstructing vision significantly. However, it is possible that the opacities will recur after keratectomy. Therefore, surgery is recommended for corneal dystrophy only as a last resort measure in dogs with significant visual deficit [38].

### **Chronic superficial keratitis/pannus**

The most frequent ocular disease in German Shepherds is chronic superficial keratitis (CSK) [17]. It is a progressive, bilateral, inflammatory, and potentially blinding disease of the canine cornea. It is also known as: German Shepard pannus, Überreiter's syndrome, and degenerative pannus. Clinically, CSK is manifested initially at the temporal or inferior temporal limbus as a red, vascularized, conjunctival lesion. Early in the disease, vascularization and pigmentation occur at the temporal cornea and progress centrally (Fig. 1). As the disease progresses, it spreads as a fleshy, well-vascularized lesion that migrates toward the central cornea (Fig. 2) [38].

The German Shepherd Dog has a higher incidence of pannus than any other breed. The MHC (Major Histocompatibility Complex) class II risk haplotype has been



**Fig. 1. Chronic superficial keratitis/pannus in a 5-year-old female German Shepherd** (Source: Pavol Zubrický)



**Fig. 2. Chronic superficial keratitis/pannus in a 3-year-old male German Shepherd** (Source: Pavol Zubrický)

shown. Although there are likely several other genes and environmental factors that contribute to CSK, a recent paper suggested that MHC class II is a major genetic risk factor. It is not recommended to use a dog with pannus in breeding [2].

Pannus cannot be cured. The therapeutic goal should be control and some-times regression of the lesions so that blindness can be avoided. Treatment consists of topical application of a potent and penetrating corticosteroid eyedrop [24]. The prognosis for achieving disease control and preserving vision may be poorer in those animals that show rapid onset of disease in early adulthood [10].

### **Persistent pupillary membranes**

The persistent pupillary membrane (PPM) is a remnant of foetal mesodermal tissue that is responsible for providing nutrition to the forming lens during embryonic development. In dogs, it usually atrophies within six weeks of life. Clinical symptoms can be extremely variable—from insignificant clinical conditions to visual disturbances or the development of cataracts at an older age [40]. In general, small remnants spanning from one portion of the iris to another (iris-to-iris persistent pupillary membranes—PPMs) sometimes cross the pupil, but they have no discernible visual consequences. A visual impairment may occur, however if the strand contacts the cornea (iris-to-cornea PPMs) or lens (iris-to-lens PPMs) it may create an opacity within the visual axis [28]. A therapy is rarely necessary for PPMs [20]. The most commonly diagnosed PPMs in German Shepherds are iris-to-iris PPMs. In this form, heredity is not yet defined. The breeding use of a dog with iris-to-iris PPMs is a breeder option [2].

### **Cataracts**

A cataract is a partial or complete opacity of the lens and/or its capsule. In cases where cataracts are complete and affect both eyes, blindness results [2]. Cataracts are divided according to aetiology into primary and secondary. Primary cataracts: all bilateral or unilateral cataracts and especially cortical cataracts are known or presumed hereditary eye diseases (KP-HED). Secondary cataracts: cataracts known to be caused by physical influences (trauma, electric, irradiation), ocular inflammation, metabolic diseases, nutritional deficiencies, age, intoxication or another KP-HED. We recognize cataracts congenital and non-congenital. A congenital cataract is considered a cataract, if it is diagnosed by the age of 8 weeks of age, or if it is diagnosed later in life, but there is a distinct indication whether the cataract is congenital in origin (e.g., in microphthalmos, adjacent to PPM, or persistent hyaloid artery). Non-congenital cataracts are divided according to the location to cortical cataracts, posterior polar cataracts, nuclear cataracts and other lens opacities [32]. The treatment of cataracts is clearly a surgical condition in dogs. There are many different surgical procedures to remove lenses and cataracts in veterinary ophthalmology. Phacoemulsification cataract surgery is the most common type of cataract surgery performed in all species of animals performed by veterinary ophthalmologists world-wide [15].

The most commonly diagnosed cataract in the German Shepherd is a cortical cataract with presumed autosomal recessive inheritance. Affected individuals are not recommended for use in breeding [2].

### Cone degeneration

Cone degeneration is an autosomal recessively inherited early degeneration of the cone photoreceptors. Afflicted puppies develop day-blindness and colour blindness, but they remain ophthalmoscopically normal their entire life. Electroretinography is required to definitively diagnose the disorder [2]. Inheritance is autosomal recessive [12], affected dogs are not recommended for breeding [2]. The predominant causes of achromatopsia are mutations of the CNGA3 gene [22].

### Retinal dysplasia

The term retinal dysplasia embraces several congenital/neonatal conditions resulting from disorderly proliferation and atypical differentiation of the retina during embryonic life. In addition to genetically determined hereditary retinal dysplasia, a wide variety of extraneous insults (for example, infectious agents such as canine herpes virus and irradiation) to the developing retina may cause acquired, non-inherited, retinal dysplasia [5].

Retinal folds are linear, triangular, curved or curvilinear foci of retinal folding that may be single or multiple. When seen in puppies, this condition may partially or completely resolve with maturity. Its significance to vision is unknown. There are two other forms of retinal dysplasia (geographic, and detached) which are known to be inherited in other breeds and, in their most severe form, cause blindness. The breeding use of a dog with retinal folds is possible. The genetic relationship between folds and more severe form of retinal dysplasia is undetermined [2].

### Optic nerve hypoplasia/Micropapilla

Optic nerve hypoplasia is a congenital defect of the optic nerve which causes blindness and abnormal pupil response in the affected eye [2], and it is usually diagnosed early in life. There is no therapy [18]. Affected dogs must not be used in breeding [2]. Although most cases happen to be sporadic isolates in families, it is now clear that many cases are caused by mutations in genes involved in eye development. [8]. Micropapilla refers to a small optic disc which is not associated with vision impairment. Inheritance is not defined and the usability of an individual is the breeder's option [2].

### Limbal melanoma

Epibulbar or limbal melanocytic neoplasms appear

clinically as darkly pigmented masses arising from the limbus and expanding into the adjacent cornea and sclera or as masses arising posterior to the limbus and expanding into the adjacent sclera [9], [23]. Rare histologically malignant limbal melanomas have been described, and some otherwise benign neoplasms may include areas with cells that are less pigmented or amelanotic and mitotically active [9, 11, 30, 31, 39]. Surgical treatment of limbal and scleral diseases are usually directed toward the removal of neoplastic and inflammatory masses. The German Shepherd breed appears most frequently affected [16]. Dogs with limbal melanoma should not be included in the breeding [2]. The inheritance of limbal melanoma has not been defined [12].

## CONCLUSIONS

The German Shepherd is predisposed to several inherited eye diseases affecting different eye structures. As the heredity of most of them have not been yet precisely defined, they can currently be identified on the basis of an ophthalmological finding. Therefore, it is recommended to perform a preventive clinical examination of the eyes before placing the dog in the breed.

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## INTERVERTEBRAL DISC DISEASE IN DOGS – THE RELATIONSHIP BETWEEN RECOVERY AND TIMING OF SURGERY

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### ABSTRACT

The aim of this study was to evaluate the relationship between functional recovery and timing of surgery in dogs diagnosed with intervertebral disc disease treated surgically. Intervertebral disc disease is the most common spinal disease in dogs; it plays a significant role in the scientific field by its high prevalence. There is also an existing hypothesis that the faster the surgery is performed, the better the outcome will be. The data were collected during two years at one institution. The patients were neurologically assessed using the modified Frankel score when they were first diagnosed with intervertebral disc disease at the clinic and later after the surgery and during the following weeks. A total of 36 dogs were included in this study, represented by 13 different dog breeds and crossbreeds. In total 17 were females and 19 males. The mean age of the patients was  $6.9 \pm 2.97$  years (range 2—15 years) and the mean body condition score was 3.5 within a scale of 1—5. Out of the 36 evaluated dogs, surgery was performed on 25 of them. The mean time of duration of clinical signs before surgery was  $9 \pm 13$  days. The main limitation of this study was the small group of investigated dogs. Although the study was based on a small number of participants, the findings suggested that the

timing of the surgery and recovery had a positive Pearson correlation coefficient of 0.39; implying that the timing of the surgery may have affected the recovery.

**Key words:** dog; herniation; intervertebral disc; recovery; surgery; timing

### INTRODUCTION

Intervertebral disc disease (IVDD) is the most common spinal disease in dogs, and the surgery performed for IVDD is the most common spinal surgery in veterinary medicine. IVDD is a degenerative and age-related condition, but there is a certain predisposition in chondrodystrophic breeds, which can experience this condition also as young adults. Chondrodystrophic breeds are characterized by long bodies and disproportionally short and often curved legs; examples of these are: Dachshund, Basset Hound, Welsh Corgi, Shi Tzu, Beagle, Cavalier King Charles Spaniel, French and English Bulldog, Miniature Schnauzer, Lhasa Apso, Pekingese, American Cocker Spaniel and Tibetan Spaniel [14]. In these breeds, the IVDD mainly occurs in the cervical and thoracolumbar spinal segments, and typically around the age of 3—7 years [10, 14].

Intervertebral disc degeneration results in reduced

shock-absorbing capacity and it can thereby lead to intervertebral disc herniation, spinal cord compression and damage to the associated nerves [5]. The chondrodystrophic breeds are predisposed through abnormal maturation of the intervertebral disc, which causes premature dehydration and mineralization of the *nucleus pulposus*. When the *nucleus pulposus* is mineralized this can cause splitting of the *anulus fibrosus* and result in acute herniation of the disc material into the spinal canal [10].

The veterinary pathologist Hans-Jürgen Hansen first classified IVDD as type I and type II in 1951. This classification was mainly based on the type of intervertebral disc herniation (IVDH) and the types of breeds affected [3, 4]. It was assumed that the chondrodystrophic (CD) dogs were associated with Hansen I and suffered from chondroid degeneration, while fibroid degeneration associated with Hansen II occurred in the non-chondrodystrophic (NCD) dogs. A recent study from 2017, compared the histopathology of the degenerated intervertebral disc in NCD and CD dogs, and found that the histopathological changes were similar in both categories and appeared as chondroid metaplasia [4].

