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PREVALENCE AND PHYLODIVERSITY OF ESBL-PRODUCING COLIFORMS ISOLATED FROM RUMINANT MASTITIS IN NIGERIA

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ABSTRACT

The public health threat posed by Extended-spectrum beta-lactamase producing *E. coli* (ESBL-EC) in food animal production systems has attracted global attention. Data on the prevalence, diversity and genetic characteristics of ESBL-producing coliforms are key to advocacy on promoting responsible antimicrobial stewardship and proper planning of control strategies. The coliforms were isolated from 1052 milk samples of 160 cows, 103 ewes and 103 does with mastitis in Plateau State, Nigeria and analysed for ESBL production by phenotypic, biochemical, antimicrobial sensitivity and genetic characterization. The percentage of occurrence of clinical mastitis in cows, ewes, and does were 0.2 %, 0 %, 1.5 % respectively, while the percentage occurrence of subclinical mastitis in ruminants were 18.1 %, 28.2 % and 38.3 % respectively. From the 677 isolates, 31.3 % (n = 212) were ESBL producing coliforms, with a prevalence of 48.6 %, 18.4 %, 12.7 %, 8.9 %, 5.7 %, 3.8 % and 1.9 % for *E. coli*, *K. pneumoniae, C. freundii, K. aerogenes, S. marcescens, K. oxytoca* and *E. cloacae*, respectively. The genetic characterization revealed a higher prevalence of bla_{CTX-M} than bla_{TEM} in the samples analysed (24.39 % *vs.* 12.19 %). High pairwise identity was observed among the bla_{CTX-M} and bla_{TEM} gene sequences obtained in this study, but they displayed high phylodiversity with sequences from ruminants and humans from other climes. The bla_{SHV} gene was not detected. Multidrug resistances especially to the commonly used antimicrobials; ofloxacin, gentamycin and streptomycin in veterinary practice in Nigeria were observed. This has public health implications considering the fact that consumption of raw unpasteurized milk is a common practice in some cultures in Nigeria. Such practise will facilitate the transfer of multidrug resistant coliforms to humans resulting in the complications of treatment outcomes. To the best of our knowledge this is the first genetic characterization of ESBL-producing agents from ruminant mastitis in Nigeria.

Key words: antibiogram; β-lactamases; coliform; mastitis; phylogeny; ruminants

INTRODUCTION

Antibiotics have been used successfully over the years in the treatment and prevention of diseases, or as growth promoters in livestock, thereby, promoting animal production and safeguarding public health [1, 36]. However, the development of resistance poses a serious challenge to the continued use with desirable outcomes [28]. More so, the inordinate use of antimicrobials especially in veterinary practice in developing nations where the enforcement of regulations is weak or non-existent, which makes the situation worse [20, 37].

Most Gram-negative bacteria possess naturally occurring chromosomally mediated β-lactamases brought about by selective pressure exerted by β-lactam producing soil organisms found in the environment [18]. These β-lactamases provide an extended resistance to β-lactam antibiotics and are usually produced by a variety of coliforms such as *Escherichia coli*, *Klebsiella* spp. and *Citrobacter* spp., that are commonly called Extended-spectrum β-lactamase (ESBL) bacteria [15, 40]. ESBL-producing Enterobacteriaceae are now considered among the "priority pathogens" of emerging global health threat because of their increasing prevalence in livestock [6, 13, 34].

The first β-lactamase resistance gene in *E. coli* was isolated from a patience called Temoniera in Greece, hence was named TEM [10]. TEM is among the most frequently isolated ESBL genes usually in association with CTX-M genes [3]. *E. coli* and other Enterobacteriaceae species producing CTX-M-type extended-spectrum-lactamases have been more frequently isolated from humans, their environment and their companion animals [22], from food-producing animals [9], and from retail meats, including chicken, beef, and pork [32], globally.

Mastitis is a common disease of ruminants, especially cows [7]. Coliforms like *E. coli* and *K. pneumoniae* have been incriminated as life-threatening cause of clinical mastitis [9]. Other Gram-negative commonly isolated bacteria from mastitis in cows include *Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., *Serratia* spp., and *Proteus* spp. [29]. Therefore, food-animals have been incriminated as reservoirs of ESBL-producing *E. coli*, with zoonotic potential [12, 17, 27]. In Nigeria, several studies have reported the isolation of different bacteria from mastitis in ruminants with varying prevalence. However, most of these studies relied on the classical methods for the identification of the bacterial causes of mastitis [2].

To the best of our knowledge there is no reported study on the genetic characterization of coliforms associated with mastitis in ruminants in Nigeria. Therefore, the objective of this study was to determine the prevalence and characterize by amplification and sequence analyses the bla_{TEM} and $bla_{\text{CTX-M}}$ genes in ESBLs producing coliforms from milk of ruminants suffering from mastitis in Nigeria.

MATERIALS AND METHODS

Study location and demography

The study was conducted on milk samples obtained from ruminants in Plateau State, Nigeria from March 2018 to October 2019. Plateau State (9°00′—10°30′ N and Latitude 8°30—09°30′ E) occupies an area of approximately 26,899 km^2 in the central part of Nigeria. Located at an altitude of 1217 m above sea level, the state enjoys a more temperate climate than other parts of Nigeria. The temperature in most of the state ranges from 13 and 22 °C and the mean annual rainfall of 131.75 cm (52 in) in the southern part and 146 cm (57 in) on the Plateau. Plateau State has an estimated population of about 5.5 million inhabitants, most of them are engaged in agricultural activities. Milk from ruminants is consumed as raw or pasteurized or processed into milk products such as cheese, yoghurt or ice cream.

Ethical clearance

In line with the guidelines of the Animal Use and Care Committee (AUCC) of National Veterinary Research Institute (NVRI), Vom, Nigeria, the procedure involved in the milk sampling was non-invasive and does not inflict pain or discomfort to the animals, Therefore, no ethical approval was required.

Milk Sampling

This is a cross-sectional survey on lactating cows, ewes and does in Plateau State, Nigeria. By Snowball Random Sampling, herds who met the inclusion criteria were identified, and those identified recommended other farms within the LGA who meet the inclusion criteria. Due to different strata in each herd, such as meat ruminants, lactating but dry ruminants, and lactating ruminants, the Stratified

Random Sampling was used to select ruminants be included in the study. The lactating ruminants comprising of 160 cows, 103 ewes and 103 does were assessed both clinically and using the California Mastitis Test (CMT) for evidence of subclinical mastitis. About 10 ml of milk was aseptically collected from all the affected quarters and halves into labelled sterile universal bottles as described earlier [44]. The samples were kept on ice and transported to the Microbiology Laboratory of Federal College of Animal Health and Production Technology Vom for further processing.

Culture and biotyping

Bacteriological examination was done according to Geser et al. [17]. Briefly, one ml of each milk sample was inoculated into 9 ml of sterile Peptone Water for enrichment and incubated overnight at 37 °C. A loopful of broth culture was streaked on sterile MacConkey Agar (Oxoid, UK) and Eosin Methylene Blue (EMB) Agar (Oxoid, UK) plates using the quadrant streaking method and incubated at 37 °C under aerobic condition. The plates were checked for bacterial growth after 24, 48 and 72 hours to rule out slow growing bacteria. The colonies were examined for morphological features such as size, shape, and colour. Pink colonies on MacConkey Agar, and greenish metallic sheen, purple, pink, blue-black, and orange colonies on EMB were sub-cultured respectively on freshly prepared MacConkey Agar and EMB Agar plates and incubated at 37 °C for 24 hours to obtain pure culture of coliform isolates. Representative colonies were stored on slant of Nutrient Agar and kept 4 °C until required for further work [11].

Presumptive coliforms by Gram stain were subjected to conventional biochemical tests, namely gelatine liquefaction, nitrate reduction, urease production, oxidase, indole-methyl-red-Voges-Proskauer (IMVP), catalase, citrate agar, and sugar fermentation tests [31].

Identities of the presumptive coliforms were further confirmed using Oxoid™ Microbact™ GNB 24E according to the manufacturers' instructions. Briefly, 1—3 distinct colonies were picked from an 18—24 hours old culture and emulsified in 5.0 ml sterile saline and mixed thoroughly to obtain a homogeneous suspension. The plate containing the substrates was placed in a holding tray and using a sterile Pasteur pipette 4 drops (approximately 100 µl) of the bacterial suspension were added. The substrates underlined in the black wells were overlaid with sterile mineral oil except for well 20 which was used for the detection of oxidase-positive and miscellaneous Gram-negative bacteria. Incubation was done at 37 °C for 18—24 hours [5]. The results were read and interpreted according to the manufacturers' instructions.

Antimicrobial susceptibility test

An antibiotic sensitivity test was carried out on the coliforms isolated using the disc diffusion method. The antibiotics employed were ofloxacin (5 µg), pefloxacin (5 µg), ciprofloxacin (5 µg), amoxicillin/clavulanic acid (30 µg), gentamycin (10 µg), streptomycin (10 µg). Colonies of the isolated coliforms were suspended in bottles of 9ml sterile peptone water, standardized to 0.5 McFarland and incubated for 4 hours. A sterile swab stick was dipped into the standardized inoculum and excess fluid removed from the swab by pressing it on the side of the bottle. The swab was used to spread on the entire surface of dried Mueller Hinton agar plate and allowed to stand for 30 minutes. The antibiotic discs were placed on the surface of the seeded plates aseptically 15 mm apart. The plates were then incubated at 37 °C for 24 hours. The diameter of the zone of inhibition around each disc was measured in millimetres (mm) and compared against a reference standard [8].

Phenotypic assay for ESBL production

The phenotypic assay for ESBL production was assessed as earlier described [14]. Inocula of the isolated coliforms that have already been standardized to 0.5 McFarland standards were inoculated on Brilliance ESBL Chromogenic Culture Medium (Oxoid, UK). Inoculated plates were incubated at 37 °C under aerobic condition for 24 hours and 48 hours. Change in the colour of colonies was observed and interpreted according to the guidelines provided by Oxoid, UK. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as negative and positive controls, respectively.

PCR assays for TEM and CTX-M beta-lactamase genes

Conventional PCR was used for the amplification and detection of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} genes as described previously [24, 38, 42]. The primers (Table 1) and expected product sizes are listed in table S1. The reaction was performed on a GeneAmp PCR System 9700 thermocycler

Table 1. Primers used for amplification of ESBL genes

Target	Primer name	Sequence (5′—3′)	Product size	References
bla_{TEM}	TEM-F	TCCGCTCATGAGACAATAACC	931 bp	[22]
	TEM-R	TTGGTCTGACAGTTACCAATGC		
bla_{CTX-M}	CTX-F	CGC TTT GCG ATG TGC AG	550 bp	[34]
	CTX-R	ACC GCG ATA TCG TTG GT		
bla_{SHV}	SHV-F	TGGTTATGCGTTATATTCGCC	868 bp	[22]
	SHV-R	GGTTAGCGTTGCCAGTGCT		

(Applied Biosystems, USA) under the following conditions: Initial denaturation at 95 °C for 4 minutes followed by 35 cycles of denaturation were at 95 °C for 1 minute, annealing at 48 °C for bla_{TEM} and 60 °C for bla_{SHV} and bla_{CTX-M} for 1 minute. Primer extension was at 72 °C for 1 minute and the final extension step at 72 °C for 5 minutes for all the genes. Amplified genes were separated by gel electrophoresis in 1 % agarose gel in TBE 0.5X (Tris/borate/EDTA) buffer. *Klebsiella pneumoniae* ATCC 700603 containing bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes was used as positive control while *Escherichia coli* ATCC 25922 not containing the bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes was used as negative control. PCR products were detected with ethidium bromide fluorescence using the Bio-imaging system (VWR-Syngene, USA).

Sequencing and sequence analyses

Amplicons of the appropriate sizes were sequenced in both directions at a commercial sequencing company (Macrogen, Netherlands) using the PCR primers. The raw sequences were manually edited using the BioEdit v.7.2.3 program [21] and were compared and matched to annotated sequences in the GenBank™ database using the BLASTn search algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences generated in this study were submitted to GenBank under the accession numbers; bla_{TEM} (MZ310390—MZ310395); bla_{CTX-M} (MZ310396—MZ310406).

Pairwise identities and phylogeny

The bla_{TEM} and bla_{CTX-M} gene sequences obtained in this study and those within the database were aligned using Clustal W [41]. The analysis of pairwise similarities between the sequences from Nigeria was performed using SDT v.1.2 [30] with pairwise gap deletions. Phylogenetic relationships were inferred using the maximum likelihood method based on the Jukes-Cantor model implemented in MEGA6 with bootstrap replicate values set at 1,000 [39].

Analyses of population genetics

The genetic structure and diversity of the two genes within the populations were estimated via nucleotide and haplotype diversity, the number of polymorphic sites, number of mutations, population mutation estimates based on the number of segregating sites and mutations, and the number of nucleotide differences between sequences. These were estimated in DnaSP v5.10.01 [26]. The NAsp v5.10.01 was used to calculate the Tajima's D, Fu and Li's F* and D*, and Fu's Fs to determine the deviation of the two antimicrobial genes from neutrality assuming a constant population size, with zero recombination and migration [33].

Data analysis

The Statistical Package for Social Science (SPSS) version 23 software was used to analyse data collected. Chi-square test (χ^2), and P values < 0.05 were considered statistically significant.

RESULTS

Isolation and phenotypic identification of coliforms

The percentage occurrence of clinical mastitis in cows, ewes, and does were 0.2 %, 0 %, 1.5 % respectively, while the percentage occurrence of subclinical mastitis in ruminants were 18.1 %, 28.2 % and 38.3 % respectively. A total of 677 coliforms were isolated from 1052 milk samples

collected from ruminants in Plateau State of Nigeria. The high number of coliforms isolated may due to the type of housing system practiced by the pastoralists in this part of the world. Table 2 showed that *Escherichia coli* was the most commonly (44.0%) isolated coliform from the milk samples analysed. This was followed by *K. pneumoniae* (23.6%), *C. freundii* (11.2%), *K. aerogenes* (10.1%), *K. oxytoca* (7.1%), *E. cloacae* (2.2%) and *S. marcescens* (1.8%). Based on the study location, 183 (72.9%) of the positive samples were isolated from Plateau North, 160 (20.5%) from Plateau Central and 334 (82.3%) from Plateau South (Table 3).

Beta-lactamase production

From the total of 677 coliforms isolated, 212 (31.3%) were ESBL producers. *E. coli* had the highest prevalence of 48.58%, followed by *K. pneumoniae* (18.40%), *C. freundii* (12.74%), *K. aerogenes* (8.96%), *S. marcescens* (5.66%), *K. oxytoca* (3.77%) and *E. cloacae* (1.89%). The milk samples obtained from Plateau South had the highest number (n = 132; 62.3%) of ESBL-producing coliforms while Plateau Central had the least (n = 38; 17.9%) (Table 7).

Antibiotic susceptibility testing of ESBL producing coliforms

Four out of the seven coliforms, *E. coli*, *C. freundii, K. aerogenes* and *S. marcescens* isolated from ruminant mastitis in this study showed the highest resistance to gentamycin (Table 4). *K. pneumonia* showed the highest resistance equally to amoxicillin-clavulanic acid and streptomycin. These are antibiotics commonly used in veterinary practice in Nigeria.

Occurrence of ESBL variant genes in ruminant mastitis

ESBL producing genes were identified in 30 coliforms isolated from milk samples obtained from ruminants with mastitis, by conventional PCR. The $bla_{\text{CTX-M}}$ gene had a higher prevalence of 24.39% than bla_{TEM} with 12.19% in the samples screened in this study. However, the bla_{SHV} gene was not detected (Table 8).

Clonal diversity of CTX-M and TEM genes of isolated coliforms

The pairwise identities within the bla_{TEM} gene sequences in this study ranged from 93.3% to 97.6% (Fig. 1). However, the $bla_{\text{CTX-M}}$ gene sequence similarities ranged from 86.0% between to 99.5% (Fig. 2). Phylogenetic analyses showed that the bla_{TEM} and $bla_{\text{CTX-M}}$ gene sequences in this study formed four clusters along with sequences in GenBank indicating putative taxonomic and evolutionary inferences within the populations (Figs. 3 and 4).

Further evaluation of the genetic diversity within the bla_{TEM} and $bla_{\text{CTX-M}}$ genes form ruminants in Plateau State show that 6 haplotypes were identified within the bla_{TEM} isolates while 11 were present within the $bla_{\text{CTX-M}}$ sequences. This shows that each isolate represented individual haplotypes. The numbers of segregating sites within the bla_{TEM} isolates (86) and $bla_{\text{CTX-M}}$ (88) were almost similar while analogous trends were also observed for the total number of mutations and nucleotide diversity (Table 5). Both sets of genes had haplotype diversity at exactly 1.000.

Table 2. Frequency of coliforms from milk samples of ruminants

Bacteria	n = 677	
	Number	[%]
Escherichia coli	298	44.0
Klebsiella pneumoniae	160	23.6
Klebsiella oxytoca	48	7.1
Citrobacter freundii	76	11.2
Klebsiella aerogenes	68	10.1
Enterobacter cloacae	15	2.2
Serratia marcescens	12	1.8

$\chi^2 = 747.925$; $P < 0.000$—statistically significant

Table 3. Distribution of coliform isolates from different locations in the study area

Zone	Number samples	Number of coliforms	[%]
Plateau North	251	183	72.9
Plateau Central	395	160	20.5
Plateau South	406	334	82.3
Total	**1,052**	**677**	**64.4**

$\chi^2 = 747.925$; $P < 0.000$—statistically significant

Table 4. Percentage of resistant ESBL coliforms isolated from ruminant mastitis to some antibiotics

Isolate	No. of isolate	OFX [%]	PEF [%]	CPX [%]	AMC [%]	CN [%]	S [%]
E. coli	124	69 (55.64)	53 (42.74)	46 (37.09)	59 (47.58)	89 (71.77)	80 (64.51)
K. pneumoniae	66	36 (54.54)	31 (46.97)	33 (50.00)	44 (66.67)	34 (51.51)	44 (66.67)
K. oxytoca	8	5 (62.50)	3 (37.50)	4 (50.00)	2 (25.00)	5 (62.50)	6 (75.00)
C. freundii	45	28 (62.22)	29 (64.44)	22 (48.89)	27 (60.00)	33 (73.33)	29 (64.44)
K. aerogenes	14	11 (78.57)	7 (50.00)	7 (50.00)	5 (35.71)	12 (85.71)	11 (78.57)
E. cloacae	4	3 (75.00)	1 (25.00)	2 (50.00)	1 (25.00)	2 (50.00)	3 (75.00)
S. marcescens	11	3 (27.27)	1 (9.09)	3 (27.27)	3 (27.27)	9 (81.82)	7 (63.63)

OFX—Ofloxacin; PEF—Pefloxacine; CPX—Ciprofloxacin; AMC—Amoxicillin-Clavulanic acid; CN—Gentamycin; S—Streptomycin

Table 5. Genetic variability determinants of two antimicrobial resistance genes amplified from bacteria isolates obtained from ruminant animals in Plateau State, Nigeria

Population	N	H	S	Hd	Eta	Π	K	θ-W	θ-Eta
***bla*$_{TEM}$ gene** (n = 6)	935	6	86	1.000	90	0.04501	37.0000	0.04795	39.4161
***bla*$_{CTX-M}$ gene** (n = 11)	551	11	88	1.000	98	0.05131	28.2182	0.06083	33.4589

N—Number of nucleotide sites, h—Number of haplotypes, S—Number of variable sites, Hd—Haplotype diversity, Eta—Total number of mutations, π—Nucleotide diversity, k—Average number of nucleotide differences between sequences, θ-W—Waterson's estimate of population mutation rate based on the total number of segregating sites, θ-Eta—Waterson's estimate of population mutation rate based on the total number of mutations

Table 6. Neutrality tests on the two antimicrobial resistance genes amplified from bacteria isolates obtained from ruminant animals on Plateau State, Nigeria

Population	Tajima's D	Fu and Li's D	Fu and Li's F
***bla*$_{TEM}$ gene** [n = 6]	–0.3958	–0.2485	–0.3076
***bla*$_{CTX-M}$ gene** [n = 11]	–0.7504	0.2167	–0.0365

Table 8. Distribution of PCR-identified ESBL genes from ruminants with mastitis in Nigeria

Coliform	No. examined	No. of ESBL genes [%]	
		***bla*$_{TEM}$**	***bla*$_{CTX-M}$**
E. coli	30	4 (13.33)	4 (13.33)
K. pneumoniae	22	1 (4.55)	9 (40.90)
K. oxytoca	4	1 (25.00)	2 (50.00)
K. aerogenes	14	2 (14.28)	1 (7.14)
C. freundii	8	1 (12.50)	2 (25.00)
S. marcescens	4	1 (25.00)	2 (50.00)
Total	**82**	**10 (12.19)**	**20 (24.39)**

Table 7. Occurrence of ESBL-producing coliforms isolated from ruminant mastitis in Plateau State, Nigeria

Sampling location	No. of positive samples [%]	Isolate [%]						
		E. coli	*K. pneumoniae*	*K. oxytoca*	*C. freundii*	*K. aerogenes*	*E. cloacae*	*S. marcescens*
P. North	42 (19.8)	21 (50)	9 (21.43)	1 (2.38)	4 (9.52)	6 (14.28)	0 (0.0)	1 (2.38)
P. Central	38 (17.9)	28 (73.68)	6 (15.79)	0 (0.0)	2 (5.26)	2 (5.26)	0 (0.0)	0 (0.0)
P. South	132 (62.3)	54 (40.90)	24 (18.18)	7 (5.30)	21 (15.90)	11 (8.33)	4 (3.03)	11 (8.33)
Total	**212**	**103 (48.58)**	**39 (18.40)**	**8 (3.77)**	**27 (12.74)**	**19 (8.96)**	**4 (1.89)**	**12 (5.66)**

P. North—Plateau North; P. Central—Plateau Central; P. South —Plateau South

Table 9. Properties of *bla*$_{TEM}$ and *bla*$_{CTX-M}$ gene sequences obtained from bacterial species isolated from ruminants with mastitis in Plateau State, Nigeria

Gene	Organism	Study location	Sequence ID	Host	Accession Number	Number of nucleotides
***bla*$_{TEM}$**	*K. pneumoniae*	North	NG-JN-sh1	Sheep	MZ310390	870
***bla*$_{TEM}$**	*K. aerogenes*	North	NG-JS-cw1	Cow	MZ310391	870
***bla*$_{TEM}$**	*E. coli*	South.	NG-LS-cw	Cow	MZ310392	877
***bla*$_{TEM}$**	*C. freundii*	South	NG-QP-shp	Sheep	MZ310393	861
***bla*$_{TEM}$**	*K. aerogenes*	South	NG-QP-sh1	Sheep	MZ310394	837
***bla*$_{TEM}$**	*K. pneumoniae*	North	NG-JN-gt	Goat	MZ310395	935
***bla*$_{CTX-M}$**	*E. coli*	South	NG-LS-cwa	Cow	MZ310396	551
***bla*$_{CTX-M}$**	*K. pneumoniae*	North	NG-JS-cw	Cow	MZ310397	550
***bla*$_{CTX-M}$**	*E. coli*	South	NG-LS-cwb	Cow	MZ310398	551
***bla*$_{CTX-M}$**	*E. coli*	South	NG-QP-gt	Goat	MZ310399	551
***bla*$_{CTX-M}$**	*K. pneumoniae*	South	NG-QP-gt	Goat	MZ310400	550
***bla*$_{CTX-M}$**	*K. pneumoniae*	North	NG-JN-gt	Goat	MZ310401	550
***bla*$_{CTX-M}$**	*E. coli*	Central	NG-KN-cwa	Cow	MZ310402	551
***bla*$_{CTX-M}$**	*E. coli*	South	NG-LS-cw	Cow	MZ310403	551
***bla*$_{CTX-M}$**	*K. pneumoniae*	North	NG-JN-cw	Cow	MZ310404	551
***bla*$_{CTX-M}$**	*C. freundii*	South	NG-QP-cw	Cow	MZ310405	551
***bla*$_{CTX-M}$**	*E. coli*	Central	NG-KN-cw	Cow	MZ310406	551

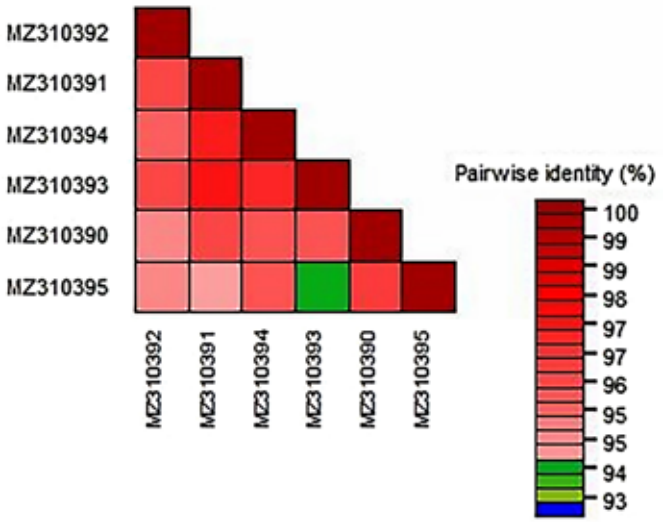

Fig. 1. Pairwise of all *bla*$_{TEM}$

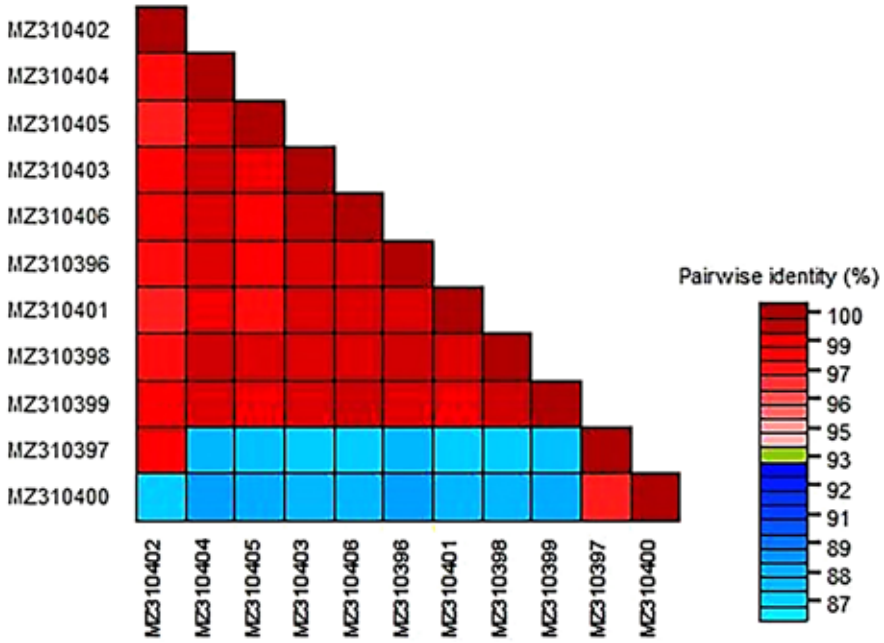

Fig. 2. Pairwise of all *bla*$_{CTX-M}$

Nucleotide polymorphisms within the two gene population, as showing via the test of neutrality, show negative Tajima's D (–0.3958), Fu and Li's D (–0.2485) and Fu and Li's F (–0. 3076) statistic values for the *bla*$_{TEM}$ isolates (Table 6). Similar observations were obtained from the *bla*$_{CTX-M}$ genes, except for Fu and Li's D with positive values of 0.2167. Neutrality tests revealed negative values for the worldwide isolates, which did not statistically deviate from zero ($P > 0.10$). Negative Tajima's D statistic indicates superfluous low-frequency polymorphism triggered by background selection, genetic hitchhiking, or population expansions.

DISCUSSION

In this study, coliforms of veterinary and public health importance were isolated from milk samples of ruminants

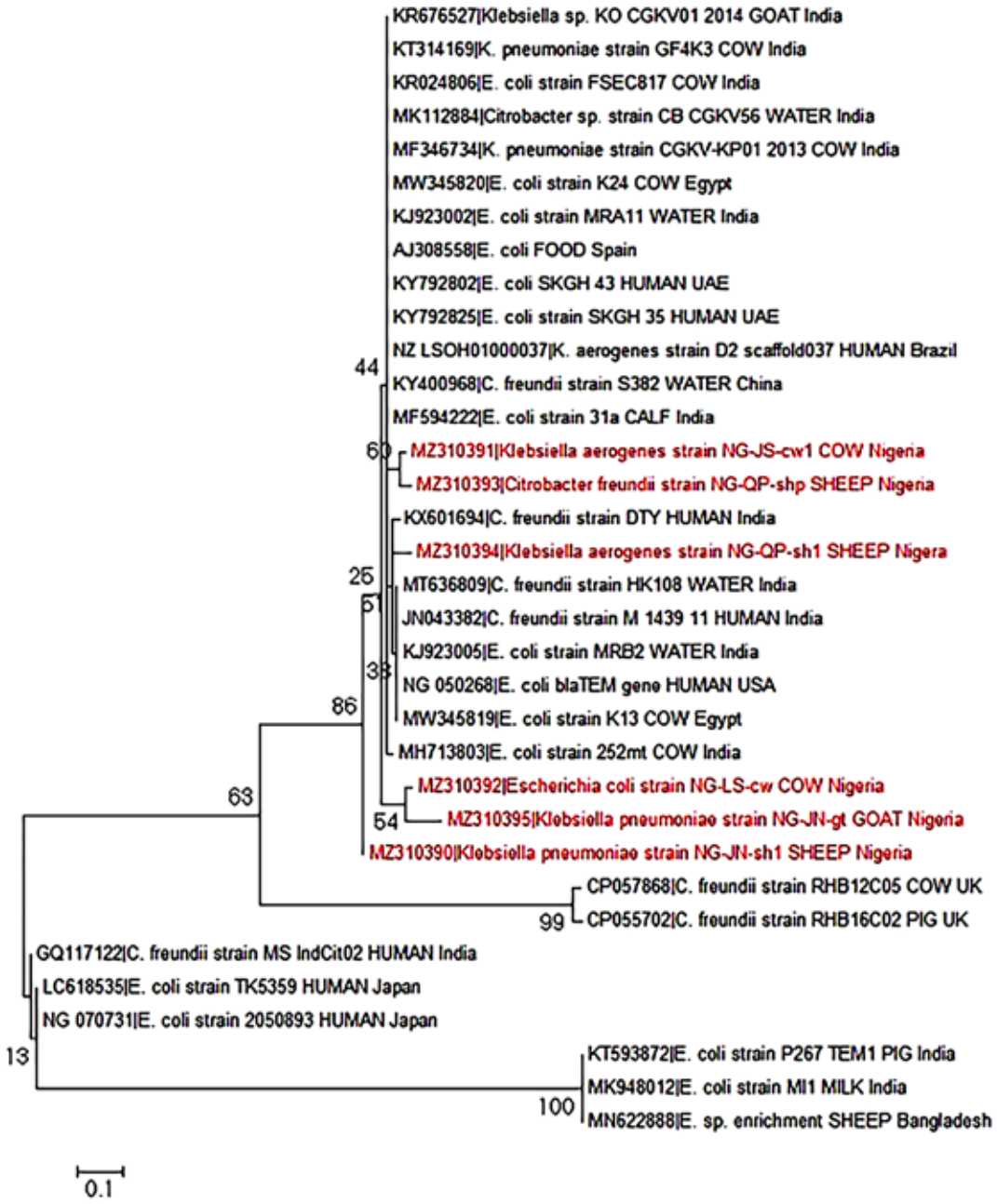

Fig. 3. Phylogenetic analysis of *bla*$_{TEM}$ genes of coliforms isolated from milk obtained from ruminants with mastitis in Nigeria

with mastitis. Among the ESLB- producing coliforms isolated, *E. coli* had the highest prevalence of 48.6% in all the milk samples from the various ruminants examined in this study. Similar findings have been reported for milk obtained from mastitic cows from different regions of the world [3, 17, 25, 27]. However, the prevalence of *E. coli* in this study is higher than the 32.2% for samples obtained from apparently healthy poultry workers, chickens and environment in Abuja, a neighbouring city to this study area [4]. But, the prevalence reported herein is within the range of 32.2 to 75.5% from previous studies involving different samples from various animal species and man, thus, underscoring the role of *E. coli* as the leading cause of antimicrobial resistance along the food value chain [4, 12, 20].

