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DETECTION OF NEWCASTLE DISEASE VIRUS (NDV) IN LAUGHING DOVES AND THE RISK OF SPREAD TO BACKYARD POULTRY

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ABSTRACT

Newcastle disease (ND) is a highly infectious viral disease of birds caused by the Newcastle disease virus (NDV) and doves have been incriminated in previous outbreaks of the disease that have discouraged backyard poultry productions. This survey was done to detect and characterize the NDV from 184 swabs from the cloacae and pharynxes of 67 trapped laughing doves and 25 backyard poultry birds. The study utilized haemagglutination assay (HA) followed by haemagglutination inhibition (HI) tests on HA positive samples to screen field samples. Conventional reverse transcriptase polymerase chain reaction (RT-PCR) was conducted on the HI positives to characterize the NDV. This study revealed that of 134 dove samples screened, 88 (65.7 %) were HA positive. Of these HA positives subjected to HI testing, 37 (42.1 %) were HI positive. Interestingly, 21 (56.8 %) of the HI positives were also RT-PCR positive: 8 lentogenic, 12 velogenic, while one had both lentogenic and velogenic NDV. Comparatively, of the 50 chicken samples screened, 23 (46 %) were HA positive; and of these, HA positives subjected to HI testing, 16 (69.6 %) were HI positive. Only 4 (25 %) of the HI positives were RT-PCR positive: 3 lentogenic and a velogenic NDV. From this study it was concluded that laughing doves were demonstrated to be infected with either lentogenic or velogenic NDV or both. The use of red blood adsorption-de-adsorption concentration of NDV enhanced the RT-PCR detection using the fusion gene primers NDV-F 4829 and NDV-R 5031. The detection of not only lentogenic but velogenic NDV in laughing doves poses a great risk to backyard poultry production.

Key words: backyard poultry; laughing doves; molecular pathotyping; Newcastle disease virus

INTRODUCTION:

Laughing doves belong to the Kingdom—Animalia, Phylum—*Chordata*, Class—*Aves*, Order—*Columbiformes*, Family—*Columbidae*, Genus—*Streptopelia*, Species—*senegalensis* and subspecies—*senegalensis*. They are small Af-

rican pigeons so named because of their distinct cooing sounds which they make especially in the mornings that mimic human laughter [12]. Laughing doves are commonly called Senegal dove, laughing turtle dove, or little brown dove, depending upon the region in which they are found. Laughing doves are widely distributed throughout most of tropical West Africa, Asia and Arabia. They inhabit desert scrub land from the guinea savannah to the rain forests. Laughing doves usually move in pairs, although monogamous pairs may form large groups of up to 20 to 50 birds in a particular home range. Laughing doves are seldom migratory and may be resident in a particular home range for up to seventeen years.

Adult doves measure about 25 cm long and have an average wing span of 45 cm. Their purplish brown plumage is unique with a pinkish brown breast and a black chequered neck band, purple pink legs and a black bill. The bird is flighty although they may be found nesting very close to human dwellings particularly in garden trees, flower hedges and window ledges. In Nigeria the laughing dove is freely trapped or hunted with catapults and sold as pets or meat to customers from live bird markets. The bird is prolific in the wild and unlike pigeons, do not breed in captivity. Laughing doves (*Streptopelia senegalensis*) in foraging for food, visit households to pick up grains, flour, chaff, and chicken feed spilled on the ground around backyard poultry houses, especially when feeding their nestlings (squabs) and when grains are scarce in the fields [15]. Laughing doves also invade human dwellings in search of drinking water during the dry season [18]. These increase the chances of dove droppings contaminating the premises of households in backyard poultry operations. The soles of footwear of poultry farmers may get contaminated with these wild bird droppings and be transferred into the backyard poultry houses [18]. Also, with broken chicken wire fencing of poultry houses, doves may actually invade such pens and contaminate poultry feed and water with oro-nasal discharges and faeces. Dove droppings on rooftops during the rainy season may be washed down with runoff water into drums and tanks which backyard poultry farmers use to water birds during periods of water scarcity.

Newcastle disease (ND) caused by avian paramyxovirus serotype 1 (APMV-1) viruses can be diagnosed in specimens from both live and dead birds. Most commonly in live birds, swabs of the pharyngeal area and/or cloacae (or faeces) may be tested at appropriate diagnostic laboratories using virus isolation [5, 10]. During seasonal outbreaks of ND, laughing doves and pigeons are usually knocked down and may be found dead under trees or in gutters [17]. These wild doves may act as sentinel animals for the monitoring of ND in an area known for the production of poultry by backyard and large scale poultry farms [10]. The trapping of these doves routinely and sampling for Newcastle disease virus (NDV) may assist avian disease monitoring and surveillance teams predict the eventual outbreak of ND in poultry farms in a region [5, 10]. The "gold standard" for the identification of NDV involves isolation and cultivation in embryonated chicken eggs followed by hemagglutination test, hemagglutination inhibition test and pathotyping of the virus [13]. Pathogenicity is determined by the intracerebral pathogenicity index [13]. These assays are labour intensive and time-consuming, requiring up to ten days to complete. This hinders the authorities in undertaking adequate measures in a timely manner to limit the spread and eradicate the infection [10]. The real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) is a test that satisfies the requirements for high sensitivity and specificity, coupled with a short turnaround time [4, 6, 7, 11, 17].

This study was designed to by-pass the isolation and cultivation of the Newcastle disease virus in embryonated chicken eggs which are time consuming, by utilizing the HA and HI tests to screen field samples and to detect the virus from the oropharyngeal and cloacal swab samples of laughing doves and then confirm the pathotype by RT-PCR and gel-electrophoresis [16, 17, 18].

MATERIALS AND METHODS

Study area

The study areas fell within latitude 11°10'38"N, longitude 7°37'43"E and latitude 11°8'18"N, longitude 7°041'3"E, occupying a land area of 26.59 km^2. The residential areas used for this study were selected by convenience and comprised of the residential clusters in Zaria, especially the staff quarters of A. B. U. Samaru, Zaria, Kaduna State, Nigeria in West Africa (Fig. 1).

Study design

A survey for NDV was carried out on trapped doves and backyard poultry birds from the premises of backyard

poultry farmers in Areas BZ, E, G and C (R1), and areas H, Silver Jubilee of A. B. U. Staff quarters, and Palladan (R2), and from the live bird market in Samaru (R3). These areas are located in Zaria, Kaduna State, Nigeria, West Africa (Fig. 1).

Sampling

Laughing doves are not an endangered species in Nigeria and the birds are usually trapped or shot with catapults by hunters for sale as food or pets. No special ethical clearance was needed therefore in this study, since the birds were treated humanely and were released back into their natural habitats after the sample collections. A total of sixty-seven laughing doves were randomly trapped and sampled along with twenty-five chickens sampled from the backyard poultry from the residential areas based on convenience. This was subject to the number of doves that were trapped during the six month survey period from September 2014 to February 2015. Thus, a sum total of 184 oropharyngeal and cloacal swabs were collected and tested. We purposed to sample at least ten doves and five chickens from each residential area.

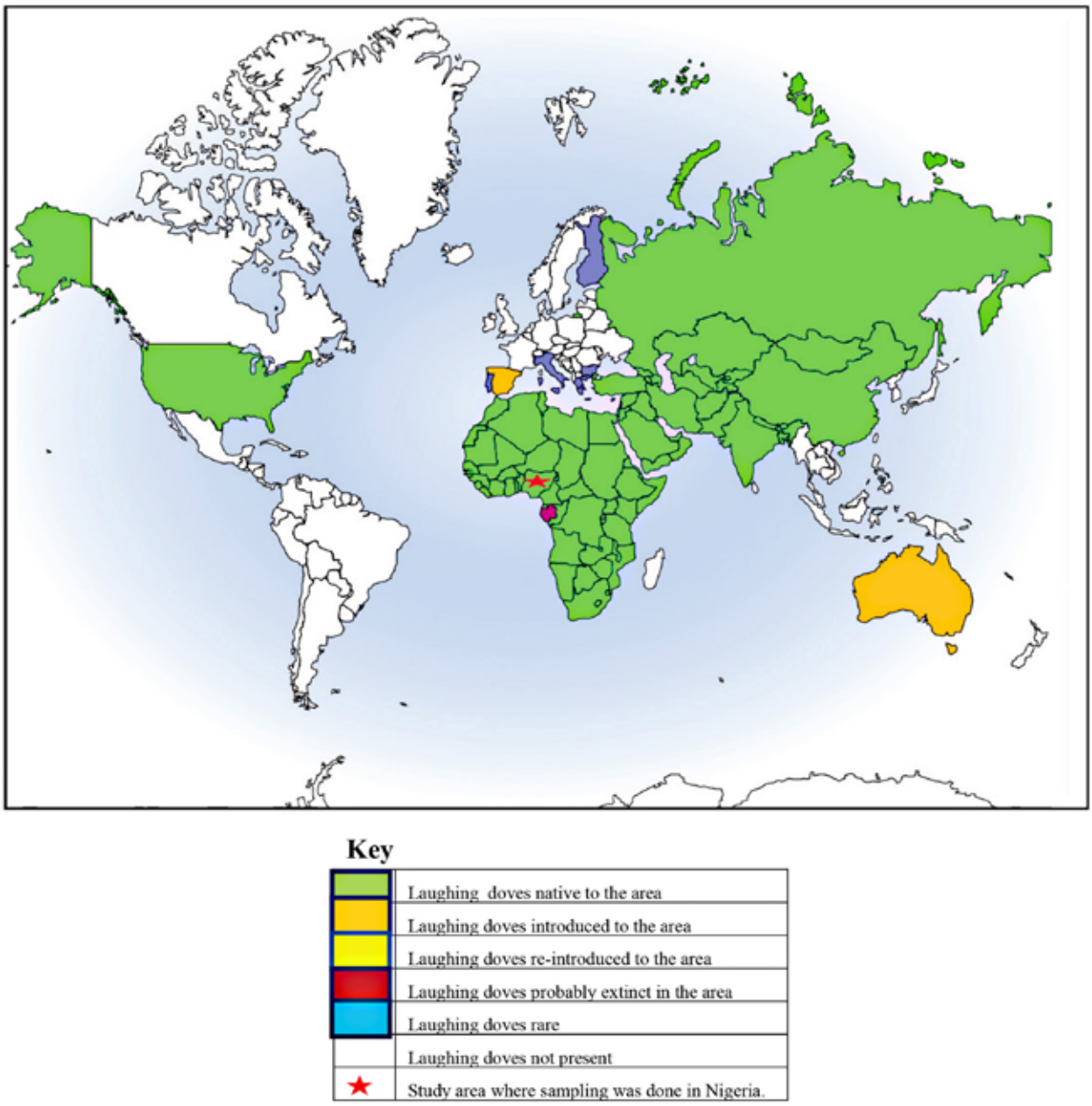

Fig. 1. Worldwide distribution of the Laughing dove [oiseaux.net/maps/laughingdove.html]

Eligibility criteria of participants

All residents with backyard poultry farms who had raised broilers, layers or local chickens for commercial or domestic purposes in the last three years or more were considered as backyard poultry farmers. Farmers with up to 300 birds stocking capacity were considered to have standard backyard poultry farms.

Trapping of laughing doves

Three locally constructed bamboo cages with apparently healthy laughing doves and grains of guinea corn (sorghum) and millet, placed around backyard poultry farms, were used to lure and trap laughing doves into the study areas. The best trapping successes were obtained between 10 a. m.—12 noon and 4 p. m.—6 p. m.; times at which laughing doves foraged from the ground. Between 12 noon—4 p. m. the doves were either watering themselves or resting high up in the trees.

Each dove trapped and sampled was tagged by clasping a non-corroding metallic colour-coded bracelets and rubber bungs on their shanks. Each residential area had a different colour code: Area BZ-green, Area E/G-blue, Area C-golden, Silver Jubilee and Area H-red/pink, and Palladan-silver; while subject doves from the Samaru live bird market were marked on their shanks with black indelible ink. This was to prevent double sampling of subjects and in a follow up future study assess whether laughing doves migrated from one residential area to another area or were limited to the areas in which they were trapped initially (Fig. 2).

Sample collection

Sixty-seven pairs of oropharyngeal and cloacal swabs were collected from randomly trapped laughing doves close to backyard poultry farms. Each dove trapped, ringed and sampled was released back into the wild but was not sampled again even if re-trapped. Fourteen, twenty-eight and twenty-five doves were sampled from clusters 1, 2 and the Samaru live bird market respectively. A similar collection was done on five chickens conveniently selected from three out of four residential areas in cluster 1 and two out of three residential areas in cluster 2, making a total of twenty-five pairs of samples. Oropharyngeal and cloacal swabs from doves trapped close to backyard poultry farms and the Samaru live bird market were eluted in 2.0 ml of viral transport medium in screw cap tubes, and frozen at –20 °C until analysis.

Fig. 2. Trapping (top) and tagging (below) of laughing doves

Preparation of washed chicken red blood cells

Whole blood was collected into EDTA-coated tubes from apparently healthy local chickens with no known history of Newcastle disease vaccination or clinical disease. The whole blood was washed with dextrose gelatine veronal (DGV) solution (pH 7.4), and 10 %, 1 % and 0.1 % suspensions of the washed chicken red blood cells were prepared and stored at 4 ^{0}C until needed [16].

Antigen

Newcastle disease virus antigen was obtained by reconstituting commercially sold Newcastle disease La Sota vaccine and using it fresh for the HI test [13, 15, 18]. The antigen had a titre of 1:256 with a 4 HAU of 64. Sterile PBS was prepared with a pH of 7.4 to serve as a diluent.

Positive control serum

Hyper-immune serum with antibodies to Newcastle disease was obtained by raising ten broilers to 8 weeks

of age, and vaccinating them at week 1, 3, 6 and 8 with La Sota vaccine. Ten millilitres (10 ml) of whole blood was collected from these ten broilers at 10 weeks of age, pooled together, kept to stand, and allowed to coagulate to obtain the serum. The serum was decanted into a screw cap sterile container and stored frozen at –20 °C [16]. The HI titre was determined to be 1:64. Sterile PBS with pH 7.4 served as a diluent for the negative control which was a blank of negative serum.

Laboratory analysis for detection of Newcastle disease virus

Haemagglutination Assay and Haemagglutination Inhibition assay (HA & HI):

Each oropharyngeal and cloacal swab was subjected to HA and HI tests in U-shaped micro-well titre plates. To each drop (0.02 ml) of test sample placed in a well, one drop of one percent (1 %) washed chicken red blood cells was added. The sides of the plate were tapped lightly, and then the plate covered with a piece of paper, and left to stand for 30—45 minutes. The result was read as positive for haemagglutination if a diffuse mat of red blood cells was observed in the well and negative if a button of clumped red blood cells settled at the base of the well. These were compared with the reactions of the negative (RBC and PBS only) and positive (RBC, La Sota NDV and PBS) control wells. Then 0.02 ml of the positive control serum was added to each haemagglutination positive well and the positive control wells to observe for the inhibition of the haemagglutination. HI positive samples transformed from a diffuse mat of red blood cells to a button of clumped cells at the bottom of such a well, while HI negative samples remained as diffuse mats of red blood cells [16]. This was compared with the positive and negative control wells.

RBC adsorption-de-adsorption concentration of NDV

Prior to the RNA extraction and RT-PCR, the NDV was concentrated by adsorption and de-adsorption of the viral particles to and from washed red blood cells respectively [8]. This was done by centrifuging 1 ml of oropharyngeal or cloacal swab HI positive samples in a micro-centrifuge tube for 10 minutes at 12,000 rpm twice. Next, 300 µl of supernatant was transferred into a new centrifuge tube and incubated with 50 µl of washed chicken RBC, then spun at 150 rpm for 30 minutes at room temperature to adsorb the NDV to the RBC (adsorption step). The mix was further spun at 2,110 rpm for 5 minutes at room temperature to concentrate the suspended RBC into a pellet at the base of the tube. The supernatant was decanted. The pellet of RBC with adsorbed NDV was re-suspended in 300 µl of PBS and then 50 µl of 5 mM EDTA and 50 µl of beta mercaptoethanol were added to the mix and incubated at 37 °C for 5 minutes (de-adsorption step). The tube was spun at 2,400 rpm for 5 minutes to separate the NDV from the RBC. Finally, 200 µl of the NDV-rich supernatant was pipetted for the RNA extraction.

RNA extraction

The concentrated 200 µl of the NDV-rich test samples were subjected to the RNA extraction using a BIONEER AccuPrep® Viral RNA extraction kit. To every 200 µl of NDV-rich test sample, 400 µl of a binding buffer (VB) were added, and then transferred into 1.5 ml micro-centrifuge tubes and vortexed for 5 seconds. The tubes were incubated for 10 minutes at room temperature. Then, 100 µl of isopropanol was added to the tube, lightly vortexed for 5 seconds, and then spun for 10 seconds. The binding column was fitted into the 2 ml collection tube. The liquid was transferred into the binding column. The lids were closed carefully and centrifuged for 1 minute at 8,000 rpm. Following the centrifugation, the binding column was transferred to another 2 ml collection tube. Then 500 µl of washing buffer 2 was added and centrifuged for 1 minute at 8,000 rpm. After centrifugation, the binding column was transferred to a 2 ml collection tube. Thereafter, 500 µl of washing buffer 2 was added and centrifuged for 1 minute at 8,000 rpm. The collection tube was spun down once more at 13,000 rpm for 1 minute to remove the ethanol completely. The binding column was transferred to a 1.5 ml collection tube, 50 µl of elution buffer was added, and allowed to stand for 1 minute to allow the buffer to permeate the column. The eluted RNA was retrieved by spinning down at 8,000 rpm for 1 minute and used directly for the RT-PCR.

Reverse transcription—polymerase chain reaction (RT-PCR) test

The RNA extracted from fifty-three of the HI positive samples were subjected to reverse transcriptase polymerase chain reaction (RT-PCR) technique using the primer set NDV-F 4829 (5'-GGTGAGTCTATCCGGARGATACAAG-3') and NDV-R 5031 (5'-TCATTGGTTG-

CRGCAATGCTCT-3'). These primers amplified the Fusion-gene segments of the NDV antigen that coded for lentogenic, mesogenic or velogenic NDV.