Dogs suffering from IVDD can experience varying neurological clinical signs, mostly with acute onset in the cases of Hansen type I or less often they can develop progressively in some cases of Hansen type II. These clinical signs include neck and/or back pain and varieties of neurological dysfunctions such as paresis or paralysis in one or more limbs, and/or the tail. This can present as a: loss of balance, co-ordination, ability to walk and in some cases, a loss of control of defecation and urination [3]. Indications for surgical treatment of IVDD include severe or progressive neurological deficits, irreversible pain and failure of previous non-surgical treatment [13]. It remains controversial which prognostic factors that are best to use for evaluation of the prognosis. These factors commonly include: duration of clinical signs, speed of onset, and the presence of deep pain perception [1, 8, 12, 15]. As an example, the study done by Ferreira et al. [2] involving 71 dogs, showed that the duration of the clinical signs seemed less significant for the clinical outcome for the patients but did affect the length of recovery time. It also showed that the rate of onset of the clinical signs significantly influences the outcome.

Our study was focused on a very important aspect of the surgical therapy, which is timing of the surgical de-

compression. When the period of time from the development of the clinical signs until the surgery is prolonged, it can have a negative effect on the outcome [8]. This is unluckily influenced by the approach of the owners, when they wait sometimes for a very long period until the dog is presented to the veterinarian. The goal of this study was to investigate how the timing of the surgery affects the outcome and prognosis of the patients.

## MATERIALS AND METHODS

The data used in this study were collected from patients' records of dogs with a confirmed diagnosis of IVDD during a two year period. The inclusion criteria were: age, gender, breed, body condition score, body weight, modified Frankel score prior to surgery, duration of clinical signs prior to surgery, diagnostic imaging, type of surgery, outcome by means of the modified Frankel score recorded during observational period 6 months after surgery.

The modified Frankel score (MFS) is a neurological scoring system used to assess the severity and prognosis for spinal cord injury. It ranges from the favourable MFS grade 1 to the more severe neurological grade of MFS grade 5 (Table 1). Grade 1 is characterized by spinal hyperesthesia and no neurological dysfunction, grade 2 by ambulatory paraparesis, grade 3 by non-ambulatory paraparesis, grade 4 where the patient is paraplegic with intact pain perception in either pelvic limb and/or tail and lastly grade 5 with the paraplegic patients with absent deep pain perception in both pelvic limbs and tail [8].

The obtained data were expressed as Min-Max values and Mean  $\pm$  SD. The Pearson correlation coefficient was

**Table 1. Modified Frankel Score used for neurological scoring of the dysfunction**

Modified Frankel Score	Neurological grade
<b>Grade 1</b>	Spinal hyperaesthesia, no neurological dysfunction
<b>Grade 2</b>	Ambulatory paraparesis
<b>Grade 3</b>	Non-ambulatory paraparesis
<b>Grade 4</b>	Paraplegic, with intact pain perception in either pelvic limb and/or tail
<b>Grade 5</b>	Paraplegic, with absent pain perception in both pelvic limb and/or tail

**Table 2. Breed distribution of the 36 dogs with neurological dysfunction**

Breed	Number of affected dogs
Cocker Spaniel	1
Dogo Argentino	1
German Shepherd	1
Great Swiss Mountain dog	1
Poodle	1
Short Hair Collie	1
Doberman	1
Lhasa Apso	1
Slovak Hound	2
Dachshund	3
Yorkshire Terrier	3
Maltese	4
Crossbreed	5
French Bulldog	11

**Table 3. Age distribution of the 36 dogs with neurological dysfunction at the time of the diagnosis**

Age in years	Number of affected dogs
2	2
3	5
4	2
5	4
6	8
7	4
8	0
9	4
10	2
11	3
12	1
13	0
14	0
15	1

used to express correlation between the timing of the surgery and the outcome, as well as the size of the patients and outcome. The timing of surgery was expressed in days while the size categories were divided according to the individual body weight in kilograms.

## RESULTS

A total of 36 dogs were included in this study. The patients were represented by 13 different dog breeds in addition to a few crossbreeds. Breed distribution of the 36 dogs with neurological dysfunction are presented in Table 2.

In total, 17 of the dogs were females (10 castrated, 7 intact) and 19 males (5 castrated, 14 intact). The age of the patients ranged from 2 to 15 years (Table 3) with the mean age  $6.9 \pm 2.97$  years and a mean body condition score (BCS) of 3.5 within a 5-degree scale. By means of the course of the disease, 15 dogs (41.6%) were presented with acute onset of the clinical signs, while the remaining 21 dogs (58.3%) were characterized with a chronic course.

Out of the 36 dogs, nine dogs (3%) were presented with grade 2 MFS, 13 dogs (36%) were presented with grade 3 MFS, seven dogs (19.5%) were presented with grade 4 MFS, and seven dogs with grade 5 MFS.

Most of the dogs were diagnosed with myelography (25 dogs, 69.4%), the remaining five dogs with MRI (Magnetic Resonance Imaging) (13.8%) and three dogs with x-rays (8.3%). Three of the dogs were euthanized before any attempt to diagnosis at the owners request; these were paralyzed chondrodystrophic dogs with possible intervertebral disc disease.

Out of the 33 dogs that were diagnosed with imaging diagnostics, 8 dogs (24.2%) had the lesion located in the cervical segments, 16 dogs (48.5%) were affected in the thoracic segments, 8 dogs (24.2%) in the thoracolumbar segments and 1 dog (3%) in the lumbosacral segment.

Localization of lesions in acute and chronic patients is shown in Table 4.

Surgical intervention was performed on 25 of the dogs. The remaining 3 dogs were euthanized, 3 dogs ended up with the wheelchair as a solution and 2 dogs had owners that declined surgery with no further information about the dogs. Three dogs were operated within a day, 5 dogs after 2 days, 4 dogs after 3 days, 8 after 4—10 days and the remaining 5 dogs 14 days or more after the first onset of clinical signs.

The mean MFS at the time of diagnosis put most of the patients in grade 3, non-ambulatory paraparesis. Already one day post-surgery the mean MFS was below 3, placing



**Table 4. Localization of lesions in acute and chronic patients**

Localisation of lesions	Total	Acute	Chronic
Cervical	8	2	6
Thoracic	16	9	7
Thoracolumbar	8	2	6
Lumbosacral	1	0	1

the patients in grade 2, ambulatory paraparesis. Two weeks post-surgery the mean MFS of treated dogs dropped below 2 and improved even closer to 1 at 6 months post-surgery. Patients with MFS grade 1 were characterized by spinal hyperesthesia and no neurological dysfunction, which was characteristic for all dogs at 6 months follow up.

The outcome of the surgery according to the size of the dog shows that all sizes improved to a MFS of grade 1 within 6 months after the surgery. The medium sized breeds of dogs had the best total improvement of 2.0 MFS after 6 months. This group of patients also showed the fastest recovery, except at 1 week post-surgery where large breeds had the best MFS improvement, followed by the small breeds. Based on the MFS score recorded 6 months post-surgery and the size of the breed this gave a negative Pearson correlation coefficient of  $-0.87$ ; meaning that the bigger dogs had the lower MFS and thereby the better outcome at 6 months post-surgery.

According to the localization of the lesion, the patients with a thoracic localization showed the best improvement at 1 day and 1 week post-surgery, and they also had the best total improvement from the time of diagnosis until 6 months post-surgery. The dogs with thoracolumbar lesions started off with the highest and thereby more severe MFS grade at the time of diagnosis, with 1.67 MFS improvement they still ended with the highest average MFS grade (1.83) at 6 months post-surgery, placing them closer to grade 2 when the other two categories improved to grade 1 at the end of the follow-up period.

## DISCUSSION

The chondrodystrophic breeds of dogs have a certain predisposition to IVDD, usually occurring in animals from

3—7 years of age and involving mostly the cervical and thoracolumbar region [10, 11, 14]. The findings in our study showed a mean age of  $6.9 \pm 2.97$  (2—15) years, placing it in the upper limit.

There were 13 different breeds represented in this study, 6 of these breeds were considered chondrodystrophic, 23 of the 36 (63.9%) diagnosed dogs. The French bulldog accounted for 30% of all diagnosed dogs, while it was characterized as chondrodystrophic this result may also be explained by the high popularity of this breed. The American Kennel Club reported this breed as the 3rd most popular dog breed in Europe in 2018. The Highland Canine breed ranked as the 7th most popular dog breed in the world by January 2022, based on google searches.

According to Nelson and Couto [9], most of disc extrusions occurred in the caudal thoracic or lumbar spine. Lemarié et al. [7] reported that 15% of the reported canine IVDD cases have cervical localization. In acute thoracolumbar disc extrusions, 65% are localized between Th11/12, for cervical discs, the C2/3 intervertebral disc was most frequently affected [9]. Our study included 33 patients where the exact localization of the lesion was determined through the imaging diagnostics. The thoracic lesions were most common, accounting for 16 of the patients, 9 of these with acute onset. We found the exact same number of patients with cervical as with the thoracolumbar lesions, representing 8 patients each, both with 6 chronic and 2 acute onsets. Only one patient was presented with a lumbosacral lesion and was considered chronic.

Martin et al. [8] found, that when the period from the first occurrence of clinical signs until the surgery is delayed overnight, the patient has an increased risk of deterioration. Their study was focused on dogs with thoracolumbar disc extrusions, which had spinal decompressive surgery. It has been shown by several studies [6, 11], that neuronal death can continue for hours to days after initial spinal cord injury, as well as further extrusion of disc material constitutes a risk of deterioration of the clinical signs with worsening prognosis.

Our results showed a positive correlation coefficient of 0.39 for the duration in days from the onset of clinical signs until the surgery and the MFS evaluated at 6 months post-surgery. This indicated that these two variables moved in the same direction, stating the duration from onset of clinical signs until surgery decreases, so does also the MFS at 6 months post-surgery. It also indicated that

in the case of the opposite scenario, when the duration of this period increases, so does also the MFS at 6 months post-surgery. It was the group of patients that were operated within 1 day and after 2 days that had the highest MFS of improvement after 2 weeks post-surgery, with the group operated after 2 days having improvements that were more favourable and earlier.

Controversially, our study demonstrated that both the group operated after 2 days and after 3 days had a better total improvement at 6 months post-surgery than the group of patients operated within 1 day. There can be several reasons for this, also as both these groups had a higher mean MFS at the time of diagnosis and as recovery is individual and depends on more factors than timing, like for example etiology and severity.