Additionally, there was an association between the prevalence of *E. coli* and the animal species and study location. The prevalence was 43.1%, 40% and 38% for does, cows and ewes, respectively. Similarly, the preva-

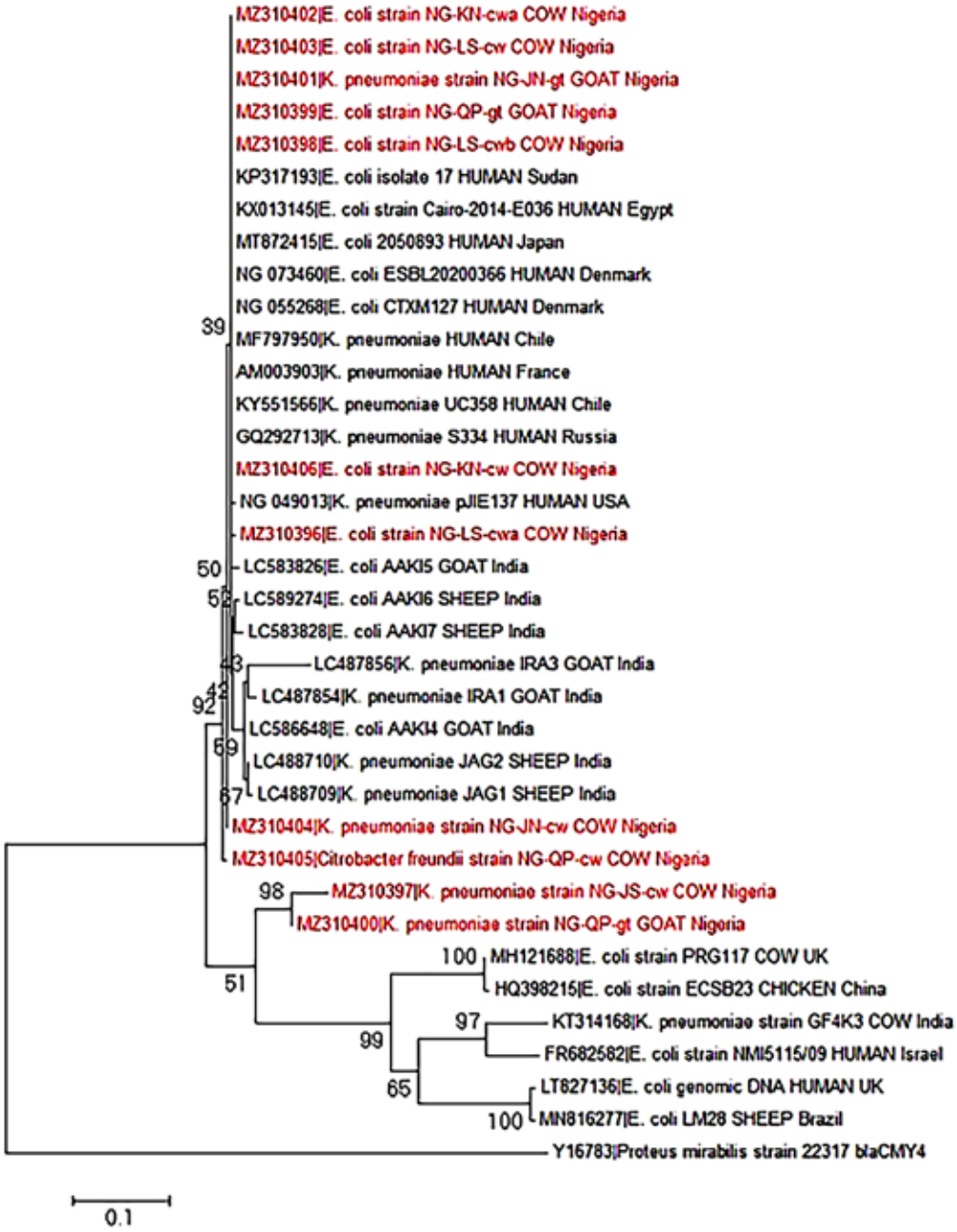

Fig. 4. Phylogenetic analysis of *bla*$_{\text{CTX-M}}$ genes of coliforms isolated from milk obtained from ruminants with mastitis in Nigeria

lence of *E. coli* was higher in the Southern (82.3%), than the Northern (72.9%) and the central (20.5%) regions of Plateau State, Nigeria. These variations can be attributed to environmental conditions, socio-cultural practices, species variation and differences in type and use of antimicrobials. Generally, the profile of coliforms recorded in this study is similar to earlier reports from various regions: Africa [16], Asia [38], and South America [35]. However, the prevalence and diversity of the coliforms differed according to the source of samples analysed and animal host.

Two resistant genes *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ were characterized from coliforms isolated from milk samples in this study. The overall prevalence of *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ in ruminant mastitis was 24.39% and 12.19% respectively. Higher prevalence of *bla*$_{\text{CTX-M}}$ reported herein is in agreement with the report of Ali et al. [3], but contrary to that of Yu et al. [43] where *bla*$_{\text{CTX-M}}$ was more prevalent in milk samples from bovine mastitis. On a general note, recent studies have reported the detection of CTX-M genes from different sources [4, 12, 20]. This could be attribut-

ed to the ability of this gene to undergo point mutation in the environment [12]. Phylogenetic analyses showed that some of the bla_{CTX-M} and bla_{TEM} gene sequences from the ruminants in this study have common ancestry with genes from human from different parts of the world. The high-level of phylodiversity observed among ruminants means that there is transfer of coliforms harbouring the CTX-M and TEM genes which might have been transmitted from clones of varying origins [23]. Surprisingly, the bla_{SHV} gene was not detected in any of the samples screened in this study. Similar to our results, a recent study that analysed samples from chickens, humans and the environment did not report the bla_{SHV} gene [4]. Furthermore, lower prevalence of bla_{SHV} (14.2%) than bla_{TEM} (26.1%) and bla_{CTX-M} (73.0%) was reported in isolates from animals in Pakistan [12], a similar trend, 27.54% (bla_{SHV}), 57.97% (bla_{TEM}) and 72.46% (bla_{CTX-M}) was reported for isolates from broiler in Philippines [20]. The reason for the non-detection of the bla_{SHV} gene in isolates in this study is not readily deducible and, is the subject of further studies in this country.

Multidrug resistance was observed in most of the coliforms tested in this study. High resistance by most of the coliforms was particularly recorded for ofloxacin, gentamycin and streptomycin. The majority of the isolates exhibited the highest prevalence (72—85%) of resistance to gentamycin. This is not surprising as gentamycin is one of the commonest antimicrobials used in veterinary and medical practice in Nigeria and may be subject to abuse. However, lower resistance was shown against the drugs that are rarely used in veterinary practice in Nigeria such as pefloxacine, ciprofloxacin and amoxicillin-clavulanic acid. Therefore, veterinarians should consider the use of these compounds whenever handling cases that are unresponsive to routinely used antimicrobials. Of concern is the public health implications of this finding, considering the fact that consumption of raw unpasteurized milk is a common practice in some cultures in Nigeria. Such practise will facilitate the transfer of multidrug resistant coliforms to humans resulting in complications of treatment outcomes.

CONCLUSIONS

A high prevalence of ESBL-producing coliforms was detected in milk samples from ruminants with mastitis in Nigeria. Two resistance genes; bla_{CTX-M} and bla_{TEM} associated ESBL producing agents were characterized revealing a high phylodiversity among the isolated from Nigeria in comparison to sequences from other parts of the world. Future studies should consider other resistance genes associated with agents of ruminant mastitis to provide a holistic picture for effective control strategies. A high resistance to commonly used antibiotics in veterinary practice is worrisome and calls for urgent steps to reverse this trend. Public enlightenment coupled with advocacy on responsible antimicrobial stewardship are needed to sensitize the general public on the dangers associated with the abuse of antimicrobials along the food value chain in Nigeria. Also some socio-cultural practices such as consumption of raw milk should be discouraged.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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DEWORMING SCHEMES' EFFICACY FOR ADULT DOGS WITH MIXED GASTRO-INTESTINAL HELMINTHOSES

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ABSTRACT

Mixed gastrointestinal helminthoses, which combines *Toxocara canis, Trichuris (T.) vulpis, Toxascaris leonina, Uncinaria stenocephala, Ancylostoma caninum*, and *Dipylidium caninum* in various combinations, are very common pets' problems worldwide. It is unlikely necessary to choose between 100 % efficiency and for the patient's body to heal the infected animals safely. The present work aims to develop an affordable scheme for adult dogs' deworming, which will create a minimum load on the body due to the low drugs' toxicity. Mixed breed dogs, 1—5 years old, representing both sexes, spontaneously infected with *T. vulpis* (100 % prevalence) in combination with other gastrointestinal helminths (from 12.7 to 45.1 %) were selected for study. Regimens combining Caniquantel® Plus (fenbendazole + praziquantel) and fenbendazole with a 24-hour interval were tested. After a single treatment of experimental animals with Caniquantel® Plus, no helminth eggs were detected in their faeces after three days, except for *T. vulpis*. Seven days after the start of the experiment, the intensity of infection of this nematode decreased by only 22.0 % ($P < 0.001$). Bodies and fragments of dead helminths were found in faeces 1—4 days after deworming, with *T. vulpis* isolated only in 2 days in small quantities (4.54 ± 0.21 specimens per 100 g of faeces). Two-stage deworming with Caniquantel® Plus and fenbendazole after 24 hours resulted in 100 % efficiency against eggs of all parasites after five days. Helminths' bodies stopped excreting after four days, and *T. vulpis* was detected within three days in substantive quantities (from 10.03 ± 0.45 to 36.8 ± 1.2 specimens per 100 g of faeces).

Key words: canine; co-infection; fenbendazole; parasites; praziquantel; prevalence; *Trichuris vulpis*; *Toxocara canis*

INTRODUCTION

Mixed gastrointestinal helminthoses are one of the most common problems in pet veterinary medicine world-

wide. However, such helminthoses are rare in the form of mono-infections. The main component of co-infections is usually *Toxocara (T.) canis* (Werner, 1782) in puppies or young dogs and *Trichuris (T.) vulpis* (Froelich, 1789) in adult dogs [10, 14, 31, 48]. Other members of helminthic parasitocenoses in dogs are usually *Toxascaris (T.) leonina* (Linstow, 1902), representatives of Ancylostomatidae family—*Uncinaria (U.) stenocephala* (Railliet, 1884) and *Ancylostoma (A.) caninum* (Ercolani, 1859), *Dipylidium (D.) caninum* (Linnaeus, 1758). According to the analysis, the worldwide prevalence of *Toxocara* infection in dogs is 11.1% (10.6—11.7%). The regional distribution of this nematode varies from 6.4% to 19.2%: Eastern Mediterranean (19.2%, 13.7—25.5%), Africa (18.5%, 13.7—23.9%), South-East Asia (11.9%, 6.8—18.2%), North America (11.1%, 10.6—11.7%), South America (10.9%, 7.6—14.6%), Europe (10.8%, 8.9—12.9%), and Western Pacific (6.4%, 3.3—10.2%).

Young animals under 12 months, stray, rural and male animals have a significantly ($P < 0.001$) higher prevalence of *T. canis* than older, pet, urban or female dogs [43]. According to a similar monitoring of *T. leonina* spread among dogs, the pooled prevalence is 7.2% (3.5—12.0%) in the Eastern Mediterranean region, 5.7% (1.4—12.2%) in South-East Asia, 3.6% (1.2—6.9%) in Africa, 2.6% (1.6—3.8%) in Europe, 2.0% (1.1—3.2%) in North America), 1.0% (0.1—3.4%) in the Western Pacific and 0.6% (0.1—2.1%) in South America [44].

Infestation of dogs with the nematode *T. vulpis*, according to various authors, is 15.1% in Bulgaria [19], 9.9% in Italy [49], 9.6% in Serbia [18], 3% in Palestine [34] from 2.43% [52] to 2.74% in the USA [33], 1.31% in Pakistan [22]. Thus, this indicator has the highest values in Europe.

Strongylatoses incidence in dogs is 31.6% in India [34], 31.4% in Australia [5], 30.23% in Kenya [32], 18.33% in Indonesia [39], 15—16.5% in Serbia [18], 8.3% in Brazil [2], 8% in Palestine [34], from 5.63% [52] to 8.23% in USA [33], and 3.94% in Pakistan [22]. Infestation with the tapeworm *D. caninum* is characteristic of 23% of dogs in Palestine [34] 21% in Ethiopia [15], 11.8% in Pakistan [22] 5.4% in Serbia [18], 1.67% in Indonesia [39].

The stability of geohelminths' eggs in the environment contributes to their spread among dogs. According to studies conducted in the Kharkiv region (Ukraine), the soil contamination level in urban areas of cities by exogenous stages of helminths is 5—55%, and in residential areas 20.0—23.3%. In general, in soil samples, the authors identified eggs of Strongylata, Ascaridata, *Trichocephalus*, and Cestoda. The researchers also point out that dogs and cats infected with helminths in the environment pollute from faeces with eggs of *Toxocara* spp. (75 ± 4 eggs.g^{-1}) and *D. caninum* (6 ± 1 eggs.g^{-1}) [36]. According to a survey in the Poltava region, in 31.18% of the dogs, fleas of the genus *Ctenocephalides* mostly parasitize in the form of associations with Nematoda (*T. canis, T. vulpis, U. stenocephala*), Cestoda (*D. caninum*), protozoa and other ectoparasites [56]. The prevalence of *T. vulpis* in the Kyiv region, according to previous studies, was high enough at 27.1% [47]. The trematode *Alaria (A.) alata* (Goeze, 1792) deserves special attention. Researchers say this helminth is widespread among domestic and wild carnivores: in Germany (25% of red foxes) [55], Serbia (28% of dogs) [29], Denmark (32.9% of raccoon dogs) [23] and many others in Europe. But, there is not any data about its prevalence in Ukraine.

Effective deworming is a necessary condition for maintaining the health of not only pets but also their owners [45]. Control of gastrointestinal tract helminths in dogs requires the use of anthelmintics containing various active substances. Thus, benzimidazole compounds [27, 52] or macrocyclic lactones [16, 20, 40] are usually used to release nematodes. Praziquantel is the most widely used substance to control trematodes and cestodes in dogs [24, 28]. Niclosamide is much less commonly used today [8]. Parasites' morphological and biological features require not only the correct choice of anthelmintics' active substance. The mode of its use has to be chosen correctly. Thus, *T. vulpis* feed periodically, and they are firmly fixed on the mucous membrane of the large intestine. Therefore, a single application of anthelmintics (benzimidazoles) does not give sufficient effectiveness [3, 41]. The use of long-acting drugs (macrocyclic lactones) allows holding a sufficient concentration in patients for the *T. vulpis* death. But, it's ineffective against trematodes and cestodes in mixed infections [1, 16, 25]. Excess doses or multiplicity of antiparasitic drugs can be justified, strictly dictated by the need [42]. Thus, it is grave to find a maximum effective treatment regimen that will not harm the patient [9, 51]. Fenbendazole is a low-toxic substance with a safety factor of 200 [7]. It is highly effective against nematodes. Although, it has low solubility and reaches low concentrations in the blood, which leads to low bioavailability [21].

The combination of fenbendazole and praziquantel is optimal for combating mixed-infection (trematodes, cestodes and most nematodes) in pets. It was weighty to research the fenbendazole + praziquantel (as Caniquantel® Plus) combination followed by fenbendazole (with 24-hour interval), taking into account the need to destroy *T. vulpis* in dogs.

Given the above, the present work aimed to develop an affordable deworming scheme for adult dogs. It was grave for us to create a minimum load on patients' bodies due to the low toxicity of the selected drugs. Such data will allow us to safely free adult dogs from all members of the combined helminth infection.

MATERIALS AND METHODS

Animal materials and study design

The research took place in 2020—2021 in private yards of villages of the Bila Tserkva district of the Kyiv region. Laboratory studies were conducted in the Laboratory of the Department of Parasitology and Pharmacology, Bila Tserkva National Agrarian University, Ukraine. To set up the experiment, two groups of adult dogs were formed (n = 35 and n = 36). Animals of both groups were 1—5 years old, 10—25 kg weight, mixed breeds and sexes. Animals of both groups were spontaneously infected with *T. vulpis* nematode or its combination with other helminths (*T. canis*, *T. leonina*, Ancylostomatidae family, *D. caninum*) in different variations (Fig. 1).

Parasitological study

The faeces examination method using the "Counting Chamber for Ovoscopic Researches" was used to make the initial diagnosis [48]. During the 7-day experiment, both ovoscopic (with a determination of the content of parasites' eggs or packets in 1 g of faeces) and helminthoscopic examinations (with a determination of the number of parasites' eggs or proglottids in 100 g of faeces) were performed daily. Faeces helminthoscopy was performed by sequential lavage [30, 37]. According to the results of helminthoscopy after deworming, infection of 9 from 71 experimental dogs with *A. alata* Trematoda was also established. A sorting of experimental animals between two experimental groups was carried out on the principle of analogues.

Caniquantel® Plus (Euracon Pharma GmbH, Germany) and Fenbendazole ultra 5 % (O. L. KAR Animal Health, Ukraine) were used in the deworming scheme (Fig. 2). According to results of further parasitological studies of faeces' samples, the efficiency of animal treatment was established. Results indicate the frequency, which assumes that one day is 24 hours after the start of the previous stage, not the current day.

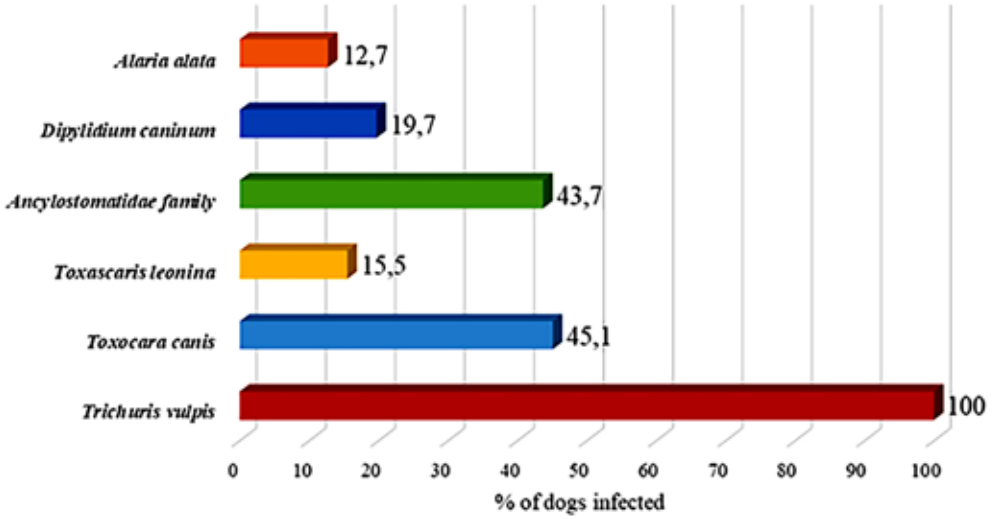

Fig. 1. Prevalence of helminths in dogs of experimental groups, n = 71

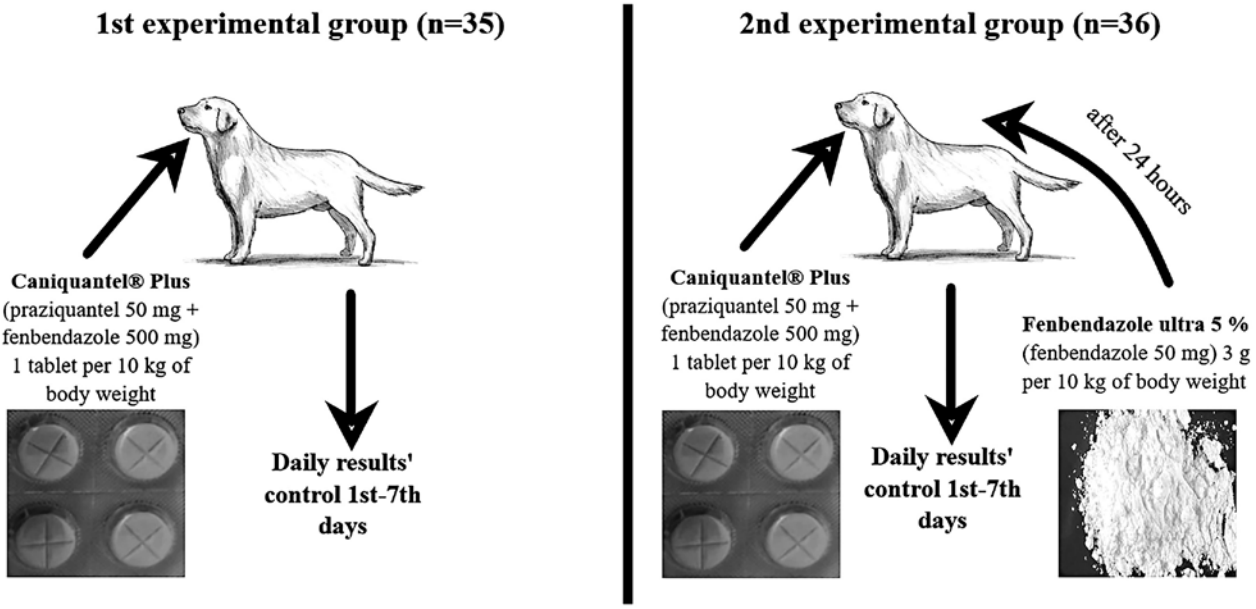

Fig. 2. Experimental deworming scheme

Statistical analysis

Datasets of helminths eggs' or packets' content were expressed as mean (x) ± standard error of the mean (SE). Mathematical analysis of study results was conducted by means of Statistica 13.3 IT Application (StatSoft Inc., USA). Differences between average values were considered statistically significant at $P < 0.05$ (ANOVA).

Ethical considerations

The research protocol of the present study was approved by the Ethic Committee of Bila Tserkva National Agrarian University in Ukraine (Approval №7, conclusion 3/1, 21. 05. 2020).

RESULTS

The dynamics of helminth eggs' content in faeces of the 1st experimental group's dogs (treated with Caniquantel® Plus once) shows an increase in this indicator (from 11.8 % for *T. vulpis* to 3.2 times for Ancylostomatidae family) one day after the study start (Fig. 3). After two days, last portions of *T. canis* eggs (73.7 ± 2.6 specimens.g^{-1} of faeces) and *D. caninum* packets (12.64 ± 0.51) were detected. Eggs of *T. leonina* (2.08 ± 0.06 specimens.g^{-1} of faeces) and Ancylostomatidae family (11.13 ± 0.58 specimens.g^{-1} of faeces) were last recorded three days after an anthelmintic administration. Regarding the excretion of *T. vulpis* eggs, after two days, their content in the faeces significantly decreased by 19.4 % compared to the previous indicator ($P < 0.01$), no longer having a significant difference from the data before the experiment. Subsequently, by the end of the study, a concentration of eggs of this pathogen fluctuates slightly, reaching after seven days a decrease relative to the initial value of 22.0 % ($P < 0.001$).

The use of a combination of drugs in dogs of the 2nd experimental group (Fig. 4) allowed to record the last presence *T. canis* eggs (91.1 ± 3.9 specimens.g^{-1} of faeces) and packets of *D. caninum* (7.26 ± 0.42 specimens.g^{-1} of faeces) 2 days after the start of deworming. Eggs of *T. leonina* (5.26 ± 0.22 specimens.g^{-1} of faeces) and Ancylostomatidae family (3.25 ± 0.17 specimens.g^{-1} of faeces) last appeared in faeces of dogs after three days. The concentration of *T. vulpis* eggs gradually increased, almost doubling after three days relative to baseline ($P < 0.001$). After four days, their content in faeces decreased significantly. The last *T. vulpis* eggs appeared five days after the start of the experiment (12.32 ± 0.45 specimens.g^{-1} of faeces).

Regarding the dynamics of helminth bodies' excretion with faeces of experimental dogs from the 1st group, *T. vulpis* appeared in insignificant quantity two days after the beginning of the experiment (4.54 ± 0.21 specimens

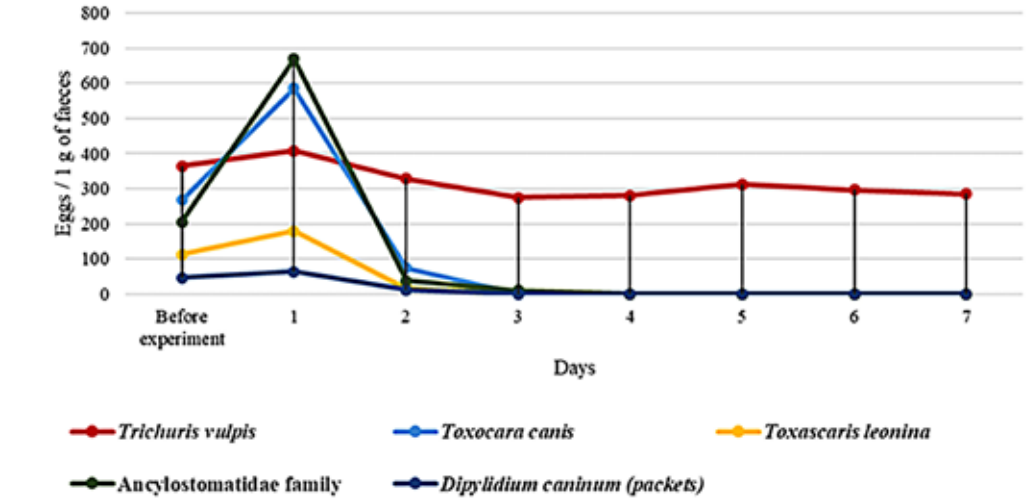

Fig. 3. Helminths eggs' excretion with canine faeces (n = 35) after deworming with Caniquantel® Plus

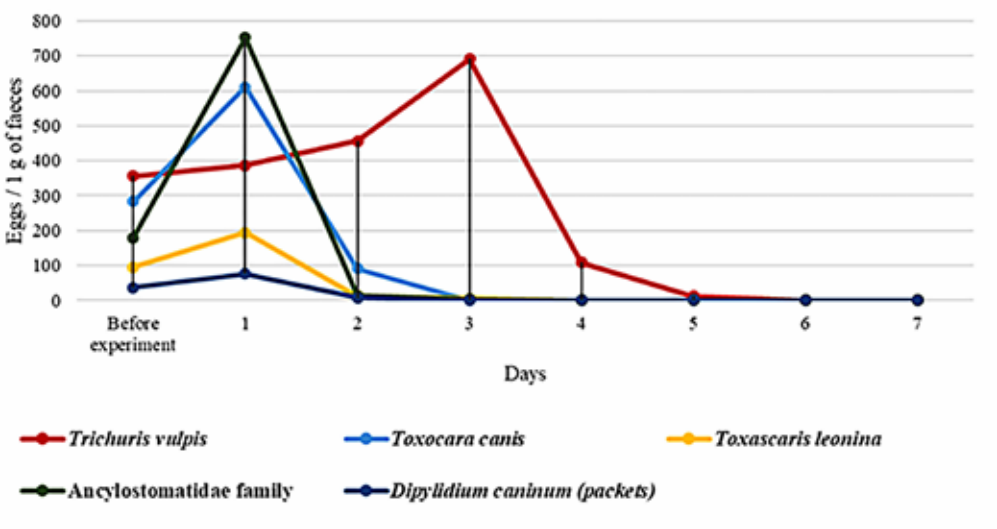

Fig. 4. Helminths eggs' excretion with canine faeces (n = 36) after deworming with Caniquantel® Plus and fenbendazole (24 h interval)

per 100 g of faeces), Fig. 5. Subsequently, parasites of this species were absent in faeces samples. Adult bodies of *T. canis* appeared in faeces samples for the first time after one day from the beginning of the experiment (19.50 ± 0.72 specimens per 100 g of faeces), and last—after two days (3.32 ± 0.14 specimens per 100 g of faeces). Excretion of *T. leonina* nematodes occurred only one day after deworming. Ancylostomatidae family helminth bodies appeared two days later (179.5 ± 9.1 specimens per 100 g of faeces) and last time after four days from the start of the experiment (5.39 ± 0.25 specimens per 100 g of faeces). A similar gradual decrease in faeces content was characteristic of *D. caninum* proglottids, but they were isolated in the period from 1 to 3 days. Adults of *A. alata* trematodes also appeared after one (18.76 ± 0.77 specimens per 100 g of faeces) and two days (28.4 ± 0.93 specimens per 100 g of faeces) from the study start.