The PCR reaction mix per extracted RNA sample contained 13 µl RNase-free water, 50 µl PCR Buffer 2 ($MgSO_4$ 2.4 mM; dNTPs 1.6 mM), 1 µl Primer NDV-F 4829, 1 µl Primer NDV-R 5031, 1 µl Enzyme mix (Taq polymerase enzyme) making a total reagent volume of 45 µl. The mixture was vortexed for a few seconds, then transferred into 0.2 ml PCR tubes. Next, 5 µl of extracted RNA was added making a final volume of 50 µl. The reverse transcription and amplification of cDNA was achieved following opti-

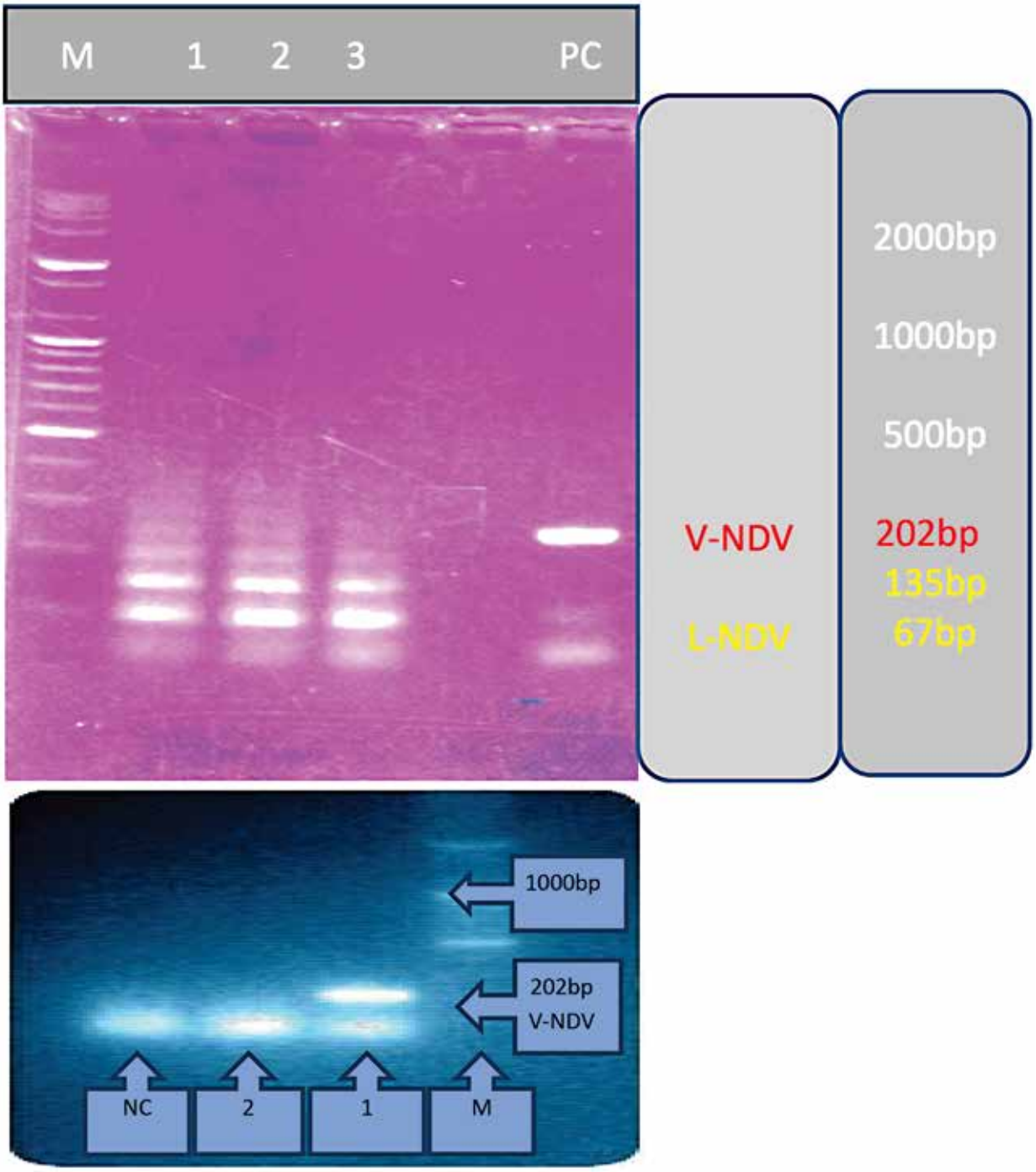

Fig. 3. Gel electrophoretogram of RT-PCR products from dove and chicken samples

Lane M—marker; Lanes 1, 2 and 3—NDV samples from doves in cluster 1, 2 and the Samaru live bird market respectively; PC—positive control from optimized velogenic NDV compared with lentogenic NDV from La Sota vaccine; NC—negative control; bp—base pair; V-NDV—velogenic Newcastle disease virus; L-NDV—lentogenic Newcastle disease virus. Swabs 1 and 3 were oropharyngeal swabs while swab 2 was a cloacal swab

mization of the cycling conditions using the NDV RNA extracted from the La Sota vaccine which also served as a positive control. Placing the reaction mix in the thermocycler, cDNA was produced by heating to 42 °C for 60 minutes, followed by raising the temperature to 94 °C for 5 minutes to inactivate the enzymes. cDNA was amplified in 40 cycles, consisting of 95 °C for 15 seconds, 49 °C for 30 seconds, and then 72 °C for 30 seconds. After that, the 40 cycles enzymes were inactivated by maintaining the temperature at 72 °C for 7 minutes. Amplicons were stored at 4 °C until use.

Agarose gel electrophoresis

Amplicons were transferred into the wells of a 2 % agarose gel for gel electrophoresis. The results were visualized after gel electrophoresis as follows: Lentogenic NDV appeared as 2 bands of approximately 135 bp and 67 bp, while mesogenic or velogenic NDV appeared as a single band of approximately 202 bp, respectively (Fig. 3).

Data analysis

The percent positives and frequency tables were calculated and drawn for the detection rates obtained by HA, HI and RT-PCR tests on oropharyngeal and cloacal swabs obtained from the doves and chickens from the field survey.

RESULTS

Detection of Newcastle disease virus

Out of 134 oropharyngeal and cloacal swab samples from the laughing doves examined, 88 (65.7 %) were HA positive. Of the 88 HA positives, 37 (42.1 %) were HI positive. On subjection of the 37 samples that were both HA and HI positive to conventional RT-PCR, 21 (56.8 %) of them were positive. The detection rate was higher in the cloacal (45.7 %) than the oropharyngeal (38.1 %) samples (Table 1).

Meanwhile, of the 50 samples from chickens subjected to HA test, 23 (46 %) showed haemagglutination and sixteen (69.6 %) of the 23 HA positives were HI positive. In order to determine the pathogenicity of the NDV, the sixteen samples that showed HA that were inhibited by the positive control hyper-immune serum were subjected to conventional RT-PCR. Only four (25 %) of such HA and HI positive samples were positive by RT-PCR. The detection rate was higher in the oropharyngeal (75.0 %) than the cloacal (63.6 %) samples (Table 2).

Cluster 1 (Areas BZ, C, E, and G) had 23 (82.1 %) of the 28 samples from the doves trapped close to backyard poultry houses, being HA positive. Nine (39.1 %) of the 23 HA positives were also HI positive. However, four (44.4 %) of the nine samples that were both HA and HI positive were RT-PCR positive. While cluster 2 (Areas H, Silver Jubilee and Palladan) from which 56 samples from doves were examined, 41 (73.2 %) were HA positive. Twelve (29.3 %) of the HA positives were HI positive. Four (33.3 %) of the twelve HA and HI positives were RT-PCR positive. Cluster 3 (Samaru live bird market) had 50 swabs from the doves and 24 (48 %) were HA positive. Of the 24 HA positives 16 (66.7 %) were HI positive also. Interestingly, 13 (81.3 %) of the 16 HA and HI positives were detectable by conventional RT-PCR (Table 3).

For comparison with the dove swabs, fifty swabs were obtained and examined from chickens from backyard poultry farms in clusters 1 and 2. Thirty swabs obtained from chickens in cluster 1 (Areas BZ, C, E and G) were subjected to HA tests and fifteen (50 %) were HA positive. Nine (60 %) of the fifteen HA positives were also HI positive. However, only three (33.3 %) of the HA and HI positives were detectable by the conventional RT-PCR. Of the twenty swabs from chickens in cluster 2 (Areas H, Silver Jubilee and Palladan), eight (40 %) were HA positive. Seven (87.5 %) of the HA positives were also HI positive. Upon subjection of these seven HA and HI positives to RT-PCR, only one (14.3 %) was positive (Table 4).

Table 5 summarizes the pathogenicity of the detectable NDV from HA and HI positive swabs by RT-PCR from doves and chickens from the three sampling clusters (R1, R2 and R3). Of the four swabs from fourteen doves trapped, sampled and examined from cluster 1 (Areas BZ, C, E and G) that were HA, HI and RT-PCR positive, three were velogenic while one was lentogenic NDV. Of the four swabs from twenty-eight doves from cluster 2 (Areas H, Silver Jubilee and Palladan) that were HA, HI and RT-PCR positive, two were velogenic, one was lentogenic and one was a mixture of both velogenic and lentogenic NDV. Following the trend, thirteen swabs from the twenty-five doves in cluster 3 (Samaru live bird market) were HA, HI and RT-PCR positive. Of these thirteen positives, seven were velogenic, while six were lentogenic NDV. In compar-

Table 1. Detection of Newcastle disease antigen in Laughing doves trapped around backyard poultry farms and the live bird market sampled in Samaru, Zaria, Nigeria

Subject	Swab type	No. tested	HA positive (%)	HI positive (%)	PCR positive (%)
Dove	Oropharyngeal	67	42 (62.7)	16 (38.1)	11 (68.8)
Dove	Cloacal	67	46 (68.7)	21 (45.7)	10 (47.6)
Total		**134**	**88 (65.7)**	**37 (42.1)**	**21 (56.8)**

Table 2. Detection of Newcastle disease antigen in chickens from backyard poultry farms sampled in Samaru, Zaria, Nigeria

Subject	Swab type	No. tested	HA positive (%)	HI positive (%)	RT-PCR positive (%)
Chicken	Oropharyngeal	25	12 (48.0)	9 (75.0)	2 (22.2)
Chicken	Cloacal	25	11 (44.0)	7 (63.6)	2 (28.6)
Total	**–**	**50**	**23 (46.0)**	**16 (69.6)**	**4 (25.0)**

Table 3. Detection of NDV in laughing doves using HA, HI and RT-PCR tests according to sample area sub-units, in Samaru, Zaria, Nigeria

Subject	Area	Sample type	No. tested	HA positive (%)	HI positive (%)	PCR positive (%)
Dove	Cluster 1	OPS/CS	28	23 (82.1)	9 (39.1)	4 (44.4)
Dove	Cluster 2	OPS/CS	56	41 (73.2)	12 (29.3)	4 (33.3)
Dove	Cluster 3	OPS/CS	50	24 (48.0)	16 (66.7)	13 (81.3)
Total	**–**	**–**	**134**	**88 (65.7)**	**37 (42.0)**	**21 (56.7)**

OPS/CS—Oropharyngeal swab and cloacal swab; Cluster 1—Areas BZ, C, E and G
Cluster 2—Areas H, Silver Jubilee and Palladan; Cluster 3—Samaru live bird market

Table 4. Detection of NDV in chickens from backyard poultry farms using HA, HI and RT-PCR tests according to sample area sub-units, in Samaru, Zaria, Nigeria

Subject	Area	Sample type	Number tested	HA positive (%)	HI positive (%)	PCR positive (%)
Chicken	Cluster 1	OPS/CS	30	15 (50.0)	9 (60.0)	3 (33.3)
Chicken	Cluster 2	OPS/CS	20	8 (40.0)	7 (87.5)	1 (14.3)
Total			**50**	**23 (46.0)**	**16 (69.6)**	**4 (25.0)**

OPS/CS—Oropharyngeal swab and cloacal swab; Cluster 1—Areas BZ, C, E and G; Cluster 2—Areas H, Silver Jubilee and Palladan

Table 5. Pathotype of NDV detected in subjects based on RT-PCR and gel electrophoretyping in Samaru, Zaria, Nigeria

Subject	Area	Sample	PCR positive (%)	Lentogenic (%)	Velogenic (%)	Mixed Pathotype (%)
Dove	Cluster 1	OPS/CS	4 (44.4)	1 (25.0)	3 (75.0)	0
Dove	Cluster 2	OPS/CS	4 (33.3)	1 (25.0)	2 (50.0)	1 (25.0)
Dove	Cluster 3	OPS/CS	13 (81.3)	6 (46.2)	7 (53.8)	0
Chicken	Cluster 1	OPS/CS	3 (33.3)	2 (66.7)	1 (33.3)	0
Chicken	Cluster 2	OPS/CS	1 (14.3)	1 (100)	0	0
Total			**25 (47.2)**	**11 (44)**	**13 (52)**	**1 (4)**

OPS/CS—Oropharyngeal swab and cloacal swab; Cluster 1—Areas BZ, C, E and G
Cluster 2—Areas H, Silver Jubilee and Palladan; Cluster 3—Samaru live bird market

ison of the three swabs from chickens in cluster 1 that were HA, HI and RT-PCR positive, two were lentogenic while one was velogenic NDV. The single HA, HI and RT-PCR positive swab from chickens in cluster 2 was lentogenic NDV (Table 5).

DISCUSSION

NDV detection and characterization

This study was able to demonstrate that there were high levels of the NDV circulating in wild laughing doves and backyard chickens. C h a n t a l et al. [2] had reported that chickens were more often infected by the NDV than other birds. They also reported that chickens from live bird markets were significantly more often positive for the NDV than birds at commercial or backyard farms which contributed to the enzootic circulation of the NDV. This is in agreement with the findings from this study in which the detection of the NDV in doves was significantly higher in birds from the Samaru live-bird market than trapped wild doves from the residential areas in clusters 1 and 2. This could be attributed to the poor biosecurity measures at the live-bird market. Doves trapped from within and outside Zaria were kept with chickens and other birds in small cages at the live bird market. This increased the possibility of infection between susceptible and infected birds shedding the virus due to the close proximity of birds in such small cages, poor hygiene, poor nutrition, transportation stress and overstocking. Residents from the two clusters 1 and 2 who buy live doves or chickens from the live-bird market and return home with them may help in the enzootic circulation of NDV in Samaru-Zaria. C h a n t a l et al. [2] explained that chickens in live-bird markets in Nigeria were sourced from both vaccinated (commercial or backyard poultry) and unvaccinated (free-range local chickens) flocks. Vaccination likely reduced the expression of clinical signs of Newcastle disease. This did not necessarily suppress viral shedding from asymptomatic chickens. The relatively high detection of the NDV from apparently healthy backyard chickens from the two clusters 1 and 2 in our study was not unexpected as farmers vaccinated their chickens as often as once every month. C h a n t a l et al. [2] had also reported that outbreaks of Newcastle disease in vaccinated flocks have been increasingly reported from Nigeria suggesting a suboptimal protection by vaccination.

In this study, our detection of NDV from oropharyngeal swabs was higher than from cloacal swabs of chickens. This agreed with H a q u e et al. [7] who reported that the viral isolation rate from clinical samples was found highest in the tracheal swabs (90 %) compared to cloacal swabs (85 %) and serum (65 %). However, in our own study, the reverse was the case for samples from doves with detection higher in cloacal swabs than oropharyngeal swabs.

Also, it was discovered that the NDV from doves were slightly more of the mesogenic or velogenic [3] than the lentogenic pathotypes. This agrees with the findings from K e r r i et al. [9] who reported that Pigeon Paramyxovirus-1 (PPMV-1) from doves were typically mesogenic by intra-cerebral pathogenicity index (ICPI) in chickens. It has also been reported that PPMV-1 isolates from doves increased their virulence in chickens after passage and therefore represented a genuine threat to poultry production [12]. The isolation of a virulent strain of the NDV from field samples required reporting to the Office International des Epizooties (OIE) [13]. With the detection of virulent or mesogenic NDV from laughing doves in Zaria in this study there is a need for reporting to the OIE by the Veterinary Agencies of the Nigerian Government following standard reporting protocol.

Newcastle disease virus could be detected by subjecting oropharyngeal swabs (OPS) and or cloacal swabs (CS) from laughing doves and backyard poultry to haemagglutination assay as a screening test since the NDV has haemagglutinating properties [14]. Subjecting positive haemagglutinating samples to haemagglutination inhibition assay using hyper-immune serum allowed us to be able to narrow down the confirmatory detection of NDV cheaply. Running the reverse-transcription polymerase chain reaction on such HI positives we were able to confirm the presence of the Newcastle disease virus RNA, classify the NDV into lentogenic or mesogenic/velogenic pathotypes and reduce the number of samples to be subject to RT-PCR which is quite expensive in developing economies. The sensitivity of RT-PCR could be increased by concentrating the virus in swabs by a red blood cell adsorption-de-adsorption technique [8]. The higher detection of the ND antigen from cloacal swabs than oropharyngeal swabs of laughing doves may be attributed to the fact that birds were primarily exposed to the NDV by the oro-nasal routes. The localization of the virus in the gastrointestinal tract is common in the lentogenic Newcastle disease as the lentogenic

NDV has a monobasic amino acid motif at the F-cleavage site 112 G-R/K-Q-G-R*L 117 which is cleaved extracellularly by trypsin-like proteases found in the respiratory and more so in the intestinal tract [9]. It has been reported that the Newcastle disease virus that was detected in pigeons was mostly the lentogenic strains which could mutate to the mesogenic/velogenic strains after passaging in chickens [12]. The vaccination of chickens with La Sota vaccine produces a mild respiratory form of Newcastle disease [16] and may have influenced the detection pattern, with higher detection rates from the oropharyngeal swabs than from the cloacal swabs of chickens. This study reaffirmed that HA and HI tests can be used to detect NDV from field samples. Also, other haemagglutinating avian pathogens not limited to Avian Influenza (H5N1) virus, infectious bronchitis virus [1] and Egg-drop syndrome ad enovirus, and unlikely agents like *Mycoplasma gallisepticum*, *M. septicum* and Avian Pathogenic *Escherichia coli* with haemagglutinating properties could be diagnosed similarly. In our study, the HA positives that were not HI positive could be due to the avian influenza virus or any of the haemagglutinating pathogens mentioned previously.

W a m b u r a [18] had reported that the NDV had only been demonstrated in Nigerian pigeons by serological evidence and this posed a risk to village chickens [15]. Our study revealed that the NDV could be demonstrated directly from oropharyngeal and cloacal swabs from doves and chickens. The subjection of such swabs to HA tests followed by HI tests demonstrated the presence of the NDV. The conventional RT-PCR using the primer set NDV-F 4829 and NDV-R 5031 was able to pathotype the NDV demonstrated from swabs that were both HA and HI positive, as either lentogenic and or velogenic [19]. The "gold standard" for the diagnosis of the NDV involves isolation and cultivation in embryonated chicken eggs, followed by HA, HI and ICPI [13] which are labour intensive and time consuming. The methods employed in our study used HA to screen field swab samples rapidly. HA positives were then subjected to HI test which was also easily done. And then samples that were both HA and HI positive were subjected to RT-PCR for pathotyping. In order to improve the sensitivity of the RT-PCR, the HA and HI positives were subjected to a red blood cell adsorption-de-adsorption concentration prior to the RT-PCR which mimicked the results reported by J i a n z h o n g and C h e n g q i a n [8]. The use of HA, HI and RT-PCR in our study seemed cheaper, faster, easier, convenient and more feasible for backyard poultry outbreak investigations than the gold standard for the NDV detection. There was also no need to sample the birds twice as we would have done if we depended on serology to differentiate between the active infection and previous exposures.

CONCLUSIONS

From our study the following conclusions were made:

About 65.7 % of the oropharyngeal and cloacal swabs from the trapped laughing doves sampled in Samaru-Zaria exhibited haemagglutination, 42.1 % of which was due to Newcastle disease virus. Further, studies should be geared towards screening laughing doves for haemagglutinating avian pathogens particularly: Avian Influenza virus, Egg-drop syndrome adenovirus, and *Mycoplasma gallisepticum*; using HA, HI, and RT-PCR for monitoring and surveillance purposes.

About 38.1 % of the NDV detected in the trapped laughing doves sampled in Samaru-Zaria were demonstrated to be lentogenic, 57.1 % were mesogenic/velogenic while 4.8 % were a mixture of both lentogenic and velogenic NDV. The sequencing and phylogenetic blasts of the isolated samples from doves and chickens would be able to confirm whether the NDV in the doves and chickens were of the same origin.

There may be enzootic circulation of the NDV between chickens and laughing doves. There may be a need to consider the vaccination of laughing doves with thermostable I-2 Newcastle disease vaccine so that velogenic NDV in doves does not wipe off a large number of laughing doves or result in an epizootic of Newcastle disease in wild birds.