The main source of error was the small number of patients in this study. The number of patients were also unequal in the different groups, as not all of the dogs that were diagnosed with IVDD had surgery and therefore could not be included in the group that focused on recovery. The real statistical value from this study is therefore decreased and the correlation coefficients are mostly informational as the group is too small to be representative for all dogs with IVDD.

All patients that had surgery had the same treatment with: massage, passive range of motion exercises and hydrotherapy following the surgery. However, some physiotherapy exercises and movement restrictions were to be performed by the owners at home and are therefore considered as possible sources of error and differing outcomes.

## CONCLUSIONS

The main limitation of this study was the small group of patients. Although the study was based on a small number of participants, the findings suggested that the timing of the surgery and recovery had a positive Pearson correlation coefficient of 0.39; implying that the timing of the surgery affected the recovery.

When investigating the most affected groups of patients, this study found a negative Pearson correlation coefficient of  $-0.87$  between the size of the patient and the recovery, implying that the larger breeds had better improvement of MFS after surgery than the medium and small sized breeds.

The relevance of anatomical phenotypes implying that chondrodystrophic breeds are predisposed remains clearly supported by our study, accounting almost 64% of all patients, with French Bulldogs as the most prominent breed. This study also found that sex and castration status had less impact in the development of IVDD, while the BCS of the patients implied that obesity was a common factor among the affected dogs without necessarily being a predisposing factor of IVDD development.

Taken together these findings suggest that the duration from onset of clinical signs of IVDD until surgery affects the recovery rate of these dogs. Intervertebral disc disease is the most common spinal disease in dogs, further research with larger groups of patients are needed to estimate and validate the exact degree of importance this factor has on the recovery.

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## CYTOGENETIC ANALYSIS OF A MARE AND HER FOAL WITH SUSPECTED GENETIC CAUSES OF DISABILITY

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### ABSTRACT

Hereditary diseases represent a serious problem in horses, especially in terms of sport use and breeding. Nowadays, we know the genetic basis of several breed-specific inherited diseases. In this study, we focused on the cytogenetic analysis of the clinical case of a healthy mare and her foal with numerous malformations in order to confirm or disprove the suspicion of genetic causes of a disability in this offspring. We used conventional metaphase staining to analyse chromosomal aberrations—breaks and gaps. In general, the number of breaks exceeding the norm (2—3 breaks/100 metaphases) may indicate the influence of the external environment with a potential teratogenic effect on the offspring during its mother gravidity. Compared to the norm, we found a slightly increased percentage of chromosomal aberrations in both the mother and the foal. As another method, we used karyotyping to assess the number and morphology of chromosomes, where in addition to conventional staining, we also applied differential staining of metaphases (G-banding). Multiplication, loss or rearrangement of chromosome segments are almost always associated with pathology. In the karyotypes we constructed, we observed changes

in both individuals, compared to the international standard; in the mare, we probably recorded the mosaic form of her karyotype. In the foal, we found 64, XX with a suspected morphological change which was probably related to autosomal chromosome pair 31. The cytogenetic analysis of suspected individuals is also very beneficial for horse owners and breeders. Thanks to the combination of cytogenetic and modern molecular-genetic methods, we were able to identify individuals unsuitable for breeding.

**Key words:** cytogenetics; chromosomal aberrations; hereditary diseases; horse genetics

### INTRODUCTION

Understanding the genome of the horse helps us in various areas such as: prophylactic, diagnostic and therapeutic. Currently, DNA tests can be used for 21 genetic diseases in horses. Genetic tests for predisposing genetic diseases of a particular breed are required for registration of individuals and for licensing of breeding stallions, and last but not least, the results of these tests have relevance in the: breeding, nutritional management, or training pro-

gram of an individual [9]. Progress in the study of equine genomics and the development of molecular methods have qualitatively improved clinical genetics, but as indicated by S z c z e r b a l and S w i t o n s k i [19], cytogenetic testing is still one of the first steps in suspecting an individual's genetic defects. Cytogenetics deals with the study of the organization of genetic material at the chromosomal level. The basis of classical cytogenetic analysis is the arrest of mitosis at the metaphase stage, when chromosomes are best observed. Chromosomes can be further classified according to different schemes, based on the differential staining techniques used [12]. Part of cytogenetics is also karyotyping [17]. The karyotype reflects copies of chromosomes from both the mother and the father arranged according to size and shape (morphology). All horses have a diploid number of autosomal chromosomes, i.e. each chromosome contains a copy from both the dam and the sire. The normal karyotype of a mare is referred to as 64, XX and that of a stallion as 64, XY [2]. Karyotypes can be used, for example, to study chromosomal aberrations. As mentioned by I a n u z z i et al., [11], chromosomal aberrations are more often detected in horses than in other domestic species and are normally associated with the sex chromosome pair in more than 95 % of the cases.

The aim of our study was the cytogenetic analysis of a clinical case of a mare and her foal, which showed a suspected genetic disability.

## **MATERIALS AND METHODS**

### **Blood collection and animals**

The peripheral blood of a foal, the foal's dam and a healthy mare (negative control) collected in sterile tubes with anticoagulant (mostly heparin) were used as biological material for cytogenetic analysis. The dam of the foal was a 3.5 years old English Thoroughbred mare (race-horse), first-born, phenotypically normal, without clinical signs or trauma. The foal (female) appeared healthy after birth, but after a few days there was a significant deterioration in her condition; she bumped into objects, was unable to locate the mammary gland independently, did not respond to sound stimuli, appeared blind and deaf, did not urinate, and did not defecate. Joint swelling and general apathy were observed. After approximately 17 days, euthanasia was performed. As a negative control, the blood

of a healthy, 13-year-old mare of the Norik of Muráň breed was used.

### **Blood cultivation**

The culture medium (RPMI [Roswell Park Memorial Institute] 1640, foetal bovine serum, phytohaemagglutinin/pokeweed, and antibiotics/antimycotics) was prepared in sterile tubes; 0.2 ml of blood was added and incubated for 72 hours at 37 °C. Approximately 90 min. before the final termination of incubation, colchicine (0.5 mg.ml<sup>-1</sup>) was applied to the tubes to stop cell division at the metaphase stage. At the end of the culture, the cells were centrifuged and the pellets were hypotonised with 0.075 M KCl solution and fixed several times with a fixative solution composed of methanol and acetic acid in a 3 : 1 ratio.

### **Preparation of slides for observation**

For conventional staining of slides, the Giemsa stain in phosphate buffer pH 7 was used (3—4 minutes). Where possible, chromatid and chromosome breaks/gaps were evaluated in 100 metaphases under the light microscope (Nikon).

For the G-banding method, the slides were allowed to stand for 1, 2, and 5 days at laboratory temperature or 7 days at -20 °C. The slide groups thus prepared were treated with 0.1 % trypsin solution (PAA Laboratories/Fisher Scientific), cooled to 4 °C for 5, 15, 30, and 60 seconds. Subsequently, the slides were rinsed briefly in PBS (pH 6.8) and transferred to Giemsa's dye solution for 4 minutes. The quality of G-bands was assessed using a light microscope with a camera (Motic Scientific, T025A) and photographic documentation obtained. At least 15 metaphases were examined for each of the days and time mentioned above.

### **Construction of karyotypes**

Karyotypes were constructed according to an international standard based on well-defined species-specific criteria, according to which each chromosome pair has a clearly defined banding pattern [3].

### **Statistical evaluation**

The frequencies of chromatid and chromosome breaks and gaps were statistically evaluated using chi-square test (Graph Pad Prism).

## Ethical considerations

All procedures concerning the animals were performed in compliance with the national guidelines for animal care.

## RESULTS

### Evaluation of chromosomal aberrations (breaks, gaps)–Giemsa staining

In the first part of the paper, we focused on chromosome instabilities: chromosome and chromatid breaks, and gaps (Fig. 1, Fig. 2). In Table 1, we present the results of these analyses as a percentage of the aberrant cells. Compared to the norm, we found a slightly increased number of observed chromosomal aberrations in both the dam and the foal. These aberrations partially indicated the influence of external factors with a possible teratogenic effect on the offspring (foal) only in the case when breaks were considered together with the gaps (Table 1).

### Metaphase staining by differential staining–G-banding method

In this method, we evaluated 15–30 metaphases of individuals in differentially stained slides where, based on the different affinities of heterochromatin and euchromatin for the dye, the chromosome appears as a continuous series of light and dark bands [12]. This method is very challenging as it involves biological material that responds individually to the denaturing effects of trypsin. It means that it is not always possible to use a one-size-fits-all procedure. Also, the biological material in the case of the foal was suboptimal, more difficult to collect and of lower quality due to the adverse health status of the individual (Fig. 3a, b).

### Compilation and assessment of karyotypes

The karyotypes of the mother and her foal are shown in the Fig. 4 (Fig. 4a, b). In the mare we examined 100 Giemsa-stained metaphases and 30 G-banded metaphases from 5-days old slides (Fig. 3b). In one of the convention-

Table 1. Results of the evaluation of unstable chromosome aberrations in the dam and foal

Investigated individual	The number of examined metaphases	% Aberrant cells (Breaks $\pm$ SD)	% Aberrant cells (Breaks and Gaps $\pm$ SD)
Mother	100	3.0 $\pm$ 0.171a	4.0 $\pm$ 0.196a
Foal	70	4.3 $\pm$ 0.203a	7.1 $\pm$ 0.258a
Negative control	100	2.0 $\pm$ 0.140	3.0 $\pm$ 0.171



Fig. 1. Metaphase of a foal with a chromosome gap on an acrocentric chromosome. The gap is marked with an arrow  
Photo: Kuliková (2022); 1000 $\times$



Fig. 2. Metaphase of a foal with a chromatid break on the metacentric chromosome. The break is marked with an arrow  
Photo: Kuliková (2022); 1000 $\times$

ally stained metaphase we observed suspected Y chromosome (163 % of metaphases) (Fig. 4a). This could suggest that the mother could be mosaic in her lymphocytes (64, XX/64, XY). In the foal, we assume a karyotype  $2n=64$ , XX, with a presumed aberration of the 31st chromosome (Fig. 4b).

