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RESTORATIVE EFFECTS OF ALOE VERA GEL ON ALCOHOL INDUCED HEPATO-NEPHROCELLULAR DYSFUNCTION

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ABSTRACT

Excessive alcohol intake is associated with pathological conditions that are detrimental. Aloe vera is a plant that possesses antimicrobial and anti-oxidant properties. This study investigated the effects of Aloe vera gel on alcohol induced hepato-nephrocellular dysfunction in rats using the specific activities of glutathione-S-transferase (GST), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH) as well as concentration of some electrolytes as indices. Six groups of male albino rats containing 5 rats each were used in the experiments. Groups A and B were administered distilled water and 50 % (v/v) alcohol for 21 days respectively. Groups C and D were administered 50 % (v/v) alcohol, while groups E and F were administered distilled water for the first 14 days, followed by co-administration (without stopping alcohol or distilled water administrations) of 125 mg and 250 mg.kg^{-1} body weight Aloe vera gel respectively for 7 days. The administration of Aloe vera gel extract significantly modulated serum electrolytes imbalances with concomitant lowering of ALT, AST, ALP, GGT, LDH and GST rates when compared to group B. These results suggested the restoration of alcohol induced dysfunction by Aloe vera gel.

Key words: alcohol; Aloe vera; electrolytes; restoration effect; tissue damage; toxicity

INTRODUCTION

Alcohol consumption has a widespread social tradition among many populations worldwide because its moderate intake has been regarded beneficial to cardiovascular health [15, 33]. However, excessive alcohol intake is also associated with a variety of pathological conditions varying from simple intoxication, to severe life-threatening states which are increasingly becoming important causes of morbidity and mortality globally [15, 17, 27].

The liver is the site of essential biochemical reactions and has the function to detoxify xenobiotics [24]. It is also

the primary organ for the metabolism of alcohol which makes it susceptible to alcohol related injuries [17, 32]. The site for elimination of reactive metabolites which is the kidney, may also be affected by excessive alcohol consumption and could cause impairment in its ability to regulate the volume and composition of fluid and electrolytes in the body [7, 30].

The developments of interest in the therapeutic potentials of medicinal plants against alcohol induced toxicity have become an increasing attractive area of research [8, 13]. Aloe vera (*Aloe barbadensis* Miller) is one of the four species among over 360 species known to have medicinal properties [5]. It is a tropical succulent plant that belongs to the liliaceous family [18, 24]. Its common names include: Aloe vera, Barbados aloe, Mediterranean aloe, True aloe and Curacao aloe [4, 18, 21]. The leaf extract and/or the juice has been shown to possess: laxative, antimicrobial, wound healing, anti-inflammatory, anti-cancer, anti-diabetic and anti-oxidant properties [4, 18, 22, 26, 34, 35].

Many studies have shown the pharmacological evidence and the support of the traditional folklore medicinal usage of Aloe vera in the treatment of different toxicant induced tissue damages [4, 8, 16, 24, 28]. Most of the medicinal properties of Aloe vera leaves have been attributed to the presence of the polysaccharides found in the inner part of the parenchymatous tissue [4, 8]. Thus, a continuous screening in the laboratory for its biochemical and pharmacological properties against liver and kidney impairment is necessary. Akinloye et al. [2] have already reported the antioxidative potential of *Aloe barbadensis* on alcohol induced oxidative stress in rats. However, the restorative effects of Aloe vera on serum electrolyte disturbances induced by alcohol administration have not been completely explored. This study was therefore designed to investigate the restorative effect of lyophilized Aloe vera gel extract on alcohol induced hepatocellular and nephrocellular dysfunction in male albino rats.

MATERIALS AND METHODS

Collection and authentication of plant materials

The collection and authentication of plant materials were done as previously carried out by Akinloye et al. [2]. Fresh Aloe vera plants were obtained from the Botanical garden of the department of Pure and Applied Botany and authenticated by a botanist (Professor D. A. Agboola) in the department of Pure and Applied Botany, Federal University of Agriculture, Abeokuta, Nigeria. The plant was identified and authenticated with herbarium number FUNAABH0028.

Extraction of Aloe vera gel

The Aloe vera plants were washed thoroughly with clean water, rinsed with distilled water and the inner part (the gel) of the leaves was removed into a clean container and blended (homogenized) using ROSPEC 7.4 V 2000 rpm electric blender. The homogenized gel was stored in a frozen state (at –10 °C) and lyophilized at Covenant University, Ota, Ogun State, Nigeria using LTE Scientific Lyotrap ultra freeze dryer with ice capacity of 10 kg (6 kg in 24 hours) and temperature –55 °C (heat extraction rate of –40 °C).

Experimental animals

The approval of the Departmental Animal Ethical Committee (FUNAAB-BCH) was obtained prior to the experiment with ethical no FUNAAB-BCH-DI 017. All of the protocols and the experiments were conducted in strict compliance according to the guidelines approved by the committee. Forty-five (45) male albino rats weighing between 180 g and 250 g were purchased from the Department of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Nigeria. The animals were acclimatized for two weeks before the commencement of the experiments. They were housed in plastic cages with good ventilation and were supplied with standard pellet and clean water *ad libitum*.

Experimental design

The experimental design was carried out as described by Akinloye et al. [2]. Forty-five (45) male albino rats weighing 180—250 g were first divided into two (2) groups of 15 animals and 30 animals. The 15 animals were given orally, 4 mg.kg^{-1} body weight of distilled water for the first two weeks (14 days) of the experiments, while the other 30 animals were orally administered 4 ml.kg^{-1} body weight of 50 % (v/v) ethanol for the first two weeks (14 days) of the experiments. After the first two weeks (14 days), the 15 animals were further divided into three (3) groups (A, E and F) of 5 animals each; group A served as the positive control and were further administered 4 ml.kg^{-1} body weight of distilled water for another one week (7 days);

Table 1. Experimental Design

Dosage administered	Groups
Distilled water for 21 days	A
50 % Alcohol for 21 days	B
50 % Alcohol (14 days) followed by alcohol + Aloe vera (7 days)	C (125 mg.kg^{-1}) D (250 mg.kg^{-1})
Distilled water (14 days) followed by alcohol + Aloe vera (7 days)	E (125 mg.kg^{-1}) F (250 mg.kg^{-1})

while groups E and F were given 125 mg and 250 mg.kg^{-1} body weight Aloe vera gel respectively for another one week (7 days) of the experiments. Twenty-four (24) animals were selected from the 30 alcohol treated group after the first two weeks (14 days) of alcohol administration. From these, 10 animals were further administered the same quantity and concentration of alcohol for the next one week (7 days) and served as the negative control group (B). The remaining 14 animals were also divided into 2 groups (C and D) of 7 animals each; which were then treated with 125 mg and 250 mg.kg^{-1} body weight Aloe vera gel extract respectively after 30 minutes of alcohol administration for another one week (7 days) of the experiment. On the 21st day of the experiment, after the last treatment, the animals were fasted for 12 hours overnight and were sacrificed after anaesthetized slightly. Blood samples were then collected via the abdominal vein into clean plain tubes, allowed to stand and clot for 15 minutes and then centrifuged at 3000 rpm for 10 minutes to collect serum using the refrigerated centrifuge (BK-THRI16, Biobase Biodustry Shagdong). Kidney and stomach were excised for biochemical examination, using 10 % homogenate preparation.

Biochemical assays

Glutathione-S-transferase (GST, EC: 2.5.1.18) specific activity was determined according to the method described by Habig et al. [14]. The specific activities of alanine aminotransferase (ALT, EC: 2.6.1.2), aspartate aminotransferase (AST, EC: 2.6.1.1), alkaline phosphatase (ALP, EC: 3.1.3.1), gamma glutamyl transferase (GGT, EC: 2.3.2.21) and lactate dehydrogenase (LDH, EC: 1.1.1.27) as well as the concentration of the: total protein, creatinine, uric acid, bilirubin, urea, albumin, sodium, magnesium, calcium, potassium and chloride were determined using the Randox standard kit methods of Randox Laboratory Limited (United Kingdom).

Statistical analysis

The results were expressed as the mean ± SEM (n = 5). The level of homogeneity among the groups was assessed using one-way analysis of variance (ANOVA). Where homogeneity occurred, Duncans' test was used to differentiate between the groups. All analyses were done using the Statistical Package for Social Sciences (SPSS 20.0) and $P < 0.05$ was considered the statistically significant.

RESULTS

Table 2 shows the effects of the Aloe vera gel extract on the serum tissue damaged enzymatic markers of alcohol in the intoxicated male albino rats. The administration of alcohol significantly ($P < 0.05$) elevated the serum specific activities of: ALT, AST, ALP, GGT, LDH and GST, when compared to the controls. However, co-treatment with 125 mg and 250 mg.kg^{-1} body weight Aloe vera gel extract (groups C and D) significantly lowered the elevated specific activities of ALT (with the exception of group D that showed a slight reduction), AST, ALP, GGT, LDH and GST, as compared to the alcohol treated group (group B). The administration of 250 mg.kg^{-1} body weight Aloe vera gel extract only, showed no significant difference in the specific activities of ALT, LDH and GGT, as compared to the control group (A).

Alcohol significantly ($P < 0.05$) increased the activities of renal AST, GST and LDH in the alcohol treated groups when compared to the control group as shown in Table 3. Also, significant reductions were observed in the specific activities of renal ALT and ALP of the alcohol treated group when compared to the control group. Treatment with 125 mg and 250 mg.kg^{-1} body weight Aloe vera gel significantly reduced the elevated activities of AST and LDH with a concomitant increase in the specific activities of ALT and ALP kidney of the Aloe vera treated group when compared to the alcohol treated group (B). However, there was no significant difference in GST specific activity of group C (group administered 125 mg.kg^{-1} body weight Aloe vera gel extract) when compared to group B.

The administration of alcohol resulted in a marked increase in the stomach specific activities of GGT, ALP, GST and LDH when compared to the control group (Table 4). But, after co-treatment with Aloe vera gel extract (groups C and D), the elevated specific activities of GGT, ALP, GST

Table 2. Effects of Aloe vera gel extract on tissue damaged enzyme markers in the serum of alcohol intoxicated male albino rats

Parameter	Serum (U.mg^{-1} protein)					
	ALT	AST	ALP	LDH	GGT	GST
Control (A)	4.46 ± 0.05^{b}	5.16 ± 0.18^{b}	0.61 ± 0.01^{a}	0.96 ± 0.01^{a}	0.79 ± 0.01^{a}	0.49 ± 0.08^{b}
ALC (B)	5.37 ± 0.26^{c}	6.92 ± 0.38^{c}	16.92 ± 0.05^{e}	7.20 ± 0.03^{c}	9.14 ± 0.02 ^{c}	0.79 ± 0.01^{c}
ALC + 125 mg A. V (C)	2.41 ± 0.08^{a}	5.37 ± 0.31^{b}	13.86 ± 0.35^{d}	3.00 ± 0.03^{b}	3.71 ± 0.03^{b}	0.35 ± 0.03^{b}
ALC + 250 mg A. V (D)	3.46 ± 0.13^{bc}	4.81 ± 0.09^{b}	7.26 ± 0.58^{c}	1.44 ± 0.09^{s}	0.64 ± 0.14^{s}	0.44 ± 0.07^{b}
125 mg A. V (E)	1.69 ± 0.10^{a}	1.97 ± 0.13^{a}	0.45 ± 0.01^{a}	0.72 ± 0.22^{a}	0.66 ± 0.01^{a}	0.42 ± 0.01^{b}
250 mg A. V (F)	2.5 ± 0.37^{b}	2.55 ± 0.01^{a}	1.51 ± 0.03^{b}	1.10 ± 0.08^{a}	0.34 ± 0.04^{a}	0.18 ± 0.00^{a}

These data are expressed as the mean ± SEM (n = 5); Values with different superscript letters along the column for each parameter differed significantly (P < 0.05); ALC + 125 mg A. V—alcohol + 125 mg.kg^{-1} body weight Aloe vera; ALC + 250 mg A. V—alcohol + 250 mg.kg^{-1} body weight Aloe vera; 125 mg A.V—125 mg.kg^{-1} body weight Aloe vera; 250 mg A. V—250 mg.kg^{-1} body weight Aloe vera

Table 3. Effects of Aloe vera gel extract on tissue damaged enzyme markers in the kidney of alcohol intoxicated male albino rats

Parameter	Kidney (U.mg^{-1} protein)					
	ALT	AST	ALP	LDH	GGT	GST
Control (A)	5.97 ± 0.15^{b}	4.35 ± 0.19^{a}	4.36 ± 0.41^{b}	3.68 ± 1.15^{a}	2.40 ± 0.22^{ab}	0.17 ± 0.00^{a}
ALC (B)	2.42 ± 0.06^{a}	18.75± 0.35^{a}	1.30 ± 0.12^{a}	28.04 ± 1.84^{d}	2.21 ± 0.02^{b}	0.58 ± 0.00^{b}
ALC. + 125 mg A. V (C)	6.06 ± 0.44^{b}	13.19 ± 0.27^{bc}	1.41 ± 0.09^{a}	15.32 ± 0.95^{bc}	2.75 ± 0.08^{c}	0.17 ± 0.02^{a}
ALC. + 250 mg A. V (D)	6.80 ± 0.44^{bc}	12.68 ± 0.65^{b}	2.09 ± 0.32^{a}	11.06 ± 2.94^{ab}	2.50 ± 0.14^{ab}	0.56 ± 0.03^{b}
125 mg A. V (E)	7.61 ± 0.59^{cd}	14.18 ± 0.49^{c}	2.82 ± 0.21^{ab}	23.45 ± 0.80^{cd}	1.23 ± 0.04^{a}	0.23 ± 0.01^{a}
250 mg A. V (F)	8.11 ± 0.28^{d}	16.38 ± 0.28^{d}	3.41 ± 1.54^{c}	24.12 ± 5.66^{d}	1.53 ± 0.15^{a}	0.37 ± 0.03^{a}

These data are expressed as the mean ± SEM (n = 5). Values with different superscript letters along the column for each parameter differed significantly at P < 0.05; ALC + 125 mg A. V—alcohol + 125 mg.kg^{-1} body weight Aloe vera; ALC + 250 mg A. V—alcohol + 250 mg.kg^{-1} body weight Aloe vera; 125 mg A. V—125 mg.kg^{-1} body weight Aloe vera; 250 mg A. V—250 mg.kg^{-1} body weight Aloe vera

Table 4. Effects of Aloe vera gel extract on the specific activities of GGT, ALP, GST and LDH in the stomach of alcohol intoxicated rats

Parameter	Stomach (U.mg^{-1} protein)			
	GGT	ALP	GST	LDH
Control (A)	6.93 ± 0.67^{d}	80.82 ± 0.65^{e}	1.50 ± 0.12^{a}	9.11 ± 0.43^{d}
ALC (B)	9.06 ± 0.32^{e}	176.74 ± 0.01^{f}	2.90 ± 0.49^{b}	12.96 ± 0.01^{e}
ALC + 125 mg A. V (C)	7.74 ± 0.73^{d}	52.93 ± 0.74^{c}	1.60 ± 0.01^{a}	2.13 ± 0.02^{a}
ALC + 250 mg A. V (D)	4.09 ± 0.21^{c}	55.65 ± 0.22^{d}	1.21 ± 0.06^{a}	2.59 ± 0.01^{b}
125 mg A. V (E)	1.58 ± 0.55^{a}	22.55 ± 0.68^{a}	1.44 ± 0.14^{a}	3.37 ± 0.20^{c}
250 mg A. V (F)	2.49 ± 0.2^{b}	40.79 ± 1.22^{b}	1.13 ± 0.07^{a}	3.68 ± 0.02^{c}

These data are expressed as the mean ± SEM (n = 5). Values with different superscript letters along the column for each parameter are significantly different at P < 0.05; ALC + 125 mg A. V—alcohol + 125 mg.kg^{-1} body weight Aloe vera; ALC + 250 mg A. V—alcohol + 250 mg.kg^{-1} body weight Aloe vera; 125 mg A. V—125 mg.kg^{-1} body weight Aloe vera; 250 mg A.V —250 mg.kg^{-1} body weight Aloe vera

Table 5. Effects of Aloe vera gel extract on serum—tissue damaged markers in the serum of alcohol intoxicated male albino rats

Parameter	Bilirubin [μmol.l^{-1}]	Urea [μmol.l^{-1}]	Uric acid [μmol.l^{-1}]	Albumin [μmol.l^{-1}]	Creatinine [mmol.l^{-1}]
Control (A)	11.17 ± 1.12^{c}	1.15 ± 0.10^{b}	0.11 ± 0.01^{b}	2.29 ± 0.11^{b}	99.46 ± 3.21^{b}
ALC (B)	15.54 ± 1.23^{d}	2.58 ± 0.13^{c}	0.15 ± 0.02^{c}	1.62 ± 0.10^{a}	108.2 ± 4.02^{c}
ALC + 125 mg A. V (C)	13.88 ± 1.10^{bc}	1.32 ± 0.11^{ab}	0.12 ± 0.01^{b}	1.70 ± 0.10^{a}	85.74 ± 2.10^{a}
ALC + 250 mg A. V (D)	4.40 ± 0.23^{a}	0.96 ± 0.01^{a}	0.04 ± 0.01^{a}	2.53 ± 0.13^{b}	86.95 ± 2.40^{s}
125 mg A. V (E)	5.18 ± 0.43^{a}	1.17 ± 0.10^{b}	0.08 ± 0.01^{a}	3.33 ± 0.25^{c}	101.4 ± 5.40^{b}
250 mg A. V (F)	8.78 ± 0.55^{b}	1.46 ± 0.14^{ab}	0.10 ± 0.01^{b}	3.79 ± 0.32^{c}	100.2 ± 6.10^{b}

These data are expressed as the mean ± SEM (n = 5). Values with different superscript letters along the column for each parameter differ significantly at P < 0.05; ALC + 125 mg A. V—alcohol + 125 mg.kg^{-1} body weight Aloe vera; ALC + 250 mg A. V—alcohol + 250 mg.kg^{-1} body weight Aloe vera; 125 mg A. V—125 mg.kg^{-1} body weight Aloe vera; 250 mg A. V—250 mg.kg^{-1} body weight Aloe vera

Table 6. Effects of Aloe vera gel on serum electrolytes levels of alcohol intoxicated male albino rats

Parameter	Serum electrolytes [mmol.l^{-1}]				
	Na^{+}	K^{+}	Cl^{-}	Mg^{2+}	Ca^{2+}
Control (A)	135.02 ± 0.06^{bc}	4.88 ± 0.42^{a}	91.51 ± 1.67^{ab}	0.68 ± 0.04^{b}	0.34 ± 0.04^{s}
ALC (B)	88.27 ± 0.79^{a}	6.37 ± 0.41^{c}	89.81 ± 1.27^{a}	0.33 ± 0.03^{a}	0.35 ± 0.07^{a}
ALC + 125 mg A. V (C)	120.94 ± 0.09^{ab}	5.88 ± 0.20^{bc}	94.26 ± 1.16^{ab}	0.63 ± 0.04^{b}	0.34 ± 0.04^{a}
ALC + 250 mg A. V (D)	97.45 ± 0.09^{a}	4.73 ± 0.45^{b}	93.56 ± 0.67^{ab}	0.57 ± 0.01^{b}	0.34 ± 0.03^{a}
125 mg A. V (E)	167.61 ± 0.26^{bb}	5.87 ± 0.41^{bb}	90.93 ± 2.14^{bb}	0.63 ± 0.04^{b}	0.34 ± 0.03^{b}
250 mg A. V (F)	175.10 ± 0.34^{d}	5.31 ± 0.28^{b}	95.40 ± 1.67^{b}	0.64 ± 0.03^{b}	0.35 ± 0.05^{a}

These data are expressed as the mean ± SEM (n = 5). Values with different superscript letters along the column for each parameter differ s ignificantly at P < 0.05; ALC + 125 mg A. V—alcohol + 125 mg.kg^{-1} body weight Aloe vera; ALC + 250 mg A. V—alcohol + 250 mg.kg^{-1} body weight Aloe vera; 125 mg A.V—125 mg.kg^{-1} body weight Aloe vera; 250 mg A. V—250 mg.kg^{-1} body weight Aloe vera

and LDH were reduced to a comparable level to the normal control (P < 0.05). No significant effect was observed in the specific activities of GST following the treatment with 125 mg and 250 mg.kg^{-1} body weight Aloe vera gel extract when compared to group A.

The effects of Aloe vera gel extract on serum: bilirubin, urea, uric acid, albumin and creatinine levels in alcohol intoxicated rat are depicted in Table 5. The administration of alcohol resulted in a significant increase in serum: bilirubin, uric acid, urea and creatinine concentrations, while a significant (P < 0.05) decrease was observed in the albumin level when compared to group A. Co-treatment with Aloe vera gel extract (groups C and D) caused a marked decrease when compared to group B with the exception of group C albumin concentration. More so, the administration of Aloe vera gel extract only (groups E and F) showed no significant difference in the concentration of creatinine when compared with group respectively.

The effects of Aloe vera gel extract on serum electrolytes levels of alcohol intoxicated rats are depicted in Table 6. Alcohol ingestion lowered the levels of serum Na^{+} and Mg^{2+}, while a marked increase was observed in K^{+} level in rats when compared to the control group (P < 0.05). No significant difference was observed in serum Ca^{2+} and Cl^{-} levels. However, co-treatment with Aloe vera gel extract (125 and 250 mg.kg^{-1} body weight) resulted in a marked increase in the serum Mg^{2+} and reduced the serum K^{+} levels as compared to the alcohol treated group (B). The administration of 125 mg and 250 mg.kg^{-1} body weight Aloe vera extract only (groups E and F), showed no significant

difference in the concentrations of magnesium and calcium as compared to control group (A) respectively.

DISCUSSION

The liver and kidney are two important organs which play important roles in physiological processes and excessive alcohol consumption damages these organs, and may lead to alterations in their metabolic activities [12, 23]. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH) are the most sensitive biomarkers directly implicated in the extent of tissue damage and toxicity [4, 24, 29, 32]. These enzymes are considered as indicators of liver and kidney malfunction and damage [16]. The leakages of these marker enzymes out from the necrotic hepatocytes into the blood stream in abnormal amounts are diagnostic of liver and kidney dysfunctions [4, 9, 20, 24]. More so, glutathione-S-transferase (GST) is an important enzyme that plays a crucial role in the detoxification and metabolism of many foreign and endobiotic compounds [17, 19].

This study demonstrated that, co-treatment with different concentrations (125 mg and 250 mg.kg^{-1} body weight) of Aloe vera gel extract significantly ($P < 0.05$) restored the elevated specific activities of serum: AST, ALT, ALP, LDH, GGT and GST, when compared to group administered alcohol only (group B). These restorative effects of Aloe vera on the specific activities of these tissue damage marker enzymes might be due to its hepato-renoprotective activities as reported by Cui et al. [8], Hussain et al. [16], Raksha et al. [25] and Saka et al. [28] which enable it to protect the liver and kidney against the deleterious effects of alcohol.

The significant decrease ($P < 0.05$) in the specific activities of ALT and ALP in the kidney observed in this study further supports reports that ethanol causes the leakage of the biomarker enzymes into the serum [4, 17]. Whereas, the elevated specific activities observed in the stomach of these biomarker enzymes might be due to the amount and duration of ethanol exposure which might influence the results as observed. The significant increase in the specific activities of the tissues (liver, kidney and stomach) GST might be the response of the enzyme to detoxify the toxic metabolites produced by alcohol [17]. The reduction observed in the specific activities of kidney and stomach GST as a result of Aloe vera gel co-administration, may be attributed to the anti-toxicity properties of bioactive substances in the extract which enable it to restore the tissues from the debilitating effects of alcohol.

Serum albumin is produced in the liver and it is the most abundant blood plasma protein which plays a key role in the transport of hormones, anaesthetics, endogenous ligands and free fatty acid [10], whereas, bilirubin is a breakdown product of haeme [31]. Both are recommended for the assessment of hepatobiliary injury in preclinical studies and are useful clinical clues to the severity of hepatic necrosis [31]. Creatinine is a waste product of creatine phosphate by muscle metabolism, while urea and uric acid which are excreted in urine are the breakdown products of protein and purine metabolism respectively [16]. The observed concentrations of serum bilirubin, creatinine, urea, uric acid as well as albumin in this study are consistent with previous reports [3, 31] that alcohol ingestion causes the accumulation of bilirubin, creatinine, urea, uric acid and decrease in albumin concentration in the serum. Co-treatment with 125 mg and 250 mg.kg^{-1} body weight Aloe vera gel however, significantly increased the serum albumin concentration and decreased the serum levels of bilirubin, creatinine, urea and uric acid to a comparable level to that of the controls. Modulations of serum bilirubin and albumin by Aloe vera gel extract might be due to the enhancement of the activity of the microsomal enzyme UDP-glucuronyl transferase (UDPGT), which is responsible for the conjugation reaction and thus controls hyperbilirubinaemia [31].

Hypomagnesaemia is the most common electrolyte disturbance in alcoholics and the mechanisms for development of hypomagnesemia in alcoholism include: increased transfer of magnesium from extracellular to intracellular fluid [1, 12]. This is usually due to respiratory alkalosis and increased magnesium excretion which may occur in hypophosphatemia that causes reduced magnesium reabsorption at the loop of Henle and the distal tubules [1]. The serum sodium (Na^+) level is determined by the balance of fluid in relation to the availability of sodium in the system [12]. Hyponatremia does not only constitute a biochemical abnormality but also has clinical consequences [12]. Normally the kidneys are a major route of potassium ion (K^+) excretion and serve as an important site of potassium regulation [6]. Alcohol consumption historically has been found to reduce the amount of potassium excreted by the

kidneys [6]. The levels of potassium, like those of sodium, also can affect the way the kidneys handle fluid elimination or retention and its depletion has been proposed to exacerbate hyponatremia [11]. Chloride as a major anion is important in the maintenance of the cation/anion balance between the intracellular and extracellular fluids and is also essential to the control of proper hydration, osmotic pressure and acid/base equilibrium [7]. The observed significant ($P < 0.05$) hypomagnesemia, hyponatremia and hyperkalemia ensued after alcohol administration in this study, were completely reversed back to normal following co-treatment with Aloe vera gel. The modulatory effect of this extract on the serum Na^+ level is consistent with the study of S a k a et al. [28] where Aloe vera gel extract elevated the serum Na^+ level in rats. Although, to the best of our knowledge, little or no report is known of the ameliorative effect of Aloe vera gel on alcohol induced hypomagnesemia. R a k s h a et al. [25] recognized the presence of Mg^{2+} in the Aloe vera gel extract as part of its phytochemical screening and this might be responsible for the reversal of hypomagnesemia. Moreover, the general restorative effects of the extract on alcohol induced electrolytes imbalances could be attributed to the bioactive substances present in the polysaccharides of the extract. These bioactive components are said to be responsible for Aloe vera gel therapeutic effects and could account for its modulatory ability to ameliorate electrolytes disturbances [4, 7].

CONCLUSIONS

This study demonstrated that Aloe vera gel (250 $mg.kg^{-1}$ body weight) could alleviate alcohol induced liver and kidney injury by decreasing the level of bilirubin, uric acid and urea and increasing the albumin level as well as lower the elevated marker enzymes and modulate the electrolytes disturbances. It is therefore suggested that Aloe vera gel could be used as part of decoction in the management of alcohol induced liver and kidney dysfunction.

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REFERENCES

1. **Adewale, A., Ifudu, O., 2014:** Kidney injury, fluid, electrolyte and acid-base abnormalities in alcoholics. *Nig. Med. J.*, 55, 2, 93—98. DOI: 10.4103/0300-1652.129631.
2. **Akinloye, D. I., Ugbaja, R. N., Dosumu, O. A., 2019:** Appraisal of the antioxidative potential of *Aloe barbadensis* M. on alcohol induced oxidative stress. *Folia Veterinaria*, 63, 3, 34—46. DOI: 10.2478/fv-2019-0025.
3. **Aldalou, A. R., Abdel-Aziz, I., Shahwan, O., 2013:** Effects of chromium on some haemato-biochemical parameters in domestic rabbits. *Int. J. Biol. Pharm. Res.*, 4, 847—854.
4. **Al-Shinnawy, M. S., Hassan, A. R., Ismail, D. A., Shahin, M. A., 2014:** The potential protective and therapeutic effects of Aloe vera juice on malathion induced hepatotoxicity in rabbits. *Egypt. J. Hosp. Med.*, 55, 146—158. DOI: 10.12816/0004500.
5. **Ashafa, A. O. T., Sunmonu, T. O., Abass, A. A., Ogbe, A. A., 2011:** Laxative potential of the ethanolic leaf extract of Aloe vera (L.) Burm. f. in Wistar rats with loperamide-induced constipation. *J. Nat. Pharm.*, 2, 3, 158—162. DOI: 10.4103/2229-5119.86268.
6. **Cecchin, E., De Marchi, S., 1996:** Alcohol misuse and renal damage. *Addict. Biol.*, 1, 1, 7—17. DOI: 10.1080/13556219610000124656.
7. **Chandini, P., 2017:** Serum electrolytes levels in patients with alcohol dependence syndrome. *J. Contemp. Med. Sci.*, 4, 5, 992—997.
8. **Cui, Y., Wang, H., Li, W., Yao, W., Qian, H., 2014:** Hepatoprotective potential of Aloe vera polysaccharides against chronic alcohol induced hepatotoxicity in mice. *J. Sc. Food Agric.*, 94, 1764—1771. DOI: 10.1002/jsfa.6489.
9. **Das, S. K., Vasudevan, D. M., 2008:** Alcohol induced effects on kidney. *Indian J. Clin. Biochem.*, 23, 1, 4—9. DOI: 10.1007/s12291-008-0003-9.
10. **Devi, V. S., Chidi, O. O., Coleman, D., 2009:** Dominant effect of ethanol in thermal destabilization of bovine serum albumin in the presence of sucrose. *Spectroscopy*, 23, 5—6, 263—270. DOI: 10.3233/SPE-2009-0400.
11. **Epstein, M., 1992:** Alcohol and the kidney. In **Lieber, C. S. (Ed.):** *Medical and Nutritional Complications of Alcoholism:*

Mechanisms and Management. Plenum Medical Book Company, New York, 495—513. DOI: 10.1007/978-1-4615-3320-7_16.

12. **Epstein, M., 1997:** Alcohol's impact on kidney function. *Alcohol, Health Res. World*, 21, 1, 84—93.
13. **Gopinathan, S., Naveenraj, D., 2013:** Gastroprotective and anti-ulcer activity of Aloe vera juice, papaya fruit juice and Aloe vera and papaya fruit combined juice in ethanol induced ulcerated rats. *Intl. J. Drug Dev. Res.*, 5, 4, 300—311.
14. **Habig, W., Pabst, M. J., Jakoby, W. B., 1974:** The first enzymatic step in mercapturic acid formation. Glutathione-S-transferase. *J. Biol. Chem.*, 249, 7130—7139.
15. **Heier, C., Hao, X., Robert, Z., 2016:** Nonoxidative ethanol metabolism in humans—from biomarkers to bioactive lipids. *IUBMB*, 68, 12, 916—923. DOI: 10.1002/iub.1569.
16. **Hussain, N., Chaudhary, M. N., Anjum, A. A., Abbas, N., Khan, M. N., Nadeem, S. M., 2016:** Investigating the ameliorative potential of *Aloe barbadensis* aqueous fraction on oxidative stress markers and biochemical parameters in cadmium intoxicated rats. *Pol. J. Environ. Stud.*, 25, 6, 2423—2433. DOI: 10.15244/pjoes/64239.
17. **Ighodaro, O. M., Omole, J. O., 2012:** Ethanol-induced hepatotoxicity in male Wistar rats: Effects of aqueous leaf extract of *Ocimum gratissimum*. *J. Med. Med. Sci.*, 3, 8, 499—505.
18. **Imaga, N. O. A., Ebuechi, O. A. T., Edom, J. J., Obomniru, G. E., Oyadina, O. O., 2016:** Evaluation of the therapeutic effects of Aloe vera gel on alloxan-induced diabetic rats. *Nig. Q. J. Hosp. Med.*, 26, 2, 377—386.
19. **Ji, X., Zhang, P., Armstrong, R. N., Gilliland, G. L., 1992:** The three-dimensional structure of a glutathione S-transferase from the mu gene class. Structural analysis of the binary complex of isoenzyme 33 and glutathione at 22 ANG. *Resolution. Biochem.*, 31, 42, 10169—10184. DOI: 10.1021/bi00157a004.
20. **Lee, H. I., McGregor, R. A., Choi, M. S., Seo, K. I., Jung, U. J., Yeo, J., et al., 2013:** Low doses of curcumin protect alcohol induced liver damage by modulation of the alcohol metabolic pathway, CYP2E1 and AMPK. *Life Sci.*, 93, 693—699. DOI: 10.1016/j.lfs.2013.09.014.
21. **Manvitha, K., Bidya, B., 2014:** Aloe vera: a wonder plant, its history, cultivation and medicinal uses. *J. Pharmacogn. Phytochem.*, 2, 5, 85—88.
22. **Ni, Y., Yates, K. M., Tizard, I. R., 2004:** Aloe polysaccharides. In **Reynolds, T., (Ed.):** *Aloes the Genus Aloe*. CRC Press, Boca Raton, USA, 75—87 pp.
23. **Niemela, O., 2007:** Biomarkers in alcoholism. *Clin. Chim. Acta*, 377, 39—49. DOI: 10.1016/j.cca.2006.08.035.
24. **Padmanabhan, P., Jangle, S. N., 2014:** Hepatoprotective activity of herbal preparation (HP-4) against D-Galactosamine induced hepatotoxicity in mice. *Intl. J. Pharm. Sci. Drug Res.*, 6, 1, 31—37. DOI: 10.3126/ijasbt.v2i1.9346.
25. **Raksha, B., Pooja, S., Babu, S., 2014:** Bioactive compounds and medicinal properties of Aloe vera L.: An update. *J. Plant Sci.*, 2, 3, 102—107. DOI: 10.11648/j.jps.20140203.11.
26. **Reddy, U., Reddy, C. H., Reddy, J., 2011:** Aloe vera — a wound healer. *AJOHAS*, 1, 91—92.
27. **Rukkumani, R., Aruna, K., Suresh, V. P., Menon, V. P., 2004:** Influence of ferulic acid on circulatory prooxidant. *J. Physiol. Pharmacol.*, 55, 3, 551—561.
28. **Saka, W. A., Akhigbe, R. E., Ishola, O. S., Ashamu, E. A., Olayemi, O. T., Adeleke, G. E., 2012:** Hepatotherapeutic effect of Aloe vera in alcohol induced hepatic damage. *J. Biol. Sci.*, 14, 14, 742—746. DOI: 10.3923/pjbs.2011.742.746.
29. **Saritha, V., Anikulamar, K. R., 2010:** Toxicological evaluation of methanol extract of Aloe vera in rats. *IJPBR*, 1, 5, 142—149.
30. **Scott, R. B., Reddy, K. S., Husain, K., Schlorff, E. C., Rybak, L. P., Somani, S. M., 2000:** Dose response of ethanol on antioxidant defence system of liver, lung, and kidney in rat. *Pathophysiol.*, 7, 1, 25—32. DOI: 10.1016/S0928-4680(99)00034-6.
31. **Senthilkumar, T, Asha, S. K., 2015:** Selective and sensitive sensing of free bilirubin in human serum using water-soluble Polyfluorene as fluorescent probe. *Macromol.*, 48, 11, 3449—3461. DOI: 10. 1021/acs.macromol.5b00043.
32. **Shanmugam, K. R., Ramakrishna, C. H., Mallikarjunaa, K., Sathyavelu, R. K., 2010:** Protective effect of ginger against alcohol induced renal damage and antioxidant enzymes in male albino rats. *Indian J. Exp. Biol.*, 48, 143—149.
33. **Shield, K. D., Charles, P., Jürgen, R., 2014:** Chronic diseases and conditions related to alcohol use. *Alcohol Res.: Current Reviews*, 35, 2, 155—173.
34. **Soni, H., Sharma, S., Singhai, A. K., 2011:** Qualitative and quantitative profile of aloin isolated from Aloe vera. *Int. Res. J. Pharm.*, 2, 9, 121—122.
35. **Talmadge, J., Chavez, J., Jacobs, L., Munger, C., Chinnah, T., Chow, J. T., 2004:** Fractionation of Aloe vera L. inner gel, purification and molecular profiling of activity. *Int. J. Immunopharmacol.*, 4, 1757—1773. DOI: 10.1016/j.intimp.2004.07.013.

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INFLUENCE OF DIFFERENT HERBAL-MIX FEED ADDITIVES ON SEROLOGICAL PARAMETERS, TIBIA BONE CHARACTERISTICS AND GUT MORPHOLOGY OF GROWING PIGS

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ABSTRACT

A sixteen-week study was carried out in order to investigate the effects of different herbal-mix feed additives (Moringa, Basil and Neem) on serological parameters, tibia bone characteristics and gut morphology of growing pigs. Eighty-one Large White breed of pigs with a mean weight of 15.35 ± 1.07 were grouped on weight equalization basis into three treatments with 9 replicates per treatment and 3 pigs per replicate in a completely randomized design. Pigs on treatment 1 were fed a ration without integration of herbal-mix feed additives, those in treatments 2 and 3 were offered a diet that contained moringa-basil leaf meal and neem-basil leaf meal respectively. Blood samples were collected from the experimental pigs on the 12th week of the study for the determination of serum levels, while 1 pig from each replicate group whose weight was close to the mean replicate weight was chosen at the end of the study, fasted for 12 hours, sacrificed and the small intestine and left tibia bone were harvested and used for gut and bone morphology evaluation. These data generated from the study were subjected to a 1-way analysis of variance (ANOVA). The results indicated that herbal-mix feed additives influenced some of the serological parameters, i.e., globulin, glucose, total cholesterol, high- and low-density lipoproteins, and aspartate aminotransferase. These parameters decreased with integration of different herbal-mix in the pigs' diets except high density lipoprotein that increased statistically. The tibia characteristics such as tibia breaking strength, medial thickness and lateral thickness were also positively impacted by the dietary inclusion of the herbal-mix. Likewise, the villus height of the jejunum was positively enhanced by dietary inclusion of the herbal-mix feed additives. This study concluded that herbal-mix feed additives can be utilized in pigs' nutrition in enhancing some serological parameters, tibia bone and gut characteristics of growing pigs.

Key words: blood indices; feed additives; growing pigs; small intestine

INTRODUCTION

The calls for the promotion of variable alternatives to modern health management practices need to be intensified as the rate of shortfalls in the supply of animal protein to the teeming world population continues to widen especially among resource-poor citizens of developing countries. The high cost and non-availability of chemically synthesized allopathic drugs among the local people that constitute the majority of the livestock farmers promote these shortfalls as pathogenic microbes prevails, leading to a high rate of mortality and morbidity [50]. It has been documented that about 80 % of livestock producers in developing countries depends on traditional medicines as a means of animal health management and disease control [28, 51]. Traditional medicine is an integral part of the ethnic group that have been passed from one generation to the next generation through practical experiences. The major advantages of this health practice are that they are cheap, readily available in pastoral areas and solve the problem of microbial resistance unlike commercial synthetic allopathic drugs. The development of resistant pathogenic microbes due to abuse of synthetic drugs, results in great concern over human health and environmental toxicity that leads to partial withdrawal and total ban in some parts of the world [42].