A similar pattern to results of group 1 was observed for all helminths except *T. vulpis* (Fig. 6). Thus, *T. canis* bodies appeared in samples one (20.4 ± 0.85 specimens per 100 g

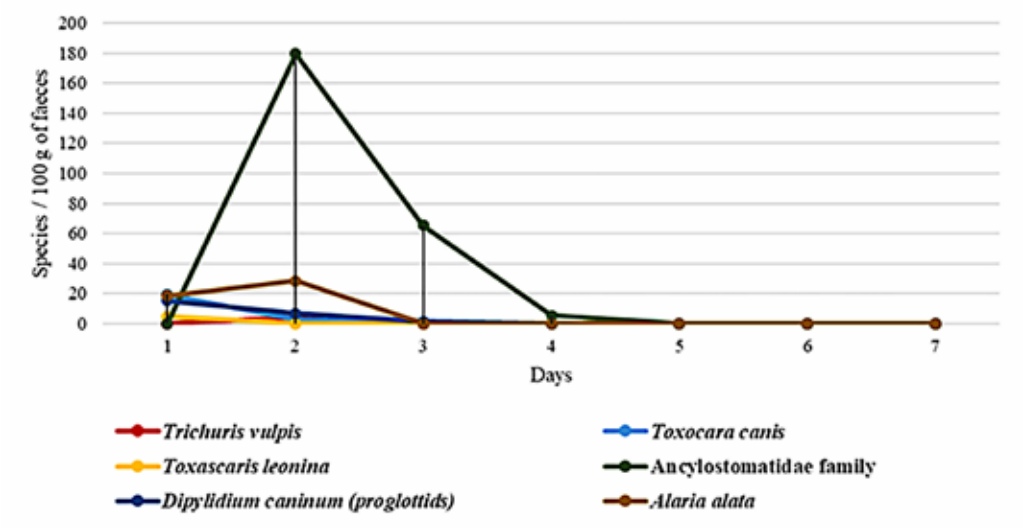

Fig. 5. Helminths' excretion with canine faeces (n = 35) after deworming with Caniquantel® Plus

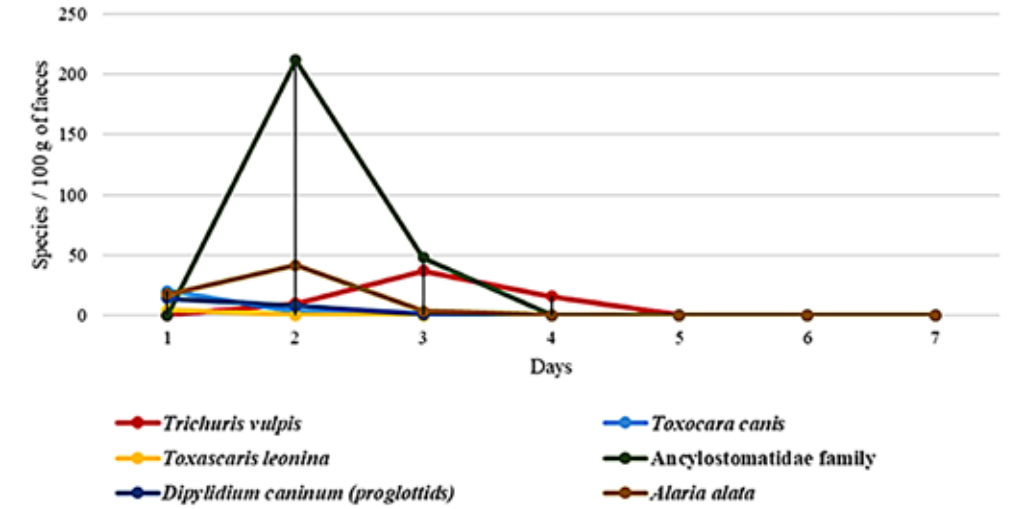

Fig. 6. Helminths' excretion with canine faeces (n = 36) after deworming with Caniquantel® Plus and fenbendazole (24 h interval)

of faeces) and two days (2.92 ± 0.11 specimens per 100 g of faeces) after the study started, *T. leonina* after one day (4.30 ± 0.18 specimens per 100 g of faeces), Ancylostomatidae family appeared after two (211.6 ± 12.4 specimens per 100 g of faeces) and three days (47.9 ± 2,3 specimens per 100 g of faeces), *A. alata* from one (17.30 ± 0.76 specimens per 100 g of faeces) to three days (3.37 ± 0.19 specimens per 100 g of faeces), and proglottids of *D. caninum* from one (14.14 ± 0.55 specimens per 100 g of faeces) to three days (1.05 ± 0.04 specimens per 100 g of faeces). Nematodes *T. vulpis* began excreted in faeces of dogs two days after the experiment started (10.03 ± 0.45 specimens per 100 g of faeces). Their number increased sharply 3.67 times after three days ($P < 0.001$). The last time these parasites were isolated after four days in the amount of 15.71 ± 0.80 specimens per 100 g of faeces.

Helminth bodies were not excreted with faeces of dogs in both groups after 5—7 days from the beginning of the experiment.

DISCUSSION

In previous decades, manuscripts have been published stating that Caniquantel® Plus can be used three times with an interval of 24 hours to successfully deworm dogs with mixed helminthoses [53]. Fenbendazole is needed to control parasitic nematodes in dogs (*T. vulpis, T. canis, T. leonina*, Ancylostomatidae family), and praziquantel—with cestodes (*D. caninum*, Taeniidae). Both of these substances are considered as low-toxic. However, more recent studies have shown that a single dose of praziquantel-containing drugs is sufficient to kill mature cestodes in dogs [24, 28] and there is no urgent need to re-feed this substance. But fighting some parasitic nematodes (*T. vulpis*, and in some cases *T. canis*), a single treatment with benzimidazoles may not be enough and should be duplicated with a daily interval [12, 57]. This evidence prompted us to investigate the effectiveness of a deworming regimen involving a single application of praziquantel and a double application of fenbendazole. This regimen has to be the most effective against mixed helminthiasis in adult dogs and the least toxic to patients, i.e. the golden mean of canine deworming.

The result of the present study was the identification of differences in the excretory of eggs and bodies of helminths with faeces of two experimental groups' dogs. Significantly, these differences were manifested in the indicators related to the nematode *T. vulpis*. Thus, a single application of the anthelmintic drug Caniquantel® Plus led to the effective release of the dogs' body at the 1st experimental group from helminths *T. canis, T. leonina*, Ancylostomatidae family, *D. caninum, A. alata*. It manifested itself in the cessation of their eggs' excretion (or packets) and/or the bodies of adults with dogs' faeces. However, such a deworming scheme proved to be ineffective against *T. vulpis*. The number of eggs of this nematode in canine faeces only partially decreased at the end of the experiment by 22.0 % ($P < 0.001$) compared to the initial data. The bodies of adult parasites were excreted in faeces only after two days and in small quantities (4.54 ± 0.21 specimens per 100 g of faeces). Such results give the right to speak only about the partial release of the host from parasites because the continued excretion of eggs indicates the presence of alive adult *T. vulpis* in the intestine.

Results of the presented study about the insufficient effectiveness of standard regimens for anthelmintics' use against *T. vulpis* echo the scientific reports from different European countries. Thus, Hinaidy from Austria describes that the usual deworming carried out by owners for bitches and their puppies were only partially effective [17]. The presence of *T. vulpis* eggs in faeces of dogs from Finland, which are dewormed at least once a year were indicated by Pullola [38]. Data published in Switzerland emphasize that even in the case of canine deworming every 3 months (with a combination of pyrantel embonate, praziquantel and febantel), *T. vulpis*, *Capillaria* spp. and Taeniidae eggs were detected in faeces with a prevalence 11—22 % [46] The authors also emphasize that the frequency and mode of deworming directly depend on the characteristics of the environment, nutrition and lifestyle of pets [11].

Significantly better results in the present study were obtained in a comprehensive parasitological study of canine faeces at the 2nd experimental group. Thus, the excretion of dead specimens of *T. vulpis* lasts three days and stops four days after the experiment start, and their eggs after five days. The excretion of eggs (packets) of helminths *T. canis, T. leonina*, Ancylostomatidae family, *D. caninum* stopped in 2—3 days from the beginning of deworming, and bodies (proglottids) of *T. canis, T. leonina, Strongylata* sp., *D. caninum*, and *A. alata*—after 1—3 days. It means that the gastrointestinal tract is free of helminths.

A significant increase of helminth eggs' concentration in faeces of experimental dogs in the first days after the use of anthelminthics is due to the lysis of the bodies of female parasites after death. The decay of bodies leads to the mass release of helminth eggs (as well as somatic toxins) into the environment, which is the intestine's content [4]. The same applies to the situation with *T. vulpis*, when four days after the beginning of deworming of animals of the 2nd experimental group, these parasites were no longer excreted with faeces, and their eggs—yes. Similarly, in *T. leonina*, the release of eggs stopped after three days, and bodies of these parasites stopped after one day.

A surprise result of the present study was the isolation of mature individuals of *A. alata* with faeces of dogs in both experimental groups, as there are no reliable data on the spread of this parasite in Ukraine. In addition, as a result of ovoscopic examination of canine faeces, previous researchers didn't find eggs of this trematode. Although in Ukraine's neighbouring countries, this parasite is quite common among domestic and wild Canids, as well as

among wild boars in Belarus [50], Poland [6, 54], and Latvia [35]. This phenomenon is easy enough to explain by the method most often used to prepare samples for ovoscopic research. Thus, Ukrainian parasitologists classically use flotation or combined methods of laboratory examination for canine faeces [56]. Just for samples from cattle, it is considered appropriate to use sedimentation techniques to diagnose the fasciolosis [13]. However, eggs of *A. alata*, as in many other trematodes, are quite large, 0.115—0.130 × 0.068—0.093 mm [26]. Therefore, the detection of these pathogens by flotation methods is unlikely. This situation may lead to further study of this trematode's prevalence among domestic and wild animals in Ukraine.

CONCLUSIONS

Deworming of adult dogs with Caniquantel® Plus allows fighting successfully the helminths *T. canis, T. leonina*, Ancylostomatidae family, *D. caninum*, and *A. alata*. However, such treatment is ineffective in controlling the nematode *T. vulpis*. Alternate use of Caniquantel® Plus and fenbendazole (with 24-hour interval) allows to completely rid the body of adult dogs against the causative agents of mixed gastrointestinal tract helminthoses. It is manifested by the complete cessation of the release of eggs (packets) and bodies (proglottids) of mature helminths of different species with animal faeces five days after the deworming start. Therefore, such a scheme has the maximum efficiency and safety due to the combination of low-toxic substances.

Further study could usefully investigate *A. alata* prevalence among domestic and wild Canids' populations in the Kyiv region particularly, and Ukraine generally.

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OBSERVATIONS ON SOME BIOLOGICAL CHARACTERISTICS OF *RHIPICEPHALUS SANGUINEUS* SENSU LATO (ACARI: IXODIDAE) UNDER NATURAL AND LABORATORY CONDITIONS IN NIGERIA

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ABSTRACT

The brown dog tick, *Rhipicephalus sanguineus* sensu lato (s.l.) is a globally distributed tick of medical and veterinary significance. This study reports some observations on the biological characteristic of the brown dog ticks kept under natural and laboratory conditions in Nigeria. Variation in biological parameters was observed within the Nigerian *R. sanguineus* s.l. population kept under various conditions. A higher number of *R. sanguineus* exhibited the type 1 oviposition pattern than the type 2 pattern. The type 1 pattern was observed in 84.2 % and 80 % of *R. sanguineus* s.l. reared under laboratory and natural conditions, respectively. The pre-oviposition period was significantly shorter in ticks maintained under laboratory conditions than under natural conditions. Similarly, ticks kept under laboratory conditions laid significantly more eggs with higher egg mass weight than ticks kept under natural conditions. Furthermore, the incubation period and the total weight loss by female ticks throughout the oviposition period differed significantly between the two groups ($P < 0.05$). The engorged *R. sanguineus* s.l. females kept at 4 °C survived for up to 21 days and remained viable producing fertile eggs. Egg of *R. sanguineus* s.l. kept at 4 °C for up to 63 days retain viability and were able to hatch to larvae after variable incubation periods when returned to ambient temperatures depending on the duration of storage at 4 °C. The results showed that the tropical lineage of the brown dog tick can survive and remain fecund at low temperatures, suggesting that it can be translocated and established in temperate regions.

Key words: fecundity; hatching; refrigerator; *R. sanguineus* s.l.; survival; zoonoses

INTRODUCTION

Rhipicephalus sanguineus sensu lato (s.l.) is reputed as the most widespread ticks of dogs and is a well-recognized putative vector of numerous pathogens to dogs and humans worldwide [7, 30]. Based on available bio-

logical, molecular, and genetic data, the *R. sanguineus* s. l. is a group of at least 17 morphologically closely related species [20, 21]. Therefore, a lot of studies have been conducted using morphological, biological, and genetic analyses to generate data that may assist in resolving the taxonomic ambiguity associated with this tick [3, 4, 12, 14, 20, 22, 23]. Specifically, variations in the biological characteristics of *R. sanguineus* s. l. strains obtained from different climes have been reported, attesting to the complexity within this taxon [10, 19, 27].

Although the origin of the brown dog tick has been traced to Africa [1, 9, 29], little has been reported on its biological characteristics in Nigeria. Therefore, the aim of this study was to highlight some observations on the biological characteristics of the brown dog tick under natural and laboratory conditions in Nigeria in order to update available data on this enigmatic tick from the continent.

MATERIALS AND METHODS

Study location and ticks sampling

Ticks were collected from owned dogs in Nigeria. The details of the tick sampling locations and climatic conditions are available in the study by K a m a n i [15]. Naturally infested dogs were properly restrained and the ticks were removed using forceps. The ticks were placed in labelled ventilated tubes and transported to the Entomology Research Laboratory, National Veterinary Research Institute (NVRI), Vom, Nigeria.

Ticks identification

The morphological and molecular identification of the ticks used in thus study have been previously described [15]. Only ticks identified as *R. sanguineus* s. l. were used in this study.

Oviposition in *R. sanguineus* s. l. under natural and laboratory conditions

Partially or fully engorged female ticks were selected for this study. The selected female *R. sanguineus* s. l. ticks were weighed individually and placed in coded sterile ventilated tubes. Nineteen ventilated tubes each containing one female tick were placed in an incubator at 27 °C, relative humidity of 85 % with 12-hour photoperiods, while another 19 tubes were placed on a bench in the laboratory at 22 °C (range 18.3—25.1 °C), and humidity 65 % (range 42—76 %). The ticks were monitored for the commencement of egg laying. Daily, at 7 : 00 hrs the ticks and the eggs laid were weighed and the number of eggs counted. Ten eggs were randomly picked from each clutch daily and measured (length and width) under a calibrated microscope (Nikon, Eclipse E100). This was continued until egg laying ceased evidenced by constant weight of the ticks for five consecutive days. Biological parameters; engorged female weight (FW), pre-oviposition period (POP), duration of oviposition (DO), total number of eggs produced (TNEP), egg mass weight (EMW), egg size (ES), tick final weight after oviposition (TFW), percent of initial weight lost during oviposition (WLO), incubation period (IP), efficiency of conversion of feed reserve to produce eggs (ERCE), oviposition efficiency (OPE), mass conversion rate (MCR), and mass conversion efficiency (MCE) were calculated according to D i p e o l u, et al. [6] and S z a b ó, et al. [28].

Effect of low temperature (4 °C) on the survival and fecundity of *R. sanguineus* s. l.

The engorged female *R. sanguineus* s. l. (n = 30) collected from the dogs in Nigeria were weighed and individually placed in labelled ventilated tubes. The tubes were place on a rack in a group of five and kept in a refrigerator (4 °C). A group of five ticks were removed weekly and assessed for viability. Ticks that were viable were placed in an acaridium at conditions earlier mentioned and assessed for fecundity.

Effect of low temperature (4 °C) on the survival and hatchability of *R. sanguineus* s. l. eggs

Eggs laid by seven *R. sanguineus* under natural conditions were pooled and gently mixed on a clean petri-dish. Thereafter, 100 eggs were counted and kept in ventilated tubes. The tubes containing the eggs were kept on a rack in a refrigerator (4 °C). Two tubes containing the eggs were removed weekly and placed in an acaridium at conditions earlier mentioned and assessed for hatchability. Percent hatching was obtained by subtracting the number of unhatched eggs from 100.

Ethical statement

The study protocol was approved by the Animal Use and Care Committee (AUCC), National Veterinary Re-

search Institute (NVRI) Vom, Nigeria. Oral consent was obtained from dog owners before ticks were collected from the dogs.

RESULTS

Four of the ticks kept under natural condition escaped from the vials, hence data was only available for 15 ticks in this group. A higher number of *R. sanguineus* s.l. exhibited the type 1 oviposition pattern than the type 2 pattern. Type 1 pattern was observed in 16 out of 19 (84.2%) and 12 out of 15 (80%) of the ticks kept under laboratory and natural conditions, respectively. The type 1 oviposition pattern is characterized by initial low oviposition and attainment of peak after a few days, while the type 2 is characterized by early attainment of peak oviposition usually within the first set of eggs (Fig. 1). The pre-oviposition period was significantly shorter in ticks maintained under laboratory condition than those kept under natural condition. Similarly, the ticks kept under laboratory conditions laid significantly more eggs and had higher egg mass weight than the ticks kept under natural conditions (Table 1). Furthermore, the incubation period and the total weight loss by female ticks throughout the oviposition period differed significantly between the two groups (Table 1). There was higher POP, DO, OPE and MCR for ticks kept under natural condition while those kept under laboratory condition had higher WLO and ERCE, although the differences were not significant ($P > 0.05$) (Table 1).

Effects of low temperature (4 °C) on adult *R. sanguineus* s.l. females

The engorged *R. sanguineus* females kept at 4 °C survived for up to 21 days and remained viable. There was an increase in the percent loss of the initial tick weight with

Table 1. Biological parameters of female *R. sanguineus* s.l. under natural and laboratory conditions in Nigeria

Parameters		Natural conditions (n = 15)		Laboratory conditions (n = 19)		F	P
		Mean ± SD	Range	Mean ± SD	Range		
FW [mg]		170.3 ± 119.7	21—406	184.1 ± 154	34—533	0.6	0.15
POP [days]		6.9 ± 1.8	5—10	5.8 ± 0.9	4—7	3.8	0.003*
DO [days]		12.4 ± 5.4	1—21	10.5 ± 3.9	3—20	1.89	0.09
TNEP		1687 ± 1274	38—4041	2179 ± 1952	162—7084	0.43	0.04*
EMW [mg]		73.7 ± 57	2—185	95.8 ± 89.8	8—317	0.4	0.03*
ES [µg]	**L**	454 ± 39.5	409—502	472 ± 28.2	418—525	0.98	0.5
	B	361 ± 18.1	334—399	364 ± 21	334—426	1.35	0.3
TFW [mg]		44.4 ± 25.3	12—112	34.9 ± 24	10—91	1.19	0.37
IP [days]		31.3 ± 3.7	24—36	26.7 ± 1.7	24—29	4.8	0.00*
ERCE [%]		39.7 ± 12.7	6.7—55.8	49.9 ± 13.7	19.7–67.4	0.9	0.37
WLO [%]		62.1 ± 19.8	17.6—80	75.1±8.1	55.9—87.5	6.02	0.00*
OPE		0.13 ± 0.11	0.06—0.5	0.11 ± 0.07	0.05—0.3	0.85	0.37
MCR		0.14 ± 0.11	0.06—0.5	0.09 ± 0.05	0.05—0.25	1.82	0.12
MCE		0.07 ± 0.03	0.05—0.18	0.07 ± 0.01	0.05—0.1	0.95	0.46

F—Fisher test; P—significance value; FW—engorged female weight; POP—pre-oviposition period; DO—duration of oviposition; TNEP—total number of eggs produced; EMW—egg mass weight; ES—egg size; TFW—tick final weight after oviposition; WLO—percent of initial weight lost during oviposition; IP—incubation period; ERCE—efficiency of conversion of feed reserve to produce eggs; OPE—oviposition efficiency; MCR—mass conversion rate; MCE—mass conversion efficiency; values with * are statistically significant

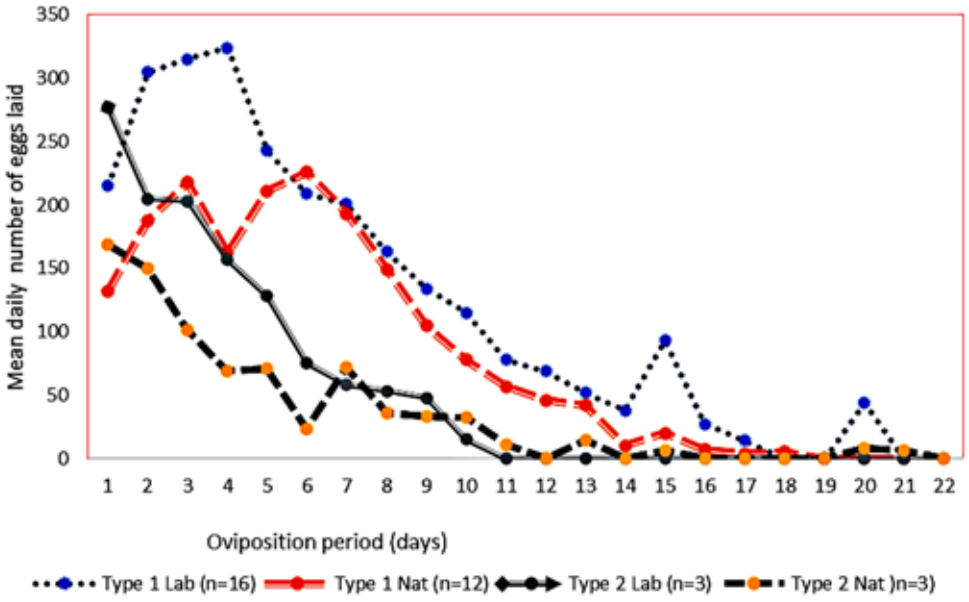

Fig. 1. Oviposition patterns exhibited by *R. sanguineus* s.l. under natural and laboratory conditions

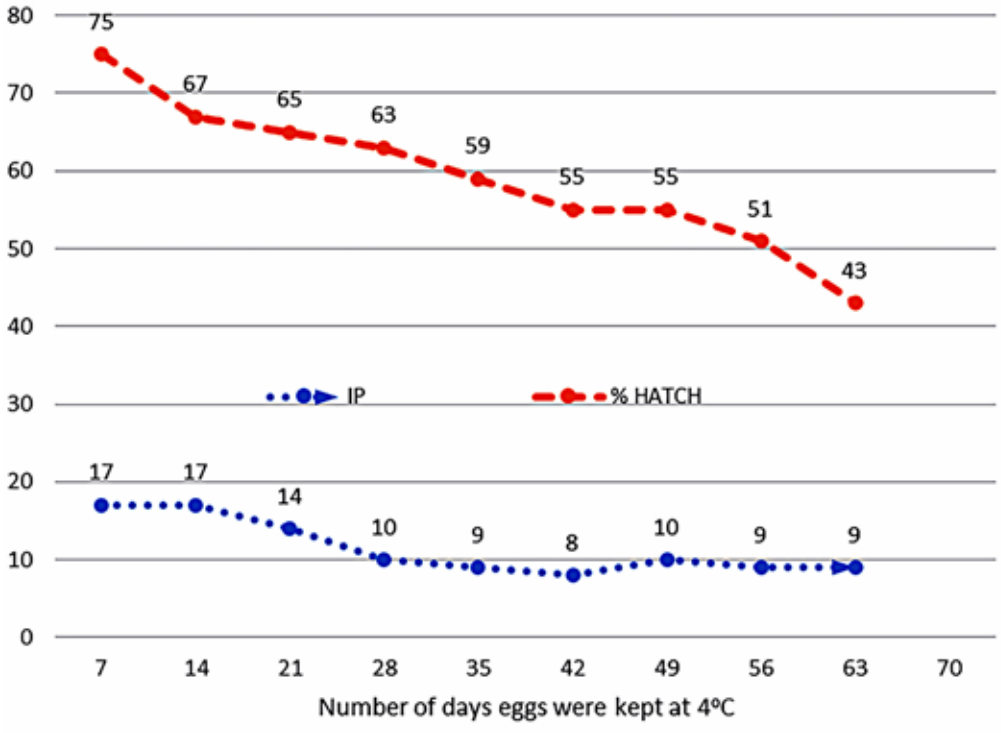

Fig. 2. Effect of low temperature (4 °C) on the survival and hatchability of *R. sanguineus* s.l. eggs

the duration of stay at 4 °C before moving them to the ideal environmental conditions for tick breeding. However, they were able to produce fertile eggs when returned to the ideal conditions. The IP was 26, 28 and 30 days respectively for ticks kept for 1, 2 and 3 weeks at 4 °C before returning them to ambient conditions. The POP decreased with increased duration of stay at 4 °C; from six days for 7 and 14 days to 5 days for ticks kept for 21 days at 4 °C. Similarly, the EM and ERCE decreased with longer storage at 4 °C.

Effects of low temperature (4 °C) on survival and hatchability of *R. sanguineus* s. l. eggs

Egg of *R.sanguineus* kept at 4 °C for up to 63 days retained viability and were able to hatch to larvae when they were returned to the ideal environmental conditions after variable IP depending on the duration of storage at 4 °C. The IP and percent hatching were inversely related to the period of storage at 4 °C (Fig 2). Although no hatching was recorded during the period of egg storage at 4 °C for up to 63 days, there might be some biological activities taking place in the eggs, which may explain the shorter IP in eggs stored for longer period. Equally the low percent hatch in eggs kept for longer period at 4 °C could be due to exhaustion or depletion of nutrient reserve in the egg leading to embryonic death.

DISCUSSION

This study reports on the observations of some biological parameters of *R. sanguineus* s. l. removed from naturally infested dogs in Nigeria. The FW and EMW for *R. sanguineus* s. l. recorded in this study were higher than the values reported for the same tick species from Brazil and Indonesia but lower than that of Argentina [10, 27]. Most of the biological parameters assessed in this study varied from other studies in different climes [10, 12, 25, 27]. These differences can be attributed to variation in climatic, as well as, the intra species variation among the diverse *R. sanguineus* complex [24, 25, 27]. Interestingly, varying the environmental conditions among the same tick population in this study resulted in significant differences in some of the biological parameters like the POP, IP, TNEP, EMW and WLO, confirming the role of abiotic factors on tick fecundity. In addition, a wide variation in the sizes of *R. sanguineus* s. l. was recorded in this study similar to reports from other countries [2, 10—13, 27, 28]. The female engorgement weight is a key determinant of the number of eggs laid and most of the other biological parameters of *R. sanguineus* s. l. in this study which is in agreement with reports from other studies [5, 16, 26]. Furthermore, the type 1 oviposition pattern was recorded in >80 % of the *R. sanguineus* s. l. in this study similar to the earlier report of 78 % in Nigeria [5]. It has been established that environmental temperature and humidity are critical factors in the survival and fecundity of ticks [7, 8]. Some studies have reported that temperatures of 20—35 °C and humidity of 35—95 % are ideal for the survival and optimum fecundity of *R. sanguineus* s. l. [18, 26]. Differences were observed in the fecundity of *R. sanguineus* s. l. reared under laboratory (T = 27 °C; RH = 85 %) and natural condition (T = 22 °C; RH = 65 %) in this study. This is in agreement with the results from other studies [18, 26]. Environmental temperature has been suggested as the key determinant for tick survival and establishment in a locality [30]. Indeed, it has been reported that an increment of 2—3 °C in the mean annual temperature favors the establishment of the tropical lineage of *R. sanguineus* s. l. in temperate climes [8]. In this study the ability of *R. sanguineus* s. l. tropical lineage to survive at low temperature (4 °C) was investigated. Interestingly, engorged females and eggs survived at 4 °C for over 21 and 63 days, respectively and maintained their viability. This finding agrees with the reported ability of *R. sanguineus* s. l. to overwinter in the environment and even infest dogs during winter in some regions of temperate climates [17]. Arguably, the ticks may not be able to perform full biological activities like: oviposition, egg hatching, or moulting at the low temperature [11, 18], but the ability to survive and remain viable for weeks at 4 °C have implication for its translocation, introduction and establishment into new climes, as well as, for research. Adult *R. sanguineus* and eggs can be stored in the fridge for several weeks for later use.

CONCLUSIONS

The variation in biological parameters of *R. sanguineus* s. l. in Nigeria is indicative of the existence of the diversity of haplotypes under this taxon. This has veterinary and public health implications taking into cognizance the var-

iations in vector capacity for different pathogens by the various haplotypes. Therefore, efforts should be geared towards elucidating the epidemiology and vector competence of this tick for various pathogens of veterinary and public health significance in Nigeria.

CONFLICT OF INTEREST

There is no conflict of interest.

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POTENTIAL ROLE OF *AGRIMONIA EUPATORIA* L. EXTRACT IN CELL PROTECTION AGAINST TOXICITY INDUCED BY BISPHENOL A

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ABSTRACT

The aim of this study is to reveal the potentially protective role of ethanolic extract of agrimony (*Agrimonia eupatoria* L.) against the cytotoxic effect of bisphenol A (BPA) *in vitro*, using an intestinal porcine epithelial cell line (IPEC-1). The cells were exposed to different concentrations of BPA: 12.5, 25, 50, 100, and 200 µg.ml^{-1} alone and in combination with agrimony extract (250 µg.ml^{-1}). The proliferative cell response was monitored for 72 h by a xCELLigence system or real-time cell analyser (RTCA), recorded as the cell index (CI) and expressed as a proliferative activity (% PA) compared to the control cells without treatment. The metabolic activity was measured by a MTS colorimetric test, performed after 48 h of treatment with the tested substances. The cytotoxic effect on cells exposed to BPA alone, in comparison to the control cells without treatment, was observed in both assays ($P < 0.0001$). It was confirmed that BPA reduces both the metabolic activity and the proliferation of cells. After the cell treatment with agrimony, the metabolic activity had increased to reach over the control (101.52 %), while reducing the proliferation of the cells. The protective role of agrimony against cytotoxicity caused by BPA was observed after cell treatment with agrimony in combination with lower concentrations of BPA (12.5; 25 and 50 µg.ml^{-1}). The slight improvement in the adherence was observed in cells treated with these combinations, in comparison to the cells treated with BPA alone. On the other hand, the metabolic activity was slightly improved in cells treated with a combination of agrimony and BPA at higher concentrations (50 a 100 µg.ml^{-1}). This supported our assumption that agrimony can protect a model organism against cytotoxicity caused by BPA.