It is likely that frequent passaging of lentogenic NDV from doves in chickens resulted in mesogenic or virulent NDV being shed to doves again; hence the detection pattern observed in this study. Further studies should be done on the tagged doves to evaluate the migratory patterns of laughing doves amongst the different residential areas. The veterinary agencies of governments should also encourage more research on the role of laughing doves in the spread of Newcastle disease to poultry.

Running HA-HI on field samples from doves and chickens as a screening test before subjecting positive HA-HI samples to RT-PCR cut down costs that would have

been incurred if all field samples were subject to RT-PCR directly. This modified HA-HI screening method for field samples from wild and domestic birds should be made into a commercial kit that could easily be used by backyard poultry farmers to screen for the NDV in their flocks so as to know when to give booster La Sota NDV vaccines.

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RESPONSE OF THREE NIGERIAN BREEDS OF SHEEP EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA VIVAX* TO DIMINAZENE ACETURATE THERAPY

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ABSTRACT

The efficacy of diminazene aceturate in three Nigerian breeds of sheep [West African Dwarf (WAD), Yankassa and Ouda] experimentally infected with *Trypanosoma vivax* was studied. Five rams of each breed were administered 0.5 ml of goat blood containing 2.5 × 10^6 *T. vivax* per millilitre intravenously, while three rams of each breed served as uninfected controls. The treatment with diminazene aceturate was intramuscularly administered to the infected sheep, when their packed cell volume (PCV) fell to 15 %, at a dosage of 7 mg.kg^{-1} b. w. The parameters measured were parasitaemia, live weight gain and PCV. By 24 hours post treatment (pt.), no trypanosomes were detected by either the Haematocrit Concentration Technique (HCT) or the Polymerase Chain Reaction (PCR) in the blood of any of the treated sheep. However, a relapse of parasitaemia occurred 17 to 32 days pt. in 46.7 % of the treated rams and these were retreated with 14 mg.kg^{-1} b. w. diminazene aceturate. There were gradual increments in the live weight gain and the PCV of the treated rams until the resurgence of parasitaemia. Ouda had the highest cases of relapse (80 %), the least mean live weight gain and was the only breed in which mortality was recorded despite the treatment. In conclusion, diminazene aceturate administered at 7 mg.kg^{-1} b. w. cleared the trypanosomes in the blood of all the treated sheep within 24 hours and this was accompanied by the restoration of lost weight and the reversal of anaemia. However, the subsequent resurgence of parasitaemia indicated that a dosage of 7 mg.kg^{-1} b. w. was no longer reliable for complete elimination of trypanosomes from all the tissues of the host.

Key words: breeds; diminazene aceturate; sheep; therapy; *Trypanosoma vivax*

INTRODUCTION

Trypanosomiasis is a vector-borne haemoprotozoan disease that affects man and other animals. It is caused by *Trypanosoma* species which are commonly transmitted cyclically by tsetse flies (*Glossina* spp.) and at times mechanically by other biting flies [10, 25, 33]. African animal trypanosomiasis is caused primarily by: *Trypanosoma congolense* (*T. congolense Savannah, T. congolense Forest, T. congolense Kilifi*), *T. vivax* and *T. brucei*. *Trypanosoma vivax* is one of the most pathogenic species of trypanosomes affecting a wide host range including cattle, sheep, goats, horses and donkeys; and can be transmitted both cyclically and mechanically. The disease is characterized by: undulating fever, anorexia, anaemia, body weight loss, lymphadenopathy, growth retardation, abortion and reduced fertility [9, 12, 16, 31].

In Africa, trypanosomiasis is controlled principally by means of chemotherapeutics or chemoprophylactic agents [19]. Other control methods are: reducing or eliminating tsetse flies with traps or insecticides and by selection of trypanotolerant breeds [37]. In Nigeria, the available trypanocides include diminazene aceturate, isometamidium, and homidium; but most times diminazene aceturate and isometamidium are the drugs used extensively [8, 11, 34]. There are widespread reports of resistance to trypanocides, particularly diminazene aceturate in the treatment of animal trypanosomiasis and several authors have reported it in different animals such as calves, sheep, dogs and rats [1, 3, 13, 14]. Some authors have attributed the resistance to sub-standard drug preparations, under dosing of the drug and overuse of the same type of trypanocide [15, 27]. D e K o n i n g [7] and D e K o n i n g et al. [8] associated the resistance to diminazene aceturate with reduced uptake of the drug by the trypanosomes. In Nigeria, the most common breeds of sheep are the West African Dwarf (WAD) in the forest zone, Yankassa in the grasslands and Ouda in the sahel/arid region. They serve as valuable supplements to cattle as sources of animal protein towards food security of the nation. Attempts to develop vaccines against trypanosomiasis have not succeeded mainly due to the continuous change of the protective surface antigen of the parasites [4, 21]. Also, efforts to control the disease through elimination of the tsetse vectors have largely been abandoned in sub-Saharan Africa due to fund constraints. No new veterinary trypanocide has been developed for over five decades and there is a very slim probability of the production of new trypanocides in the near future. Furthermore, these challenges are compounded by injudicious administration of the available trypanocides by unqualified personnel, including the animal rearers themselves. Consequently, there are increasing number of reports of resistance of trypanosomes to the existing therapeutic agents [15, 27, 32].

There is a dearth of information on the susceptibility to drugs of trypanosomes in sheep in Nigeria. This study was undertaken to evaluate the therapeutic efficacy of diminazene aceturate at a dosage of 7 $mg.kg^{-1}$ in three Nigerian breeds of sheep experimentally infected with *T. vivax*, as judged by parasite clearance, and the effects of treatment on the live weight and (PCV) of the infected animals.

MATERIALS AND METHODS

Experimental Design

Eight rams each from West African Dwarf (WAD), Yankassa and Ouda were obtained from Akinyele sheep markets, Ibadan, Oyo State and Teaching and Research farm, Alabata, Ogun State, and were acclimatized for a period of 30 days. The animals were housed in a pen partitioned into six units and a tsetse fly trap was positioned in the holding yard. Five rams of each breed served as the infected group while three rams of each breed served as the uninfected control group. Each breed of infected and control was housed separately in a fly-proof house. The sheep were screened for endo- and ecto-parasites and treated accordingly with albendazole at a dose of 10 $mg.kg^{-1}$ b. w. and cypermethrin pour-on at 6 $mg.kg^{-1}$ b. w. An isolate of trypanosomes obtained from naturally infected cattle was found to be *Trypanosoma vivax* [by PCR using internal transcribed spacer (ITS) primers]. The result was positive for *T. vivax*. This was further confirmed using a specie specific primer set (ILO 1264 and ILO 1265) for *T. vivax*. A goat that screened negative for worms and trypanosomes was used for the final passaging. The parasitaemia was estimated using a rapid matching method as described by H e r b e r t et al. [20]. The blood was diluted with normal saline to bring the parasitaemia to 5×10^6 per ml of the blood. Each sheep was infected with 0.5 ml of goat blood (containing approximately 2.5×10^6 *Trypanosoma vivax* organisms) through the jugular vein.

The parasitaemia was confirmed by both the haematocrit centrifugation technique (HCT) [28] and the PCR.

Thereafter, the PCR products obtained from experimentally infected sheep with high *T. vivax* parasitaemia were sent for sequencing to the Core laboratory of Cornel University (NY, USA). Each animal was treated with a freshly prepared 7 % solution of diminazene aceturate (Nozomil® Kepro B.V., Holland) intramuscularly at the rate of 7 $mg.kg^{-1}$ b. w. when the PCV fell to 15 %. The PCV limit of 15 % before initiating treatment was chosen because M u r r a y et al. [29] had shown that it was at this PCV value or less that death might occur. Relapsed cases were treated with 14 $mg.kg^{-1}$ b. w. of diminazene aceturate (Nozomil®). Where needed, a third treatment was effected with isometamidium at the rate of 1 $mg.kg^{-1}$ b. w.

About 3 ml of blood was collected through the jugular vein into an EDTA bottle from each of the experimental animals twice weekly for 6 weeks following the treatment with diminazene aceturate. The parasitaemia, live weight gain and PCV were measured and documented.

Determination of live weight gain

The live weight of all the animals were measured twice weekly using a hanging scale.

Determination of PCV

Blood was drawn into heparinized capillary tubes, one end of the tubes was sealed with plasticine and the tubes were centrifuged at 10,000 g for 5 min and the PCV was measured with a microhaematocrit reader [24].

Estimation of parasite centrifugation method of W o o [40] was used as described by M u r r a y et al. [28]. Briefly, the area immediately above the buffy coat in the haematocrit capillary tube was examined directly under the microscope for the presence of trypanosomes at 400× magnification. Afterwards, the buffy coat and upper most layers of red blood cells of the capillary tube were extruded onto a microscopic slide and examined with a phase—contrast microscope at ×400 magnification [28] for the presence of trypanosomes.

Detection of trypanosomes by polymerase chain reaction (PCR)

The DNA was extracted from the blood using QuickgDNATM Mini-Prep (Zymo Research Corporation, Irvine, CA, USA) as described by the manufacturer. Briefly, 400 µl of genomic lysis buffer was added to 100 µl of blood, thoroughly mixed and incubated at room temperature for 5—10 minutes. The mixture was transferred to a spin column in a collection tube and centrifuged at 10,000 g for 60 seconds after which the collection tube with the flow through was discarded and the spin column transferred to a new collection tube. A volume of 200 µl of prewash buffer was added to the spin column and centrifuged at 10,000 g for 60 seconds, after which 500 µl of genomic DNA wash buffer was added to the spin column and centrifuged at 10,000 g for 60 seconds. The soluble DNA was eluted into a clean 1.5 ml micro-centrifuge tube, incubated at room temperature for 2—5 minutes and centrifuged at 16,000 g for 30 seconds. The eluted DNA was stored at –20 °C until use.

The PCR amplification was performed in 20 µl final reaction volume containing the equivalent of 20 ng of genomic DNA 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 µM KCl, 200 µM each of dNTPs, 40 ng of each of the primers and 1 unit of Taq DNA polymerase (Bioneer, Inc. Alamada, CA, USA). The generic primers set that target the internal transcribed spacer 1 (ITS 1) used was (CF: 5'-CCGGAAGTTCACCGATATTG-3' and BR: 5'-TGCTGCGTTCTTCAACGAA-3' [30]. The primer set used for *T. vivax* was ILO 1264 and ILO 1265 and its primer sequence 5'-3" was CAGCTCGCCGAAGGCCACTTGGCTGGG and TCGCTACCACAGTCGCAATCGTCGTCTCAAGG [26].

The reactions were placed in a MJ MINITM Personal Thermocycler model PTC-1148 (Biorad, Hercules, CA, USA). The reaction conditions were as follows: For ITS initial denaturation of the DNA at 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 1 minute, 58 °C for 1 min and 72 °C for 1 min with the final extension at 72 °C for 10 min. For *T. vivax* initial denaturation at 94 °C for 4 minutes followed by 35 cycles of 94 °C for 4 minutes followed by 35 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 30 s followed by the final extension at 72 °C for 5 minutes.

The gel electrophoresis procedure; 10 µl of the PCR products were electrophoresed through 1 % agarose gel in 1× TBE (89 mM Tris, 89 mM boric acid 1 mM EDTA) at 90 V for 80 minutes, along with 10 µl of GENEMate Quanti-Marker 100bp DNA ladder (BioExpress, Kaysville, UT, USA). The gels were stained with GelRed® Nucleic Acid stain (Phenix Research Products, Candler, NC, USA) at 5 µl.100 ml^{-1} of the agarose gel suspension. After electrophoresis, the PCR products were visualized on a UV transilluminator and were photographed using an Alphalmager HP System (Protein Simple, Santa, Clara, CA, USA).

The PCR products obtained were sent for partial sequencing to the Core laboratory of Cornell University using a forward primer. The obtained sequences were BLAST for homology search in GenBank.

Data Analysis

The data obtained were summarized as the means ± standard deviation and the groups were compared by one-way ANOVA.

RESULTS

Parasitaemia before treatment

Parasitaemia was confirmed in all infected sheep by both the HCT and PCR techniques before the commencement of treatment with diminazene aceturate. The sequencing of the isolate of *T. vivax* used in this study revealed 99 % homology with the *T. vivax* with accession numbers U43183 and L25129 in GenBank (Fig. 1).

The phylogenetic tree was inferred using an unweighted pair group method with arithmetic mean (UPGMA) algorithm, involving a bootstrap procedure with 1000 replicate and evolutionary distance adjusted using the Kimmura-2 parameter.

Duration of infection prior to and outcome of the treatment

The mean (and range) period before the PCV of *T. vivax* infected sheep declined to 15 % (and hence required therapy) were 25.0 ± 12.1 days (18—39 days), 28.3 ± 12.9 days (14—39 days), and 23.5 ± 7.1 days (18—34) for WAD, Yankassa and Ouda, respectively. All sheep treated with 7 $mg.kg^{-1}$ b. w. of diminazene aceturate recovered, except one Ouda sheep that died one-day pt. Relapses of infection occurred in seven of the remaining 14 sheep: four (80 %) in Ouda breed, two (40 %) in the WAD and one (20 %) in the Yankassa. The mean (range) days to relapse were 21.75 ± 7.09 (17—32), 26.0 ± 7.07 (21—31) and 21 ± 00 (21) for Ouda, WAD and Yankasa, respectively.

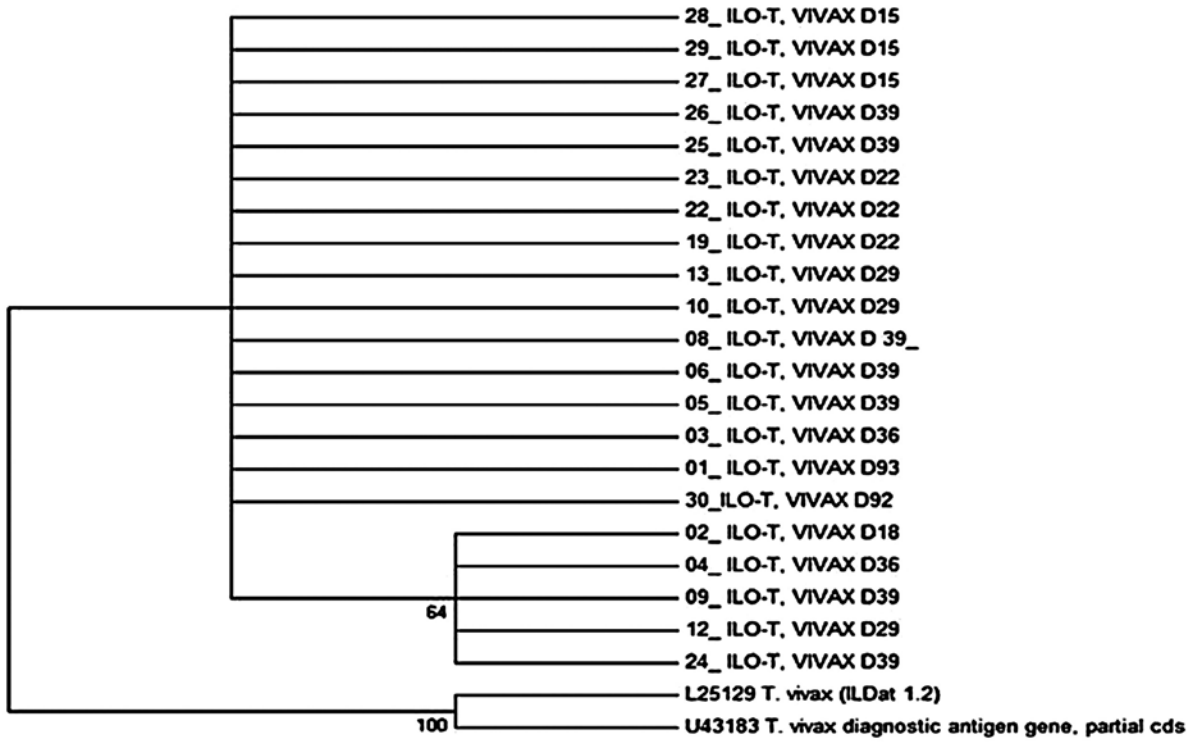

Fig. 1. Phylogenetic Tree of *T. vivax* isolates from this study (01_ILO-*T. vivax* to 30_ILO-*T. vivax*) and two *T. vivax* sequences obtained from GenBank

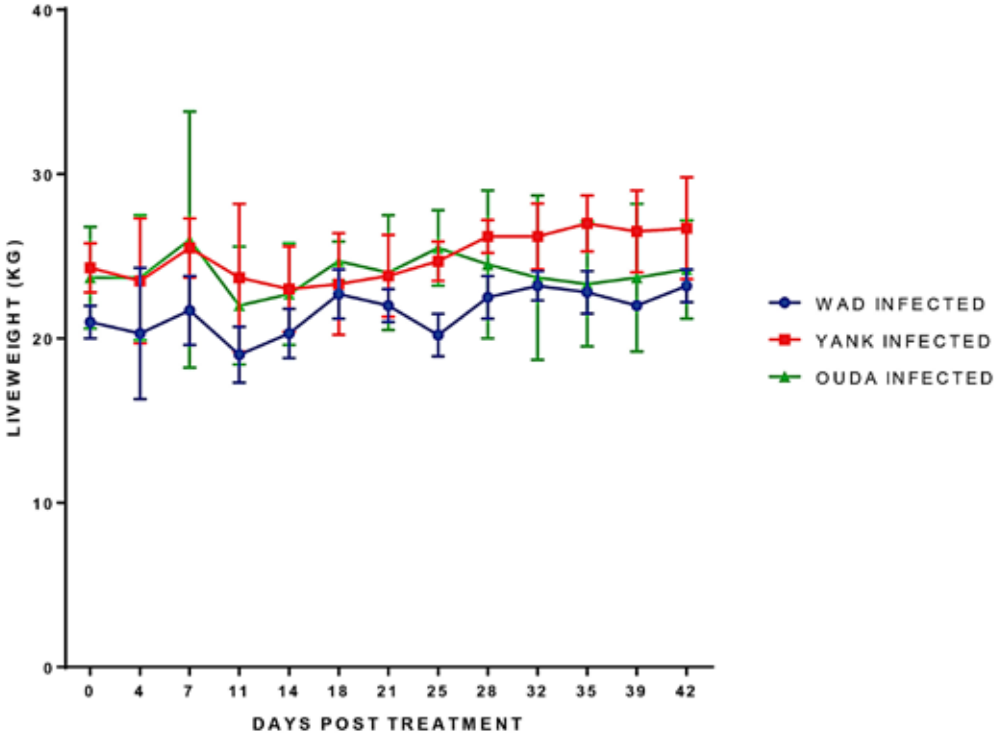

Fig. 2. Live weight values (means ± SD kg) of sheep following the treatment of experimental *T. vivax* infection with diminazene aceturate

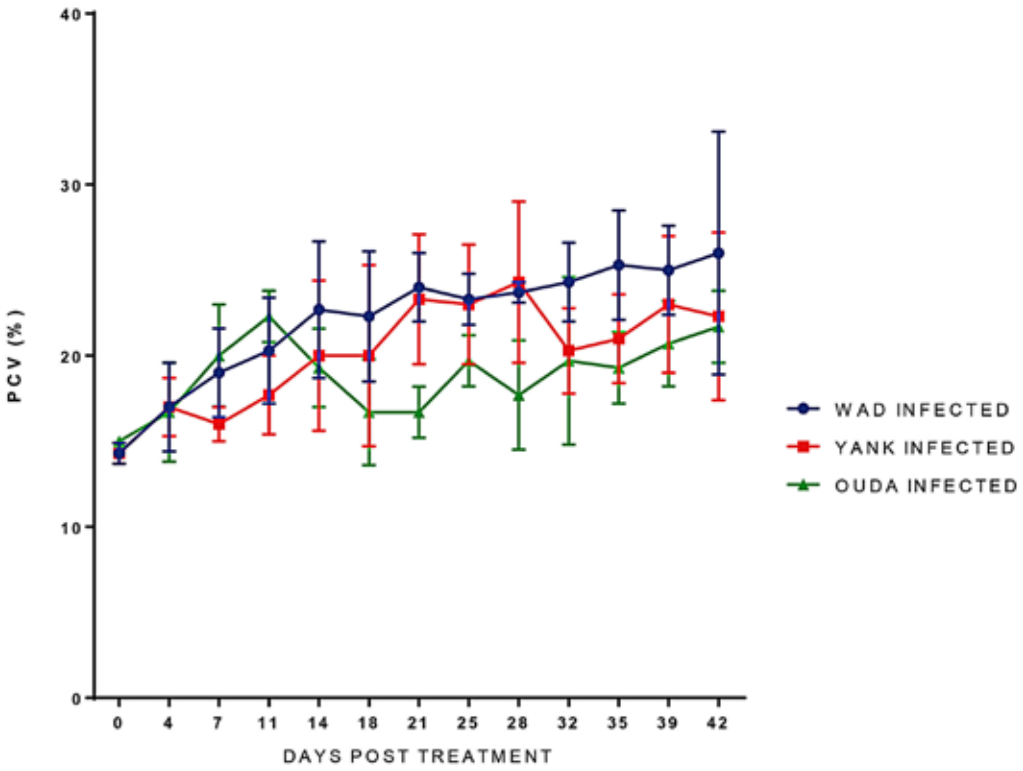

Fig. 3. Packed cell volume values (%; means ± SD) of the sheep following the treatment of experimental *T. vivax* infection with diminazene aceturate

These relapsed infections were re-treated with 14 mg.kg^{-1} diminazene aceturate. Of these, one sheep had a relapse of infection 27 days post second treatment and was treated with isometamidium (Trypamidium-samorinR) at a dose of 1 mg.kg^{-1}. The diminazene aceturate therapy study was finally terminated at day 42 pt. Throughout the period of the experiment, all of the uninfected controls were negative for trypanosomes.