## DISCUSSION

In our study, we focused on cytogenetic analysis of cultured lymphocytes of a foal with multiple congenital malformations and her mother—a young mare who was a first-born. Congenital malformations are defined as defects in morphogenesis, which developed during intrauterine life, and are observed at birth [20]. Unlike inborn errors that are genetically determined, congenital defects may or may not be of genetic origin (Fig. 5). Therefore, in the first part of our work, we focused on the evaluation of chromosomal instability (breaks, gaps) as one of the possible causes of malformations in the foal. In general, the percentage of breaks (aberrant cells) exceeding the norm (2—3 breaks/100 metaphases) may indicate the presence of the environmental agent with a potential teratogenic effect on the offspring during intrauterine life (Fig. 5). Compared to the norm, we found only a slightly increased percentage of chromosomal aberrations in both

mother and the foal. This suggested that the environmental influence was probably not significant as a cause of malformations in the foal. In the second part of the study, we focused on the construction of karyotypes using standard Giemsa staining of chromosomes and G-banding method that allowed the precise identification of individual chromosomes with their subsequent arrangement into specific homologous pairs in the karyotype. Our cytogenetic analysis revealed that 99 per cent of the blood lymphocyte metaphases contained two X chromosomes and one per cent a probable Y chromosome. This unusual mosaic karyotype of mare 64, XX/64, XY should be interpreted very carefully because for confirmation of the Y chromosome presence, it is necessary to use fluorescently labelled specific Y chromosome painting probe [1, 8, 16] and/or molecular methods such as PCR (Polymerase Chain Reaction).

In 1999, Bugno et al. [4] found karyotype 64, XX/64, XY in a fertile mare of the Wielkopolska breed, but it was not described as a mosaicism, but as a leukocytic chimerism (87% XX:13% XY). This mare was phenotypically normal and gave birth to a normal foal at the seven years of age. Later, Bugno-Poniewierska et al. [5] reported about a case of sex chromosome mosaicism 64, XX/65, XXY/66, XXYY in a mare. The main difference between mosaicism and chimerism is that mosaicism is a condition when cell lines are derived from one embryo and are produced by unbalanced sister chromosome

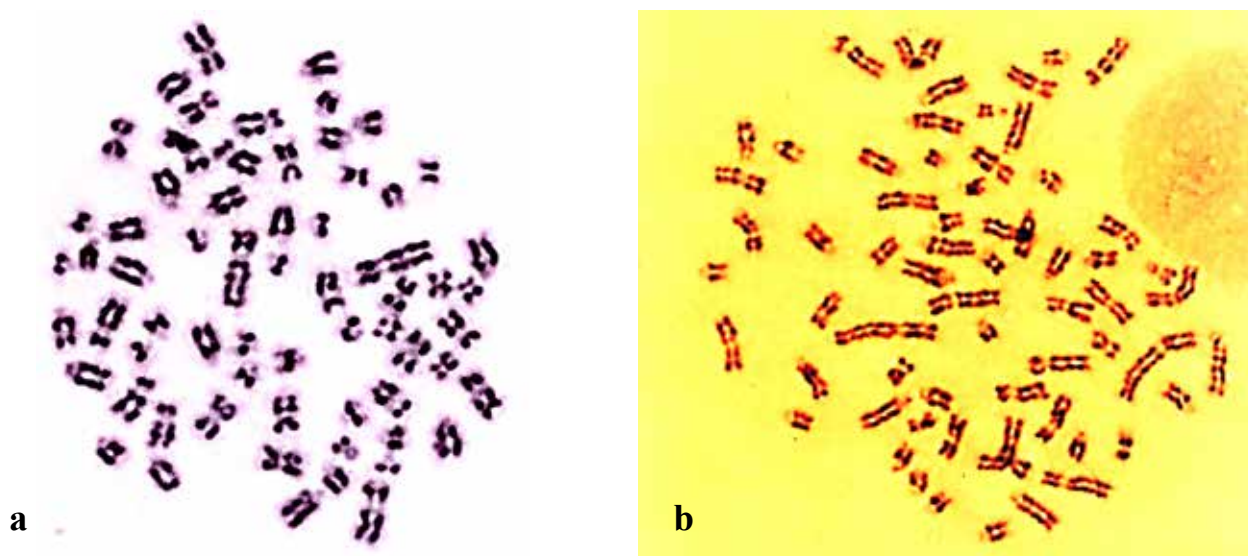
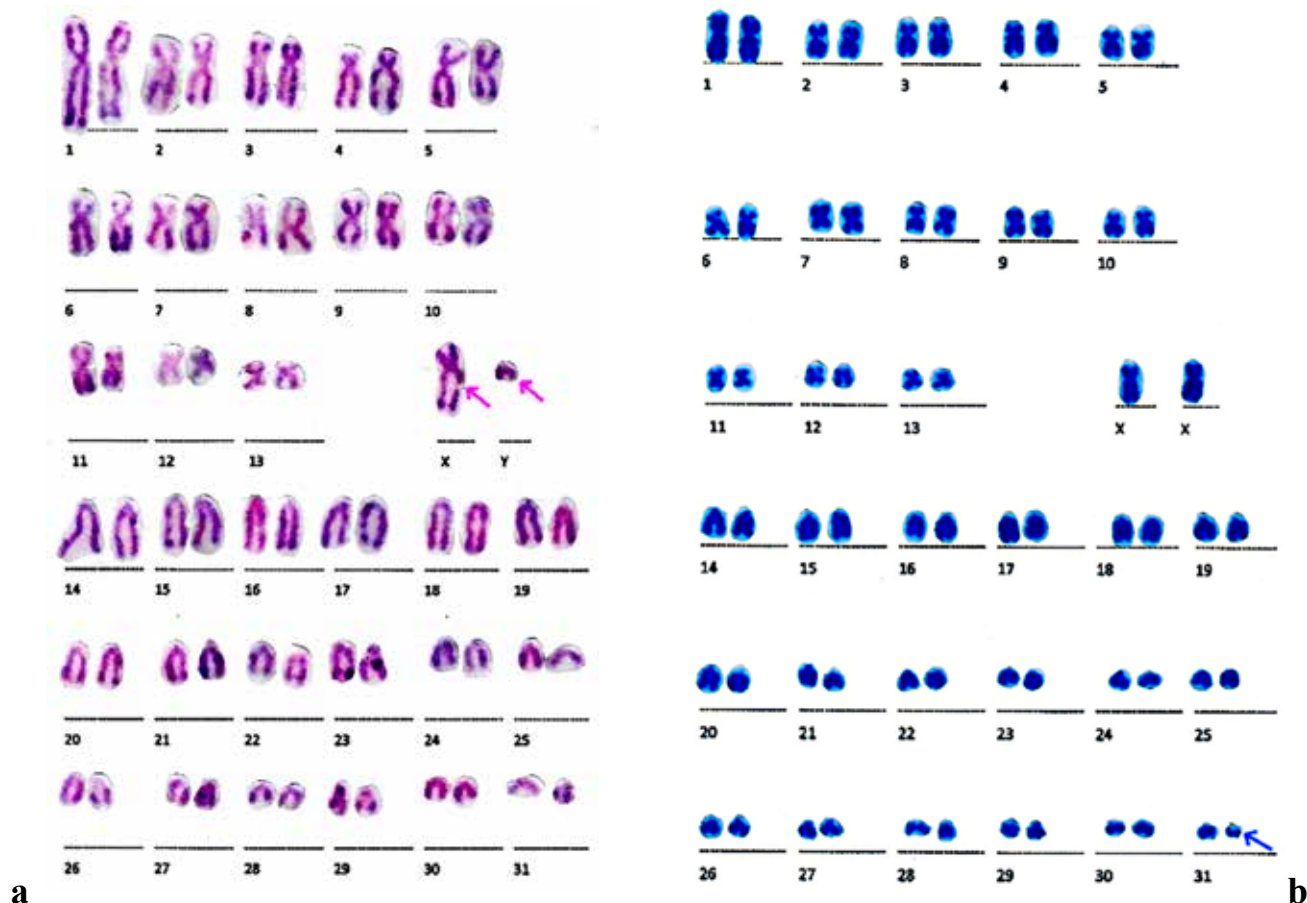


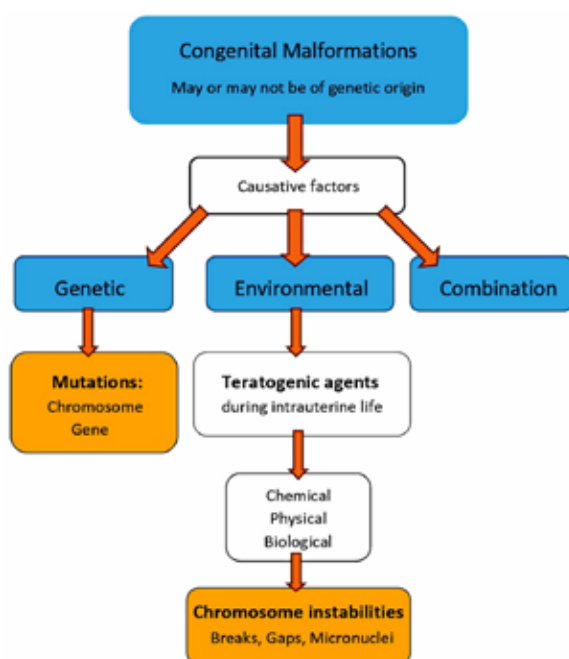
Fig. 3. The G-banding pattern of mare chromosomes, 1000×  
a) 2-day old slide, 5 seconds of trypsin exposure; b) 5-day old slide, 20 seconds of trypsin exposure  
Photo: Kulíková (2022) and Holečková (2022)





**Fig. 4. Karyotypes of mother and her foal**

a) Unusual mare karyotype—metaphase with a suspected Y chromosome (indicated by an arrow on the right). Only one per cent of the blood lymphocyte metaphases contained a probable Y chromosome and 99 per cent two X chromosomes. This indicated unusual mosaic karyotype of mare 64, XX/64, XY. Also, a chromatid gap on X chromosome was detected (indicated by an arrow on the left); b) Karyotype of a foal ( $2n = 64$ , XX) with a presumed aberration of the 31st chromosome (indicated by an arrow)



**Fig. 5. Origin of congenital malformations**  
adapted from the paper by Corseillo and Giuffr  [7]

segregation during the mitotic anaphase. On the contrary, chimerism is the presence of cell lines derived from different embryos (e.g. lymphocyte chimerism in heterosexual twins) [19]. Both conditions are usually linked with fertility troubles. As reported by Bugno-Poniewierska et al. [6], large-scale cytogenetic surveys show that almost 30% of horses with reproductive or developmental problems have chromosome aberrations, whereas abnormal karyotypes are found in only 2—5% of the general population. Most chromosomal abnormalities described in horses are rare and occur in only one or a small number of individuals. One of the most recurrent abnormalities typical of horses is the occurrence of 63, XO (instead of 64, XX) in sterile mares [6], which has also been found in the 63, X / 64, XX mosaic, and the occurrence of 64, XY (SRY-negative) in sterile mares (disorder of sexual development—DSD—with sex reversal) [16]. Animals phenotypically appear female, but the karyotype corresponds to males [15]. Other rare chromosomal abnormalities are



trisomies 65, XXX and 65, XXY and also autosomal trisomies, which are usually the result of unequal segregation of chromosomes during meiosis. In horses, cases of trisomy of chromosomes 1, 3, 15, 20, 23, 24, 26 and 27 have been reported [6, 10] that have caused early pregnancy loss of the foetus, or congenital defects in live born foals. Trisomy of chromosome 31 (65, XY, +31) was reported in 1999 by Lear et al. [14] in a thoroughbred colt with severe congenital defects involving musculoskeletal, urogenital and nervous systems.