Most of Materia medica used in ethno-veterinary medicine is derived from plants [32]. The integration of herbal plants and their extracts into the ration of farm animals have been reported to enhance the productivity of the animal by improvement of the diet properties, quality of the produce from the animal, welfare of the animal and the general well-being of the animals [21]. Shahidi et al. [47] and Frankic et al. [10] promoted the prospects that herbs and spices with their extracts may serve as possible replacement of synthetic antibiotic growth enablers. Herbs and spices can be utilized as feed additives in livestock nutrition to improve feed intake, flavour and anti-oxidant properties due to the presence of multiple active ingredients and their mode of actions [9]. The integration of herbal plant parts in the form of leaf meal in the ration of livestock can assist in the reduction of feed cost, and total cost of production, thereby widening the profit margin of the rural livestock farmers. A study by Sugiharto et al. [50] revealed that leaf meal contains high protein that can be used for partial replacement of expensive conventional plant protein sources like soya bean meal, groundnut cake, cotton seed meals, etc., in the diets of farm animals, especially among the resource poor rural dwellers of developing countries. Hence, the incorporation of herbal leaf meal in the diets of pigs will not only lead to higher profitability to pig farmer but also promote the health of the pigs.

There is a need to investigate and validate the properties of medicinal plants that have been used in traditional medicine with antimicrobial properties which can be integrated easily into the ration of farm animal in order to reduce the microbial loads and feed cost, and increase the livestock productivity by inserting its growth promoting effects in the animals. To this end, this study was carried out in order to evaluate the effects of herbal-mix feed additives on serological parameters, tibia bone and gut morphology of growing pigs.

MATERIALS AND METHODS

Animal use and care

The study protocol was approved by Animal Welfare Committee guideline of the Federal University of Agriculture, Abeokuta, Nigeria [11].

Collection and preparation of herbal leaf blends

Fresh matured moringa (*Moringa oleifera*), basil (*Ocimum gratissimum*) and neem (*Azadirachta indica*) leaves were harvested within the environment of Federal University of Agriculture, Abeokuta. The leaves were separated from the stems, spread in the shade and allowed to dry for 3—5 days without direct exposure to sunlight and turned constantly to avert the growth of fungi until they were crispy when touched, while still maintaining their greenish coloration. The leaves were packaged in jute sacks, sealed and kept within room temperature. They were later transported to commercial feed mill where they were milled into leaf meals and integrated into the various experimental diets. Blends of moringa-basil and neem-basil were obtained from mixing 5 % moringa or neem with 1 % basil. Three diets were formulated to meet the nutrient requirements of pigs such that diet 1 had no herbal leaf meal additives, while diets 2 and 3 contained moringa-basil and neem-basil leaf meals, respectively. The diets were formulated to meet with the recommended nutrient requirements for growing pigs

as stipulated by NRC [37] with direct replacement of soybean meal and groundnut cake with equivalent herbal-mix leaf meals (weight for weight). The gross compositions and calculated analysis of the experimental diets are presented in Table 1.

Table 1. Percentage composition of experimental diets of growing pigs

Ingredients [%]	**Diet 1**	**Diet 2**	**Diet 3**
Maize	50	50	50
Palm kernel cake	10	10	10
Basil leaf	0	2	2
Moringa leaf	0	0	10
Neem leaf	0	10	0
Soya bean meal	18	8	8
Groundnut cake	9	7	7
Fish meal	2	2	2
Wheat offal	6.9	6.9	6.9
Bone meal	3	3	3
Lysine	0.3	0.3	0.3
Methionine	0.25	0.25	0.25
***Premix**	0.3	0.3	0.3
Salt (NaCl)	0.25	0.25	0.25
TOTAL	**100**	**100**	**100**
Calculated analysis			
Metabolizable energy ([kcal ME.kg^{-1}]	2834.59	2541.99	2805.9
Crude protein [%]	20.9	18.87	17.6
Ether extract [%]	4.56	4.84	4.49
Crude fibre [%]	4.68	6.4	5.56
Ash [%]	3.05	3.54	3.1

*Premix to supply the following per kg diets; vitamin A 12 600 IU; vitamin D_3 2800 IU; vitamin E 49 IU; vitamin K 32.8 mg; vitamin B_1 1.4 mg; vitamin B_2 5.6 mg; vitamin B_6 1.4 mg; vitamin B_{12} 0.014 mcg; niacin 21 mg; pantothenic acid 14 mg; folic acid 1.4 mg; biotin 0.028 mg; choline chloride 70 mg; manganese 70 mg; zinc 140 mg; iron 140 mg; copper 140 mg; iodine 1.4 mg; selenium 0.28 mg; cobalt 0.7 mg; antioxidant 168 mg

Study area

The study was carried out in 2019 at the piggery unit of the Teaching and Research Farm of the College of Animal Science and Livestock Production, Federal University of Agriculture, Abeokuta, Nigeria. The area lies between longitude 3°2' East and latitude 7°10' North [14]. The site is found in the derived savannah vegetation zone of South-Western Nigeria. The annual mean precipitation rate of about 1100 mm with optimum rainfall recorded in the months of June to September. The ambient temperature ranges from 28 °C in December to 36 °C in February with the annual mean of relative humidity of 82 %.

Experimental pigs and management

A total of eighty-one (81) Large White weaner pigs with a mean weight of 15.35 ± 1.07 kg were obtained from the existing stock in the Teaching and Research Farm of Federal University of Agriculture, Abeokuta. The pigs were grouped into 3 treatments based on the weight equalization method and later subdivided into 9 replicates of 3 pigs per replicate. The pigs were housed in individual pens with floor dimensions of 2 m by 2 m and equipped with concrete feeders and drinkers. The pens were open sided dwarf-walled of about 1.4—1.6 m high, which allowed for the adequate movement of air. All good management practices applicable to pigs were adhered to strictly.

DATA COLLECTION

Serum parameters

Blood samples were collected from 9 pigs per treatment (1 pig per replicate) on the 12th week of the study and used for serological measurements. Serum was obtained after blood in plane sample bottle was allowed to stand for 2 hours at room temperature and centrifuged at 2 000 r. p. m. for 10 minutes. The total serum protein was determined by the Biuret method and serum cholesterol was assessed by the enzymatic colorimetric technique [38]. The serum albumin was analysed by the colorimetric method according to Douma and Brigs [7]. The globulin was estimated as the difference between the total serum protein and albumin. Serum glucose was estimated using a commercial glucose colorimetric assay kit (Cayman® Chemical Company, Ann Arbor, USA) and the protocol was followed according to the manufacturer's recommendation.

Gut morphology

At the end of the sixteenth week of the experiment, a total of twenty-seven (27) pigs consisting of nine (9) pigs per treatment whose weight were close to the replicate weight were selected, slaughtered and the small in-

testines were removed for gut morphology. The pigs were weighed and fasted for 12 hours, and the fasted weight of each pig was taken before they were stunned by the percussion method and bled by incision using a sharp knife cutting through the jugular vein between the skull and the atlas. The complete bleeding and dehairing were done. The stomach of the pig was opened along the greater curvature and emptied. The digestive tract was removed and sections of the duodenum (approximately 10 cm from the stomach sphincter), the jejunum (5.5 m from the stomach sphincter), and the ileum (10 cm prior to the ileo-caecal orifice) were excised and opened along their length at the mesenteric border and a portion of each section was cut and put in a universal bottle containing 10 % formalin and embedded in paraffin wax. These tissue samples were cut in transverse section in 5 µm thick slices and were stained with haematoxylin and eosin. The villus height and width, were measured at 50× magnification using a calibrated eyepiece graticule earlier outlined by H a l a s et al. [16].

Tibia bone morphology

The left tibia bones of the pigs slaughtered and used for gut evaluation were collected and the adhering muscles and connective tissues were manually removed after they were dipped in boiling water for 5 minutes. The tibia length, medial thickness, and lateral thickness were determined with the aid of Vernier calliper, while the wet tibia bone weight and dry tibia bone weight were determined with the aid of a sensitive scale. While loss in tibia weight was obtained as difference between wet tibia weight and dry tibia weight. The bone breaking strength was determined using instron materials tester.

RESULTS

Effects of herbal-mix feed additives on serological parameters of growing pigs

Table 2 shows the effects of herbal-mix feed additives on serum parameters; globulin, glucose, total cholesterol, high density lipoprotein, low density lipoprotein and aspartate amino transferase were significantly ($P < 0.05$) influenced by the herbal-mix feed additives. Pigs on diet without herbal-mix feed had the highest globulin value of 3.23 $g.dl^{-1}$ followed by 3.08 $g.dl^{-1}$ documented for pigs on neem-basil leaf meal supplemented ration while the least value of 2.43 $mg.dl^{-1}$ was recorded for pigs on moringa-basil leaf meal blend. Growing pigs on the control ration recorded the highest serum glucose value of 102.00 $mg.dl^{-1}$, followed by 92.83 $mg.dl^{-1}$ noted for pigs on the moringa-basil dietary treatment, while those on the ration containing neem-basil leaf meal had the least glucose

Table 2. Effects of herbal-mix feed additives on serological parameters of growing pigs

Parameters	No herbal-mix	Moringa-basil	Neem-basil
Total protein [$g.dl^{-1}$]	7.00 ± 0.32	5.77 ± 0.85	6.90 ± 0.27
Albumin [$g.dl^{-1}$]	3.77 ± 0.23	3.33 ± 0.57	3.82 ± 0.14
Globulin [$g.dl^{-1}$]	3.23 ± 0.23^{a}	2.43 ± 0.32^{b}	3.08 ± 0.24^{ab}
Glucose [$mg.dl^{-1}$]	102.00 ± 0.77^{a}	92.83 ± 2.29^{b}	86.67 ± 1.63^{c}
Urea [$mg.dl^{-1}$]	5.43 ± 0.49	5.70 ± 0.38	5.23 ± 0.15
Creatinine [$mg.dl^{-1}$]	0.63 ± 0.67	0.78 ± 0.13	0.82 ± 0.90
Total cholesterol [$mg.dl^{-1}$]	94.83 ± 1.56^{a}	86.83 ± 1.30^{b}	85.67 ± 3.21^{b}
Triglycerides [$mg.dl^{-1}$]	101.17 ± 2.15	97.83 ± 1.58	103.17 ± 2.67
High density lipoprotein [$mg.dl^{-1}$]	40.68 ± 1.43^{b}	52.47 ± 0.86^{a}	45.73 ± 3.59^{b}
Low density lipoprotein [$mg.dl^{-1}$]	24.97 ± 1.42^{a}	21.17 ± 1.21^{ab}	18.93 ± 1.28^{b}
Very low density lipoprotein [$mg.dl^{-1}$]	20.78 ± 0.41	19.53 ± 0.58	19.83 ± 0.68
Aspartate aminotransferase [$\mu U.l^{-1}$]	41.83 ± 1.40^{a}	40.33 ± 1.23^{a}	33.33 ± 0.95^{b}
Alanine aminotransferase [$\mu U.l^{-1}$]	23.17 ± 1.28	22.92 ± 2.10	21.50 ± 2.14

$^{a, b, c}$—Means with different superscript across the row differed significantly ($P < 0.05$)

level of 86.67 mg.dl^{-1}. Pigs on a diet without herbal-mix feed additive had the highest significant total cholesterol value of 94.83 mg.dl^{-1}, while pigs on the ration containing moringa-basil and neem-basil leaf blends had comparable mean values of 86.83 mg.dl^{-1}, respectively. The high-density lipoprotein was significantly ($P < 0.05$) influenced by the herbal-mix feed additives with pigs on the ration containing moringa-basil feed additive had the highest value of 52.47 mg.dl^{-1} and comparable mean high density lipoprotein values of 40.68 and 45.73 mg.dl^{-1} were recorded for the pigs on the control diets and neem-basil leaf meals.

Effects of herbal-mix feed additive on tibia bone morphology and strength of growing pigs

Table 3 shows the effects of herbal-mix feed additives on tibia bone morphology and strength of growing pigs. Bone medial thickness, lateral thickness and bone breaking strength were significantly ($P < 0.05$) influenced by

Table 3. Effects of herbal-mix feed additive on tibia bone characteristics of growing pigs

Parameters	No herbal-mix	Moringa-basil	Neem-basil
Wet tibia bone weight [g]	97.84 ± 5.50	99.24 ± 8.22	99.24 ± 6.35
Dry tibia bone weight [g]	95.13 ± 3.34	96.53 ± 4.11	96.64 ± 4.54
Loss in tibia bone weight [g]	2.71 ± 0.18	2.71 ± 0.91	2.60 ± 0.16
Tibia bone length [cm]	15.38 ± 0.31	15.03 ± 0.57	15.55 ± 0.32
Medial thickness [mm]	26.72 ± 1.32^{a}	21.92 ± 1.91^{b}	22.10 ± 0.99^{b}
Lateral thickness [mm]	43.42 ± 1.84^{a}	40.39 ± 1.93^{ab}	38.02 ± 1.70^{b}
Ash weight [g]	34.17 ± 3.00	32.09 ± 3.14	27.86 ± 0.43
Bone breaking force [N]	20.02 ± 0.73^{a}	17.52 ± 0.28^{b}	13.55 ± 1.14^{c}

$^{a, b, c}$—Means with different superscript across the row differed significantly ($P < 0.05$)

Table 4. Effects of herbal-mix feed additives on the gut morphology of growing pigs

Parameters	No herbal-mix	Moringa-basil	Neem-basil
	Duodenum [µm]		
Villus height	667.67 ± 46.09	691.67 ± 30.05	608.33 ± 39.62
Apical width	30.83 ± 1.54	29.83 ± 1.17	35.00 ± 2.58
Basal width	103.33 ± 6.15	95.83 ± 5.54	110.83 ± 4.90
Lamina propria depth	251.67 ± 14.00	236.67 ± 13.82	271.67 ± 21.51
	Jejunum [µm]		
Villus height	595.00 ± 15.86^{b}	641.67 ± 30.05^{ab}	695.00 ± 27.89^{a}
Apical width	42.50 ± 1.71	39.83 ± 2.95	40.00 ± 2.24
Basal width	115.00 ± 6.19	107.50 ± 10.47	115.83 ± 5.23
Lamina propria depth	240.00 ± 14.38	233.33 ± 12.56	238.33 ± 14.00
	Ileum [µm]		
Villus height	686.67 ± 26.03	606.67 ± 29.06	715.00 ± 43.57
Apical width	36.67 ± 3.07	38.33 ± 2.11	39.17 ± 2.39
Basal width	103.33 ± 6.15	116.67 ± 6.15	105.00 ± 7.53
Lamina propria depth	240.00 ± 15.28	256.67 ± 24.04	256.67 ±22.61

$^{a, c}$—Means with different superscript across the row differed significantly ($P < 0.05$)

the herbal-mix feed additives. Pigs on the diet without herbal-mix feed additive had the highest breaking force of 20.02 N followed by 17.52 N documented for pigs on the moringa-basil leaf meal supplemented ration while the least value of 13.55 N was recorded for pigs on the ration containing neem-basil leaf meal. The growing pigs on the control ration recorded the highest medial thickness of 26.72 mm followed by 22.10 mm documented for pigs on the neem-basil, while those on the ration containing moringa-basil leaf blend had the least medial thickness of 21.92 mm. The highest lateral thickness (43.42 mm) was recorded for pigs on the diet with no herbal leaf meal while those on neem-basil leaf meal supplemental diet had the least lateral thickness of 38.02 mm.

Effects of the herbal-mix feed additives on the gut morphology of growing pigs

The effects of the dietary inclusion of the herbal-mix on gut morphology of growing pigs is shown in Table 4. The dietary inclusion of the herbal-mix feed additive had no significant effect in all gut morphological parameters considered in the present study except for the jejunum villus height. Growing pigs subjected to dietary inclusion of the neem-basil blend recorded the highest jejunum villus height followed by their counterparts on the ration that contained moringa-basil leaf meal while those on the diet that had no herbal-mix feed additive recorded the least villus height of 595.00 μm.

DISCUSSION

The health conditions of farm animals like pigs can be estimated from the blood parameters. The blood indices indicate the physiological state and nutritional status of the pig's body. The result of this study revealed variations in the dietary crude protein fed to the growing pigs. This must have resulted due to the significant lower globulin values recorded for pigs on diets containing herbal leaf blends which had lower dietary crude protein contents with range values of 17.60 % to 18.87 % compared to 20.90 % of the control ration. The lower dietary crude protein content of the ration with herbal-mix feed additives must have resulted to deficit intake of amino acids [6]. According to the observation of Awosanya et al. [4], the level of blood protein is contingent upon the quality and quantity of the dietary protein intake of the farm animal. The observation of this study is consistent with the studies of Attia et al. [3] and Liu et al. [27] that reported significantly lower plasma total protein and globulin levels with decreasing levels of the dietary crude protein content. In contrast, Mahmoud et al. [29] observed a decrease in alpha-globulin and albumin to globulin ratio with increasing levels of dietary crude protein contents of laying birds. These differences in this present result with that of Mahmoud et al. [29] may be due to genotype or genotype x diet interactions [30].

The inclusion of herbal leaf blend in the ration of pigs had remarkable serum glucose reduction in pigs when compared with the value noted for pigs on the control ration. This reduction in the serum glucose points to the presence of hypoglycaemic properties of the herbal plants. According to Kappel et al. [20] and Oleforuh-Okoleh et al. [39] the presence of phytochemical compounds in leaf meal enhances the carbohydrate metabolism of farm animals. Sauvaire et al. [45] and Schryver [46] linked the reduction of serum glucose in animals fed fenugreek seed to direct S-cells production that secrete secretin from the duodenum and jejunum which stimulate exocrine pancreatic secretion of amino acid (4-hydroxy isoleucine) that improves insulin production, thereby resulting to higher glucose tolerance levels.

Our results showed a significant reduction in total cholesterol and low-density lipoprotein, but increased the high-density lipoprotein. The significant lower total cholesterol and low-density lipoprotein levels in pigs fed rations containing herbal-mix feed additives compared with the value documented for their counterparts on control diet, could indicate the presence of hypolipidemic agent in the leaf meal. It has been reported that moringa has high phytosterol that reduces the cholesterol contents of meat, egg and serum [19]. It contains bioactive compounds like flavonoids and phenolic compounds with antioxidant potentials that assist in the synthesis of bile salts needed for fats emulsification and the reduction in lipids absorption thereby decreasing serum cholesterol [19, 36, 49]. Also, Ghazaiah and Ali [12] associated the hypocholesterol properties in animals fed diets containing leaf meals to defatted components of the leaves with high fibre contents that can obstruct intestinal cholesterol absorption.

Serum enzymes like Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) are involved in the

metabolism of amino acid and can be used in determining the functionality status of liver [13]. Our study showed a significant reduction in the serum AST levels of the pigs on herbal-mix feed additives compared to the results obtained for the pigs on the control diet. This result implies that the herbal-mix feed additives exerts a protective influence on the liver without inducing visible toxicity. Choudhury et al. [5] observed that herbal extracts reduced liver enzymes (Alanine transaminase and Aspartate transaminase) in the plasma and attributed this fact to the existence of numerous hepatoprotective constituents in the plants.

The state of the bone is unswervingly linked to the bioavailability of calcium and phosphorus levels. The commonest indices utilized in determining the bioavailability of calcium and phosphorus are the ash contents and bone breaking strength [48]. Our study showed that the medial thickness, lateral thickness and breaking strength of the bone decreased statistically with the integration of different herbal-mix feed additive; with the most impact observed in pigs on ration containing blends of neem-basil. The decreasing tibia morphological values with different integration of blends of herbal plants suggests poor mineralisation of the bone due to the presence of anti-nutritional factors that must have stalled protein synthesis, digestion and mineral absorption in the digestive tract of the animal [31] resulting in the increased excretion of calcium from its body [41]. Another possible explanation for the decreasing tibia values as observed in our study could be linked to the quantity of crude protein available in the diets fed to the pigs which appeared to be least in the ration with neem-basil leaf blends as depicted by the calculated composition of the experimental diets. The health status of the bone can be assessed through bone length, weight and the percentage of ash content [52], which in turn is subject to the mineral composition of the bone [15]. Heaney and Layman [18] depicted that many factors have been identified to impact the health of bone; among them are dietary protein content and the source of the protein which could have positive or negative impact on: the bone, calcium intake, weight loss and acid/base of the diet. High dietary protein intake has been said to have an effect on calcium homeostasis which can influenced the excretion of calcium. Albright et al. [2] as quoted by Heaney and Layman [18] articulated that diets with insufficient protein results in a negative nitrogen balance that could have impaired the possibility of osteoblasts to deposit the necessary organic matrix required in bone formation. The authors argued that some of the osteopathies linked with deficiencies of calcium and phosphorus in the ration of farm animals could have resulted from dietary protein shortage rather than the minerals. Leach and Lilbum [23] observed that long bones like the tibia experience cellular sensitivity to multiple dietary inadequacies. The statistical differences noted in the tibia bone breaking strength which reduced with dietary integration of herbal-mix feed additives is at variance with the study of Nkukwana et al. [35] that observed comparable means in bone breaking strength of broiler chickens fed diets with different inclusion levels of *Moringa oleifera* leaf meals. On the other hand, our study concurred with the summation of Rehman et al. [43] that dietary inclusion of herbal plants improved the indices of tibia bone of broiler chickens. The statistical variation in bone breaking strength in our study could be linked to the presence of caffeic acid in herbal plants used in the formulation of the diets fed to the pigs which must have resulted in higher osteoporosis that leads to less tensile strength of the bone, thereby requiring less breaking force [43].

Digestion and absorption of nutrients taken into the body of farm animals, occurs mostly in the small intestine of the animal. From the study of Ruttanavut et al. [44], it was stated that the growth rate and intestinal performance were a result of the functionality of the intestinal villus and epithelial cell structures. Likewise, Mekbungwan et al. [33] and Pappenheimer and Michel [40] stated that there is a positive correlation between the height of the villi and the rate of intestinal digestibility and absorption of nutrients. The statistical higher difference noted in the jejunum villus height of pigs on rations containing herbal-mix feed additives points to the fact that herbal plants contained an active ingredient that could have initiated cell proliferation [17, 22]; hence, improving the absorptive capacity of the intestine by increasing the surface area for nutrient absorption. Liu et al. [26] affirmed that the integration of *Capsicum oleoresin*, *Turmeric oleoresin* or garlic in the diet of weanling pigs improved the gut health by increasing intestinal villi height, gut barrier function, and integrity. Likewise, Michiels et al. [34] concluded that the inclusion of carvacrol and thymol in pig's rations led to a decline in the number of intra-epithelial lymphocytes and improvement in the villus height/crypt depth in the distal

small intestine. The improvement in the jejunum height could also be linked to higher fibre content in the diets with range values of 4.68—6.40 %; higher values were obtained in diets with herbal-mix feed additives which must have enhanced the gut health of the pigs. Le Sciellour [24] surmised that there is an increasing evidence indicating that fibre could have prebiotic properties in pigs as a result of its interaction with gut micro-environment and the associated gut immune systems. Lindberg [25] listed the merits of dietary fibre which includes its ability to stimulate gut health, increase the satiety in pigs, and affect pig behaviour and general well-being of the pigs. Fang et al. [8] and Adebiyi et al. [1] reported statistical increases in villus height in animals on dietary inclusion of photogenic plant parts.

CONCLUSIONS

Our study showed that dietary inclusion of different herbal-mix feed additives had an impact on serum parameters (Globulin, glucose, total cholestero, high- and low-density lipoproteins and aspartate aminotransferase), tibia morphometric (medial and lateral thickness and bone breaking strength) and jejunum villus height of growing pigs. It is therefore recommended that for sustainable food security in developing countries of the world, herbal-mix feed additives should be integrated in the ration of growing pig in order to enhance their general well-being and as well as reduce the cost of feeding among rural livestock keepers; however, caution must be taken as it can lead to poor bone mineralization at higher inclusion levels.

REFERENCES

1. **Adebiyi, O. A., Ajayi, O. S., Adejumo, I. O., Osungade, T. O., 2014:** Performance, microbial load and gut morphology of weaned pigs fed diets supplemented with turmeric, ginger and garlic extracts. *Trop. Anim. Prod. Invest.*, 17, 1, 25—31.
2. **Albright, F., Smith, P. H., Richardson, A. M., 1941:** Postmenopausal osteoporosis: Its clinical features. *Am. Med. Assn.*, 116, 2465—2474.
3. **Attia, Y. A., Bovera, F., Abd-El-Hamid, A. E., Tag EL-Din, A. E., Al-Harthi, M. A., Nizza, A., Elharidy, R. M., 2017:** Effect of dietary protein concentrations, amino acids and conjugated linoleic acid supplementations on productive performance and lipid metabolism of broiler chicks. *Ital. J. Anim. Sci.*, 16, 4, 563—572. DOI: 10.1080/1828051X.2017.1301228.
4. **Awosanya, B., Joseph, J. K., Apata, D. F., Agboola, M. A., 1999:** Performance, blood chemistry and carcass quality attribute of rabbits fed raw and processed pueraria seed meal. *Trop. J. Anim. Sci.*, 2, 2, 89—96. DOI: 10.4314/tjas.v212.49687.
5. **Choudhury, S., Rahaman, C. H., Mandal, S., 2009:** Studies on leaf epidermal micromorphology, wood element characters and phytochemical screening of three medicinally important taxa of the family *Convolvulaceae*. *J. Environ. Sociobiol.*, 6, 2, 105—118. DOI: 10.1055/s-0029-1235011.
6. **Corzo, A., Loar II, R. E., Kidd, M. T., 2009:** Limitations of dietary isoleucine and valine in broiler chick diets. *Poult. Sci.*, 88, 1934—1938. DOI: 10.3382/ps.2009-00109.
7. **Douma, B. T., Briggs, H. G., 1972:** Serum albumin by bromocresol green binding stand. *Meth. Clin. Chem.*, 7, 175—179.
8. **Fang, J., Yan, F. Y., Kong, X. F., Ruan, Z., Liu, Z. Q., Huang, R. L., Li, T. J., Geng, M. M., et al., 2009:** Dietary supplementation with *Acanthopanax senticosus* extract enhances gut health in weaning piglets. *Livest. Sci.*, 123, 268—275. DOI: 10.1016/j.livsci.2008.11.020.
9. **Figen, K., Bora-Unlu, H., Guven, O., 2011:** Effects of oregano and garlic essential oils on performance, carcass, organ and blood characteristics and intestinal microflora of broilers. *Livest. Sci.*, 137, 219—225. DOI: 10.1016/j.livsci.2010.11.010.
10. **Frankic, T., Voljg, M., Salobir, J., Rezar, V., 2009:** Importance of medical herbs in animal feeding: A review. *Acta. Agric. Slovenica*, 92, 95—95.
11. **FUNAAB, 2013:** *Policy on Research of the Federal University of Agriculture*. Abeokuta. Nigeria. http://www.unaab.edu.ng (04/06/2016).
12. **Ghazalah, A. A., Ali, A. M., 2008:** Rosemary leaves as a dietary supplement for growth in broiler chickens. *Intern. J. Poult. Sci.*, 7, 234—239. DOI: 10.3923/ijps.2008.234.239.
13. **Giannini, E. G., Testa, R., Savarino, V., 2005:** Liver enzyme alteration: a guide for clinicians. *Canad. Med. Assn. J.*, 172, 3, 367—379. DOI: 10.1503/cmaj.1040752.
14. **Google Earth, 2018:** http;//www.google.com.
15. **Hafeez, A., Mader, A., Boroojeni, F. G., Ruhnke, I., Röhe, I., Männer, K., Zentek, J., 2014:** Impact of thermal and organic acid treatment of feed on apparent ileal mineral absorption, tibial and liver mineral concentration, and tibia quality in broilers. *Poult. Sci.*, 93, 1754—1763. DOI: 10.3382/ps.2013-03750.

16. **Halas, D., Hansen, C. F., Hampson, D. J., Kim, J. C., Mullan, B. P., Wilson, R. H., Pluske, J. R., 2010:** Effects of benzoic acid and inulin on ammonia-nitrogen excretion, plasma urea levels, and the pH in faeces and urine of weaner pigs. *Livest. Sci.*, 134, 243—245. DOI: 10.1016/j.livsci.2010.06.153.
17. **Hanczakowska, E., Swiatkiewicz, M., 2012:** Effect of herbal extracts on piglet performance and small intestinal epithelial villi. *Czech J. Anim. Sci.*, 57, 9, 420—429. DOI: 10.17221/6316-CJAS.
18. **Heaney, R. P., Layman, D. K., 2008:** Amount and type of protein influences on bone health. *Am. J. Clin. Nutr.*, 87 (Supplement), 1567—1570. DOI: 10.1093/ajcn/87.5.15675.
19. **Hussain, W., Ullah, A., Hussain, J., Hussain, S., Shinwari, Z. K., Ibrar, M., 2014:** Ethno-medicinal plants of Tahsil Barawal Bandi Dir Upper Khyber Pakhtunkhwa Pakistan. *J. Appl. Pharma. Sci.*, 4, 7, 94—97. DOI: 10.7324/JAPS.2014.40716.
20. **Kappel, V. D., Zanatta, L., Postal, B. G., Silva, F. R. M. B., 2013:** Rutin potentiates calcium uptake via voltage-dependent calcium channel associated with stimulation of glucose uptake in skeletal muscle. *Arch. Biochem. Biophys.*, 532, 55—60. DOI: 10.1016/j.abb.2013.01.008.
21. **Kołodziej-Skalska, A., Rybarczyk, A., Matysiak, B., Jacyno, E., Pietruszka, A., Kawęcka, M., 2011:** Effect of dietary plant extracts mixture on pork meat quality. *Acta Agric. Scand. Sect. A. Anim. Sci.*, 6, 80—85. DOI: 10.1080/09064702.2011.599860.
22. **Lauronen, J., Pakarinen, M. P., Kuusanmakai, P., Savilahti, E., Vento, P., Paavonen, T., Halttunen, J., 1998:** Intestinal adaptation after massive proximal small-bowel resection in the pig. *Scand. J. Gastroentero.*, 33, 152—158. DOI: 10.1080/00365529850166879.
23. **Leach Jr., R. M., Lilburn, M. S., 1992:** Current knowledge on the etiology of tibial dyschondroplasia in the avian species. *Poult. Sci. Rev.*, 4, 57—65.
24. **Le Sciellour, M., Labussière, E., Zemb, O., Renaudeau, D., 2018:** Effect of dietary fibre content on nutrient digestibility and faecal microbiota composition in growing-finishing pigs. *PLOS ONE*, 13, 10, e0206159. DOI: 10.1371/journal.pone.0206159.
25. **Lindberg, E., 2014:** Fibre effects in nutrition and gut health in pigs. *J. Anim. Sci. Biotech.*, 5, 1, 15—23. DOI: 10.1186/2049-1891-5-15.
26. **Liu, Y., Song, M., Che, T. M., Almeida, J. A. S., Lee, J. J., Bravo, D., et al., 2013:** Dietary plant extracts alleviate diarrhoea and alter immune responses of weaned pigs experimentally infected with a pathogenic *Escherichia coli*. *J. Anim. Sci.*, 91, 11, 5294—5306. DOI: 10.2527/jas.2012-6194.
27. **Liu, S. K., Niu, Z. Y., Min, Y. N., Wang, Z. P., Zhang, J., He, Z. F., et al., 2015:** Effect of dietary crude protein on the growth performance, carcass characteristics and serum biochemical indices of Lueyang black-boned chickens from seven to twelve weeks of age. *Rev. Bras. de Cienc. Avic.*, 17, 1, 103—108. DOI: 10.1590/1516-635x1701103-108.
28. **Luseba, D., Tshisikhawe, M. P., 2013:** Medicinal plants used in the treatment of livestock diseases in Vhembe region, Limpopo Province, South Africa. *J. Med. Plants Res.*, 7, 10, 593—601. DOI: 10.5897/JMPR012.1213.
29. **Mahmoud, A., Mohamed, E. A. E., Mayada, R. F., Ruchi, T., Swan, S., Kumaragurubaran, K., Kuldeep, D., 2016:** Positive and negative impacts of dietary protein levels in laying hens. *Asian J. Anim. Sci.*, 10, 2, 165—174. DOI: 10.3923/ajas.2016.165.174.
30. **Marks, H. L., 1990:** Genotype by diet interactions in body and abdominal fat weight in broilers. *Poult. Sci.*, 69, 879—886. DOI: 10.3382/ps.0690879.
31. **Mbikay, M., 2012:** Therapeutic potential of *Moringa oleifera* leaves in chronic hyperglycemia and dyslipidaemia: A review. *Front. Pharmacol.*, 3, 1—12. DOI: 10.3389/fphar.2012.00024.
32. **McCorkle, C. M., 1986:** An introduction to ethno-veterinary research and development. *J. Ethno-biol.*, 6, 129—149.
33. **Mekbungwan, A., Yamauchi, K., Sakaida, T., Buwjoom, T., 2008:** Effects of a charcoal powder-wood vinegar compound solution in piglets for raw pigeon pea seed meal. *Animal*, 2, 366—374. DOI: 10.1017/S1751731107001243.
34. **Michiels, J., Missotten, J., Van Hoorick, A., Ovyn, A., Fremaut, D., De Smet, S., Dierick, N., 2010:** Effects of dose and formulation of carvacrol and thymol on bacteria and some functional traits of the gut in piglets after weaning. *Arch. Anim. Nutr.*, 64, 136—154. DOI: 10.1080/17450390903499915.
35. **Nkukwana, T. T., Muchenje, V., Masika, P. J., Hoffman, L. C., Dzama, K., 2014:** The effect of *Moringa oleifera* leaf meal supplementation on tibia strength, morphology and inorganic content of broiler chickens. *South African J. Anim. Sci.*, 44, 3, 228—239. DOI: 10.4314/sajas.v44i3.4.
36. **Nobakht, A., Mogaddam, M., 2013:** The effects different levels of customary (*Tanacetum balsamita*) medicinal plant on performance, egg traits and blood biochemical parameters of laying hens. *Iranian J. Appl. Anim. Sci.*, 3, 307—312. http://ijas.iaurasht.ac.ir/article_516037_0f15ee00f75e1215b288c-faa589a7253.pdf.
37. **National Research Council (NRC) 1998:** *Nutrient Requirement of Swine* (10th Revised Edn.). Subcommittee on Animal Nutrition, National Research Council, 210 pp.

38. Odoh, L. I., Bratte, L., 2015: Effect of varying level of neem (*Azadirachta indica*) leaf meal in layer diet on the haematological and serological indices and faecal bacterial counts of layers. *J. Natural Sci. Res.*, 5, 4, 37—44. https://core.ac.uk/download/pdf/234655629.pdf.

39. Oleforuh-Okoleh, V. U., Ndofor Foleng, H. M., Olorunleke, S. O., Uguru, J. O., 2015: Evaluation of growth performance, haematological and serum biochemical response of broiler chickens to aqueous extract of ginger and garlic. *J. Agric. Sci.*, 7, 4, 167—173. DOI: 10.5539/jas.v7n4p167.

40. Pappenheimer, J. R., Michel, C. C., 2003: Role of villus microcirculation in intestinal absorption of glucose: Coupling of epithelial with endothelial transport. *J. Physiol.*, 553, 2, 561—574. DOI: 10.1113/jphysiol.2003.043257.

41. Parikh, P., Patel, C., Rangrez, A., 2015: Osteoprotective effect of different components of *Moringa oleifera* in ovariectomy induced osteoporosis model of Wistar rats. *Europ. J. Biomed. Pharma. Sci.*, 2, 3, 1179—1196.

42. Rajput, Z. I., Xiao, C. W., Hu, S. H., Arijo, A. G., Soomro, N. M., 2007: Improvement of the efficacy of influenza vaccination (H5N1) in chicken by using extract of *Cochinchina momordica* seed (ECMS). *J. Zhejiang University Sci. B.*, 8, 331—337. DOI: 10.1631/jzus.2007.B0331.

43. Rehman, H. F., Masood, S., Yousaf, M. S., Ashraf, S., Khan, I., Shah, M., et al., 2018: Effect of *Moringa oleifera* leaf powder supplementation on pectoral muscle quality and morphometric characteristics of tibia bone in broiler chickens. *Braz. J. Poult. Sci.*, 20, 4, 817—824. DOI: 10.1590/1806-9061-2017-0609.

44. Ruttanavut, J., Yamauchi, K., Goto, H., Erikawa, T., 2009: Effects of dietary bamboo charcoal powder including vinegar liquid on growth performance and histological intestinal change in Aigamo ducks. *Inter. J. Poult. Sci.*, 8, 229—236. DOI: 10.3923/ijps.2009.229.236.

45. Sauvaire, Y., Petit, P., Broca, C., Manteghetti, M., Baissac, Y., Fernandez-Alvarez, J., et al., 1998: 4-Hydroxyisoleucine, a novel amino acid potentiator of insulin secretion. *Diabetes*, 47, 206—210. DOI: 10.2337/diab.47.2.206.

46. Schryver, T., 2002: Fenugreek. *Total Health*, 24, 42—44.

47. Shahidi, F., Janitha, P. K., Wanasundara, P., 1992: Phenolic antioxidants. *Crit. Rev. Food Sci. Nutr.*, 32, 67—102. DOI: 10.1080/10408399209527581.

48. Shaw, A. L., Blake, J. P., Moran, E., 2010: Effects of flesh attachment on bone breaking and of phosphorus concentration on performance of broilers hatched from young and old flocks. *Poult. Sci.*, 89, 295—302. DOI: 10.3382/ps.2009-00402.

49. Srinivasan, K., 2005: Plant foods in the management of *diabetes mellitus*: Spices as beneficial antidiabetic food adjuncts. *Inter. J. Food Sci. Nutr.*, 56, 399—414. DOI: 10.1080/0963748 0500512872.

50. Sugiharto, S., Isroli, I., Yudiarti, T., Widiastuti, E., Wahyuni, H. I., Sartono, T. A. 2018: Performance, physiological and microbiological responses of broiler chicks to *Moringa oleifera* leaf powder, garlic powder or their combination. *Livest. Res. Rur. Dev.*, 30, 12. http://www.lrrd.org/lrrd30/12/sgh_u30209.html.

51. World Health Organization (WHO), 2002: *World Health Organization Monographs on Selected Medicinal Plants*, Volume 2, Geneva. 358 pp. https://apps.who.int/iris/handle/10665/42052.

52. Ziaie, H., Bashtani, M., Karimi-Torshizi, M. A., Naeeimipour, H., Farhangfar, H., Zeinali, A., 2011: Effect of antibiotic and its alternatives on morphometric cha racteristics, mineral content and bone strength of tibia in Ross broiler chickens. *Global Vet.*, 7, 315—323.

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EFFECTS OF SELENIUM ON BULL'S SPERM OXIDATIVE STRESS AND VIABILITY UNDER *IN VITRO* CONDITIONS

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ABSTRACT

The aim of this study was to determine the effects of sodium selenite on the level of oxidative stress and viability of spermatozoa in fresh bull ejaculate in *in vitro* conditions at different temperatures. Samples of the bull's ejaculates with a concentration of 7×10^5 spermatozoa per ml were placed into the commercial semen extender containing 0 (control), 1 (1Se), 3 (3Se) and 5 (5Se) μg.ml^{-1} of sodium selenite. The following analyses were performed by flow cytometry after 1, 3, 6, 8, 24, 48 and 72 hours of incubation at 4 °C and 37 °C. All analyses were carried out in triplicate. The level of oxidative stress at both temperatures were significantly lower in the experimental groups in comparison to the control group. However, a significant decline of live sperm concentration and a rise of damaged sperm concentration were recorded, especially in groups 1Se and 3Se in comparison to the control group. Only in group 5Se was there observed a positive effect on the damaged spermatozoa level in comparison with groups C, 1Se and 3Se at 4 °C. In conclusion, the applied concentrations of sodium selenite had a positive effect on the level of oxidative stress in all experimental groups, but mainly at concentration of 5 μg.ml^{-1} of sodium selenite, especially at 4 °C. However, the effect of selenium was not sufficient for improving the sperm viability.