Parasitaemia and live weight after treatment

By 24 hours pt., trypanosomes were not detected by HCT or PCR in the blood of any of the treated live sheep. The live weight of sheep after treatment with diminazene aceturate is presented in Fig. 2. The pre-treatment live weight of WAD, Yankassa and Ouda (21.0 ± 1.0, 24.3 ± 1.5, 23.7 ± 3.1, respectively) when compared with post-treatment live weight (23.2 ± 1.0, 26.7 ± 3.1, and 24.2 ± 3.0, respectively) at day 42 pt. were not significantly ($P > 0.05$) different.

Packed Cell Volume

The mean PCV of sheep before and after treatment with diminazene aceturate is presented in Fig. 3. In the WAD breed, there was a significant ($P < 0.001$) increase in the PCV from day 14 to day 42 while the significant ($P < 0.05$) increase in Yankassa breed was noticeable from day 21 to day 42. But in the Ouda breed, there was no significant increase in the PCV until day 42. There were significant ($P < 0.05$) increments when the pre-treatment PCV of WAD, Yankassa and Ouda (14.3 ± 0.6, 14.3 ± 0.6, 15.0 ± 0.0, respectively) were compared with post-treatment PCV (26.0 ± 7.1, 22.3 ± 4.9, and 21.7 ± 2.1, respectively) at day 42 pt.

DISCUSSION

The recommended dosage of diminazene aceturate for the treatment of trypanosomiasis in domestic animals is 3.5 to 7 mg.kg^{-1} b. w. [5, 18]. Due to the widespread twin problem of drug resistance and marketing of substandard drug preparations, the employment of higher dosages is necessary for effective therapy. This is especially so with severe disease that commonly follow experimental infections.

In this study, by 24 hours post treatment with diminazene aceturate at 7 mg.kg^{-1} b. w., there was disappearance of parasitaemia completely from the blood of the infected sheep (except for the one that died before the clearance of parasitaemia was checked at 24-hour pt.). This was confirmed by the HCT and PCR techniques which demonstrated the efficacy of the drug used. The subsequent relapse infections recorded in the various breeds (80 % in Ouda, 40 % in WAD and 20 % in Yankassa) may be an indication of the development of a level of resistance of the trypanosome isolates to diminazine aceturate. The relapse infections recorded in this study agree with the findings of B e n g a l y et al. [1] in which one sheep infected with *T. vivax* had a relapse infection after treatment with diminazene aceturate. Drug resistance to diminazene was also reported in *T. vivax* infected cattle [2] and in experimental *T. vivax* infected goats [38] in which relapse cases were recorded after treatment. The treatment of the infected sheep was not commenced immediately when the parasitaemia set in, but was delayed till the PCV declined to 15 %. This PCV limit of 15 % before initiating treatment was chosen because M u r r a y et al. [29] had shown that it was at this PCV value or less that the death of animals might occur and also to forestall unnecessary suffering of the experimental sheep. The set PCV limit of 15 % occurred at the mean of 25.0 ± 12.1 (18—39) days, 28.3 ± 12.9 (14—39) days, and 23.5 ± 7.1 (18—34) days post infection for WAD, Yankassa and Ouda, respectively. According to the reports of J e n n i n g s et al. [23] on *T. brucei* in mice and J a t a u et al. [22] on *T. evansi* in rats, the longer the duration of trypanosomiasis infection before treatment, the greater the chances of relapse. Therefore, the delay in the treatment of the sheep could have been responsible for the relapse infections that were recorded in this study. All infected sheep recovered except one Ouda, which died in spite of the chemotherapy in less than 24-hour post treatment. This might have resulted from an overwhelming of the host by the severe infection.

Following the treatment, the live weight gains and PCV of sheep showed a gradual increase until when parasitaemia reappeared in seven (7) of the remaining 14 infected sheep at different days post treatment (between days 17 and 32). This corroborated the report of E z e h et al. [14] that there was improvement in haematological parameters after treatment with diminazene aceturate. As a result of the incidence of relapses of the infections (Ouda, WAD and Yankassa had 80 %, 40 % and 20 %, respectively) at different days in the three breeds of sheep, the mean live

weight and mean PCV did not follow a particular trend (that is, they fluctuated). Ouda breed that had 80 % relapse of infection had the least increase in live weight gain and PCV compared to WAD (40 %) and Yankassa (20 %) breeds. This suggests that, of the three sheep breeds, the Ouda was the most susceptible to the *T. vivax* infection.

Following re-treatment of the relapsed infection with 14 mg.kg^{-1} b. w. diminazene aceturate, no parasitaemia was seen again except in 1 Ouda sheep 27 days post second treatment. The relapses of the infections might have resulted from the localization of the trypanosomes in a site which the drugs could not reach. Whitelaw et al. [39] and Osório et al. [33] reported that in the aparasitaemic phase, *T. vivax* can be found in extravascular foci such as lymph nodes, the aqueous humour of eyes and cerebrospinal fluid; and these could serve as potential sources of the relapsed infections after the chemotherapy of trypanosomiasis. It is important to note that this study was not actually planned for a trypanosome drug resistance test in a ruminant model; therefore, graded dosages of diminazene aceturate and the required 100-day pt. duration for observation for complete recovery or relapse were not incorporated [6, 36]. However, the results of this study could serve as a preliminary test for further study on trypanosome drug resistance.

We also wish to point out that the *T. vivax* used for the experimental infection before treatment with diminazene aceturate was successfully passaged in mice and had 99 % homology with those *T. vivax* sequence (accession numbers; U43183 and L25129) in the GenBank. There were very few similar reports of this rather unusual phenomenon which provides increased opportunity for experimental investigations with laboratory strains of *T. vivax*. *Trypanosoma vivax* is known to be difficult to cultivate in the laboratory and only a few *T. vivax* strains have been isolated that readily infect rodents. The most published *in vivo* work on this species comprises the very few mouse-infective strains; the main one being Y486 and its derivatives [17].

CONCLUSIONS

Diminazene aceturate administered at 7 mg.kg^{-1} b. w. cleared trypanosomes in the blood of all of the treated sheep in the three sheep breeds within 24 hours (except for one sheep that died in less than 24 hours pt.) and this was accompanied by restoration of lost weight and reversal of anaemia. However, the subsequent resurgence of parasitaemia indicated that a dosage of 7 mg.kg^{-1} b. w. is no longer reliable for complete elimination of trypanosomes from all of the tissues of the host.

From the results of this study, it can therefore be recommended that for a successful treatment without relapse using diminazene aceturate at a dosage of 7 mg.kg^{-1} b. w., the treatment should be carried out early enough before the PCV declined to 15 %.

In endemic environments, the treatment with diminazene aceturate at 7 mg.kg^{-1} b. w. should be followed up with isometamidium 1 mg.kg^{-1} two weeks after, to forestall the relapse or drug resistance as the latter have prophylactic properties. The toxicity of diminazene aceturate at 14 mg.kg^{-1} b. w. should be studied in sheep. New trypanocides should be developed to put an end to the problem of trypanosome drug resistance.

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ULTRASTRUCTURE OF GRANULOSA CELLS OF BOVINE OVARIAN ANTRAL FOLLICLES IN RELATION TO ATRESIA

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ABSTRACT

The aim of this study was to describe the most common forms of ovarian follicle atresia in large antral follicles of cows and quantify the occurrence of different cell structures in each form. Atresia of antral follicles in ovaries was determined on the basis of ultrastructural images acquired by electron microscopy of ultrathin sections contrasted with uranyl acetate and lead citrate to visualize cell organelles. All forms of atresia in large follicles are accompanied by regressive changes of the granulosa cells. The initial form of atresia is characterized by enlarged intercellular spaces after the disruption of the gap junctions and desmosomes. Small collapsed cells with pyknotic nuclei, substantially reduced the cytoplasm and a higher incidence of lysosomes are located on the surface of the granulosa layer. The *stratum granulosum* wall collapses and the basal membrane is swollen with a rupture of the *lamina basalis*. Obliterative atresia is characterized by a multiplied loose connective tissue consisting of collagen fibers, fibroblasts, histiocytes, blood capillaries and sporadically granulocytes. The cystic form of atresia is characterized by small collapsed, pyknotic granulosa cells settled in one or two layers. In luteinization-associated atresia, granulosa cells are hypertrophied, their cytoplasm contains smooth endoplasmic reticulum and mitochondria with tubules. In conclusion, the initial atresia of large antral ovarian follicles is associated with processes of cell death, followed by multiplication of the loose connective tissue cells, its dystrophy and hyalinization of the collagen fibers. Ultrastructural examination could be used as a complementary method to improve histopathological diagnostics of cow reproductive organs in veterinary practice.

Key words: atresia; granulosa; ovarian follicle; ultrastructure

INTRODUCTION

In mammalian ovaries, more than 90 % of the follicles undergo a degenerative process known as atresia [9, 13]. Follicular atresia in mammals is a process that destroys ovarian follicles and significantly eliminates from the ovary the oocytes prepared for selection, ovulation and potential fertility [19].

Using morphological assessments, Byskov [2] and Blondin et al. [1] described the occurrence of pyknotic nuclei in the *stratum granulosum*, or in the *antrum* of the atretic ovarian follicles of cows. Later on, the antral and basal forms of atresia were described as two basic patterns of bovine antral follicle atresia [6]. Basal atresia occurs in small follicles (≤ 5 mm) and antral layer atresia occurs in large antral follicles (≥ 5 mm), including dominant, which can be identified histologically and biochemically [17]. It has also been found that the occurrence of morphological, phenotypic changes in *granulosa basal lamina* in the form of loops and openings is related to the functional competence of bovine oocytes [7].

The concentrations of follicular fluid steroid hormones can be used to classify atresia and distinguish some of the different types of atresia. However, this method is unlikely to identify follicles early in atresia, and hence misclassify them as healthy. Other biochemical and histological methods can be used, but since cell death is a part of normal homeostasis, deciding when a follicle has entered atresia remains somewhat subjective [17].

The aim of this study was to describe the ultrastructural changes in the granulosa cells of non-ovulated antral follicles (≥ 5 mm) of cows. In addition to the morphological description, we attempted to quantify the incidence of different cell structures related to each status of follicles: normal, initial atresia, atresia with luteinization, cystic and obliterative atresia.

MATERIALS AND METHODS

The non-pregnant cows of Slovak spotted breed and Holstein cross-breed (n = 30), slaughtered at a local abattoir at the age of 4—8 years, were used as a source of ovaries without previous ovary examination. Ovaries were isolated following 20 min after the slaughter of cows and transported to the laboratory in thermos at a temperature of 26 °C. Antral follicles (n = 98) of 5—10 mm (1—5 follicles per ovary) were picked out of the ovary and the follicular wall was cut off in a size of 1.1—2.0 mm and processed for transmission electron microscopy.

The ovarian tissue samples containing antral follicles were fixed in the aldehyde mixture (2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium cacodylate; all from Fluka, Buchs, Switzerland) at 4 °C for 1 hour and then post-fixed in 1 % OsO_4 (Fluka, Buchs, Switzerland) in 0.1 M sodium cacodylate for 1 hour. Samples were dehydrated in acetone for 30 min and then embedded into Durcupan ACM (Fluka, Buchs, Switzerland). Ultrathin sections (70 nm) were contrasted with uranyl acetate and lead citrate to visualize cell organelles. The contrasted sections were analysed under a JEM100 CXII transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

The follicles were classified as normal (N) without pyknotic nuclei or atretic, which had pyknotic nuclei and ≤ 4 granulosa cell layers. Atresia was determined on the

Table 1. Ultrastructure of granulosa cells from normal or atretic bovine ovarian follicles

Granulosa cell structures	Forms of ovarian follicle atresia				
	N	IA	LA	CA	OA
Cell contacts	+++	+	+	++	+
Intracellular spaces	small	large	large	small	large
Pyknotic nuclei	-	+++	++	+	+
Endoplasmic reticulum					
rough	+++	+	+	+++	++
smooth	+	+	+++	+	-
Mitochondria					
with cristaes	+++	+	+	+++	++
with tubules	+	+	+++	+	-
Lipid droplets	+	++	++	+	+
Lysosomes	+	+++	+	+	++
Autophagic vacuoles	+	+++	+	+	++

N—normal follicle; IA—initial atresia; LA—atresia with luteinization; CA—cystic atresia; OA—obliterative atresia (initial phase)
Occurrence: „-"—none; „+"—small, less than 30 %; „++"—moderate, 30—50 %; „+++"—abundant, more than 50 %

basis of ultrastructural features and assigned to the following forms: initial (IA), atresia with luteinization (LA), cystic (CA) and obliterative (OA). The presence of important cell structures (in %) is shown in Table 1, as a small (less than 30 %), moderate (30—50 %) or abundant (more than 50 %) occurrence.

RESULTS

In the wall of normal (N) antral follicles—*stratum granulosum*, we observed granulosa cells as polyhedral cells with heterochromatic nuclei; their cytoplasm contained rough endoplasmic reticulum in the form of short seg-

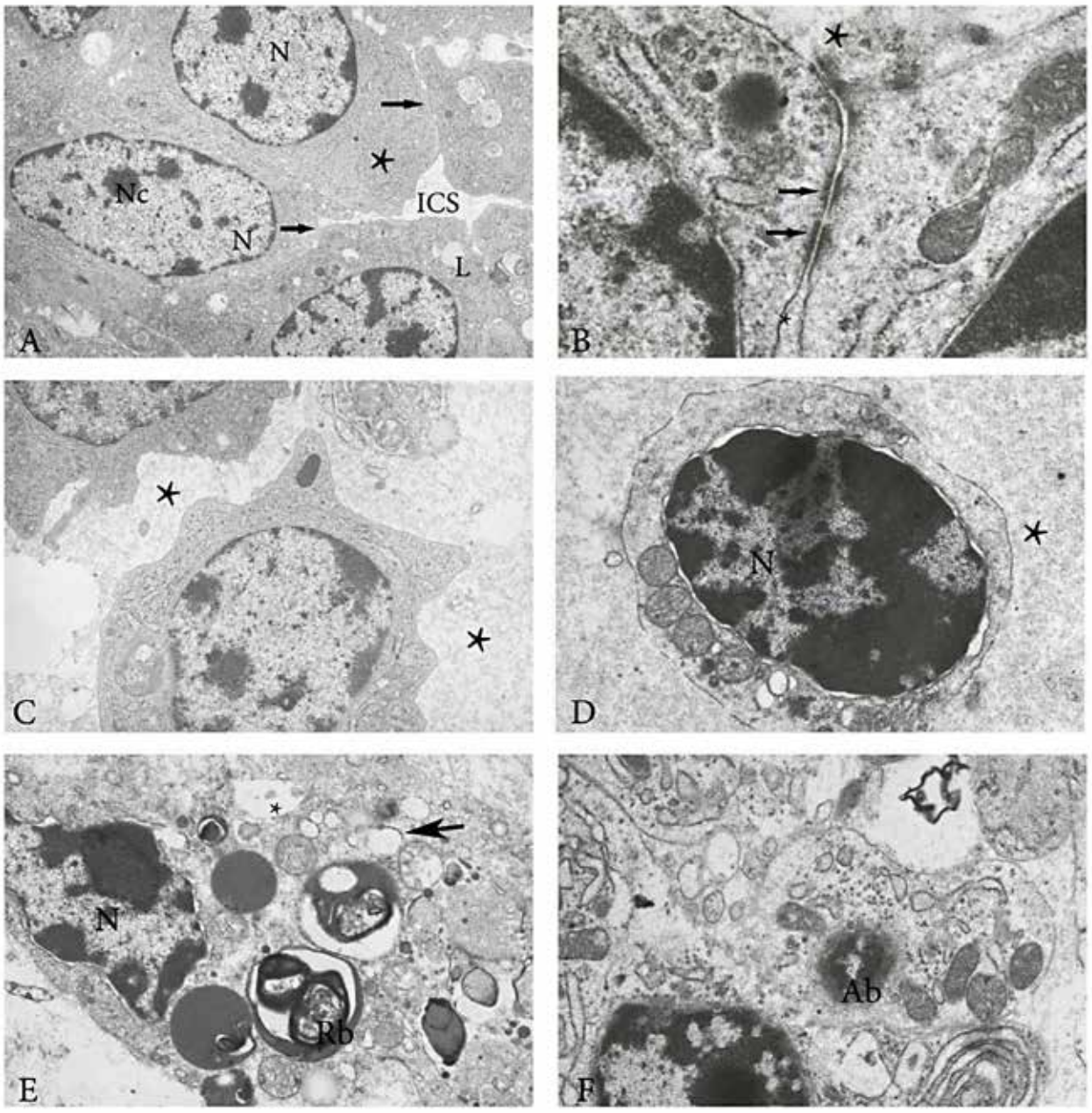

Fig. 1. Ultrastructure of granulosa cells of normal and atretic ovarian follicles of cows

A—Granulosa cells with hyperchromatic nuclei (N) and nucleoli (Nc) in close contact coupled by gap junctions (arrows), rough endoplasmic reticulum (asterisk) and lipids (L). Intercellular spaces (ICS) are small. Magn. ×4800; B—Intercellular junctions of the desmosomal type (*macula adherentes*; arrows), connecting two adjacent granulosa cells, broken gap junctions and enlarging intercellular space (asterisks). Magn. ×29 000; C—Granulosa cell after gap junction loosening. The large intercellular space is filled with finely granulated extracellular matter (asterisks). Magn. ×7200; D—Pyknotic cells and enlarged intercellular spaces in *stratum granulosum*. Pyknotic nucleus (N) in the antral cavity of the atretic follicle and enlarged intercellular spaces (asterisks). Magn. ×14 000; E—Necrotic granulosa cells with pyknotic nucleus (N), cytoplasmic breakdown and cytoplasmic membrane rupture. Dilated tubules of rough endoplasmic reticulum (arrow), vesicles (asterisk) and residual bodies (Rb). Magn. ×10 000; F—Apoptotic bodies (Ab) in the cytoplasm of granulosa cell. Magn. ×10 000