Key words: agrimony; bisphenol A; cytotoxicity; intestinal cell line; MTS assay; xCELLigence

INTRODUCTION

Environmental pollution adversely affects human health and the stability of an ecosystem. Various pollutants produced by human industrial activity (automobile exhaust, heavy metals, radioactive compounds, etc.) remain in nature and pose a great threat to the life on Earth [13]. One of the important chemical components used in the manufacturing of plastic materials is bisphenol A (BPA), commonly referred to as 2,2-(4,4-dihydroxydiphenol) propane, which is present in different ecological media, such as water, sediment, soil, biomass and air [20]. BPA is a chemical substance obtained by condensation of phenol with acetone in the presence of a strongly acidic ion-exchange resin, in the gel form, as a catalyst [11]. It was first synthesised in 1905. Since the 1960s BPA is the main ingredient used for the production of a variety of polymers such as polycarbonate plastics, epoxy resins, or thermal papers and is therefore found in a wide range of consumer products, including plastics and food packaging, medical equipment (for dialysis and blood oxygenation), bottles for feeding infants, and kitchen dishes [11]. The polycarbonates have wide utilization for their advantageous properties, such as: durability, light weight, high tensile strength, high modulus of elasticity, high melting point and high vitrification temperature [2, 5]. BPA was considered as neutral to human health for many years, but its detection in the natural environment, in drinking water and food products (since 1990) induced the interest of many researchers and the negative effects of this compound on human health was established. Many authors have studied the migration of BPA and its derivatives into food from polymer packaging in which it was stored, especially at elevated temperatures (e. g. microwave heating or other thermal process) [11]. In 1996 BPA was classified by the European Commission as a substance of external origin with a harmful effect on human health. It was confirmed that BPA had estrogenic properties and an agonistic effect toward the estrogenic receptors. In recent studies, BPA has been characterised as an endocrine disruptor, which disturbed the hormonal balance in humans and animals [3, 7]. The effects of exposure to BPA can be particularly harmful to foetus, infants and young children, because of a lack of feedback regulating the activity, synthesis and elimination of hormones [11]. It has also been reported that the cytotoxicity of BPA may not be linked to endocrine disruptors, but induced by the production of reactive oxygen species that cause oxidative stress leading to cell damage.

Herbs play an important role in the protection against oxidative stress. *Agrimonia eupatoria* L. is a herb of the Rosaceae family, which is used in traditional (folk) medicine for its beneficial effects. Common agrimony is a perennial herbaceous plant with small yellow star-shaped flowers, a short rhizome and a hard, hairy stem inhabiting pasturelands across Europe. The historical documents about the beneficial effects of agrimony date back as far as the 4th to 5th centuries and this herb was mentioned in the Old English Herbarium from the 10th century [4, 18, 19]. Agrimony contains polyphenol-enriched fractions (tannins, phenolic acids, flavonoids and terpenoids), which has a very important role in antioxidative properties [12]. Its water extracts (infusions and decoctions) or hydroalcoholic extracts (tinctures) has also been used in traditional medicine to treat lungs, inflammation, liver diseases, cholecystitis, cholestasis, intestinal or bladder atony, pyelonefrititis, bleeding disorders, skin defects, and inflammatory of oral mucosa [8].

The aim of our study was to evaluate the potential protective effects of *Agrimonia eupatoria* L. ethanolic extracts against harmful cytotoxic effects of BPA using a model porcine intestinal epithelial cell line (IPEC-1).

MATERIAL AND METHODS

Plant extracts

Lyophilised ethanol extract *Agrimonia eupatoria* L. (Calendula, Nová Ľubovňa, Slovakia) was diluted shortly before the experiment with sterile water in order to reach a final concentration of 250 $\mu g.ml^{-1}$. This concentration was selected based on preliminary study (data not shown) in which the effects of different concentrations (0.01—1000 $\mu g.ml^{-1}$) were evaluated.

Bisphenol A—solution

Bisphenol A purchased from Sigma Aldrich (Germany) was diluted with sterile water and tested at the final concentrations of: 12.5, 25, 50, 100, and 200 $\mu g.ml^{-1}$.

Cell cultivation

For this experiment porcine intestinal epithelial cells (IPEC-1, CVCL 2245) were used. The cells were cultivat-

ed in Earl's Minimal Essential Medium (EMEM; Lonza, Valais, Switzerland) supplemented with 10 % (v/v) foetal bovine serum (FBS; Lonza, Valais, Switzerland), 1 % L-glutamine, 0.1 % gentamicin and 1 % penstrepten. The cells were grown in a humidified atmosphere of 37 °C and 5 % CO_2, subcultured each for 3—4 days and were regularly checked for the absence of mycoplasma contamination.

xCELLigence system

In the experiment, the real-time cell analyser xCELLigence system (RTCA; ACEA Biosciences Inc., San Diego, CA, USA) was used to monitor the changes in cell proliferation or adherence. This system allows label-free monitoring of cell behaviour (adhesion, proliferation, growth and morphology) throughout the treatment. It is based on measurement of impedance on gold electrodes at the bottom of microplate wells. The more cells are attached the higher the impedance is recorded. The values are expressed as dimensionless cell index (CI) and recorded in curves each hour throughout the experiment [6, 15, 16].

For our experiment, the cells were seeded at 5×10^3 cells per 16-well E-plate (Acea Bioscience, San Diego, CA, USA). After 22 hours (cells were still in a log phase) BPA at concentrations of 12.50 $\mu g.ml^{-1}$—200 $\mu g.ml^{-1}$ alone, and in combination with agrimony extract (at concentration 250 $g.ml^{-1}$) were added to the cells. The cell response was monitored for 48 h. The cells without treatment served as the control and their activity was considered as 100 %. Change in the proliferative activity (PA) was calculated using the following formula:

$$\% PA = CIsample \times 100/CIcontrol$$

MTS assay

For the measurements of changes in the metabolic activity of the cells MTS colorimetric assay (CellTiter 96®AqueousOne Solution Cell Proliferation Assay, Promega, Madison, WI, USA) was performed. This method is based on the viable cells ability to reduce MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulphophenyl)-2H-tetrazolium) compound to form a coloured formazan product that is soluble in cell culture media. The cells were seeded into two 96-well plates (Greiner-Bio-One, Kremsmünster, Austria) in an amount of 8.6×10^3 cells per well in a 100 µl medium. After 22 h cultivation, the cells were treated the same way as in RTCA and incubated for 48 hours. Then 2 µl of the MTS solution was added to each well. The absorbance was measured at 490 nm after 4 h incubation using a microplate reader (Synergy HT; Biotek, Winooski, VT, USA). The absorbance of the control cells was considered as 100 %.

Statistical analysis

The data were statistically evaluated by the GraphPad Prism 8.3.1 software (USA), using one-way ANOVA analysis of variance followed by Dunnett's multiple comparison test. The results are presented as the means ± SD. The significance level was set to $P < 0.05$. All experiments were conducted in triplicate.

RESULTS

Real-time monitoring of cell proliferation using the xCELLigence system

The cell response to the exposition to the tested substances was monitored in real time using the xCELLigence system (RTCA). We observed significant changes in proliferative activity (PA) expressed as CI values of the treated cells in comparison to the control cells without treatment ($P < 0.05$). Figure 1 illustrates CI changes in wells containing IPEC-1 cells and BPA of different concentrations, in comparison to control cells without treatment. The lowest CI values were recorded at the highest concentrations 100 $\mu g.ml^{-1}$ and 200 $\mu g.ml^{-1}$. Within the first 2 hours after treatment with BPA, CI values drop from 1.0 (set as normalised cell index) to less than 0.1, indicating that these concentrations are the most effective. Cells treated with BPA at concentrations 12.5, 25 and 50 $\mu g.ml^{-1}$ show significantly lower CI values ($P < 0.05$) compared to the control cells.

The following figures (Figs. 2, 3, 4, 5 and 6) illustrate changes in the CI after treatment with agrimony extracts and BPA alone and in mutual combinations. The most significant change can be seen in Figure 2 in which the growth of CI or cell proliferation treated with the combination of BPA at 12.5 $\mu g.ml^{-1}$ and agrimony is higher in comparison with cells treated with BPA alone. Similarly, the concentrations 25 and 50 $\mu g.ml^{-1}$ in combination with agrimony caused slight improvement in CI (Figs. 3 and 4). On the other hand, the highest tested concentrations 100 and 200 $\mu g.ml^{-1}$ caused a fast decline in the adherence im-

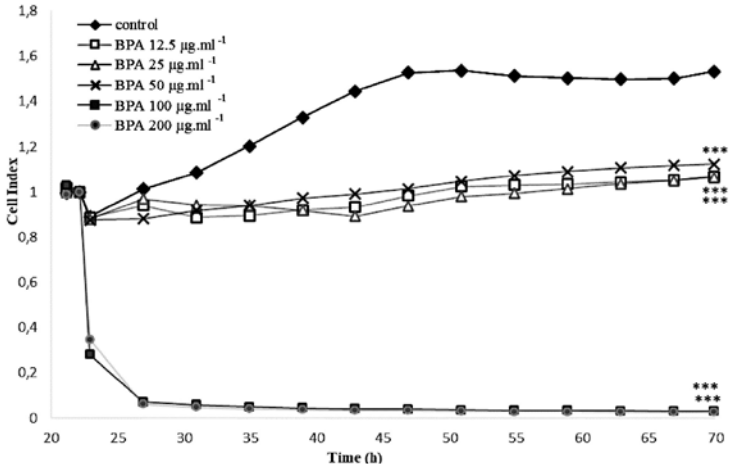

Fig. 1. Real-time monitoring of proliferation of cells treated with different concentrations of bisphenol A (12.5—200 µg.ml^{-1}) compared to control without treatment. Statistically different to control *—P < 0.0001**

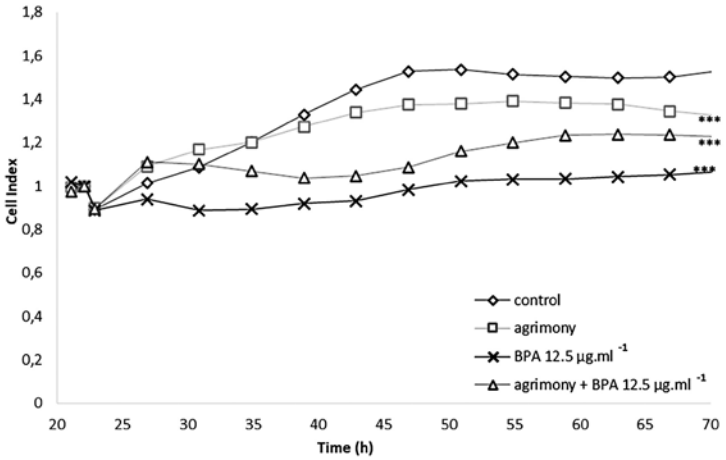

Fig. 2. Real-time monitoring of proliferation of cells after treatment with bisphenol A at 12.5 µg.ml^{-1} and agrimony in comparison to control cells without treatment. Statistically different to control *—P < 0.0001**

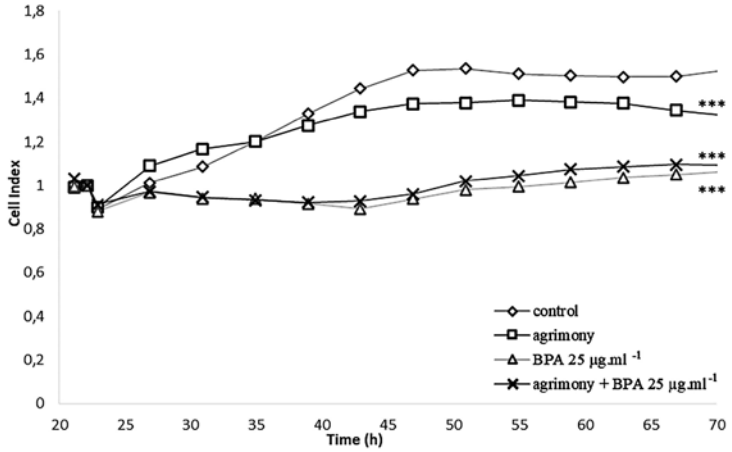

Fig. 3. Real-time monitoring of cell proliferation after treatment with bisphenol A at 25 µg.ml^{-1} and agrimony in comparison to control cells without treatment. Statistically different to control *—P < 0.0001**

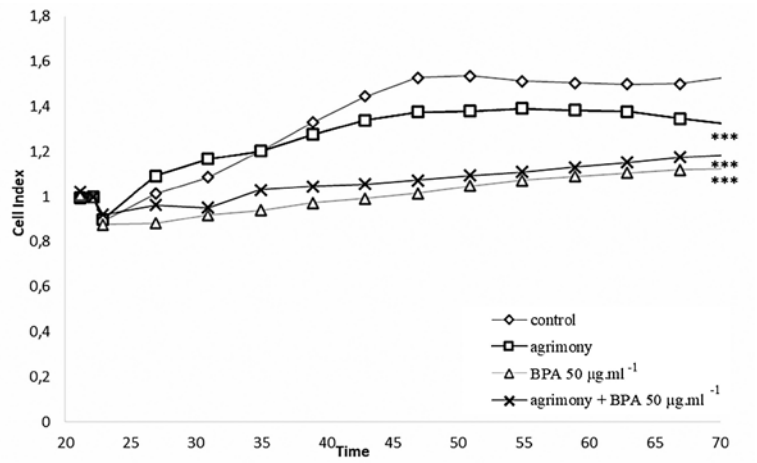

Fig. 4. Real-time monitoring of cell proliferation after treatment with bisphenol A at 50 µg.ml^{-1} and agrimony in comparison to control cells without treatment. Statistically different to control *—P < 0.0001**

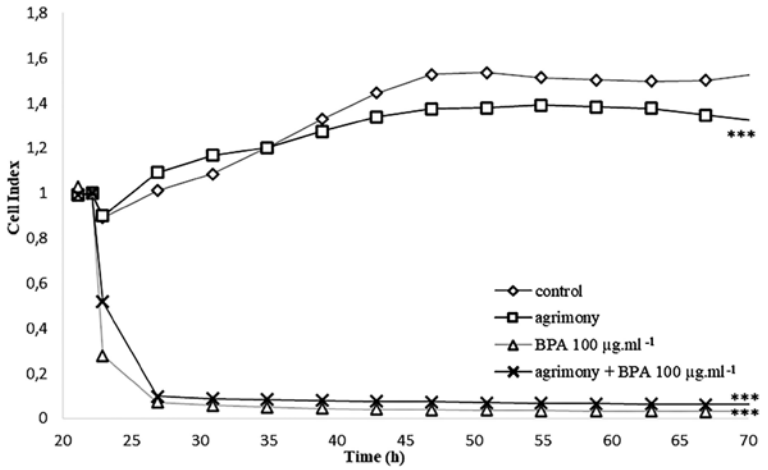

Fig. 5. Real-time monitoring of cell proliferation after treatment with bisphenol A at 100 µg.ml^{-1} and agrimony in comparison to control cells without treatment. Statistically different to control * —P < 0.0001**

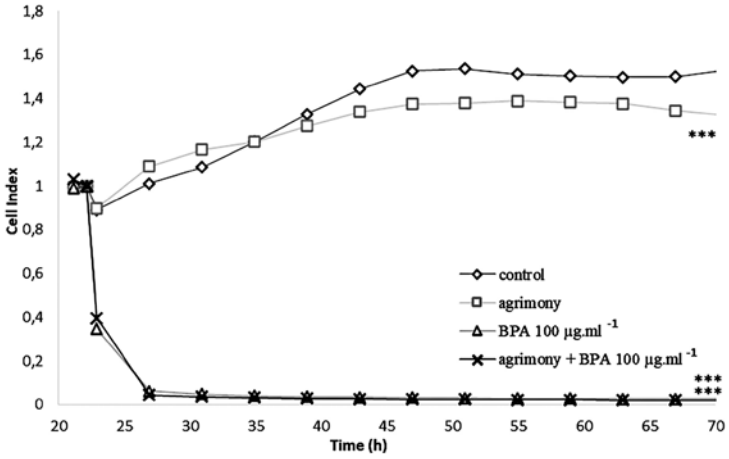

Fig. 6. Real-time monitoring of cell proliferation after treatment with bisphenol A at 200 µg.ml^{-1} and agrimony in comparison to control cells without treatment. Statistically different to control *—P < 0.0001**

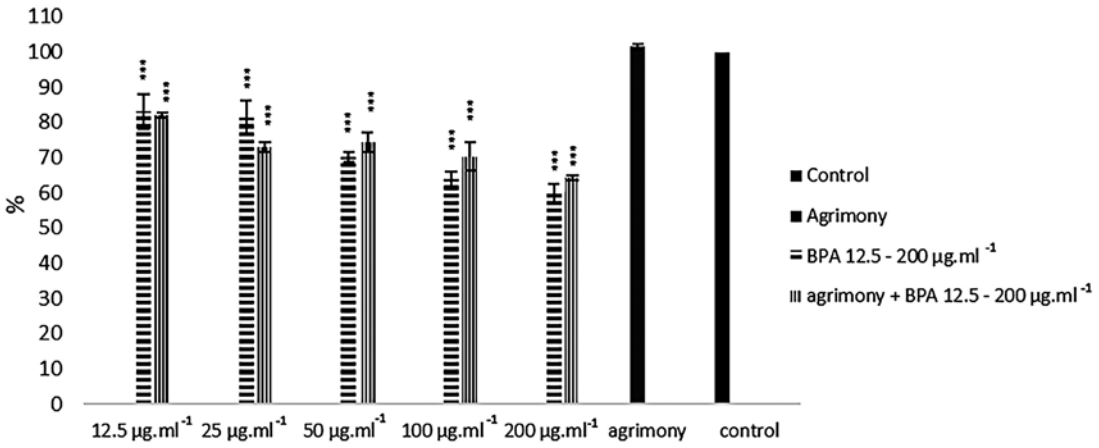

Fig. 7. Change in metabolic activity of cells treated with bisphenol A, agrimony and their mutual combinations. *—P < 0.0001 significantly different to control**

Tab. 1. Comparison of metabolic activity and cell adherence [%]

	% MA	± SD	% PA	± SD
BPA 12.5 µg.ml^{-1}	83.22***	4.84	66.83***	1.23
BPA 25 µg.ml^{-1}	81.38***	4.97	66.27***	1.79
BPA 50 µg.ml^{-1}	70.34***	1.38	73.28***	2.52
BPA 100 µg.ml^{-1}	63.91***	2.11	1.98***	0.26
BPA 200 µg.ml^{-1}	59.77***	2.87	2.43***	3.15
BPA 12.5 µg.ml^{-1} + agrimony	82.07***	0.69	80.14***	2.02
BPA 25 µg.ml^{-1} + agrimony	73.10***	1.38	71.42***	1.95
BPA 50 µg.ml^{-1} + agrimony	74.48***	2.76	77.33***	0.94
BPA 100 µg.ml^{-1} + agrimony	70.34***	4.14	4.08***	0.27
BPA 200 µg.ml^{-1} + agrimony	64.14***	0.69	1.30***	0.26
Agrimony (250 µg.ml^{-1})	101.52	1.90	75.13***	3.62
Control	**100.00**	**0.20**	**100.00**	**0.50**

BPA—bisphenol A at concentrations 12.5—200 µg.ml^{-1}; % MA—change in metabolic activity; % PA—change in proliferative activity; significant difference compared to control cells ***—P < 0.001

mediately after addition to cells and the protective effect of agrimony was not observed (Figs. 5 and 6).

MTS results

The metabolic activity (MA) of the IPEC-1 cells was measured 48 hours after the treatment with the tested substances using the MTS test. The results are recorded in Fig. 7. Cells treated with agrimony showed metabolic activity comparable with the control cells (101.5%; P > 0.05). A negative effect of BPA was observed in a dose-dependent manner. Interestingly, there was an improvement in values of MA when treated with combina-

tions of agrimony and higher concentrations of BPA (50; 100 and 200 μg.ml^{-1}) in comparison to MA of cells treated with corresponding concentrations of BPA alone.

Comparison of RTCA and MTS results

The results of changes in the metabolic activity (MA) and the proliferative activity (PA), measured 48 hours after addition of tested substances, expressed in percentage are summarized in Table 1. All the recorded values after cell treatments were significantly different to values observed in control cells ($P < 0.05$). Improvement in PA was recorded after the treatment with agrimony in combination with BPA at 12.5, 25, 50 μg.ml^{-1} (compared to cells treated with BPA at corresponding concentrations alone) and MA was slightly but not significantly improved after the treatment with agrimony in combination with BPA 50, 100 and 200 μg.ml^{-1} in comparison to cells treated with BPA alone.

DISCUSSION

BPA is known to act as an endocrine-disrupting chemical, by mimicking estrogen and compatibility binding to its receptors in the body. It has also been discovered that BPA has several other negative effects on the organism [10]. The cytotoxicity of BPA may be mediated by an increase of reactive oxygen species, thus BPA could directly cause oxidative stress through their release, causing cell damage [19]. The reason for its wide use is based on chemical properties, low adsorption of moisture, and thermal stability [14]. The main exposition is through oral intake with food and water (it represents more than 90 %) [9]. BPA is only one of many substances known to have negative effects on an organism, therefore it is relevant to find the way how to protect live organism and reduce negative impact of toxicants.

Plants containing phytochemicals, which are the biologically active part of the plants, have several positive health-bringing effects. For the plant itself, phytochemicals are produced as part of their defences against environmental stress [17]. *Agrimonia eupatoria* L. is a plant that is well known for its positive effects on numerous health problems. In early times before modern medicine, this plant was used to treat: eye-infections, gastrointestinal problems, and liver- and kidney-related problems. Recent studies have shown that this plant also potentially acts with: inherited anti-tumour, anti-mutagenic, hepatoprotective, anti-viral, anti-oxidant, and anti-inflammatory qualities. All of these properties are related to its: chemical composition, agrimony is rich in flavonoids, aromatic-rings, triterpenes, tannins, coumarin, glycosides, and vitamins B and K [1]. It makes this plant attractive in research and searching for the new ways of utilization of these positive and protective abilities.

This study focused on revealing the potential role of medicinal plant agrimony in protection against the toxic effect caused by BPA. For the experiment, porcine intestinal epithelial cells IPEC-1 were employed and exposed to agrimony at 250 μg.ml^{-1} and BPA at concentrations between 12.5—200 μg.ml^{-1} alone and in their mutual combinations. After 48 h exposition we could observe a decrease in the proliferative activity of cells that were monitored in real-time by RTCA. The two highest concentrations (100 and 200μg.ml^{-1}) caused a decrease in the CI immediately after addition to cells that indicated the highest toxicity. Loss of adherence may lead to cell death of adherent cells. On the other hand, lower concentrations (12.5—50 μg.ml^{-1}) did not cause complete loss of cell adherence and the CI did not drop which indicated inhibition of cell proliferation. The potential protective role of agrimony was observed after the treatment with combination of agrimony and BPA at lower concentrations (12.5—50 μg.ml^{-1}) due to which we could observe an increase in the PA in comparison to cells treated with BPA alone.

RTCA is useful in *in vitro* studies, such as toxicology studies, cell function, and potential drug effects. Since the RTCA alone will not give any results about the changes inside the cell, this experiment was performed simultaneously with the MTS test that measure the metabolic activity (MA) of cells. The addition of the tested substances caused a significant decrease in MA except for agrimony ($P > 0.05$). An interesting finding was observed after the addition of higher concentrations of BPA (50—200 μg.ml^{-1}) in combination with agrimony that caused a slight increase in the metabolic activity in comparison to cells exposed to BPA alone. This indicated a potential protective role of agrimony against the cytotoxic effect of BPA. BPA is only one of many potentially harmful substances in the environment. Medicinal plants could perhaps aid to eliminate such negative effects and additional experiments are desirable to establish the true role of these plants.

CONCLUSIONS

The positive effects of medicinal plants (phytochemicals) have been known for a long time, and the plants are still in popular use today. In the form of tea or other supplements we can make use of beneficial effects of these plants with minimal negative side effects. This is important not only in treating diseases, but also in preventing them. Agrimony is known to contain several phytochemicals belonging to the group of polyphenols that are known for their antioxidant effect and ability to protect cells from oxidative stress. On the other hand, BPA cytotoxicity may be induced by oxidative stress caused by the release of reactive oxygen species. This could explain why agrimony could be beneficial in protection against toxic effects of BPA.

Our study revealed a potential cytoprotective effect of ethanolic extract of *Agrimonia eupatoria* L. against the environmental toxin BPA at different concentrations by using porcine intestinal epithelial cells as a model organism. Improvement in the proliferative activity of cells treated with a combination of the agrimony and BPA was recorded at lower concentrations in comparison to cells treated with BPA alone. Interestingly, the metabolic activity was higher after the treatment with a combination of agrimony and a higher concentrations of BPA. This could be ascribed to different principles of the methods used. However, further studies are needed, especially *in vivo*, to confirm the beneficial effects of the agrimony and its bioactive compounds.

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A 3-YEAR CASE REPORT ON AN ATOPIC PATIENT WITH A CANINISED MONOCLONAL ANTIBODY TREATMENT

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ABSTRACT

A 6-year-old, male, neutered, English Mastiff dog was presented for a second opinion due to chronic pruritus. The patient had been on methylprednisolone and chlorphenamine for four years. The diagnostic investigations included: swabs for bacterial and mycology culture, hair plugs for dermatophyte culture, acetate tape strips and deep skin scrapes, skin biopsies for dermatohistopathology, biochemistry, haematology, endocrinology, serology for canine scabies using enzyme-linked immunosorbent assay (ELISA) testing and serology for allergen specific IgE antibodies. The history and the diagnostics confirmed the diagnosis of canine atopic dermatitis. This case report details the treatment with lokivetmab, which is a caninised monoclonal antibody drug. At periods of flare ups, additional treatments were prescribed, such as systemic glucocorticoids and oclacitinib in order to manage the clinical signs. The treatment showed good response to the overall treatment management during the three year period of this atopic patient.

Key words: canine atopic dermatitis; CAD; caninised monoclonal antibody; lokivetmab; mAb

INTRODUCTION

Canine atopic dermatitis (CAD) is a common skin disorder defined as a hereditary predisposition to develop pruritic inflammatory skin disease associated with type I hypersensitivity [21]. Whilst the disease can be present in dogs from as early as 4 months old to as late as 7 years old [21], it mainly affects those between 6 months old and 3 years old, with 75 % of the cases within this age range [2].

CAD is characterised by pruritus and secondary skin lesions. Common skin lesions involve localised pruritus of the ears, lips, peri-palpebral region, digits, axillae, inguinal and perineal regions. The primary lesions include erythema and papules and salivary staining of hairs caused by licking. Many secondary changes lead to chronic lesions which mainly appear due to chronic self-trauma. Such lesions include excoriation, alopecia, lichenification and hyperpigmentation [9].

IL-31 is a known cytokine for its pruritogenic effects [19] in dogs [7] monkeys [17] and mice [1]. There are many treatment options to manage CAD, however there is no cure. Topical treatments include: glucocorticoid (triamcinolone) sprays [4] and ointments, tacrolimus [3], shampoos, emollient emulsifiers, moisturisers, occlusive dressings, cleaners and diets (either home prepared or commercially prepared). Systemic treatments include: glucocorticoids, specific immunotherapy, cyclosporin A (CsA), antihistamines, nutraceuticals such as essential fatty acids [9] oclacitinib [16] and lokivetmab [22]. Lokivetmab is a caninised monoclonal antibody (mAb) that binds and neutralizes canine Interleukin (IL)-31. Recent studies have shown good efficacy of lokivetmab for the management of CAD [2, 18, 22, 25].

CASE PRESENTATION

Signalment, history and presenting complaint

A 6-year-old, male, neutered, English Mastiff dog, with body weight of 45 kg and a body condition score 6/9 was presented for a second opinion due to chronic pruritus. The onset of pruritus started at the age of 2 years old by licking the paws, scratching and shaking of the ears. The owner estimated the pruritus score to be 6/10 and was described as: moderate pruritus that itches/scratches often [14]. The patient had been on methylprednisolone (Medrone; Zoetis) 0.09 $mg.kg^{-1}$ *per os* (*po*) once daily and chlorphenamine (Piriton; GlaxoSmithKline) 4 mg per dog *po* twice daily for four years. There were periods of good pruritus control and periods of flare ups. The patient was not up to date with a preventive parasitic treatment. An acute diarrhoea was only present at the age of 1 and was fully resolved with symptomatic treatment.

Physical examination

The physical examination was performed while the patient was muzzled, therefore the mouth was not assessed.

General physical examination

The patient appeared nervous but alert and responsive. The heart rate was found to be normocardic 80 bpm, the respiratory rate was normopnoeic 20 brpm and the rectal temperature was normothermic 38.5 °C. The rest of the general examination was unremarkable.

Dermatological examination

During the consultation the patient occasionally scratched his ears and tried to reach the paws and the medial aspect of the thighs bilaterally through the muzzle. On otoscopic examination, both auricles had moderate diffuse erythema on the convex and concave aspects. The external auditory canals appeared moderately erythematous and the lateral aspect of the tympanic membranes were normal and intact. There were no signs of stenosis, cerumen accumulation nor signs of purulent otitis. The clinical examination of the thoracic and pelvic ventral carpal, interdigital spaces and ventral pedal and the medial aspect of the thighs revealed spotty hyperpigmentation, lichenification, partial alopecia and diffuse scarlatiniform erythema of the skin. No ectoparasites were seen and there were no signs of pyoderma. There were no other primary or secondary dermal lesions on the rest of the body.

ETHICAL CONSIDERATIONS

Written consent for permission to publish this case report was obtained from the owner of the patient. The author declares no known conflicts of interest.

CASE MANAGEMENT

Problem list and differential diagnoses

The main problem was the pruritus with the accompanied skin lesions. The initial differential diagnoses considered included with descending order of importance with CAD to be top on the list due to its chronicity and clinical presentation followed by: food hypersensitivity, contact dermatitis, folliculitis, infectious or parasitic otitis, fungal and yeast infections such as dermatophytosis, malassetic dermatitis and candidiasis. Apart from CAD, another hereditary disease was considered, i.e., the familial canine dermatomyositis. Lastly, parasitic infestations such as mites (*Sarcoptes scabiei* var. *Canis* and *Demodex*), fleas (*Ctenocephalides felis*), and lice (*Cheyletiella* and H*eterodoxus spiniger*) were also considered.