Key words: bull's sperm; flow cytometry; oxidative stress; selenium; viability.

INTRODUCTION

Oxidative stress occurs when there is a disbalance between reactive oxygen species (ROS) and antioxidants, often occurring in spermatozoa with defective mitochondria [5]. ROS have both a physiological and a pathological role in spermatozoa [15]. Under physiological conditions only small amounts of ROS are produced, and it is used for capacitation and acrosomal reaction of the spermatozoa when fertilizing the egg [20].

The plasma membrane in spermatozoa differentiate from somatic cells in the body, and have a much higher

content of polyunsaturated fatty acids (PUFA), therefore they are highly susceptible to lipid peroxidation (LPO) caused by ROS [26]. The result of the ROS-induced damage is the disruption of sperm function and decreased semen quality [16]. Oxidative stress in spermatozoa also affects DNA fragmentation of damage in the chromatin, despite the tight packaging of DNA in spermatozoa [6]. They can affect the fertility and cause sub- and infertility, therefore the evaluation of oxidative stress of spermatozoa is important.

Several defence mechanisms, including antioxidants and antioxidant enzymes, have been developed to prevent the LPO of sperm and maintain sperm motility and viability [32]. Many reports have suggested that selenium (Se), as a component of mammalian enzymes such as glutathione peroxidases (GPx) and selenoproteins, is an important nutrient that can affect many biological processes including spermatogenesis and semen quality [17, 18]. Regarding male fertility, Se is important as an antioxidant, for the maintenance of testosterone biosynthesis and formation and normal development of spermatozoa [2]. According to Zubair et al. [32] the significance of Se in male reproduction is proven by the fact, that Se supplementation decreases the release of lipids from the sperm cell during long time storage, and thus contributes to better quality over time. The insufficiency of Se has been associated with reproductive complications and decreased sperm quality in different animal species [7].

Much information is available on Se in relation to cryopreservation, but little in relation to fresh semen. Therefore, we decided to study the effects of selenium on sperm oxidative stress and viability in fresh semen under *in vitro* conditions.

MATERIALS AND METHODS

A semen sample was obtained from one bull by collection with an artificial vagina. Immediately after collection, the semen was investigated for concentration by means of counting beads in flow cytometry. Subsequently, 7×10^5 spermatozoa per ml were placed into a commercial medium Kruuse BTS Plus (Kruuse, Denmark) supplemented with 0 (control), 1 (1Se), 3 (3Se) and 5 (5Se) $\mu g.ml^{-1}$ of sodium selenite (Na_2SeO_3) (Sigma-Aldrich Co., USA) and analysed for viability and level of oxidative stress by flow cytometry with the use of specific fluorescent dyes. The following analyses of oxidative stress and viability were performed 1, 3, 6, 8, 24, 48 and 72 hours after dilution in medium with selenium. All analyses were performed in triplicate at temperatures 4 °C and 37 °C.

Flow cytometry

The concentration, viability and the level of oxidative stress of the spermatozoa were determined by flow cytometry using a BD FACS CantoTM cytometer (Becton Dickinson Biosciences, USA) equipped with blue (488 nm) and red (633 nm) lasers and 6 fluorescence detectors. BD FACS DivaTM Software was used to analyse the data.

Determination of concentration of the spermatozoa

Semen concentration was determined by counting beads, 123 count eBeadsTM (eBioscience, ThermoFisher Scientific, USA) according to Evenson et al. [10]. Twenty-five µl of the diluted sample was mixed with 100 µl of well-resuspended counting particles. The spermatozoa position was determined on the FSC-A base dot plot graph opposite to SSC-A (the cells size opposite their granularity or inner complexity) (Fig. 1). This delimitation also includes particle counts which have a high granularity. The position of particle was then determined on the FITC-A dot plot graph against to PE-A. The numbers of spermatozoa were determined according to the formula:

$$\underset{[\text{sperm/ml}]}{\text{Absolute number}} = \frac{\text{sperm count} \times \text{used particle volume}}{\text{particle count} \times \text{used sperm volume}} \times \underset{[10^4/\text{ml}]}{\text{concentration of particles}}$$

The cell count was expressed as $\log_{10}$ numbers.ml^{-1} ± standard deviation.

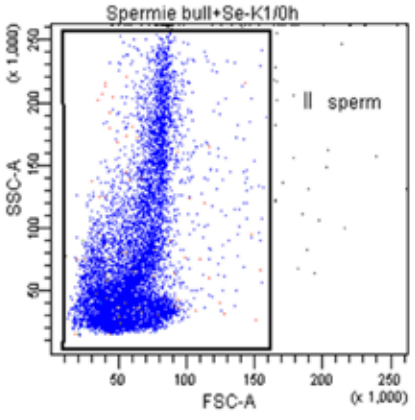

Fig. 1. Determination of the sperm position on the FSC-A base dot blot graph

Determination of spermatozoa viability

Spermatozoa viability was determined by fluorescent dyes, carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) according to Ricci et al. [25]. The cFDA staining principle is based on the ability of nonfluorescent cFDA to penetrate all cells, however only in live, metabolically active cells it can be cleaved by means of enzymes into fluorescent green carboxyfluorescein (cF). The PI can pass only through damaged membranes of cells and colour the DNA to red. This combination of fluorescent dyes allows us to distinguish between three types of cells:

1. Dead spermatozoa emitting red fluorescence as a result of positive staining with PI.

2. Living spermatozoa emitting green fluorescence by positive staining with cFDA.

3. Damaged spermatozoa emitting fluorescence of both dyes [6].

The staining procedure: 2.5 µl of 1 mM cFDA and 222.5 µl of phosphate-buffered saline (PBS) were added to 25 µl of the diluted semen samples. The sample was then incubated for 15 minutes at 37 °C. Consequently, 1.5 µl of PI (1 mg.ml^{-1}) was added and incubated for another 15 minutes at 37 °C. The position of the spermatozoa was determined on the FSC-A dot plot graph opposite to SSC-A and the live, damaged and dead proportions on the dot plot graph FITC-A opposite to the PE-A (Fig. 2).

Determination of oxidative stress level

The oxidative stress of the spermatozoa membrane was investigated using the C11-BODIPY fluorescence

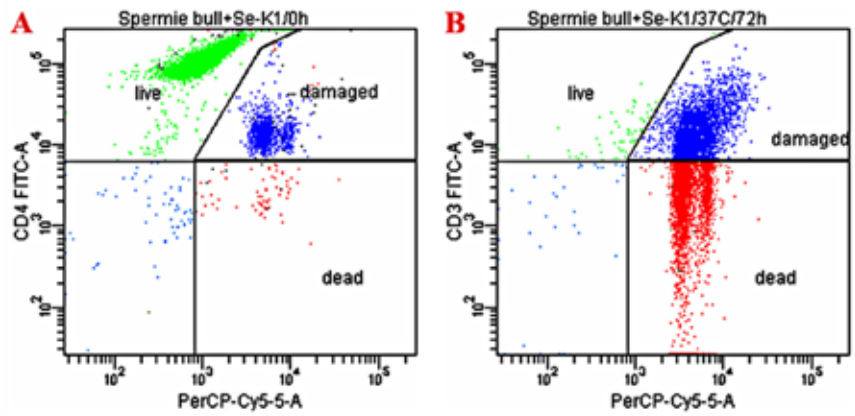

Fig. 2. Dot plot from sperm viability analysis evaluated by flow cytometry

Green colour represents live sperms stained with CFDA, red represents dead sperms stained by PI and dying sperms are represented by blue colouration as a result of staining by both fluorescent dyes (cFDA and PI). **A:** Spermatozoa of control group with good viability at 0 h. **B:** Damaged and dead spermatozoa of the same group at 72 h

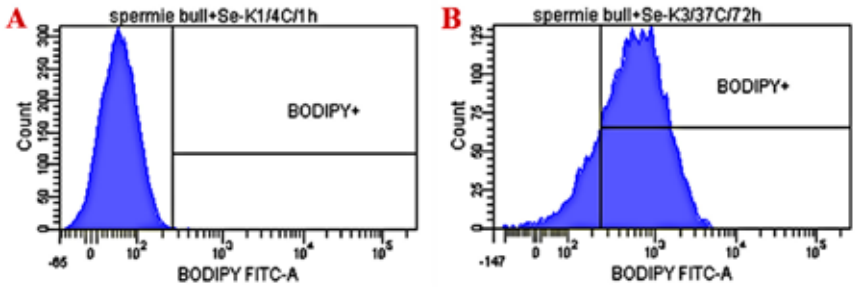

Fig. 3. Illustration of cytometric examination of lipoperoxidation

Stain: C11-BODIPY 581/591; **A:** ejaculate with low level of lipoperoxidation and **B:** ejaculate with high level of oxidative stress

probe according to Brouwers and Gadella [5]. C11-BODIPY581/591 is an analogue of fatty acid which can be incorporated into the membrane of the spermatozoa. The reactivity of the probe to peroxidation is comparable to the reactivity of unsaturated fatty acids which are abundant in the spermatozoa membrane. This fluorescent dye emits red fluorescence in an unoxidized state which changes to bright green after peroxidation [6].

Ten µl of 20 µM C11-BODIPY581/591 solution (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) (Molecular Probes, ThermoFisher Scientific, USA) was added to 50 µl of spermatozoa diluted 1 : 100 and incubated for 30 minutes at 37 °C. After incubation, 200 µl of PBS was added and the samples were analysed. In the assessment, the spermatozoa position was determined at first, similarly to the investigation of viability and consequently, the oxidative stress level was assessed on a histogram (FITC-A for BODIPY opposite counts) (Fig. 3).

Oxidation stress

For the statistical analysis, the two-way analysis of variance (ANOVA) was used, with the supplementary Tukey's test to compare differences between groups and time differences. The significance of the results is marked by stars (*). We found significant differences between groups at the following levels: *P < 0.05; **P < 0.01; ***P < 0.001.

RESULTS

Oxidative stress

The level of oxidative stress was significantly lower (P < 0.05 – P < 0.001) in the experimental groups in comparison with the control group at 3 h onwards at 4 °C. At 37 °C significantly increased levels of oxidative stress were detected in the experimental groups, mainly in 1Se and 3Se, in comparison to the control group at 3 h to 8 h (P < 0.01 – P < 0.001). Subsequently, significantly lower levels of oxidative stress were observed from 48 h in 3Se and 5Se in comparison with groups C and 1Se (P < 0.05 – P < 0.001). Within the time dynamics, a significant rise of oxidative stress at 4 °C was detected mostly in the control group mainly between 1 h and 3 h (P < 0.05) and between 48 h and 72 h (P < 0.001). At 37 °C there was recorded a significant increase of oxidative stress mainly after 24 h (P < 0.001) in groups C and 1Se and after 48 h (P < 0.001) in the groups 3Se and 5Se in comparison to the previous analyses (Fig. 4 and 5).

Viability at 4 °C

At 4 °C we recorded a significant decline of the proportion of live spermatozoa and rise of damaged spermatozoa in the experimental groups 1Se (P < 0.05 – P < 0.001) and 3Se (P < 0.01 – P < 0.001) in comparison with the control group from 3 h onwards, but especially markedly in all experimental groups at 48 h and 72 h (P < 0.001). Concerning the dead spermatozoa, we detected significantly lower numbers of dead sperm in the experimental groups

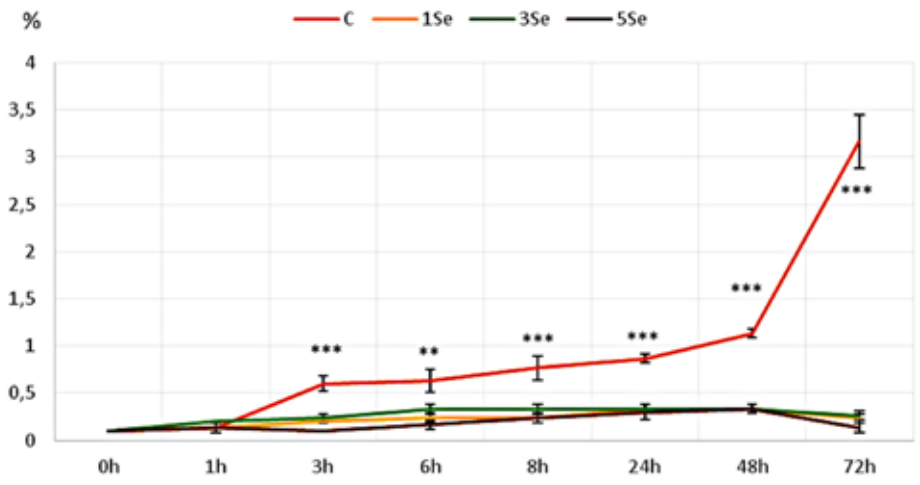

Fig. 4. Oxidative stress at 4 °C
**P < 0.01; ***P < 0.001

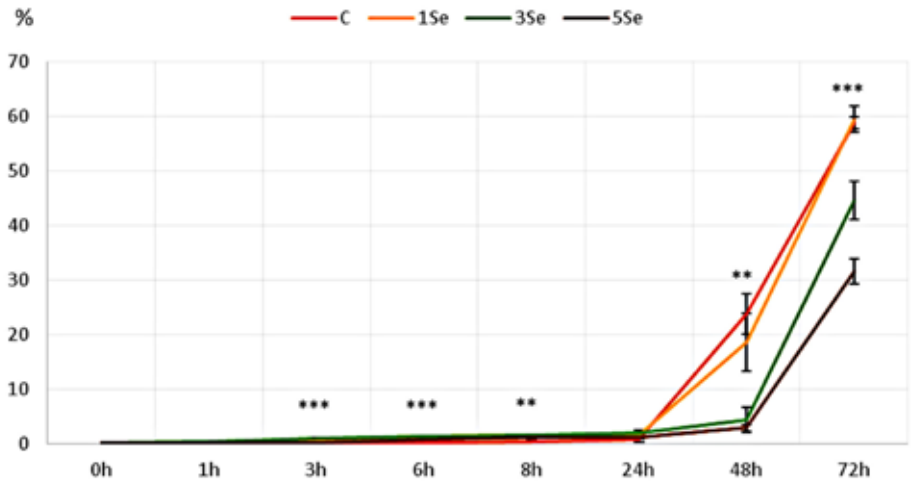

Fig. 5. Oxidative stress at 37 °C
****P < 0.01; *** < 0.001**

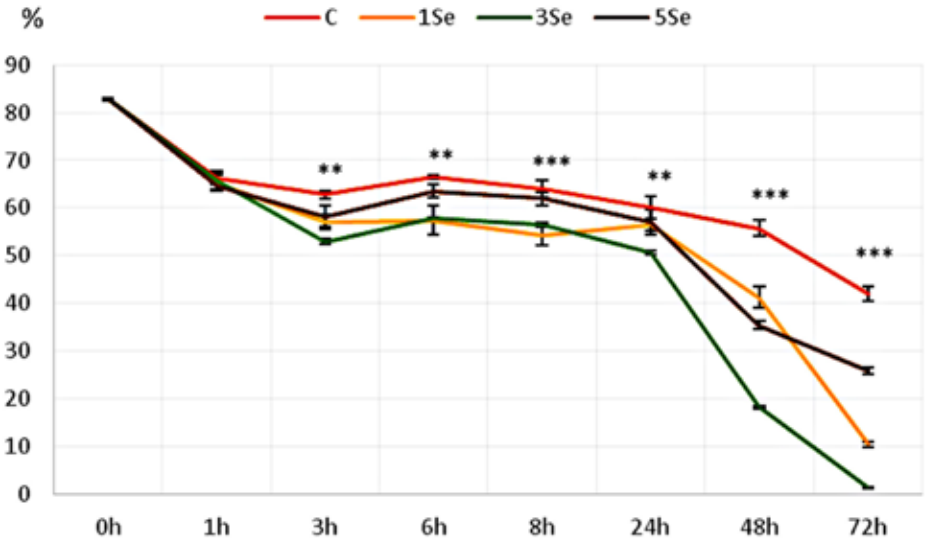

Fig. 6. Live spermatozoa at 4 °C
**P < 0.01; ***P < 0.001

than in the control group at 1 h (P < 0.05) and subsequently a significant increase of dead sperm in 1Se in comparison to all other groups (P < 0.01) (Fig. 6, 7 and 8).

Viability at 37 °C

At 37 °C, a significant decrease of live sperm concentration and an increase of damaged sperm concentration were detected in 1Se at 3 h (P < 0.01 – P < 0.001), in 3Se at 1 h (P < 0.001) and in 5Se at 24 h (P < 0.001) in comparison with the control group. A significant decline of live spermatozoa and a rise of damaged spermatozoa were recorded in groups 1Se and 3Se as compared to group 5Se from 1 h to 8 h (P < 0.05 – P< 0.001). Significantly higher numbers of dead spermatozoa were found in groups C and 5Se than in groups 1Se and 3Se at 24 h (P < 0.05 – P < 0.01) and 72 h (P < 0.05 – P < 0.001) (Fig. 9, 10 and 11).

DISCUSSION

Artificial insemination and *in vitro* fertilization are very important in cattle breeding nowadays. Their efficacy is considerably affected by the poor quality of ejaculate

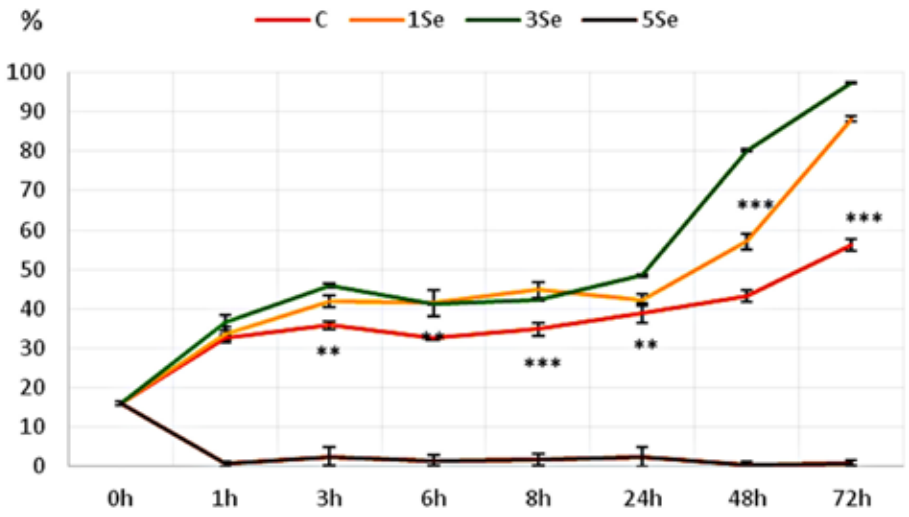

Fig. 7. Damaged spermatozoa at 4 °C
**P < 0.01; ***P < 0.001

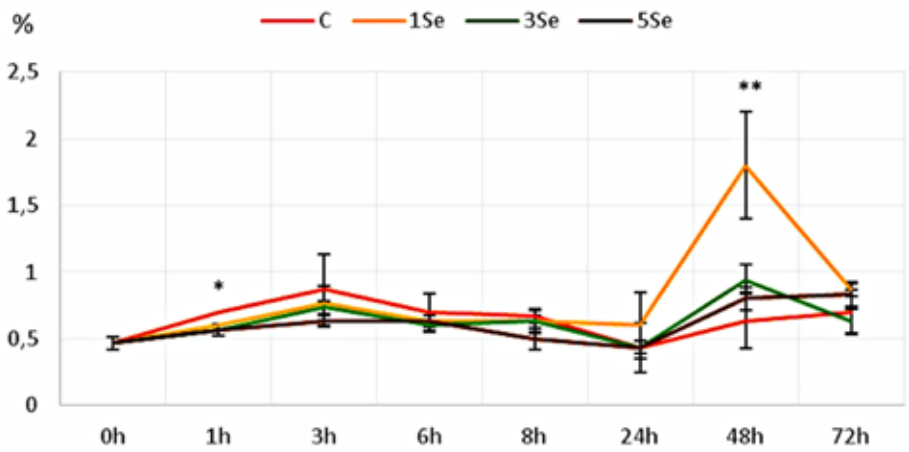

Fig. 8. Dead spermatozoa at 4 °C
*P < 0.05; **P < 0.01

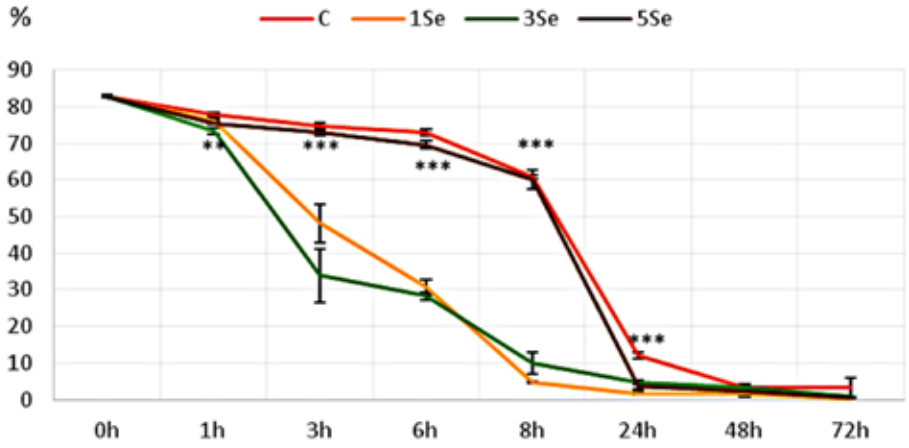

Fig. 9. Live spermatozoa at 37 °C
**P < 0.01; ***P < 0.001

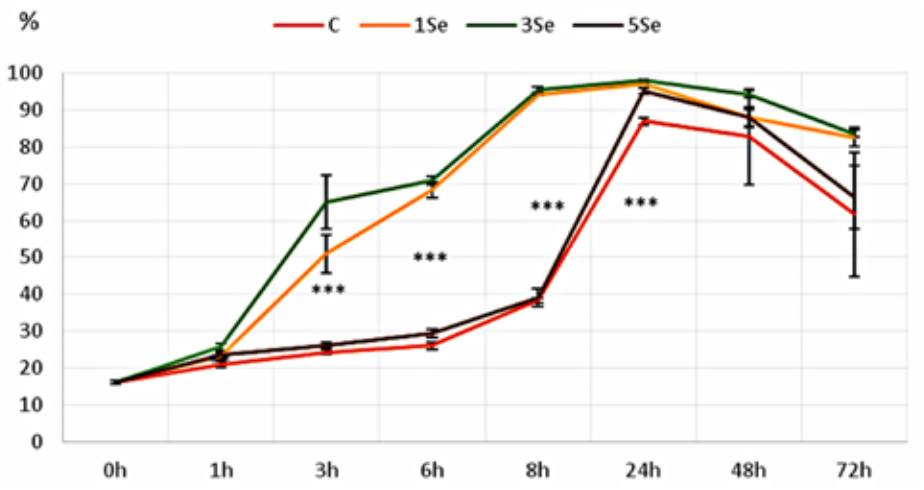

Fig. 10. Damaged spermatozoa at 37 °C
***P < 0.05; **P < 0.01; ***P < 0.001**

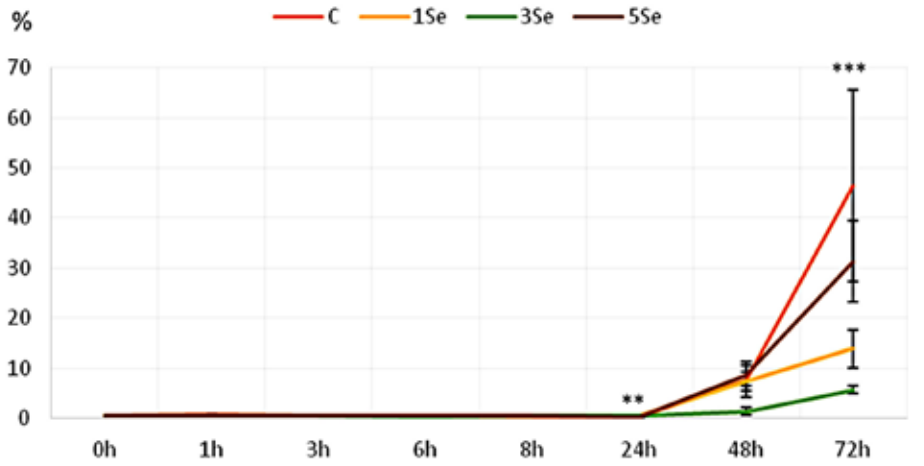

Fig. 11. Dead spermatozoa at 37 °C
****P < 0.01; ***P < 0.001**

caused by insufficient sperm fertilization properties [19]. Spermatozoa are very sensitive cells. During handling with the purpose of fertilization or storage, they are exposed to stress. From the results of some studies it is obvious that during manipulation and storage of semen there is an increase of reactive oxygen species (ROS) concentration and imbalance of antioxidant capacity. Subsequently, spermatozoa can be damaged, or their function can be reduced [21].

Therefore, the examination of the ejaculate quality is important and manipulation with spermatozoa must be done carefully. The use of various extenders is necessary for sperm protection and the maintaining of their optimal fertilization characteristics [13]. Several studies are known, that have investigated the effects of different antioxidants added to the extenders for improvement of the antioxidant activity and maintaining spermatozoa in a functional and healthy condition during semen processing [3, 8, 12, 27].

Selenium is an essential trace element and their antioxidant properties are necessary for maintaining the fertilization characteristics of spermatozoa. The positive effect of selenium on ejaculate quality has been reported by many studies [9, 13, 19, 23]. The most of them deal with the effect of dietary selenium in regard to fertilization characteristics of spermatozoa, but information about the *in vitro* effects of selenium are limited.

The reduction of semen antioxidant capacity and the following increase in vulnerability of spermatozoa to oxidative stress occurs during semen processing [14]. Spermatozoa contain large amount of unsaturated fatty acids in their plasma membranes and are very susceptible to lipoperoxidation caused by ROS. In addition to lipid membrane damage, increased levels of ROS have detrimental effects on chromatin quality as well as on motility and morphologic parameters [30].

In our experiments, we recorded the positive effects of selenium on the level of oxidative stress in all experimental groups in comparison to the control group at both temperatures, while better results were detected at 4 °C. According to Zubair et al. [32] this is due to the antioxidant effect of selenium. Our results are in accordance with the results of Dorostkar et al. [7], where the positive effect of sodium selenite in concentration 1 and 2 $\mu g.ml^{-1}$ on total antioxidant capacity of semen at 4 °C was demonstrated. Similarly, Ghafarizadeh et al. [11] found a decrease of lipoperoxidation in semen at 37 °C after the addition of sodium selenite, which had a positive effect on the level of oxidative stress. The studies determined a dose of 2 $\mu g.ml^{-1}$ of sodium selenite as the most appropriate for improving the fertilizing characteristics of spermatozoa. In our study, significant differences among the doses of selenium at 4 °C were not detected. They were recorded only at 37 °C, where lower oxidative stress was detected in groups with higher concentration of selenium, mainly in the group supplemented with 5 $\mu g.ml^{-1}$ of sodium selenite. These results can indicate that selenium at concentration 1, 3 and mainly 5 $\mu g.ml^{-1}$ are beneficial to reduce oxidative stress. Thus, our findings are not completely consistent with Dorostkar et al. [7]. According to them, a dose of 5 $\mu g.ml^{-1}$ sodium selenite is toxic for spermatozoa of water buffaloes.

At 37 °C a significant increase of oxidative stress was observed in the control and 1Se group after 24 hours. This could indicate that the concentration of 1 $\mu g.ml^{-1}$ of sodium selenite at 37 °C can be beneficial for storage of fresh semen to 24 hours. According Seremak et al. [27], 1 $\mu g.ml^{-1}$ of sodium selenite had a positive influence on sperm freezing ability and survival at the temperature of 38 °C.

The increase of oxidative stress in all 37 °C groups from 24 h indicates poor viability. This is in accordance with our results for viability, where the number of live spermatozoa decreased, while dead and damaged spermatozoa increased at 24 h in 37 °C. This has also been proven by Parham et al. [22].

Agarwal et al. [1] demonstrated that the increased production of ROS caused a reduction in intracellular adenosine triphosphate (ATP) levels and the subsequent increase of membrane permeability led to a decrease of motility and viability. Therefore, the antioxidant properties of selenium in preventing cellular damage from free radicals could have protective effect on sperm motility and viability.

However, in our experiments we recorded rather a negative effect of administered doses of selenium on sperm viability. The most deleterious effect was observed after application of 1 and 3 $\mu g.ml^{-1}$ of sodium selenite at both temperatures. It is not in agreement with other *in vitro* studies, which demonstrated a dose of 2 $\mu g.ml^{-1}$ of sodium selenite as the optimum concentration for improving sperm viability and motility [7, 11]. However, in the study by Dorostkar et al. [7] even this dose was not sufficient for improving sperm viability in fresh semen. According to Siegel et al. [28] the supplementation of selenium in doses exceeding 2 $\mu g.ml^{-1}$ caused a decline of sperm motility in bovines and doses of over 5 $\mu g.ml^{-1}$ of selenium caused a significant decrease of semen quality. Similarly, Dorostkar et al. [7] demonstrated, that doses 4 and 8 $\mu g.ml^{-1}$ of selenium significantly decreased sperm viability and motility as compared to the control group. However, this negative effect was observed only at higher selenium concentrations after processing (equilibration, freezing and thawing) of the samples, which could be due to increased sperm handling. In their study, similar to our results were recorded at a concentration of 4 $\mu g.ml^{-1}$ in the fresh ejaculate. Even, our results point to the effect of 5 $\mu g.ml^{-1}$ of selenium as more positive than the lower selenium concentrations and at 4 °C had a positive effect on the percentage of damaged spermatozoa in experimental group after three hours from the selenium supplementation. These results indicate that the addition of 5 $\mu g.ml^{-1}$ of selenium selenite gives better protection of viability than the other experimental groups. The results of Rezaeian et al. [24] also showed a significant increase in human sperm viability and motility after treatment with selenium at a dose of 5 $\mu g.ml^{-1}$.

At both temperatures a more significant decline of live sperm counts and rise of damaged sperm counts were recorded after three hours, but mainly in groups 1Se and 3Se at temperature 37 °C. Better results were observed at 4 °C,

where the most significant decrease of live sperm concentration was detected after 48 hours from selenium supplementation in comparison with 37 °C, where a similar decrease was detected after 24 hours and subsequently also with higher percentages of dead spermatozoa. These findings are in agreement with other studies, which demonstrated better effects of lower temperatures on sperm fertilizing properties during their storage [4, 29]. During sperm incubation at lower temperatures, they adopt a resting state, which allows them to decrease their metabolism, preserve their energy and decrease the concentration of toxic metabolic products such as reactive oxygen species, which can have deleterious effects on sperm viability [31].

CONCLUSIONS

The aim of our study was to determine the effects of sodium selenite on the level of oxidative stress and viability of spermatozoa in fresh bull ejaculate in conditions *in vitro* at different temperatures. The selected concentrations of selenium had a positive effect on the level of oxidative stress in all experimental groups, but mainly the concentration of 5 $\mu g.ml^{-1}$ of sodium selenite. However, the effects of selected selenium concentrations were not sufficient for improving the sperm viability. Lower levels of oxidative stress and lower concentrations of damaged spermatozoa during longer periods were detected at 4 °C. Therefore, this temperature seems more beneficial for the storage of spermatozoa.

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REFERENCES

1. **Agarwal, A., Virk, G., Ong, Ch., du Plessis, S. S., 2014:** Effect of oxidative stress on male reproduction. *World J. Men's Health*, 32, 1, 1—17. DOI: 10.5534/wjmh.2014.32.1.1.
2. **Ahsan, U., Kamran, Z., Raza, I., Ahmad, S., Babar, W., Riaz, M. H., et al., 2014:** Role of selenium in male reproduction — *A review. Anim. Reprod. Sci.*, 146, 1, 55—62. DOI: 10.1016/j.anireprosci.2014.01.009.
3. **Ansari, M. S., Rakha, B. A., Andrabi, S. M. H., Ullah, N., Iqbal, R., Holt, W. V., et al., 2012:** Glutathione-supplemented tris-citric acid extender improves the post-thaw quality and *in vivo* fertility of buffalo (*Bubalus bubalis*) bull spermatozoa. *Reprod. Biol.*, 12, 3, 271—276. DOI: 10.1016/j.repbio.2012.10.001.
4. **Appell, R. A., Evans, P. R., 1977:** The effect of temperature on sperm motility and viability. *Fertil. Steril.*, 28, 12, 1329—1332. DOI: 10.1016/s0015-0282(16)42978-x.
5. **Brouwersa, J. F., Gadella, B. M., 2003:** *In situ* detection and localization of lipid peroxidation in individual bovine sperm cells. *Free Radic. Biol. Med.*, 35, 11, 1382—1391. DOI: 10.1016/j.freeradbiomed.2003.08.010.
6. **Dolník, M., Mudroňová, D., Pošivák, J., Lazar, G., Mudroň, P., 2019:** Flow cytometry in assessment of sperm integrity and functionality — a review. *Acta Vet. Brno*, 88, 1, 169—175.
7. **Dorostkar, K., Alavi-Shoushtari, S. M., Mokarizadeh, A., 2012:** Effects of *in vitro* selenium addition to the semen extender on the spermatozoa characteristics before and after freezing in water buffaloes (*Bubalus bubalis*). *Vet. Res. Forum*, 3, 4, 263—268.
8. **Ejaz, R., Ansari, M. S., Rakha, B. A., Ullah, N., Husna, A. U., Iqbal, R., et al., 2014:** Arachidic acid in extender improves post-thaw parameters of cryopreserved Nili-Ravi buffalo bull semen. *Reprod. Domest. Anim.*, 49, 1, 122—125. DOI: 10.1111/rda.12239.
9. **El-Sheshtawy, R. I., Ahmed, W. M., Zaabal, M. M., Ali, G. A., Shalabyet, S. I., 2014:** Effect of selenium and/or vitamin E administration on semen characteristics, plasma testosterone level and some immunogenetic constituents in seminal plasma proteins of *Baladi bucks. Glob. Vet.*, 12, 6, 878—884. DOI: 10.5829/idosi.gv.2014.12.06.9478.
10. **Evenson, D. P. Parks, J. E., Kaproth, M. T., Jost, L. K., 1993:** Rapid determination on sperm cell concentration in bovine semen by flow cytometry. *J. Dairy Sci.*, 76, 1, 86—94. DOI: 10.3168/jds.s0022-0302(93)77326-9.
11. **Ghafarizadeh, A. A. Vaezi, G., Shariatzadeh, M. A., Malekirad, A. A., 2018:** Effect of *in vitro* selenium supplementation on sperm quality in asthenoteratozoospermic men. *Andrologia*, 50, 2, e12869. DOI: 10.1111/and.12869.
12. **Iqbal, S., Riaz, A., Andrabi, S. M. H., Shahzad, Q., Durrani, A. Z., Ahmad, N., 2016:** L-cysteine improves antioxidant enzyme activity, post-thaw quality and fertility of Nili-Ravi buffalo (*Bubalus bubalis*) bull spermatozoa. *Andrologia*, 48, 9, 855—861. DOI: 10.1111/and.12520.

13. **Jamali, N. U., Kaka, A., Khatri, P., Malhi, M., Naeem, M., Memonet, A. A., et al., 2019:** Effect of *in vitro* selenium addition to the semen extender on the spermatozoa characteristics before and after freezing in Kundhi buffalo bull and *in vitro* fertility rate. *Pak. J. Zool.*, 51, 1, 317—323. DOI: 10.17582/Journal.Pjz/2019.51.1.317.323.
14. **Kadirvel, G., Kumar, S., Kumaresan, A., 2009:** Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. *Anim. Reprod. Sci.*, 114, 1, 125—134. DOI: 10.1016/j.anireprosci.2008.10.002.
15. **Kothari, S., Thompson, A., Agarwal, A., du Plessis, S. S., 2010:** Free radicals: Their beneficial and detrimental effects on sperm function. *Indian J. Exp. Biol.*, 48, 5, 425—435.
16. **Li-guang, S., Yang, R. J., Yue, W. B., Xun, W. J., Zhang, C. X., Ren, Y. S., et al., 2010:** Effect of nano-selenium on semen quality, glutathione peroxidase activity and testis ultrastructure in male Boer goats. *Anim. Reprod. Sci.*, 118, 2—4, 248—254. DOI: 10.1016/j.anireprosci.2009.10.003.
17. **Mahima, K., Verma, A. K., Kumar, A., Rahal, A., Kumar, V., Roy, D., 2012:** Inorganic versus organic selenium supplementation: *A review. Pak. J. Biol. Sci.*, 9, 15, 418—425. DOI: 10.3923/pjbs.2012.418.425.
18. **Mahmoud, G. B., Abdel-Raheem, S. M., Hussein, H. A., 2013:** Effect of combination of vitamin E and selenium injections on reproductive performance and blood parameters of Ossimi rams. *Small Rum. Res.*, 113, 1, 103—108. DOI: 10.1016/j.smallrumres.2012.12.006.
19. **Maidin, M. S., Adanan, N. F., Aminudin, M. T., Tawang, A., 2014:** *In vitro* supplements improves motility and progressive score of spermatozoa in jermasia goats. *APCBEE Procedia*, 8, 3/4, 329—333. DOI: 10.1016/j.apcbee.2014.03.049.
20. **Martínez-Pastor, F., Mata-Campuzano, M., Álvarez-Rodríguez, M., Álvarez, M., Anel, L., De Paz, P., 2010:** Probes and techniques for sperm evaluation by flow cytometry. *Reprod. Domest. Anim.*, 45, 2, 67—78. DOI: 10.1111/j.1439-0531.2010.01622.x.
21. **Nabi, A., Khalili, M. A., Halvaei, I., Roodbari, F., 2014:** Prolonged incubation of processed human spermatozoa will increase DNA fragmentation. *Andrologia*, 46, 4, 374—379. DOI: 10.1111/and.12088.
22. **Parham, A., Arshami, J., Naserian, A. A., Kandelousi, M. A. S., Azizzadeh, M., 2013:** Quality of bovine chilled or frozen-thawed semen after addition of omega-3 fatty acids supplementation to extender. *Int. J. Fertil. Steril.*, 7, 3, 161—168.
23. **Piagentini, M., Silva, D. C., Dell'Aqua, Cpf., Moya-Araujo, C. F., Codognoto, V. M., Ramos, A. A., et al., 2016:** Effect of selenium supplementation on semen characteristics of Brazil's ram. *Reprod. Domest. Anim.*, 100, 1, 1—4. DOI: 10.1111/rda.12903.
24. **Rezaeian, Z., Hossein, Y., Sima, N., Zahra, R., Parvin, F., Fardin, A., 2016:** Effect of selenium on human sperm parameters after freezing and thawing procedures. *Asian. Pac. J. Reprod.*, 5, 6, 462—466. DOI: 10.1016/j.apjr.2016.11.001.
25. **Ricci, G., Perticarari, S., Fragonas, E., Giolo, E., Canova, S., Pozzobon, C., et al., 2002:** Apoptosis in human sperm: its correlation with semen quality and the presence of leukocytes. *Hum. Rep.*, 17, 10, 2665—2672. DOI: 10.1093/humrep/17.10.2665.
26. **Sanocka, D., Kurpisz, M., 2004:** Reactive oxygen species and sperm cells. *Reprod. Biol. Endocrinol.*, 2, 1, 1—7. DOI: 10.1186/1477-7827-2-12.
27. **Seremak, B., Udala, J., Lasota, B., 1999:** Influence of selenium additive on ram semen freezing quality. *Electron. J. Pol. Agric. Univ.*, 2, 1, 1—2.
28. **Siegel, R. B., Murray, F. A., Julien, W. E., Moxon, A. L., Conrad, H. R., 1980:** Effect of *in vitro* selenium supplementation on bovine sperm motility. *Theriogenology*, 13, 5, 357—367. DOI: 10.1016/0093-691X(80)90048-5.
29. **Thijssen, A. Klerkx, E., Huyser, C., Bosmans, E., Campo, R., Ombelet, W., 2014:** Influence of temperature and sperm preparation on the quality of spermatozoa. *Reprod. Biomed. Online*, 28, 4, 436—442. DOI: 10.1016/j.rbmo.2013.12.005.
30. **Weir, C. P., Robaire, B., 2007:** Spermatozoa have decreased antioxidant enzymatic capacity and increased reactive oxygen species production during aging in the brown Norway rat. *J. Androl.*, 28, 2, 229—240. DOI: 10.2164/Jandrol.106.001362.
31. **Wusiman, A., Wang, Y. P., Ren, K., Zhou, G. B., Fu, X. W., Suo, L., et al., 2012:** Semen storage at 23, 4 or –196 °C and its application to artificial insemination in small-tail han sheep. *Asian J. Anim. Vet. Adv.*, 7, 4, 299—308. DOI: 10.3923/ajava.2012.299.308.
32. **Zubair, M. Ali, M., Ahmad, M., Sajid, S. M., Ahmad, I., Gul, S. T., 2014:** Effect of selenium and vitamin E on cryopreservation of semen and reproductive performance of animals (A review). *J. Entomol. Zool. Stud.*, 1, 3, 82—86.