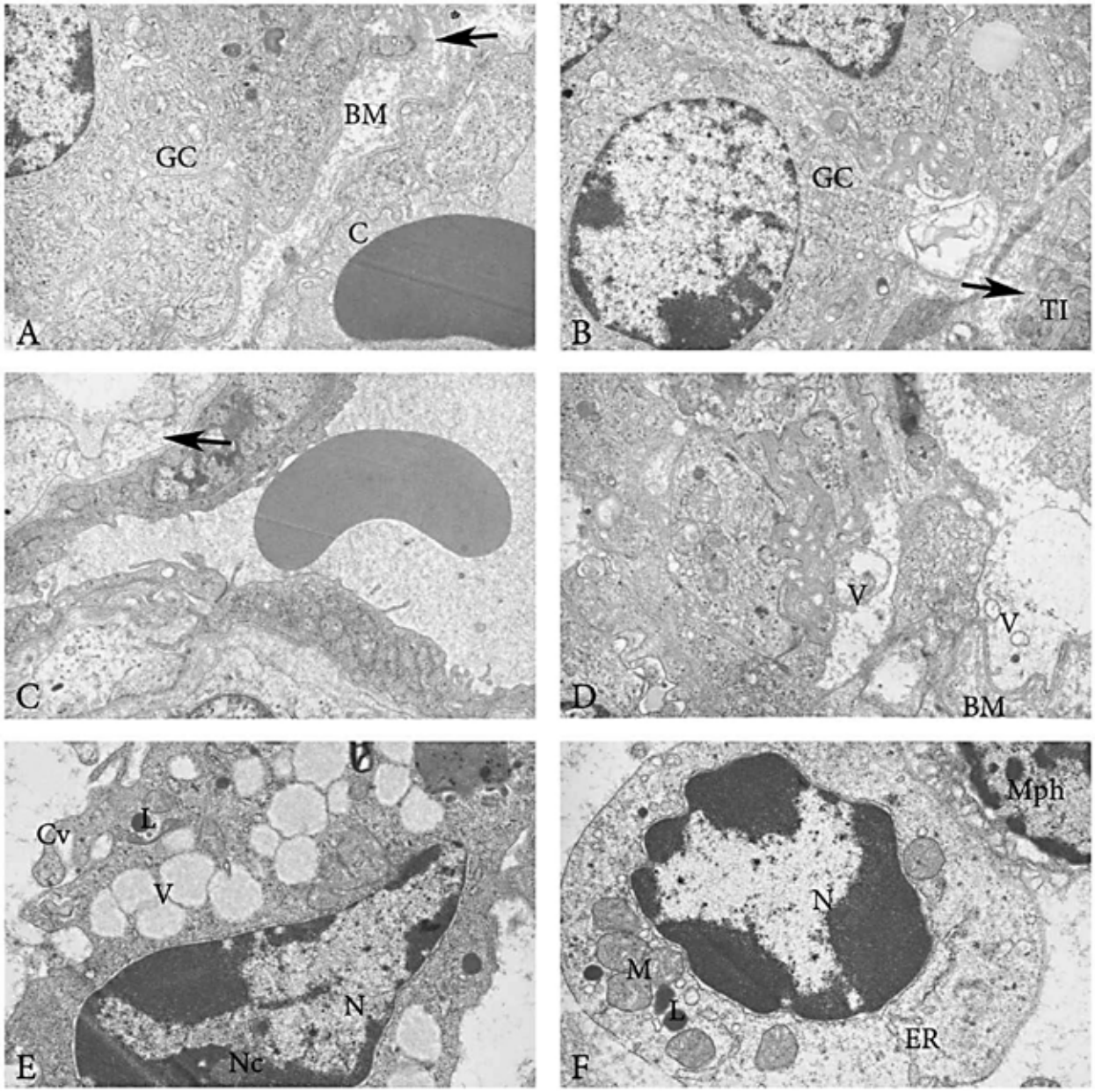

Fig. 2. Ultrastructure of the basal lamina of cow ovarian follicles at the initial form of atresia

A—The basal layer of granulosa cells (GC) adheres to the waved and swollen basal membrane (BM). Disrupted *lamina basalis* from the side of *stratum granulosum* cells (arrow); capillary (C). Magn. ×7200; B—Disrupted *lamina basalis* (arrow) from the side of the *theca interna* (TI) cells. Granulosa cells (GC) are present on the left side. Magn. ×7200; C—Disrupted *lamina basalis* in contact with the sub-endothelial space (arrow). Magn. ×7200; D—Vesicles (V) between granulosa cells and disintegrated plasma membranes. Basal membrane (BM) is located on the right lower part. Magn. ×7200; E—Macrophage in the *stratum granulosum* of the antral follicle, heterochromatic nucleus (N) and nucleolus (Nc). Numerous occurrence of large vacuoles after endocytosis (V), lysosomes (L), cytoplasmic projections (Cv). Magn. ×10 000; F—Luteinized granulosa cell (*stratum granulosum*) of the cow antral follicle in contact with macrophages (Mph). The heterochromatic nucleus (N) of a cell. Cytoplasm containing mitochondria with tubules (M), small amounts of rough endoplasmic reticulum (ER) and several lysosomes (L). Magn. ×10 000

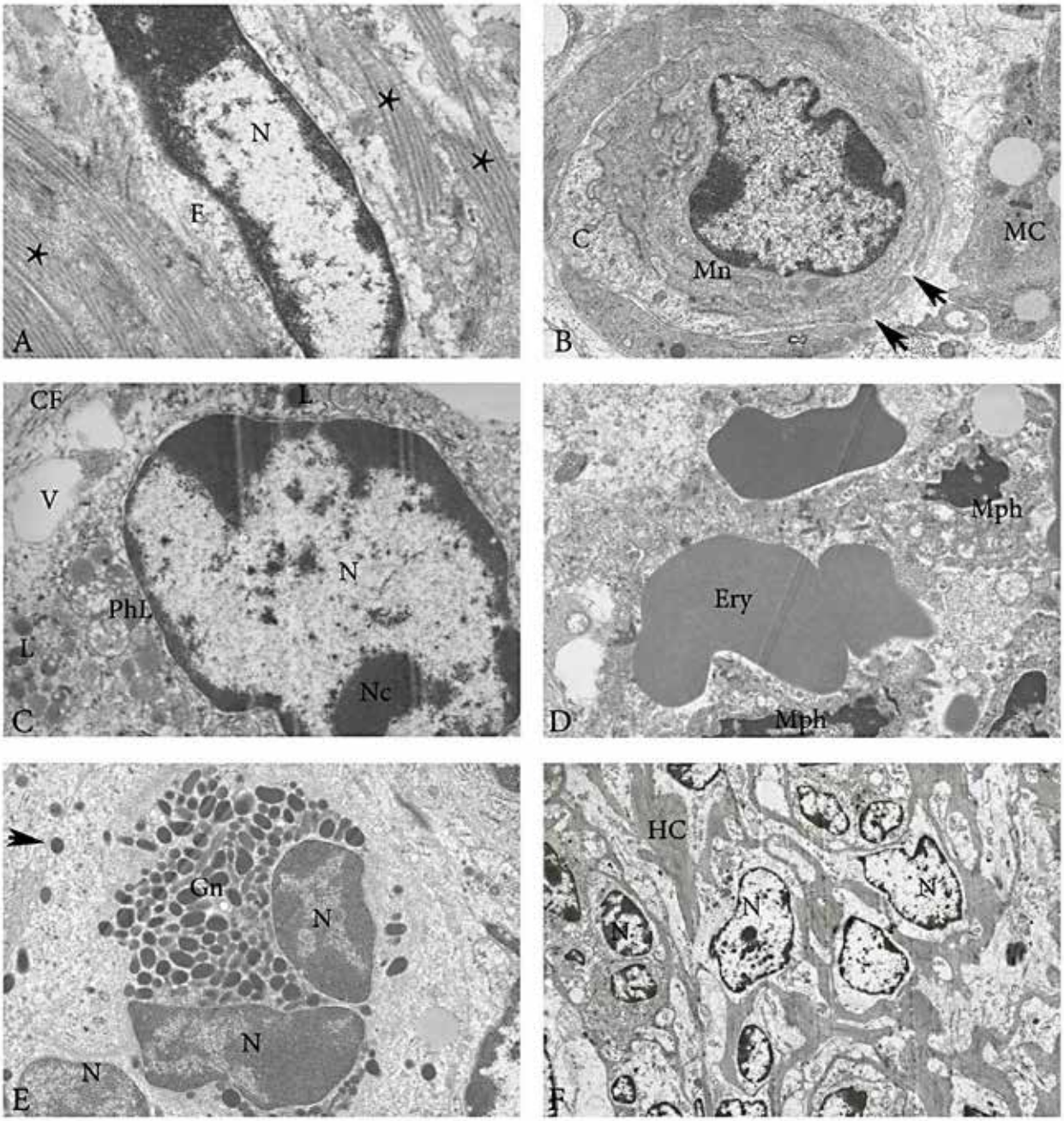

Fig. 3. Ultrastructure of cells from the antral ovarian follicles of cows at obliterative form of atresia

A—Initial stage of obliterative antral follicle atresia. In close proximity to the fibroblast (F) with oval-shaped nucleus (N) there are numerous fibrils with periodic transverse striations forming bundles of normal collagen (asterisk). Magn. ×14 000; B—Capillary (C) of a loose connective tissue with a monocyte (Mn) at the process of diapedesis (arrow). On the right side - part of the mast cell (MC) in close proximity to the capillary wall. Magn. ×7200; C—Histiocyte-macrophage in loose connective tissue with hyperchromatic nucleus (N) and compact nucleolus (Nc). The cytoplasm contains smaller amounts of rough endoplasmic reticulum, mitochondria and a number of vacuoles (V), lysosomes (L) and phagolysosomes (PhL), with larger vacuoles on the surface of the cytoplasm in contact with collagen fibrils (CF); (top left). Magn. ×1000; D—Phagocytosis of degraded connective cell elements, including erythrocytes (Ery), by macrophages (Mph). Magn. ×7200; E—Basophilic granulocyte in the loose connective tissue of the antral follicle. Large three-lobed nucleus (N). Numerous round granules (Gn); some of them with fine granulation and many of them in close contact with the cytoplasmic membrane (arrow). Magn. ×10 000; F—Advanced collagen hyalinization (HC) at obliterative follicular atresia with numerous necrotic connective tissue cells in hyalinized follicle. Hyperchromatic nuclei (N) of necrotic cells. Magn. ×1900

ments. Small rod-shaped mitochondria were mainly with cristae and to a lesser extent with tubules. The Golgi complex was located near the nucleus. Small amounts of lipid droplets, multi-vesicular bodies and autophagic vacuoles were observed. The cell-to-cell contacts were provided by frequently occurred gap junctions and desmosomes (*maculae adherentes*). Intercellular spaces were small (Table 1; Fig. 1A). The granulosa cells by their basal layer were settled on a thin intact extracellular *lamina basalis* layer.

In antral follicles with initial atresia (IA), enlarged cell spaces filled with intercellular mass (Fig. 1C) were observed due to the broken intercellular junctions of the desmosomal and gap junction types (Fig. 1B). On the surface of the *stratum granulosum*, we observed the frequent occurrence of collapsed granulosa cells with pyknotic nuclei (Fig. 1D). In the cytoplasm of granulosa cells of atretic follicles we found frequent dilation of tubules of the rough endoplasmic reticulum and formation of vesicles, autophagic and residual bodies (Fig. 1E). The mitochondria number decreased, they were diminished in size and often dark-pyknotic; the numbers of autophagic vacuoles and lysosomes were increased (Table 1). In some granulosa cells of antral follicles the apoptotic bodies had been found (Fig. 1F). The *lamina basalis* underwent disintegration and, to a greater extent, created openings into the intercellular area of *stratum granulosum* (Fig. 2A) and into the *theca interna* area (Fig. 2B). We also observed a break in the integrity of the capillary sub-endothelial basal lamina (Fig. 2C) and the formation of vesicles between the granulosa cells following the plasma membrane disintegration (Fig. 2D). Among granulosa cells of the basal part of the atretic follicles the macrophages were frequently evident (Fig. 2E).

At the atresia with luteinization (LA), we observed the hypertrophied polygonal cells among the granulosa cells, which contained a nucleus with 1—3 nucleoli. The cytoplasm contained smooth endoplasmic reticulum, overspread tubules and vesicles of Golgi complex, numerous mitochondria with tubules and the lysosomes (Fig. 2F).

The cystic form of atresia (CA) was characterized by several pyknotic granulosa cells grouped in one or two layers, similar as at the initial form of atresia (data not shown). There were intercellular connections; intercellular spaces were small, endoplasmic reticulum was rough and the mitochondria had cristaes (data not shown).

Granulosa cells in the initial phase of the obliterative form of atresia (OA) substantially lost intercellular connections, intercellular spaces were large, endoplasmic reticulum was rough and mitochondria had cristaes (Fig. 3A—F). In older atretic follicles, at an advanced phase of an obliterative form of atresia we observed an increased incidence of fibroblasts (Fig. 3A). At this stage, a diapedesis of monocytes that penetrate the capillary wall into the loose connective tissue was present (Fig. 3B). The macrophages in the loose connective tissue of atretic follicles had a typical hyperchromatic nucleus and contained smaller amounts of rough endoplasmic reticulum, mitochondria, a prominent Golgi complex, a number of vacuoles, lysosomes and phagolysosomes (Fig. 3C). The macrophage activity gradually increased, and phagocytosis of all degraded elements of connective cells including erythrocytes occurred (Fig. 3D). The polymorphonuclear neutrophil granulocytes with numerous typical granules in the cytoplasm were also observed in the loose connective tissue (Fig. 3E). We have observed that the loose connective tissue gradually became dense, and its fine-fiber tangle of collagen fibrils underwent hyaline dystrophy converting to a strongly hyalinized connective tissue, manifested as an unstructured homogeneous dense mass (Fig. 3F).

DISCUSSION

In mammals during the development of ovarian follicles most of the antral follicles undergo atresia at different stages of their development [17], however, the mechanisms controlling this selective process are not known. T e e r d s and D o r r i n g t o n [18] reported histological differences in atresia of preantral and antral follicles: oocyte fragmentation, disordered granulosa layer and hypertrophied theca layer were characteristic for atresia in preantral follicles, whilst massive apoptosis of granulosa cells in the presence of a more or less intact oocyte being characteristic of atresia in antral follicles.

The basic physical mechanism of follicular atresia is granulosa cell apoptosis [22]. Atretic changes may affect cells of both antral and non-antral follicles but apoptotic changes only occur in the antral follicles [5, 21]. The degeneration of cattle oocytes, however, may occur at any stage of atresia. Several studies have shown that apoptosis in granulosa cells may occur much earlier than the morphological changes in follicular atresia [11, 12]. M e n g et al. [13] studied the ovarian atresia, besides immuno-

histochemistry, also from the functional viewpoint using certain molecular markers of autophagy and apoptosis: microtubule-associated light-chain protein 3 (LC3), sequestosome 1 (SQSTM1/P62), Beclin1, autophagy-related protein 7 (ATG7) and cleaved caspase 3 (cCASP3). These authors had found that preantral and antral follicular atresia was the result of the activation of different cell-death pathways. Antral follicular degeneration was initiated by massive granulosa cell apoptosis, while preantral follicular atresia occurred mainly via enhanced granulosa cell autophagy [13].

The granulosa cells of the antral ovarian follicles are interconnected by the gap junctions and desmosomes—*maculae adherentes*, and the whole ovarian follicle is surrounded by the *lamina basalis* [16]. At the initial form of follicular atresia, some extracellular changes lead to the breakdown of the intercellular junctions and the enlargement of the extracellular space, which is filled with the granularly structured extracellular matrix.

One form of cow ovarian atresia is also cystic atresia of ovarian follicles. This form of cysts affects the antral follicles of 3—5 mm in size and it must not be misinterpreted with the cystic ovarian disease of the antral follicles 15—20 mm in size. Cystic atresia does not affect the estrous cycle of cows, while cystic-diseased cows have an abnormal estrous cycle. At cystic atresia, the *stratum granulosum* is absent, and the internal part of the cyst is lined with one or two layers of small cubic, partially pyknotic granulosa cells.

Atresia with luteinization may be caused by the action of serum luteinization-controlling factors, which, after changing the permeability of the basal membranes of atretic follicles, act untimely even with the involvement of capillaries penetrating between granulosa cells [14]. The fact, that even non-ovulated follicles can luteinize, had been confirmed by Westfahl [23], when he found that ovulation was not necessary for the follicle luteinization process. According to Elfont et al. [4], also the lysosomal cell system may participate in ovarian steroidogenesis. Similar results were observed in our study, when there were more lysosomes in the cytoplasm of atretic and luteinizing granulosa cells.

We also observed that when the process of antral follicle atresia progressed over time, fibroblasts began to appear in the follicles; the follicle became occupied with the loose connective tissue and the process of obliterative atresia was initiated. This process was accelerated especially by the formation of cracks of the granulosa epithelium, its separation from the theca cells, as well as with the increased incidence of pyknotic nuclei of the granulosa cells associated with the oocyte degeneration.

In addition to necrotic and apoptotic processes, also macrophages were involved in the regression of the antral follicle cells [8]. Macrophages penetrated the wall of the atretic follicles between the granulosa and theca cells, infiltrated into the loose connective tissue of obliterated follicles, phagocytosed necrotic cells and gradually disappeared. The abundance of rough endoplasmic reticulum in their cytoplasm suggests an increased synthesis of proteins, e.g. cytokines required for the apoptotic process. We assume that macrophages can play an important role in fibrosis and, by secreting fibroblast activation factor [20], accelerate the process of obliterative atresia. Various mediators, in particular histamine, released by the mast cells, may be greatly involved in the onset of the local immunopathological response by increasing the permeability of the *theca interna* capillaries, thus creating the conditions for the formation of edema and diapedesis of neutrophils, that we observed in the loose connective tissue of atretic follicles in our study.

In general, atresia is considered a functional ovarian disorder in which maturing follicles do not ovulate, undergo various forms of regression and luteinization and gradually vanish. The development of atresia is influenced by various factors, the most common being internal factors, especially hormonal misbalance of gonadotropins. For example, Bitecourt et al. [3] found that endogenous gonadotropins in rats promoted ovulation and protected pre-ovulatory follicles from atresia.

Among the external factors, this may be, for example, the body condition of cows and level of milk productivity. In our previous study [15] the emaciated cows with BCS (body condition score) 1 or BCS 2 had a higher incidence of late (cystic) and lutenization-associated atresia in the antral ovarian follicles compared to the normal (BCS 3) body conditioned cows. Moreover, cows from the higher milk lactation group showed a higher incidence of non-ovulated antral ovarian follicles and increased incidence of cystic or luteinization-associated atresia, than cows with low milk yield [10]. These factors affect the ovarian status and ultimately lead to an increased incidence of various forms of atresia, which may affect the regularity of the estrous cycle and adversely influence the fertility of dairy cows.