According to Demyda-Peyrás et al. [8], most of sex chromosome aberrations remain undiagnosed because of the complexity of the horse karyotype and because a number of animals carrying these anomalies have normal phenotypes. Cytogenetic methods are useful diagnostic tools for revealing chromosomal aberrations. However, to refine and confirm cytogenetic findings, the methods of fluorescent in situ hybridisation (FISH) using chromosome-specific probes [13, 18] and PCR (e.g., evidence of SRY gene) are very useful.

## CONCLUSIONS

In our study of a foal with multiple defects, we found a slightly increased number of aberrant cells with chromosomal breaks and gaps compared to the negative control. This result only partially indicates a potential teratogenic effect of the external environment on the health status of the foal, as it was not possible to evaluate a sufficient number of metaphases. We also used the karyotyping method to assess the number, size and morphology of examined horse's chromosomes. Compared to the karyotype standard, we found variations in both karyotypes of the mare and the foal. This indicates the probable influence of genetic factors that could have caused the congenital malformations observed in the foal. However, further methods such as fluorescence in situ hybridization and modern molecular biology approaches would be needed to definitively confirm our findings.

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## EFFECT OF XYLENE ORAL EXPOSURE ON THE MOUSE UTERUS – A PRELIMINARY STUDY

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### ABSTRACT

Xylene is one of the environmental pollutants with a negative impact mainly on several organ systems. The aim of this study was to determine the effect of xylene on the uterus of mice. The study was performed on 12 adult female mice. Control mice (n=6) were fed shredded pellets at a dose of 4 g per day. Xylene mice (n=6) were fed the same diet at the same dose and orally administered xylene at 10 µl per day for 14 days. The mice were synchronized using the Whitten effect and introduced to males before the end of the procedure. Mice of both groups with no copulation plug were euthanized by cervical dislocation. The uteri were collected for routine histological and immunohistochemical analysis. The endometrial epithelium demonstrated vacuolar degeneration, mitotic cell activity, and the presence of leukocytes typical of metoestrus. Reductions of the endometrium, stroma, and myometrium were observed in the xylene mice. The xylene application did not have a significant effect on the superficial epithelium, or the size and number of uterine tubular glands. The immunohistochemical analysis of a proliferation marker PCNA revealed that

the xylene increased its expression in the stroma, endometrial and myometrial cells, but did not significantly affect the superficial epithelial cells. The expression of an anti-apoptotic marker Bcl-xl in the xylene mice was stronger in the superficial epithelial, stromal, and endometrial cells. The Bcl-xl expression in the myometrial cells was similar to the controls. The results showed that the application of xylene stimulated the proliferation and exerted an anti-apoptotic effect on the uterine cells. However, the increased proliferation can lead to the malignant transformation of cells, resulting in their uncontrollable division.

**Key words:** apoptosis; mouse; proliferation; uterus; xylene

### INTRODUCTION

The uterus is an important part of the female reproductive system; especially the endometrium is a highly dynamic tissue playing a crucial role in the establishment and maintenance of normal pregnancy [3]. Like other organ systems, the reproductive system is subject to various

changes due to various chemicals. Such an effect disrupts the structure of the organs, which in turn affects their function. Therefore, it is appropriate to perform various scientific analyses and then apply the results to various scientific areas.

Xylene or dimethyl benzene is used as a: solvent, steel cleaner, pesticide, or thinner for paints and varnishes. Xylene is a liquid volatile organic compound considered an environmental pollutant produced by the oil industry [6, 8]. It can exist in three isomeric forms: ortho-, meta-, and para-xylene. By mixing these isomeric forms of xylene with ethylbenzene, the so-called technical xylene is made [16]. Many studies have shown that xylene has adverse effects on mammalian organism. Short-term xylene exposure has been found to irritate especially chemoreceptors on the mucous membranes of the conjunctiva, nasal cavity, and the throat leading to neurological, gastrointestinal, and reproductive disorders. In addition, long-term exposure to xylene can affect respiratory, circulatory, and urinary system functions [5]. As with those organ systems, there have been studies investigating the effects of xylene on reproduction. In women who have been exposed to aromatic hydrocarbons, such as xylene or toluene, the main adverse effect on the production of hormones important for reproduction has been found [1, 6, 17]. The *in vitro* studies have shown no effect of xylene on the release of progesterone and oestradiol-17 $\beta$  from the rat ovaries [17], while stimulatory (release of insulin-like growth factor I and cell proliferation) or inhibitory (release of testosterone and progesterone) have an effect on porcine ovarian cells [15]. Studies in pregnant animals have shown that xylene may increase foetal mortality or delay growth and development of foetuses [16]. The subject of our research was to determine the possible structural and functional changes in the mouse uterus after oral intoxication by xylene because nowadays the effect of xylene on the uterus is unknown.

## MATERIALS AND METHODS

### Experimental animals, diet, and additives

The CDR-1 ICR female mice (n = 35; Velaz, Czech Republic) 35 days of age were used in this study. The mice were transported from the breeding facility to the experimental facility (Institute of Animal Physiology, Slovak Academy of Sciences, Slovak Republic) at the age of

28 days spending 7 days in the quarantine. Animals were allocated into two groups as follows: control group (C; n = 20) was fed with ground pellets (M3; BONAGRO a.s. Blažovice, Czech Republic) at a dose of 4 g per day (divided into 2 doses per day) and the xylene group (X; n = 15) was fed with M3 at the same dose + xylene (1 : 10; 10  $\mu$ l per day using p.o. cannula). Water was available *ad libitum* for the animals. Xylene was administered for 14 days. Three days before the end of the application, the mice were induced to oestrus by adding male urine contaminated litter (Whitten effect) and then transferred to males (the end of xylene application). Females without a formed copulatory plug were removed from the males, euthanized by cervical dislocation (n = 6 for each group) and their uteri were removed.

### Routine histology and morphometry

Uterine horn excisions were fixed in 4 % paraformaldehyde for 24 h. Subsequently, they were dehydrated with an ascending series of alcohols and with xylene, embedded in paraplast, and cut into 5  $\mu$ m sections (Leica RM2255 microtome, Leica Germany). Paraffin sections were stained with haematoxylin and eosin (H-E) for uterine morphometry. The slides (n = 6 per group) were evaluated using NIS Elements Br software (Nikon Laboratory Imaging, Japan) for an Eclipse E200 microscope (Nikon) connected to a digital camera (ProgRes Capture Pro 2.7.7, Jena Germany). Photomicrographs obtained at a magnification  $\times$ 100 were observed and the endometrial and myometrial thickness, superficial cylindrical epithelium and stroma heights, as well as tubular gland length were measured (expressed in  $\mu$ m). The endometrial glands were counted in four quadrants expressing the average number per 1 mm<sup>2</sup>.

### Immunohistochemistry

Paraffin sections of uterine horns were deparaffinised and rehydrated in xylenes and descending series of alcohols, followed by the antigen retrieval (boiling of the slides in 10 mM citrate buffer, pH 6.0, for 6 min), blocking endogenous peroxidase activity (incubation in TBS, 0.05 M Tris-HCl + 0.15 M NaCl, pH 7.6 with 1 % H<sub>2</sub>O<sub>2</sub> for 10 min) and non-specific binding (incubation with 1 % bovine serum albumin for 30 min). Mouse monoclonal antibodies to proliferating cell nuclear antigen (PCNA) and Bcl-xl (both diluted at 1 : 250; Santa Cruz Biotechnology Inc., USA) were applied on tissue sections and incubated

for about 20 h at 4 °C. Subsequently, sections were washed in TBST (TBS enriched with 0.1 % Tween 20; Santa Cruz) and goat anti-mouse secondary antibodies (Dako REAL-EnVision®/HRP, Rabbit/Mouse (ENV), ready-to-use, Dako, Denmark) were added and incubated for 2 h. Then washed again with TBST and TBS. Diaminobenzidine (Dako REAL™ DAB+ Chromogen, Dako) was used for visualization of a colour reaction. The sections were counterstained with haematoxylin, then dehydrated in ascending series of alcohols and in xylenes, and mounted in DPX (Distyrene Plasticizer and Xylene; Buchs, Switzerland). After drying, photomicrographs were taken using a light microscope connected to the computer and camera (same as for the routine histology). The intensity of the IHC response from 6 photomicrographs of the uteri from each animal and group was quantified using ImageJ software (National Institutes of Health, Bethesda, USA). A positive immune, DAB+ brown, reaction was rated on a greyscale and the intensity of the immune reaction was expressed by the relative optical density index (ROD) using the formula according to S m o l e n [14].

## Statistical analysis

All results were statistically analysed using the Graph-Pad Prism 3 (USA) from 6 samples per animal and each parameter, 6 animals per group with the arithmetic mean and standard error of the mean (Mean ± SEM). Differences between the groups were compared using unpaired t-test with the indication of statistical significance at  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ .

## Ethical consideration

The experiment was carried out under the approval of the State Veterinary and Food Administration of the Slovak Republic (Approval No. 598/18-221/3) in accordance with the EU regulations concerning animal experiments.