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OCCURRENCE OF *CRYPTOSPORIDIUM* OOCYSTS AND HELMINTH OVA ON DRIED CRAYFISH (*PROCAMBARUS CLARKII*) SOLD IN KADUNA STATE, NIGERIA

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ABSTRACT

***Cryptosporidium* spp. and helminths are responsible for diarrhoal illness in humans and many other animals. The transmission routes of these parasites suggest a risk for human infection through contaminated foods. In order to determine the occurrence of *Cryptosporidium* oocysts and helminth ova on dried crayfish (*Procambarus clarkii*) sold in Zaria and Kaduna Central market, Kaduna State, 100 crayfish samples were examined using the modified Ziehl-Neelsen staining technique for *Cryptosporidium* oocyst and the flotation technique for helminth ova. The measurements of the oocysts from the positive samples were estimated by using a calibrated microscope eyepiece. The prevalence of *Cryptosporidium* oocysts in dried crayfish was 20.0 % while the location-based prevalence was 43.3 %, 5.0 %, and 12.0 % for Sabo, Samaru and Kaduna central market, respectively. Micrometry revealed that the oocysts size ranged from 3.68 µm to 4.7 µm. Also, the prevalence of helminth ova in dried crayfish was 19 %, while the specific prevalence based on location showed a higher prevalence in Sabo (30.0 %) than in Kaduna central market (18.0 %) and Samaru (5.0 %). The helminth eggs recovered from this study included: *Toxocara* spp. (4.0 %), *Ascaris* spp. (7.0 %), *Trichuris* spp. (4.0 %), *Schistosoma* spp. (2.0 %), *Ancylostoma* spp. (1.0 %), and *Capillaria* spp. (1.0 %). The analysis of the questionnaires revealed that 71 (71.0 %) of the respondents ate uncooked crayfish and 44.0 % used their bare hands to handle the crayfish. There was no statistically significant association ($P > 0.05$) between the prevalence of *Cryptosporidium* oocysts or helminth ova and the locations sampled. This study has shown that dried crayfish obtained from markets within the study area were contaminated with parasite ova and oocysts, thus public enlightenment on the dangers of the consumption of raw or undercooked dried crayfish should be well publicized.**

Key words: crayfish; *Cryptosporidium*; helminth ova; oocysts; Ziehl-Neelsen

INTRODUCTION

Cryptosporidium is an intracellular protozoan parasite that causes gastrointestinal pathologies in all vertebrates such as mammals, reptiles, birds and fish; thus, it is one of the most predominant enteric parasitic pathogens in humans [23]. *Cryptosporidium* is an important cause of diarrhoeal diseases alongside of *Giardia* in humans globally and is included in the WHO neglected disease initiatives [20]. The organism affects the epithelial cells of both the digestive and respiratory tracts and multiplies in them [6]. *Cryptosporidium* is also a relevant food borne pathogen resulting in diseases of socioeconomic importance globally [17].

Cryptosporidiosis is ubiquitous in its infectivity, and has an undesirable worldwide reputation [4]. The growing concern of cryptosporidiosis affecting immunocompromised individuals has led to the introduction of control measures of the disease in humans and other animals; which is of public health significance [9]. Humans are susceptible to infection by *Cryptosporidium* via many transmission pathways. Infection occurs through the consumption of contaminated food or physical contact with faeces of infected individuals [8].

Helminthosis is also of great concern because scientists have discovered that rivers are infected with numerous helminths [1]. There is a high health risk associated with helminth infections because helminths persist for longer periods in the environment, its host immunity is usually low to non-existent and the infective dose is small. Helminth infections are estimated to be the cause of 135,000 deaths annually around the globe [11].

Crayfish are crustaceans resembling tiny lobsters. They are colloquially called crawfish or crawdads. Taxonomically, they are members of the superfamilies *Astacoidea* and *Parastacoidea*. Some species of crayfish are found in brooks and streams where freshwater is running, while others thrive in swamps, ditches and paddy fields [16]. *P. clarkii* has been eaten in the United States, Cambodia, Europe, China, Africa, Australia, Thailand, Canada, New Zealand, and the Caribbean [13]. This species is typically dark red, with long claws and head, small or no spines on the sides of its carapace just below the head, and rows of bright red bumps on the front and side of the first leg [15].

Several "external" nematodes have been reported on crayfish, and these can be considered to be free-living epibionts with limited impact on host survival [7, 10]. Truly parasitic nematodes are normally found within crayfish internally, either encysted in the muscles or encapsulated on the intestinal wall; few parasitic nematodes such as *Gnathostoma spinigerum*, *Angiostrongylus cantonensis* have been reported in crayfish, with crayfish acting as paratenic hosts in all cases [14]. Taylor et al. [21] also identified crayfish as the intermediate host for *Paragonimus westermani* and *Polymorphus boschadis*. Furthermore, Abraham et al. [1] detected *Porospora gigantica* (sporozoa), *Nicothoic astaci* (copepod), *Hysterothylaactum* spp. (nematode), *Paragonimus uterobilateralis* (trematode), Leech-like parasite (*hirudinea*) and *Polymorphus botulus* (*acanthocephalan*) on crayfish and lobsters in Great Kwa river of Cross river State, Nigeria. Reports presented at the Infectious Disease Society of America's (IDSA's) annual conference revealed that contaminated crayfish can cause severe illnesses such as salmonellosis and *Vibrio mimicus* infections [16].

Cryptosporidiosis and helminthosis affect humans and other animals; hence their public health significance. The literature on parasitic zoonoses of crayfish in Kaduna State was unavailable at the time this research was done. Therefore, there was a need for research in this field. Research in parasitic zoonoses of crayfish is important since crayfish is a common seasoning/condiment added to soup and food in general. This study may reveal possible risk factors associated with contamination of crayfish sold at the market. The aim of this study was to determine the occurrence of *Cryptosporidium* oocysts and helminth ova and also the knowledge and practices of dried crayfish sellers in relation to its handling and sales in Zaria and Kaduna Central market, Kaduna State, Nigeria.

MATERIALS AND METHODS

Study design

This was a cross-sectional study in which three markets within Kaduna State were selected, i. e. Sabo (30), Samaru (20) and Kaduna central markets (50), which gave a total sample size of 100. The sampling was achieved using the simple random sampling method from consenting sellers from June to July, 2017.

Questionnaire administration

By using oral interviews, questionnaires (100) were administered to the crayfish sellers within the selected markets to determine the relationship between knowledge and practices of dried crayfish sellers in relation to their handling and sales within the study areas.

Sample collection

Smoked dried crayfish were bought randomly from the sellers who consented to the questionnaire. Each sample was packaged in small polythene bags, labelled with a number and then transported to the Parasitic Zoonosis Laboratory, Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

SAMPLE PROCESSING

Preparation of homogenate

Ten grams of each sample of the crayfish was weighed on a measuring scale. It was then put in a sterile polythene bag and 90 ml of peptone water was added. The contents were tied up in the polythene bag and put in a stomacher for homogenization. The homogenate was then centrifuged and the supernatant was decanted. Part of the sediment was used as a flotation technique to detect the helminth ova. The other portion of the sediment was used to make a thin smear on glass slides in order to stain for *Cryptosporidium* oocysts, using a modified Ziehl-Neelsen staining technique as described by Cheesbrough [5].

The detection of the *Cryptosporidium* oocysts used the modified Ziehl-Neelsen staining method

Briefly, the slides were fixed with ethanol for 2—3 minutes, then stained with carbol-fuschin for 15 minutes and washed with water. The slides were then counter-stained with 0.3 % methylene blue for 30 seconds, then washed with water and allowed to air dry. The stained slides were viewed using the light microscope at 40 objective lens. *Cryptosporidium* oocysts appeared red on a blue background. The degree and proportion of staining varied between oocysts. The internal structures took up various degree of stain from amorphous to eccentric. *Cryptosporidium* oocysts appeared as spherical discs with diameters ranging from 2.94 to 9.0 μm [21]. The measurements of all oocysts from the positive samples were conducted using a calibrated microscope eyepiece.

Detection of helminth ova

This was achieved using the flotation technique as described by Arora and Arora [2]. The homogenates of crayfish were filtered using gauze and a funnel into a centrifuge test tube. The filtrate was centrifuged at 2,000 revolutions per minute for 5 minutes, the supernatant was decanted and Zinc sulphate solution was added up to the brim of the test tube until a convex meniscus was formed. Cover-slips were gently placed on top of the flotation medium and allowed to stay for ten minutes. After some time, the cover-slips were gently taken off and the flotation smear homogenized; the flotation medium was discarded and the sediment at the bottom of the sample bottle was smeared at another corner of the glass slide, thus each slide had two smears: one for flotation and the other for sedimentation. The slides were allowed to air dry for 24 hours at room temperature and then viewed at x10 on the microscope. The eggs were identified on the basis of their morphological features as described by Urquhart et al. [22].

Data analysis

Using Graph pad prism version 5, Pearson's correlation (r) and Chi square (χ^2) were calculated to determine the association between independent and dependent variables. P-values less than or equal to 0.05 were considered statistically significant. The relationship between knowledge and practices of dried crayfish sellers in relation to their handling and sales were determined from the questionnaires administered using SPSS version 21.

RESULTS

The overall prevalence of *Cryptosporidium* oocysts in the dried crayfish from the markets within the study area was 20 %. Location-based prevalences were 43.3 %, 5.0 %, and 12.0 % for Sabo, Samaru and the Kaduna central market, respectively. Although the prevalence was higher in Sabo market than Samaru and Kaduna central market, there was no statistically significant association (P = 0.08) between the prevalence of *Cryptosporidium* oocysts and the location sampled upon analysis (Table 1). Micrometry

Table 1. Locational distribution of *Cryptosporidium* oocysts on dried crayfish sold in Sabo, Samaru, and Kaduna Central Market

Location	Number examined	Number positive	Specific rate [%]	χ^2	P value
Sabo	30	13	43.3	9.766	0.08
Samaru	20	1	5.0		
Central market	50	6	12.0		
Total	**100**	**20**	**20**		

Table 2. Locational distribution of ova of helminths detected on dried crayfish sold in Sabo, Samaru and Kaduna Central markets using Floatation Technique

Location	Number examined	Number positive	Specific rate [%]	χ^2	P value
Sabo	30	9	30.0	4.938	0.085
Samaru	20	1	5.0		
Central market	50	9	18.0		
Total	**100**	**19**	**19**		

results revealed that the oocysts sizes ranged from 3.68 µm to 4.7 µm. The helminth eggs recovered from this study included: *Toxocara* spp. (4.0 %), *Ascaris* spp. (7.0 %), *Trichuris* spp. (4.0 %), *Schistosoma* spp. (2.0 %), *Ancylostoma* spp. (1.0 %), and *Capillaria* spp. (1.0 %). The overall prevalence of helminth ova in the dried crayfish from the study was 19 %, while the specific prevalence based on the location showed a higher prevalence in Sabo (30.0 %) than Samaru (5.0 %) and Kaduna central market (18.0 %). Upon analysis there was no statistically significant association (P = 0.085) between the prevalence of helminth ova and the locations sampled (Table 2).

Evaluation of the respondents on knowledge and practices in the handling and sales of dried crayfish in the markets showed that 52 % were within the age of 20—39 years, most of the sellers were females (64 %) and 50 % of the respondents have been selling dried crayfish for 6—7 years. Seventy-one (71 %) of the respondents eat uncooked crayfish and 44 % use their bare hands to handle crayfish, while only 4 % knew that vomiting could be a symptom associated with consumption of uncooked contaminated dried crayfish (Table 3).

The results shown in Table 4 indicated the extent to which the listed variables were responsible or contributing to contraction of helminth infection and cryptosporidiosis. There was a significant association between the source of dried crayfish and the occurrence of helminth ova and *Cryptosporidium* oocysts on crayfish (P = 0.031).

DISCUSSION

The occurrence of *Cryptosporidium* oocysts and helminth ova on smoked dried crayfish sold at the market may have been due to poor handling during harvesting, processing, storage and display which predisposed the commodity to contamination as reported by K u m o l u - J o h n s o n et al. [12]. Poor handling further exposed the commodity to insect infestation and the growth and proliferation of moulds which in turn reduced the quality of the commodity.

The higher prevalence of *Cryptosporidium* oocysts and helminth ova on dried crayfish sampled in Sabo than those from Samaru and Kaduna central market may imply poor environmental hygiene. Furthermore, a mechanical vector (houseflies) may have been attracted by the aroma of crayfish and their enormous number in this location could probably be due to the activities of meat sellers and those

Table 3. Demography of respondents in the study of the knowledge and practices of dried crayfish sellers in Samaru, Sabo and Kaduna central market

Variables	Categories	Samaru	Sabo	Central market	Total
Age (years)	< 20	3 (15 %)	3 (10 %)	1 (2 %)	**7 (7 %)**
	20—39	12 (60 %)	12 (40 %)	28 (56 %)	**52 (52 %)**
	40—59	4 (20 %)	15 (50 %)	18 (36 %)	**37 (37 %)**
	≥ 60	1 (5 %)	0 (0 %)	3 (6 %)	**4 (4 %)**
Gender	Male	4 (20 %)	10 (33 %)	22 (44 %)	**36 (36 %)**
	Female	16 (80 %)	20 (67 %)	28 (56 %)	**64 (64 %)**
Duration of Selling (years)	< 1	2 (10 %)	2 (6.67 %)	4 (8 %)	**8 (8 %)**
	1—5	7 (35 %)	1 (3.33 %)	9 (18 %)	**17 (17 %)**
	6—7	7 (35 %)	12 (40 %)	31 (62 %)	**50 (50 %)**
	11—15	2 (10 %)	15 (50 %)	6 (12 %)	**23 (23 %)**
	>16	2 (10 %)	0 (0 %)	0 (0 %)	**2 (2 %)**
Eating of uncooked crayfish	Yes	13 (65 %)	30 (100 %)	28 (56 %)	**71 (71 %)**
	No	7 (35 %)	0 (0 %)	22 (44 %)	**29 (29 %)**
Tasting dried crayfish	Yes	9 (45 %)	10 (33 %)	19 (38 %)	**38 (38 %)**
	No	11 (55 %)	20 (67 %)	31 (62 %)	**62 (62 %)**
Source of buying the crayfish	Source (Fishermen)	1 (5 %)	6 (20 %)	16 (32 %)	**23 (23 %)**
	Wholesaler	13 (65 %)	24 (80 %)	29 (58 %)	**66 (66 %)**
	Retailer	6 (30 %)	0 (0 %)	5 (10 %)	**11 (11 %)**
Packaging	Source (Fishermen)	3 (15 %)	3 (10 %)	4 (8 %)	**10 (10 %)**
	Wholesaler	5 (25 %)	15 (50 %)	22 (44 %)	**42 (42 %)**
	Retailer	2 (10 %)	12 (40 %)	4 (8 %)	**18 (18 %)**
	Self	10 (50 %)	0 (0 %)	20 (40 %)	**30 (30 %)**
Measuring	Bare hands	6 (30 %)	8 (27 %)	30 (60 %)	**44 (44 %)**
	Measuring cup	13 (65 %)	21 (70 %)	7 (14 %)	**41 (41 %)**
	Hand covered with polythene bag	1 (5 %)	1 (3 %)	13 (26 %)	**15 (15 %)**
Symptoms associated with consumption of uncooked dried crayfish	Vomiting	2 (10 %)	1 (3 %)	1 (2 %)	**4 (4 %)**
	Diarrhoea	0 (0 %)	0 (0 %)	0 (0 %)	**0 (0 %)**
	Fever	0 (0 %)	0 (0 %)	0 (0 %)	**0 (0 %)**
	Stomach ache	0 (0 %)	0 (0 %)	0 (0 %)	**0 (0 %)**
	None	18 (90 %)	29 (97 %)	49 (98 %)	**96 (96 %)**

Table 4. Multi-linear regression of the knowledge and practices of crayfish sellers in Samaru, Sabo and Kaduna central market

Model	Unstandardized coefficients		Standardized coefficients	T	Significance
	Beta	Std. Error	Beta		
(Constant)	1.614	0.148		10.912	0.000
Processing of dried crayfish	0.026	0.027	0.097	0.959	0.340
Eating of uncooked dried crayfish	0.001	0.023	0.003	0.029	0.977
Tasting dried crayfish before buying	0.039	0.021	0.188	1.822	0.072
Source of buying the crayfish	–0.045	0.020	–0.256	–2.189	0.031
Packaging of the dried crayfish	0.004	0.010	0.041	0.387	0.699
Supplies of dried crayfish	–0.037	0.027	–0.162	–1.367	0.175
Storage of dried crayfish	–0.017	0.020	–0.086	–0.869	0.387
Measuring and package of dried crayfish	0.016	0.015	0.114	1.083	0.282
Other uses of dried crayfish	0.166	0.039	0.408	4.219	0.000
Consumption of uncooked dried crayfish	0.010	0.014	0.072	0.728	0.469

Beta—Regression coefficient; T—Test statistic

selling dried crayfish who are in very close proximity as observed during sampling in Sabo; enhancing contamination through the transfer of parasite eggs as they perch from one point to another. A z z a et al. [3] reported a lower prevalence of 6 % for *C. parvum* oocysts on crayfish in Egypt. Also, A b r a h a m et al. [1] described the distribution of parasitic infections in crayfish from 4 sampling zones of Great Kwa river, Cross river State, Nigeria, to have a prevalence of 6.0 %, 8.7 %, 15.3 % and 10.0 %. The higher prevalence observed in our study could be due to the difference in points of sampling of the product thus, sampling for this study was done at the point of sale; which have undergone some level of unhygienic handling, while other studies sampling was mostly at the point of harvest.

From this study, respondents indicated sourcing the dried crayfish mostly from the wholesalers where this product was displayed in large quantities in an open field and sometimes on bare ground which creates a greater chance of being contaminated with helminth ova and *Cryptosporidium* oocysts.

The responses from the questionnaires depicted that, there were: poor handling of dried crayfish with unclean hands, eating of uncooked dried crayfish, tasting of dried crayfish, poor packaging, storage, measuring of the dried crayfish, thereby serving as the major contributing factors of helminthosis and cryptosporidiosis. Sometime the products were packaged in unclean bags such as cement paper bags and old newspapers. It should be noted that the crayfish were mostly used and tasted in their dried form as such, which served as the dominant factor for helminth infection and cryptosporidiosis. The concept of eating raw food stuffs is becoming common in our society more or less everywhere these days and crayfish is not an exception. This study has shown that eating of undercooked or raw crayfish could be a possible risk factor to parasitic infections. This was also affirmed by R o t h s c h i l d [19] who reported that six residents of Missouri and Illinois came down with a rare parasitic disease; paragonimiasis caused by *Paragonimus* trematodes, or lung flukes, following consumption of uncooked crayfish, between September 2009 and September 2010.

CONCLUSIONS

This study has established the occurrence of *Cryptosporidium* oocysts and helminth ova in dried crayfish sampled from Sabo, Samaru and Kaduna central market. This study has also revealed that there is poor handling and packaging of crayfish at the markets thus, there should be public awareness of the possibility of contamination of dried crayfish by zoonotic organisms detrimental to public health if eaten uncooked. Proper hygiene should be ensured when packaging dried crayfish and there should be proper storage of dried crayfish to avoid contamination by persons, environment, insects or other animals.

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REFERENCES

1. **Abraham, J. T., Oloko, G. O., Ikpeme, E. M., 2013:** Parasites of crayfish (*P. clarkia*) and lobsters (*Macrobrachium vollenhovenic*) as indicators of metallic pollution in Great Kwa river, Nigeria. *Global J. Agric. Sci.*, 14, 27—36. DOI: 10.4314/gjas.v14i1.4.
2. **Arora, D. R., Arora, B., 2006:** *Medical Parasitology*. 10th edn., B. S. Publishers, New Delhi, India, 166 pp.
3. **Azza, M. R., Amro, M., Mohamed, E. M., Abd El-Maksoud, S. A., 2003:** Further studies on the role of crayfish (*Procambarus clarki*) in transmission of some zoonotic parasites in East Delta. In *The 3rd International Science Conference,* Faculty of Veterinary Medicine, Mansoura University, Egypt, 151—168.
4. **Checkley, W., White, C., Jaganath, D., Arrowood, M. J., Chalmers, R. M., Chen, X. M., et al., 2014:** A review of the global burden, novel diagnostics, therapeutics and vaccine targets for cryptosporidiosis. *The Lancet Infect. Dis.*, 15, 85—94. DOI: 10.1016/S1473-3099(14)70772-8.
5. **Cheesbrough, M., 2009:** *District Laboratory Practices in Tropical Countries*, Part 1, 2nd edn., Cambridge University Press, New York, USA, 206—207.
6. **Chen, X. M., Keithly, J. S., Paya, C. V., LaRusso, N. F., 2002:** Cryptosporidiosis. *N. Engl. J. Med.*, 346, 22, 1723—1731. DOI: 10.1056/NEJMra013170.
7. **Edgerton, B. F., 2002:** Hazard analysis of exotic pathogens of potential threat to European freshwater crayfish. *Bull. Fr. Pêche Piscic.*, 367, 813—820. DOI: 10.1051/kmae:2002069.
8. **Goldstein, S. T., Juranek, D. D., Ravenholt, O., Hightower, A. W., Martin, D. G., Mesnik, J. L., et al., 1996:** Cryptosporidiosis: An outbreak associated with drinking water despite state-of-the-art water treatment. *Ann. Intern. Med.*, 124, 5, 459—468. DOI: 10.7326/0003-4819-124-5-199603010-00001.
9. **Jerlyn, K., Sponseller, A., Jeffrey, K., Griffiths, B., Saul, T., 2014:** The evolution of respiratory cryptosporidiosis: Evidence for transmission by inhalation. *Clin. Microbiol. Rev.*, 27, 3, 575—586. DOI: 10.1128/CMR.00115-13.
10. **Jones, J. B., Lawrence, C. S., 2001:** Diseases of yabbies (*Cherax albidus*) in western Australia. *Aquaculture*, 194, 221—232. DOI: 10.1016/S0044-8486(00)00508-1.
11. **Jourdan, P. M., Lamberton, P. H., Fenwick, A., Addiss, D. G., 2018:** Soil-transmitted helminth infections. *Lancet*, 391, 10117, 252—265. DOI: 10.1016/S0140-6736(17)31930-X.
12. **Kumolu-Johnson, C. A., Aledetodun, N. F., Ndimole, P. E., 2010:** The effect of smoking on nutritional qualities and shelf-life of *Clarias gariepinus*. *Afr. J. Biotechnol.*, 9, 1, 073—076. DOI: 10.5897/AJB09.548.
13. **Larry, W. B. Jr., Robert, P. R., 1990:** *Crawfish production: harvesting, marketing and economics.* Southern Regional Aquaculture Center. Publication No. 242. Aquaculture Curriculum Guide, 95—114.
14. **Moravec, F., 2007:** Some aspects of the taxonomy and biology of adult spirurine nematodes parasitic in fishes: a review. *Folia Parasitol.*, 54, 239—257. DOI: 10.14411/fp.2007.033.
15. **Nagy, R., Fusaro, A., Conard, W., 2016:** "*Procambarus clarkii*". *Nonindigenous Aquatic Species Database, Gainesville*, FL. USGS. Retrieved from Wikipedia on June 14, 2017.
16. **Needon, C., Petermann, J., Scheffel, P., Scheibe, B., 1971:** Plants and animals (Pflanzen und Tiere). Leipzig, Urania Verlag. In *Conference paper 2D Animation Design of Freshwater Lobster Cultivation,* KnE Social Sciences. DOI: 10.18502/kss.v4i12.
17. **Putignani, L., Menichella, D., 2010:** Global distribution, public health and clinical impact of the protozoan pathogen *Cryptosporidium*. *Interdiscip. Perspect. Infect. Dis.*, 4, 753512. DOI: 10.1155/2010/753512.
18. **Robert, R., 2010:** Disease threats from crayfish, frogs described at IDSA. *Center for Infectious Disease Research and Policy (CIDRAP) News*. Oct 22, 2010. https://www.cidrap.

umn.edu/news-perspective/2010/10/disease-threats-crayfish-frogs-described-idsa.

19. **Rothschild, M., 2010:** Don't eat raw crayfish. *Food Safety News*. Breaking news for everyone's consumption. December 17, 2010. https://www.foodsafetynews.com/2010/12/raw-crayfish-cause-rare-parasitic-disease.
20. **Savioli, L., Smith, H., Thompson, R. C. A., 2006:** *Giardia* and *Cryptosporidium* join the "Neglected Diseases Initiative". *Trends Parasitol.*, 22, 203—208. DOI: 10.1016/j.pt.2006.02.015.
21. **Taylor, M. A., Coop, R. L., Wall, R. L., 2015:** *Veterinary Parasitology*, 4th edn., Wiley-Blackwell, Ho boken, NJ, 1032 pp.
22. **Urquhart, G. M., Armour, J., Duncan, J. L., Dunn, A. M., Jennings, F. W., 2003:** *Veterinary Parasitology*, 2nd edn., University of Glasgow, Scotland, 307 pp.
23. **Xiao, L., 2010:** Molecular epidemiology of cryptosporidiosis: an update. *Exp. Parasitol.*, 124, 80—89. DOI: 10.1016/j.exppara.2009.03.018.

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SPATIAL DISTRIBUTION AND SEROPREVALENCE OF NEWCASTLE DISEASE IN KADUNA STATE, NIGERIA

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ABSTRACT

Newcastle disease is one of the greatest constraints to the development of poultry production in Nigeria. In this study, the spatial distribution of Newcastle disease antibodies was determined using the Geographic Information System. A total of 400 serum samples were collected from chickens in districts around Kaduna Metropolis and screened for Newcastle disease virus antibodies using the haemagglutination inhibition test done according to the procedure of OIE (2002). The spatial distribution demonstrated that the highest antibody titre level for Newcastle disease was closely associated with communities that were at entry points (Zaria—Kaduna road; Nnamdi Azikiwe bypass road; Television garage; Abuja—Kaduna expressway and Kachia road) to the metropolis and houses that are closely situated near live bird markets signifying the importance of bird movements in the spread of the disease. About 31 % (124 of 400) of chickens had antibodies to Newcastle disease virus, with exotics breeds (32.0 %) with more Newcastle disease virus antibodies than local breeds (29.8 %). The Newcastle disease prevalence was 33.9 % (39 out of 115), 31.8 % (41 out of 129) and 28.2 % (44 out of 156) for Kaduna North, Kaduna South and Chikun Local Government Areas (LGA), respectively. A Newcastle disease prevalence of 29.8 % (54 out of 181) was recorded in the local chickens, while 32.0 % (70 out of 219) was obtained from the exotic chickens.

Key words: chickens; entry points; haemagglutination inhibition test; Kaduna; Newcastle disease virus antibodies; Nigeria; spatial distribution

INTRODUCTION

Newcastle disease is a highly contagious viral disease caused by an avian paramyxovirus that produces pneumoencephalitis in young chickens, turkeys and other domesticated and wild birds [12]. Newcastle disease is enzootic within individual regions and even villages in Nigeria. Newcastle disease is said to be a major factor affecting the development of the poultry industry in Nigeria and Africa in general [32]. Newcastle disease remains enzootic in states surrounding Kaduna State such as Zamfara and Plateau States, including the Federal Capital Territory [7], with a similarity in climatic and ecologic conditions in these areas. Kaduna metropolis serves as an intersection for the movement between the aforementioned states, as road network plays a major role in the spread of diseases serving as epicentres of disease spread [29]. There have been continuous outbreaks of ND since it was first reported in Nigeria [16]. It has been found to be an epizootic problem in the different parts of Nigeria and continue to cause serious losses in poultry despite vaccination of birds [23]. There has been an emergence of new antigenic lineage of Newcastle disease virus (NDV) in Africa [10].

There have been increasing reports of ND outbreaks in commercial farms in Kaduna metropolis by private veterinarians but the number reported may actually not be representative of the total number of outbreaks due to under-reporting in the current conventional animal disease reporting system and poor veterinary infrastructures [28]. Newcastle disease is particularly devastating for small scale poultry farmers who usually have limited means of protecting their flocks based on the level of their biosecurity practices [32].

Urbanization and increased movement in poultry transportation have resulted in the exposure of chickens to the Newcastle disease virus from infected areas. Since Kaduna Metropolis is an intersection between rural communities and other states due to trade, it could serve as a point of infection for ND in Kaduna State and Nigeria as a whole.

Newcastle disease remains a constant threat to poultry production and has a devastating effect on the productivity of the poultry industry [18]. The current system of disease investigation and reporting is inefficient [11]. There are increasing numbers of ND cases compared to other poultry diseases [4], with a paucity of data about the burden of ND in chickens in Kaduna Metropolis.

Live bird markets contribute to the persistence and spread of ND virus (NDV) [14]. These markets which are found in strategic locations around major road intersections in Kaduna Metropolis usually do not follow appropriate cleaning and disinfecting techniques which allows for the possibility of environmental spread. Live birds are also exposed to birds from multiple sources [29]. These birds run the risk of disseminating Newcastle disease virus as they leave the markets posing a health threat to household chickens [21].

Vaccination has been reported as the only safeguard against endemic ND [2]. In order to formulate appropriate vaccination schedules and control measures, the serological status of NDV among chickens in Kaduna State need to be elucidated. Also, Kaduna State, especially the metropolis, has witnessed massive increase in the demographic population in the last few years and to meet the increased protein demand, poultry and poultry products are being moved to live bird markets across the state for sales [3]. The preference of consumers for this type of meat and the readily available markets justifies the need for this study.

MATERIALS AND METHODS

Study area

Our study was carried out in the Kaduna metropolis of Kaduna State. Kaduna Metropolis is accessible via major transportation links from all regions of the country. Kaduna Metropolis lies in three different Local Government Areas (LGAs): Kaduna North, Kaduna South, and Chikun; located between latitude 10°34'57.4" N and 10°22'56.6" N and longitude 7°38'12.2" E and 7°23'29.0" E. According to the latest census, the city's population in 2006 was 1,128,694 [24].

Study population

For serology, the study population consisted of mostly backyard poultry and free-ranging (extensively managed) poultry kept and having the possibility to mix with other birds. Commercial poultry farms were excluded while exotic breeds managed as backyard poultry with local chickens were included.

Study design

A cross-sectional study was carried out consisting of two separate overlapping descriptive and analytical stud-

ies on data obtained on spatial distribution and seroprevalence of Newcastle Disease in Kaduna Metropolis from October 2014 to April 2015.

Sampling methods

Sample frame and sample size determination

The sampling frame consisted of a list of localities and communities obtained from the Kaduna State chapter of the National Population Commission and local government secretariats that form the Kaduna city area.

Sample size was determined using the formula [33]:

$$n = \frac{Z^2 \times P_{exp}(1 - P_{exp})}{d^2}$$

where:

- n = required sample size;
- P_{exp} = expected prevalence (using 57 % prevalence [7]);
- Z = standard normal deviation of 95 % confidence level (1.96);
- d = desired absolute precision (0.05).

Substituting these values in the formula:

$$n = \frac{(1.96)^2 \times 0.57 \times (1 - 0.57)}{(0.05)^2} = 376.63$$

A total number of 400 chicken blood samples were collected to increase the level of precision.

Kaduna North LGA had the minimum number of districts having 6 districts in total. Six districts each were then selected randomly from the remaining LGAs in the metropolis based on this minimum number, giving a total of 18 districts. A total of 400 chicken blood samples were collected from household poultry farms from the 18 selected districts across the metropolis using convenience sampling. Sampling location was geo-referenced using GIS mapping system to get coordinates.

Sample collection and transportation

Two millilitres of blood were collected from the chickens through the brachial vein using 21-gauge needles and 5 ml syringes after carefully observing asepsis to avoid any contamination of the blood. The blood was allowed to clot in a cool place to allow the serum to separate from the blood clot. The serum was then decanted into another tube, stored in the refrigerator at 4 °C and transported to the Viral Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, ABU Zaria using cooler packs for haemagglutination inhibition tests to detect antibodies to ND virus.

The vaccination status of chickens sampled were not determined during this study in order to get a picture of the level of flock immunity and to control any form of bias towards the protective antibody titre levels to ND virus in the study area.

Sample analyses

Preparation of chicken red blood cell suspension

A total of 5 ml of chicken blood was pooled from 3 separate apparently healthy chickens aseptically into a disposable syringe containing 1 ml of sodium citrate (4 % solution) as an anticoagulant. The blood was centrifuged at 1,500 rpm for 10 minutes and the plasma and buffy coat were removed with a pipette. After washing three times with phosphate buffered saline (PBS), the 1 % suspension was ascertained to be free of ND antigen or any related antigen capable of cross-reactions. This was done by observing under normal conditions in a U-bottomed microtitre plate and testing for agglutination and agglutination inhibition using ND antigen (La Sota) and ND antibody-positive serum obtained from the National Veterinary Research Institute Vom, respectively, on the same sample.

In the 1st stage, 25 µl of the 1 % RBC suspension was put in a microtitre plate and 25 µl of PBS solution was added to it. The plate was tapped gently and kept at room temperature for 30 minutes and observed for settling of the red blood cells. The 2nd stage was done by adding 25 µl of the ND antigen into a well to which 25 µl of PBS solution was added, followed by 50 µl of the 1 % chicken red blood cell suspension. The plate was tapped at the side repeatedly before it was covered and kept at room temperature for 45 minutes and observed for haemagglutination. The 3rd stage involved the addition of 25 µl of ND antibody serum to the well in the 2nd stage, tapped at the side repeatedly before it was covered and kept at room temperature for 45 minutes and observed for haemagglutination inhibition. The pooled chicken blood sample which was ascertained to be free of ND antigen or any related antigen capable of cross-reaction, by expressing the 1st, 2nd, and 3rd reaction as stated in the procedure above was used to prepare the 1 % chicken red blood cell suspension in PBS used for the HI test.

Haemagglutination test

Haemagglutination test was carried out according to the method described by Grimes [13]. Twenty-five microlitre of PBS was dispensed into the each well in the first row of the microtitre plate. Twenty-five microlitre of the ND antigen suspension (La Sota vaccine) was then dispensed into the first well. A two-fold dilution of 25 µl of the suspension was done across the wells up to the 11th well. Twenty-five microlitre of PBS solution was further added to each well in the first row. Then 25 µl of 1 % chicken RBC was dispensed into each of the wells. The 12th well would serve as control, containing only PBS and red blood cells. The plate was tapped gently before it was covered and kept for 45 minutes at room temperature. The HA was determined by observing for a sharp button of RBCs (negative) or hazy film and no button of RBCs (positive) at the bottom of the plate. The end point was the last well to show complete haemagglutination and contained one haemagglutination unit (1 HAU). The cut-off titre was calculated using the range of initial dilutions from which 4 HAU was arrived and this was used for the HI test.

Haemagglutination inhibition test

The sera collected from the chickens were tested for NDV specific antibody by the haemagglutination inhibition test (HI) as described by Hosain et al. [15] and done according to the procedure of OIE [26]. The HI test was performed using the beta technique (constant virus and varying serum) against 4 HA units of the virus computed from the HA titration. Two-fold serial dilution of 25 µl serum was made with phosphate buffer saline (PBS) in U-bottomed microtitre plates up to 10th well in the 2nd row. Twenty-five microlitres of 4 haemagglutinating (HA) units of NDV virus or antigen (La Sota) was added up to the 11th well. The plates were kept at room temperature for 30 minutes to facilitate antigen-antibody reaction. Then 50 µl of 1 % (v/v) chicken RBC suspension was added to each well. The 11th well contained antigen and RBCs as the positive control and the 12th well contained only RBCs as the negative control. After gentle mixing, the RBCs were allowed to settle at room temperature for 45 minutes and agglutination was assessed by tilting the plates. The samples showing peculiar central button shaped settling of RBCs were recorded as positive and maximum dilution of each sample causing haemagglutination inhibition was considered as the end point, which was used to estimate the HI titre. The HI titre of each serum sample was expressed as the reciprocal of the serum dilution.

Data management and analyses

The results obtained from the HI tests were subjected to descriptive statistics to determine the frequency and distribution of the ND antibody titre of Kaduna metropolis (Tables 2 and 3). Categorical values were evaluated using the Chi Square test to check for association; odds ratio at 95 % confidence interval was used to measure the strength of association between variables and the prevalence of ND. All data obtained were analysed using SPSS statistical software (17.0)

Values of $P < 0.05$ were considered significant. Sample prevalence was estimated using the formula:

$$\text{Sample Prevalence} = \frac{\text{Number of Positive Samples for ND Antibody}}{\text{Total Number of Samples Tested}} \times 100$$

RESULTS

Spatial distribution of Newcastle disease antibodies

There were variations in the poultry distribution patterns between various communities. The poultry sampled were more concentrated in communities that had a cluster settlement pattern, while communities that had a linear settlement pattern had a low population of poultry. The antibody titre level of Newcastle disease was higher in communities that had entry points to the metropolis and houses that were closely situated with the live bird markets. The entry points included:

A. Zaria—Kaduna road;
B. Nnamdi Azikiwe bypass road;
C. Television garage;
D. Abuja Kaduna expressway and E. Kachia road (Fig. 1).