Currently, miRNA-mediated regulation is widely valued in ovarian function studies. In particular, Zhang et al. [24] reported about bioinformatic prediction of key miRNA-mediated pathways in the follicular atresia, which suggests a causal link between the levels of certain miRNAs and the fate of granulosa cells or the whole ovarian follicles. A deep understanding of the roles of miRNA networks will not only help elucidate the mechanisms of granulosa cell apoptosis, follicular development, atresia and their disorders, but also offer new diagnostic and treatment strategies for infertility and other ovarian dysfunctions.

CONCLUSIONS

On the basis of our electron microscopy examination, we can conclude that the atresia of the ovarian follicles in cows appears in several forms. It is manifested by varying incidence of initial, obliterative and cystic forms of atresia, as well as luteinization-associated atresia of granulosa cells. Granulosa cells degenerate along with the oocyte, and the *stratum granulosum* of the antral follicles collapses after swelling of the basal membrane and disintegration of the *lamina basalis*. A loose connective tissue with macrophages grows among the degenerated granulosa cells, and the macrophages phagocytose them. Subsequently, the loose connective tissue of the vanishing atretic follicle hyalinizes and turns into a dense connective tissue that persists for a time in the form of a small white body—*corpus albicans*. Using the ultrastructural analysis, we obtained a morphological image as well as a sequence of regression processes characterizing some forms of atresia that take place in the atretic and luteinized antral follicles of cows. Ultrastructural examination could be used as a complementary method to improve histopathological diagnostics of cow reproductive organs in veterinary practice.

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THE EFFECT OF LOW DOSE OF CADMIUM ON GROWTH, REPRODUCTION AND CHICKEN VIABILITY

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ABSTRACT

Cadmium (Cd) is considered to be a highly polluting heavy metal with toxic effects on health and reproduction. In this study, the effects of low dose cadmium on growth, reproductive properties, and egg properties were studied. Cadmium as cadmium chloride ($CdCl_2$) at a dose of 3 mg $Cd.kg^{-1}$ was added as a feed supplement. The following parameters were evaluated: body weight, egg production, fertility, hatching, embryo and chick mortality, and egg quality characteristics. After 2 months of exposure to low doses of cadmium, the body weight increased in the cocks and did not change in the laying hens. Egg production was not affected, while fertility increased. The values of egg weight and hardness, thickness and shell weight were significantly higher ($P < 0.05$). The embryo mortality in the experimental group was lower (6.5 % vs. 12.8 %) compared to the control group. The hatching was significantly higher ($P < 0.05$) and the loss in 7 day old chickens was similar to that in the control group. Although the toxic effects of cadmium on reproduction and accumulation in poultry bodies have been generally described, low-doses of cadmium given to adult hens and cocks has improved the reproductive parameters and qualitative properties of laying hen eggs. The results related to the reproduction and quality of eggs obtained in this study have the characteristics of the hormetic effects of low cadmium uptake.

Key words: cadmium; chicken; growth; low dose; reproductive performance

INTRODUCTION

Cadmium affects the structure, physiological and biochemical functions of various organs [1, 2, 27]. Cadmium has a diversity of toxic effects including nephrotoxicity, carcinogenicity, teratogenicity and endocrine and reproductive toxicities. At the cellular level, cadmium affects cell proliferation, differentiation, apoptosis and other cellular activities [20, 23]. The exposure of rats to low or moderate doses of Cd by the intraperitoneal route stimulated lipid

peroxidation in all of the tissues investigated. The comparison of lipid peroxidation to various tissue indicators suggested that low doses of Cd stimulated lipid peroxidation without any evidence of acute damages [13].

Cadmium has been shown to exert significant effects on the ovarian and reproductive tract morphology, with extremely low dosages reported to stimulate ovarian luteal progesterone biosynthesis and high dosages inhibiting it [5]. When adult male Sprague-Dawley rats were injected with cadmium in doses ranging from 1.6 to 152 μmol Cd.kg^{-1} body weight in the testis, changes due to disruption of the blood-testis barrier and oxidative stress have been noted, with onset of widespread necrosis at higher dosage exposures [26]. A single dose of cadmium chloride (1.2 mg.kg^{-1}b. w.) injected i. p. in adult rats, and killed after 7 or 56 days resulted in the significant reduction in testis and epididymis weights, gonadosomatic index and length of seminiferous tubule after 7 and 56 days [17].

The various experiments showed that the toxicity of cadmium on reproductive functions of chickens responded to the amount of cadmium ingested. Studies about the toxic effects of cadmium on reproduction and accumulation of cadmium in the bodies of poultry have been widely conducted [3, 4, 5, 8, 21, 22] and the results showed that the exposure to cadmium has been linked to a wide range of detrimental effects. Studies with higher dose (30—60 mg.kg^{-1}) made in broilers showed, that prolonged dietary cadmium exposure resulted in: abnormal embryonic development, prenatal death, a decrease of body weight and sexual dysfunction on the fowl reproduction. The egg production of the experimental groups, particularly of those exposed to a high (150 mg.kg^{-1}) cadmium load rapidly decreased in Japanese quail [19]. The exposure to cadmium induced changes in biochemical parameters and decreased the reproductive performance, including fertility [4, 7, 14, 16, 17, 28]. The adult cocks receiving 20 mg.kg^{-1}Cd displayed structural changes in the testis and in qualitative properties of the semen. In the fowl, cadmium intake decrease spermatogenesis [15].

Although the acute and chronic effects of poisoning by high dose of cadmium is known, the effect of chronic low-dose of cadmium is less well characterized. While high-level exposures to toxic chemicals is well known and generally is detrimental to human or other animal health, studies regarding the effects of chronic low-level of cadmium on reproduction in mammals are sporadic and in fowl are absent. The purpose of this study was to evaluate the effect of low dose of cadmium on the growth in hens and cocks and reproductive parameters in laying hens including mortality of embryos and chicks after hatching and the qualitative properties of eggs.

MATERIALS AND METHODS

Animal model

Four groups, each comprised of 12 laying hens (hybrids Shaver Starcross 288, line 589) in their first year of egg-laying period, and four groups of 5 cocks each (hybrids Shaver Starcross 288), age 25—28 weeks were involved in this experiment. Except for the controls, cadmium chloride ($CdCl_2$) was added as a supplement to the feed mixture NVRM (Tab. 1). The mean feed intake for hens and cocks was 120 and 140 g per bird and per day, respectively. The

Table 1. Composition of commercial feed supplied

	Value [mg.kg^{-1}]	Minerals	Value [mg.kg^{-1}]	Vitamins	Value
Nitrogen matter	153	Phosphorus	5	A	10 000 IU.kg^{-1}
Crude protein	60	Calcium	35	B2	4.0 mg. kg^{-1}
Ash	160	Manganese	60	B12	10 mg.kg^{-1}
Lysine	7	Copper	4	D3	2000 IU kg^{-1}
Methionine	3.5	Sodium	2.5	E	10 mg.kg^{-1}
Methionine + Cystine	6	Iron	40		–
		Zinc	40		–

experimental birds were kept in three-floor cages. The birds were exposed to cadmium for 8 weeks. The chicks hatched in the individual groups were reared up to 7 days of age. In the experiment, the dose applied to laying hens and cocks was as follows: 0 = control group, 1 = experimental group received 3 mg.kg^{-1} Cd in the feed. The body weights of the hens and cocks were determined at the start and at the end of the application period. The mortality of the experimental birds and of the chicks up to 7 days of age was monitored daily. The observation of individual indices and sampling were carried out as follows: egg-laying intensity was recorded daily, and egg weight was recorded 3 times at 7-day intervals. The index of egg shape, hardness, thickness and weight of the shell were examined during 7 days of the first and the second months of application. The colour of egg yolk was determined according to the paper scale (Hoffman La Roche, Switzerland). The index of the egg shape was determined by measuring the length and width of the egg. Eight batches of eggs were used to follow the hatching and fertilization of the eggs. The eggs were collected at 7-day intervals. The chickens hatched in the individual groups were reared up to 7 days of age. The calculation of hatchability was made by observation of the morphological features of the developed embryos. The analysis of variance was used for the statistical evaluation of the data. The performance indices were evaluated by Duncan's test.

RESULTS

Body weight of hens and cocks

The results showed (Table 2) that after administration of low doses of cadmium in the feed for 8 weeks, the body weight of the hens did not change before or after the treatment, while it was higher in the cocks after treatment (100 % vs. 106.6 %). In the control body weight, both the hen and the cocks were lower (96.5 % vs. 105.8 %). Weight gain in laying hens was higher (3.5 %) than in roosters (1.2 %).

Egg production

During the two-month exposure to 3 mg Cd.kg^{-1} in the feed, the egg production (Table 3) did not change before or after the experiment and the values in both samples were similar (90.7 %).

Fertility and mortality of embryos

The fertilization rate in the experimental group was significantly higher (98.8 %) than in the control group (97.8 %). Embryo mortality after the first and second sampling in the group receiving 3 mg Cd.kg^{-1}, alternated between one quarter and one third of the control group. After incubation, embryo mortality was lower and reached 6.5 %, compared to 12.8 % in the control group.

Table 2. Body weight of hens and cocks

Group	Hens body weight [kg]		%	Cocks body weight [kg]		%
	Before treatment	After treatment		Before treatment	After treatment	
Control	1.43 ± 0.15	1.38 ± 0.18	96.5	2.04 ± 0.20	2.16 ± 0.35	105.8
3 mg Cd	1.58 ± 019	1.58 ± 0.23	100.00	2.10 ± 0.32	2.24 ± 0.28	106.6

Table 3. Eggs production before (28 days) and on (56 days) the experiment

Group	Total eggs produced		Daily mean of eggs		Production of eggs [%]	
	28 days before exp.	56 days on exp.	28 days before exp.	56 days on exp.	28 days before exp.	56 days on exp.
Control	315	635	11.2 ± 0.6	11.3 ± 0.7	93.7 ± 2.4	94.4 ± 4.1
3 mg Cd	305	610	10.8 ± 0.7	10.8 ± 0.8	90.7 ± 1.9	90.7 ± 3.8

Table 4. Fertility and mortality of the embryos

Group	Eggs incubation	Unfertile eggs	Fertility [%]	Dead embryos			
				1st sampling	2nd sampling	After incubation	Total (%)
Control	476	10	97.8 ± 8.4	8	10	42	12.8
3 mg Cd	433	5	98.8 ± 9.6	2	3	23	6.5

Table 5. Hatching and mortality of 7 day old chicks

Group	Embryos hatched	Embryos hatched [%]	Number of chicks died	Chicks died [%]
Control	406	87.1 ± 9.8	1	0.25
3 mg Cd	400	93.4 ± 7.2*	1	0.25

*—Statistically significant difference at P < 0.05

Table 6. Weight of eggs [g]

Group	28 days on experiment	56 days on experiment	After experiment	Comparison [%]		After experiment
				28 days on experiment	56 days on experiment	
Control	58.94 ± 6.9	60.34 ± 9.2	59.63 ± 18.7	100.0	100.0	100.0
3 mg Cd	62.36 ± 13.4	62.27 ± 7.8	62.28 ± 16.4*	105.7	103.1	104.4

*—Statistically significant difference at P < 0.05

Table 7. Qualitative properties of eggs

[%]	Index of eggs	Colour of yolk	Hardness of shell	Thickness of shell [µm]	Weightof shell [g]
Control	1.53 ± 0.37	6.48 ± 1.22	7.87 ± 0.83	327.6 ± 45.7	4.70 ± 0.82
3 mg Cd	1.58 ± 0.19	6.50 ± 0.83	8.14 ± 1.2*	346.4 ± 43.1*	5.40 ± 0.79*

*—Statistically significant differences at P < 005

The hatching and mortality of 7 days old chicks

The hatching in the experimental group was 93.4 % and was significantly higher (P < 0.05) than in the control group where the hatching was 87.1 %. The mortality rate of chicks hatched from treated hens at day 7 of life was similar in the control group and was 0.25 %.

Weight of eggs

After 28 days, the weight of the eggs in the cadmium group was higher (105.7 %), than after the experiment (104.4 %). Compared to the control group (100 %), it was statistically significant (P < 0.05).

Index of the eggs and colour of yolk

The values of egg index and yolk colour in the experimental group were higher than in the control group (1.58 vs. 1.53 and 6.50 vs. 6.48). Data on eggshell—hardness, thickness and weight in the experimental group were significantly higher (P < 0.05).

DISCUSSION

The results of various experiments with high doses of cadmium have shown high toxicity to the reproductive function. The results obtained in this experiment with low doses of cadmium showed that the body weight of the hens before and after the treatment was similar but higher than in the control group. The weight of the cocks was higher than that of the hens and higher than that of the control group. Egg production did not change after the experiment, while fertility increased slightly. Embryo deaths were remarkably lower in the experimental group. These results are quite contradictory due to the toxicity of metals in chicken embryos [2]. The dose of cadmium ingested by hens appears to be too low to affect embryonic development. In contrast, cadmium in this experiment had a stimulating effect on embryonic development. The positive effects of cadmium were also observed with lower embryo mortality and a higher number of hatched embryos. Similar results were observed in another study of phenylmercury [18] and methylmercury in wild ducks (*Anas platyrhynchos*) [9, 10].

The stimulatory effects of cadmium were also observed in the parameters related to the properties of the eggs. The results of the shell show that cadmium is in some way associated with the formation of the shell. According to R a h m a n et al. [19] cadmium can positively affect the secretory mechanisms of membrane proteins and glycogenes in the mucosa of the fallopian tubes. The properties of the eggshell can affect the mineral properties of the eggshell in the mother glands through mineral metabolism. The results regarding the propagation and quality of the eggs obtained in this experiment have the characteristics of the hormetic effect of low cadmium absorption and a reduced amount transferred to the eggs.

The role of cadmium in cell viability and growth is still debated. Some reports have shown that cadmium at concentrations above 1 mM inhibits cell growth and DNA synthesis in a wide variety of cell types [12, 24]. Very low concentrations of cadmium have also been found to stimulate cell growth and DNA synthesis [29]. The inhibitory and stimulatory effects can be explained by differences in the conditions of exposure and sensitivity of the respective cells. The range of stimulation of cadmium doses is specific to the cell type. Cadmium generally induces both harmful and protective signalling pathways, but the exact mechanisms on which it is based remain unresolved.

The phenomenon known as fever has been described by various authors. In several comments and reviews, fever has been defined as stimulation at low doses and inhibition at high doses. Cd has been shown to exert significant effects on ovarian and reproductive tract morphology, with extremely low dosages reported to stimulate ovarian luteal progesterone biosynthesis and high dosages inhibiting it. Exposure to low levels of known toxic chemicals could be "beneficial" to human health [5, 11]. According to C a l a b r e s e et al. [6] this phenomenon is considered to be an "adaptive" and frequently observed response resulting from exposure to an interfering agent. Because hormesis may be associated with an adaptive response, it may only be observed at certain times after or during exposure.

The hormetic effect depends on the dose that determines the stimulation (and poison). Low-dose cadmium appears to have a positive effect on some reproductive functions. Our findings regarding the growth, reproduction and quality of eggs obtained after a low dose of cadmium have the character of a hormetic effect. There is sufficient data in the original hormesis database to suggest that this phenomenon is functional and similarly important in many biological, pharmacological and other biomedical disciplines [25]. Despite the presented results, we do not rule out that low doses of suspected toxic substances with stimulatory responses are not always beneficial and some may be harmful even at low concentrations.

CONCLUSIONS

The results obtained in this experiment showed that cadmium at a low dose administered orally did not affect body growth and reproductive parameters, but positively affected most of them, including fertility, hatching, dead embryos and egg quality. The results also showed that a small amount of cadmium transferred to eggs from laying hens was not toxic to the developing embryo and did not adversely affect the further physiological condition of the hatched offspring. The dose of cadmium and the time we used in the experiment showed a hormetic effect on the growth and reproductive function of poultry.

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MORPHOMETRIC STUDY OF MICROSCOPIC CYSTS OF *SARCOCYSTIS* SP. IN SHEEP CARCASSES

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ABSTRACT

Muscular samples of the oesophagus and diaphragm of 335 sheep collected from the slaughterhouse of El Harrach were analyzed by the histopathological method to describe the morphology of two species of *Sarcocystis*: *S. arieticanis* and *S. tenella*. The cysts were counted and measured with a micrometer for their dimensions. A total of 895 cysts were measured. The width, length, shape index (length/width), the thickness of the wall and the length of the projections were recorded. The thick-walled cysts of *S. tenella* were 10—450 µm long (the mean ± SD value was 50.35 µm ± 1.380) and 1—110 µm (the mean ± SD value was 27.51 µm ± 0.533) wide. The cyst walls were 0.5—4 µm thick (the mean ± SD value was 1.547 µm ± 0.020) and provided with radial striations. The shape index was 1—14 µm (the mean ± SD value was 1.93 ± 0.045). While in the *S. arieticanis*, the cyst wall was thin and had long hair-like protrusions, the cysts measured 8—780 µm (the mean ± SD value was 123.13 µm ± 12.799) in length and 5—100 µm (the mean ± SD value was 37.00 µm ± 1.68) in width. The hair-like protrusions reached a length of 3—14 µm (the mean ± SD value was 5.428 ± 0.353) and the shape index was 1—17 µm (the mean ± SD value was 3.10 ± 0.281). For the length, the width and the shape index, the differences were statistically significant between thin-walled cysts and thick-walled cysts. The general structural features of the cysts, previously described by other authors, were confirmed.

Key words: histopathological method; *Sarcocystis arieticanis*; *S. tenella*; sheep

INTRODUCTION

Sarcocystis is a common coccidian parasite of a wide range of animals and is of worldwide distribution. It is commonly found in the cystic form in the musculature of slaughtered food animals [8].

Four species of *Sarcocystis* commonly infect sheep: *Sarcocystis ovifelis* (formerly *gigantea*) and *Sarcocystis medusiformis* are transmitted by felids and develop into macro-

scopically visible cysts in sheep, while *Sarcocystis ovicanis* (formerly *tenella*) and *Sarcocystis arieticanis* are transmitted by canids and develop into microscopic cysts in sheep [8]. Recently other species of *Sarcocystis* have been reported to occur in sheep including *Sarcocystis gracilis*-like [11] and *Sarcocystis mihoensis* [30].

Two *Sarcocystis* species are known to be pathogenic in sheep: *Sarcocystis tenella* and *Sarcocystis arieticanis* [8]. Upon primary exposure to pathogenic species, acute sarcocystiosis may cause death of the host, abortion, premature parturition in pregnant ewes [18], neurologic signs known as ovine protozoal myeloencephalitis (OPM) [3] and respiratory disease [31]; while chronic infections can lead to reduced quality of wool, meat and milk [21, 22].

The size, shape, and cyst wall morphology of sarcocysts are important in the identification and differentiation of *Sarcocystis* sp. [19, 24]. The species with the sheep-canid life cycle have far smaller cysts than the sheep-felid species [15, 25, 28, 29]. The cyst wall of *S. tenella* is observed thick by light microscopy but thin in the other 3 species, *S. arieticanis*, *S. gigantea* and *S. medusiformis*; *S. mihoensis* had the far thicker cyst wall than all the above 4 species [7, 8, 15, 19, 28, 29].

Many studies have shown that the sarcocyst wall varies structurally between species and it could be used as a useful criterion for the identification of species [2, 7, 12, 20, 25, 28]. There are numerous detailed investigations on the morphology of *Sarcocystis* sp. from domestic sheep [15, 26, 27, 28].

This paper describes the morphology of two microscopic species of sarcocysts; *S. arieticanis* and *S. tenella* in the muscles of two organs (oesophagus and diaphragm) of sheep slaughtered in the slaughterhouse of El Harrach in the north of Algeria. The present description is the first report from sheep in Algeria. There are different data on the size of the sarcocysts of both *Sarcocystis* species described here.