Initial treatment plan

Given the long history of the patient, it was advised to proceed with diagnostics in order to get a diagnosis, thus

Table 1. Results of haematological and biochemical examination

HAEMATOLOGY		
Parameter	**Value**	**Reference interval**
Platelets	$62 \times 10^9.l^{-1}$ ↓	$160—500 \times 10^9.l^{-1}$
WBC	$5.3 \times 10^9.l^{-1}$ ↓	$6.0—15.0 \times 10^9.l^{-1}$
HAEMATOLOGIST'S COMMENTS		
WBC	Morphology unremarkable	
RBC	Normocytic normochromic	
Platelets	Moderate clumping, count would be higher	
Sample quality	Good	
BIOCHEMISTRY		
Parameter	**Value**	**Reference interval**
Creatinine	111 $\mu mol.l^{-1}$↑	$40—106\ \mu mol.l^{-1}$
Creatine kinase	210 $U.l^{-1}$ ↑	$0—190\ U.l^{-1}$
Pancreatic lipase (DGGR)	83 $U.l^{-1}$ ↑	$0—50\ U.l^{-1}$

WBC—white blood cells; RBC—red blood cells
DGGR—1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester

Table 2. The IgE and IgG positivity for food allergens

FOOD ALLERGENS		
Item	**IgE**	**IgG**
Beef	Negative	Positive
Lamb	Negative	Positive
Chicken	Negative	Positive
Turkey	Negative	Positive
Egg	Positive	Negative
Soya been	Negative	Positive
Corn	Negative	Positive
Wheat	Negative	Positive
Rice	Positive	Positive
Carrot	Negative	Positive

enabling it to be managed appropriately. Initially it was advised to taper the methylprednisolone to $0.045\ mg.kg^{-1}$ po for a week and then to cease, whilst continuing with the chlorphenamine treatment at a higher dosage, at 8 mg per dog *po* three times daily, and perform diagnostics a month later in order to do a washout period before the diagnostics. Additionally, it was advised to bathe the patient with a soothing hypoallergenic shampoo (Ermidra; Vetruus) twice per week. Lastly, an endo-parasitic preventive treatment was prescribed with milbemycin oxime $0.5\ mg.kg^{-1}$ *po* and praziquantel $5\ mg.kg^{-1}$ *po* (Milbemax; Elanco) and an ecto-parasitic preventive treatment was prescribed, i. e., Fluralaner (Bravecto; MSD) $31.1\ mg.kg^{-1}$ *po* every three months.

Diagnostic investigations and results

Due to the temperament of the dog all the procedures were done under a general anaesthetic.

Pre- and intra-operative details

The patient was premedicated with medetomidine (Sedastart; Animalcare) at $5\ \mu g.kg^{-1}$ intramuscularly (*im*) and methadone (Synthadon; Animalcare) $0.25\ mg.kg^{-1}$ *im*. After ten minutes, sedation was judged to be good and propofol (PropoFlo Plus; Zoetis) at $4\ mg.kg^{-1}$ was used by slow intravenous injection. The trachea was intubated with a size 12.0 mm cuffed endotracheal tube. The anaesthesia was maintained with sevoflurane (SevoFlo; Zoetis) vaporised in 100 % oxygen delivered via a non-rebreathing breathing circuit.

Blood work was advised for haematology, biochemistry and endocrinology in order to rule out systemic dermatoses, but also to look for possible hepatopathy due to the long term use of glucocorticoids. A mild increase of the creatinine, creatine kinase and pancreatic lipase were not significantly important (Table 1). The platelets count was low, however there were clumps on the microscopic examination which ruled out a possible thrombocytopenia and the mild leukopenia was not significant (Table 1). The remaining haematology, biochemistry, total thyroxine (T4) and canine thyroid stimulating hormone (TSH) were within the normal range. A serum sample was obtained for canine scabies using enzyme-linked immunosorbent assay (ELISA) testing and it was ruled out.

Serum samples were obtained for the initial allergy screen for allergen specific IgE- antibodies which included testing for allergy to fleas, food, Malassezia IgE, perennial (indoor) and pollen (outdoor) allergens. On the basis of positivity in the initial screen, the serum samples were tested for pollen allergens and food allergens (Table 2). The serology allergy testing for the pollen showed positives for privet and borderline positivity for oak. For food allergens, the IgE positives were egg and rice and the IgG positives included beef, lamb, chicken, turkey, soya bean, corn, wheat, rice and carrot (Table 2).

Skin sampling

Swabs were taken for bacterial and mycology cultures, and hair plugs for dermatophyte cultures. Acetate tape strips (scotch test) and deep skin scrapes for cytology were taken from the interdigital spaces, from the external auditory canals and from the medial aspect of the thighs.

The aerobic, anaerobic, mycotic and dermatophyte cultures did not grow any pathological microorganisms apart from normal cutaneous microbiota. In cytological microscopy examination, no ectoparasites, arthrospores, bacteria, yeasts, nor nucleated material were seen.

Full thickness skin samples were taken by 6 mm punch biopsy technique from each medial thigh for dermatohistopathology. The skin was closed with a single cruciate pattern using nylon (Ethilon; Ethicon) size 3.0 metric sutures which were removed 10 days postoperatively without complications. The histological diagnosis for both samples was perivascular dermatitis, lymphoplasmacytic, multifocal, mild with epidermal hyperplasia, orthokeratotic hyperkeratosis and mild dermal oedema which was consistent with secondary chronic degenerative changes of the skin due to poor control of the disease.

Upon completion of the surgery, the sevoflurane was discontinued and the patient was moved to recovery. The patient was injected with atipamezole (Sedastart; Animalcare) 25 $\mu g.kg^{-1}$ *im* in order to reverse the sedative effects of medetomidine. Once the swallowing reflex had returned the trachea was extubated. Following the general anaesthetic, meloxicam (Metacam; Boehringer Ingelheim) 0.2 $mg.kg^{-1}$ subcutaneously (*sc*) was administered for analgesia.

The patient was discharged from the hospital on the same day with an Elizabethan collar to protect the wounds. Oral formulation of meloxicam 0.1 $mg.kg^{-1}$ *po* was prescribed for three days starting the first dose 24 hours after the initial injectable dose.

DIAGNOSIS

Taking into consideration the patient's long history of pruritus, the positive response to glucocorticoids, the histological report and the positive results on serological ELISA testing, the author reached the diagnosis of CAD.

TREATMENT AND OUTCOME

For the management of environmental allergens, the patient was prescribed lokivetmab (Cytopoint; Zoetis) 1.11 $mg.kg^{-1}$ *sc*, every four weeks. Additionally, a highly hydrolysed hypoallergenic diet (Hill's prescription z/d Dog Food) was advised by gradual introduction in order to manage the food allergens. Lastly, the owner was advised to keep up to date with preventive parasitic treatment and hypoallergenic shampoo twice a week. The patient was reviewed four weeks later, where the clinical signs were fully resolved and the same lokivetmab dose was repeated. It was proposed to repeat the next lokivetmab dose five weeks later, however the pruritus and subsequently the dermal signs returned soon after the fourth week. Therefore, it was agreed with the owner to administer the lokivetmab every four weeks in order to manage the clinical signs sufficiently. It was advised to stop the chloramphetamine treatment at that point.

Later on, in springtime, there was a flare up of CAD due to the increased pollen levels, despite having the lokivetmab every four weeks. The clinical signs were resolved with a short acting glucocorticoid, dexamethasone (Dexadreson; MSD) 0.08 mg.kg^{-1} *im*.

For three years and up until the time of writing this case report, the patient has been on the same dose of lokivetmab injections every four weeks, hydrolysed diet, preventive parasitic treatment and regular hypoallergenic shampoo baths. In periods of flare ups and in particular during the springtime, the clinical signs are managed with short acting systemic glucocorticoid injections such as dexamethasone at a dose of 0.16 mg.kg^{-1} *im* and in some cases with oclacitinib (Apoquel; Zoetis) 18 mg.kg^{-1} *po* once daily for two weeks.

DISCUSSION

The methylprednisolone was replaced with lokivetmab due to the side effects that glucocorticoids can cause in long term use, but also due to the inadequate response to the former treatment. Lokivetmab has high efficacy with 87.8 % reduction of pruritus associated with CAD and can be administered every 4—8 weeks depending on the patient's needs, with rare side effects [25]. A study by Moyaert et al. demonstrated that lokivetmab at a minimum dose of 1 mg.kg^{-1} *sc*, repeated at monthly intervals, provided onset of effect in reducing pruritus within one day and continued efficacy for one month [22]. In the Marsella et al. [18] study, prednisolone, CsA, oclacitinib and lokivetmab were compared for the clinical efficacy on severity of dermatitis and pruritus, and also the effects on trans epidermal water loss and hydration. Lokivetmab showed that it can prevent flare ups when given prior to challenge and also that it has some positive effects on skin barrier parameters. A more recent study by Fleck et al. showed that a single subcutaneous injection of 2 mg.kg^{-1} lokivetmab produces a significant suppression of pruritus starting 3 hour post-treatment and be sustained for 42 days [5].

Another treatment option for management of CAD is CsA. This treatment was not considered by the author due to the high cost, the need for daily medicating, the immunosuppressive component and that CsA should be reserved for severe CAD cases where pruritus is inadequately controlled by standard antipruritic treatments [9]. Furthermore, lokivetmab's antipruritic efficacy is more pronounced than CsA, with fewer adverse effects [22].

Flare ups were managed by the author with dexamethasone injections and oral oclacitinib. Takahashi et al. demonstrated topical glucocorticoid treatment and systemic oclacitinib (inhibitor of proinflammatory cytokines) combination therapy for CAD patients is more effective than oclacitinib monotherapy [27]. A human comparative study showed that topical glucocorticoids achieved effective skin concentrations greater than the effective concentration achieved by oral corticosteroid treatment [20]. Based on these two studies, the author will consider the use of topical glucocorticoids instead of systemic ones in order to increase the skin concentrations by using fewer glucocorticoids in shorter periods of time.

Allergen-specific IgE serological (ASIS) testing was used by the author to aid the diagnosis due to the lack of in-house intradermal testing (IDT). Both techniques have identical interpretation of the results, however in a serological approach it is better to use techniques employing standardised procedures and high thresholds to ensure good specificity [9]. Both ASIS and IDT are designed to aid in the diagnosis of CAD and also to aid in the formulation of allergen specific immunotherapy (ASIT). It is also very important to remember that ASIS should not be used for screening, as false positive results are found in non-atopic patients [10]. Furthermore, there is poor correlation between ASIS and IDT testing [6] and the success rate of ASIT based on both methods is not significantly different [24].

ASIT, also known as anti-allergen vaccination or desensitisation, is a treatment option for managing the aeroallergens but not the food allergens [9]. This treatment approach was not considered due to the seasonal positive pollen allergens. It would not be beneficial for the patient to receive this treatment all year round when the pollen aeroallergens are only present during the spring and summer. Moreover, immunotherapy takes considerably longer to be effective. After 9—18 months, only 10—20 % of the patients showed a complete cure and 50—80 % of the animals showed significant improvement [8].

CAD shows identical clinical signs whether caused by food or environmental allergens [11], therefore the feeding diet that the patient receives plays an important role in the management of this disease. Cutaneous adverse food reactions and in this case the immune IgE and IgG mediated

hypersensitivity revealed positive results in serological allergy testing. Although many laboratories offer serological testing for IgE and IgG antibodies for food allergens, it is good to bear in mind that many studies have shown that this method is not a reliable way of diagnosing adverse food reactions [23]. Currently allergen specific IgE serology and intradermal allergy testing do not confirm canine adverse food reactions [12, 13]. The gold standard for diagnosis and management of food allergy is an elimination home cooked diet trial. However, this method can be very time consuming and extremely difficult to meet the nutritional requirements [26] of a dog and therefore the author did not consider this approach. The author, instead, recommended a commercial highly hydrolysed diet where the allergenicity is decreased [23]. Johansen et al. study showed some promising results for patch testing on the diagnosis of food allergy, however this is still in the experimental stage [15].

This case was managed with a good outcome. Lokivetmab was the main drug that kept the CAD under control, along with the diet and the baths. Flare ups are expected with CAD and it is a matter of managing them in the best possible way. There are many available treatment options, however each treatment must be tailored to the patient's need and to the owner's compliance.

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VACCINES FOR LYME BORRELIOSIS: FACTS AND CHALLENGES

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ABSTRACT

Lyme borreliosis (LB) is a multisystem infectious disease abundant in the northern countries of the world and is caused by *Borrelia* species. Vaccination against LB is an effective way to prevent and reduce the number of diseases in endemic areas. Several vaccines have been developed and tested in the past, but no human LB vaccine is currently available on the market. This review aims to uncover and delineate various strategies and diverse technological approaches related to vaccine production. Furthermore, we characterize already tested vaccines, possibilities for their future development, and reasons for their failure.

Key words: *Borrelia*; LB prevention; Lyme borreliosis; vaccination options

INTRODUCTION

Lyme borreliosis (LB) is one of the most common infectious diseases transmitted by arthropods in temperate regions of northern America, Asia, and Europe. LB is caused by spirochetes of the *Borrelia* species, in the USA it is *B. burgdorferi*, while in Europe and Asia it is *B. afzelii*, *B. garinii*, or *B. bavariensis* [20, 40]. There is currently a debate on the feasibility of separating LB into a separate genus *Borreliella*, including *B. burgdorferi* sensu stricto (s. s.) and other genospecies *B. burgdorferi* sensu lato (s. l.) complex [1]. The Centre for Disease Control and Prevention estimates that there are approximately 300,000 cases of LB per year in the United States, and the incidence in Europe is 230,000 cases per year [41, 44, 58].

Lyme borreliosis, one of the most common seasonal diseases transmitted by tick bites, is essentially a bacterial infection that is successfully treated with antibiotics (the fatal consequences of LB are very rare) [2]. However, the most important is the early detection of a tick bite (at the acute phase) and immediate antibiotic treatment. If the infection occurs without primary manifestations, the disease often progresses to a chronic stage, when antibiotic treatment is less effective and undesirable health complications can occur [48, 55].

Usually the symptoms of the disease range from erythema migrans (which is a typical manifestation of LB) to infections of the nervous system (neuroborreliosis), heart (Lyme carditis), joints (Lyme arthritis), or skin (acrodermatis chronica atroficans) [40] (Table 1).

Ticks *Ixodes* spp. go through a life cycle in three stages: a larva, a nymph, and an adult. Although some species of *Borrelia* can be transmitted transvariously, in *B. burgdorferi* s.l complex this does not happen (each generation of ticks must be re-infected) [47]. The life cycle of ticks *Ixodes* spp. is shown in Fig. 1.

VACCINES FOR LYME BORRELIOSIS

The vaccination against various infectious diseases is considered to be a highly effective means of controlling the spread of a given infection in a population [11]. Prophylactic vaccination against LB is a relatively attractive approach in preventing the risk of LB infection [12].

The development of new strategies in LB vaccination focuses on the host and its immunity, and the reservoirs of bacteria (including ticks and vertebrates themselves). There is a need to address how bacteria can be targeted via

Table 1. Stages and clinical features of Lyme borreliosis. Adapted from [57]

Stage		Clinical features	
Stage I (days-weeks)	Early and localized infection	Erythema migrans	Headache, fatigue, myalgia, arthralgia, malaise, fever
		Borrelial lymhocytoma	Skin manifestations occurring on the earlobe in children or on the nipple in adults (rare)
Stage II (weeks-months)	Systemic symptoms, disseminated infection	Lyme neuroborreliosis	Lymphocytic meningitis, headache, mild stiff neck, facial paralysis and radiculoneuritis (motor or sensory) Rarely cerebellar ataxia or encephalomyelitis
		Lyme carditis	Atrioventricular nodal blockade Pancarditis or cardiomegaly Acute myopericarditis or mild left ventricular dysfunction (unusual)
Stage III (months- years)	No systemic syndromes, localized infection	Lyme arthritis	Pain and swelling of the weight-bearing joints (knee), Persistent synovitis sometimes occurs
		Acrodermatitis chronic atrophicans	Slowly progressive lesion located on the acral parts of the limbs relatively rare and especially in older women.

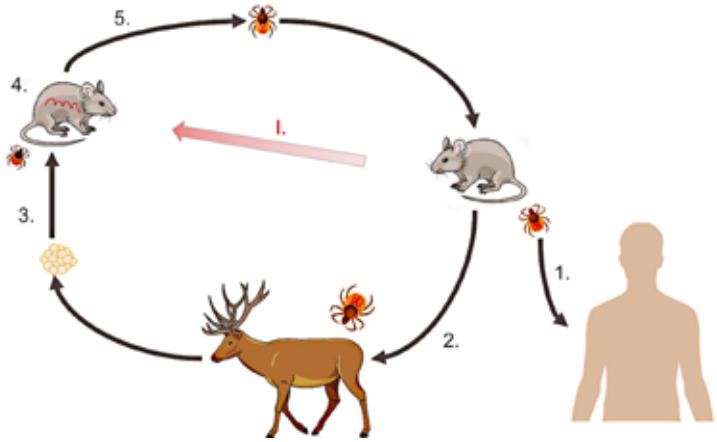

Fig. 1. The Enzootic cycle of *B. burgdorferi*

I—*B. burgdorferi* is kept in its reservoir host; 1—The infected nymphal tick can transmit *B. burgdorferi* to animals including humans, during feeding; 2—The nymphal tick moults to the adult stage, which feeds and mates on large mammals and lays eggs; 3—Eggs hatch into uninfected larval ticks; 4—*B. burgdorferi* is transmitted by larval tick feeding on an infected small mammal; 5—The larval tick moults to a nymph

various vaccine candidates for direct application in human and veterinary medicine. Importantly, it is crucial to disrupt the transmission of substances (agents) that maintain the enzootic cycle of *B. burgdorferi* and study how these indirect strategies will affect the incidence of an infection in a random host (human and domestic animals) [15].

Vaccines directed against spirochetes in the vector: outer surface protein A

Outer surface protein (OspA) is considered a leading vaccinogen against LB. OspA (31.5—33 kDa) is a surface protein expressed in the unfed midgut of ticks. OspA expression allows spirochete colonization and persistence in the vector by TROSPA (Tick receptor of OspA) binding [12]. Thus, antibodies directed against OspA can recognize and neutralize spirochetes in the vector in a complementary manner [8].

Intensive research into LB vaccines took place in the United States in the 1990s, when two different vaccines against LB appeared [39]. Both types of vaccines: LYMErix™ (SmithKline Beecham, now GlaxoSmithKline) and ImuLyme™ (Pasteur Mérieux Connaught, now Sanofi Pasteur) used recombinant OspA as the immunogen [42]. The mechanism of action was the same for both vaccines: the OspA vaccine-induced host antibodies, neutralized the OspA expressed by the spirochetes in the vector and thus blocked the transmission of pathogens from the vector to the host [26].

The LYMErix™ vaccine was marketed in 1998 and was voluntarily withdrawn from the market in 2002. The vaccine consisted of 30 µg of recombinant OspA lipoprotein expressed in *Escherichia coli* as an adjuvant with aluminum hydroxide. The specific OspA strain used in the vaccine was *B. burgdorferi* s. s. ZS7. The vaccine was administered intramuscularly at a dose of 0.5 ml in three doses (0, 1, and 12 months). The third phase of the clinical study involved more than 10,000 individuals from endemic areas, aged 15—70 years, the study was placebo-controlled. The efficacy of the vaccine was set at 76 % (after three doses), with no significant side effects observed at this stage [56]. However, the LYMErix™ had several disadvantages:

Its effectiveness was less than 80 %, which meant that the remaining 20 % of fully vaccinated people could still be infected with LB [42].

- Three doses were needed to gain full protection.
- There was a lack of vaccine testing in young children as a population at high risk of developing LB [59].
- The vaccine was only effective against a North American *Borrelia* strain (*B. burgdorferi*).
- It was not clear what the final length of vaccine-induced immunity would be whether the vaccine will not need to be re-administered each year [42].

The second vaccine developed was ImuLyme™. The vaccine itself consisted of recombinant OspA from *B. burgdorferi* s. s. strain B31 with no adjuvant used. ImuLyme™ has undergone the third phase of clinical trials (more than 10,000 subjects aged 18—92 years from LB endemic areas) [42]. The efficacy of the vaccine was determined to be 68 % after two doses and 92 % after three doses [53]. However, the vaccine manufacturer did not apply for its approval, nor did he specify exactly the reasons why he did so [42].

A second-generation OspA vaccine, VLA 15 (Valneva, France), has recently been developed and is currently in clinical trials. Clinically relevant OspA serotypes that cause most LB infections in Europe and North America have been used to develop the VLA 15 vaccine, as follows: *B. burgdorferi* (serotype 1-ST 1), *B. afzelii* (serotype 2-ST 2), *B. garinii* (serotype 3,5,6-ST 3,5), and *B. bavariensis* (serotype 4-ST4) [13, 38]. It is a multivalent protein LB subunit vaccine consisting of three OspA heterodimers (each heterodimer is a fusion of the C-terminal portion of OspA from two different serotypes with a 21 amino acid long linker) [8].

The vaccine is currently in the second phase of clinical trials, a randomized, observer-blinded, placebo-controlled study in endemic areas of the United States and Europe. The clinical trial involved 452 healthy subjects aged 18 to 65 years who received the vaccine (dose level 135 µg and 180 µg) intramuscularly in three doses on days 1, 29, and 57. Immunogenicity was determined as the number of IgG antibodies against each of the six most prevalent OspA LB serotypes at day 85 (i. e. one month after the end of the primary immunization) [62]. Preliminary results published on the vaccine manufacturer's website are as follows:

Vaccine VLA 15 is immunogenic in all tested groups (in the group from 18—49 years, the seroconversion rate ranged from 85.6—97 %). This vaccine is also safe at all doses tested and in all groups. The tolerability profile, including the occurrence of fever, is comparable to other lipid recombinant vaccines. Reactogenicity decreased af-

ter the first dose. The seroconversion rate (SCR) showed similar responses after primary vaccination and ranged from 93.8—98.8% [62]. The next step in clinical trials is a further randomized, placebo-controlled phase II under the following conditions: 600 healthy participants, aged 5—65 years (including the pediatric population aged 5—17), who will be vaccinated at a dose of 180 µg (based on the results already published). The aim is to compare two types of dose plans: initial vaccination (0—2—6 months) and reduced (0—6). The initial data from this study are expected in the second quarter of 2022 [61].

Vaccines directed against spirochetes in the host—outer surface protein C

There may be several strains of *B. burgdorferi* s. s. in one endemic area, and a high proportion of reservoirs may be infected by more than one strain, which means that patients with LB can be infected with another strain of *Borrelia* [6, 52] even after successful antibiotic treatment [21]. The outer surface protein—OspC (22 kDa) has long been considered a suitable candidate for an LB vaccine, because it's a highly polymorphic surface protein that is recognized by strain-specific neutralizing antibodies in infected hosts. Anti-OspC antibodies protect against infection, but only against OspC-expressing strains [9]. However, the scope of prevention is relatively narrow due to the specific intertype variation of epitopes, but on the other hand, these specific OspC epitopes allow the development of chimeric polyvalent recombinant OspC vaccines [10, 54].

In 1999, Immuno-AG (now Baxter, Austria) produced a 5-valent adjuvanted OspC vaccine composed of a recombinant OspC strain of *B. burgdorferi* and two strains from *B. afzelii* and *B. garinii*. After three administrations of the multivalent OspC vaccine, more than 95% of the subjects showed an antibody response. It was relatively well tolerated in phase I clinical trials, but approximately half of the subjects showed erythema and swelling at the injection site. Phase II safety and preliminary efficacy data have never been disclosed due to the reactogenicity of the skin at the site of application [54].

The reason for the failure of OspC vaccines was probably the fact that there is significant heterogeneity of OspC between *Borrelia* spp. Therefore, it is necessary to develop a vaccine that contains several heterologous epitopes, which would increase its efficacy [17].

Chimeric vaccines against LB

Chimeric vaccines can be characterized as subunit vaccines containing chimeric proteins that consist of linear epitopes derived from different proteins, i. e., their variants. The advantage of chimeritope vaccinogens is that they can be designed to induce a robust antibody response [18]. One of the chimeric vaccines that has already come into clinical use is the canine vaccine VANGUARD®crLyme (Tab. 2). This subunit chimeric vaccine consists of the outer surface protein OspA and 14 different linear epitopes-chimeritope of OspC. Combining the OspA and chimeritope OspC into a single vaccine has led to the development of a humoral immune response that has a synergistic effect on targeting spirochetes, both in ticks and mammals [35].

A six-component vaccine has been developed containing the outer surface protein OspA, which has been combined with bacterial ferritin to form self-assembling nanoparticles. OspA-ferritin nanoparticles have elicited high antibody titres for more than six months (in mice, and rhesus macaques) against infection by American, European, or Asian strains of *Borrelia* and offer their potential to prevent the spread of LB [20].

Vaccines based on blocking LB transmission

Transmission-blocking vaccines are divided into LB reservoir-targeted vaccines and anti-tick vaccines. The implementation of effective bacterial transmission-blocking strategies as a tool to control the incidence of LB depends on several factors: an understanding of the eco-epidemiological determinants (informing about the risk of potential LB in a certain geographical location) and the development of suitable vaccine carriers [15].

Vaccines targeting animal reservoirs affect the natural enzootic cycle and reduce the risk of LB by reducing the number of infected vectors. This hypothesis was tested as follows: mice (*Peromyscus leucopus*) were subcutaneously administered a recombinant OspA vaccine, which resulted in a reduction in the prevalence of nymphal infection after one year. OspA-based vaccines are effective against most species and strains of *B. burgdorferi* [36]. Various potential candidates for a transmission-blocking vaccine were evaluated:

- Surface proteins of *B. burgdorferi* (BB0405, BBA52, BBI39),

- Tick antigens (subolesin, salivary proteins, tick salivary lectin pathway inhibitor, tick histamine factor) [4—27].

The introduction of vaccines targeting LB reservoirs as a part of integrated pest management depends on the development of oral carriers for immunogen delivery [16, 50].

LB vaccines based on outer surface membrane vesicles

Outer surface membrane vesicles (OMVs) can be characterized as bilayer, spherical and membranous nanostructures with a size of 20—250 nm, which are released during the growth of various gram-negative bacteria [19, 45]. The OMVs are composed of lipopolysaccharides, phospholipids, outer membrane proteins, and entrapped periplasmic components. Due to their natural properties such as: immunogenicity, self-adjuvant and absorption by immune cells, they are considered to be attractive for vaccines development against various pathogenic bacteria, including *B. burgdorferi* [14].

A great advantage of OMVs is the modulation of the host immune response through the transfer of antigens (including heterologous ones) from other pathogens that have been expressed on the vesicular structure. Antigens generated by exposure to the surface of OMVs are capable of forming specific binding through B cells as well as antigens without surface exposure are capable of inducing an antibody response [34].

Recently, a vaccine against LB was developed based on native OMVs (nOMVs), which were isolated by EDTA extraction from *N. meningitidis* serogroup B OMVs with OspA *B. burgdorferi* B 31. The vaccine (40 µg meningococcal nOMVs) was tested in mice (females, from six to eight weeks) to which the vaccine was administered according to the following schedule: 0, 14, and 28 days, antibody titres were determined from blood serum on day 42 of the experiment. Subsequently (two weeks after vaccination with the last dose), mice were administered subcutaneously with *B. burgdorferi* N40 (1×10^5 spirochetes). Mice were sacrificed on day 62 and samples were collected from various tissues (skin, bladder, ear, heart, and ankle) that were cultured to detect spirochetes. The results of the experiment documented that the vaccine based on native OMVs induced high antibody titres. Furthermore, vaccinated mice were protected from infection revealing significantly lower amounts of spirochetes in analysed tissue [22].

Vaccines based on the principle of DNA against LB

The DNA vaccines against LB represent one of the innovative and alternative approaches as standard research on LB vaccines focuses on recombinant proteins [5, 23]. The DNA vaccines can be characterized as vaccines containing purified plasmid particles that contain transgenes encoding proteins or peptides that elicit an immune response (via T and B cell activation) against various diseases. The DNA vaccine can be considered immunogenic, safe (without significant side effects), and it can also be modified in a relatively short time [28]. One of the disadvantages of this vaccine is its lower efficiency in producing antibodies [43].

A codon-optimized bb0405 DNA vaccine was prepared according to the procedure published in [64] using the plasmid pVAC, which was tested as follows: The DNA vaccine was administered to mice, and the vaccine was found not to induce sufficient antibody production or protection against strain *B. afzelii* (mice were stimulated with a tick nymph infected with this spirochete). This phenomenon has been explained by the fact that *B. afzelii* spirochetes drastically reduce the expression of the BB0405 protein and thus the DNA vaccine does not provide cross-protection against *B. afzelii* in a mammalian host [23].

Recently a study was published in which the authors evaluated several DNA tattoo vaccines against borrelial OspC or tick antigens. One group of mice received an OspC DNA vaccine capable of generating a robust IgG response, with no positive culture of *B. burgdorferi* s.l. (after infection of mice with *I. scapularis* nymphs) present in the skin and bladder tissues of most mice. Tick Salivary Gland DNA vaccine (against TSLPI, Salp15, tHRF, as well as Tix-5) did not induce robust IgG responses in mice, and spirochetes were also detected in the skin and bladder tissues in *B. burgdorferi* infection in all individuals. The DNA tattoo vaccination can be considered as an effective vaccination platform aimed at evaluating new candidates of *B. burgdorferi* antigens in a relevant model [24].

Development of new LB vaccinogens on the omics approach

The availability of *in silico* methods and "omics" technologies such as genomics, proteomics, transcriptomics, or immunnomics have simplified and accelerated the development and selection of suitable vaccinogens for various diseases (including LB) [37]. The most likely com-

ponent of the vaccine is the protein expressed during the infection of the host. Not only the identification of a suitable vaccinogen, but also the definition of immunological correlates is important for vaccine development due to the choice of a suitable carrier—vectors, adjuvants and also the vaccination schedule [25]. Benc úrová et al. [3] identified the following proteins as potential candidates for the LB vaccine by interactome and orthological reconstruction: ErpX, ErpL, ErpY, and also VLP (Variable large protein). These proteins also have a suitable antigenic profile as well as a positive compartment localization, making them good vaccine candidates. [3].

Canine LB vaccination

LB is a tick-borne disease often found in dogs, but the majority of dogs do not become ill after infection [33, 51]. There are currently several commercially available vaccines for dogs on the market, such as VANGUARD®cr-Lyme (Zoetis, USA), Nobivac® (Merck Animal Health, Germany), Borrelym (Bioveta, Czech Republic), Duramune Lyme (Elanco, USA), which are recommended for vaccination of dogs in endemic areas of America and Europe. A comparison of several commercially available OspA vaccines was performed, where it was shown that the administration of LB vaccine in three doses significantly increased antibody titres more than administration of the vaccine in two doses [60]. High protection was also found in dogs given a bivalent vaccine consisting of a strain of *B. burgdorferi* and negative strains OspA and OspC. This approach resulted in high antibody titres against OspA and OspC [30]. One year after vaccine administration, OspA antibody titres decreased and OspC antibody titres disappeared. After the addition of the infected tick, 40 % of the vaccinated dogs were infected with *Borrelia*, and the infection was removed within two months and there was no dissemination of LB to other organs. Thus, vaccination of dogs with multiple Osp proteins is effective [29].

A summary of LB vaccines (for human and veterinary use) is in Table 2.