Seroprevalence of ND antibodies

The overall seroprevalence of Newcastle disease antibodies in the study area was 31.0 % (124 out of 400). Among the positive Newcastle antibody sera, 103 out of 400 (25.75 %) had titres between 1/64 and 1/512 and 21 out of 400 (5.25 %) had titres ≥ 1/4096 (Table 1). The ND prevalence was 33.9 % (39 out of 115), 31.8 % (41 out

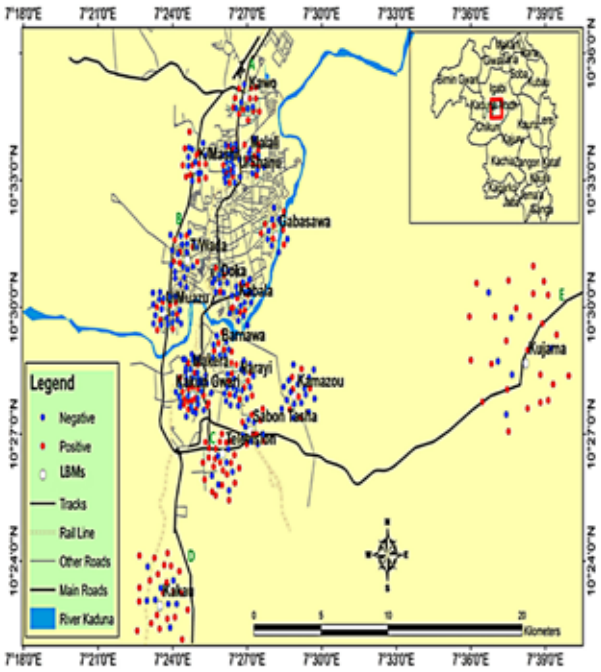

Fig. 1. Map of Kaduna Metropolis showing the coordinates of the positive and negative samples of Newcastle disease antibodies in districts where the study was carried out, Live bird markets (LBMs) and entry points A—E (ArcGIS 10.1)

of 129) and 28.2 % (44 out of 156) for Kaduna North, Kaduna South and Chikun local governments, respectively (Table 2).

The prevalence of 29.8 % (54 out of 181) was recorded in local chickens, while 32.0 % (70 out of 219) was obtained from the exotic chickens, with local chickens having the odds ratio of 0.905 times likely of being positive for ND antibody than the exotic chickens (Table 3).

DISCUSSION

This serological study revealed the presence of circulating antibodies of NDV among chickens in three different districts of the Kaduna Metropolis. Also, the occurrence of detectable ND haemagglutinating (HI) antibodies in chickens tested in all of the three LGAs of Kaduna studied, with an overall seroprevalence of 31 %, was diagnostic evidence that ND was prevalent and likely enzootic in the study areas [17]. The antibodies detected were most likely due to natural infections in the local chickens rather than vaccination since local chickens were rarely vaccinated in

Table 1. Distribution of Newcastle disease antibody titre levels in the sera collected from chickens in the Kaduna metropolis of Kaduna State, Nigeria

Local government area	HI titre												
	≤ ½	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024	1/2,048	≥ 1/4,096	Total
Kaduna North	14	10	18	14	20	10	10	7	5	5	1	1	115
Kaduna South	14	17	15	25	17	5	9	10	10	4	1	2	129
Chikun	17	29	23	25	18	10	8	11	8	3	3	1	156
Total	**45**	**56**	**56**	**64**	**55**	**25**	**27**	**28**	**23**	**12**	**5**	**4**	**400**
%	**11.25**	**14**	**14**	**16**	**13.75**	**6.25**	**6.75**	**7**	**5.75**	**3**	**1.25**	**1**	**100**

Table 2. Distribution of Newcastle disease titre levels in sera collected from chickens in the three Local Government Areas of the Kaduna metropolis of Kaduna State, Nigeria

Local Government Area	HI titre		Total	%
	< 1/64 (-ve)	≥ 1/64 (+ve)		
Kaduna North	76	39	115	33.9
Kaduna South	88	41	129	31.8
Chikun	112	44	156	28.2
Total	**276**	**124**	**400**	**31**

Chi square value (χ^2) = 1.063; P-value = 0.588; (-ve)—Negative ND antibody sera; (+ve)—Positive antibody sera

Table 3. Seroprevalence of Newcastle disease in the breed of chickens in Kaduna metropolis of Kaduna State, Nigeria

Breed	No. of samples tested	No. of samples positive for ND antibody	No. of samples negative for ND antibody	Prevalence [%]	Odds Ratio	95 % CI
Local chickens	181	54	127	29.8	0.905	0.591–1.387
Exotic chickens	219	70	149	32.0	1.00	
Total	**400**	**124**	**276**	**31.0**		

95 % CI = Confidence Interval; Chi square value (χ^2) = 0.210; P-value = 0.647

Nigeria [1], while most likely due to vaccination in the exotic chicken flock [27].

Our study demonstrated a relatively comparable seroprevalence rate of ND in chickens obtained by other investigators in other countries, i. e., with an overall seroprevalence rate of 33 % in Pakistan [20], and 27.86 % in backyard and small-scale chicken poultry farms in Ethiopia [8].

However, our study revealed a relatively lower seroprevalence rate of ND compared to the overall seroprevalence rate of 66.5 % in commercial poultry farms, village households and live bird markets in Benue State [1]; 63.5 % in local and exotic chickens in the Gwagwalada [5]; 38.5 % recorded in both Bauchi and Gombe States [22]; 79.6 % in North Gondar zone of Ethiopia [9] and 40.13 % reported in unvaccinated backyard poultry in Iran [30]. On the other hand, our study showed a higher seroprevalence rate as compared to the overall seroprevalence rate of 22.3 % in cross-bred chickens in Afikpo [25], and 6.2 % in the Barda region of Azerbaijan [34].

These observed differences in the seroprevalence of ND may be as a result of differences of ecological factors in NDV survival and transmission, and variations in sampling methods [31]. This could also be as a result of local preventive methods used against ND in the study area [6]. Although no advanced field and laboratory research was done in this study to differentiate ND antibodies due to vaccination from those from active infections.

Also, the titre of serum antibodies to ND virus in chickens could be affected if the chickens were infected with IBD virus, since it has been reported to be significantly lower than that of birds infected with ND virus alone [19]. It has also been reported that IBD virus affects ND virus excretion from chickens as ND virus was more frequently isolated from chickens infected with IBD virus, without the IBD virus infection altering the pathogenicity of ND virus in chickens [19].

CONCLUSIONS

The spatial distribution of ND from this study showed a higher concentration of ND antibodies at entry routes to the metropolis, signifying the importance of bird movement in the spread of the disease. The entry points include:

A. Zaria—Kaduna road;
B. Nnamdi Azikiwe bypass road;
C. Television garage;
D. Abuja—Kaduna expressway and
E. Kachia road.

Serological study showed a 31 % prevalence (124 out of 400) of ND antibodies in the study area indicating the enzootic nature of the disease. The ND prevalence was 33.9 % (39 out of 115), 31.8 % (41 out of 129) and 28.2 % (44 out of 156) for Kaduna North, Kaduna South and Chikun local governments respectively. Newcastle disease prevalence of 29.8 % (54 out of 181) was recorded in local chickens while 32.0 % (70 out of 219) was obtained from the exotic chickens, with local chickens having the odds ratio of 0.905 times likely of being positive for ND antibody than exotic chickens.

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REFERENCES

1. **Abah, H. O., Ochola, P. F., Ishaya, V., 2020:** Survey for Newcastle disease virus antibodies in local chickens, ducks and pigeons in Makurdi, Nigeria. *Anim. Vet. Sci.*, 8, 3, 55—59 DOI: 10.11648/j.avs.20200803.12.
2. **Abdisa, T., Tagesu, T., 2017:** Review on Newcastle disease of poultry and its public health importance. *J. Vet. Sci. Technol.*, 8, 3, 441. DOI: 10.4172/2157-7579.1000441.
3. **Abdullahi, J., Shaibu-Imodagbe, E. M., Mohammed, F., Sa'id, A., Dahiru, I. U., 2009:** Rural-urban migration of the Nigerian work populace and climate change effects on food supply: A case study of Kaduna city in Northern Nigeria. In *Fifth Urban Research Symposium: Cities and Climate Change: Responding to the Urgent Agenda,* Marseille, France, June 28—30, 16 pp.
4. **Aliyu, H. B., Sa'idu, L., Abdu, P. A., Oladele, S. B., 2015:** Retrospective analysis of Newcastle disease diagnosed at the poultry clinic of Ahmadu Bello University, Zaria, Nigeria. *Sok. J. Vet. Sci.*, 13, 3, 42—48. DOI: 10.4314/sokjvs.v13i3.7.
5. **Ameh, J. A., Mailafia, S., Olatunde, H., Olabode, H. O., Adah, B. J., Okoh, G. J., et al., 2016:** Sero-prevalence of Newcastle disease virus antibodies in local and exotic chickens in Gwagwalada, Nigeria. *J. Vet. Med. Anim. Health*, 8, 11, 193—198. DOI: 10.5897/JVMAH2016.0482.
6. **Anebo, Z. G., Teklemichael, K., Belachew, B., Tadios, Addisalem, H., 2014:** Evaluation of the Newcastle disease antibody level after vaccination regimes in chickens in Debrezeit Agricultural Research Center, Ethiopia. *J. Vet. Med. Anim. Health*, 6, 1, 7—12. DOI: 10.5897/JVMAH2013.0229.
7. **Anzaku, S. A., Umoh, J. U., Abdu, P. A., Kabir, J., Bala, A., 2017:** Serological survey of Newcastle disease in free ranging local chickens in the Federal Capital Territory, Abuja, Nigeria. *New J. Sci.*, 2017, 9646138, 1—5. DOI: 10.1155/2017/9646138.
8. **Asfaw Geresu, M., Elemo, K. K., Kassa, G. M., 2016:** Newcastle disease: Seroprevalence and associated risk factors in backyard and small scale chicken producer farms in Agarfa and Sinana Districts of Bale Zone, Ethiopia. *J. Vet. Med. Anim. Health*, 8, 99—106. DOI: 10.5897/JVMAH2015.0427.
9. **Birhan, M., Birhan, M. I., Tesfaye, S., Tariku, A., 2019:** Detection of antibodies against Newcastle and infectious bursal disease on chicken in north Gondar zone, Ethiopia. *Online J. Anim. Feed Res.*, 9, 2, 51—58.
10. **Cattoli, G., Fusaro, A., Monne, I., Molia, S., Le-Menach, A., Maregeya, B., et al., 2010:** Emergence of new genetic lineage of Newcastle disease virus in West and Central Africa-Implications for diagnosis and control. *Vet. Microbiol.*, 142, 3—4, 168—176. DOI: 10.1016/j.vetmic.2009.09.063.
11. **Gary, F., Diop, B., Barbosa, H., 2010:** *Performances of Veterinary Services (PVS) Gap Analysis Report: Federal Republic of Nigeria*. World Organisation for Animal Health, Paris, France, 22—23.
12. **Getabalew, M., Alemneh, T., Akeberegn, D., Getahun, D., Zewdie, D., 2019:** Epidemiology, diagnosis and prevention of Newcastle disease in poultry. *Am. J. Biomed. Sci. Res.*, 2019, 3, 1. AJBSR. MS.ID.000632. DOI: 10.34297/AJBSR.2019.03.000632.
13. **Grimes, S. E., 2002:** *A Basic Laboratory Manual for the Small-scale Production and Testing of I-2 Newcastle Disease Vaccine*. Food and Agricultural Organisation (FAO) Publication No. 22, Regional Office for Asia and the Pacific (RAP), Bangkok, Thailand, 136 pp.
14. **Haile, B., Fentie, T., 2020:** The role of live chicken markets as a source of replication and dissemination of Newcastle disease virus in chickens, northwest Ethiopia. *Poult. Sci.*, 99, 11, 5415—5421. DOI: 10.1016/j.psj.2020.08.025.
15. **Hossain, K. M., Ali, M. Y., Yamato, I., 2010:** Antibody levels against Newcastle Disease Virus in chickens in Rajshahi and

surrounding districts of Bangladesh. *Int. J. Biol.*, 2, 2, 102—106. DOI: 10.5539/ijb.v2n2p102.

16. **Ibitoye, E. B., Jimoh, A. A., Mungadi, H. U., 2013:** A retrospective (2007—2011) analysis of Newcastle disease diagnosed at Avian clinic of Veterinary Teaching Hospital, Usmanu Danfodiyo University Sokoto, Nigeria. *Curr. Res. Poult. Sci.*, 3, 1, 12—17. DOI: 10.3923/crpsaj.2013.12.17.
17. **Jibril, A. H., Umoh, J. U., Kabir J., Saidu, L., Magaji, A. A., Bello, M. B., et al., 2014:** Newcastle disease in local chickens of live bird markets and households in Zamfara State, Nigeria. *ISRN Epidemiology 2014*, Article ID 513961. DOI: 10.1155/2014/513961.
18. **Kapczynski, D. R., Afonso, C. L., Miller, P. J., 2013:** Immune responses of poultry to Newcastle disease virus. *Dev. Comp. Immunol.*, 41, 3, 447—453 DOI: 10.1016/j.dci.2013.04.012.
19. **Kashif-ur-Rehman, Ahmad, M., Raza, M. A., Ahmad, M. J., Joiya, M. H., Imtiaz-ul-Haq, et al., 2014:** Effects of infectious bursal disease vaccine on the immunity induced by Newcastle disease vaccine in broiler birds. *Sci. Int.* (Lahore), 26, 1, 243—247.
20. **Khan, M. Y., Arshad, M., Hussain, I., 2011:** Epidemiology of Newcastle disease in rural poultry in Faisalabad, Pakistan. *Int. J. Agric. Biol.*, 13, 491—497. DOI: 10–705/RNK/2011/13–4–491–497.
21. **Lawal, J. R., El-Yuguda, A. D., Ibrahim, U. I., 2016:** Survey on prevalence of Newcastle disease in village poultry at live bird markets in Gombe, Nigeria. *J. Anim. Sci. Livest. Prod.*, 1, 1, 2—9. DOI: 10.21767/2577-0594.100001.
22. **Musa, I. W., Abdu, P. A., Sackey, A. K. B., Oladele, S. B., Lawal, S., Yakubu, I. U., 2010:** Outbreak of velogenic viscerotropic Newcastle disease in broilers. *Int. J. Poult. Sci.*, 9, 1116—1119. DOI: 10.3923/ijps.2010.1116.1119.
23. **Musa, W. I., Abdu, P. A., Sa'idu, L., Bello, M., 2016:** Survey for avian influenza and Newcastle disease antibodies and viruses in domestic and wild birds in Bauchi and Gombe States, Nigeria. In *17th International Congress on Infectious Diseases*, Hyderabad, India, March 2—5/*Int. J. Infect. Dis.*, 45S: 1–477 (472). DOI: 10.1016/j.ijid.2016.02.997.
24. **National Population Commission (NPC), 2006:** *Social Statistic in Nigeria, National Bureau of Statistics: Federal Republic of Nigeria*, 300 pp.
25. **Nworie, O., Ezeifeka, G. O., Ogbu, O., Ogbu, M. A., Anyim, C., Ugwu, J. I., et al., 2012:** Sero-prevalence of Newcastle disease in humans and apparently cross-breed of chickens in Nigeria. *Cent. Eur. J. Exp. Biol.*, 1, 2, 69—73.
26. **Office International des Epizooties (OIE), 2008:** *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. 6th edn., OIE, Paris, France, 1, 581—582.
27. **Orsi, M. A., Doretto, Jr., L., Camillo, S. C. A., Reischak, D., Ribeiro, S. A. M., Ramazzoti, A., et al., 2010:** Prevalence of Newcastle disease virus in broiler chickens (*Gallus gallus*) in Brazil. *Braz. J. Microbiol.*, 41, 2, 349—357. DOI: 10.1590/S1517-83822010000200014.
28. **Perry, B. D., Randolph, T. F., Bett, B., Grace, D., Globig, A., Fadiga, M., et al., 2011:** *Independent Impact Assessment of the World Bank-funded Nigeria Avian Influenza Control and Human Pandemic Preparedness and Response Project (NAICP).* Nigeria, 258 pp.
29. **Rivas, A. L., Chowell, G., Schwager, S. J., Fasina, F. O., Hoogesteijn, A. L., Smith, S. D., et al., 2010:** Lessons from Nigeria: The role of roads in the geo-temporal progression of avian influenza (H5N1) virus. *Epidemiol. Infect.*, 138, 192—198. DOI: 10.1017/S0950268809990495.
30. **Saadat, Y., Ghafouri, S. A., Tehrani, F., Langeroudi, A. G., 2014:** An active serological survey of antibodies to Newcastle disease and avian influenza (H9N2) viruses in the unvaccinated backyard poultry in Bushehr province, Iran, 2012–2013. *Asian Pac. J. Trop. Biomed.*, 4 (Suppl. 1), S213—S216. DOI: 10.12980/apjtb.4.2014c1293.
31. **Salihu, A. E., Chukwuedo, A. A., Echeonwu, G. O. N., Ibu, J. O., Chukwuekezie, J. O., Ndako, J., et al., 2012:** Seroprevalence of Newcastle disease virus infection in rural household birds in Lafia, Akwanga and Keffi Metropolis, Nasarawa State Nigeria. *Int. J. Agric. Sci.*, 2, 2, 109—112.
32. **Shittu, I., Joannis, T. M., Georgina, N., Odaibo, O., Olufemi, D., 2016:** Newcastle disease in Nigeria: Epizootiology and current knowledge of circulating genotypes. *Virus Dis.*, 27, 4, 329—339. DOI 10.1007/s13337-016-0344-6.
33. **Thrusfield, M. V., 2005:** *Veterinary Epidemiology*, 3rd edn., Blackwell Science Limited, Oxford, UK, 233 pp.
34. **Zeynalova, S., Guliyev, F., Vatani, M., Abbasov, B., 2015:** Biosurveillance of avian influenza and Newcastle disease viruses in the Barda region of Azerbaijan using real time RT-PCR and hemagglutination inhibition. *Front. Microbiol.*, 6, 1128. DOI: 10.3389/fmicb.2015.01128.

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ULTRASTRUCTURAL MORPHOLOGY OF THE EPENDYMA AND CHOROID PLEXUS IN THE AFRICAN GIANT RAT (*CRICETOMYS GAMBIANUS*)

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ABSTRACT

Ependymal cells line the interface between the ventricular surfaces and the brain parenchyma. These cells, in addition to the choroid plexus, form the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) and serve important functions in the protection and regulation of brain metabolism. The African giant rat (AGR) has been used as sentinels to detect potential neuropathology arising from ecotoxicological pollutions. This study examined the lateral ventricular lining by using histology, immunohistochemistry and electron microscopy. Marked variations were observed in some regions of the ventricles which showed multi-layering of ependymal cells that differed from the typical single layered ependymal cells at the apical surface, while subependymal structures revealed indistinctive neuropil and glia following histological examinations. The ependymal cells which form the epithelial lining of the ventricles were comprised of cuboidal or low columnar cells, with the plasmalemma of abutting cells forming intercellular bridge appearing links by: tight junctions (*zonula occludens*), intermediate junctions (*zonula adherens*), desmosomes (*macula adherens*) and infrequent gap junctions. The choroid plexus revealed cells of Kolmer with several cilia and microvilli. The possible functional components of the ependyma and choroid plexus morphology of the AGR are discussed and thus provide a baseline for further research on the AGR brain.

Key words: African Giant Rat; choroid plexus; ependymal; tight junctions; ultrastructure

INTRODUCTION

The African giant rat (*Cricetomys gambianus*) is a nocturnal, sub-Saharan African rodent which shares close dwellings with human societies. For this reason, they are currently being utilized as sentinel models to study ecotoxicological pollutions and disease burdens [37]. This continued use as a research model for biomedical research has also spurred studies into their morphophysiology and especially that of the nervous system [15, 24, 25, 27]. The information generated has been progressively helpful in proposing better concepts in the adoption of the AGR as a convenient laboratory animal for investigating diseases of great significance to humans and their domestic animals [39].

One of the more recent uses of the AGR has been the use of their olfactory prowess to sniff out landmines and diagnose tuberculosis infected sputum samples [30, 39]. This attribute therefore requires the proper understanding of the neural systems in the AGR. The gross and cellular central nervous system (CNS) profile of the African giant rat have been elucidated with various studies covering: adult neurogenesis, astrocyte heterogeneity and oligodendrocyte morphology [25—29]. The ependyma and choroid plexus are yet to be described for this rodent.

Morphologically, the ependyma is typically known as a single-layered, cuboidal to columnar-celled, ciliated epithelium on the surface of the ventricles of the brain and central canal of the spinal cord [10] and is responsible for important functions related to: morphogenesis, brain physiology, neuro-protection and water transport [9, 16]. The typical rodent ependyma morphology has been shown to consist of multi-layering populations, sub-ependymal cells and the existence of abundant gap junctions [12, 21]. The choroid plexus meanwhile, is an epithelial–endothelial vascular convolute within the ventricular system of the vertebrate brain which consists of fenestrated blood vessels and synthesizes the cerebrospinal fluid [7, 40]. Adjoining choroid plexus cells have tight junctions that form the blood- cerebrospinal fluid barrier (BCSFB). This barrier along with the blood-brain barrier (BBB), play a major role in the homeostatic regulation of the brain by protecting the CNS from the inflammatory molecules, pathogens, and toxins which may be circulating in the blood stream [8, 31].

This paper therefore seeks to add to the body of knowledge on the neuro-cellular profile of the African giant rat by describing the morphology of ependymocytes and choroid plexus using the electron microscopy and allude to the functional basis of maintaining the integrity of the BBB and BCSFB.

MATERIALS AND METHODS

A total of 6 male African giant rats (*Cricetomys gambianus*) randomly assigned into two groups (A and B; n = 3/group) were used for this study. All rats were anaesthetized with intraperitoneal injection of Ketamine/Xylazine (90/10 $mg.kg^{-1}$) and were subsequently perfused transcardially with 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. (for animals in group A) and freshly prepared Karnovsky fixative (for animals in group B). The brains harvested from group A were processed for histology (with H&E and Cresyl violet stain for anatomical orientation) while immunohistochemistry (IHC) with anti-glial fibrillary acidic protein (anti-GFAP) was performed using the free-floating method for tanycytic astrocytes of the subependyma in accordance with the methods described by Olude et al. [27] and Mustapha et al. [24]. The brain samples from group B were prepared for both scanning and transmission electron microscopy as described by Olude et al. [25]. The images were captured using Zeiss Axioskop (Carl Zeiss Microscopy GmbH, Jena, Germany) and Axiovision software (Carl Zeiss Microscopy GmbH, Jena, Germany).

RESULTS

The ependymal cells with variable numbers of cilia, formed the epithelial lining of the lateral ventricles comprised of cuboidal or low columnar cells in shape. Regional variations were observed in some regions of the ventricles with multi-layering of ependymal cells which differed from the typical single layered ependymal cells at the apical surface. The sub-ependymal structures reveal indistinctive neuropil and glia following histological examination (Fig. 1a, b). Anti-GFAP immunohistochemistry revealed a clear zone of ependymal cells and brush borders (arrowhead in Fig. 1c) and a sub-ependymal zone with sequential extensions of processes (black arrows in Fig. 1d) into the subependymal hypocellular level (Fig. 1c, d).

The examination of the ultrastructure revealed that the

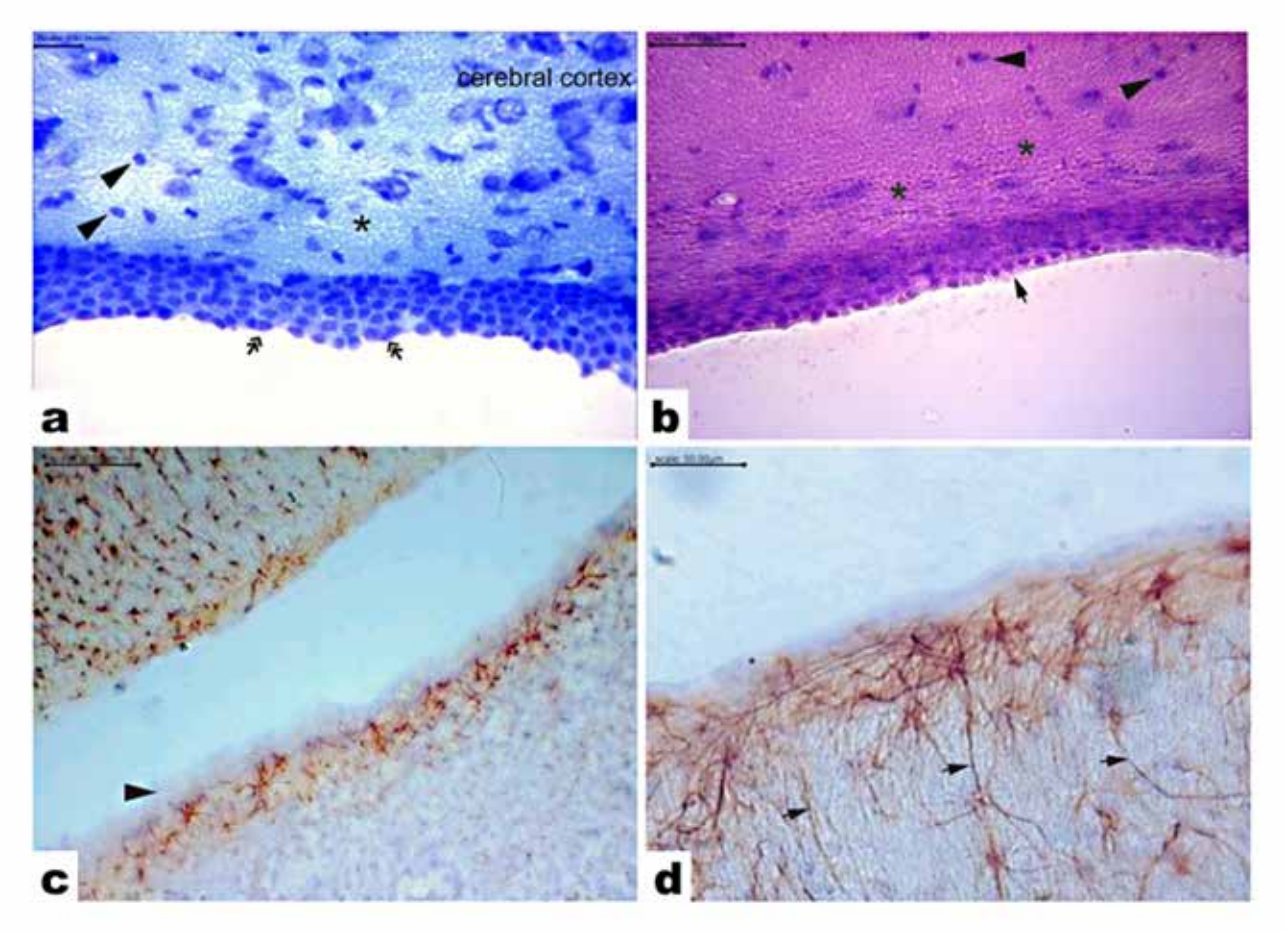

Fig. 1. Histology of the ependymal and sub-ependymal structures: [a] Nissl stain and [b] H&E stain

Note the multi-layered ependymocytes (double arrowheads, **a)** compared to the single-layered ependymocytes (black arrow; **b)** and also the indistinct neuropil (asterisks) and glia (arrowheads, a & b) in the subependymal layer; **c)** Anti-GFAP showing distinguishable ependymal cell and brush borders (arrowhead). **d)** Anti-GFAP showing zone of extensive processes projecting to the sub-ependymal hypocellular layer (arrows). Scale bars: 100 µm (a); 50 µm (b—d)

epithelial surface presented broad ridges with finger-like projections of ciliary shafts and numerous microvilli. The nucleus was simple, regularly oval and occupied large portions of the cell close to the wall while the nuclear envelope had patches of ribosomes on the outer cytoplasmic surface (Fig. 2a). Several mitochondria were seen in the supranuclear region and fewer in the basal aspects. They were typically elongated with internal transverse cristae structure (Fig. 2a, 2b). The endoplasmic reticulum was widely spread, presenting smooth surfaced vesicles with short canaliculi (Fig. 2b), while the Golgi complexes which were also found supranuclear in the cytoplasmic region with dense cluster of vesicles and flattened cisternae in a stack arrangement (Fig. 2b). These were very closely associated with mitochondria and endoplasmic reticulum while within the cytoplasm of the ependyma cells (Fig. 2b). Few supraependymal intraventricular macrophages were sighted at the apical surface of non-ciliated ventricular ependymocytes, with few microvilli (Fig. 2c). Cilia were observed as typical as in protozoans with 9 + 2 arrangement (nine peripheral doublets and a central pair) enclosed within the ciliary membrane (Fig. 2d). The structural layout of the crowning ependymal cilia was built on a typical ciliary complex with basal body, rootlets within the surrounding granular zones in the subapical zone. Cone-shaped striated basal feet were noted to be in contact with the rootlets of the basal bodies (Fig. 2e). Tight junctions (*zonula occludens*), intermediate junctions (*zonula adherens*) and desmosomes (*macula adherens*) and infrequently gap junctions were identified along the adjoining borders of neighbouring cells. *Zonula occludens* and *zonula adherens* were mostly found close to the luminal junctions while desmosomes were seen largely on the distal adjoining lateral portions of the plasmalemma of contiguous ependymocytes (Fig. 2b).

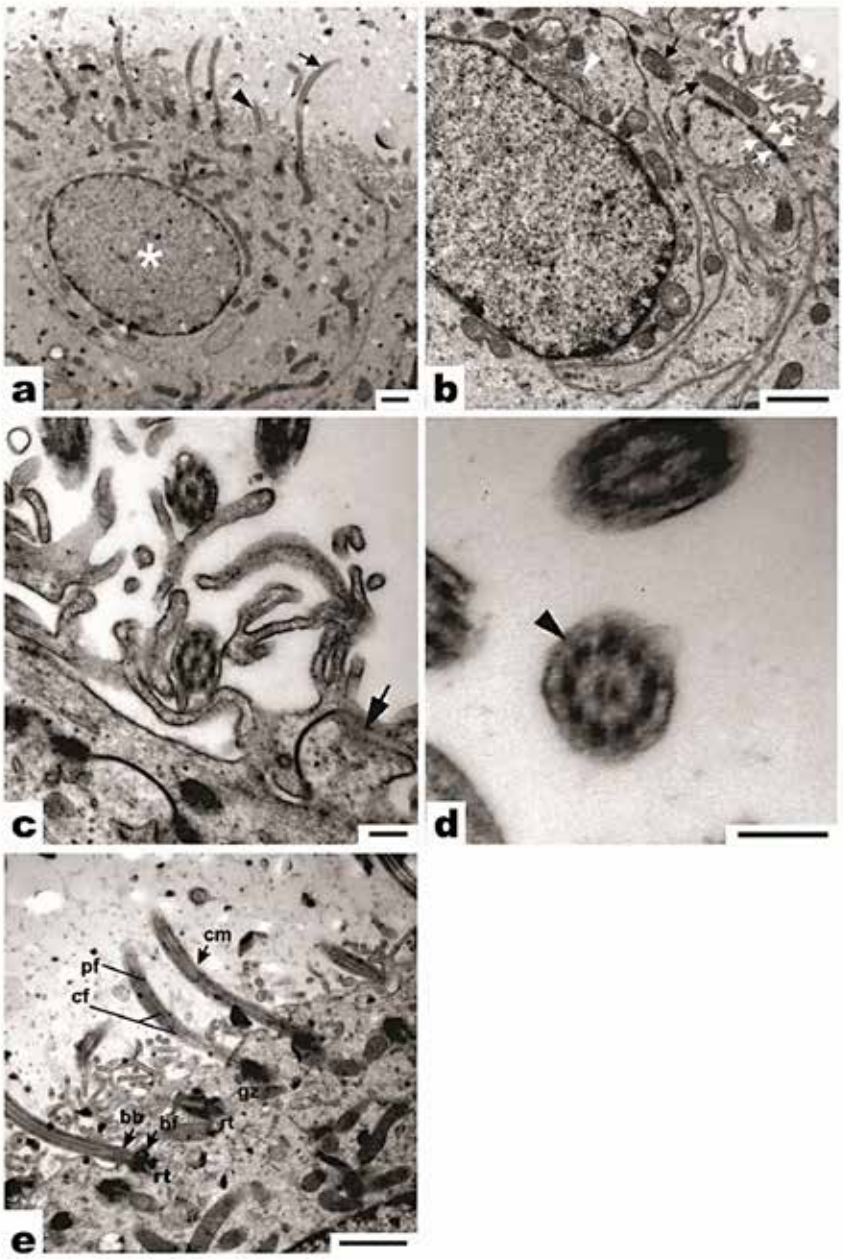

Fig. 2. Transmission electron micrograph of the AGR ependymal layer showing: a) distinct intracellular details of an ependymocyte

Note the oval shaped nucleus (white asterisk), apical cilia (black arrow) and microvilli (black arrowhead); **b)** cellular junctional complexes formed by two abutting plasmalemmae (white arrows) with numerous mitochondria (black arrows) at the apical membrane and Golgi complexes (white arrowhead) within the cytoplasm; **c)** A non-ciliated portion of the ependymal layer with a supra-ependymal intraventricular macrophage (black arrow) at the apical surface with scanty microvilli; **d)** Cross section of the ciliary shafts showing the typical 9 + 2 arrangement of ependymal cilia (black arrowhead); **e)** The ciliary complex of the AGR ependyma highlighting the basal body (bb), rootlets (rt), basal foot (bf), and granular zone (gz). cf, central fibres; cm, ciliary membrane; pf, peripheral subfibre. Scale bars: 500 nm (a—b, e); 200 nm (c—d)

The scanning electron micrographs of the roof and floor of the lateral ventricles were viewed and presented minimal differences with slight morphological patterns. The surface appearance and modifications of the roof were almost always more populated and denser while the floor showed areas of patches with the cilia slightly thicker and less clumped than those of the roof (Fig. 3a—d). The wall epithelium looked matted and slightly scantier with domains of "baldness" with regular patterns.

The surface of the choroid plexus of the lateral ventricles showed varied folds with homogeneous vesicle-like projections which represent choroid cells (Fig. 4a, 4b).

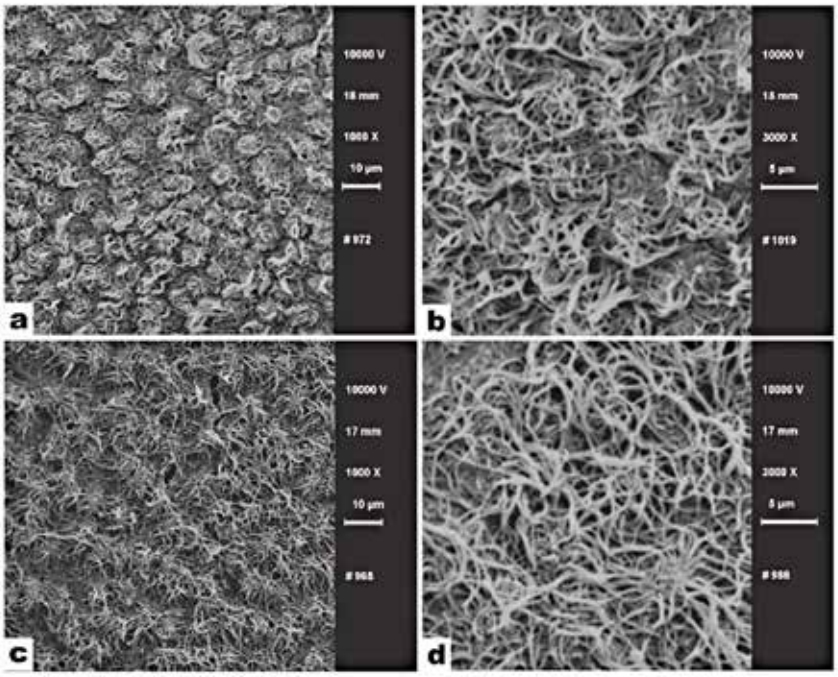

Fig. 3. Scanning electron micrographs of the lateral ventricles showing slight variations in the morphology in the walls of the lateral ventricles at a) low magnification; b) high magnification and in the roof of the lateral ventricles at c) low and d) high magnification

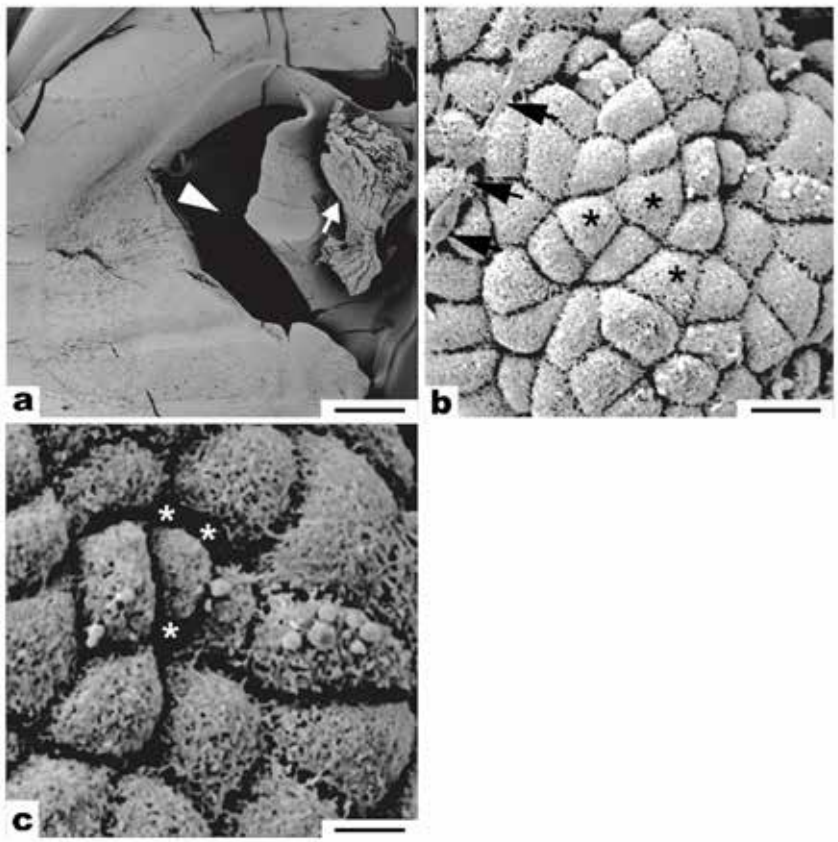

Fig. 4. Scanning electron micrograph showing: a) the choroid plexus (white arrow) at lower magnification projecting into the lateral ventricle (white arrowhead); b) Epiplexus cell of Kolmer with distinct protoplasmic extensions (black arrows) as well as choroid cells (black asterisks) and c) junction sites separating one choroid cell from another (white asterisks). Scale bars: a) 1000 µm; b) 10 µm; c) 5 µm

There were electron-dense areas surrounding each cell which were the junction sites from one choroid to another (Fig. 4c.). The surfaces of the choroid cells had cilia and microvilli. Epiplexus cells of Kolmer were observed with their protoplasmic extensions (Fig. 4b). The lateral ventricle is demonstrated by the white arrowhead (Fig. 4a).

DISCUSSION

The basic morphology of ependymal cells ranges from flattened, cuboidal to columnar cells. In the AGR, we identified cuboidal or low columnar cells in shape. Other features presented in the AGR, including the regional multi-layering of ependymal cells are reported as typical rodent features [10, 21]. The abundant occurrence and arrangement of mitochondria, Golgi complex and perinuclear ribosomes indicates active energy metabolism in the production and circulation of cerebral spinal fluid (CSF) in the ventricles along with the various functions of the ependymal cells in water regulation, and disease monitoring [38].