## RESULTS

Morphometric data on the uteri of mice with or without xylene application are shown in Figure 1. Murine uteri of both groups revealed the vacuolar degeneration and

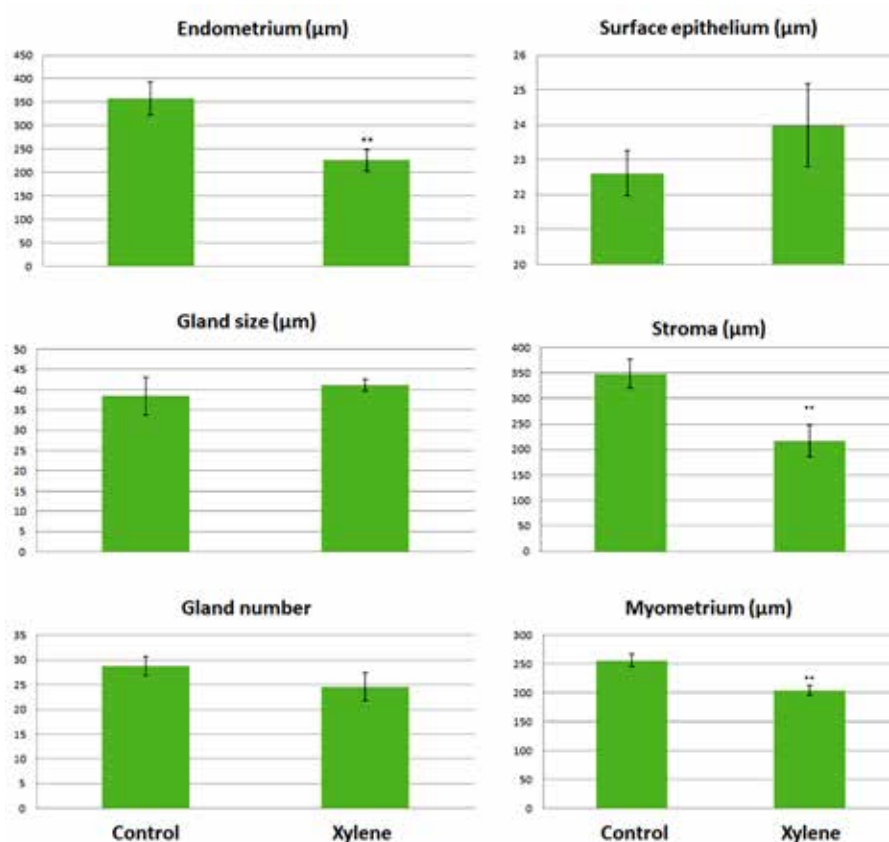
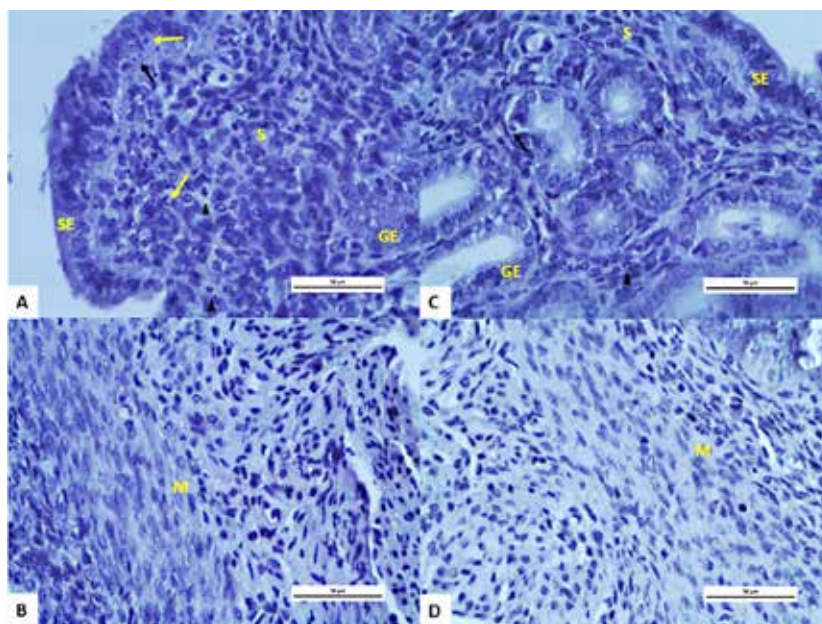


Fig. 1. Morphometric analysis of the uteri in mice after administration of xylene and in control animals  
\*\* $P < 0.01$  – significant difference to the control



**Fig. 2. Histology of the uteri in mice after administration of xylene and in the control animals**  
A, B—control mice; C, D—xylene mice; SE—surface epithelium; S—stroma; GE—glandular epithelium; M—myometrium; yellow arrow—mitotic figures; black arrow—vacuolar degeneration; black arrowhead—leucocyte. Scale bar= 50  $\mu$ m; all pictures at a magnification of  $\times 400$

**Table 1. The effect of *per oral* application of xylene on the proliferation and apoptosis in the mouse uterus**

Uterine area	Marker	ROD	
		Control	Xylene
Surface epithelium	PCNA	10.94 $\pm$ 0.19	11.76 $\pm$ 0.51
	Bcl-xl	11.99 $\pm$ 0.03	13.20 $\pm$ 0.06***
Stroma	PCNA	6.42 $\pm$ 0.45	8.64 $\pm$ 0.29**
	Bcl-xl	12.25 $\pm$ 0.08	12.92 $\pm$ 0.06***
Endometrial glands	PCNA	10.66 $\pm$ 0.29	13.83 $\pm$ 0.39***
	Bcl-xl	12.58 $\pm$ 0.04	12.88 $\pm$ 0.05**
Myometrium	PCNA	6.62 $\pm$ 0.23	7.44 $\pm$ 0.23*
	Bcl-xl	12.48 $\pm$ 0.04	12.64 $\pm$ 0.08

ROD—relative optical density; PCNA—proliferative cell antigen; Bcl-xl—B-cell lymphoma-extra large;

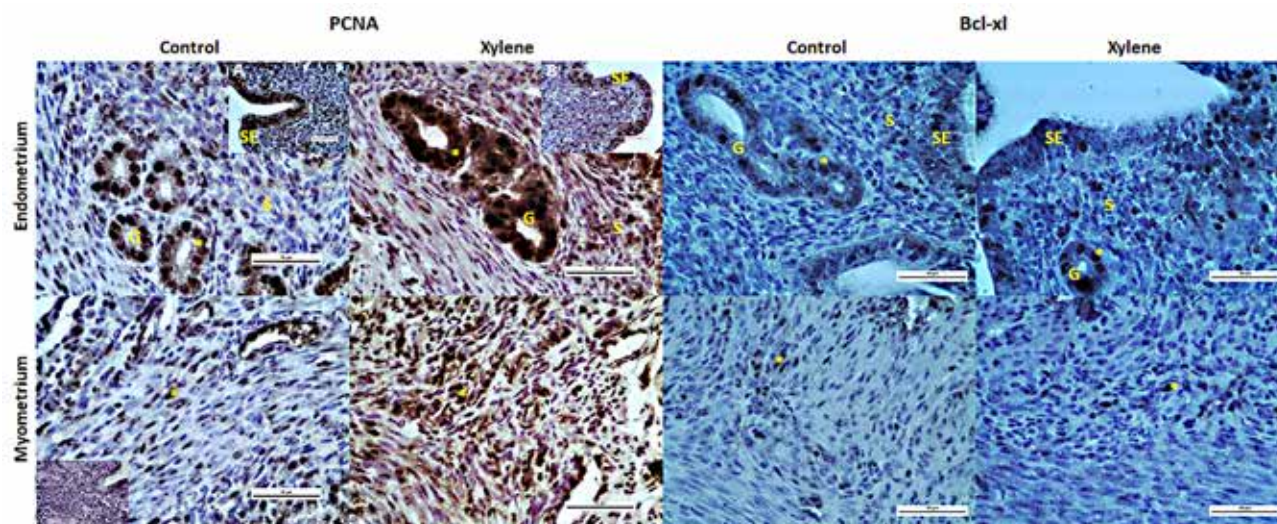
\*— $P < 0.05$ , \*\*— $P < 0.01$ , \*\*\*— $P < 0.001$ —significant difference with the control

presence of numerous mitotic patterns and leukocytes in the endometrial epithelium (Figure 2). The uteri of the xylene-treated mice had significantly thinner endometrium, stroma, and myometrium ( $P < 0.01$ ) compared to the controls. Two weeks of xylene oral application had no significant effect on the uterine epithelial height, endometrial gland size and number when compared to the control mice.

The localization (Figure 3) and amount (relative optical density, ROD; Table 1) of proliferating cell nuclear antigen

(PCNA) and anti-apoptotic antigen Bcl-xl were monitored using an immunohistochemical analysis. A positive immunoreaction to PCNA marker was present in cell nuclei. Oral administration of xylene significantly increased the stromal ( $P < 0.01$ ), endometrial ( $P < 0.001$ ) and myometrial ( $P < 0.05$ ) cell proliferation, but ROD PCNA of the superficial epithelium was not significantly affected by the xylene administration.





**Fig. 3. Immunohistochemical reaction of PCNA and Bcl-xl in the uterus of mice after administration of xylene and in control animals**  
 PCNA—proliferating cell nuclear antigen; Bcl-xl—B-cell lymphoma-extra large; SE—surface epithelium; S—stroma; G—endometrial gland;  
 \*—positive DAB+ reaction; A and B—incorporated pictures of the surface epithelium. Incorporated picture in the left lower corner is an example of  
 a negative control where primary antibodies were omitted. Scale bar = 50  $\mu$ m; all pictures at a magnification of  $\times 400$

A positive immunoreaction to Bcl-xl was noted in the cytoplasm of cells of all uterine parts in both groups. In the xylene-treated mice, the immune reaction was more pronounced in the superficial epithelial cells ( $P < 0.0001$ ), stroma ( $P < 0.001$ ), and endometrial glands ( $P < 0.01$ ). Xylene had no significant effect on the anti-apoptotic activity of myometrial cells compared to the control animals.

## DISCUSSION

The increasing burden of the environment on chemical waste [6] and the growing problems with the reproduction of humans and animals; force us to analyse their adverse effects on the body. Xylene as a part of solvents, plastics, or pesticides significantly affects the human as well as other animal organisms [16].