CONCLUSIONS

The development of an effective vaccine to prevent LB remains a major challenge, as the non-adoption of the first LYMErix™ vaccine by the public has hampered their further development.

Various potential candidates and strategies against LB are currently being discussed and researched. The leading vaccinogen against LB is considered to be the OspA expressed by bacteria of *Borrelia* species. Several vaccines have been developed on this approach: the first-generation vaccines, LYMErix™ and ImuLyme™, were based on the full-length OspA isolated from *B. burgdorferi* s.s. (serotype 1), which limited their use to the North American region. However, the effectiveness of the vaccine was at the level of 80 %. The recently developed VLA 15 vaccine (Valneva, France) is a multivalent OspA vaccine containing 6 different serotypes of *Borrelia* species. This vaccine is currently in clinical trials; partial results are given in the article. Other approaches to vaccination are based on the principle of blocking spirochetes in the host (on this principle a polyvalent OspC vaccine was developed which, however, due to the reactogenicity of the skin at the injection site did not get into practice) or blocking LB transmission at the reservoir, which, however, is conditioned by the development of a suitable oral carrier. New approaches in the development of anti-LB vaccines are chimeric vaccines, such as DNA-based vaccines, and OMVs-based vaccines. These perspectives are currently at the stage of intensive research but have not yet entered clinical practice.

There are several reasons why the LB vaccine is still not available. One reason is that vaccine production is time-consuming and technically demanding, as well as the fact that anti-LB vaccine production has been a subject of discussion from the beginning (insufficient documentation of serious adverse reactions in the first vaccines). Well, there is still a great need to develop vaccines against this disease, because several thousand cases of LB are reported every year. Among other reasons why human vaccines were not very successful was the fact that the disease against which the vaccine was supposed to protect is relatively well manageable even with antibiotic treatment (the fatal consequences of LB are very rare).

The aim is to develop a vaccine with the efficiency of at least 80 % for two years, which prevents the transfer of LB in Europe as well as in North America, that is tolerated (without cross-reaction epitopes and human proteins) and is also approved for the use in children.

Table 2. Comparison of human and available canine vaccines against Lyme borreliosis

Vaccine	Use	Principle	Vaccination schedule	Efficacy	Side effects	References
LYMErix™ (SmithKline Beecham, now GlaxoSmithKline)	Human	30 µg of lipidated recombinant OspA of *B. burgdorferi* s.s. strain ZS7 adsorbed onto the aluminum adjuvant	0 —1—12 months (dose 0.5 ml)	50 % after 2 doses 78 % after 3 doses	• muscle and joint pain • pain and redness at the injection site • fatigue • numbness and tingling • backache and headache	[7] [49]
ImuLymeTM (Pasteur Mérieux Connaught, now Sanofi Pasteur)	Human	30 µg recombinant OspA of *B. burgdorferi* s.s. strain B31 was used in the manufacture of the vaccine, without adjuvant	0—1—12 months (dose 0.5 ml)	68 % after 2 doses 92 % after 3 doses	No significant side effects were noted (in clinical trials)	[46] [53] [42]
VLA 15 (Valneva France)	Human	135/180 µg C-terminal part of OspA serotypes: *B. burgdorferi* (ST 1) *B. afzelii* (ST 2) *B. garinii* (ST3, ST5, ST6) *B. bavariensis* (ST 4) with aluminium hydroxide	0—2—6 or 0—6 months	SCRs showed similar responses after primary vaccination and ranged from 93.8—98.8 %	No related serious adverse events	[63] [62]
Multivalent OspC vaccine (Immuno-Ag, now Baxter)	Human	Recombinant OspC of: *B. burgdorferi* *B. afzelii* and *B. garinii* adsorbed onto the aluminum adjuvant	No information	Preliminary efficacy and safety data have not been disclosed	Erythema and swelling on injection side (in clinical trials)	[54]
VANGUARD®cr-Lyme (Zoetis, USA)	Veterinary	OspA and recombinant chimeric OspC of *B. burgdorferi*	0—21 days (dose 1 ml)	The efficacy of the vaccine was determined as an absence of development of antibodies to peptide C6, non-manifestation of clinical symptoms of LB	No information	[35]
Nobivac® Lyme (Merck Animal Health, Germany)	Veterinary	Two inactivated isolates of *B. burgdorferi*	0—14 (28) days	The efficacy of the vaccine was determined as induction of borreliacidal activity (OspA and OspC)	Swelling of soft tissue (temporary)	[29] [30]
Borrelym (Bioveta, Czech Republic)	Veterinary	Inactivated *B. burgdorferi* sensu lato (*B. garinii, B. afzelii*) and *B. burgdorferi* s. s.	0—14 (21) days	The efficacy of the vaccine was determined as induction anti-OspA antibodies against *Borrelia* spp.	Transient swelling (rare) Temporary increase of body temperature Allergic reaction (very rare cases)	[65]
Duramune Lyme (Elanco, USA)	Veterinary	Multiple types OspA, OspB, and OspC of *B. burgdorferi*	0—14 (21) days	92.2 % against natural infection in endemic areas	No information	[31] [32]

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CANINE INTRACRANIAL VENOUS SYSTEM: A REVIEW

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ABSTRACT

The intracranial venous system (ICVS) represents in mammals a complex three-dimensional structure, which provides not only for adequate brain perfusion, but has also a significant impact on: cerebrospinal fluid (CSF) resorption, maintaining of the intracranial pressure (ICP), and brain thermoregulation. An intimate understanding of the anatomy and physiology of ICVS is fundamental for neurological diagnostics, selection of therapeutic options, and success of neurosurgical procedures in human and veterinary medicine. Since the intracranial interventions in dogs are recently performed more frequently than twenty or thirty years ago, the authors decided to review and report on the basic knowledge regarding the complex topic of morphology and function of the canine ICVS. The research strategy involved an NCBI/NLM, PubMed/MEDLINE, and Clarivate Analytics Web of Science search from January 1, 1960, to December 31, 2021, using the terms "canine dural venous sinuses" and "intracranial venous system in dogs" in the English language literature; also references from selected papers were scanned and relevant articles included.

Key words: dog; function; intracranial venous system; morphology

INTRODUCTION

The fact that the central nervous system in humans, apes, and other mammals receives: a prominent part of the cardiac output, demonstrates the key role which the cerebral blood circulation plays in maintaining adequate perfusion of the brain, and copes with its high metabolic demands is very important [16, 43]. The intracranial vasculature consists of arterial and venous parts. The intracranial venous system (ICVS) is composed of: the dural venous sinuses (DVSs), dorsal cerebral veins (DCVs), ventral cerebral veins (VCVs), cerebellar veins (CbVs), meningeal veins (MVs), emissary veins (EVs), and diploic

veins (DVs). In comparison with the arterial compartment, the venous one contains about 70—80 % of the circulatory volume inside the skull [12, 16, 34, 43, 49]. Detailed knowledge of: the morphology, composition, and function of DVSs, their tributaries, important connecting veins, and potential occurrence of their anomalies is essential for neurological diagnostics, assessment of therapeutic options, and the success of neurosurgical interventions in both, human as well as in veterinary health care practice [1, 3, 7, 12, 15, 26, 32, 39, 49]. Since traumatic events as well as different pathological processes involving the skull, meninges, brain, vertebral column, and spinal cord in humans, also occur in big mammals, the structure and function of their ICVSs are similar; the porcine, canine, ovine, and feline models are often used in experimental and clinical neuroscience for translational research [10, 13, 14, 22, 24, 26, 28, 30, 34, 35, 37, 47, 51, 53—56, 58, 59]. Besides, there are owners willing to pay considerable sums of money for the treatment of their pets, the intracranial procedures in cats and dogs are performed more and more frequently during the last decades [18, 22, 31, 40, 46, 50, 52, 53]. One of the significant hazards of craniotomy and brain surgery is an injury of important venous structures. It may cause profuse bleeding, air embolism, thrombosis, development of brain oedema and/or venous haemorrhagic infarction, i. e., serious complications potentially leading to a permanent neurological deficit, even fatality [2, 48, 62]. The above-mentioned reasons inspired the authors to concentrate on the complex subject of the canine intracranial venous system.

MATERIALS AND METHODS

English language literature published between January 1, 1960, and December 31, 2021, was searched in the PubMed/MEDLINE, and Clarivate Analytics Web of Science databases using specific strings of terms, for example: "canine dural venous sinuses", "intracranial venous system in dogs", "cerebral venous system in domestic mammals"; also references from selected papers were scanned and relevant articles included. The titles and abstracts were screened by two independent reviewers (I. L., M. G.) and selected publications were critically evaluated and assessed for their quality and relevance. The literature search yielded 362 articles of which 63 met the inclusion criteria given by the 62-year time interval, by the above-mentioned string of terms, and by the preference of the sources that underwent the peer-review process. Papers published in almanacs, eventually in collections of abstracts were excluded. Any disagreements between reviewers were resolved by the consensus of all authors involved.

Venous drainage of the canine skull and intracranial structures

The venous blood from the canine cerebrum, cerebellum, meninges, and skull bones is collected by DCVs, VCVs, CbVs, EVs, DVs, and MVs to DVSs which transport it into the systemic circulation via paired maxillary, internal jugular, and vertebral veins, as well as via the internal vertebral venous plexus [3—5, 12, 34, 39, 49]. The vascular structures (besides DVSs), are valveless, thin-walled vessels lacking *tunica media* and *tunica adventitia* [12, 49]. By contrast, the walls of DVSs (*sinus durae matrix*) in mammals are thicker, but the structure and quality of individual sinus walls may significantly differ, which is particularly important for neurosurgeons [7, 8].

The dural venous sinuses

The DVSs are usually situated between the periosteal and meningeal layers of the *dura mater*; in dogs, they also can run inside the canals within the cancellous layer of skull bones (*diploë*). The walls of DVSs are composed of connective tissue, that consists of collagen and elastin fibres with fibrocytes, sometimes also myocytes, i. e., plain muscle cells. The inner surface of the DVSs is covered by a smooth layer of endothelial cells [1, 17, 20, 45]. Inside DSS and TrSs in humans and in some animals (sheep) occur *chordae Willisii* [7, 26]. Besides the draining function, the dorsal sagittal sinus (DSS) also participates in the resorption of *liquor cerebrospinalis* (cerebrospinal fluid—CSF), which is accomplished via villi of arachnoid granulations situated inside the DSS and TrSs [21, 26, 31, 38, 58]. Since the intracranial veins have no valves, they are able to develop alternative channels, e. g. in the case of their obstruction [42, 43, 49]. Generally, there are two main systems providing for venous outflow from the canine cerebrum—a dorsal and ventral one [12, 15, 19, 23, 39].

The dorsal system of dural venous sinuses is composed of the DSS (*sinus sagittalis dorsalis* in Latin), the straight sinus (SS—*sinus rectus*), and usually paired (right and

left) transverse sinuses (TrSs—*sinus transversus dexter et sinister*). The TrSs are usually paired, but one may be dominant, the other recessive, even absent [12, 13, 49].

The DSS begins at the cribriform plate of the ethmoid bone by the confluence of the right and left nasal veins draining the venous blood from the nasal septum, its mucous membranes, the olfactory bulbs, and adjacent meninges, and external ethmoid veins draining the right/left ophthalmic plexus [12, 14, 26]. The DSS runs caudally within the dorsal margin of the *falx cerebri* ventral to the sagittal suture between the parietal bones and the interparietal process of the occipital bone. Before the DSS enters the occipital bone through a special foramen (*foramen sinus sagittalis dorsalis*) and subsequently terminates by merging with transverse sinuses (TrSs) in the confluence of sinuses (CoSs) inside the *diploë* of the occipital bone (*os occipitale*), it is usually joyned by StS. The venous blood from the cortex of frontoparietal lobes of cerebral hemispheres is drained by three to five irregularly located pairs of DCVs (*venae cerebri dorsales*) entering the rostral segment of the DSS. The venous blood from the cortex of occipital lobes of cerebral hemispheres is usually drained by two pairs of DCVs entering the caudal segment of the DSS [4, 12, 39, 49]. The DCVs (also termed the bridging veins) traverse the subdural space, i. e. the gap between the cerebral cortex and the *dura mater*, which they penetrate and enter the DVSs. The walls of the bridging veins are composed of connective tissue with circumferentially arranged collagen fibres, but they lack the outer reinforcement by arachnoid trabeculae [20]. This feature explains why their subdural portion is more fragile and prone to laceration, which usually results in the development of subdural hematoma [16, 20, 30, 32].

The venous blood from the dorsal cerebellar cortex is drained by several dorsal cerebellar veins (DCbVs—*venae cerebellares dorsales*) into the right or left TrS. The venous blood from the ventral parts of the cerebellar hemispheres is collected by tiny ventral cerebellar veins (VCbVs—*venae cerebellares ventrales*), also draining into the TrSs [4, 39, 49].

The venous blood from the white matter of: the cerebral hemispheres, the caudate nucleus (*nucleus caudatus*), the diencephalon (the small parts of the brain located on either side of the 3rd ventricle, composed of the *thalamus, hypothalamus, epithalamus,* and *subthalamus*), the choroid plexus (*plexus chorioideus*) of the 3rd ventricle, and the mesencephalic tectum (*tecum mesencephali*, i. e., the dorsal part of the midbrain), is drained by the right or left thalamostriate vein (TsV— *vena thalamostriata*) to the paired, i. e., right or left internal cerebral vein (ICV—*vena cerebri interna*) [6, 19]. The right and left ICVs merge, affiliate the unpaired vein of the corpus callosum (VCC—*vena corporis callosi*), and drain the venous blood into the unpaired great cerebral vein (GCV—*vena cerebri magna*). The GCV joins the unpaired straight sinus (StS—*sinus rectus*), which enters the DSS, the principal constituent of the confluence of the sinuses (CoSs—*confluens sinuum*). However, the StS also can enter the occipital bone via an accessory foramen and merge separately with the CoSs [3, 13, 15, 19, 39, 41].

The ventral system of dural venous sinuses is composed of the right and left cavernous sinus (CS—*sinus cavernosus dexter et sinister*) interconnected by the rostral and caudal intercavernous sinus (IcS—*sinus intercavernosus rostralis et caudalis*), right and left temporal sinus (TS—*sinus temporalis dexter et sinister*), right and left dorsal petrosal sinus (DPS—*sinus petrosus dorsalis dexter et sinister*), right and left ventral petrosal sinus (VPS—*sinus petrosus ventralis dexter et sinister*), right and left sigmoid sinus (SS—*sinus sigmoideus dexter et sinister*), as well as right and left basilar sinus (BS—*sinus basilaris dexter et sinister*) interconnected usually by an interbasilar sinus (IBS—*sinus interbasillaris*), located at the rostral rim of the great foramen of the occipital bone (*foramen magnum ossis occipitalis*). Exceptionally there are two (rostral and dorsal) interbasillar sinuses. The right/left BS is connected with the cranial segment of the ipsilateral ventral vertebral vein (*vena vertebralis*) by the emissary veins of the hypoglossal canal, i. e. *vena emissaria canalis hypoglossi* [1, 3, 12, 15, 39, 49].

The venous blood from the cortex of the temporal lobes is drained by VCVs (*vena cerebri ventralis dextra* and *sinistra*) into the right or left DPS, which joins the right or left TS. TSs pass through the right or left temporal meatus (*meatus temporalis*) to the retro auricular foramen (*foramen retroauriculare*). Here the TSs become emissary veins of the foramen (*vena emissaria foraminis retroauricularis dextri* or *sinistri*) draining into the right or left maxillary vein (MaV—*vena maxillaris dextra et sinistra*) [3, 5, 12, 14, 21, 41].

The venous blood from the ventral parts of the midbrain and the pons is drained into the right or left SS; the

venous blood from the dorsal medulla and choroid plexus of the 4th ventricle (*ventriculus quartus*) also is drained into the SSs. The SSs after merging with the VPSs continue as the right or left BS caudally, draining venous blood into the ventral internal vertebral venous plexus. The venous blood from the ventral medulla is drained via the tiny medullary veins directly into the BSs, which merge with the ventral internal vertebral venous plexus [3, 6, 12, 49].

The cavernous sinus (CaS—*sinus cavernosus*) is the paired venous sinus, actually trabecular dural venous structure, located parallel to either side of the hypophyseal fossa (*fossa hypophyseos*) on the floor of the cranium, which contains the pituitary gland. The CaS extends from the orbital fissure (*fissura orbitalis*) to the apex of the pyramid of the temporal bone (*pyramis ossis temporalis*), where it joins the right or the left VPS. The VPSs, passing through the right or left intraosseous petrooccipital canal (*canalis petrooccipitalis*), connect the caudal parts of the right or left CaS with the ipsilateral SSs. Each SS then fork to the internal jugular vein (IJV) rostrally, and to the vertebral vein (VV) caudally [3, 12, 31, 49]. Diagrams of the canine intracranial venous system—see Fig. 1 and Fig. 2.

The emissary veins

The EVs are valveless, thin-walled vascular structures connecting the extracranial venous system (e. g. the cutaneous or subcutaneous veins) with diploic veins and dural venous sinuses [12, 49]. Under normal physiologic conditions, deoxygenated blood is drained through EVs from extracranial to intracranial space. However, the direction of blood flow may be reversed, which helps to decrease the elevated intracranial pressure (ICP), caused by different pathological conditions, such as brain oedema, cerebral congestion, and dural venous obstruction. This way EVs (analogous to the nasal veins, the rostral tributaries of the DSS) participate in cerebral thermoregulation by protecting the brain against a rise in intracranial temperature [49]. However, the EVs also may help spread the extracranial infections into the ICVS and the brain [34]. The specific EVs get their names according to the foramina or the canals they pass through [12, 19, 44, 49].

The main emissary veins are:

a) The right/left mastoid emissary veins (MsEmVs) originate from the distal parts of TrSs or from the SSs, penetrate the occipital bone, emerge on the surface of the right or left mastoid process, and anastomose with the

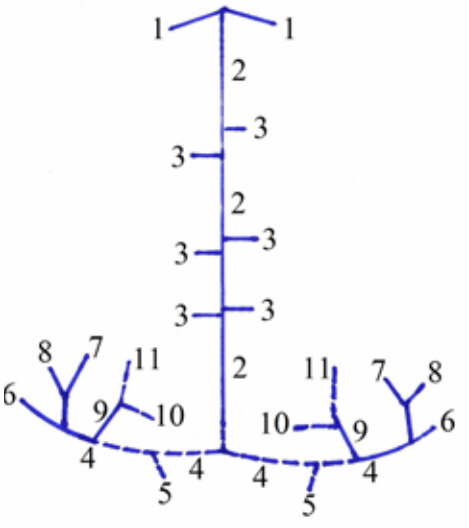

Fig. 1. The diagram of the canine intracranial venous system, dorsal view
1—veins from the nasal cavity; 2—dorsal sagittal sinus; 3—dorsal cerebral veins; 4—transverse sinuses; 5—occipital emissary veins; 6—temporal sinuses; 7—dorsal petrosal sinuses; 8—ventral cerebral veins; 9—sigmoid sinuses; 10—basilar sinuses; 11—ventral petrosal sinuses

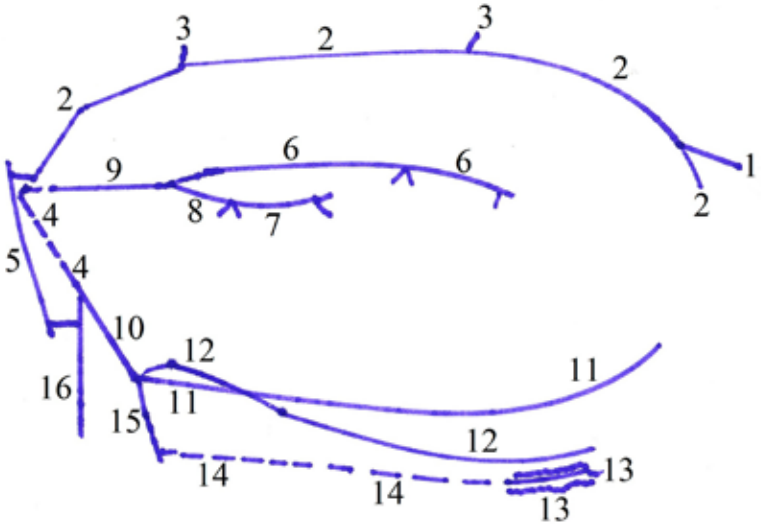

Fig. 2. The diagram of the canine intracranial venous system, lateral view
1—vein from the nasal cavity; 2—dorsal sagittal sinus; 3—frontoparietal diploic veins; 4—transverse sinus; 5—occipital emissary vein; 6—vein of corpus callosum; 7—internal cerebral vein; 8—great cerebral vein; 9—straight sinus; 10—temporal sinus; 11—dorsal petrosal sinus; 12—ventral cerebral vein; 13—sinus cavernosus; 14—ventral petrosal sinus; 15—sigmoid sinus; 16—basilar sinus

occipital or the dorsal auricular veins, the tributaries of the external jugular veins. The MsEmVs may be absent uni-, even bilaterally, but may also be multiple [12, 19, 23, 44, 49].

b) There are three sets of the condylar emissary veins (CoEmVs). The ventral CoEmVs connect the right/left internal jugular vein with the system of the internal vertebral venous plexuses. The lateral CoEmVs and the dorsal CoEmVs connect the right/left internal jugular vein with the system of the external vertebral venous plexuses [12, 19, 23, 49].
c) The right/left emissary vein of the retroauricular foramen (EmVRaF) originate in the right or left TS, leave the skull through the ipsilateral retroauricular foramen and join the right or left maxillary vein caudal to the temporomandibular joints [12, 19, 23, 44, 49].
d) The right/left emissary vein of the round foramen (EmVRdF) originate in the CaS, pass through the right or left round foramen together with the maxillary branch of the trigeminal nerve, continue through the alar canal (where it joins the ophthalmic plexus) until it caudally joins the maxillary vein and veins of the pterygoid plexus. The above-mentioned extracranial veins and EmVRdF can again act as potential passages for transmitting the infection to ICVs [12, 19, 49, 61].
e The right/left emissary vein of the oval foramen (EmVOvF) connects the CaS with the ipsilateral pterygoid venous plexus. The EmVOvF passes the oval foramen together with the mandibular branch of the trigeminal nerve [12, 19, 44, 49, 61].
f) The right/left emissary vein of the *foramen lacerum* (EmVLaF) originates in the place where join the CaS with the VPS traverse the right/left *foramen lacerum* and enters the ipsilateral maxillary vein [12, 19, 23, 44, 49].
g) The right/left emissary carotid canal venous plexus (EmCarCanVP) passes via the carotid canal together with the right/left internal carotid artery. The EmCarCanVPs) connect the CaS with the right/left internal jugular veins [12, 19, 49].
h) The right/left emissary vein of the hypoglossal canal (EmVHgC) connects the ipsilateral BSs with the internal jugular veins (IJVs), or the cranial parts of the vertebral veins (VVs) [12, 19, 49, 61].
i) There are two emissary veins connecting the ophthalmic plexus with the extracranial venous system. The first one is the right/left external ethmoidal vein (eEtmV) which passes together with the ipsilateral ethmoidal artery through the ethmoidal foramen and enters the right/left ventral ophthalmic vein. The second one is the right/left frontal diploic vein passing through the right/left supraorbital foramen which connects the venous plexus located inside the *diploë* of the frontal bone with the ipsilateral dorsal ophthalmic veins [12, 19, 44, 49].

The diploic veins

DVs are considered an integral component of the intracranial venous system [3, 19, 39]. They are formed by thin-walled, valveless vessels which collect venous blood from the skull bones and transport it to the DVSs [49]. There are three groups of diploic veins draining specific areas of the canine skull:

a) The frontal diploic veins (FDVs—*venae diploicae frontales*) drain venous blood from the cancellous layer of the frontal bone via extracranial anastomoses into the right or left angular vein of the eye, eventually via intracranial anastomoses into the rostral DCVs, or directly to the rostral segment of DSS [12, 23, 31].
b) The parietal diploic veins (PDVs—*venae diploicae perietales*) begin in the cancellous layer of the central area of the parietal bones, anastomose with the occipital diploic system and via intracranial anastomoses drain the venous blood into the DSS, or DCVs [12, 23, 34].
c) The occipital diploic veins (ODVs—*venae diploicae occipitales*) begin in the cancellous layer of the occipital bone, anastomose with parietal diploic venous system and via intracranial anastomoses drain venous blood into the right, left, or both TrS. Frequently there are several ODVs located bilaterally [12, 23, 34].

The meningeal veins

Other significant components of the ICVS are meningeal veins [19, 49]. There are usually two groups of bilaterally located, thin-walled and valveless vessels, that collect venous blood from the *dura mater*—the rostral and middle meningeal veins:

a) The rostral meningeal veins (RMVs) are tiny vessels that begin in the meninges covering the frontal lobes of the cerebral hemispheres. They perforate the inner layers of the frontal bone and drain the collected venous

blood into the frontal diploic veins in the region of the cribriform plate, i. e. *lamina cribrosa* [12, 39].

b) The middle meningeal veins (MMVs) pass together with the middle meningeal arteries (MMAs) in the *dura mater* overlying the lateral surface of the cerebral hemispheres. The impression of the MMA (*sulcus arteriae meningeae mediae*) is visible on the internal surface of the parietal and temporal bone [44]. The middle meningeal artery enters the cranium via its foramen (*foramen arteriae meningeae mediae*); the MMVs join the right or left CAS. Into the oval foramen, the MMVs issue the right or left emissary veins (*vena emissaria foraminis ovalis*) connecting the cavernous sinus with the ipsilateral maxillary vein [12, 19, 39].

Drainage of the intracranial venous blood to the systemic circulation

The SSs (also named the connecting sinuses) collect the deoxygenated blood from the dorsal intracranial vascular network via the TrSs, from the ventral intracranial vascular network via the DPSs and VPSs [19, 39]. The SSs, therefore, are the principal components of the intracranial draining system returning the venous blood from the brain and the skull via the maxillary, internal jugular, external jugular, and vertebral veins, eventually internal vertebral venous plexuses to cranial or caudal vena cava (*vena cava cranialis/vena cava caudalis*), to the right atrium (*atrium dextrum cordis*), and systemic circulation [12, 49]. This task is accomplished via several specific routes. The right maxillary vein drains to the cranial vena cava via the right external jugular vein and the right brachiocephalic trunk. The left maxillary vein drains venous blood to the cranial vena cava via the left external jugular vein. The internal jugular veins are direct tributaries of the cranial vena cava, terminating in the right atrium [12, 19, 49]. The left VVs drain via the hemiazygos vein to the azygos vein, and the right VVs drain directly to the azygos vein, the tributary of the cranial vena cava. The longitudinal internal vertebral venous sinus drains the deoxygenated blood from the spinal canal via segmentally arranged intervertebral veins, passing through the intervertebral foramina together with the spinal nerves. In the neck, the intervertebral veins drain into the right/left VVs, while in the thoracic cavity, the right intervertebral veins drain directly into the azygos vein; the left intervertebral veins, into the hemiazygos vein, and then to the azygos vein. In the cranial part of the abdomen the intervertebral veins drain via the azygos vein into the cranial vena cava; and in the caudal part of the abdomen into the caudal vena cava [12, 19, 49]. The valveless craniospinal venous system freely anastomoses with the thoracic, abdominal, sacral, as well as pelvic veins, and venous plexuses, thus providing a route for intraspinal or intracranial propagation of cancer cells or bacterial emboli and spread neoplastic or septic metastases [11, 25]. The prolonged or chronic increase of intraabdominal pressure or compression of the caudal vena cava (e. g. by gravidity, intraabdominal tumour, ascites) may also participate in the development of idiopathic intracranial hypertension [57].

The variations in size and shape of the cranium in different breeds of *Canis lupus familiaris* (e. g. in brachycephalic, mesocephalic, or dolichocephalic dogs) principally influence neither the elementary morphology nor function of the ICVS [14, 15].

Physiology of intracranial venous circulation

According to the Monro-Kellie hypothesis, the sum of brain volume plus CSF volume, plus cerebral blood volume in an intact skull remains constant [43, 60]. Therefore, any increase in one of these parameters causes a reduction in one or both of the remaining two. However, circulating arterial blood volume is not reduced, as the venous pressure is much lower and the volume of venous blood in the ICVS considerably exceeds the volume of blood inside the cerebral arteries [43]. The pulsatile arterial blood flow generates the synchronous pulsations of the brain and this phenomenon positively influences the intracranial venous blood flow. Generally, the intracranial pressure (ICP) provides for the stability of the diameter of thin-walled cerebral veins. The continuous blood flow in these vessels is maintained by the residual intracapillary pressure (*vis a tergo*), by the transmission of brain pulsations (*vis a latere*), and supported by the progressively decreasing (in an upright position negative) pressure in the intracranial venous sinuses (*vis a fronte*) [16, 43].