The lateral ventricles host the greater part of the subependymal layer [35], and our anti-GFAP staining revealed positive cells which appear as astrocytes but have extensions into the subependymal neuropil. Astrocytic heterogeneity has been described in the AGR with subependymal astrocytes identified as periventricular astrocytes [27]. Although this study did not utilize special immunostaining antibodies to delineate tanycytes, the described cells identified resemble tanycytes [23]. In the lateral ventricles, stem cells from the neuroepithelium are retained between the ependymal cells and subventricular zone, thus, regions of multi-ciliated ependyma have been reported to play an important role in neurogenic and gliogenic activities [16]. This function is alluded to in the juvenile AGR, which has been reported in one of our previous studies as having the most active adult neurogenesis profile in the AGR subventricular zone [29]. The proliferative potential of the adult forebrain ependyma and sub-ependyma has been reported [6]. However, only subependymal cells have neural stem cell characteristics as determined by their self-renewing capability (generating secondary spheres) and their ability to clonally give rise to neurons and glia [22, 32]. The existence of indistinct neuropil and glia seen with histology in the AGR may indicate AGR sub-ependymal clonal ability. The multi-layered ependyma most of the time contained tanycytes whose basal processes extend into the adjacent subependymal layer and neuropil [21]. This is believed to be in consonance with the findings in the AGR. Multilayering has also been reported as ependymal tissue response to injury [4]. It is hereby suggested that the glial populations of the subependyma be studied along ependymal studies in toxicity and neurodegenerative processes.

Tight junctions are widely demonstrated in ependymal cells and are seen as early as in the development in the neuroepithelium where they present *zonula occludens* apically and *zonula adherens* and gap junctions in the lateral plasma membrane domains [1, 17]. The mRNA coding connexins have been indicated as being important in this respect [3]. This gap junction pattern has been suggested to function in the reception of extrinsic growth factors from CSF likely through sensory apical cilia [14]. They function in electrical and metabolic couplings integrating the functioning of the cell layer. Furthermore, they are known to play a role in cilia beating synchronization and consequently in the circulation of CSF. They also integrate ependymal function with underlying astroglia, thus regulating water and ion transport [2, 16, 34].

Ependymal tight junctions in the AGR brain were tortuous and had regions of occlusion and adhesion. The ciliary arrangement 9 + 0 shows that the non-motile cilia (primary cilia) are composed of nine radially organized microtubule doublets, whereas the other 9 + 2 arrangement shows that the motile cilia contain an additional central microtubule doublet [20]. The works of Marshall and Kintner [20], has shown the existence of Dynein arms in the 9 + 2 arrangement that generate the shearing force needed for motility. Also, these types of cilia differ by the kinds of receptors on their membrane and the posttranslational modifications of their microtubules [11].

The choroid plexus cells contribute CSF secretion and serve as integral parts of the CNS barrier system which separate blood, CSF and CNS interstitial space via the BCSFB in conjunction with the BBB [33]. The morphology of individual choroid plexus in the lateral ventricles, third and fourth ventricles has been reported [36]. In addition to their protective role, the cells of the choroid plexus have been reported to act as a defence system by protecting the brain against acute neurotoxic insults, using a complex, multi-layered detoxification system [13]. The Kolmer cells, present on the AGR choroid plexus play this defensive role.

Epiplexus or Kolmer cells have been described as intraventricular macrophages associated with the choroid plexus. These intraventricular macrophages are referred to as "supraependymal" cells when found on the ependyma [17]. Entering by the transcellular pathway or the paracellular [18], these cells express complement type 3 (CR3) receptors, major histocompatibility class I and II (MHC I and II) antigens, and leukocyte common antigens under normal conditions. Thus, indicating their involvement in endocytosis similar to other macrophages and microglial cells in the brain (CR3) and antigen processing and presenting capacities for lymphocytes in case of MHC I and II [19]. These intraventricular macrophages may also be involved in sequestration of iron in certain neuropathological conditions that cause an increase in iron content in the CSF [5].

This paper has described the ultrastructure of the AGR morphology and shown possible functional components of the ependymocytes, the choroid plexus, tight junctions and subependymal tissues in view of its usefulness in research.

CONCLUSIONS

The ependymal layering of the brain of African Giant Rat showed marked variations in some regions of the ventricles with multi-layering of ependymal cells that differ from the typical single layered ependymal while subependymal structures reveal indistinct neuropil and glia with histological examination. This existence of indistinct neuropil and glia seen in this rodent may indicate sub-ependymal clonal ability. The ultrastructure of the ependymal cells and choroid seems typical depicting high secretory activity, but also a protective role against brain insults.

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REFERENCES

1. **Aijaz, S., Balda, M. S., Matter, K., 2006:** Tight junctions: Molecular architecture and function. *Int. Rev. Cytol.*, 248, 261—298.
2. **Banizs, B., Pike, M. M., Millican, C. L., Ferguson, W. B., Komlosi, P., Sheetz, J., 2005:** Dysfunctional cilia lead to altered ependyma and choroid plexus function and result in the formation of hydrocephalus. *Development*, 132, 5329—5339. DOI: 10.1242/dev.02153.
3. **Boucher, S., Bennett, S. A., 2003:** Differential connexin expression, gap junction intercellular coupling, and hemichannel formation in NT2/D1 human neural progenitors and terminally differentiated hNT neurons. *J. Neurosci. Res.*, 72, 393—404.
4. **Bruni, J. E., Anderson, W. A., 1987:** Ependyma of the rat fourth ventricle and central canal: response to injury. *Acta. Anat.* (Basel), 128, 4, 265—273.
5. **Burdo, J. R., Connor, J. R., 2003:** Brain iron uptake and homeostatic mechanisms: An overview. *Biometals*, 16, 63—75.
6. **Chiasson, B. J., Tropepe, V., Morshead, C. M., van der Kooyal, D., 1999:** Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cell characteristics. *J. Neurosci.*, 19, 4462—4471.
7. **Damkier, H. H., Brown, P. D., Praetorius, J., 2013:** Cerebrospinal fluid secretion by the choroid plexus. *Physiol. Rev.*, 93, 1847—1892. DOI:10.1152/physrev.00004.2013.
8. **Daneman, R., Prat, A., 2015:** The blood-brain barrier. Cold Spring Harb. *Perspect. Biol.*, 7, 1, 1—44. DOI: 10.1101/cshperspect.a020412.
9. **Del Bigio, M. R., 1995:** The ependyma: A protective barrier between brain and cerebrospinal fluid. *Glia*, 14, 1—13. DOI: 10.1002/glia.440140102.
10. **Del Bigio, M. R., 2010:** Ependymal cells: biology and pathology. *Acta Neuropathol.*, 119, 1, 55—73. DOI: 10.1007/s00401-009-0624-y.
11. **Delgehyr, N., Meunier, A., Faucourt, M., Grau, M. B., Strehl, L., Janke, C., et al., 2015:** Ependymal cell differentiation, from monociliated to multiciliated cells. *Methods Cell Biol.*, 127, 19—35. DOI: 10.1016/bs.mcb.2015.01.004.
12. **Gabrion, J. B., Herbuté, S., Bouillé, C., Maurel, D., Kuchler-Bopp, S., Laabich, A., et al., 1998:** Ependymal and choroidal cells in culture: Characterization and functional differentiation. *Microsc. Res. Tech.*, 41, 2, 124—157.DOI: 10.1002/(SICI)1097-0029(19980415)41:2<124::AID-JEMT3> 3.0.CO;2-U.

13. **Ghersi-Egea, J. F., Strazielle, N., 2001:** Brain drug delivery, drug metabolism and multidrug resistance at the choroid plexus. *Microsc. Res. Tech.*, 52, 183—188.
14. **Götz, M., Huttner, W. B., 2005:** The cell biology of neurogenesis. *Nat. Rev. Mol. Cell. Biol.*, 6, 777—788. DOI: 10.1038/nrm1739.
15. **Ibe, C. S., Onyeanusi, B. I., Hambolu, J. O., 2014:** Functional morphology of the brain of the African giant pouched rat (*Cricetomys gambianus*, Waterhouse, 1840). *Onderstepoort J. Vet. Res.*, 81, 1, E 1—7. DOI: 10.4102/ojvr. v81i1.644.
16. **Jiménez, A. J., Domínguez-Pinos, M. D., Guerra, M. M., Fernández-Llebrez, P., Pérez-Fígares, J., 2014:** Structure and function of the ependymal barrier and diseases associated with ependyma disruption. *Tissue barriers*, 2, e28426. DOI: 10.4161/tisb.28426.
17. **Kaur, C., Rathnasamy, G., Ling, E. A., 2016:** The choroid plexus in healthy and diseased brain. *J. Neuropathol. Exp.*, 75, 3, 198—213.
18. **Ling, E. A., 1981:** Ultrastructure and mode of formation of epiplexus cells in the choroid plexus in the lateral ventricles of the monkey (*Macaca fascicularis*). *J. Anat.*, 133, 555—569.
19. **Ling, E. A., Kaur, C., Lu, J., 1998:** Origin, nature, and some functional considerations of intraventricular macrophages, with special reference to the epiplexus cells. *Microsc. Res. Tech.*, 41, 43—56.
20. **Marshall, W. F., Kintner, C., 2008:** Cilia orientation and the fluid mechanics of development. *Curr. Opin. Cell. Biol.*, 20, 1, 48—52. DOI: 10.1016/j.ceb.2007.11.009.
21. **Mathew, T. C., 2008:** Regional analysis of the third ventricle of rat by light and electron microscopy. *Anat. Histol. Embryol.*, 37, 9—18.
22. **Morshead, C. M., Reynolds, B. A., Craig, C. G., McBurney, M. W., Staines, W. A., Morassutti, D., et al., 1994:** Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron*, 13, 1071—1082.
23. **Mullier, A., Bouret, S. G., Prevot, V., Dehouck, B., 2010:** Differential distribution of tight junction proteins suggests a role for tanycytes in blood-hypothalamus barrier regulation in the adult mouse brain. *J. Comp. Neurol.*, 518, 7, 943—962. DOI: 10.1002/cne.22273.
24. **Mustapha, O. A., Olude, M. A., Taiwo, B., Olopade, J. O., 2019:** Cytoarchitecture of the hippocampal formation in the African Giant Rat (*Cricetomys gambianus*, Waterhouse). *Niger. J. Physiol. Sci.*, 34, 1, 55—62.
25. **Olude, M. A., Bello, S. T., Mustapha, O. A., Olopade, F. E., Plendl, J., Ihunwo, A. O., et al., 2018:** Oligodendrocyte morphology in the developing brain of the African giant rat (*Cricetomys gambianus*, Waterhouse): Histology, immunohistochemistry and electron microscopy. *Anat. Histol. Embryol.*, 47, 3, 231—238. DOI: 10.1111/ahe.12348.
26. **Olude, M. A., Mustapha, O. A., Olopade, J. O., 2016:** Morphological characterization of the African Giant Rat (*Cricetomys gambianus*, Waterhouse) brain across age groups: Gross features of cortices. *Niger. J. Physiol. Sci.*, 31, 133—138.
27. **Olude, M. A., Mustapha, O. A., Aderounmu, O. A., Olopade, J. O., Ihunwo, A. O., 2015:** Astrocyte morphology, heterogeneity and density in the developing African giant rat (*Cricetomys gambianus*). *Front. Neuroanat.*, 9, 67, 1—10.
28. **Olude, M. A., Ogunbunmi, T. K., Olopade, J. O., Ihunwo, A. O., 2014a:** The olfactory bulb structure of African giant rat (*Cricetomys gambianus*, Waterhouse 1840) I. Cytoarchitecture. *Anat. Sci. Int.*, 89, 4, 224—231. DOI: 10.1007/s12565-014-0227-0.
29. **Olude, M. A., Olopade, J. O., Ihunwo, A. O., 2014b:** Adult neurogenesis in the African Giant Rat (*Cricetomys gambianus*, Waterhouse). *Metab. Brain. Dis.*, 29, 857—866. DOI: 10.1007/s11011-014-9512-9.
30. **Poling, A., Weetjens, B., Cox, C., Beyene, N. W., Bach, H., Sully, A., 2011:** Using trained pouched rats to detect land mines: another victory for operant conditioning. *J. Appl. Behav. Anal.*, 44, 351—355.
31. **Redzic, Z., 2011:** Molecular biology of the blood-brain and the blood-cerebrospinal fluid barriers: similarities and differences. *Fluids and barriers of the CNS*, 8, 1, 3. DOI: 10.1186/2045-8118-8-3.
32. **Reynolds, B. A., Weiss, S., 1992:** Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*, 255, 1707—1710.
33. **Segal, M. B., 2000:** The choroid plexuses and the barriers between the blood and the cerebrospinal fluid. *Cell. Mol. Neurobiol.*, 20, 2, 183—196.
34. **Sival, D. A., Guerra, M., den Dunnen, W. F. A., Bátiz, L. F., Alvial, G., Castañeyra-Perdomo, A., et al., 2011:** Neuroependymal denudation is in progress in full-term human foetal spina bifida aperta. *Brain Pathol.*, 21, 163—179. DOI: 10.1111/j.1750-3639.2010.00432.x.
35. **Smart, I., 1961:** The subependymal layer of the mouse brain and its cell production as shown by radioautography after thymidine-H3 injection. *J. Comp. Neurol.*, 116, 325—347.
36. **Strazielle, N., Ghersi-Egea, J. F., 2000:** Choroid plexus in the central nervous system: biology and physiopathology. *J. Neuropathol. Exp. Neurol.*, 59, 561—574.

37. **Usende, I., Emikpe, B., Olopade, J., 2017:** Heavy metal pollutants in selected organs of African giant rats from three agro-ecological zones of Nigeria: evidence for their role as an environmental specimen bank. *Environ. Sci. Pollut. Res.*, 24, 22570—22578. DOI:10.1007/s11356-017-9904-6.

38. **Vidovic, D., Davila, R. A., Gronostajski, R. M., Harvey, T. J., Piper, M., 2018:** Transcriptional regulation of ependymal cell maturation within the postnatal brain. *Neural Dev.*, 13, 2. DOI: 10.1186/s13064-018-0099-4.

39. **Weetjens, B. J., Mgode, G. F., Machang'u, R. S., Kazwala, R., Mfinanga, G., Lwilla, F., et al., 2009:** African pouched rats for the detection of pulmonary tuberculosis in sputum samples. *Int. J. Tuberc. Lung. Dis.*, 13, 6, 737—743.

40. **Wolburg, H., Paulus, W., 2010:** Choroid plexus: biology and pathology. *Acta. Neuropathol.*, 119, 75—88.

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MONITORING OF STRESS IN POLICE HORSES

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ABSTRACT

Every year, police horses undergo police training focused on passing difficult obstacles, during which their physical and mental abilities are tested. Using a method of eye movement monitoring, specifically eye blinking and eyelid twitching, we estimated if the training of horses was stressful. The results we found suggested that the training itself, *per se* does not affect the welfare of police horses; as it is not stressful for them because the gradual training allows the horses to adapt to different types of work or physical activity.

Key words: cortisol; eye-blink; eye-twitch; stress

INTRODUCTION

The selection of horses for work in the police force is challenging. Horses must undergo both selection and training, before they can then be included in the service. Horses can be exposed to various stimuli during training, which may be stressful for them. Such stimuli are: sirens sound, gunshots, simulated crowd noise, barking dogs or passing obstacles through fire. The accumulation of such stress stimuli has the potential to have a significant effect on the body when compared to the action of a one single stress stimulus.

Stress in animals can be defined as a combination of psychological and biological reactions to new or threatening circumstances. In response to new unknown external stimuli, horses undergo both homeostatic and behavioural changes. Further, changes in physiological parameters may develop when an external stimulus is perceived as a potential threat to alleviate a perceived stressor. Stress can have a negative or a positive effect on the body and may help the animal to cope with routine short-term stressors.

Stress in animals can be divided into acute and chronic stress. In acute stress, a short-term stressor acts, which initiates a physiological response in the stimulation of the recovery of disturbed homeostasis. In animals, such in horses, this response can be assessed by observing their behaviour or by analysing some physiological parameters; for example, cortisol release can be considered the most

relevant catecholamine or to examine the changes of the heart rate variability [3, 4, 5, 16, 20, 21, 25].

Researchers have examined behavioural indicators in horses that have been subjected to multiple conditions of stress. Higher head carriage, centred alignment of the ears, increased vocalizations and increased mouth movements have been demonstrated by stress challenged horses. For that purpose, by using a combination of both behavioural and physiological measures, measuring stress responses in animals seems more reliable. Young et al. [26] have produced a new scale to classify horses' stress-related behaviour. Horses displayed an improvement in oral behaviour, flattened or pinned ears and flared nostrils associated with an increase in heart rate (HR) and salivary cortisol when horses were subjected to stressful husbandry activities, such as electric coat clipper tone, social isolation and grooming procedures [12, 13, 15, 24].

In deciding the values of stress factors in horses, several authors have expressed recent interest. These can be determined from samples of blood, saliva, or faeces. For horses, taking blood samples induces tension. The measurement results could, therefore, be distorted. Other strategies that may be included among the non-invasive alternative methods used to monitor acute stress in horses have also been reported. These strategies include changes in the amounts of blinks in the eye and twitches in the eyelid. We noticed this by watching the animals and their eye expressions, whether they were stressed by the demanding training that police horses undergo. To understand the animal and eventually enhanced health, it is important to recognize valid stress indicators [7, 9, 11, 14, 18, 22, 23].

MATERIALS AND METHODS

Our research was carried out on 9 police horses, which underwent a demanding one-day training at the equestrian centre in Košice. The training consisted of overcoming challenging simulated events, during which the horses were exposed to the sound of sirens, gunshots, fire handling, barking dogs, waving banners, etc. The horses were 6 to 14 years old (five geldings and four mares). Those horses had more than 3 years of experience with police training and they were in good physical and mental conditions. The horses were transported for 450 km to Košice. They were housed together after arrival in the same stable until the next day, when they completed the training. Prior to training, in the morning, all 9 horses underwent right eye monitoring for 1 minute, which was recorded using a digital camera. The horses were held by a handler on a fairly loose lead (1 m) with just enough contact to maintain its head relatively still without restricting the movements. After completing the horses' training in the afternoon, we monitored the horses' movement of the eyes or eyelids in the same way. We played the recorded videos using a video player (in 40 × slow motion) and the eye movements were evaluated by 4 independent observers who checked the number of 3 different possible movements of the eye: full blink, half blink or eyelid twitches (Table 1). The inter-observer reliability was 91.5 %. The average number of full, half blinks and eye twitches has been documented in Table 2.

Statistical evaluation

All our data were reported as the mean ± SD (standard deviation). The differences between means were determined according to the paired t-test using Microsoft Excel software. By conventional criteria, difference ($P < 0.05$) was considered to be statistically significant.

RESULTS AND DISCUSSION

The aim of our study was to determine whether the method of measuring the number of blinks is suitable for measuring the stress level in horses. We verified this procedure by observing the movement of the eyes and twitching of the eyes. This method is considered non-invasive, and can be used to indicate stress in horses, as well as people.

Blinking is characterized as a rapid eyelid movement that opens and closes the palpebral fissure and consists of three distinct blinks: full, half and twitches of the eye. Complete blinks are total closure with concomitant vision suppression of both eyelids, whereas incomplete closure of the eyelids is half (partial) blinks. We understand the movement of the upper eyelid through the innervation of the muscles of the *levator palpebrae superioris*, without the movement of the muscles of the *orbicularis oculi*. In humans, partial blinks based on computer terminal screens have been observed and have been used as a diagnosis for dry eye disease. In both dogs and cats, partial blinks have also been recorded [1, 2, 8, 10, 17, 19].

Table 1. Ethogram of behaviours observed in horses before and after police training

Behaviour	Description
Eye- full blink	The right eye becomes momentarily but completely closed
Eye- half blink	The right upper lid moved toward the lower lid of the eye but does not cover the eye completely
Eyelid- twitch	Fine fibrillary movement of the skin involving the levator palpebrae superioris muscle of the upper eyelid

Table 2. Average number of full, half blinks and eye twitches before and after training in police horses (n = 9)

	Full eye blinks			Half eye blinks			Eye twitches		
	Mean ± SD	**P-value**	**Paired t-test**	**Mean ± SD**	**P-value**	**Paired t-test**	**Mean ± SD**	**P-value**	**Paired t-test**
Before training	9 ± 0.737	NS	1.111	13 ± 1.155	NS	1.111	2.5 ± 0.497	NS	0.666
After training	7.5 ± 0.628	NS		12 ± 1.315	NS		3.0 ± 0.146	NS	

NS—non significant

We compared our results with those of Merkies et al. [14], who found that a reduction in the number of full and half blinks and an increase in the number of eyelid twitches occurs if horses are exposed to a stressful situation. The number of full blinks decreases from an average of 5 to 8—9 blinks per minute, and the number of eye twitches increases from an average of 2 twitches per minute to 6 twitches per minute in a stressful situation. We revealed that the number of full eye blinks in the police horses decreased on average from 9 blinks per minute to 7.5 blinks per minute. The number of eye twitches did not change on average (2.5 blinks per minute before training and 3 blinks per minute after training).

According to our observations, horse training was not correlated with a decrease in eye blinking and an increase in eye twitching under stress conditions. The method of measuring eye blinking and eye twitching appeared to be a suitable alternative method for monitoring the effect of stress factors in horses. According to Hanák and Olehla [6], if a certain physical load was repeated on a regular basis and increased in length, intensity or frequency gradually, then adaptation to the corresponding physical load (training) appeared to be a fundamental biological property.

CONCLUSIONS

Monitoring eye blinking and eye twitching is considered a non-invasive procedure, and compared to the techniques used to assess cortisol levels in the blood, it can be categorized as inexpensive. The drawbacks to these approaches are the time taken to analyse the outcomes and the need to build a team of individuals. We found that no signs of stress were shown based on the assessment of eye movements in horses undergoing challenging training, supporting the hypothesis that horses can adjust to this form of load by incremental training.

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REFERENCES

1. **Bennett, V., Gourkow, N., Mills, D. S., 2017:** Facial correlates of emotional behaviour in the domestic cat (*Felis catus*). *Behav. Proc.*, 141, 342—350. DOI: 10.1016/j.beproc.2017.03.011.
2. **Cruz, A. A. V., Garcia, D. M., Pinto, C. T., Cechetti, S. P., 2011:** Spontaneous eyeblink activity. *Ocul. Surf.*, 9, 9—41. DOI: 10.1016/S1542-0124(11)70007-6.
3. **Fazio, E., Medica, P., Cravana, C., Ferlazzo, A., 2013:** Cortisol response to road transport stress in calm and nervous stallions. *J. Vet. Behav. Clin. Appl. Res.*, 8, 4, 231—237. DOI: 10.1016/j.jveb.2012.10.010.
4. **Ferlazzo, A., Medica, P., Cravana, C., Fazio, E., 2012:** Circulating β-endorphin, adrenocorticotropin, and cortisol concentrations of horses before and after competitive show jumping with different fence heights. *J. Equine Vet. Sci.*, 32, 11, 740—746. DOI: 10.1016/j.jevs.2012.02.021.
5. **Fowler, V., Kennedy, M., Marlin, D., 2012:** A comparison of the Monty Roberts technique with a conventional UK technique for initial training of riding horses. *Anthrozoos*, 25, 301—321. DOI: 10.2752/175303712X13403555186217.
6. **Hanák, J., Olehla, Č., 2010:** Clinical physiology of horses and their training (Online in Czech). Cit. 2019-03-28. Available on Internet: < cit.vfu.cz&gt.
7. **Hovey, M. R., Davis, A., Chen, S., Godwin, P., Porr, C. A. S., 2020:** Evaluating stress in riding horses: Part one-behaviour assessment and serum cortisol. *J. Equine Vet. Sci.* (In press – Journal pre-proof). DOI: 10.1016/j.jevs.2020.103297.
8. **Jie, Y., Sella, R., Feng, J., Gomez, M. L., Afshari, N. A., 2019:** Evaluation of incomplete blinking as a measurement of dry eye disease. *Ocul. Surf.*, 17, 3, 424—433. DOI: 10.1016/j.jtos.2019.05.007.
9. **Jung, A., Jung, H., Choi, Y., Colee, J., Wickens, C., Lee, J. W., Yoon, M., 2019:** Frequent riding sessions daily elevate stress, blood lactic acid, and heart rate of thoroughbred riding horses. *J. Vet. Behav.*, 32, 5, 1—5. DOI: 10.1016/j.jveb.2019.03.012.
10. **Koo Lin, L., 2013:** Eyelid anatomy and function. In **Holland, E. J., Mannis, M. J., Lee, W. B., (Eds.):** *Ocular Surface Disease: Cornea, Conjunctiva and Tear Film*. Elsevier, Cambridge, UK, 11—15.
11. **Krueger, K., Marr, L., Dobler, A., Palme, R., 2020:** Preservation of faecal glucocorticoid metabolites and immunoglobulin A through silica gel drying from field studies in horses. *Conserv. Physiol.*, 7, 1, 1—8. DOI: 10.1093/conphys/coz065.
12. **McKinney, C., Mueller, M. K., Frank, N., 2015:** Effects of therapeutic riding on measures of stress in horses. *J. Equine Vet. Sci.*, 35, 922—928. DOI: 10.1016/j.jevs.2015.08.015.
13. **Merkies, K., McKechnie, M. J., Zakrajsek, E., 2018:** Behavioural and physiological responses of therapy horses to mentally traumatized humans. *Appl. Anim. Behav. Sci.*, 205, 61—67. DOI: 10.1016/j.applanim.2018.05.019.
14. **Merkies, K., Ready, Ch., Farkas, L., Hodder, A., 2019:** Eye blink rates and eyelid twitches as a non-invasive measure of stress in the domestic horse. *Animals*, 9, 8, 562. DOI: 10.3390/ani9080562.
15. **Merkies, K., Sievers, A., Zakrajsek, E., MacGregor, H., Bergeron, R., König von Borstel, U., 2014:** Preliminary results suggest an influence of psychological and physiological stress in humans on horse heart rate and behaviour. *J. Vet. Behav.*, 9, 242—247. DOI: 10.1016/j.jveb.2014.06.003.
16. **Nagel, C., Erber, R., Bergmaier, C., Wulf, M., Aurich, J., Möstl, E., Aurich, C., 2012:** Cortisol and progestin release, heart rate and heart rate variability in the pregnant and postpartum mare, foetus and new-born foal. *Theriogenology*, 78, 4, 759—767. DOI: 10.1016/j. Theriogenology.2012.03.023.
17. **Nakajima, S., Takamatsu, Y., Fukuoka, T., Omori, Y. 2011:** Spontaneous blink rates of domestic dogs: A preliminary report. *J. Vet. Behav.*, 6, 95. DOI: 10.1016/j.jveb.2010.08.015.
18. **Peeters, M., Sulon, J., Serteyn, D., Vandenheede, M., 2010:** Assessment of stress level in horses during competition using salivary cortisol: preliminary studies. *J. Vet. Behav.*, 5, 4, 216—216. DOI: 10.1016/j.jveb.2009.10.043.
19. **Portello, J. K., Rosenfield, M., Chu, C. A., 2013:** Blink rate, incomplete blinks and computer vision syndrome. *Optom. Vis. Sci.*, 90, 482—487. DOI: 10.1097/OPX.0b013e31828f09a7.
20. **Schmidt, A., Möstl, E., Wehnert, Ch., et al., 2010a:** Cortisol release and heart rate variability in horses during road transport. *Horm. Behav.*, 57, 2, 209—215. DOI: 10.1016/j.yhbeh.2009.11.003.
21. **Schmidt, A., Biau, S., Möstl, E., Becker-Birck, M., Morillon, B., Aurich, J., Faure, J. M., Aurich, C., 2010b:** Changes in cortisol release and heart rate variability in sport horses during long-distance road transport. *Domestic Anim. Endocrinol.*, 38, 3, 179—189. DOI: 10.1016/j.domaniend.2009.10.002.

22. **Strzelec, K., Kankofer, M., Pietrzak, S. 2011:** Cortisol concentration in the saliva of horses subjected to different kinds of exercise. *Act. Vet. B.*, 80, 101—105.
23. **Valera, M., Bartolomé, E., Guerrero, M. J. S., et al., 2012:** Changes in eye temperature and stress assessment in horses during show jumping competitions. *J. Equine Vet. Sci.*, 32, 827—830, DOI: 10.1016/j.jevs.2012.03.005.
24. **Visser, E. K., van Reenen, C. G., Hopster, H., Schilder, M. B. H., Knaap, J. H., Barneveld, A., Blokhuis, H. J., 2001:** Quantifying aspects of young horses' temperament: Consistency of behavioural variables. *Appl. Anim. Behav. Sci.*, 74, 241—258. DOI: 10.1016/S0168-1591(01)00177-0.
25. **Von Lewinski, M., Biau, S., Erber, R., et al., 2013:** Cortisol release, heart rate and heart rate variability in the horse and its rider: different responses to training and performance. *Vet. J.*, 197, 2, 229—232. DOI: 10.1016 /j.tvjl. 2012.12.025.
26. **Young, T., Creighton, E., Smith, T., Hosie, C., 2012:** A novel scale of behavioural indicators of stress for use with domestic horses. *Appl. Anim. Behav. Sci.*, 140, 33—43. DOI: 10.1016/j.applanim.2012.05.008.

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INFECTIONS OF THE URINARY TRACT OF BACTERIAL ORIGIN IN DOGS AND CATS

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ABSTRACT

The diseases of the urinary tract in small animal medicine, especially in dogs and cats, occur frequently with different etiologies. The most common cause of urinary tract infections (UTIs) are bacteria which enter upwards through the urethral opening. UTIs occur in hosts with compromised defence mechanisms in which the virulent microbes can adhere, multiply and persist in the urinary tract. In addition to bacteria, also viruses or fungi may infect the urinary tracts. Bacterial infection can develop in the upper (kidneys and ureters) or lower (bladder, urethra) urinary tract and are manifested by various clinical signs such as: frequent or difficult and painful urination, presence of blood in the urine, or its foul odour. The symptoms in sick animals are often accompanied by fever, discomfort, and loss of appetite. The UTIs in dogs and cats are caused by both Gram-negative (e. g. *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp., *Proteus* spp.) and Gram-positive (e. g. *Staphylococcus* spp., *Streptococcus* spp., and *Enterococcus* spp.) bacteria. The properly performed aseptic sampling of the urine is one of the most important steps in bacteriological diagnostics. In this study, 105 urine samples were collected from dogs and cats. The cultivation, microscopy and biochemical examinations were used for species identification. Finally, the bacteriological examination included also determining the susceptibility of pathogens against antibiotics by the disc-diffusion method. The results were processed and expressed as a percentage according to identified pathogens, animal species, sex, breed and age. The uropathogens were diagnosed mainly in males aged 7—10 years. The most commonly identified pathogens were *Escherichia coli*, *Streptococcus* spp. and *Staphylococcus* spp. Tests of bacterial susceptibility to antibiotics showed that enrofloxacin, cefotaxime, and cephazolin were the most effective against uropathogens.

Key words: bacterial pathogens; cats; dogs; urinary infections; urinary tract

INTRODUCTION

Urinary tract infections (UTIs) in the veterinary literature are used to describe both bacterial cystitis and subclinical bacteriuria. They are caused mainly by bacterial pathogens, but urinary disorders correlate very often with the occurrence and the development of urinary bladder stones, insufficient protection of the urogenital tract (UGT) against external influences due to immunosuppression, tumours and others. Also, viruses, fungi or parasites can cause UTIs. The age, gender, immunity, dietetic principles or trauma are important predisposing factors contributing to urinary tract disorders in small animals. Persistent or recurrent UTIs can involve refractory bacterial isolates and may also prove difficult to resolve using conventional antimicrobial therapy [8].

From the anatomical point of view, it is necessary to mention that the urethra in the females of dogs and cats is shorter and rather wide in comparison to males and this is the reason why urethritis develops more often in males than in females. The urethra in tomcats is particularly very narrow and long which is a frequent cause of their urinary disorders [6].

Another risk factor that is significant in the development of UTIs is the age of the animals. UTIs are more common in older female dogs. The mean age at diagnosis regardless of sex is approximately 7—8 years [16].

Dogs and cats suffering from urinary tract problems show symptoms such as dysuria, even anuria, stranguries, enlarged abdomen or haematuria. During urination the animals can adopt a hunched posture which indicates painfulness. The symptoms may suggest inflammatory processes in both lower and upper parts of the urinary tract that may be induced by various factors whether primary infectious agents or secondary issues.

Composition, concentration and the volume of eliminated urine directly affect the occurrence of uroinfections. Pathological processes in the body leading to the loss of fluids and electrolytes result in excessive multiplication of uropathogens [5]. Physiologically, urine must not contain blood, proteins, glucose or microorganisms. It should be sterile without any contamination. The presence of any of the above-mentioned components indicates pathological status. Collection of urine samples and their examination is an important part of basic clinical examinations.

Frequently, the problems associated with urination in dogs and cats are related to the composition of feed consumed in the long term by these animals. The feed with high levels of proteins that exceeds the requirements of animals results in increased synthesis of urea that increases the burden on kidneys. Excessive intake of animal fat aggravates the course of chronic failure of kidneys which is manifested by increased concentration of lipids and cholesterol in animal blood. Feed deficient in fibres suppresses the progress of microbial fermentation in the large intestine. This results in reduced elimination of urea in the faeces and, on the contrary, increased excretion via urine that again puts considerable load on the kidneys. In this way, the renal insufficiency is exacerbated [17].

A diet, age or hormone imbalance can influence the creation of urinary stones. The urinary stones (uroliths) are defined as the solid crystalline masses and the most common urinary stones in cats and dogs are struvite and calcium oxalate (Fig. 1).

The phosphate (magnesium-ammonium-phosphate or struvite) stones form at alkaline pH and thus the intake of nutrients, particularly those of plant origin that increase pH, stimulates formation of such stones that are a conglomeration of bacteria, crystals and protein matrix. Deficient production of the active form of vitamin D by kidneys results in hyperphosphataemia, demineralization of bones and subsequent deposition of crystals in the renal parenchyma. The crystals irritate the tissue and lead to inflammatory processes. This type of stones occurs most frequently in dogs. Struvite stones usually develop in dogs due to a

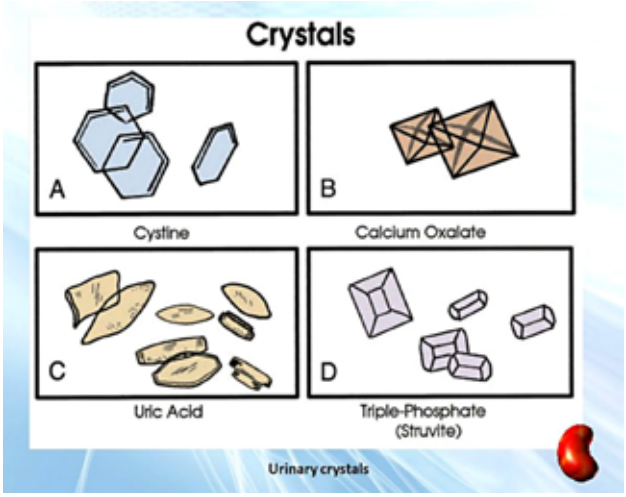

Fig. 1. Types of urinary stones
Source: https://allhealthpost.com/crystals-in-urine/

UTI with urease-producing bacteria while in cats, they are typical as sterile stones [15]. These bacteria create alkaline urine, the ideal environment for struvite to form in.

Acidic pH predisposes for formation of oxalate stones. Calcium oxalate is the most common type of stone found in the upper urinary tract of both species of cats and dogs [7]. Feeding the acidifying compounds, hypercalcemia and increased intake of vitamins A and C result in the formation of oxalate stones that are typical of cats, particularly tomcats.

The generally reduced intake of water increases the concentration of urine and contributes to increased occurrence of stones in the urinary tract. The uncontroversial recommendation for preventing the recurrence of calcium oxalate stones is to increase the urine volume. Dogs that produce more concentrated urine and that urinate less frequently might be predisposed to calcium oxalate formation [14].

The samples of urine for bacteriological examination can be collected in several ways (spontaneous miction, catheterization, cystocentesis). The optimum method is cystocentesis which prevents undesirable contamination of urine during sampling. The aim of the bacteriological examination of urine is the identification of the agent of infection and the determination of suitable therapy based on the determination of the susceptibility of the infectious agent to antibiotics. The pathogens that occur most frequently in the urinary tract are microorganisms of the family *Enterobacteriaceae* (*E. coli*, *Proteus* spp., *Klebsiella* spp.). However, even the presence of Gram-positive bacteria, such as staphylococci, streptococci or enterococci in the urinary system is also frequent [8].

Currently, due to excessive and frequently imprudent use of antibiotics, resistance to them increases and represents a serious issue not only in veterinary but also in human medicine. The resistance of enterobacteria, particularly of multiresistant strains of *Escherichia coli* and *Klebsiella pneumoniae*, the frequent causative agents of uroinfections in animals and humans, has become a worldwide problem. The multiresistant strains are those that are resistant to the effective antimicrobials of at least 3 different groups [9].

The aim of this study was to identify bacteria that most frequently induce the inflammation of the urinary tract and to provide information about the percentage of occurrence of these pathogens in dogs and cats. Urine samples were taken lege artis from dogs and cats that showed clinical signs of urinary problems. The disc-diffusion method was used to determine the susceptibility or resistance of the isolated bacteria to clinically related antibiotics. The obtaining of an antibiogram is an important step in the struggle against increased antibiotic resistance. The study also included the evaluation of the data with respect to the most affected individuals according to animal species, gender, age and breed.

MATERIALS AND METHODS

Samples of urine must be collected into sterile vessels, preferably in the morning. Urine can be obtained during spontaneous micturition, by manual compression of the urinary bladder, catheterization or cystocentesis [19]. In this study, 105 samples were collected into sterile vessels mainly during spontaneous micturition of the animal patients. All procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Animals (Protocol No. 3323/16-221/3) which was approved by the State Veterinary and Food Administration of the Slovak Republic and by Ethics Commission of the University of Veterinary Medicine and Pharmacy in Košice, Slovakia. Respective aliquots (100 µl volume) of undiluted samples were inoculated onto the surface of solid nutrient media and cultivated at 37 °C for 24 hours under aerobic conditions. The following nutrient media were used: blood agar, Endo agar, CLED agar, Bile-esculin agar (BEA), Baird-Parker agar (BPA) and Mueller-Hinton agar (HiMedia, India). The blood agar was used for the detection of haemolytic activity of potential pathogens such as *E. coli*, staphylococci, and streptococci. Endo agar and CLED agar were used as selective media for Gram-negative bacteria of the *Enterobacteriaceae* family and Mueller-Hinton agar was used for the determination of the susceptibility to antibiotics. Also, 2 diagnostic selective media were used, Bile-esculin agar for the enterococci identification and Baird-Parker agar for the identification and confirmation of coagulase-positive staphylococci, particularly *S. aureus* and *S. pseudintermedius.*

The solitary colonies were subjected to Gram staining, catalase and oxidase testing and some isolates were also subjected to the extended biochemical examination that included the proof of sugar fermentation and other relevant tests. Commercially available test as EnteroTest 24,

StaphyTest 24, StreptoTest 24 (Lachema, Brno, Czech Republic) were used and the results obtained after biochemical examinations were evaluated by means of software TNW ProAuto 7.0. The susceptibility of the isolates to antibiotics was determined by the disc-diffusion method based on the measurement of the inhibition zones on Mueller-Hinton agar (HiMedia, India) according to the Kirby-Bauer's method and the results were interpreted according to EUCAST. For the determination of antibacterial effectiveness, the following antibiotic discs were used: enrofloxacin (ENR), 5 µg/disc; ciprofloxacin (CIP), 5 µg/disc; amoxicillin with clavulanic acid (AMC), 30 µg/disc; cephalotin (CLT) 30 µg/disc; cephazolin (CZL), 30 µg/disc; cefuroxime (CXM), 30 µg/disc; cefoxitin (FOX), 30 µg/disc; cefotaxime (CTX), 30 µg/disc; and trimethoprim-sulfamethoxazole (COT), 25 µg/disc (Thermo Fisher Scientific, UK).