MATERIALS AND METHODS

Collection of tissue samples

Muscle samples were collected from 335 sheep carcasses slaughtered at a slaughterhouse of El Harrach in the north of Algeria. A total of 670 muscle samples from 335 oesophagi and 335 diaphragms were sampled and placed into separate bags. Samples were taken to the Laboratory of Parasitology and Mycology in the Superior National Veterinary School, Algiers and were previously fixed in 10 % neutral buffered formalin for histological analysis of microscopic *Sarcocystis* cysts.

Histological technique

Tissue samples were prepared in the Laboratory of Anatomy and Cytopathology of the University Hospital of PARNET, Algeirs, and in the Laboratory of Anatomy Pathological at the Superior National Veterinary School, Algiers. The fixed tissue samples were cut into 0.5 cm-thick sections, dehydrated with serial dilutions of ethanol and xylene, processed into paraffin, sectioned of 4 to 5 microns, stained with haematoxylin and eosin (H & E), and examined for microcysts with the light microscope at various magnifications (×100, ×400, ×1000). The wall morphology of the microcysts in the muscle sections were examined by the light microscope with oil immersion (×1000). Sarcocystis species present in the muscle samples were identified on the basis of the differences in the wall morphologies of sarcocysts, as described by Dubey et al. [11]. The cysts were counted and measured with a micrometer under a light microscope in order to determine their dimensions. The width, the length, the shape index (length/width), the thickness of the wall and the length of the projections were recorded.

Statistical analysis

The data were processed using IBM SPSS Statistical version 20 software. Means, standard errors and 95 % confidence intervals were determined for the different morphological characteristics. For the comparison, the Mann-Whitney U-test and the Chi-square test were used for the study of homogeneity. A difference was considered statistically significant at $P < 0.05$.

RESULTS

Sarcocystis sp. infections were diagnosed in 315 (94.03 %) of the sheep. Sarcocysts were located within the muscle cells. Two types of microcysts were differentiated on the basis of their cyst wall morphologies under the light microscope (×1000). Some cysts were bounded by a thin cyst wall, with the hair-like projections identified as *S. ar-*

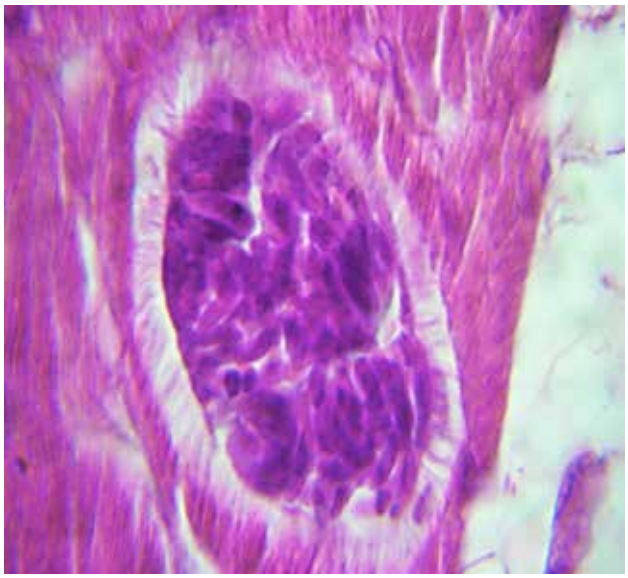

Fig. 1. *S. arieticanis*, thin-walled with hair-like projections in oesophagus (H & E, Magn. ×1000)

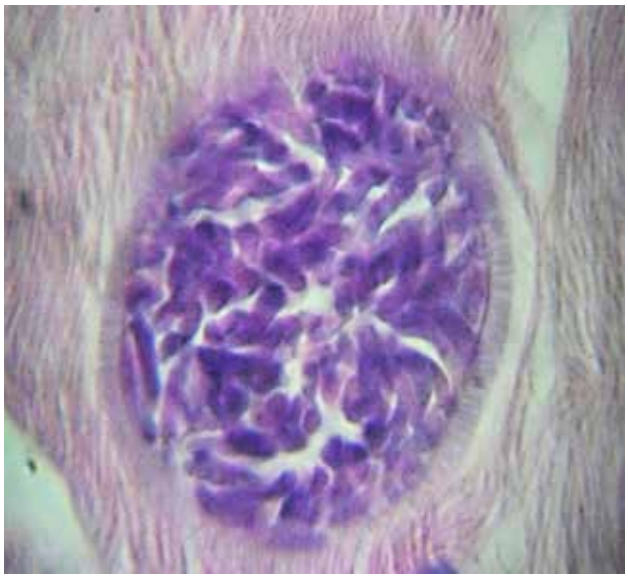

Fig. 2. *S. tenella*, thick-walled with radial striations in oesophagus (H & E, Magn. ×1000)

ieticanis (Fig. 1). Other cysts were bounded by a relatively thick cysts wall with radial striations which were identified as *S. tenella* (*S. ovicanis*) (Fig. 2). Among the cysts found, 895 cysts were measured (772 thick-walled cysts of *S. tenella* and 123 thin-walled cysts of *S. arieticanis*).

Structural features of the cysts

The means of length, width, shape index, thickness of the wall (*S. tenella*) as well as the length of the projections (*S. arieticanis*) are reported in Table 1. For the length, the width and the shape index, the U de Mann-Whitney test was very significant ($P < 0.01$) between thin-walled cysts and thick-walled cysts.

The means of length, width, shape index, thickness of the wall (*S. tenella*) and the length of the projections (*S. arieticanis*) according to the organ are found in Tables (2) and (3). For each *Sarcocystis* species, the U Mann-Whitney test showed no significant differences ($P > 0.05$) between the two organs for each parameter measured.

Thickness of the wall (thick-walled cysts) and length of the projections (thin-walled cysts)

The U Mann-Whitney test demonstrated that the distribution of the number of thin-walled cysts of *S. arieticanis* according to the length of the projections (villi) was not significant between the 3 classes, while for the thick-walled cysts, the distribution according to the thickness of the walls was highly significant (Table 4).

Length of the cysts

For the thick-walled cysts, the highest number of cysts was in the class ≤ 50 µm, followed by the class [51—100 µm], and ≥ 100 µm; a highly significant difference was found between the 3 classes. For the thin-walled cysts, the class ≤ 50 µm represented the highest number of cysts, followed by the class ≥ 100 µm, then the class [51—100 µm]; the test of U Mann-Whitney showed that there was not a difference between the 3 classes (Table 5).

Width of the cysts

Regarding the width of the cysts, a very large number of cysts belonged to the class ≤ 50 µm, followed by the class [51—100 µm] for the two species of *Sarcocystis*; for the class ≥ 100 µm, we founded only one cyst of *S. tenella*, while no cyst was found for *S. arieticanis*. The statistical analysis showed that there was a highly significant difference between the three classes for each *Sarcocystis* species (Table 6).

Shape index of the cysts

The sarcocysts in the oesophagus and diaphragm were round (Fig. 3), ovoid (Fig. 4), fusiform (Fig. 5), or linear (Fig. 6). The distribution of cysts according to the shape index showed that a very large number of cysts were found in

Table 1. Morphological characteristics of thick-walled and thin-walled cysts

	Parameters	Length [µm]	Width [µm]	Shape index [µm]	Wall thickness of thick-walled cysts [µm]	Length of the projections of thin-walled cysts [µm]
Thin-walled cysts	n	123	123	123	–	35
	Mean ± SD	123.13 ± 12.79	37.00 ± 1.68	3.10 ± 0.28	–	5.42 ± 0.35
	95 % CI	[97.79—148.47)	[33.68—40.33]	[2.54—3.66]	–	[4.71—6.14]
	Min	8	5	1	–	3
	Max	780	100	17	–	14
Thick-walled cysts	n	772	772	772	772	–
	Mean ± SD	50.35 ± 1.38	27.51 ± 0.53	1.93 ± 0.04	1.547 ± 0.02	–
	95 % CI	[47.64—53.06]	[26.47— 28.56]	[1.84—2.01]	[1.50—1.58]	–
	Min	10	1	1	0.5	–
	Max	450	110	14	4	–
P value		**0.000**	**0.000**	**0.01**	**–**	**–**

CI—Confidence Interval

Table 2. Morphological characteristics of thick-walled cysts according to the organ

Thick-walled cysts	Parameters	Length [µm]	Width [µm]	Shape index [µm]	Wall thickness [µm]
Oesophagus (n = 330)	Mean ± SD	47.61 ± 1.65	27.82 ± 0.81	1.84 ± 0.063	1.54 ± 0.032
	95 % CI	[44.35—50.87]	[26.22—29.42]	[1.71—1.96]	[1.483—1.610]
	Min	10	1	1	1
	Max	300	89	13	4
Diaphragm (n = 442)	Mean ± SD	52.40 ± 2.064	27.28 ± 0.707	1.99 ± 0.062	1.548 ± 0.026
	95 % CI	[48.34—56.46]	[25.89—28.67]	[1.87—2.11]	[1.49—1.59]
	Min	10	4	1	0.5
	Max	450	110	14	4
P value		**0.932**	**0.579**	**0.496**	**0.656**

CI—Confidence Intervals

Table 3. Morphological characteristics of thin-walled cysts depending on the organ

Thin-walled cysts	Parameters	Length [µm]	Width [µm]	Shape index [µm]	Villous length [µm]
Oesophagus (n = 42)	Mean ± SD	132.79 ± 21.35	39.64 ± 3.18	3.20 ± 0.46	5.27 ± 0.51
	95 % CI	[89.66—175.91]	[33.22—46.07]	[2.26—4.13]	[4.17—6.36]
	Min	8	5	1	3
	Max	500	100	13	10
Diaphragm (n = 81)	Mean ± SD	118.12 ± 16.04	35.64 ± 1.94	3.05 ± 0.35	5.55 ± 0.49
	95 % CI	[86.20—150.04]	[31.77—39.51]	[2.35—3.76]	[4.51—6.59]
	Min	8	7	1	4
	Max	780	80	17	14
P value		**0.402**	**0.247**	**0.763**	**0.705**

CI—Confidence Interval

Table 4. Distribution of the number of thin-walled cysts according to the length of the projection and of thick-walled cysts according to the thickness of the wall

Villous length [µm]	N	P	Wall thickness [µm]	N	P
[3—4]	12		[0.5—1]	311	
[5—6]	17	0.074	[1.5—2]	397	0.00001
[7—14]	6		[2.5—4]	64	
Total	**35**	**Not significant**	**Total**	**772**	**Very significant**

Table 5. Distribution of the number of thin-walled cysts and thick-walled cysts according to the lengths of cysts

Length of thick-walled cysts [µm]	N	P	**Length of thin-walled cysts** [µm]	N	P
≤ 50	499		≤ 50	52	
[51—100]	226	0.0001	[51—100]	30	0.052
≥ 101	47		≥ 101	41	
Total	**772**	**Very significant**	**Total**	**123**	**Not significant**

Table 6. Distribution of the number of thin-walled cysts and thick-walled cysts according to the width of the cysts

Width of thick-walled cysts [µm]	N	P	**Width of thin-walled cysts** [µm]	N	P
≤ 50	718		≤ 50	103	
[51—100]	53	0.00001	[51—100]	20	0.00001
≥ 101	1		≥ 101	0	
Total	**772**	**Very significant**	**Total**	**123**	**Very significant**

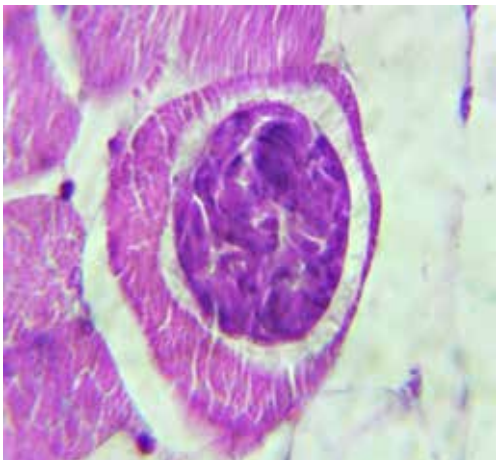

Fig. 3. *S. arieticanis*, round, thin-walled with hair-like projections in diaphragm (H & E, Magn. ×1000)

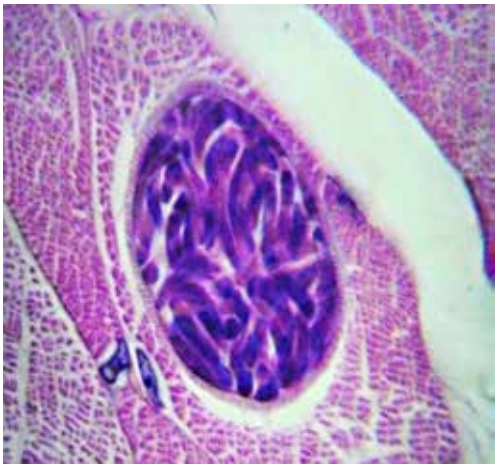

Fig. 4. *S. tenella*, ovoid, thick-walled with radial striations in oesophagus (H & E, Magn. ×1000)

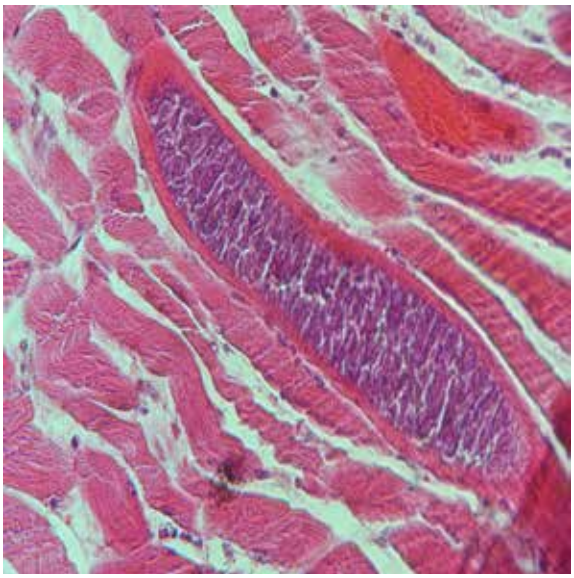

Fig. 5. *S. arieticanis*, fusiform, in diaphragm (H & E, Magn. ×400)

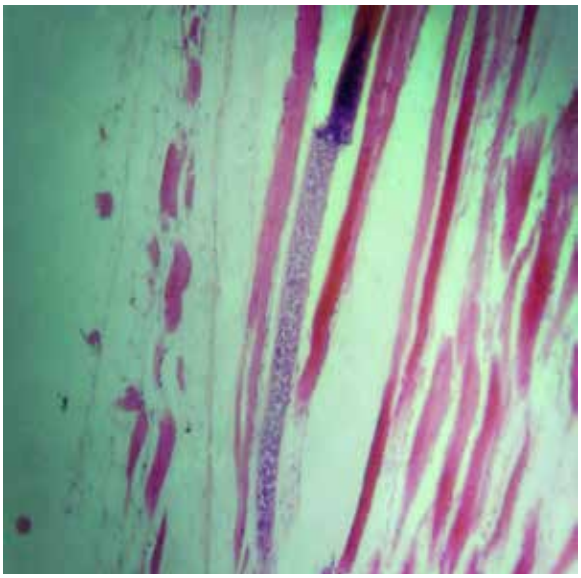

Fig. 6. *S. arieticanis*, linear, in diaphragm (H & E, Magn. ×100)

Table 7. Distribution of the number of thin-walled cysts and thick-walled cysts according to the index of forms cysts

Index of forms of thick-walled cysts [µm]	**N**	**P**	**Index of forms of thin-walled cysts** [µm]	**N**	**P**
≤ 1.19	169		≤ 1.19	30	
[1.2—2]	389	0.00001	[1.2—2]	41	0.052
≥ 2.01	214		≥ 2.01	52	
Total	**772**	**Very significant**	**Total**	**123**	**Not significant**

the class [1.2—2 µm] for *S. tenella* and the class ≥ 2.01 µm for *S. arieticanis*; the statistical tests showed that there was a highly significant difference between classes for the thick-walled cysts, while this difference was not significant for the thin-walled cysts (Table 7).

DISCUSSION

Sarcocystis species parasitize almost all vertebrates and are widely distributed around the globe. Data from various studies suggest different variations depending on the species and the area where the research was performed; for sheep the prevalence is between 18 % and 100 % [4]. In Algeria, the prevalence of microscopic cysts of *Sarcocystis* has been reported to be between 64.38 % and 99.14 % [5, 23]. Two types of microscopic cysts were detected with differences in their cyst walls morphologically: *S. tenella* and *S. arieticanis*. Sheep become infected with *S. tenella* or *S. arieticanis* by ingesting sporocysts in contaminated food or water [8].

In our study, the morphometric studies of the microscopic cysts of *Sarcocystis* (length, width, shape of cysts [length/width], and wall thickness of cysts) from slaughtered naturally infected sheep were carried out.

In the 335 sheep examined, cysts were detected in the oesophagus and the diaphragm in great numbers. The *sarcocystis* were located within the muscle fibers and there was no evidence of any pathological changes or reaction against the cysts. The interior of the mature cyst was divided into

many chamber-like compartments by septa. A great number of bradyzoites were observed in the cyst together with a small number of metrocytes which were situated along the cyst wall.

There is remarkable diversity in cyst wall zone morphology between different *Sarcocystis* species and this variation has been used as a means of species identification [13, 19].

In our study, the cyst wall of *S. tenella* was thick and striated; the same as that of *S. tenella* described by Heydron et al. [14] and Vlemmas et al. (35). No secondary cyst wall was observed in our study which was in agreement with Vlemmas et al. [35]. While in *S. arieticanis*, the cyst wall was thin and had long hair-like protrusions, which was similar to that described previously for this parasite [12, 15, 26].

In Algeria, there is very little data on the occurrence of the species of ovine *Sarcocystis* spp. Indeed, Dahmani et al. [5] recorded that 92.54 % of the sheep examined were infested with the thin-walled cysts of *S. arieticanis*, and 43.88 % of sheep with the thick-walled cysts of *S. tenella*. Nedjari [23] noted the predominance of *S. tenella* (60.63 %) compared with *S. arieticanis* (39.36 %).

There has been different data reported on the size of the sarcocysts of both *Sarcocystis* species described here. Some authors (Vercruysse et al. [34], Kudi et al. [17]) have identified the *Sarcocystis* species based on the dimensions of the cysts. Thus, the measurements of the different dimensions (length, diameter, thickness) of the cysts obtained, in our study, are compatible with the species of *S. tenella* and *S. arieticanis*.

Indeed, the cysts of *S. arieticanis* measured 8—780 μm (the mean ± SD value was 123.13 μm ± 12.799) in lengh and 5—100 μm (the mean ± SD value was 37.00 μm ± 1.68) in width. Tenter [32] reported that cysts of *Sarcocystis arieticanis* are 900 μm or less in length. Our results are almost similar to those found by some authors. In fact Al Quraishy et al. [2] found in the heart muscles that the cysts measured 38.5—64.4 μm (averaged 42.66 μm) in width and 62.4—173.6 μm (averaged 82.14 μm) in length, also within cardiac muscle of naturally infected sheep. Haziroglu et al. [12] recorded that the cysts were ranging over 35.0—62.5 μm in width and 52.5—162.5 μm in length. On the other hand, in a study conducted by Odening et al. [26] on fresh samples of muscle tissue (head, neck, larynx, heart and loin: *Musculus psoas major*) from European mouflon and domestic sheep, the sarcocysts were up to 1.8 mm long and 286 μm wide.