The veins in the superficial layers of the neck collapse when the surrounding pressure exceeds the venous pressure. However, veins inside the vertebral canal do not collapse. When a dog is in the sitting position, the venous pressure in the neck is usually less than atmospheric pressure. In this situation, the superficial neck veins collapse, but deeply located ones do not. Therefore, the venous blood from the head of mammals is drained via the

collapsible as well as noncollapsible channels. The blood flow in the collapsed vessels is governed by the Starling resistor principle, in the non-collapsed vessels by Poiseuille's law [27, 29, 33, 36, 60]. Both mechanisms help to maintain steady brain perfusion as well as the stability of intracranial pressure (ICP). Several experiments in dogs have shown that CSF pressure is higher than the pressure within the DSS and that an acute elevation of CSF pressure is not followed by increased pressure in the sinus. These data imply that there is a pressure gradient encouraging the transport of CSF to the sinus. It also demonstrates that the ICVS is able to compensate for the acute changes in CSF pressure by acting as a low-pressure runoff for the subarachnoid veins [47]. Poiseuille's law defines the velocity of the steady movement of a liquid flowing through a narrow tube (e. g. blood vessel). The value of this quality is directly proportional to the pressure gradient of the liquid between both ends of the tube and to the fourth power of its radius and is inversely proportional to the viscosity of the tube and its length [36]. The Starling resistor maintains constant flow through collapsible tubes (e. g. cortical draining veins) located in the subarachnoid space surrounded by CSF subjected to the influence of the fluctuating pressure in the rigid skull, as well as prevents siphoning of venous blood from the ICVS to the systemic circulation, and over drainage of CSF from the cranial to the spinal compartment [33, 60]. The Starling resistor is a site of compression (a so-called choke point) at the junction between the bridging vein and the DSS when the pressure in the DSS drops and becomes negative during the upright positioning of a man or an animal. In this situation, the higher CSF pressure compresses the downstream connection to the DSS and prevents venous over drainage [27, 33]. Concurrently, the pressure in the cerebral veins proximal to the choke point is maintained at a higher level than CSF pressure due to the created back force. Thus the Starling resistor helps to maintain pressure hierarchy among the liquid compartments in the cerebrum, i. e. the arterial inflow pressure is higher than the cerebral venous pressure, it is higher than the subarachnoid CSF pressure, and this is still higher than the dorsal sagittal sinus pressure [27, 60]. By contrast, this mechanism creates a pressure gradient that supports CSF drainage into the DSS via capillaries in arachnoidal villi [12, 27, 38, 60]. In the upright position of the body, the internal jugular veins collapse due to the influence of surrounding atmospheric pressure, but the rigid vertebral column seems to prevent epidural veins to collapse, and the negative epidural pressure may promote drainage of the venous blood from the skull via the noncollapsible vertebral venous plexuses [9, 29, 33, 43]. The place where the craniospinal axis gets atmospheric (the zero point) is usually at the level of the *foramen magnum* [33]. The intracranial and spinal CSF compartments freely intercommunicate through the *foramen magnum*. Under physiologic conditions, the CSF pressure is approximately 10—12 cm H_2O in the dog in a horizontal position. In the upright (e. g. in sitting) position of a dog, is the CSF pressure at the level of *foramen magnum* about 5 mm H_2O, but at the caudal end of the spinal canal, it can reach 30—40 cm H_2O, due to the hydrostatic pressure of CSF [27, 43]. The *dura mater* in the skull adheres to the inner surface of the cranium. By contrast, there is the epidural space between the spinal dural sack (*saccus dura matris spinalis*) and the vertebral canal, filled with compressible fatty tissue and venous epidural plexuses, and its capacity in dogs varies between 100 and 300 ccm. This reservoir helps to absorb the CSF (and intracranial) pressure fluctuations [43]. Even during expiration is the intrathoracic (intrapleural) pressure subatmospheric, i. e. about 5 cm H_2O. An inspiration usually causes the further decrease of the intrathoracic pressure (to approximately 8 cm H_2O). This negative pressure helps to keep dilated the intrathoracic veins and supports the return of deoxygenated blood from the head to the right atrium [43, 63].

In most quadrupeds, including dogs, the longitudinal axis of the skull and the centreline of the vertebral canal, as well as centre lines of major veins in the atlantooccipital region meet at an obtuse angle [44]. When the animal is placed in a non-physiological supine position (e. g. for abdominal surgery or imaging procedure), its head is reclined. Accordingly, jugular and vertebral veins become stretched and/or kinked, which significantly reduces cerebral venous outflow. This may cause venous congestion and a concomitant increase of the intracranial pressure, potentially damaging the brain [27, 33].

CONCLUSIONS

The canine cerebral venous system is composed of DVSs, as well as DCVs, EVs, DVs. DVSs are located between a periosteal and meningeal layer of the *dura mater*,

eventually inside the diploic canals of cranial bones. The intracranial veins are thin-walled, valveless vessels, DVSs are noncollapsible channels. The ICVS is divided into the dorsal and ventral systems. The dorsal system consists of the DSS, StS, TrSs, and their tributaries, i. e. GCV, dorsal and ventral CbVs, DVs, and EVs. The ventral system consists of the CsS, ICsS, DVPs, VPSs, TSs, SSs, BS, IBS with their tributaries, i. e. VCVs and MVs. The ICVS drains the venous blood to the systemic circulation via MaVs, IJVs, EJVs, and vertebral venous plexuses. The steady brain perfusion as well as CSF resorption, and stability of ICP in the rigid canine skull are maintained by the Starling resistor mechanism, the Poiseuille's law, and the Monro-Kellie doctrine. The presented paper reports on basic knowledge regarded the complicated topic of ICVS. Further studies dealing with the canine ICVS variations as well as with the canine dural sinus wall microstructure are planned.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ZEARALENONE BIODEGRADATION BY THE *LACTOBACILLUS* SPP. AND *BACILLUS* SPP. *IN VITRO*

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ABSTRACT

In this study, the biodegradation of zearalenone (ZEN) by cell suspensions of various *Lactobacillus* species (*Lb. fermentum* 2I3 (L1), *Lb. reuteri* L26 (L3), *Lb. plantarum* L81 (L4), *Lb. reuteri* 2/6 (L5), *Lb. plantarum* CCM 1904 (L6)), *Bacillus subtilis* CCM 2794 (Bs), and *Bacillus licheniformis* CCM 2206 (Bl); was investigated *in vitro*. All lactobacilli cell suspensions showed very good degradation efficiency (57.9—100 %) for zearalenone at the concentration 0.01 ppm. At higher concentrations of zearalenone, their biodegradation activity decreased significantly (0—13.9 %). *Bacillus subtilis* CCM 2794 was able to degrade zearalenone at concentrations of: 0.01 ppm (100 %), 0.1 ppm (74.5 %), and at higher concentrations of ZEN (1 ppm; 10 ppm), the degradation was 11.7 % and 0 %, respectively. For *Bacillus licheniformis* CCM 2206, no biodegradation of zearalenone was observed at the concentration of 10 ppm, but slight degradation (4.5—8.8 %) was found at lower zearalenone concentrations.

Key words: *Bacillus* spp.; degradation; *Lactobacillus* spp.; mycotoxins

INTRODUCTION

Mycotoxins are a large group of secondary metabolites of microscopic filamentous fungi. Fungal secondary metabolites threaten human and animal health worldwide by contaminating food and feed. More than 300 kinds of mycotoxins with different structures have been identified so far [5, 18]. Mycotoxins have no biochemical significance for fungal growth or development [19]. Mycotoxin production depends on environmental factors, such as: relative humidity, ambient pH, temperature and season. Other factors that affect mycotoxin production include: the presence of oxygen, thermal damage, mechanic damage to grain integrity during crop harvesting, damage to grain integrity by insects, plant variety, plant density, soil structure, fungal inoculum quality and vegetation stage [4]. One of the most important mycotoxins in the world is zearalenone (ZEN). It is synthesized by the microscopic

filamentous fungi *Fusarium* spp. (mainly by *F. graminearum, F. culmorum* and *F. sporotrichioides*) belonging to the field fungi.

Chemically, zearalenone is a 6-(10-hydroxy-6-oxy-trans-1-undecenyl)-beta-resorcylic acid lactone with the general formula C18H22O5. ZEN is a non-steroidal oestrogen mycotoxin which is biosynthesized via the polyketide pathway. Optimal conditions for the production of ZEN by fungi are characterized by temperatures between 20 and 25 °C and humidity above 20 %. The high levels of zearalenone are frequently found in countries with a mild and wet climate [15]. Zearalenone has immunotoxic, hepatotoxic, xenogenic, hematotoxic and other effects.

The most significant negative effect of zearalenone is its strong estrogenic activity. This activity in living organisms depends on the immune status of the organism and the state of the reproductive system. The estrogenic effects of zearalenone include: reduced fertility or infertility, vaginal prolapse, vulvar swelling and breast enlargement in females, feminization of testicular atrophy, and enlargement of the mammary glands in males [1]. Pigs and ruminants are the most sensitive to the effects of ZEN while the most resistant ones are birds, such as chickens and poultry [13].

In humans, zearalenone toxicity is predominantly chronic. ZEN and its metabolites can stimulate the growth of mammary gland cells and may be involved in breast cancer. However, ZEN is included in noncarcinogenic agents to humans, Group 3, according to the International Agency for Research on Cancer (IARC) [1, 3].

Various methods are employed to eliminate mycotoxins. Decontamination methods are divided into three basic groups: physical, chemical and biological [12]. The biological methods based on the degradation and transformation of mycotoxins by different strains of bacteria and yeasts are currently preferred [14]. Yeasts and lactic acid bacteria are involved in the biological adsorption of mycotoxins and prevent their absorption from the digestive tract into the blood of humans and other animals [10]. *Lactobacillus* spp. has a high ability to suppress the growth of microscopic filamentous fungi and inhibit the production of mycotoxins [8]. Microorganisms such as *Bacillus* spp. which are isolated from soil and other environments have been reported to be able to degrade mycotoxins [18]. *Bacillus* strains produce and secrete large quantities of extracellular enzymes, and are under investigation of mycotoxin biodegradation. However, these enzymes have been reported to degrade only a certain type of mycotoxins [10, 16]. The aim of this research was to determine the ability of cell suspensions by various species of the genera *Lactobacillus* and *Bacillus* (*Bacillus subtilis* and *Bacillus licheniformis*) in zearalenone degradation *in vitro*.

MATERIALS AND METHODS

Biodegradation of zearalenone (Sigma Aldrich, Schnelldorf, Germany) was performed by selected strains of *Lactobacillus* spp. and *Bacillus* spp. The experiment was set up similar to that described by Harkai et al. [9].

Tested microorganisms

In the experiments, seven strains of microorganisms were used (Table 1). Strains of *Lactobacillus plantarum* CCM 1904, *Bacillus subtilis* CCM 2794 and *Bacillus licheniformis* CCM 2206 were purchased from the Czech Collection of Microorganisms (Masaryk University, Faculty of Science, Institute of Experimental Biology, Brno, Czech Republic). Other strains of the genus *Lactobacillus* were acquired from the Department of Microbiology and Immunology of the University of Veterinary Medicine and Pharmacy in Košice.

Table 1. Tested microorganisms

Species	Abbreviations used
Bacillus subtilis CCM 2794	Bs
Bacillus licheniformis CCM 2206	Bl
Lactobacillus plantarum CCM 1904	L6
Lactobacillus plantarum L81	L4
Lactobacillus fermentum 2I3	L1
Lactobacillus reuteri 2/6	L5
Lactobacillus reuteri L26	L3

Cultivation media

Commercial MRS medium (HiMedia, Laboratories Pvt. Ltd., Mumbai, India) was used to cultivate lactobacilli strains. *Bacillus* spp. strains were cultured on Medium B10 prepared according to a recipe obtained from the Czech Collection of Microorganisms (Masaryk University, Faculty of Science, Institute of Experimental Biology, Brno, Czech Republic) (Table 2).

Table 2. Composition of nutrient media in a volume of 1000 ml of distilled water

MRS broth	Yeast extract 5 g, meat broth 10 g, peptone 10 g, glucose 20 g, Tween 80 5 ml, KH_2PO_4 2 g, sodium acetate 5 g, ammonium citrate 2 g, $MgSO_4 . 7H_2O$ 0.2 g, $MnSO_4 . 4H_2O$ 0.05 g
MRS agar	Yeast extract 5 g, meat broth 10 g, peptone 10 g, glucose 20 g, Tween 80 5 ml, KH2PO4 2 g, sodium acetate 5 g, ammonium citrate 2 g, $MgSO_4 . 7H_2O$ 0.2 g, $MnSO_4 . 4H_2O$ 0.05 g, agar 12 g
Medium B10 broth	Peptone 5 g, meat broth 3 g, $MnSO_4 . H_2O$ 0.01 g
Medium B10	Peptone 5 g, meat broth 3 g, $MnSO_4 . H_2O$ 0.01 g, agar 20 g

Preparation of cell suspensions of *Lactobacillus* spp. and *Bacillus* spp.

Twenty-four hours old cultures of *Bacillus subtilis* (Bs) and *Bacillus licheniformis* (B1) grown on B10 agar at 35 °C were used to prepare the cell suspensions. Using a sterile inoculation loop (mesh volume 10 µl), the colonies of bacteria were picked up and transferred to 50 ml of B10 broth in an Erlenmeyer flask. Cell suspensions, from individual lactobacilli, strains grown under anaerobic conditions using a Gas pack system on MRS agar at 35 °C for 48 h, were prepared by inoculating 50 ml of MRS broth. Media inoculated with *Lactobacillus* spp. and *Bacillus* spp. were incubated at 35 °C under aerobic conditions by shaking the Erlenmeyer flasks on a horizontal shaker (Orbital Shaker—Biosan) at 170 rpm. After 24 hours, cell suspensions of *Bacillus subtilis* (Bs) and *Bacillus licheniformis* (B1) were centrifuged for 20 minutes at 6000 rpm and the supernatants were removed. The cell pellets were then washed twice with 5 ml of PBS buffer and again centrifuged under the same conditions. Subsequently, the cell pellets were resuspended and adjusted with saline solution/PBS buffer to a density of McFarland 4, (corresponding to 108 CFU.ml^{-1}) using a densitometer.

Zearalenone biodegradation testing procedure

The stock solution of 5000 ppm (5000 mg.l^{-1}) of zearalenone was prepared by dissolving the powder (25 mg) in 100 % of DMSO (dimethyl sulfoxide). This solution was used to prepare zearalenone concentrations of 10 ppm (10 mg.l^{-1}), 1 ppm (1 mg.l^{-1}), 0.1 ppm (0.1 mg.l^{-1}), and 0.01 ppm (0.01 mg.l^{-1}) by dissolving in a liquid cultivation medium (MRS broth for *Lactobacillus* spp. and B10 broth for *Bacillus* spp.) so that the final DMSO concentration was 0.2 %. The test was performed in test tubes. 100 µl of cell suspension of the strains were added to 4.95 ml of medium containing various concentrations of zearalenone. The tubes containing only the strains in the cultivation medium (without zearalenone) served as a negative control, thus monitoring their multiplication. The positive control was represented by tubes with individual concentrations of zearalenone in the culture medium, without inoculum of the bacterial suspension. The samples were incubated at 35 °C depending on the strains 24 h for bacilli and 48 h for lactobacilli. The residual zearalenone was extracted from the samples using Neocolumn for Zearalenone (Neogen Corporation, USA). An ELISA method was used to determine the zearalenone, using the Veratox for Zearalenone ELISA kit (Neogen Corporation, USA). Based on the measured concentrations, the percentage of biodegradation was calculated according to the formula:

$$\% \text{ biodegradation} = \frac{C_{PC} - C_S}{C_{PC}} \times 100\ \%$$

where: C_s— zearalenone concentration in the sample
C_{PC}—zearalenone concentration in the positive control

RESULTS

The efficacy of *Lactobacillus* spp. and *Bacillus* spp. cell suspensions in the biodegradation of zearalenone at the various concentrations are presented in Table 3. As the results demonstrated, the lactobacilli cell suspensions possessed a very good ability to degrade zearalenone at lower concentrations (0.01 ppm), with an efficacy in the range of 57.9—100 %. However, at higher ZEN concentrations, their biodegradation efficiency decreased significantly (0—13.9 %). A different ability of zearealenone biodegradation was found for the bacilli. The capability of *Bacillus subtilis* to degrade zearalenone at concentration of 0.01 ppm reached 100 % and 74.5 % at 0.1 ppm, but at higher ZEA concentrations, its biodegradation efficacy was insufficient (11.7 % and 0 %, respectively). *Bacillus licheniformis* CCM 2206 was not effective at the highest tested zearalenone concentration (10 ppm), and also at decreasing ZEA concentrations its degradation ability was very low (4.5—8.8 %).

Table 3. Biodegradation efficacy (%) of cell suspensions of *Lactobacillus* spp. and *Bacillus* spp. at different zearalenone concentrations

Strain	ZEN concentrations			
	10 ppm	1 ppm	0.1 ppm	0.01 ppm
L1	5.4	3.9	12.5	57.9
L3	1.5	1.7	0	85.6
L4	2.8	0	13.9	100
L5	2.8	1.9	3.1	100
L6	0	6.5	8.5	100
Bs	0	11.7	74.5	100
Bl	0	4.5	6.09	8.8

L1—*Lb. fermentum* 2I3; L3—*Lb. reuteri* L26; L4—*Lb. plantarum* L81; L5—*Lb. reuteri* 2/6; L6—*Lb. plantarum* CCM 1904; Bs—*Bacillus subtilis* CCM 2794; Bl—*Bacillus licheniformis* CCM 2206; ZEN—zearalenone

DISCUSSION

Biodegradation is one of the most specific and environmentally friendly methods in reducing the possible mycotoxins from food and feed. In general, *Lactobacillus* spp. and *Bacillus* spp. are considered safe strains for detoxification of zearalenone in feed [18]. When testing the detoxifying effects of *Lactobacillus* spp. and *Bacilllus* spp. cell suspensions, the highest biodegradation ability (100 %) was found in *Lb. plantarum* L81 (L4), *Lb. reuteri* 2/6 (L5), *Lb. plantarum* CCM 1904 (L6) and *Bacillus subtilis* CCM 2794 (Bs) at the zearalenone concentration of 0.01 ppm. However, at a zearalenone concentration of 1 ppm, the degradation was significantly lower (0—11.7 %). Z h a o et al. reported that ZEN (100 $\mu g.l^{-1}$) decreased by 45 % after 48 hours of exposure to *Lactobacillus plantarum* [20]. Similarly, A d u n p h a t c h a r a p h o n et al. [2] published that ZEN degradation at concentration 0.2 $\mu g.ml^{-1}$ using *Lactobacillus* spp. were in the range of 0.5—23 %. Probably the main cause of zearalenone reduction are bacterial cell proteins and lipids [2]. *Lactobacillus* spp. cell wall is composed of glycopolymers and proteins and it consists of a dense peptidoglycan with proteins, polysaccharides and teichoic acids. The surface structure of a bacterial cell is affected by the difference between sugars and amino acids in the glycopolymer or protein structures. This difference in surface structure plays an important role in the ability of *Lactobacillus* spp. to detoxify ZEN [6]. H s u et al. [11] tested the degradation of ZEN by *Bacillus licheniformis* CK1 and recorded a reduction of 70 % (from 5 $\mu g.ml^{-1}$ to 0.5 $\mu g.ml^{-1}$). However, at higher ZEN concentrations (50 and 200 $\mu g.ml^{-1}$) the degradation effect was only 60 % and 10 %, respectively [11]. *B. licheniformis* has outstanding probiotic properties (acidic tolerance, bile salt tolerance, adherence capability, and anti-pathogenic activities), therefore, it could also be used to improve the digestibility of nutrients in feed [11, 18]. T i n y i r o et al. found that *B. subtilis* 168 was more efficient in the removal of zearalenone from liquid medium, and that more than 75 % of ZEN was eliminated after incubation [17]. C h o et al. reported that *B. subtilis* strain degraded 99 % of ZEN in a liquid medium [7]. According to H a t h o u t and A l y, decontamination of zearalenone is never complete unless the presence of its oestrogenic analogues is ruled out [10].

CONCLUSIONS

The use of microorganisms or their enzymes to detoxify mycotoxins is a new option in the production of healthy food and feed. This study points to the potential use of the cell suspensions of non-pathogenic microorganisms (*Lactobacillus* spp. and *Bacillus* spp.) for zearalenone degradation. These strains could be used as innocuous detoxifying agents. These partial results also indicate the further direction of scientific research. Furthermore, it will be necessary to investigate the properties of non-pathogenic microorganisms capable of inactivating mycotoxin in feed and food.

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CHRONIC STRESS INDICATORS IN CANINES

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ABSTRACT

With a growing number of dogs abandoned, living in shelters, and being rehomed, it is important to distinguish behavioural responses due to stress in our domestic companions. Cortisol is involved in the stress responses in animals which generally enters the individual's body into a "state of emergency". Prolonged stress can lead to exhaustion, disease, and death. Chronic stress can be detected by evaluating cortisol concentration in hair. Most domesticated dogs respond well to hair collection, thus avoiding further stressors. The method is simple, relatively inexpensive, and non-invasive. Our experiment focused on assessing multiple parameters using a modified Canine Behavioural Assessment and Research Questionnaire to evaluate their significance with cortisol in hair samples from a diverse range of dogs. Each stress parameter was tested against cortisol concentration using a t-Test, i.e., the Paired Two Sample for Means. The effect of weight on cortisol levels was statistically significant (P = 0.03). This fact revealed that an increase in body weight correlated with an increase in cortisol levels.

Keywords: canines; cortisol; hair; stress; welfare

INTRODUCTION

The body's response to a whole range of stressors is very similar, involving the release of certain hormones. M o b e r g [36] described the physiological changes elicited by these hormones as attempts to restore the balance of the body's metabolism to homeostasis. Stressors include physical trauma, pain, surgery, excessive physical effort, etc. The main hormonal mechanism of the stress reaction is the activation of the axis hypothalamus-sympathetic-adrenomedullary system which immediately induces the release of catecholamines and glucocorticoids. Catecholamines have been termed the hormones of "fight or flight" because of their effects on the heart, blood vessels, smooth muscle, and metabolism that assist the organ-

ism in responding to stress. They induce hyperglycaemia by inhibiting insulin secretion, increasing the absorption of glucose from the intestine, and glycogenolysis. They also mobilize energy sources and increase the energy uptake to the nervous system and skeletal muscles. Glucocorticoids, on the other hand, aid the animal in adapting to adverse situations and surviving. They stimulate protein catabolism, encourage the liver to take up amino acids, increase gluconeogenesis and inhibit glucose uptake by many cells, excluding the brain. Stress can also cause responses such as an increase in epinephrine or glucocorticoid release. A chronic response to these stressors acts via the amygdala to release corticotropin-releasing factor (CRF) from the hypothalamus which then increases adrenocorticotropin (ACTH) secretion from the anterior pituitary. ACTH increases the activity of both the adrenal medulla and the cortex. It enhances glucose and glycogen synthesis at the expense of lipid and protein catabolism, pain suppression, and the anti-inflammatory response. S e l y e [43] proposed the General Adaptation Syndrome (GAS) which states that a long-term response to stress acts through increased adrenal function and consists of three phases: alarm, resistance, and exhaustion. These responses disrupt normal physiological mechanisms and trigger an array of diseases. If untreated, it always leads to infection, illness, and eventually death [20].

In vertebrates, an important mechanism for coping with stressors begins with adrenally derived glucocorticoid hormones (cortisol). As previously discussed, these molecules drive gluconeogenesis, however they also: suppress the reproductive processes, alter movement and feeding rates, impact immune functions, and generally help an individual enter a "state of emergency" [8]. Cortisol acts as an immunosuppressant by suppressing protein synthesis, including the synthesis of immunoglobulin. Cortisol also reduces the peripheral blood concentrations of eosinophils, lymphocytes, and macrophages. Large doses of cortisol are known to promote the atrophy of lymphoid tissue in the thymus, spleen, and lymph nodes. Therefore, this action could account for lymphoid atrophy. Cortisol directly influences the immune response to antibodies. The mechanisms of inhibition of the immune response are multifactorial. When an antigen intrudes into the body, it is picked up by a macrophage [11]. The macrophage then presents the antigen to thymus-derived lymphocytes (T cells) and simultaneously produces and releases interleukin-1 (IL-1). Likewise, T helper cells secrete interleukin 2 (IL-2), a protein that stimulates the proliferation of still more T cells. T cells can either activate or suppress bursa-derived lymphocytes (B cells). B cells then produce antibodies directed against the original invading antigen. Cortisol inhibits the production of both IL-1 and macrophages and IL-2 by helper T cells, thus decreasing T cells responses and the generation of fever. The diminished helper T cells cause a decrease in B cells and antibody production. Once antibodies are present, neither their degeneration nor their specific reaction with antigen molecules is affected by cortisol [16].

The interaction between stress hormones and immune functions is important in the understanding of disease-coping mechanisms in wild animals [27]. For example, many urban bird populations regulate stress hormones differently than their rural or suburban counterparts [7, 14, 40]. One study reported that the presence of an introduced species impacted glucocorticoid regulation in a native species [5]. In this study, neither immunological nor disease consequences of stress hormone variation were considered, but the possibility exists that introduced species may have negative impacts on stress hormones in native species [21]. There is now evidence in both human and other animal studies that the magnitude of stress-associated immune dysregulation is large enough to have health implications [11, 38]. Lymphocytes, macrophages, and granulocytes exhibit receptors for many neuroendocrine products of the hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal medullary (SAM) axes [42]. These HPA and SAM, such as cortisol and catecholamines, can cause changes in cellular proliferation, cytokine secretion, antibody production, and cytolytic activity [28]. This can cause a shift from cell-mediated immune activities to a shift in antibody production [31]. This stress-induced decrease of immune-mediated cytokines results in dysregulation of cell-mediated immune responses [38]. A study conducted by B o r c e l et al. [9] investigated the spatial-cognitive abilities and the survival of new-born hippocampal cells in aging animals. Male Wistar rats were subjected to chronic unpredictable stress at 12 months old and then re-exposed to stress each week for a set time period. Subsequently, they were evaluated in a water maze during the early stages of aging. Chronic unpredictable stress seemed to exacerbate the spatial-cognitive decline. This was accompanied by a reduction in the survival of new-born cells and

in the number of adult granular cells in the hippocampus. P o r t e r, L a n d f i e l d [41] and M c E w e n [32] also showed that continuous exposure to stressors could accelerate cognitive decline during ageing.

The stress responses that lead to pathology and death are clearly indicative of poor animal welfare [18]. Stress indicators in animals are a valuable tool for assessing their welfare and overall health. However, as it may be difficult to define the stress of an animal, it is necessary to combine physiological and behavioural indicators of acute or chronic stress in individuals [6]. The physiological indicators of stress include the various hormonal measures mentioned above, such as: catecholamines, glucocorticoids, prolactin, gonadotrophins, thyroid-stimulating hormone, and insulin. It can also include the measures of: heart rate, blood pressure, respiratory rate, body temperature, evaluations of the immune status, and of disease incidence [6]. The serum catecholamines and glucocorticoids levels are most commonly measured in dogs as they correlate with the level of stress, however, it can be difficult to obtain accurate reading. The anticipation and immediate stress invoked by the blood sampling procedure triggers a response that may increase the plasma levels of these hormones, complicating the interpretation of the results. It can be ethically difficult to measure these physiological indicators, so it is important to choose the least invasive procedure. Thus, urine, saliva, and faeces are the most frequently used in sampling [6, 15]. Most recently, hair has been proven to be a reliable source of measuring chronic cortisol. Except for blood serum sampling of hormones, other sampling methods are focused on measuring acute stressors. However, for evaluating the long-term effects of stressors, such as kennelling, and their effects on the body and welfare of the animal, hair sampling provides a clearer picture of the stress levels. This is due to a number of reasons, including that blood-borne hormones are incorporated into the shaft during the growth phase; it is stable, and can be transported at room temperature [34]. Lastly, if the action of removing hair (e. g., in wild animals) is stressful, the stress during this event will not affect the cortisol concentration in the sample. Cortisol enters the hair shaft through two main mechanisms. Free cortisol is incorporated during the growth phase of the follicle [10]. Additionally, sweat and sebum excreted by skin glands contain cortisol which can also be incorporated into the shaft [2].

The first study examining cortisol concentration in hair was performed by K o r e n et al. [23] using hair from wild hyraxes. They determined the cortisol concentrations using a modified salivary ELISA assay. This sampling method found a positive correlation between hair cortisol and social ranking, thus supporting the hypothesis that domination is a stressful event. Other studies, such as A c o r s i et al. [1] compared cortisol in faecal samples with that in hair from domestic cats and dogs. Similarly, B e n e t, H a y s e n [3] showed that distal hair segments in dogs have a higher concentration of cortisol metabolites. In terms of assessing disease, hair cortisol levels have proven reliable. C o r a d i n i et al. [12] showed significantly higher cortisol levels in dogs with Cushing's syndrome. P a r k et al. [39] have also illustrated that dogs with atopic dermatitis have increased cortisol concentrations in hair samples [34].

MATERIALS AND METHODS

Study Design and Sampling

The experiment aimed to analyse the cortisol levels in dogs who live in various home environments and originate from different backgrounds. For the study purpose, familiar dog owners were contacted via social media/phone and asked to participate. Animals were chosen at random to include a diverse range of pedigree breeds, mixed breeds, dogs adopted from a shelter, and dogs bought from a breeder. The dogs were also randomly selected for different coat colours, home environments, size categories, and weight categories. This was in an attempt to identify possibly different stress parameters in a dogs' life. In total, there were 54 subjects (animals) analysed. A survey was made based on the Canine Behaviour and Research Questionnaire (https://vetapps.vet.upenn.edu/cbarq/) to assess individual dogs' behaviour and possible stress-related behavioural manifestations. This C-BARQ survey was modified (hereafter MCBARQ) to focus on behavioural manifestations of stress. Due to COVID-19 pandemic restrictions, it was not possible to collect the samples in person by the research team members. All participants were given the same instructions regarding collecting samples and completing the individual survey. They were instructed to shave a section of hair, close to the skin without plucking (as this could contaminate the sample with follicles and blood). It was highly recommended to shave the hair sample from the

abdominal/inguinal region or the inner hind thigh. The reasoning was to perverse cosmetic appearances of the animal and to try to have a similar region for analysis. This should have amounted to approximately 5 grams of hair. The sample was then sealed in a plastic, airtight bag and sent to the laboratory to be stored before further processing.

Sample processing and analysis

200 mg of each hair sample were weighed and washed three times with 5 ml of isopropanol using a vortex mixer. After washing the isopropanol, it was discarded and the samples were left to dry at room temperature for three days. Dry hair samples were cut into 1 mm pieces. For further processing, 100 mg of cut hair were dipped in 3 ml of ethanol, mixed on vortex, and left to be shaken at room temperature for 24 hours on a laboratory shaker. After 24 hours, 2 ml of the supernatant were transferred into a clean tube and left to dry at room temperature. Before the analysis of the samples were reconstituted by adding 200 µl of PBS (phosphate buffer) they were shaken on a vortex mixer. The samples were then placed into the ELISA well for further analysis according to the manufacturer's instructions. A DRG cortisol ELISA testing kit was used to measure the cortisol in each hair sample. The DRG Cortisol ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtitre wells are coated with a monoclonal antibody directed towards an antigenic site on the cortisol molecule. The endogenous cortisol of a sample competes with a cortisol-horseradish peroxidase conjugate for binding to the coated antibody. After incubation, the unbound conjugate is washed off. The amount of bound peroxidase conjugate is inversely proportional to the concentration of cortisol in the sample. After the addition of the substrate solution, the intensity of colour developed is inversely proportional to the concentration of cortisol in the sample.

ETHICAL STATEMENT

Consent of owners was given for obtaining and examining hair samples from their animals and for publication of the results of this study.

The authors declare no conflict of interest.

Statistical analysis

In total, 16 categories were tested against the cortisol levels to look for significance. The categories that were analysed from the MCBARQ included: training, aggression, fear and anxiety, separation anxiety, excitability, general stressors, and miscellaneous. It was decided to evaluate other factors, not just those included in the MCBARQ. The analysed categories not relating to the MCBARQ included: breed, breed size, pedigree vs mixed breed, hair colour, and age. If the dog was adopted or bought from a breeder, also, the neutered status, sex and weight were considered. This information was taken from the first section of the MCBARQ "Section 1, General". Each breed involved in the experiment was given a numerical number and plotted against cortisol to evaluate if certain breeds are more predisposed to stress. Each breed was also entered into a size category. Small < 15 kg, medium 15—25 kg, and large > 25 kg to evaluate if certain size categories were more predisposed to stress. What is more, each subject was assigned to either 'adopted' or 'bought from a breeder' to evaluate a stress response. The cortisol levels from the hair samples were compared to the above categories by using a T-Test: Paired Two Sample for Means through Microsoft Excel. The T-Test was run for each of these categories against cortisol. The P ($T \leq t$), one-tailed and two-tailed, was investigated for significance. If this number was above 0.05, the relationship between the two variables was deemed insignificant. Weight was the only category that was deemed significant with P (one-tailed) = 0.019 and P (two-tailed) = 0.038.