The uterine cycle consists of three consecutive phases – proliferative, secretory, and regressive phases. In each of these phases, the uterus undergoes physiological changes that are regulated hormonally by the action of oestrogen or progesterone, which are synthesized in the ovaries [3]. In our study, the superficial uterine epithelium showed vacuolar degeneration, mitotic activity, and variable leukocyte infiltration, the changes that can be seen in the metoestrus phase of the oestrous cycle [18] and the secretory phase of the uterine cycle [3]. The effect of xylene vapours on the uterus was studied only at the level of

its blood circulation [17] and conceptus development [4] in rats. However, no effects of xylene on the uterine structure are known. In this study, morphometric analysis of the uterine layers indicated a reduction in the endometrium, stroma, and myometrium of the xylene-treated mice. Also, adverse effects of xylene on female sex steroid hormone levels [7], gonadotropic hormones in rats [17] and women [1], and insulin-like growth factor I (IGF-I) in cows [15] have been demonstrated.

In our study, a 14-day oral application of xylene stimulated the proliferation (PCNA marker) of stromal cells, endometrial glands, and myometrium, as well as the anti-apoptotic (Bcl-xl marker) activity of superficial epithelial, stromal, and glandular cells. Some authors found a similar result in cultured porcine [15, 9–11] and bovine [13] ovarian granulosa cells where xylene has shown pro-proliferative (PCNA marker) and pro-apoptotic (marker Bax) effects, whilst in others [12] the effect was opposite. The Bcl-xl protein prevents the activation of pro-apoptotic proteins such as Bax [2]. Based on the results of the current study, it may be assumed that xylene can suppress apoptosis in the mouse uterus. The results of the current study are the first demonstration, to our knowledge, that xylene per oral intoxication can increase uterine cell proliferative and inhibit apoptotic activity in the mouse uterus that may alter the uterine structure. The increased proliferative activity of cells may represent their adaptation to toxin-induced apoptosis. On the other hand, increased proliferation may also

reflect the malignant transformation of cells that are associated with their uncontrollable division [8]. To minimise the negative impact of oil production on the environment [6] and particularly on mammals, it is very important to know its effect on individual organ systems for finding protective ways to avoid its negative impact or how to eliminate it.

## CONCLUSIONS

Orally administered xylene resulted in the thinning of the uterine wall, but immunohistochemical analysis showed increased proliferative activity in the endometrial glands, stroma, and myometrium, and anti-apoptotic activity of cells in the superficial epithelium, stroma, and glands. The results show that oral administration of xylene had a significant effect on the structure due to increased proliferation of uterine cells with a significant anti-apoptotic effect. It is important to keep in mind that xylene-induced proliferation may indicate a malignant transformation of cells, resulting in uncontrollable cell division.

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## ARE THE VARIATIONS OF THE VASCULAR SYSTEM IN NEUROLOGY UNDERESTIMATED?

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### ABSTRACT

The spinal cord issues affect dogs and cats very commonly. The right diagnostics, therapy, and patient's managements are challenging for almost all veterinarians. There exist many vascular anomalies such as: the vertebral arteries ectasia, hypoplasia and subclavian steal syndrome. These anomalies affect the patient's neurological status directly. The modern diagnostic approaches (Computed Tomography [CT], and Magnetic Resonance Imaging [MRI]) help with the diagnosis of the vascular abnormalities of the spinal cord and various other vascular anomalies. The cervical part of the spinal cord is supplied with the spinal branches from the vertebral arteries. The vertebral arteries as the first branches arise separately from the subclavian artery and they exit the thoracic aperture and enter into the transverse foramen of the sixth cervical vertebra. The arterial system of dogs was studied in 14 dogs (carcasses), the average age of which was 7 years. The carcasses were divided into two groups: 11 dogs were studied by the corrosion casting method (Duracryl Plus) and 3 dogs were studied by contrast radiography (Urografin 76 %). We confirmed the standard origin and course of the left vertebral for all but one case. The right vertebral artery originated as an independent branch in

57.14 % of the cases; in the rest of them, we reported on the variability in origin and formation of inconstant branches. The formation of anastomoses was reported also. Our work contributed new information about the thoracic and cervical arterial system in dogs.

**Key words:** arteries; corrosion casting; dog; neurology; spinal cord; vertebral artery

### INTRODUCTION

Vertebral arteries (VA), as the first branches of the subclavian arteries in dogs, are responsible for the cervical spinal cord blood supply. Vertebral arteries leave the thoracic cavity through the *apertura thoracis cranialis* and they enter into the transverse foramen of the 6th cervical vertebra between the scalene muscle and the *longus coli* muscle [15, 19]. VA provide: a blood supply for the spinal cord (*rr. spinales*, *a. spinalis ventralis*), caudal part of the brain, brainstem, meninges (*a. basilaris*), muscles, and skin (*rr. musculares*) of the neck [15]. The cervical spinal cord is supplied by the spinal branches of the vertebral arteries. These branches enter into the intervertebral foramina alongside the roots of spinal nerves and into the vertebral canal. They are then divided into the unpaired

ventral branch, and paired dorsolateral branches. The largest branch is the ventral spinal artery which runs in the ventral fissure of the spinal cord. The smaller dorsolateral branches run in the dorsolateral sulcus of the spinal cord near the dorsal roots of the spinal cord [8, 19].

Many spinal malformations do not produce overt neurologic dysfunction. It is the responsibility of the clinician to investigate and determine whether the malformation is an incidental finding or the underlying cause of a neurological clinical situation [21].

In the available anatomical books, possible variations of the vascular system are not described fairly, or they are characterised poorly [4, 8, 15, 19]. The variations of the vertebral arteries (origin, course and branching patterns) attract the same clinical interest in the humans as they do in the veterinary literature [11].

The purpose of this study is to describe the variations in the formation and course of the vertebral arteries and to contribute new knowledge about arterial system in dogs. The strong educational character of this paper is expressed by the original images and illustrations.

## MATERIALS AND METHODS

We used carcasses of 14 mix-breed dogs for this study; the average age of the dogs was 7 years. Due to an incurable stage of their diseases or trauma the dogs were euthanized at the Small animal clinic of the University of Veterinary Medicine and Pharmacy in Košice based on the owners' request.

The intravenous drugs used for the euthanasia were as follows: premedication with sedatives Butorphanol  $0.3 \text{ mg.kg}^{-1}$  (Butomidor  $10 \text{ mg.kg}^{-1}$ , Richter Pharma AG, Austria) and Medetomidine  $0.02 \text{ mg.kg}^{-1}$  (Cepetor KH  $1 \text{ mg.kg}^{-1}$ , CP-Pharma Handelsgesellschaft GmbH, Germany). Then the general anaesthetic agent Propofol  $2.0 \text{ mg.kg}^{-1}$  (Propofol  $2 \text{ mg.kg}^{-1}$ , Fresenius Kabi GmbH, Germany) was used to provide deep anaesthesia, and finally Thiopental  $1.0 \text{ g pro toto}$  (Thiopental VUAB  $1.0 \text{ g}$ , VUAB Pharma a. s., Czech Republic) was administered.

Eleven carcasses were perfused with 0.9 % sodium chloride solution (Mikrochem Trade, s.r.o., Slovakia) with sodium citrate (Mikrochem Trade, s.r.o., Slovakia) through the ascending aorta after the humane euthanasia and acrylic self-curing dental resin Duracryl Plus (Spofa-

Dental, a. s., Czech Republic) was administered through the ascending aorta after the perfusion. After polymerisation of the resin, maceration of the soft tissues was performed by a maceration unit (BM 1115, Gastrolux, s.r.o., Slovakia). Sodium hydroxide (Mikrochem Trade, s.r.o., Slovakia) at 2 % concentration was used as a maceration medium. The maceration process continued over two to four days at  $70^\circ\text{C}$ . Finally, the macerated specimen was dried at a room temperature and inspected macroscopically and in some cases microscopically under a surgical microscope Carl Zeiss Movena S7 (Carl Zeiss AG, Germany).

The remaining three carcasses underwent a contrast arteriography of the subclavian arterial branches. The contrast agent sodium amidotrizoate (UROGRAFIN 76 %, Bayer AG, Germany) was administered through the descending aorta into both subclavian arteries. The radiographs were taken using the Gierth HF 200 (GIERTH X-Ray international GmbH, Germany), immediately after application of the contrast agent in ventrodorsal (VD) and laterolateral projections.

## Ethical considerations

The owners of dogs agreed with the participation of the carcasses in this study.

## RESULTS

In all investigated cases the left vertebral artery was the first separate branch from the left subclavian artery. The course of the left vertebral artery was standard; the vessel left the thoracic inlet in the cranio-dorsal direction and it entered into the transverse foramen of the sixth cervical vertebra anatomically (Fig. 1). Only in one case, the left vertebral artery did not enter into the transverse foramen of the sixth cervical vertebra and the course of this vessel was atypical, it entered into the fifth cervical vertebra (Fig. 5).

The right vertebral artery was a separated branch from the right subclavian artery in most of the dogs. In contrast, with the left vertebral artery, the right vertebral artery originated in very closed proximity with the costocervical trunk. The right vertebral artery originated anatomically at the same level, medially from the costocervical trunk. The lateralisation of the costocervical trunk was observed. The formation of the common trunk was observed in two



**Fig. 1. The detailed view on the standard origin and course of the left vertebral artery and the left costocervical trunk**  
SA—subclavian artery; CT—costocervical trunk; VA—vertebral artery

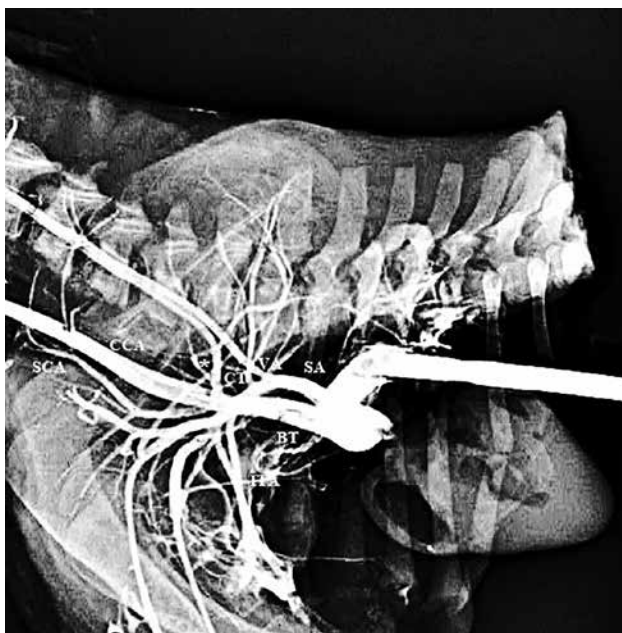


**Fig. 2. The formation and course of the right vertebral artery with communicating branch with the deep cervical artery**  
BT—brachiocephalic trunk; SA—subclavian artery; CT—costocervical trunk; DCA—deep cervical artery; VA—vertebral artery; CCA—common carotid artery; SCA—superficial cervical artery; AA—axillary artery