The results were processed and reported as the percentage of positive samples (finding/confirmation of bacterial infectious agent) to negative samples, including the species, gender, age and breed of animals from which the samples originated. In the case of samples in which the agent of infection was confirmed, we differentiated between the mono-infections (1 agent) and mixed infections/co-infections (several agents) in the sample.

RESULTS AND DISCUSSION

Dogs and cats that are kept in households and are not regularly taken out outside are exposed to higher risk of the development of UTIs. Regular and complete emptying of the urinary bladder at least four times per day acts as a preventive measure. The first important step for the correct diagnosis of UTIs is the aseptic urine sampling. The processing of freshly produced urine is suggested for microbial cultures. The cystocentesis approach is recommended as the procedure that ensures the most reliable result of microbiological examination as the sample will not become contaminated during sampling by impurities and bacteria that may be present in the efferent urinary passages. This method is a safe and simple way of obtaining the samples but requires some expertise [2]. On the other hand, even unskilled persons can collect the urine samples (like the animal's owner), but the collecting of samples via spontaneous micturition can provide dubious results. It is strongly advised that the urine sample be analysed within 60 min of the collection as it is an unpredictably unstable biological fluid. Therefore, it is recommended that urine samples be examined as soon as possible following collection [12].

In our study, the total number of the collected and examined samples was 105; 83 originated from dogs and 22 from cats. Most of the samples were collected via spontaneous micturition. Not considering the species of the patients, the results showed that 54 samples were positive for the presence of bacteria and 51 were negative. Bacterial infections of the urinary tract were detected mostly in dogs (45 of the 54 were positive samples). Only 9 samples originating from cats were positive. Our results indicated that dogs suffer more frequently from bacterial infections of the urinary system (83 % vs. 17 %) (Fig. 2). The results of this study may have been influenced by the method of sampling (spontaneous micturition). Also, the diets recommended by veterinarians are part of the prevention of the disease as they ensure the correct concentration and composition of urine and thus prevent urolithiasis.

Bacterial infections of the urinary tract were detected in 15 bitches and 28 male dogs and in 2 female cats and 7 tomcats. This indicated that males of both species were affected more frequently by UTIs than the females.

The age of most patients with the detected bacterial UTIs ranged from 10 months to 15 years. Bacterial infections were most frequent in individuals 7—10 years old. The age of the animals and the percentage of positive findings are summarised in Fig. 3.

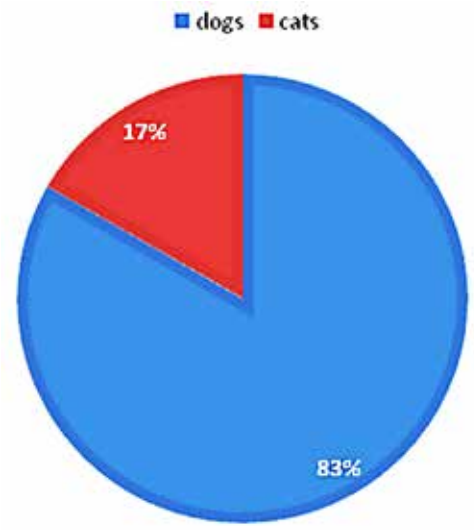

Fig. 2. Positive results for the presence of bacteria in the urine samples of the dogs and cats

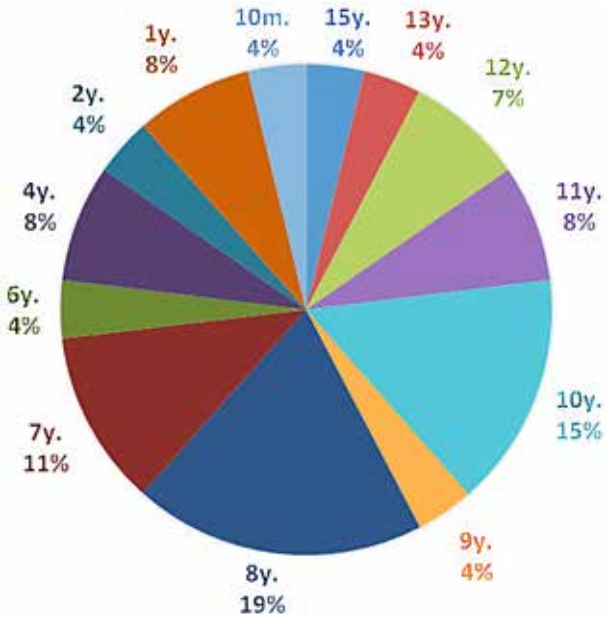

Fig. 3. Age distribution of the dogs and cats with bacterial UTIs

The breeds of all of the animals were not reported. However, of the positive samples with known breed appurtenance, most originated from the breed Chihuahua (6 of 26 samples). Other affected breeds were: Dachshund (3×), Yorkshire Terrier (2×), Bichon (2×), Cocker Spaniel (2×), Shar-Pei, Beagle, Poodle, French Bulldog, Schnauzer, Maltese dog, Viszla, German Shepherd and mongrels.

The species, age and gender of the patients, particularly the respective anatomic differences in the UGT between males and females may constitute important predispositions for the development of urinary tract infections. In dogs, UTIs were diagnosed in 2—3 % of the animals and was more frequent in females than in males; particularly affecting the older dogs. In cats, the positivity was lower than 1 % and the infections were more frequent in older individuals, at the age of 10 or more years [3]. However, tomcats suffered more frequently from urogenital infections than the females due to the anatomical structure of the urethra that is narrow and long. This results in stagnation of urine caused even by minimal obstruction, and the development of conditions that support propagation of microorganisms [13]. Sometimes more care and attention may be devoted to dogs with respect to their hygiene habits and protection against diseases in comparison with cats, but one cannot accept this as an explanation why less bacterial infections should occur in dogs. On the contrary, this can result in more frequent diagnosis of urogenital infections by veterinarians. The percentage evaluation of our results showed that UTIs affected dogs more frequently than cats. Our examinations showed that 28 male dogs were affected. A lower prevalence was detected in cats and in this species the males were affected more (7 males, 2 females). With regard to the age of the animals with positive results, the positivity was observed in various age categories. The majority of positive samples originated from animals 7—10 years old which confirms the assumption that urinary tract infections occur more frequently in older individuals.

The presence of urinary stones is closely related to the development of secondary infections. This is considered with regard to the occurrence of UTIs in various breeds of dogs, as the small breeds are more susceptible to formation of urinary stones. Thus, predisposition to this problem is expected in Yorkshire Terrier, Dachshund, Chihuahua, Miniature Schnauzer, Shi-tzu, Poodle, Bichon, but also in Dalmatian; although the latter belongs to larger breeds. The occurrence of stones in cats involves breeds such as Burma, Himalayan and Persian cats [10]. In our study, the highest number of positive samples originated from the Chihuahua breed (6 cases). A higher percentage of positive samples were found in Dachshund and Yorkshire Ter-

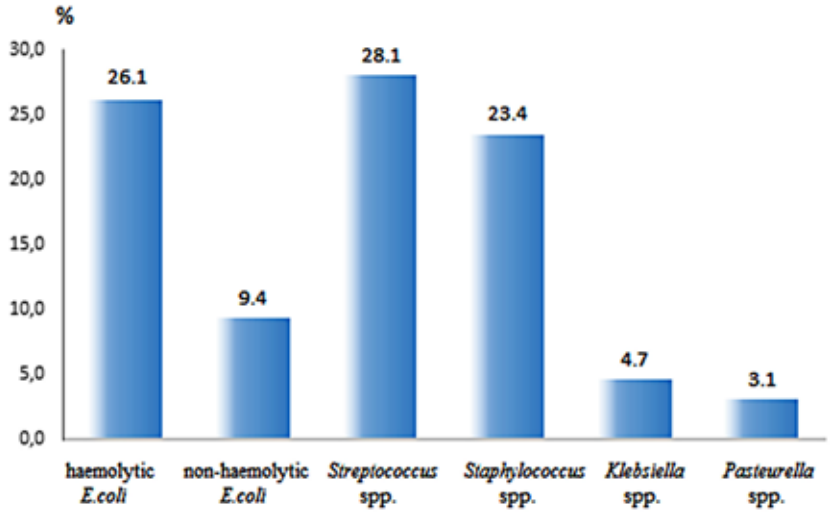

Fig. 4. Percentage proportion of the most frequently detected bacterial agents of the urinary tract.

rier breeds. One can suppose that secondary UTIs may be a consequence of the presence of urinary stones, although this has not been confirmed.

Physiologically, no microorganisms occur in the kidneys or the urinary passages. However, bacteria may be introduced to the urinary tract by blood or ascend there from the efferent ways. Generally, the most common causative agents of canine UTIs are *E. coli* bacteria [1].

Our examination of urine samples showed that in our case, the UTIs were most frequently caused by *E. coli* bacteria (haemolytic and non-haemolytic together—35.5 %) and *Streptococcus* spp. (28.1 %). The representatives of the genus *Staphylococcus* rated third (23.4 %). Streptococci and staphylococci were most abundant in the group of Gram-positive bacteria; altogether 51.5 % of the samples were positive for its presence. Other bacteria like *Serratia* spp., *Pseudomonas* spp. or *Bacillus* spp. were detected at very low counts (up to 2 %). The percentage proportion of the most frequently detected bacteria in animal patients are presented in Fig. 4.

When investigating bacterial diseases, we often encounter infections induced by several species of microorganisms. They are referred to as mixed infections or the so-called co-infections. According to our culture investigations, the proportion of mixed infections was lower (26 %) than that of mono-infections (74 %). Co-infections were confirmed in 13 samples from dogs and only 1 sample from cats. The proportions of individual bacterial pathogens in the co-infections varied. Mixed infections were relatively frequently caused by staphylococci and streptococci (28 % of all mixed infections). The second more frequent combinations of bacteria were *E. coli* and staphylococci (14 %) and *E. coli* and streptococci (14 %). Mono-infections were detected in 8 samples from cats and 32 samples from dogs.

The microbial disease agents were tested for their susceptibility to antibiotics (ATBs) using the disc-diffusion method. The antibiotics were selected on the basis of recommendation of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and also on the basis of information about drugs used in small animal practice in patients suffering from UTIs. For the therapeutic purposes recommended were those antimicrobials that ensured the biggest inhibition zone diameters in the tests of bacterial susceptibility. Bacterial colonies isolated from one sample were tested for several ATBs in order to allow the veterinarian to select the best therapeutical approach.

To alleviate the patient's signs, amoxicillin-clavulanic acid (AMC) (11—15 $mg.kg^{-1}$ p.o. every 8 h) or trimethoprim-sulphonamide (15 $mg.kg^{-1}$ p.o. every 12 h) was administered as an initial treatment until obtaining results of the tests on antibiotic resistance. The recommended length of treatment is 1—2 weeks [18].

Our determinations of the susceptibility of colonies to individual types of antibiotics showed that the most effective were enrofloxacin (ENR) and cefotaxime (CTX) that most effectively inhibited the growth of *E. coli*. Cephazolin (CZL) inhibited particularly Gram-positive bacteria. The

aim of our study was to determine the causative agent of UTI and its susceptibility to antibiotics. However, the attending veterinarian is responsible for the selection of the drugs and their dosages.

E. coli was susceptible to enrofloxacin and cefotaxime in 33 % of the cases, *Staphylococcus* spp. to cephazolin in 26 %, *Streptococcus* spp. to cefotaxime and cephazolin in 24 %, *Klebsiella* to enrofloxacin in 50 % and *Pasteurella* spp. to enrofloxacin in 75 %. Additional antibiotics subjected to the disc-diffusion testing were ciprofloxacin, cefuroxim, amoxicillin, cotrimoxazole, cephalotin and cefoxitin that were also effective against bacteria but less frequently than the antibiotics mentioned above. During the years 2002—2007, agents of UTI in dogs were tested at the Western College of Veterinary Medicine in Canada. The agents most frequently isolated from positive samples were *E. coli*, *Staphylococcus intermedius, Enterococcus* spp. and *Proteus* spp. Increasing resistance was detected in *E. coli*. In general, the most frequent UTIs inducing bacterial agents were *E. coli* [1]. The examination of urine samples in our study also showed that the UTIs were most frequently caused by *E. coli* bacteria (haemolytic and non-haemolytic strains 36 %) and *Streptococcus* spp. (28.1 %). The third most frequently identified pathogens were the members of the genus *Staphylococcus* (23.4 %). Also, other studies have confirmed the high antibiotic resistance of *E. coli* isolates from dogs presented with UTIs with high levels of resistance to penicillin-G (99 %), clindamycin (100 %), tylosine (95 %), and cephalotin (84 %). On the other hand, there were relatively low levels of resistance to enrofloxacin (16 %) and orbifloxacin (21 %) [11].

Drugs pass through the blood to kidneys, to the primary urine and are then excreted by efferent passages and in this way, they eliminate the microorganisms. Thus, it is necessary to ensure sufficient concentrations of the ATBs in the urine, kidneys and urinary bladder. When speaking about sufficient concentrations, we use the term MIC (minimum inhibitory concentration) which is the lowest concentration of an antibiotic needed for elimination of pathogens [4]. In order to alleviate the patient's symptoms, the initial treatment involves the use of amoxicillin potentiated by clavulanic acid (11—15 $mg.kg^{-1}$ p. o. every 8 h) or trimethoprim-sulphonamide (15 $mg.kg^{-1}$ p. o. every 12 h), administered in the indicated cases even before obtaining laboratory results about the susceptibility of bacteria to different ATBs. The recommended length of treatment is 1—2 weeks with the absence of side effects; the length of administration must be observed [18]. The relapse of UTIs is prevented by observation of the length of administration of drugs despite the disappearance of signs. Our determinations of susceptibility of bacteria to individual ATBs showed the highest effectiveness of enrofloxacin and cefotaxime that inhibited most effectively the growth of *E. coli*. Cephazolin inhibited particularly Gram-positive bacteria.

CONCLUSIONS

Our results allowed us to conclude that infections of the urinary tract affected more frequently males than females and this was observed for both dogs and cats. The highest number of positive samples (presence of bacteria) originated from the dogs. However, we should mention that the total number of samples obtained from dogs was higher than that from cats and thus we should consider the fact that the owners of cats, particularly those kept outside are not able to detect the UTIs problems as readily as dog owners. Thus, one should realise that this could contribute to some distortion of our results. The age range associated with the highest prevalence of UTIs was 7—10 years. The samples investigated in our study originated from animals of various breeds but included mostly small breeds of dogs, such as Chihuahua, Dachshund or Yorkshire Terrier. The most frequent agents of urinary infections were *E. coli*, *Streptococcus* spp. and *Staphylococcus* spp. According to our testing, the antibiotic enrofloxacin was the most effective against all isolated pathogens which correlated with the literary data that confirmed this antibiotic was one of the most frequently used drugs to treat urinary infections in a small animal clinical practice.

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REFERENCES

1. **Ball, K. R., Rubin, J. E., Chirino-Trejo, M., Dowling, P. M., 2008:** Antimicrobial resistance and prevalence of canine uro-

pathogens at the Western College of Veterinary Medicine Veterinary Teaching Hospital, 2002—2007. *Can. Vet. J.*, 49, 10, 985—990.

2. **Barsanti, A. J., 2005:** *Bacterial Cystitis/Urethritis DACVIM* (Specialty of Internal Medicine). Athens, GA. Retrieved from: https://www.vin.com/apputil/content/defaultadv1.aspx?id=4252856&pid=11290&print=1.
3. **Bartges, J. W., 2004:** Diagnosis of urinary tract infections. *Vet. Clin. North Am. Small Anim. Pract.*, 34, 4, 923—933. DOI: 10.1016/j.cvsm.2004.03.001. PMID: 15223209.
4. **Dowling, P. M., 2018:** *Bacterial Urinary Tract Infections*. Retrieved from: https://www.msdvetmanual.com/pharmacology/systemic-pharmacotherapeutics-of-the-urinary-system/bacterial-urinary-tract-infections.
5. **Kozák, M., et al., 2015:** *Diseases of Dogs and Cats,* Part II./A (In Slovak), 2nd edn., University of Veterinary Medicine and Pharmacy in Košice, Slovakia, 370 pp.
6. **Langston, C. E., 2011:** *Urethritis*. Saunders, an imprint of Elsevier Inc. Retrieved in January 2021 from: https://www.happypetsbahamas.com/wp-content/uploads/2018/04/0728.pdf.
7. **Low, W. W., Uhl, J. M., Kass, P. H., Ruby, A. L., Westropp, J. L., 2010:** Evaluation of trends in urolith composition and characteristics of dogs with urolithiasis: 25,499 cases (1985—2006). *J. Am. Vet. Med. Assoc.*, 236, 2, 193–200. DOI: 10.2460/javma.236.2.193.
8. **Marques, C., Belas, A., Franco, A., Aboim, C., Gama, L. T., Pomba, C., 2018:** Increase in antimicrobial resistance and emergence of major international high-risk clonal lineages in dogs and cats with urinary tract infection: 16 year retrospective study. *J. Antimicrob. Chemother.*, 1, 73, 377—384. DOI: 10.1093/jac/dkx401.
9. **Mazzariol, A., Bazaj, A., Cornaglia, G., 2017:** Multi-drug-resistant Gram negative bacteria causing urinary tract infections: A review. *J. Chemother*., 29 (Suppl. 1), 2—9. DOI: 10.1080/1120009X.2017.1380395.
10. **Mihok, T., 2010:** *Infections of the Urinary Tract* (In Slovak). Retrieved from: https://www.psickar.sk/najbeznejsie-ochorenia/mocova-sustava-psa/infekcie-mocoveho-traktu.
11. **Qekwana, D. N., Phophi, L., Naidoo, V., Oguttu, J. W., Odoi, A., 2018:** Antimicrobial resistance among *Escherichia coli* isolates from dogs presented with urinary tract infections at a veterinary teaching hospital in South Africa. *BMC Vet. Res.*, 14, 228. DOI: 10.1186/s12917-018-1552-7.
12. **Rizzi, T. E., Valenciano, A. C., Cowell, R. L., 2017:** *Atlas of Canine and Feline Urinalysis*. 1st edn., Hoboken, New Jersey, United States, Wiley-Blackwell, Inc., 113—119.
13. **Spector, D., 2017:** *Urinary Blockage in Cats: A Real Emergency*. Retrieved from: http://www.vetstreet.com/our-pet-experts/urinary-blockage-in-cats-a-real-emergency.
14. **Stevenson, A. E., Markwell, P. J., 2001:** Comparison of urine composition of healthy Labrador retrievers and miniature Schnauzers. *Am. J. Vet. Res.*, 62, 1782—1786. DOI: 10.2460/ajvr.2001.62.1782.
15. **Syme, H. M., 2012:** Stones in cats and dogs: What can be learnt from them? *Arab. J. Urol.*, 10, 3, 230—239.
16. **Thompson, M. F., Litster, A. L., Platell, J. L., Trott, D. J., 2011:** Canine bacterial urinary tract infections: new developments in old pathogens. *Vet. J.,* 190, 1, 22—27. DOI: 10.1016/j.tvjl.2010.11.013.
17. **Vajda, V., Maskaľová, I., 2016:** *Evaluation of Feed Quality and Creation of Productive Animal Health*. 1st edn., (In Slovak), University of Veterinary Medicine and Pharmacy in Košice, Slovakia, 406 pp.
18. **Weese, J. S., Blondeau, J., Boothe, D., Guardabassi, L. G., Gumley, N., Papich, M., et al., 2019:** International Society for Companion Animal Infectious Diseases (ISCAID) guidelines for the diagnosis and management of bacterial urinary tract infections in dogs and cats. *Vet. J.*, 247, 8—25, DOI: 10.1016/j.tvjl.2019.02.008.
19. **Yadav, S. N., Ahmed, N., Nath, A. J., Mahanta, D., Kalita, M. K., 2020:** Urinalysis in dog and cat: A review. *Vet. World*, 13, 10, 2133—2141. DOI: 10.14202/vetworld.2020.2133-2141.

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DIFFERENCES IN THE COMPOSITION OF CULTIVABLE AEROBIC AND FACULTATIVE ANAEROBIC ORAL MICROBIOTA IN CATS OF VARIOUS AGE GROUPS

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ABSTRACT

The feline oral cavity is naturally inhabited by various microorganisms contributing to the maintenance of its oral health. The imbalance of oral microbiota or the presence of pathogenic agents can lead to secondary oral diseases. Various factors such as sex, diet, breed, environment and even age, affect the composition of a healthy oral microbiota during the life of cats. The purpose of this study was to compare the composition of culturable aerobic and facultative anaerobic microbiota in cats in terms of different age categories. We used conventional cultivation methods in conjunction with microscopic and biochemical methods to isolate and identify the microorganisms found in the oral cavity of cats. The examination of 76 samples confirmed the dominance of the phylum *Proteobacteria* in almost all groups. Commonly occurring bacteria have been identified, i.e. *Streptococcus* spp., *Neisseria* spp. and *Pasteurella* spp. Although aerobic and facultative anaerobic oral microbiota were examined, differences between age groups were noted. The microbial diversity of the oral microbiota significantly increased with age.

Key words: age groups; cultivation methods; feline; oral microbiota

INTRODUCTION

The oral cavity is the first part of the gastrointestinal tract where the process of digestion begins, and therefore it creates a space for the action of various microorganisms [8]. The constant flow of saliva and the unique biological properties of each part of the oral cavity provide a place for attachment of microorganisms [22]. In particular, the surface of the teeth has the highest microbial load compared to other surfaces [25]. Complex, multi-species communities of resident microorganisms, from aerobic to anaerobic, play a crucial role in oral health and diseases. Important is

the beneficial defensive function or rather the competition with the pathogenic bacteria introduced with food or from the external environment [15].

The feline oral microbiota consists of several bacterial phyla such as: *Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Fusobacteria* and others [4]. Differences in the diversity and dominance of bacterial strains have been reported in terms of health and diseases [22], and it has also been disclosed that the diversity of microbiota in the oral cavity of cats is significantly greater than that in dogs [11, 15].

The feline oral microbiota may pose a risk of systemic disease if it is transmitted to other sites in the body, e.g. in the case of aspiration pneumonia [1]. Microorganisms can also be transmitted by biting, thus causing infections of wounds usually described in humans [9]. The feline oral microbiota can be a source of zoonotic pathogens (e.g. *Pasteurella* spp.), not only in terms of bites [17], but also by the potential transmission of periodontal bacteria to the oral cavity of cat owners [2].

Microbial communities inhabiting the oral cavity can be affected by various factors such as sex, environment, breed [13], diet [1] and age [13, 19]. Also, the composition of the oral microbiota may vary with the disease. Differences in compositions of the oral microbiota between healthy cats and those with a disease have been reported for periodontal disease (PD) [13, 16], feline chronic gingivostomatitis (FCGS) [6, 12] and feline immunodeficiency virus (FIV) [10, 14, 23], where the predominance of certain oral bacteria may cause or contribute to the development of the disease [18].

For the detection of feline oral microbiota, culture-independent methods (gene sequence analyses) have been increasingly used as less time-consuming than the culture methods [3]. However, conventional culture methods are still essential for the isolation and identification of microorganisms [8].

Natural colonization of the oral cavity by microorganisms occurs from birth [19]. With increasing age, the diversity of microbial communities increases significantly [1, 22] which correlates with the changes during important life stages [21]. Only a few studies have been involved in investigating the effects of age on the oral microbiota in cats. Therefore, the aim of our study was to evaluate on the basis of biochemical methods and cultures how the age of cats can affect the composition of their oral microbiota.

MATERIALS AND METHODS

Age groups of cats

An important step before sampling was the formation of age groups. Based on the scheme proposed by V o g t et al. [21], the following age groups were formed: kittens (from birth until 5 months), young adults (6 months to 2 years), adults (3 years to 7 years) and seniors (over 8 years).

Sample collection

Samples were obtained from 76 clinically healthy European Shorthair cats by inserting a sterile swab into the cat's mouth and rotating the swab for approximately 10—15 seconds. The swab included the entire oral cavity: the upper and lower dental arcade (supragingival plaque), gums, labial and buccal mucosa, palatal mucosa and sublingual region. The swabs were then placed in Amies transport medium (Sarstedt, Nümbrecht, Germany) at 8 °C and

Table 1. Characterization of feline age groups

Age groups	Mean age	Sex F/M [%]	Environment I/O/C [%]	Diet D/W/C [%]
Kittens (n = 20)	2 months	60/40	75/20/5	0/25/75
Young adults (n = 20)	1.5 years	50/50	50/25/25	0/0/100
Adults (n = 20)	4 years	55/45	35/35/30	0/0/100
Seniors (n = 16)	11 years	62.5/37.5	12.5/25/62.5	0/6.2/93.8

F—female; M—male; I—indoor; O—outdoor; C—combination; D—dry; W—wet

processed within 24 hours. The health status of each cat was assessed on the basis of anamnesis, clinical examination and oral health (normal gingiva with sharp, non-inflamed edges).

All procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Animals (Protocol No. 3323/16-221/3), approved by the State Veterinary and Food Administration of the Slovak Republic and by the Ethics Commission of the University of Veterinary Medicine and Pharmacy in Košice, Slovakia. The animals were handled in a humane manner in accordance with the guidelines established by the relevant commissions. All applicable international, national and institutional guidelines for the care and use of animals were observed. The basic data about the cats were obtained from cat owners as well as their consent to participate in the study. A summary of the data obtained on the basis of age grouping may be seen in Table 1.

Bacterial analysis of samples

Each swab was plated on blood agar, MacConkey agar (HiMedia, Mumbai, India) and Endo agar (HiMedia, Mumbai, India). The blood agar was prepared as Tryptone Soya Agar (HiMedia, Mumbai, India) supplemented with 5 % of sterile defibrinated sheep blood. The inoculated agar plates were then incubated under aerobic conditions at 37 °C for 24 hours. After incubation, the plates were examined and then different types of colonies were subcultured to obtain pure cultures. The presence of haemolytic activity and morphological characteristics such as growth, shape, size and colour of pure cultures were evaluated on blood agar. In addition to culture examinations, Gram staining was used to differentiate Gram-positive and Gram-negative bacteria and also to determine the microscopic morphology.

Bacterial isolates were then subjected to biochemical assays for oxidase and catalase activity. The indole and urease tests were used to differentiate *Pasteurella* species. Final biochemical identification of strains were done using commercial identification kits: STAPHYtest 24; STREPTOtest 24; NEFERMtest 24; ENTEROtest 16; ENTEROtest 24 (Erba Lachema, Brno, Czech Republic). Subsequently, a specific bacterial strain was determined using the software Program TNW ProAuto 7.0. The overall results were evaluated and analysed by statistical-mathematical methods in percentage terms using the Microsoft Excel programme.

Two selected isolates of *Neisseria* spp. were identified by genotyping for their species identification. DNA was isolated using DNAzol direct (Molecular Research Center Inc., Cincinnati, USA) according to the manufacturer's instructions. The 16S ribosomal RNA (rRNA) genes from the isolates were amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGYTACCTTGTTACGACTT-3'). The PCR cycling conditions comprised an initial denaturation phase of 5 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and primer extension at 72 °C for 3 min and finally a primer extension step at 72 °C for 10 min. The PCR was performed in a thermal cycler (TProfesional Basic, Biometra GmbH, Göttingen, Germany), aliquot PCR products were separated by horizontal 3 % (w/v) agarose gel electrophoresis in Tris-acetate-EDTA buffer (pH 7.8) and visualized with GelRed (Biotium, Inc., Hayward, CA, USA) under ultraviolet light. The amplification products were sent for purification and sequencing using primer 1492R (Microsynth, Wien, Austria).

RESULTS

The combination of culture evaluations and subsequent microscopic findings were the first steps in identifying most of the representatives of the oral microbiota at the level of the bacterial genus. A total of 18 different bacterial genera were isolated from the 76 samples. The most frequently isolated bacterial genera were *Streptococcus* (23.9 %), *Pasteurella* (22.4 %) and *Neisseria* (17.9 %). *Pasteurella* spp. were identified on the basis of Gram staining (Gram-negative coccobacilli), absence of haemolysis on blood agar, grey and viscous colonies, absence of growth on MacConkey and Endo agar, and positive biochemical activity of catalase and oxidase tests. Only two isolates of *Pasteurella* spp. have been accurately identified as *Pasteurella multocida* and *Pasteurella pneumotropica* based on their different biochemical characteristics (Table 2).

Escherichia coli was identified by its typical green metallic sheen on Endo agar, and some suspect isolates were confirmed by biochemical assays. *Neisseria* was identified based on the culture's characteristics (round shape, opaque, shiny, smooth colonies), microscopic morphology (Gram-negative coccoid or short rods which often oc-

Table 2. Biochemical characterization of two *Pasteurella* isolates

	Oxidase	Catalase	Urease	Indole
Pasteurella multocida	+	+	–	+
Pasteurella pneumotropica	+	+	+	+

+ — positive; – — negative

curred in pairs), positive catalase activity and positive oxidase activity. However, two isolates of *Neisseria* spp. were also subjected to sequencing analysis (16S rRNA) and were identified as *Neisseria zoodegmatis* and *Neisseria animaloris*. Both species formed non-haemolytic, round shaped, yellowish to white colonies on blood agar (Fig. 1).

The results of biochemical commercial tests of all identified species are shown in Table 3. A score of less than 50 % is reported only at the level of the bacterial genus.

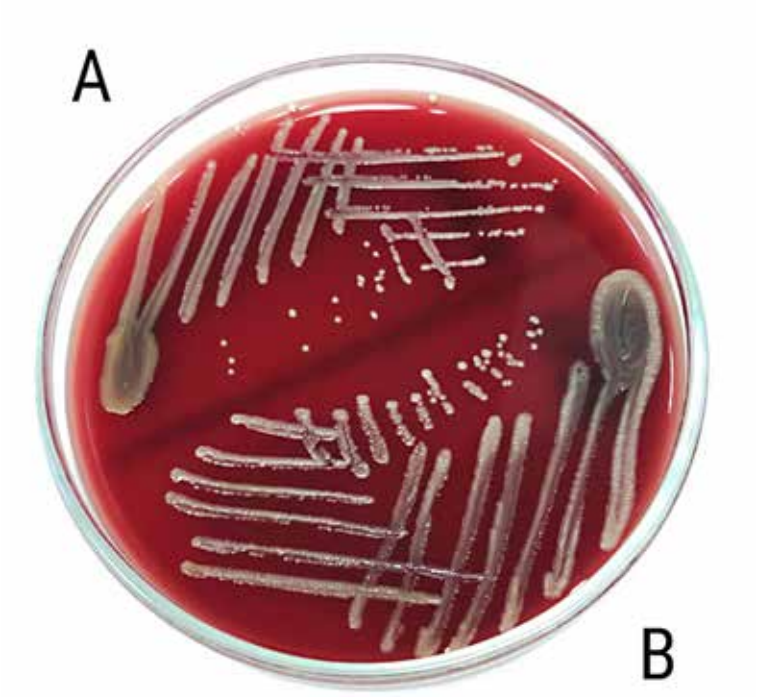

Fig. 1. Growth of *Neisseria zoodegmatis* and *Neisseria animaloris* on blood agar
A—*Neisseria zoodegmatis*; **B**—*Neisseria animaloris*

The main aim of this study was to characterize and compare the representatives of cultivable aerobic and facultative anaerobic oral microbiota of cats between age groups (Fig. 2). The members of the genus *Streptococcus* (40.7 %) in the group of kittens were predominantly identified, followed by *Pasteurella* spp. (33.3 %) and *Staphylococcus* spp. (11.1 %). In the case of young adults, the diversity of the bacteria was higher. The genera found in the highest proportions were *Pasteurella* (25.7 %) and *Streptococcus* (25.7 %), followed by *Moraxella* (8.6 %) and *Neisseria* (8.6 %). In the adult cats, the most frequently isolated bacteria were *Streptococcus* spp. (29.7 %) and *Neisseria* spp. (27 %). The genus *Pasteurella* (16.8 %) was the third most frequently identified. The highest bacterial diversity was recorded in the group of seniors. The predominant genus was *Neisseria* spp. (28.6 %) followed by *Pasteurella* spp. (17.1 %) and *Staphylococcus* spp. (11.4 %). The percentage comparison of bacterial phyla between age groups can be seen in Fig. 3.

Other bacterial genera detected in young adults were: *Enterobacter, Kocuria*; other bacterial genera detected in seniors were: *Aeromonas, Brevundimonas, Corynebacterium*, and *Sutonella, Weeksella*.

Table 3. Bacterial species identified by commercial identification kits

Bacterial species	Number of isolates	Average Percentage Identification Score [%]
Aerococcus spp.	1	48.15
Aerococcus viridans	2	95.11
Aeromonas caviae	1	90.33
Aeromonas ichthiosmia	1	100
Brevundimonas vesicularis	1	78.12
Citrobacter braaki	2	95.38
Enterobacter spp.	3	48.97
Escherichia coli	3	99.76
Klebsiella oxytoca	2	99.34
Kocuria kristinae	1	99.02
Moraxella osloensis	2	54.69
Moraxella spp.	7	49.88
Shewanella algae	2	92.11
Staphylococcus felis	1	95.11
Staphylococcus hominis ssp. *hominis*	2	99.80
Staphylococcus sciuri	2	100
Staphylococcus simulans	1	60.24
Staphylococcus spp.	4	49.74
Staphylococcus warneri	1	99.16
Streptococcus sanguinis/parasanguinis	17	94.77
Streptococcus spp.	8	49.52
Streptococcus suis	2	99.78
Streptococcus uberis	5	99.90
Sutonella indologenes	2	99.38
Weeksella virosa	1	92.35

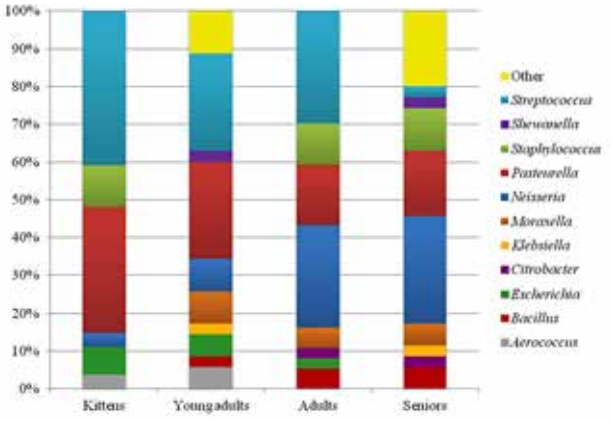

Fig. 2. Comparison of the occurrence of bacterial genera between age groups of cat

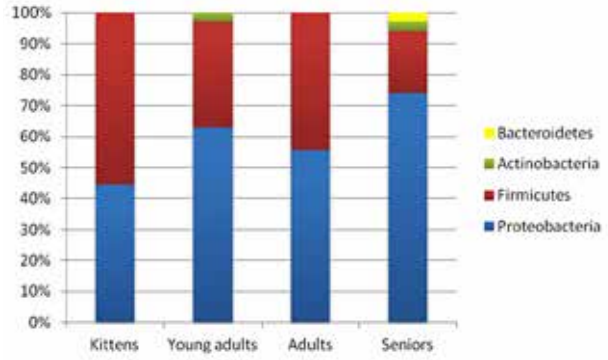

Fig. 3. Comparison of bacterial phyla between age groups

DISCUSSION

The composition of a culturable feline oral microbiota may be influenced by many factors. This study focused on various stages in a cat's life and the potential differences in the diversity of oral microbiota in the different age groups.

Oral swabs were performed without anaesthesia or sedation because the cats were calm during swabbing. This approach differed from that used in other studies [4, 18, 24] when samples were taken during anaesthesia as a part of routine dental examination. Subsequent bacteriological examinations focused mainly on the isolation and identification of the facultative anaerobic and aerobic bacteria, as the samples were transported over longer distances to the laboratory. Also, in the study by Razali et al. [17] anaerobic bacteria were not included in the investigation of the oral microbiota in the cats, as anaerobic bacteria require more complex cultivation and immediate processing after sampling.

The current trend in the analysis of the oral microbiota of cats is carried out mainly by NGS sequencing, so that the samples are subjected directly to DNA extraction without previous conventional culture [5, 20]. In addition, compared to standard culture methods, a culture-independent methodology can detect also the presence of non-culturable and unknown microorganisms in the oral microbiota [3]. On the other hand, from the point of view of veterinary practice, culture methods are used in conjunction with the determination of the antibiotic susceptibility of oral bacteria which may cause either primary or secondary infections.

Commonly occurring bacteria, for example *Streptococcus*, *Neisseria* and *Pasteurella*, were identified in this study by culture and biochemical identifications. The dominance of the *Proteobacteria phylum* was also noted. A study of the oral microflora of cats with or without disease showed that α-haemolytic *Streptococci* were the most common genus in healthy cats while in the sick cats, the genus *Staphylococcus* dominated [24]. Sturgeon et al. [20] investigated the oral microbiota from 11 clinically healthy cat and detected the predominance of *Proteobacteria phylum*, mainly the genera *Moraxella, Pasteurella, Neisseria* and unclassified bacteria of the *Pasteurellaceae* family. Another study reported that the most commonly identified genera in healthy cats were, *Enhydrobacter, Moraxella* and *Capnocytophaga* [18]. The bacterial genera *Moraxella* and *Neisseria* were obtained from a healthy feline oral cavity. These were also described as two of the most common aerobic genera identified in samples from humans bitten by cats [3]. Harris et al. [7] reported that the most dominant phylum in their study of the oral cavity of healthy cats was *Bacteroidetes,* which does not agree with our results. This may be related to other factors that may affect the composition of the oral microbiota [13].

The research of Spears et al. [19] focused on the development of oral microbiota in kittens from birth to 8 weeks of age. They examined a total of 5 litters; the number of individuals was 16. Phylogenetic analysis during their growth showed an increase in the proportion of phyla. Although a different age range was used in our study, our results indicated an increase in bacterial diversity.

CONCLUSIONS

Our study investigated changes in the composition of the oral microbiota during the life of cats. Despite the limitations of culture and biochemical methods, our results agree with the previous studies regarding the abundance and predominance of the *Proteobacteria* strains in the oral microbiota of cats. An increase in the bacterial diversity with age was observed in the older age groups. The difference in the oral microbiota may have been due to changes during the life stages, which can be associated with the change of the diet from milk to commercial or other available food for kittens. Further studies are needed to demonstrate the potential effects of other factors. A study of cultivable healthy oral microbiota of cats with respect to age can differentiate potential pathogenic bacteria not only for the cats themselves but also for the potential risk of bite infections in humans.