RESULTS AND DISCUSSION

Each subject was divided into certain groupings in order to assess breed significance on the cortisol levels of dogs. The subjects were divided into mixed or pedigree groups to evaluate if one group was more predisposed to cortisol. Some studies have reported that being adopted leads to an increased stress response in animals due to a less stable, overcrowded environment [4, 19, 33,]. Our results did not show any significant correlations.

After evaluating the MBARQ, weight, and breed results, we explored some other possibilities of stress correlation and differences among the dogs. The results from the MCBARQ were analysed under 7 separate categories

in order to assess cortisol response to behavioural traits; specifically known stress behaviours. There were no significant results from the analysis. No statistically significant difference between the sexes, neuter status, or different age categories have been shown in previous studies as well [3, 34, 44]. Some studies have shown that some hair colours have a higher concentration of cortisol [3, 34], but our results did not comply with this finding.

The results have demonstrated that there was a correlation between weight and cortisol deposited in the hair strands ($P_{\text{two-tailed}} = 0.03$). It indicated that an increase in weight correlated with an increase in cortisol. A study by Hewagalamulage et al. [17] explained that sheep which can be categorised as high cortisol responders have a higher chance of weight gain and obesity than low-cortisol responders. They found that high cortisol responding ewes ate more in response to stress than others. This was due probably to the reduced feeling of feeling full after eating (or increased ghrelin hormone) in response to stress as well as reduced thermogenesis specifically in skeletal muscles. In our experiment, the dog with the highest peak of cortisol, 68.466 $ng.ml^{-1}$, was an overweight dog. As a German Shepherd weighing 37 kg, the weight was 5 kg over the normal weight range for this breed. A study by Luno et al. [26] investigated the relationship between cortisol and ghrelin and so-called "emotional or stress-related eating". This theory supposed that there were changes in eating behaviour in response to negative stressors and suggested that it was a coping mechanism for stress. "Comfort foods" which are rich in energy, fat, or sugar are consumed to obtain a feeling of emotional wellness [13]. Another study by Luno et al. [25] conducted a survey that found that 82.7 % of owners noticed their dogs showed signs of emotional eating in relation to behavioural problems.

A study by Kuhne et al. [24] investigated dogs living in a human-dog relationship. They found that if the relationship to humans was unpleasant or if the environment was uncontrollable with unpredictable stimuli, the dog may develop chronic or recurrent stress. An unpleasant human-dog relationship was the leading cause of behaviour problems and the reason for dogs being brought to a shelter. However, previous studies have discovered that humans experience significant changes in blood pressure, heart rate (HR), oxytocin release, and immune defence as a result of petting a dog. This indicated that if the human-dog relationship is one of kindness and good welfare, the relationship can be mutually beneficial.

The experiment conducted had many parameters to compare, yet there was little significance in the results. This could have been due to several factors. Firstly, due to the COVID-19 pandemic restrictions, we were unable to collect the hair samples ourselves. This led to inconsistency in the amount and place on the body that the hair was removed from. Despite each person being given the same instructions on how to sample the hair inconsistencies occurred. It was recommended to shave the hair as close to the skin as possible on the inguinal region or inside of the thigh however there is no way to know if this was where the hair samples came from or how close to the skin the hair was removed from. If it was not cut close to the skin, there could be contaminants in the sample leading to an inaccurate cortisol value. Each hair shaft grows to a specific length, growth stops, and the final shedding cycle commences. Therefore, hair samples obtained without previous shaving will contain a mixture of hairs that have incorporated cortisol over different time periods. This can be resolved by shaving an area, then re-shaving approximately 3 months later, thus establishing a known timeline of cortisol incorporation [35]. Another possible explanation for the variations in cortisol levels may be differences in the moulting patterns and growth rate between hair from the neck and the ischiatic region [44].

Another possible issue is owner compliance. The survey comparison was a large part of the data comparison. Perhaps some owners misinterpret their animals' behaviour or perhaps they exaggerate some behaviours on the survey. This will lead to an inaccurate overall account and could account for some misevaluation of the data. Behavioural indicators of stress are rarely recognized by owners; thus only some specific situations will be noted [24, 30]. Ideally, a comprehensive evaluation of dogs' stress should be evaluated to define the human-dog relationship. Therefore, it is important to evaluate chronic stress levels and the behaviour of dogs both at home and at shelters to gain a full understanding of our pets' welfare, or indeed over a longer time period. Hair washing and shampooing could contribute to insignificant results in the effect of washing hair in dogs. Currently, there are only human studies for comparison however, damage done to human hair structure could have been caused by liquids (e. g., water and cosmetics) or the alcohol used to wash the samples before

analysis [22, 29]. The amount of cortisol after washing was decreased after a high number of shampoo washes [35]. It is yet unknown if dogs are similarly affected by shampoo washing, although dogs are usually shampooed less frequently than humans so the comparison would also have to include products and time of washing. A larger sample size would give a more accurate evaluation of the data and hopefully a clearer picture of significance. Measuring any parameters can be difficult to interpret accurately without comparative baseline values and without such a single diagnostic test; it should be remembered that the welfare of animals should be judged on how far measurements deviate from normal [37]. Nonetheless, a few studies have attempted to examine the behaviour of dogs under normal home conditions.

CONCLUSIONS

In our study, the only significant parameter was that an increase in weight (kg) led to an increase in cortisol ($ng.ml^{-1}$). It is theorized to be related to emotional comfort eating as there are papers where owners reported that their dog showed signs of emotional eating relating to behavioural issues. This is an interesting theory to consider studying in terms of both human and companion animal lifestyles. For humans, there is an increasing global risk of obesity and for understanding the importance of nutrition in companion animals, especially understanding how much 'human food' and treats to reward the animals. Interestingly, there was no significance between dogs adopted from shelters and those bought from a breeder, despite reporting behavioural issues in the MCBARQ. We believe further investigations could be done as to time adopted and specific behavioural traits in order to obtain a fuller picture. In conclusion, the topic of animal welfare in dogs relating to stressors in their life is poorly understood. This paper has provided an insight into areas that still need to be documented with more time and resources to fully understand this topic sufficiently.

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FELINE HYPERTROPHIC CARDIOMYOPATHY (FHCM)

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ABSTRACT

The feline hypertrophic cardiomyopathy (HCM) is one of many cardiac diseases known in domestic animals, and it is especially frequent in cats of all ages. HCM is the most common heart disease in cats, affecting almost 15 % of the feline population. The Maine Coon and the Ragdoll breeds of domestic cats are shown to have a special gene that is responsible for the appearance of HCM in these breeds. Hypertrophic cardiomyopathy can be detected by: echocardiography, electrocardiography (ECG), radiography, genetic testing, and a test including N-terminal prohormone of brain natriuretic peptide (NT-proBNP). The most frequently used method used for clinical purposes is echocardiography. Patients with HCM will most of the time not have any clinical signs of the disease, which makes it difficult for the owner to detect that there is something wrong with their cat. Some cats, on the other hand, will get heart arrhythmias which can cause sudden death. There are several prognosis-worsening findings that may occur due to the hypertrophy of the ventricles. These include: Feline Arterial Thromboembolism (FATE), Congestive Heart Failure (CHF), and the Dynamic Obstruction of the Left Ventricular Outflow Tract (DOLVOT). The prognosis for HCM depends on the stage of the disease. In some cats, they are well-tolerated and are associated with normal life expectancy, but in other cats they can result in congestive heart failure, arterial thromboembolism or sudden death.

Key words: cats; echocardiography; heart; heart failure; hypertrophy

INTRODUCTION

Hypertrophic cardiomyopathy, or feline hypertrophic cardiomyopathy, is a heart disease that is one of the most commonly encountered cardiac diseases found within felines [19]. HCM is the most common heart disease in cats, affecting almost 15 % of the feline population [35]. This has been known since the 1970s, but it was investigated

further and studied by scientists and veterinarians in the 1990s [10, 19]. The disease is characterized by unusual hypertrophy of the left ventricular wall and the proximal interventricular septum. The mechanism behind the growth is the growth of fibrous tissue within the cardiac muscle that makes the myocardium stiff [23, 38]. As the severity of the disease progresses, the actual structure of the heart is changed and the heart's function is also affected [15].

The thickening of the walls of the ventricle is associated with a decreased ventricular chamber volume and abnormal ventricular relaxation (diastolic function) in cats with HCM. Since the amount of blood pumped by the heart per minute (cardiac output) is the product of the amount of blood ejected per contraction (stroke volume) and the heart rate in beats per minute, this decreased chamber volume (and subsequent stroke volume) results in an increased heart rate (tachycardia) as a reflex mechanism to maintain cardiac output and blood pressure [10, 22]. Although this reflex increase in heart rate may maintain normal blood pressure in the short term, it is associated with increased consumption of oxygen by the heart muscle, to the extent that oxygen demand may exceed supply. This scenario may result in an energy-starved heart muscle, with subsequent heart cell death and worsening function [38].

Another consequence of an increased heart rate is that the ventricle has less time to fill between contractions, further diminishing the stroke volume and promoting a vicious cycle of reflex tachycardia, decreased time for ventricular filling, and so on. This decreased left ventricular filling also promotes stasis of blood in the left atrium (chamber just before the left ventricle), which ultimately contributes to the development of clinical signs.

AETIOLOGY

Although the definitive cause of feline HCM has not been identified, its prevalence within certain breeds, (i. e., Maine Coon cats, and Ragdolls) has prompted speculation that at least some forms of HCM are genetic in origin. The finding of mutations in an important cardiac protein called myosin binding protein C in affected lines of Maine Coon and Ragdoll cats supports a heritable, genetic component of HCM in these breeds [22, 23, 27]. In most cases, HCM affects mostly middle-aged cats, but cats of all ages are affected. HCM is heritable as an autosomal dominant trait [3]. In cats, this disease is more prevalent in: Ragdolls, Maine Coons, oriental breeds (Himalayan, Burmese, Sphynx, Persians), and Devon Rexes, but it is also commonly diagnosed in Domestic Short Hair cats [22]. Other common causes of HCM that may need to be excluded include: aortic stenosis, dehydration, systemic hypertension, hyperthyroidism, and acromegaly [27].

CLINICAL SYMPTOMS AND PROGNOSIS-WORSENING FINDINGS

It is common for cats with HCM to be asymptomatic, which means that the cat does not show any external and behavioural signs of the disease. The disease may be discovered by the sound of a heart murmur or arrhythmias. Murmurs in patients with HCM have been associated with SAM, which results in LVOTO and mitral regurgitation (MR), as well as with MR caused by hypertrophic remodelling and distortion of the mitral apparatus [20]. Additionally, dynamic right ventricular outflow tract obstruction has been identified as a cause of murmurs in healthy cats and cats with the noncardiac disease. Numerous types of arrhythmias can be identified on a resting ECG, including atrial and ventricular premature complexes (APCs and VPCs), atrial and ventricular tachycardia, and atrial fibrillation [6, 20]. The presence of arrhythmia and an audible gallop have been associated with a worse outcome and a detectable arrhythmia has been associated with a greater risk of sudden death [18].

In other cases, signs of congestive heart failure including laboured or rapid breathing, open-mouth breathing, and lethargy are evident. These signs occur when fluid accumulates in the lung tissue (pulmonary edema) or around the lungs (pleural effusion) secondary to the elevation of left atrial pressure [2, 33].

Prognosis-worsening findings of feline HCM

1. Dynamic obstruction of the left ventricular outflow tract (DOLVOT)

The systolic anterior motion of the mitral valve (SAM) is the most common cause of dynamic obstruction of the left ventricular outflow tract (DOLVOT) and, in turn, the most common cause of a heart murmur with feline HCM (Fig. 1).

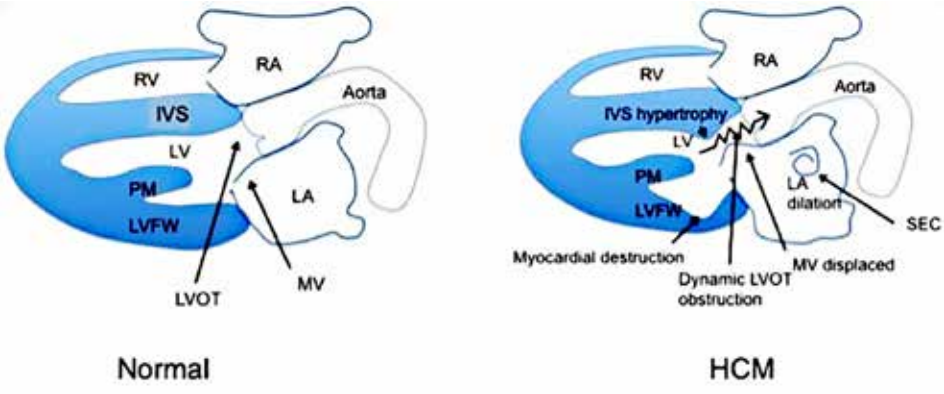

Fig. 1. Illustration of the mechanism of DOLVOT
Art by: Eric de Madron. https://www.vetspecialists.com/vet-blog-landing/animal-health-articles/2020/04/29/hypertrophic-cardiomyopathy-in-cats

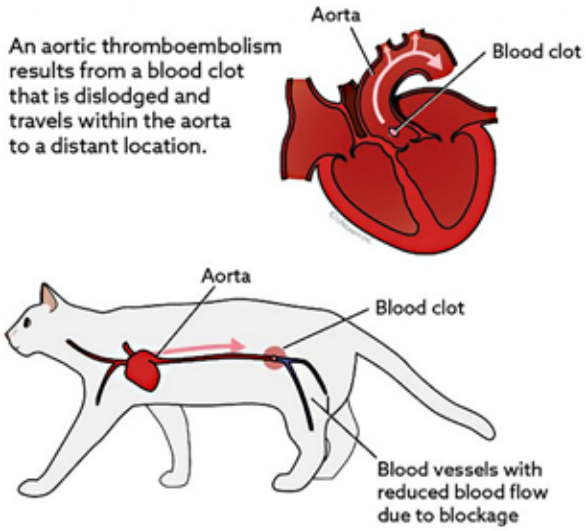

Fig. 2. Illustration of where the thrombus forms and where it clogs the arteries in FATE
Art by Malcolm Weir. https://vcahospitals.com/know-your-pet/aortic-thromboembolism-in-cats

When hypertrophic cardiomyopathy occurs, the proximal part of the interventricular septum becomes enlarged. This, as said earlier, will make for less room for the mitral valve to function, and it will be squeezed against the septum [23]. This leads to a reduction of the mitral valve's function, which again leads to obstruction of the passage.

2. Feline arterial thromboembolisms (FATE)

Diastolic dysfunction, and its consequences of abnormally increased atrial pressure leading to signs of heart failure, and sluggish atrial blood flow leading to Feline arterial thromboembolism (FATE).

A potentially devastating cloud of HCM is thromboembolism. Thromboembolism refers to the development of a clot in the heart (promoted by left atrial enlargement), with ejection of the clot to the systemic circulation [25] (Fig. 2).

When the clot lodges in the peripheral circulation, it may obstruct blood flow to the region of the heart supplied by the blocked vessel. The site of thromboembolism most commonly observed in cats with HCM is the distal aorta (termed a saddle thrombus), and clinical signs of hind limb paralysis and acute pain in the hind limbs may be observed [29]. Thromboembolism is a poor prognostic indicator in cats with HCM [22].

3. Congestive heart failure (CHF)

Congestive heart failure is a common clinical syndrome

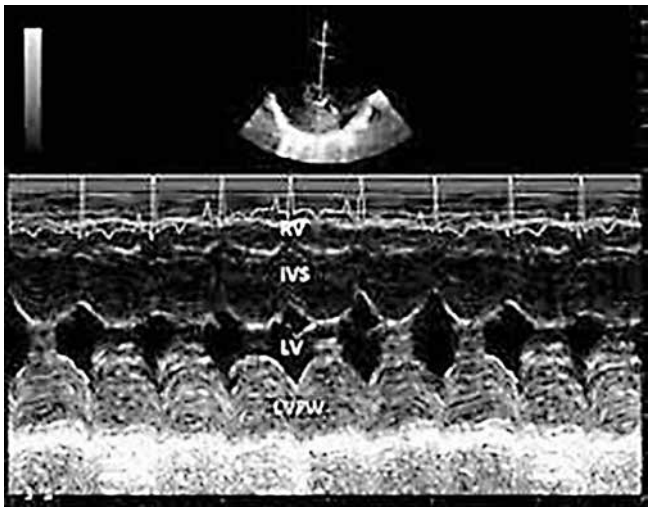

Fig. 3. Two-dimensional and M-Mode echocardiographic examination of a cat with HCM on right parasternal short axis view at the level of the *chordae tendineae*
Note interventricular septum and left ventricular free wall hypertrophy.
LVFW: left ventricular free wall; IVS: interventricular septum and RV: right ventricle.
Art by Silva et al. 2013. https://scielo.conicyt.cl/pdf/amv/v45n1/art02.pdf

that is a complication due to different causes, in this case, hypertrophic cardiomyopathy, and it manifests itself in different ways [28]. The word "heart failure", means that the function of the heart cannot meet the normal physiological demands of the body, which can in the worst-case lead to the death of the animal [23, 25]. One definition that can be used for the syndrome, is that it is a condition where the heart is unable to supply a sufficient amount of blood to the peripheral tissues to meet their metabolic needs [28]. It is a condition where the blood that usually is pumped straight into the aorta from the left ventricle can get stuck in the left atrium, and will end up streaming back to the lungs and into the thoracic cavity [30]. This is a critical condition, because the lungs and thoracic cavity can overflow and the cat will end up having trouble breathing. Due to the respiratory problems involved as a result of CHF, a clear sign of the condition would be that the cat's breathing rate is increasing, as the cat is trying hard to get as much air into its system as possible. Also, the heart rate will increase, and one can in some cases even feel the heartbeat clearly from the chest of the cat [23].

4. Systemic hypertension

Diffuse or segmental LV hypertrophy is common in cats with systemic hypertension and is observed in up to 85 % of cases, although HCM and systemic hypertension may exist concurrently. For many hypertensive cats, LV hypertrophy is only mild to moderate. Blood pressure determination should be considered for all cats with increased LV wall thickness (LOE medium) [13].

Acute stress resulting in severe tachycardia can result in acute deterioration of diastolic function and so precipitate acute left heart failure. Anaesthesia, surgery, intra venose fluid therapy and possibly corticosteroid administration can tip a cat with subclinical disease over into heart failure. However, most cats with HCM that present in heart failure have no apparent exacerbating disease or precipitating event [20, 33].

Exacerbation of clinical symptoms and sudden cat death may occur with increased exertion and stress during the clinical examination and handling. Gentle handling, reduced stress or the use of sedation can be preventive [33].

DIAGNOSTICS

The definitive diagnosis of HCM is almost always made using echocardiography. Other imaging modalities, such as CT and MRI, are used in human medicine [14]. Electrocardiography can reveal changes in some cats with HCM but is not a reliable indicator of disease [31]. Radiography cannot be used to distinguish HCM from the other cardiomyopathies but is valuable for identifying severe left atrium (LA) enlargement.

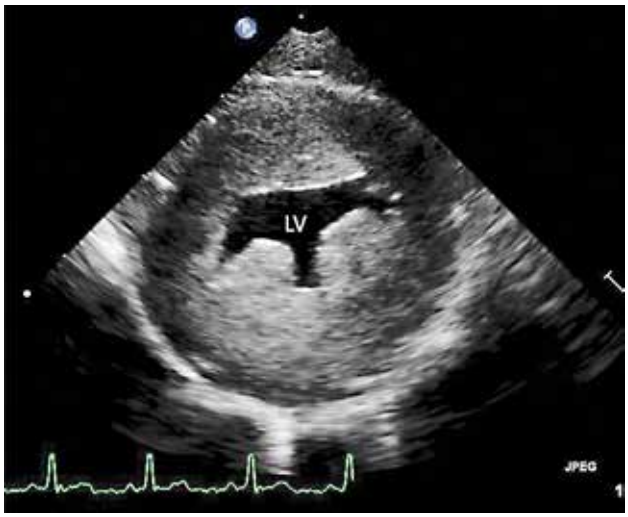

Fig. 4. Two-dimensional short-axis ultrasonographic view of Tux showing concentric left ventricular hypertrophy and papillary muscle hypertrophy, consistent with hypertrophic cardiomyopathy. His left atrium was normal in size on other images. LV, left ventricle

Art by Brent Aona et al., 2021. https://todaysveterinarypractice.com/advances-feline-cardiac-diagnostics/

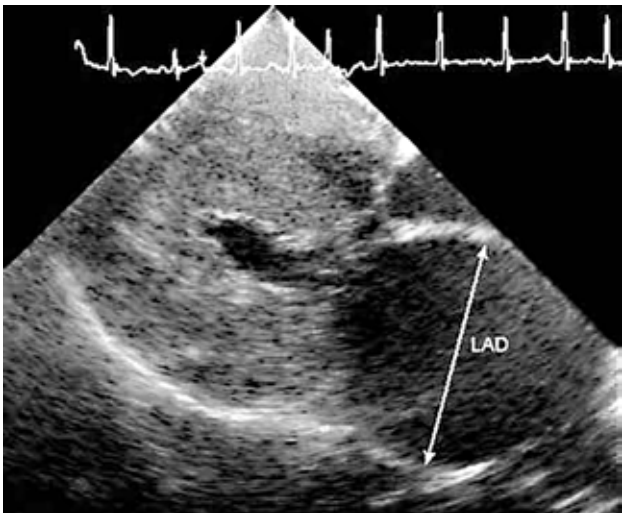

Fig. 5. Left ventricular M-mode of a cat with HCM, demonstrating systolic anterior motion (SAM) of the mitral valve

Art by Fuentes et al., 2003. https://www.vin.com/apputil/content/defaultadv1.aspx?meta=&pId=11149&id=3846601

Echocardiography

Feline HCM has been echocardiographically defined by end-diastolic measurements of the ventricular wall thickness that equals to or exceeds 6 mm (Fig. 3, 4) [10]. Hypertrophy is commonly asymmetric and in Maine coon cats with HCM, it is often the left ventricular posterior wall and papillary muscles that are most affected [19]. Ejection phase indices of the left ventricular systolic performance such as % fractional shortening are usually normal or reflect hyperdynamic ventricular emptying.

Left atrial size in cats with HCM is generally greater than in healthy cats, but left atrial enlargement is not an intrinsic feature of the disease nor required for its diagnosis (Fig. 5) [1]. However, left atrial size is a surrogate measure of hemodynamic burden, and left atrial enlargement has been associated with poor outcomes in people and cats with HCM [26, 33].

Diastolic dysfunction is a characteristic feature of HCM. Diastolic function is typically assessed in cats with HCM using tissue Doppler imaging. Most commonly a pulsed wave tissue Doppler imaging gate is placed at the lateral mitral valve annulus to measure the velocity at which it moves in early diastole (E' wave) [36]. In cats with severe HCM, the E' wave velocity is typically decreased [24].

Cardiac biomarkers

When the left ventriculus (LV) wall is severely thickened, the myocardial blood supply is compromised [34]. This results in ongoing myocyte damage and death, as evidenced by an elevation in cardiac troponin I (cTn I) in cats with HCM [5, 16]. Cardiomyocytes that die are replaced with fibrous tissue (replacement fibrosis), as evidenced by increased concentrations of circulating biomarkers of type I collagen [4]. The measurement of NT-proBNP and cTn I concentrations in serum or plasma has been evaluated extensively as a means of screening for HCM in cats without heart failure (subclinical HCM) in veterinary referral hospitals and also as a means of differentiating heart failure from primary respiratory disease in cats presented for dyspnoea [40]. The measurement of NTproBNP is widely available (through a diagnostic laboratory) which may help to identify patients with preclinical HCM at greater risk when echocardiography is not available [18].

Electrocardiography (ECG)

While the ECG is sensitive for detecting atrial fibrillation, atrial fibrillation is not an easy diagnosis to make in a cat (the ECG may not be specific for atrial fibrillation in cats) [40]. Sinus rhythm with small P waves (which may be obscured by artifact) and frequent APCs and atrial tachycardia often masquerade as atrial fibrillation in cats. The

ECG is insensitive for detecting sporadic APCs and VPCs in any species. A 24-h ambulatory ECG (Holter monitor) is a more sensitive means of identifying these arrhythmias [39].

Other common causes of HCM that may need to be excluded include: aortic stenosis, dehydration, systemic hypertension, hyperthyroidism, and acromegaly. However, there are caveats. Systemic hypertension and hyperthyroidism do not cause severe HCM, so if a cat has severe HCM (arbitrarily defined as diastolic LV wall thickness ≥ 7 mm) and systemic hypertension or hyperthyroidism, it can generally be assumed that these systemic disorders are not the sole cause of the HCM [22, 36].

TREATMENT

There is no documented reason to treat a cat with subclinical HCM that has mild to severe wall thickening and a normal to mildly enlarged LA (stage B1) if the goal is to delay the onset of heart failure. This is because there is no medication (including ACE inhibitors, beta-blockers, and spironolactone) that has been shown to reduce hypertrophy or slow progression of the disease, if it is destined to progress [36]. Therefore, the best that can be done is to:

- Monitor the cat for the development of severe LA enlargement (so that antiplatelet/ anticoagulant therapy can be started);
- Avoid treatments that can trigger heart failure iatrogenically (e. g., injudicious fluid therapy);
- Not breed the cat if it is sexually intact;
- Monitor for the onset of left heart failure, if the LA is moderate to severely enlarged [36].

The treatment of hypertrophic cardiomyopathy in cats is mostly palliative, because there is no way to reduce the enlargement of the cardiac muscle tissue. This makes the goal of the management of HCM to improve the LV ability to fill and pump out blood, together with delaying the onset of congestive heart failure and reducing the risk of further complications such as feline arterial thromboembolism. Beta-receptor blockers, calcium-channel blockers, and ACE-inhibitors are some of the methods for the treatment of HCM in felines. Beta-receptor blockers include drugs such as propranolol and atenolol that reduce the excretion of catecholamines and the influence of the sympathetic nervous system of the heart [32]. Calcium-channel blockers, such as diltiazem and verapamil, have a similar function as beta-blockers. ACE-inhibitors, on the other hand, affect the RAAS by inhibiting the production of angiotensin II and aldosterone. The function of ACE inhibitors is to minimize the fibrosis of the myocardium [7, 17, 24]. The overall function of the treatment of HCM is not to eliminate the disease, since it is chronic, but to minimize the heart rate and to improve the filling of blood in the LV in the diastole [8, 9].

The best way to do the last is to have the owner monitor the cat's sleeping respiratory rate (RR; normal is < 30 breaths.min^{-1}) and to maintain a log. in general, this should only be performed in a cat with evidence of moderate to severe LA enlargement to avoid over vigilance. The owner then needs to be instructed to call a veterinarian when the sleeping RR increases, before the onset of any severe dyspnoea and hopefully avoid the all-too-common weekend or evening visit to an emergency clinic [20].

PROGNOSIS

The prognosis for HCM depends on the stage of the disease. Many cats with mild to moderate HCM never progress to severe HCM and so have an excellent prognosis. However, if followed serially, a significant number of cats will progress to severe HCM [11, 20, 37]. Most cats with severe LV wall thickening and moderate to severe LA enlargement (stage B2) that are not in heart failure will progress to heart failure or experience ATE. For some perspective, one study examined the time from diagnosis of subclinical HCM (stages B1 and B2) to onset of heart failure and found that approximately 7 % of the cats developed heart failure within the first year, 20 % within 5 years, and 25 % within 10 years [12]. Once in heart failure, approximately half were dead within 2 months. Overall, in cats with ATE, 70 % were dead within a week. The last stands in contrast to the average survival of 11.5 months in the 37 % of cats with ATE that survive the acute episode and the 20 % of cats with ATE that lived 4 years or more in other studies [21, 33]. The exceptions to "the most will die within months, not years" rule include cats with TMT (Transient Myocardial Thickening) and those that are in heart failure due to stress, fluid administration or cortico steroid administration. These cats can stabilize after being treated for heart failure and may live for years [33].

CONCLUSIONS

Hypertrophic cardiomyopathy has been known as a common cardiac disease among both felines and humans for decades. The main sign of the disease is heart murmurs, arrhythmias, low blood pressure, and hind limb paralysis. HCM is a chronic disease, and there is no cure for it. This means that it is important to screen the cats that are especially known to carry genes for HCM. These breeds include mainly Maine Coon and Ragdoll, but also other breeds such as British Shorthair, Devon Rex, Sphynx, and other oriental breeds are known to be more commonly affected by the disease than other breeds. For that reason, it is really important with regular screening by echocardiography of cats that are used for breeding purposes to prevent the disease to inherit down later litters.

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BERGE, A. C., JOZAN, T., LEVESQUE, C., VERTENTEN, G.: A FIELD STUDY EVALUATING HUMORAL IMMUNITY IN CALVES VACCINATED WITH MULTIVALENT BOVINE RESPIRATORY PATHOGEN VACCINES

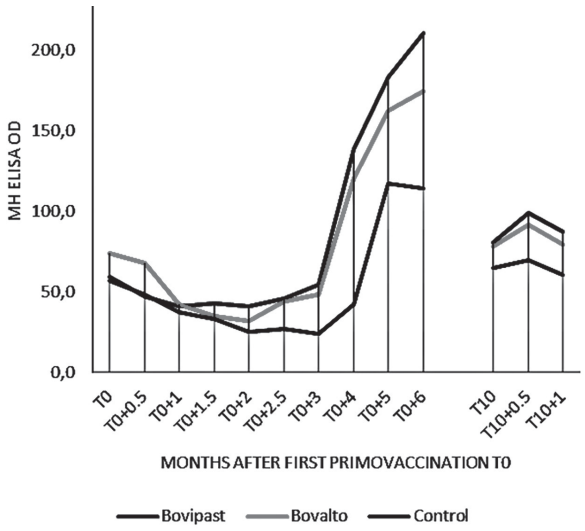

Fig. 4. ***Mannheimia haemolytica*** **optic density measures in serum from calves vaccinated with multivalent bovine respiratory disease vaccines**