**Table 1. Details of the course and formation of vertebral arteries**

No.	Left vertebral artery	Right vertebral artery	Method
1	Standard course and formation	Very close contact with the costocervical trunk	CC
2	Standard course and formation	Standard course and formation	CC
3	Standard course and formation	Standard course and formation	CC
4	Standard course and formation	Standard course and common trunk with the costocervical trunk and communicant branch with the deep cervical artery	CC
5	Standard course and formation	Standard course, send out the deep cervical artery	CC
6	Standard course and formation	Standard course and formation	CC
7	Standard course and formation	Standard course and formation	CC
8	Standard course and formation	Standard course and formation	CC
9	Standard course and formation	Standard course and formation	CC
10*	Atypical course to the transverse foramen of C5	Very close contact with the costocervical trunk	CC
11	Standard course and formation	Standard course and formation	CC
12	Standard course and formation	Very close contact with the costocervical trunk	CS
13	Standard course and formation	Common trunk with the costocervical trunk	CS
14	Standard course and formation	Standard course and formation	CS

\*—published by K o r i m et al. 2022; CC—corrosion casting; CS—contrast study; C5—fifth cervical vertebra

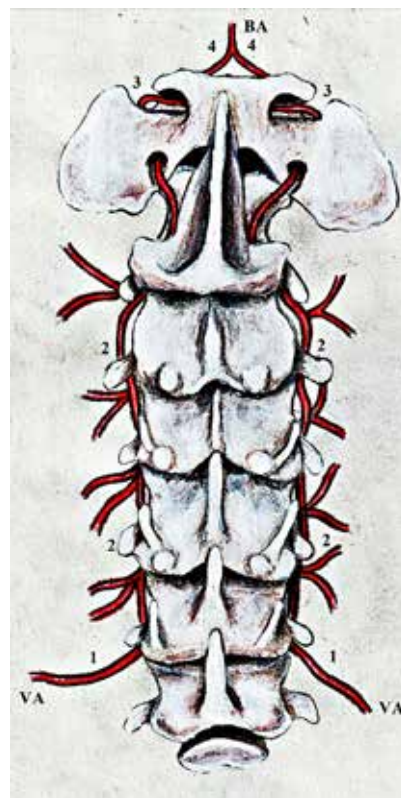


**Fig. 3. Contrast arteriogram of the branches from subclavian arteries (laterolateral projection)–presence of the common trunk of the right vertebral artery and the right costocervical trunk**  
BT–brachiocephalic trunk; SA–subclavian artery; CT–costocervical trunk; VA–vertebral artery; CCA–common carotid artery; SCA–superficial cervical artery; ITA–internal thoracic artery; \*–common trunk for origin of the right vertebral artery and the right costocervical trunk

dogs (14.29 %). The very close contact with the costocervical trunk was observed in three cases (21.43 %) (Fig. 3). However, the course of the right vertebral artery was normal and the artery entered the sixth cervical vertebra in all dogs. In one case (7.14 %), the right vertebral artery sent out the deep cervical artery. The communicating branch between the right vertebral artery and the deep cervical artery was observed in one dog (7.14 %) at the level between the first and the second thoracic vertebra (Fig. 2). The details of the course and formation of the vertebral arteries are described in Table 1.

We can divide the vertebral artery into the four segments:

1. Preforaminal or prevertebral segment which runs from the place of origin to the transverse foramen of the C6;
2. Foraminal or transverse segment which is in the transverse canal;
3. Atlantic or extradural part of the vertebral artery is at the level of the first cervical vertebra (atlas); and
4. The intracranial or intradural segment of the vertebral artery is represented by the formation of the basilar artery (Fig. 4).



**Fig. 4. The course and segmentation of the vertebral artery in dog (dorsal view)**

VA–vertebral artery; BA–basilar artery; 1–preforaminal part of the vertebral artery; 2–foraminal/transverse part of the vertebral artery; 3–atlantic/extradural part of the vertebral artery; 4–intracranial/intradural part of the vertebral artery

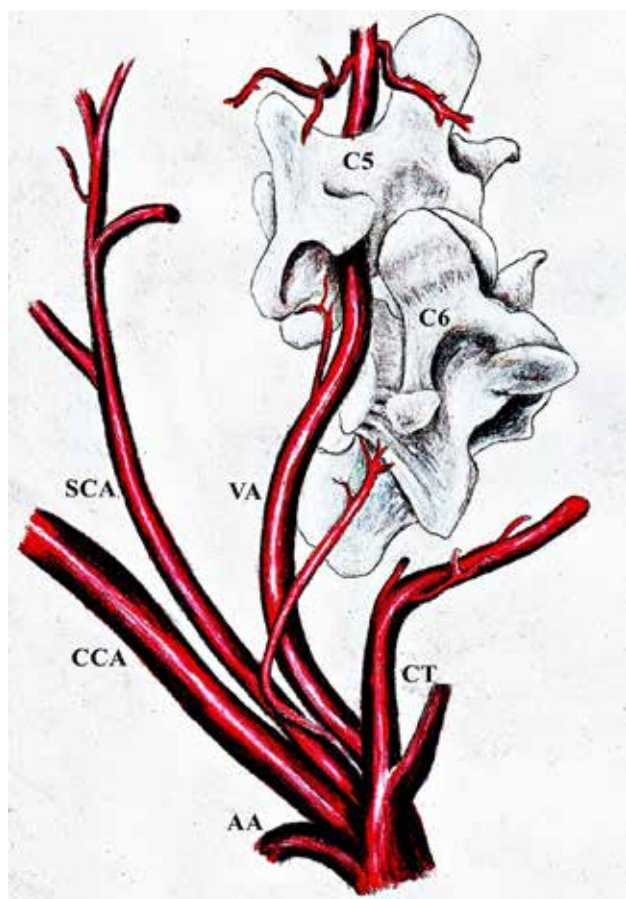
## DISCUSSION

The veterinary literature does not divide the vertebral artery into the segments [8, 16, 19]. We took the segmentation of the vertebral artery from a human anatomy [1, 11, 14], and this should also be used in veterinary medicine to characterize the lesions and their localisations more accurately.

The variability of the vertebral arteries at the place of origin was studied in laboratory animals and dogs. The course of both vertebral arteries was standard, and they entered into the sixth cervical vertebra and pierced the transverse foramen in all subjects studied [4, 5, 9, 12, 13, 15].

P o l s et al. [15] characterised the branching patterns to the I–scale in dogs. The pattern of branches from the subclavian arteries according to D a r a w i r o j et al. [4] in 58 mix-breed dogs were as follows: The vertebral artery was the first branch in the majority of the dogs. In some dogs, the vertebral artery originated in the proximity from the costocervical trunk and sometimes they formed the





**Fig. 5. The atypical course of the left vertebral artery with malformation of the sixth cervical vertebra. Scheme according to K o r i m et al. [7]**

CT—costocervical trunk; VA—vertebral artery; CCA—common carotid artery; SCA—superficial cervical artery; AA—axillary artery; C5—fifth cervical vertebra; C6—sixth cervical vertebra

common trunk. In the minimum number of cases, the vertebral artery was the second branch from the subclavian artery. K ü r t ü l et al. [9] observed a standard formation and course of both vertebral arteries in dogs. A very rare case of an unilateral left asymmetry of the sixth cervical vertebra with atypical course of the left vertebral artery in the dog has been previously described [7].

The possible arrangements of the vertebral artery in the guinea pig was studied by F l e s a r o v a et al. [5]. The vertebral artery can be formed as an independent branch from the subclavian artery, but these authors found the common trunk with the dorsal scapular artery. In some cases, the vertebral artery was formed by fusion of two separate arteries. In rabbits, the origin of the vertebral artery is similar to that in the guinea pigs. In some cases, the vertebral artery originated directly from the aortic arch [12, 13].

The ectasias of vertebral arteries in dogs were described by B o z y n s k i et al. [3] and W e s t w o r t h et al. [20].

W e s t w o r t h et al. [20] described the dilatation of the vertebral arteries (especially the left vertebral artery) at the transverse foramen of the 6th cervical vertebra and ectasias of the radicular branches at the site of C5—C6 intervertebral disc space in a 3-year-old German short-haired pointer dog. The dog suffered tetraparesis and spinal ataxia with C6—T2 myelopathy. The anastomoses between vertebral arteries were observed at the ventral aspect of the vertebral canal at the C5—C6 intervertebral space. B o z y n s k i et al. [3] described the dilatation of both vertebral arteries between C4—C6 in a 4-year-old Catahoula Leopard dog. The clinical signs were the same as in the study of W e s t w o r t h et al. [20] and ectasia and tortuosity of the spinal branches of the vertebral arteries and the ventral spinal artery were described.

The subclavian steal syndrome (SSS) is defined as a hemodynamic phenomenon characterized by retrograde blood flow in the artery. Subclavian steal syndrome affects the vertebral artery mainly and it is asymptomatic in the majority of cases. The pathophysiology of SSS is described by occlusion of the vessel lumen or cumulation of atherosclerotic plaques (stenosis) which is the most common cause of the SSS in humans. The result of occlusion or stenosis is decreasing of the blood pressure leading to retrograde vertebral artery flow from the contralateral normal vessel by the basilar artery, circle of Willis or radicular branches. The redirection of the blood flow can lead to the cerebral ischemia. [6, 10, 17, 18]. On the other hand, SSS is extremely rare in dogs. There exist only a few cases describing the SSS in dogs [2, 18, 20]; SSS can be diagnosed by Doppler ultrasonography, contrast computed tomography and contrast magnetic resonance imaging [17, 20].

## CONCLUSIONS

The findings of this study confirmed the slight variability of the vascular system of the cervical area in dogs. In all dogs, the left vertebral artery was the first branch from the left subclavian artery. The right vertebral artery reported higher variability. The importance of the arterial system (especially vertebral arteries) in veterinary neurology mainly during diagnostics has a key role and it can significantly influence the direction of therapy. However, the limitation of this study is a small sample of investigated subjects.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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