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REFERENCES

1. **Adler, C. J., Malik, R., Browne, G. V., Norris, J. M., 2016:** Diet may influence the oral microbiome composition in cats. *Microbiome*, 4, 23, 1—9. DOI: 10.1186/s40168-016-0169-y.
2. **Booij-Vrieling, H. E., van der Reijden, W. A., Houwers, D. J., de Wit, W. E. A. J., Bosch-Tijhof, C. J., Penning, L. C., et al., 2011:** Comparison of periodontal pathogens between cats and their owners. *Vet. Microbiol.*, 144, 147—152. DOI: 10.1016/j.vetmic.2009.12.046.
3. **Davis, E. M., 2016:** Gene sequence analyses of the healthy oral microbiome in humans and companion animals: A comparative review. *J. Vet. Dent.*, 33, 2, 97—107. DOI: 10.1177/0898756416657239.
4. **Dewhirst, F. E., Klein, E. A., Bennett, M., Croft, J. M., Harris, S. J., Marshall-Jones, Z. V., 2015:** The feline oral microbiome: a provisional 16S rRNA gene based taxonomy with full-length reference sequences. *Vet. Microbiol.*, 175, 294—303. DOI: 10.1016/j.vetmic.2014.11.019.
5. **Dolieslager, S. M., Riggio, M. P., Lennon, A., Lappin, D. F., Johnston, N., Taylor, D., et al., 2011:** Identification of bacteria associated with feline chronic gingivostomatitis using culture-dependent and culture-independent methods. *Vet. Microbiol.*, 148, 93—98. DOI: 10.1016/j.vetmic.2010.08.002.
6. **Dolieslager, S. M., Bennet, D., Johnston, N., Riggio, M. P., 2013:** Novel bacterial phylotypes associated with the healthy feline oral cavity and feline chronic gingivostomatitis. *Res. Vet. Sci.*, 93, 3, 428—432. DOI: 10.1016/j.rvsc.2012.11.003.
7. **Harris, S., Croft, J., O'Flynn, C., Deusch, O., Colyer, A., Allsopp, J., et al., 2015:** A pyrosequencing investigation of differences in the feline subgingival microbiota in health, gingivitis and mild periodontitis. *PLOS ONE*, 10, 11, e0136986. DOI: 10.1371/journal.pone.0136986.
8. **Kil, D. Y., Swanson, K. S., 2011:** Companion animals symposium: Role of microbes in canine and feline health. *J. Anim. Sci.*, 89, 5, 1498—1505. DOI: 10.2527/jas.2010-3498.
9. **Magaji, A. A., Saulawa, M. A., Salihu, M. D., Junaidu, A. U., Shittu, A., Gulumbe, M. L., et al., 2008:** Oral microflora of stray domestic cats (*Felis catus*) found in the premises of two human hospitals in Sokoto, Nigeria. *Sokoto J. Vet. Sci.*, 7, 1, 9—12.
10. **Miller, C., Boegler, K., Carver, S., MacMillan, M., Bielefeldt-Ohmann, H., VandeWoude, S., 2017:** Pathogenesis of oral FIV infection. *PLOS ONE*, 12, 9, e0185138. DOI: 10.1371/journal.pone.0185138.
11. **Misic, A. M., Davis, M. F., Tyldsley, A. S., Hodkinson, B. P., Tolomeo, P., Hu, B., et al., 2015:** The shared microbiota of humans and companion animals as evaluated from *Staphylococcus* carriage sites. *Microbiome*, 3, 2, 1—19. DOI: 10.1186/s40168-014-0052-7.
12. **Nakanishi, H., Furuya, M., Soma, T., Hayashiuchi, Y., Yoshiuchi, R., Matsubayashi, M., et al., 2019:** Prevalence of microorganisms associated with feline gingivostomatitis. *J. Feline Med. Surg.*, 21, 2, 103—108. DOI: 10.1177/1098612X18761274.
13. **Older, C. E., Diesel, A. B., Lawhon, S. D., Queiroz, C. R. R., Henker, L. C., Hoffmann, A. R., 2019:** The feline cutaneous and oral microbiota are influenced by breed and environment. *PLOS ONE*, 14, 7, e0220463. DOI: 10.1371/journal.pone.0220463.
14. **Older, C. E., Gomes, M. O. S., Hoffmann, A. R., Policano, M. D., Reis, C. A. C. D., Carregaro, A. B., et al., 2020:** Influence of the FIV status and chronic gingivitis on feline oral microbiota. *Pathogens*, 9, 5, 1—11. DOI: 10.3390/pathogens9050383.
15. **Özavci, V., Erbas, G., Parin, U., Yüksel, H. T., Kirkan, Ş., 2019:** Molecular detection of feline and canine periodontal pathogens. *Vet. Anim. Sci.*, 8, 100069. DOI: 10.1016/j.vas.2019.100069.
16. **Pérez-Salcedo, L., Herrera, D., Esteban-Saltiveri, D., León, R., Jeusette, I., Torre, C., et al., 2011:** Comparison of two sampling methods for microbiological evaluation of periodontal disease in cats. *Vet. Microbiol.*, 149, 500—503. DOI: 10.1016/j.vetmic.2010.11.021.
17. **Razali, K., Kaidi, R., Abdelli, A., Menoueri, M. N., Ait-Oudhia, K., 2020:** Oral flora of stray dogs and cats in Algeria: *Pasteurella* and other zoonotic bacteria. *Vet. World*, 13, 12, 2806—2814. DOI:10.14202/vetworld.2020.2806-2814.
18. **Rodrigues, M. X., Bicalho, R. C., Fiani, N., Lima, S. F., Peralta, S., 2019:** The subgingival microbial community of feline periodontitis and gingivostomatitis: characterization and comparison between diseased and healthy cats. *Sci. Rep.*, 9, 1, 12340. DOI: 10.1038/s41598-019-48852-4.
19. **Spears, J. K., Vester Boler, B., Gardner, C., Li, Q., 2017:** Development of the oral microbiome in kittens. In *Companion Animal Nutrition (CAN) Summit: The Nexus of Pet and Human Nutrition: Focus on Cognition and Microbiome*. Helsinki, Finland, May 4—7, 73—81.
20. **Sturgeon, A., Pinder, S. L., Costa, M. C., Weese, J. S., 2014:** Characterization of the oral microbiota of healthy cats using next-generation sequencing. *Vet. J.*, 201, 2, 223—229. DOI: 10.1016/j.tvjl.2014.01.024.

21. **Vogt, A. H., Rodan, I., Brown, M., Brown, S., Buffington, C. A., Forman, M. J. L., et al., 2010:** AAFP-AAHA: feline life stage guidelines. *J. Feline Med. Surg.*, 12, 43—54. DOI: 10.1016/j.jfms.2009.12.006.

22. **Wallis, C. V., Marshall-Jones, Z. V., Deusch, O., Hughes, K. R., 2017:** Canine and Feline Microbiomes. In **Singh, R., Kothari, R., Koringa, P., Singh, S.:** *Understanding Host-Microbiome Interactions—An Omics Approach*. Springer, Singapore, 279—325. DOI: 10.1007/978-981-10-5050-3_17.

23. **Weese, S. J., Nichols, J., Jalali, M., Litster, A., 2015:** The oral and conjunctival microbiotas in cats with and without feline immunodeficiency virus infection. *Vet. Res.*, 46, 21—31. DOI: 10.1186/s13567-014-0140-5.

24. **Whyte, A., Gracia, A., Bonastre, C., Tejedor, M. T., Whyte, J., Monteagudo, L. V., et al., 2017:** Oral disease and microbiota in free-roaming cats. *Top. Companion Anim. Med.*, 32, 3, 91—95. DOI: 10.1053/j.tcam.2017.07.003.

25. **Zambori, C., Tirziu, E., Nichita, I., Cumpanasoiu, C., Gros, R. V., Seres, M., et al., 2012:** Biofilm implication in oral diseases of dogs and cats. *Anim. Sci. Biotechnol.*, 45, 2, 208—212.

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THE USE OF ENDOSCOPIC DIAGNOSIS IN DOGS WITH UPPER RESPIRATORY DISEASES WITH RESPECT TO THE LOCALISATION OF PATHOGENS AND THE SUBSEQUENT THERAPY

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ABSTRACT

Bacterial diseases of the upper respiratory tract accompanied with various degrees of clinical signs are relatively frequent in a small animal clinical practice. The clinical signs are usually mild, mostly connected with clinical manifestation of nasal discharge, mild dyspnoea, sneezing, and coughing; however, in some cases they may convert to a chronic stage with serious systemic manifestations. The course and development of complications depends on the etiological agent and the success or failure of the subsequent therapy. An accurate diagnosis is of the upmost importance in order to develop an appropriate therapeutic plan for disease management. The present study focused on: endoscopic visualisation of the upper respiratory tract of the affected animals; localisation of pathological changes, and the targeted collection of the samples. This clinical study involved 26 patients with long-term signs of the affected airways that progressed to chronic stages after the failure of the prescribed therapy. Each patient was clinically examined, sedated and subjected to an endoscopic examination that involved the collection of samples. The samples were examined microbiologically, tested for antibiotic resistance, and a targeted therapy was implemented. The endoscopic and complex microbiological diagnosis enabled: more effective management of the bacterial infection; shortening of the therapy; and a convalescence period that reduced the risk of the development, or the spreading of resistant bacteria.

Key words: bacterial infection; laryngotracheitis; rhinitis; samples cultivation and endoscopy visualisation; upper respiratory diseases

INTRODUCTION

Infectious diseases of the upper canine respiratory tract constitute a significant worldwide health issue encountered in a veterinary clinical practice. The affected group includes senior dogs and also young dogs with reduced immunity, development disorders and chronic degenerative changes. Both acute and chronic infections of the up-

per respiratory airways frequently lead to a wide range of clinical signs. The most frequent are: mucopurulent nasal discharge, sniffling, as well as coughing associated with the excitation of the patient and dyspnoea that can also be related to diseases of other organ systems [9].

The etiology of the development of respiratory diseases is frequently associated with various causative agents, such as bacteria, viruses, fungi, parasites and other pathogens. The respiratory tract is constantly exposed to these infectious agents either by aerogenic or haematogenic pathways. The pathogenic invasion is arrested by physical, chemical and immunological mechanisms that include mucin, mucocilliary clearance and various inherent antimicrobial factors [15]. The impairment of the protective mechanisms caused, for example, by immunosuppression, stress, toxins, environment and infectious agents, frequently results in the development of chronic inflammatory processes affecting individual compartments of the respiratory tract.

In the majority of cases, rhinitis as a disease of the upper respiratory tract is frequently overlooked during the primary diagnosis. From the etiological point of view, rhinitis is most frequently induced by the propagation of viral infections. However, secondary bacterial contaminations may occur in long term inflammation or chronic diseases. In such conditions connected with untreated cases, inflammation from rhinitis may spread to the surrounding areas. The subsequent progressive chronic rhinitis involves infiltration of the inflammatory process into the surrounding structures and the development of *sinusitis maxillaris, frontalis* and *infraorbitalis* [11]. A non-specific infectious rhinitis is very rare in dogs. Other factors participating in the development of inflammation of the upper respiratory tract include nasal trauma, allergy, or the penetration of a foreign object [15].

Laryngotracheitis (Fig. 1) is another disease characterised by the inflammation of the upper respiratory tract. The etiology of this disease is polyfactorial. The pathogens that most frequently cause this disease include: bacteria (*Bordetella bronchiseptica, Mycoplasma cynos*, and *Streptococcus equi* subsp. *zooepidemicus*), and viruses (canine parainfluenza virus CAV-2, canine adenovirus CAV-1, canine influenza virus, and canine respiratory virus). The primary way of pathogen transfer involves aerosol and the clinical signs appear about 3—10 days after the exposure to the pathogens. Additional sources of infection are: inadequate sanitation of surfaces, contaminated examination instruments, endotracheal tubes, and toys [3].

The correct diagnosis plays an important role in the complex of prognosis, selection of therapy and its effectiveness. Endoscopic imaging diagnosis is a targeted diagnostic method. The advantages of this method are: the visualisation of the internal structures of the nasal and frontal cavities, larynx, pharynx, trachea and bronchi, the identification of anatomic abnormalities, and also the lo-

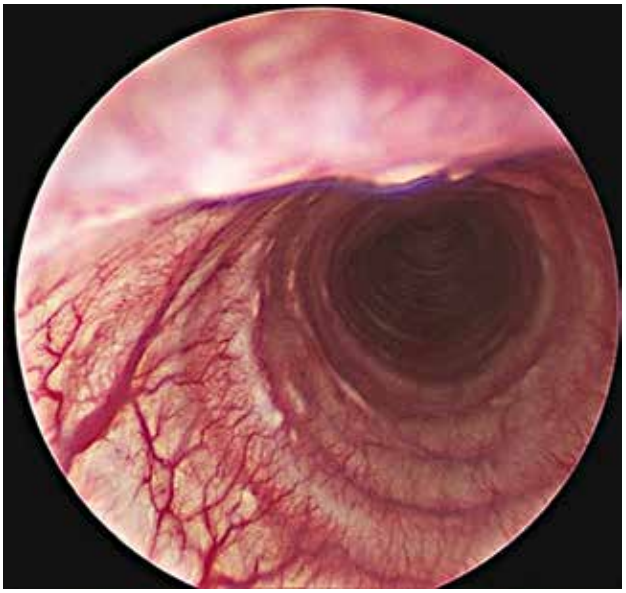

Fig. 1. Laryngotracheitis

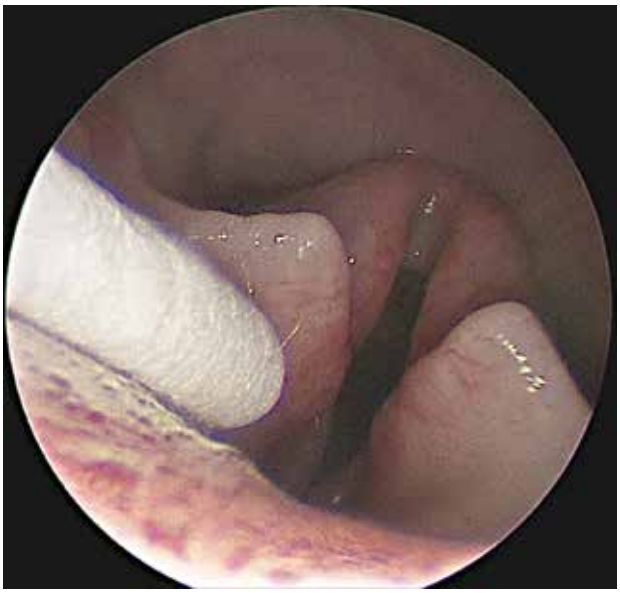

Fig. 2. Swab collecting in the area of larynx

calisation of foreign bodies [7]. The possibilities of endoscopic surgical interventions of congenital abnormalities, such as: stenotic nostrils, lengthening of the soft palate, stricture of nasopharyngeal compartments and tracheal stenosis which assist in: the therapy management complex, improvement of prognosis and the course and duration of the respiratory disease.

Endoscopic visualisation is the most suitable diagnostic method capable of revealing the sites of pathological changes and the parallel collecting of samples for cytological and microbiological examinations. One of the most frequently used ways of the collection of samples from the suspect regions of the respiratory apparatus involves swabbing under direct endoscopic control [14]. Mucosal biopsy is indicated with the presence of various pathological neoplasms, as well as suspected mycotic diseases. The most effective way of sampling is the nasal sinuses, laryngeal swab (Fig. 2), or bronchoalveolar lavage (BAL). During lavage, the appropriate volume of saline at body temperature will entrap sputum, exudate and surface cells revealing the potential for the development of respiratory infections [2].

The suspicion of the presence of a bacterial infection is frequently formulated during the primary clinical examination. When detecting signs, such as mucopurulent nasal discharge, fever, lethargy, inappetence, the best option according to the International Society for Companion Animal Infectious Diseases (ISCAID) is the empirical administration of doxycycline for 7—10 days as the first line of antibiotic effectiveness against *Mycoplasma* spp. and *Bordetella bronchiseptica*. However, much more effective is the therapy of respiratory infections of bacterial origin based on the determination of antibiotic susceptibility of isolated strains. For example, in the presence of secondary bacterial agents such as *Pasteurella* spp. and *Streptococcus* spp., the administration of amoxicillin appears to be the most adequate treatment. For the treatment of infection with *Staphylococcus* spp. it is recommended to use amoxicillin and clavulanic acid since such treatment is not effective against *Mycoplasma* spp. and *Bordetella bronchiseptica* [10].

The high prevalence of microbial contamination of the upper airways and increasing resistance to antimicrobials indicate the necessity of the utilisation of the most accurate diagnosis and targeted medicinal therapy. The aim of our clinical study was by means of the targeted endoscopic visualisation of pathological changes, to point out the increased effectiveness of the collection of samples, reliability of bacterial culture examinations and the development of the targeted therapeutic plan, based on the susceptibility of pathogens to the selected antimicrobial preparations.

MATERIALS AND METHODS

This study was carried out on patients (n = 26) exhibiting long-term clinical signs, such as: coughing, difficult breathing, asphyxia, nasal discharge, hyperaemia and depigmentation in the nostril zone. In 18 patients the disease was localised only in the upper airways, while 8 of them showed signs of a disease spreading into the tracheal zone. All patients were diagnosed and treated at the Small animal clinic of the University of Veterinary Medicine and Pharmacy (UVMP) in Košice. A rigid type of endoscope (Karl Storz, Germany) 2 mm in diameter and 15 cm in length was used for the examination of the nasal cavity. The trachea and bronchi were examined with a lavage channel endoscope, 2.7 mm in diameter and 25 cm in length.

The group of patients with a disease localised in the nasal cavity included the following breeds: Maltese dogs (n = 4), Komondors (n = 2), Dachshunds (n = 2), mongrels (n = 2), Pitbull (n = 1), Hungarian Vizsla (n = 1), Chihuahua (n = 1), and German short-haired pointer (n = 1). The mean age of the patients was 6 years. After the targeted endoscopic visualisation of the pathological changes sampled from the nasal cavities of the dogs, they were obtained by swabbing of the nasal mucosa in 14 cases (74 %): Maltese dogs (n = 4), Dachshunds (n = 2), Komondors (n = 2), mongrels (n = 2), Yorkshire terriers (n = 2), Pitbull (n = 1), and Hungarian Vizsla (n = 1). Nasopharyngeal swabs were obtained from 4 dogs (21 %): Yorkshire terriers (n = 2), German short-haired pointer (n = 1), and Chihuahua (n = 1). The sample obtained from a 9 years old Yorkshire terrier male consisted of a swab and the lavage of sinuses with saline (in 1 case—5 %).

The group of patients with clinical signs involving the trachea consisted of: Yorkshire terriers (n = 4), Dachshunds (n = 2), Maltese dog (n = 1), and mongrel (n = 1). Their mean age was 7 years. Samples from these dogs were obtained in the same way, namely by swabbing the tracheal mucosa.

All patients were subjected to a basic clinical examination which included: sampling of venous blood; evaluation of a roentgenogram of the thoracic cavity in lateral projec-

tion; and auscultation. Before each targeted endoscopic examination, the animals were sedated utilizing a combination of three anaesthetics administered to the patients intravenously, i. e. (Cepetor 1 mg.ml^{-1} inj. solution, CP-Pharma, Germany at a dose of 0.015 mg.kg^{-1} body weight), diazepam (Apaurin 5 mg.ml^{-1} inj. solution, KRKA, Slovenia at a dose of 0.3 mg.kg^{-1} b. w.), and propofol (Propofol 10 mg.ml^{-1} inj. suspension, Fresenius Kabi, Germany at a dose of 3 mg.kg^{-1} b. w.).

For the primary bacterial culture of the samples and isolation of microorganisms we used a non-selective culture medium [Columbia agar (HiMedia, India) enhanced with 5 % sheep blood]. In parallel, as another non-selective medium suitable also for propagation of pigment forming bacteria (such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*), we used meat-peptone agar (Nutrient agar No. 2, HiMedia, India). On the basis of the character of growth and haemolytic activity of the isolated bacteria, the colonies were inoculated to the specific selective Endo agar (HiMedia, India) with the focus on enterobacteria, including *E. coli*, *Klebsiella* and *Proteus*, and on to a diagnostic medium Baird-Parker agar (HiMedia, India) for identification of *S. aureus*. The basic diagnosis included microscopic examination of the native preparations and Gram-stained preparations.

Commercial biochemical tests were used for additional, more accurate identifications. The basic tests used in our study included catalase (3 % hydrogen peroxide) and oxidase test (OxiTest, Erba Lachema, Czech Republic). For the species specification we used EnteroTest 24, NefermTest 24, StaphyTest 24, and Streptotest 24 (Erba Lachema, Czech Republic). The results of the tests were evaluated by means of software TNW ProAuto 7.0.

The identified bacterial strains were subsequently subjected to tests of susceptibility/resistance to antibiotics by means of a modern automatized instrument VITEK® 2 intended for the determination of the minimum inhibition concentrations of antibiotics. The results of the tests were interpreted in agreement with the criteria determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The complex microbiological diagnosis was carried out in specialised clinical microbiology laboratories.

RESULTS

The importance of endoscopic visualisation of the respiratory tract for the diagnosis of acute and chronic diseases was confirmed. This examination revealed a broad range of pathological changes in the majority of the patients. These pathologic manifestations ranged from mucous clumps adhered to the mucous membrane of the respiratory airways, through typical macroscopic hyperaemic manifestations of inflammation, up to anatomical changes observed most frequently in the conchae, nasopharynx and trachea.

The results of the microbiological examinations of the samples collected in this study revealed the prevalence of bacterial strains involved in diseases of the upper respiratory tract. The pathogens found in the highest abundance in the nasal cavity and nasopharynx area were: *Enterococcus faecalis* (15.6 %), *Staphylococcus aureus* (12.5 %), *Staphylococcus pseudintermedius* (9.4 %), *Escherichia coli* (9.4 %), *Bacillus* spp. (6.3 %), *Pseudomonas aeruginosa* (6.3 %) and others (Table 1). In the samples from the trachea, *Escherichia coli* (20 %), *Enterobacter ludwigii* (13 %), haemolytic-*Escherichia coli* (13 %) and others (Table 2) were found in the highest abundance.

In addition to the microbiological cultivation of the samples, antibiograms were prepared illustrating the effectiveness of various groups of antibiotics used in veterinary practice. The determination of antibiotic resistance of individual pathogens provided the necessary information for the initiation of targeted individual antibiotic therapy. The results of the antibiotic resistance of contaminants of the nasal cavity and trachea isolated in our study may be seen in Figures 3 and 4. The effectiveness of individual groups of antibiotics used in the treatment of common infections of the upper respiratory tract in descending sequence were as follows: fluoroquinolones (90.0 %), aminoglycosides (89.5 %), tetracyclines (83.0 %), trimetoprim-sulphate (81.5 %), phenicols (77.0 %), cephalosporins (72.5 %), polymyxin B (63.0 %), penicillins (53.0 %), macrolides (36.0 %), fusidic acid (20.0 %), and lincosamides (11.8 %), see Figure 5.

Table 1. Bacterial species found in the nasal cavity

Bacterial species	Number of findings	%
Enterococcus faecalis	5	15.6
Staphylococcus aureus	4	12.5
Escherichia coli	3	9.4
Staphylococcus pseudintermedius	3	9.4
Bacillus spp.	2	6.3
Pseudomonas aeruginosa	2	6.3
Acinetobacter baumanii	1	3.1
Acinetobacter pittii	1	3.1
Acinobacter spp.	1	3.1
Aeromonas caviae	1	3.1
Klebsiella pneumoniae	1	3.1
Neisseria zoodegmatis	1	3.1
Pantoea agglomerans	1	3.1
Pasteurella canis	1	3.1
Pasteurella dagmatis	1	3.1
Pasteurella stomatis	1	3.1
Staphylococcus epidermidis	1	3.1
Staphylococcus intermedius	1	3.1
Staphylococcus epidermidis	1	3.1

Table 2. Bacterial species found in the trachea

Bacterial species	Number of findings	%
Escherichia coli	3	20.0
Enterobacter ludwigii	2	13.3
Haemolytical E. coli	2	13.3
Enterobacter cloacae	1	6.7
Enterobacter spp.	1	6.7
Beta haemolytic streptococci	1	6.7
Klebsiella pneumoniae	1	6.7
Pasteurella multocida	1	6.7
Pasteurella stomatis	1	6.7
Proteus mirabilis	1	6.7
Streptococcus viridans	1	6.7

Table 3. The effect of antimicrobial drugs used in the UVMP hospital on bacteria isolated in the study

Active ingredient	***Acinetobacter* spp.** (n = 3)	***Pasteurella* spp.** (n=5)	***Staphylococci* spp.** (n = 9)	***Streptococci* spp.** (n = 2)	***Bacillus* spp.** (n = 2)	**Enterobacteriacae** (n = 4)	***E. coli*** (n = 8)
Benzyl penicillin	–	0 %	28 %	0 %	–	100 %	100 %
Cephalexin	100 %	0 %	14 %	0 %	–	100 %	29 %
Enrofloxacin	0 %	0 %	13 %	0 %	–	0 %	25 %
Amoxicillin clavulanic acid	100 %	0 %	25 %	0 %	–	100 %	25 %
Amoxicillin clavulanic acid	100 %	0 %	25 %	0 %	–	100 %	25 %
Gentamycin	0 %	0 %	0 %	100 %	–	0 %	13 %

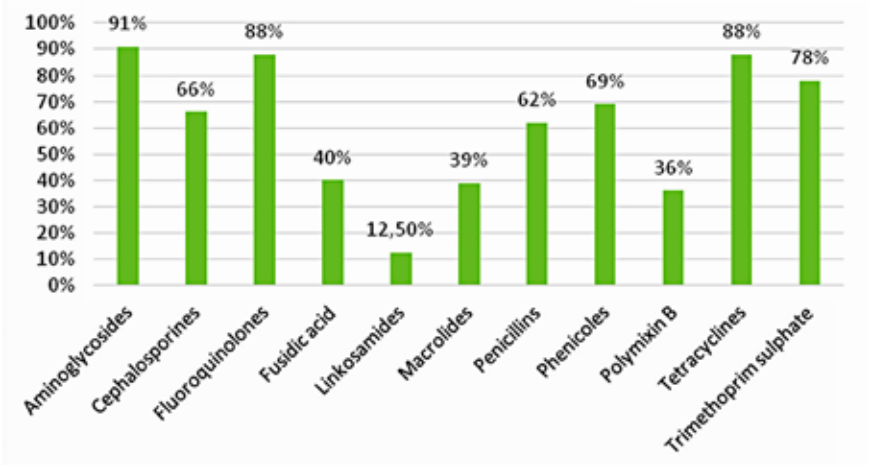

Fig. 3. The effect of antibiotics against bacterial species found in the nasal cavity

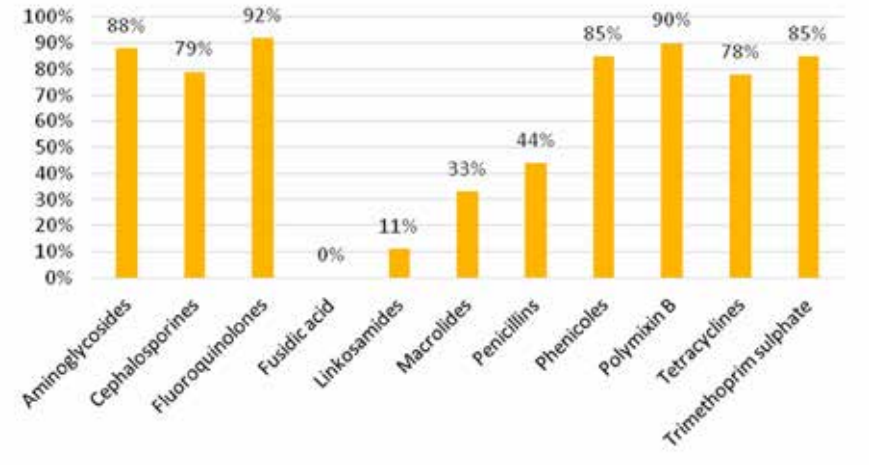

Fig. 4. The effect of antibiotics against bacterial species found in the trachea

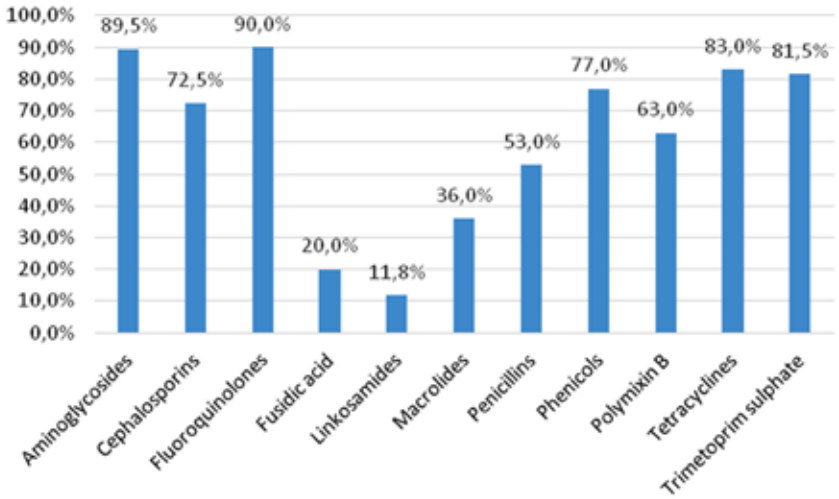

Fig. 5. The effectiveness of antibiotics used in the treatment of common infections of the upper respiratory tract

DISCUSSION

Numerous infectious agents are responsible for causing primary or secondary respiratory diseases in dogs. Clinical signs may vary from mild dyspnoea, sneezing, and coughing to severe pneumonia with systemic manifestations. Depending on the etiological agent, the gross and microscopic changes observed during these infections can be rather unspecific or have highly characteristic patterns [15].

There are many diagnostic tools available on the market that can be used for the identification of respiratory agents. The development of molecular diagnostic tools allows the rapid identification of a wide variety of pathogens and the establishment of more accurate treatments [16]. Most common bacterial cultures from the nasal cavity can be performed from specimens obtained by nasal swabbing. However nasal lavage specimens gave a higher sensitivity for bacterial growth than tissue biopsy specimens and swabs [8]. In comparison with dogs, bacterial culture results can be difficult or impossible to interpret as bacteria can also be cultured from the nasal cavity of healthy cats. However multidrug-resistant bacteria can colonize and be grown from the nasal passages in the absence of infection [10]. Bacterial cultures from the respiratory tract can be performed from specimens obtained from oropharyngeal swabs, tracheal wash, or bronchoalveolar lavage (BAL). Collection of a tracheal wash or bronchoalveolar lavage specimen is indicated in dogs with more severe clinical signs or evidence of pneumonia [13]. Some clinicians recommend quantitative or semiquantitative bacterial cultures of BAL fluid, and it has been reported that counts greater than 10^4 colony forming units ($CFU.ml^{-1}$) (or grown from primary culture) represent true infection whereas less than 103 $CFU.ml^{-1}$ (or grown from subculture) represent contamination [12].

The treatment of upper respiratory diseases is symptomatic; however, due to the common occurrence of secondary infections with a broad spectrum of bacteria, antibiotic treatment is the first therapeutic approach. Antimicrobial agents should be selected based on culture and sensitivity tests of airway specimens collected by bronchoscopy [14]. Endoscopy is a valuable diagnostic approach to the upper and lower respiratory tract, because it allows direct visualisation and sample collection. However, each anatomical region may require a range of specialized technical equipment and varying levels of experience to access and evaluate specific sites. Each procedure and normal appearance like cytology and culture results from each region will enhance the diagnostic success [5].

By means of endoscopic diagnosis and direct collection of samples from the nasal cavity (n = 18), nasopharynx (n = 4) and trachea (n = 8) of 26 animal patients, microbial cultures allowed us to identify 27 bacterial species in a total number of 47 bacterial findings. The most frequently isolated Gram-negative bacteria were: *Escherichia coli* (17.0 %), *Pasteurella* spp. (10.6 %), members of the family—*Enterobacteriacae* (10.6 %), *Acinetobacter* spp. (6.4 %), *Klebsiella pneumoniae* (4.2 %), *Pseudomonas aeruginosa* (4.2 %), *Proteus mirabilis* (2.1 %), *Aeromonas caviae* (2.1 %), and *Neisseria zoodegmatis* (2.1 %). Of the Gram-positive bacteria, we detected most frequently: *Staphylococcus aureus* (8.5 %), *Staphylococcus pseudintermedius* (6.4 %), *Streptococcus* spp. (6.4 %), *Staphylococcus* spp. (4.2 %) and *Bacillus* spp. (4.2 %). Our results resemble those published by A d a s z e k et al. [1], showing relatively frequent involvement of the pathogenic bacteria *E. coli, Klebsiella* spp. and *Staphylococcus* spp. in the respiratory tract infections in the dogs.

In the relevant study conducted by D a o d u et al. [6], the authors isolated 222 bacterial species. The most frequently identified species were: *Staphylococcus* spp. (21.7 %), *E. coli* (18.5 %), *Proteus* spp. (17.1 %), *Acinetobacter* spp. (9.0 %), *Pseudomonas* spp. (6.8 %) and *Streptococcus* spp. (5.4 %). The results of culture studies of samples from 90 nasal swabs published by A y o d h y a et al. [2] revealed various types of bacteria in mild cases: *E. coli* (63.33 %), *Klebsiella* spp. (30 %) and mixed infections (*E. coli* and *Klebsiella* spp.) (6.67 %), in 19, 9 and 2 dogs (out of 30 samples), respectively. Similarly, in the case of moderate respiratory diseases, samples revealed the presence of various bacteria: *E. coli* (30 %), *Klebsiella* spp. (33.33 %), *Streptococcus* spp. (30 %) and mixed infections (*Klebsiella* spp. and *Streptococcus* spp.) (6.67 %), in 9, 10, 9 and 2 dogs (out of 30 samples), respectively. In the case of severe respiratory diseases, various bacteria were identified: *E. coli* (6.67 %), *Klebsiella* spp. (3.33 %), *Streptococcus* spp. (46.67 %), *Staphylococci* spp. (40 %) and mixed infection (*E. coli* and *Streptococcus* spp.) (3.33 %) in 2, 1, 14, 12 dogs and 1 dog (out of 30 samples), respectively. The study conducted by C h a r k r a b a r t h i [4] showed that *Klebsiella pneumoniae* and *E. coli* spp. were the common bacteria involved in respiratory infectious diseases.

The information related to microbial contamination of the upper respiratory tract obtained in our study were subsequently used for the determination of antibiotic resistance of the isolates against 11 groups of antibiotics. The effectiveness in descending sequence was as follows: aminoglycosides (89.8 %), fluoroquinolones (89.5 %), tetracyclines (84.2 %), trimetoprim-sulphate (80.7 %), phenicols (77,0 %), cephalosporins (71.2 %), polymyxin B (67,8 %), penicillins (55,0 %), macrolides (35.6 %), fusidic acid (16.6 %) and lincosamides (11.6 %). In the study published by Daodu et al. [6] the effectiveness of fluoroquinolones on Gram-positive and Gram-negative bacteria ranged from 78.4 % to 93.3 %. The effectiveness of cephalosporines against Gram-positive bacteria reached 31.7 % while the effect of penicillins (e.g. amoxicillin), as the first line antibiotics, was paradoxically rather low. It reached 15.0 % against Gram-positive and 37.7 % against Gram-negative strains.

CONCLUSIONS

Chronic and long-term diseases of the upper respiratory tract require correct and targeted diagnosis. Direct visualisation of the respiratory airways is capable of ensuring precise sampling, accurate diagnosis and the development of the subsequent rational therapeutic plan. Direct targeted collection of samples is advantageous with respect to the elimination of the complex multifactorial influences and contribution to the therapy effectiveness. Microbiological culture methods focused on pathogenic bacteria constitute an inseparable part of the diagnosis of respiratory diseases especially with respect to prevention of increasing antibiotic resistance and incidence of chronic conditions. Although the financial demands of endoscopic diagnostic approach are moderately increased, it has been confirmed that the results obtained in this way are reliable. In practice it may result in reduced administration of therapeutically effective medications, more effective treatment and prevention of an increase in antibiotic resistance and thus, also reduced load on the patient.

REFERENCES

1. **Adaszek, L., Gorna, M., Zietek, J., Kutrzuba, J., Winiarczyk, S., 2009:** Bacterial nosocomial infections in dogs and cats. *Zycie Weterynaryjne*, 84, 805—808.
2. **Ayodhya, S., Rao, D. T., Reddy, Y. N., Sundar, N. S., Kumar, V. G., 2013:** Isolation and characterization of bacteria from canine respiratory diseases in and around Hyderabad city, Andhra Pradesh, India. *Vet. World*, 6, 9, 601-604. DOI: 10.5455/vetworld.2013.601-604.
3. **Carey, S. A., 2019:** *Canine Infectious Respiratory Disease Complex: Host, Pathogen, and Environmental Interactions.* Retrieved from https://www.michvma.org/resources/Documents/MVC/2018%20Proceedings/carey_05.pdf.
4. **Charkrabarthi, A., 2009:** *Textbook of Clinical Veterinary Medicine*. 3rd edn., Ludhiana, Kalayani Publishers, Punjub, India, 327—372.
5. **Creevy, K. E., 2009:** Airway evaluation and flexible endoscopic procedures in dogs and cats: laryngoscopy, transtracheal wash, tracheobronchoscopy, and bronchoalveolar lavage. *Vet. Clin. North Am. Small Anim. Pract.*, 39, 5, 869—880. DOI: 10.1016/j.cvsm.2009.05.001.
6. **Daodu, O. B., Amosun, E. A., Oluwayelu, D. O., 2017:** Antibiotic resistance profiling and microbiota of the upper respiratory tract of apparently healthy dogs in Ibadan, South west Nigeria. *Afr. J. Infect. Dis.*, 11, 1, 1—11. DOI: 10.4314/ajid.v11i1.4533.
7. **Elie, M., Sabo, M., 2006:** Basics in canine and feline rhinoscopy. *Clin. Tech. Small Anim. Pract.*, 21, 2, 73—77. DOI: 10.1053/j.ctsap.2005.12.011.
8. **Johnson, L. R., Kass, P. H., 2009:** Effect of sample collection methodology on nasal culture results in cats. *J. Feline Med. Surg.*, 11, 645—649. DOI: 10.1016/j.jfms.2008.12.004.
9. **Kuehn, N. F., 2013:** *Tracheobronchitis in Small Animals*. Retrieved in Sept. 2020, from https://www.msdvetmanual.com/respiratory-system/respiratory-diseases-of-small-animals/tracheobronchitis -in-small-animals.
10. **Lappin, M. R., Blondeau, J., Boothe, D., Breitschwerdt, E. B., Guardabassi, L., Lloyd, D. H., et al., 2017:** Antimicrobial use guidelines for treatment of respiratory tract disease in dogs and cats: Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases. *J. Vet. Intern. Med.*, 31, 2, 279—294. DOI: 10.1111/jvim.14627.

11. **Levkut, M., Švický, E., Lenhardt, Ľ., Ševčíková, Z., Revajová, V., Herich, R., 2007:** *Special Veterinary Pathology* (In Slovak). Vol. I. University of Veterinary Medicine and Pharmacy in Košice, Slovakia, 239 pp.
12. **Padrid P., 2000:** Pulmonary diagnostics. *Vet. Clin. North Am. Small Anim. Pract.*, 30, 6, 1187—1206. DOI: 10.1016/s0195-5616(00)06008-3.
13. **Reagan, K. L., Sykes, J. E., 2020:** Canine infectious respiratory disease. *Vet. Clin. North Am. Small Anim. Pract.*, 50, 2, 405—418. DOI: 10.1016/j.cvsm.2019.10.009.
14. **Sumner, C. M., Rozanski, E. A., Sharp, C. R., Shaw, S. P., 2011:** The use of deep oral swabs as a surrogate for transoral tracheal wash to obtain bacterial cultures in dogs with pneumonia. *J. Vet. Emerg. Crit. Care*, 21, 5, 515—520. DOI: 10. 1111/j.1476-4431.2011. 00670.x.
15. **Vieson, M. D., Pineyro, P., LeRoith, T., 2012:** A review of the pathology and treatment of canine respiratory infections. *Vet. Med.*, 3, 25—39. DOI: 10.2147/VMRR.S25021.
16. **Windsor, R. C., Johnson, L. R., Sykes, J. E., Drazenovich, T. L., Leutenegger, C. M., De Cock, H. E. V., 2006:** Molecular detection of microbes in nasal tissue of dogs with idiopathic lymphoplasmacytic rhinitis. *J. Vet. Intern. Med.*, 20, 2, 250—256. DOI: 10.1892/0891-6640(2006)20[250:MDOMIN]2.0.CO;2

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