In our study, it was demonstrated that the cyst wall of *S. arieticanis* cysts was readily identifiable by light microscopy, having a cyst wall < 1 μm characterized by hair-like protrusions. These protrusions reached a length of 3—14 μm (the mean ± SD value was 5.428—0.353). The same results were noted by Odening et al. [26] who recorded that the cyst walls were thin (0.21 to 0.62 μm) and they had unstable hair-like, 5.7 to 11.8 μm long villar protrusions. Also, Heydorn and Mehlhorn [15] found that the tissue cysts of *S. arieticanis* were characterized by hair-like protrusions reaching a length of 11 μm at the maximum. Dubey et al. [7] noted that *S. arieticanis* showed a thin wall with filiform projections with 6—12 μm.

We found *S. tenella* in the sheep we examined. The sarcocysts were 10—450 μm long (the mean ± SD value was 50.35 μm ± 1.380) and 1—110 μm (the mean ± SD value was 27.51 μm ± 0.533) wide. According to Tenter [32] the cysts *S. tenella* were 700 μm or less in length. Our results were close to those recorded by Tinak [33] who found that *S. tenella* cysts measured 68.591 μm in length and 23.260 μm in diameter. Kudi et al. [17] noted that the sarcocysts of *S. tenella* measured 35.7 to 500 μm lengthwise in tissue samples of the oesophagus and diaphragm. While according to Vercruysse and Van Marck [34], the cysts have an average length of 300 μm. Abd el-Baki et al. [1] found in the oesophagus samples that the mature sarcocysts measured 700 (670 —860) μm in length and 188 (170—200) μm in width in the longitudinal sections, while it was measured 75 (22—118) μm in the cross sections. Whereas, Erber [9] showed that *S. tenella* measured 300—650 × 20—50 μm. For Odening et al. [26] the sarcocysts were up to 2.4 mm long and 186 μm wide which was different from our results.

Fassi-Fehri et al. [10] found, that regarding the length of the cysts, the highest number of thick-walled cysts of *S. tenella* (139) was in class [100—300 μm], followed by 47 cysts in class [0—100 μm], and 29 cysts in class [300—500 μm], while 5 cysts had a length which was > 500 μm.

We recorded that the cyst walls were 0.5—4 μm thick (the mean ± SD value was 1.547 μm ± 0.020). Similar results have been noted by Odening et al. [26] who found that the cyst walls were 1.08 to 3.85 μm thick and Tinak [33] who recorded 1.081 μm thick. From Kudi et al. [17]

the cyst-wall measured 2.4 µm. Whereas K a n a k o u d i s et al. [16] recorded that the cyst wall appeared uniformly thick with numerous digit-like projections and were 3—3.5 µm thick.

From F a s s i - F e h r i et al. [10], regarding the wall thickness of the *S. tenella* cysts, a very large number of cysts (195) belonged to the class [1—4 µm], followed by the class > 5 with 30 cysts.

This difference may be related to the methods of measuring. Indeed, certain authors have resorted to the electron microscopy for this measurement [34]. This is why other techniques (molecular biology, immunohistochemistry) must be used for the more precise identification of *Sarcocystis* species [6].

The shape index (length/width) was calculated to indicate the shape of the cyst; indeed for thin-walled cysts of *S. arieticanis*, the shape index was 1—17 µm (the mean ± SD value was 3.10—0.281) while it was 1—14 µm (the mean ± SD value was 1.93—0.045) for thick-walled cysts of *S. tenella*. The sarcocysts in the oesophagus and diaphragm were round, ovoid, fusiform or linear. It is suggested that their shape varies with the arrangement of the myofibrils where they are located and they are dependent upon the section. T i n a k [33] noted that the *Sarcocystis* cysts observed were of variable shapes (elongated or ovoid) depending on the section. In longitudinal sections, the cysts were fusiform, linear, while in cross-section they were round. F a s s i - F e h r i et al. [10] found that most cysts were fusiform or linear and out of 201 *Sarcocystis tenella* cysts measured, 25 cysts had a shape index > 2, whereas for 176 cysts, this index was between 2 to 16.

CONCLUSIONS

Our investigation descibed the morphology of two microscopic species of *Sarcocystis*; *S. arieticanis* and *S. tenella* from the oesophagus and diaphragm of slaughtered naturally infected sheep for the first time in Algeria. The width, the length, the shape index (length/width), the thickness of the wall of *S. tenella* and the length of the projections of *S. arieticanis* recorded by other authors were confirmed.

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ECTOPARASITES *CTENOCEPHALIDES* (*SIPHONAPTERA, PULICIDAE)* IN THE COMPOSITION OF MIXED INFESTATIONS IN DOMESTIC DOGS FROM POLTAVA, UKRAINE

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ABSTRACT

One of the most common ectoparasites on domestic carnivores are fleas from the genus *Ctenocephalides*. This group of blood sucking insects are one of the most important in medical and veterinary terms, as they can serve as carriers of dangerous infectious and may cause other invasive diseases. Research studies have established a variety of fleas and other contagions parasitizing domestic dogs in Poltava, Ukraine. Certain peculiarities of these ectoparasitic studies, as a part of mixed infestations of dogs, have recently been determined. The results of the studies have shown that the species composition of the fleas was represented by two main species. The dominant species was *Ct. felis*, and their prevalence was 36.05 %. Another species (*Ct. canis*) was diagnosed less often and had a prevalence of 27.94 %. It was found that in 31.18 % of the dogs, the blood-sucking insects were mostly parasitizing in the form of an associations with: nematoda (*Toxocara canis, Trichuris vulpis, Uncinaria stenocephala*), *Cestoda* (*Dipylidium caninum*), protozoa (*Cystoisospora canis*), and another ectoparasite (*Trichodectes canis*). Overall, 33 types of mixed infestations were detected. Moreover, the number of different parasitic species in each dog ranged from one to seven. Fleas of the genus *Ctenocephalides* (in the composition of two species of parasites) were registered the most often (14.60 %). The infestation of dogs with other forms of mixed infestations was 0.69—8.01 %. The most frequent co-members for *Ct. felis* were *Cestoda* [*D. caninum* (13.47 %)], for *Ct. canis—Cestoda* [*D. caninum* (11.23 %)] and *Nematoda* [*T. vulpis* (8.29 %)].

Key words: cohabitants; domestic dog; fleas; mixed infestation; prevalence

INTRODUCTION

Dog breeding is gaining importance in various sectors of the economy today. The need for the development of service, decorative, sports and special dog breeding is growing, as these animals are indispensable helpers of humans. Therefore, their health is of constant concern to humanity. One of the most common groups of diseases among the population of domestic dogs are entomosis infestations caused by fleas. Their ability to use humans as an alternative host determines the importance of these parasites in the field of human health care [6, 12, 22, 29].

Most species of fleas parasitize animals of a certain species or group of species, but can easily change their hosts. Rarely, other specific non-flea parasites are registered among the fleas, tied only to the one or several close species of animals, which even after prolonged starvation can't drink the blood of their unusual host [10, 21]. Therefore, the establishment of the identity of these non-flea cohabitants of fleas that parasitize domestic dogs in different climatic regions will increase the effectiveness of measures to control and prevent them, as well as prevent the diseases they transmit.

Thus, most scientists claim that domestic dogs are parasitized by fleas of the *Ctenocephalides* genus (S t i l e s, C o l l i n s, 1930). Two species of fleas are particularly important—*Ctenocephalides felis* (B o u c h e, 1835) and *Ctenocephalides canis* (C u r t i s, 1826), which are dominant in dogs. These species are non-specific to carnivores; they can attack and feed on a variety of hosts, including humans. This feature of parasitic insects contributes to their significant stability in the environment [9, 15, 25]. At the same time, there are reports of parasitism of fleas by other species, i. e., *Echidnophagia gallinacea*, *Pulex irritans*, *Xenopsylla cheopis* and *Cediopsylla simplex* on dogs [14, 27]. The degree of infestation of domestic dogs with *Ct. felis* and *Ct. canis* varies and depends: on the climatic conditions of the region, the method of animal keeping, as well as preventive insecticide treatments [1, 2, 19, 20].

Thus, the study of the species composition of fleas parasitizing on domestic dogs in certain geographical regions of Ukraine, establishing the peculiarities of their course together with other pathogens of parasitic diseases are important in conducting comprehensive treatment measures, and control of the epizootic situations. This must take into account the sensitivity of certain species of fleas to insecticides; because it is known that the species *Ct. felis* is resistant to the most common insecticides [8, 13].

MATERIALS AND METHODS

Animals and study design

The research was conducted during 2017—2020 in the Laboratory of Parasitology of Poltava State, Agrarian Academy and Veterinary Service "Vetexpert" located in Poltava (49°34' N, 34°34' W). It is located in the north-eastern part of Ukraine. In general, the city's climate is moderately continental with cool winters and warm (sometimes hot) summers. The average annual temperature is 7.6 °C, the lowest in January (–6.6 °C), and the highest in July (20.1 °C). The relative humidity averages 74 %; it is the lowest in May (61 %), and the highest in December (88 %).

A total of 3,171 domestic dogs of different breeds and crossbreds were investigated. They were aged from 1 month to 11 years when they were brought to the Veterinary Service area for study.

Parasitological study

The isolation of fleas and chewing lice from the body of animals was performed by combing them with a plastic small-toothed special comb for 10 minutes. The collected insects were fixed in 70 % ethyl alcohol. The identification of the isolated parasitic insect species was established by microscopy according to the morphological taxonomic features [7, 17, 28].

The faeces were collected from dogs from which the fleas were isolated; coproscopic examinations were performed according to the Kotelnikov–Khrenov's method [16]. The identification of the species affiliation of helminthic eggs and oocysts of coccidian parasites were performed using recognized identifiers [11, 23].

Microphotography was performed using a digital camera and MBS-10 binocular microscope at a magnification of 8—16 times.

Statistical analysis

Standard indices of infestation of dogs with parasitic disease pathogens were calculated:

- Prevalence (P, %)—the ratio of the number of dogs infected with invasive disease pathogens to the number of animals studied;

- Intensity (I, specimens per animal)—the number of fleas of a certain species per infected host.

The datasets were expressed as mean ± standard error of the mean (SE). Statistical processing of the experimental results was carried out using Microsoft Excel software.

RESULTS

Our studies have shown that the average P of ecto-parasites *Ctenocephalides* spp. on domestic dogs at the territory of Poltava, Ukraine was 49.48 % with an average I 19.30 ± 0.31 specimens per animal. It was found that the fleas of the genus *Ctenocephalides* was represented by two species—*Ctenocephalides felis* (B o u c h e, 1835) (Fig. 1) and *Ctenocephalides canis* (C u r t i s, 1826) (Fig. 2). The dominant species was *Ct. felis*, where the P reached 36.05 % with the I 15.87 ± 0.34 specimens per animal. The *Ct. canis* species was registered less often and the P was 23.98 % with the I 13.63 ± 0.35 specimens per animal.

It was found that in 31.18 % of the dogs, the blood-sucking insects mostly parasitize in the form of an associations with the causative agents of nematodes, cestodes, protozoans and entomotic infestations. Less commonly diagnosed was the mono-infestation (14.79 %), where the infestation of dogs by *Ct. felis* only was 9.71 %, and *Ct. canis*—5.07 %. The infestation of dogs simultaneously by two species of fleas was 3.50 % (Table 1).

A total of 33 types of mixed infestations were identified, where the cohabitants of the flea *Ctenocephalides* genus were: cestodes of the species *Dipylidium caninum* (L i n n a e u s, 1758); nematodes of the species *Toxocara canis* (W e r n e r, 1782), *Trichuris vulpis* (F r ö l i c h, 1789), and *Uncinaria stenocephala* (R a i l l i e t, 1884); coccidia of the species *Cystoisospora canis* (Nemeséri, 1959); and chewing lice of the species *Trichodectes canis*

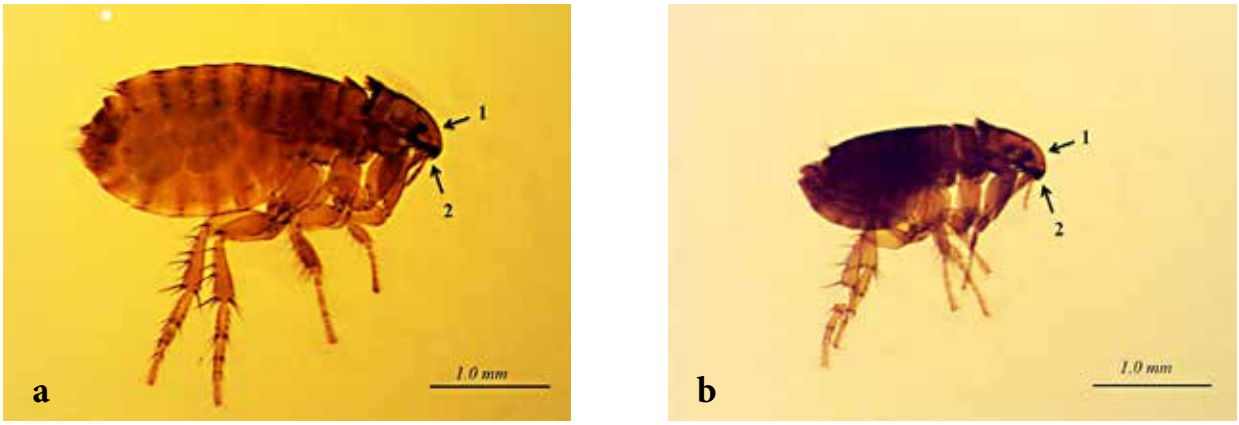

Fig. 1. *Ctenocephalides felis* fleas of *Ctenocephalides* genus: a—♀; b—♂
1— sloping forehead; 2—long teeth of oral ctenidium

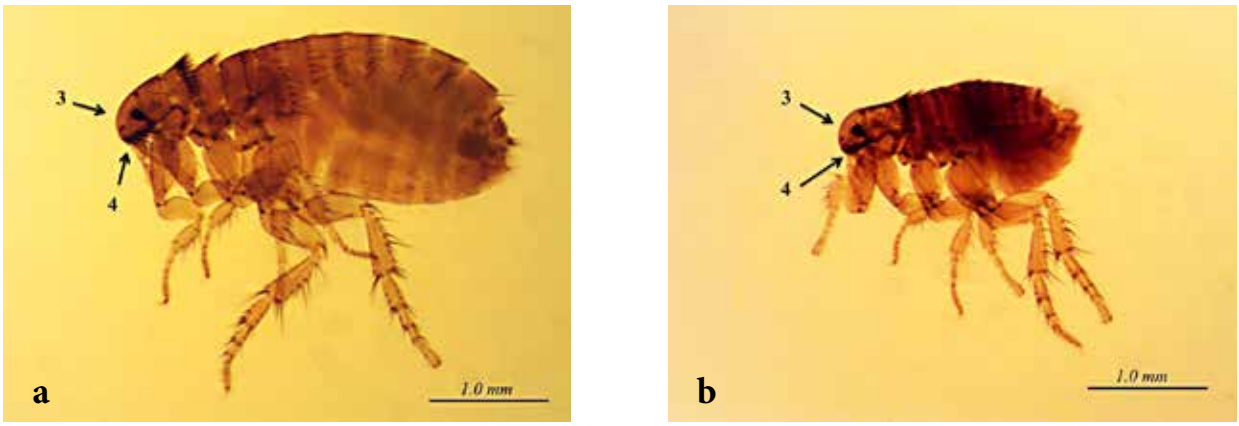

Fig. 2. *Ctenocephalides canis* fleas of *Ctenocephalides* genus: a—♀; b—♂
3—steep forehead; 4—short teeth of oral ctenidium.

Table 1. Indicators of domestic dog flea infestation of *Ctenocephalides* genus as a part of mono- and mixed infestations

Infestations' types	Number of infected animals	Prevalence % (n = 3,171)	% of infected animals (n = 1,569)
Ct. felis	308	9.71	19.63
Ct. canis	161	5.07	10.26
Ct. felis* + *Ct. canis	111	3.50	7.07
Other (mixed) infestations	989	31.18	63.03

(de G e e r, 1778). Moreover, the number of parasitic species in each animal ranged from one to seven.

The parasitizing of *Ctenocephalides* spp. in domestic dogs more often was registered in a combination with two species of agents (P = 4.60 %). Simultaneous parasitism of three (8.01 %), four (6.09 %), five (1.07 %), six (0.69 %) and seven (0. 3 %) agent species were found less often.

Nine mixed infestations with two agents were identified, of which the associations of fleas *Ct. felis* with cestodes *D. caninum* (P = 5.27 %) and with nematodes *T. vulpis* (P = 3.12 %) were most often diagnosed. Less common were the associations of fleas *Ct. felis* with nematodes *T. canis* (P = 1.86 %) and fleas *Ct. canis* with nematodes *T. vulpis* (P = 1.10 %). The infestation of dogs by association of fleas *Ct. canis* with nematodes *T. canis*, *U. stenocephala*, cestodes *D. caninum*, coccidia *C. canis* and chewing lice *Tr. canis* did not exceed 0.95 % (Table 2).

Among the associations of the fleas with two other agents, 10 types of combinations were established: *Ct. felis* with cestoda (*D. caninum*), nematoda (*T. canis*, *T. vulpis*), protozoa (*C. canis*), ectoparasites and (*Tr. canis*); and *Ct. canis* with cestoda (*D. caninum*), nematoda (*U. stenocephala, T. canis*), protozoa (*C. canis*), and ectoparasites (*Tr. canis*). The P ranged from 0.35 to 2.21 %. The mixed infestations of fleas with 3 other agents were represented by 7 types of parasitic associations. Thus, the fleas *Ct. felis* were together with cestoda (*D. caninum*), nematoda (*T. canis*, *T. vulpis*, *U. stenocephala*), and protozoa (*C. canis*), where the P ranged from 0.22 to 2.05 %. The fleas Ct. canis were together with cestoda (*D. caninum*), nematoda (*T. canis, T. vulpis*), and protozoa (*C. canis*); P ranged from 0.35 to 2.05 %. The mixed infestations with five co-members were

Table 2. The most common two-members mixed infestations in domestic dogs included *Ctenocephalides* spp.

Species of mixed infestations' co-members	Number of infected animals	Prevalence [%] (n = 3,171)	% of mixed infestations (n = 989)
Ctenocephalides canis			
Nematoda (*T. vulpis*)	35	1.10	3.54
Cestoda (*D. caninum*)	30	0.95	3.03
Ectoparasites (*Tr. canis*)	22	0.69	2.22
Protozoa (*C. canis*)	18	0.57	1.82
Nematoda (*T. canis*)	17	0.54	1.72
Nematoda (*U. stenocephala*)	16	0.50	1.62
Ctenocephalides felis			
Cestoda (*D. caninum*)	167	5.27	16.89
Nematoda (*T. vulpis*)	99	3.12	10.01
Nematoda (*T. canis*)	59	1.86	5.97

represented by three types of associations, namely: *Ct. canis, Ct. felis*, nematoda (*T. canis, T. vulpis*) and cestoda (*D. caninum*)—0.47 %; *Ct. canis, Ct. felis*, cestoda (*D. caninum*), nematoda (*T. vulpis*) and ectoparasites (*Tr. canis*)—0.28 %; *Ct. canis, Ct. felis*, protozoa (*C. canis*), cestoda (*D. caninum)* and nematoda (*U. stenocephala*)—0.32 %.

The mixed infestations that included six and seven co-members were represented by two types of parasitic associations. Thus, cases of cohabitation of six parasites were represented by *Ct. canis, Ct. felis*, nematoda (*T. canis, T. vulpis*), cestoda (*D. caninum*), and protozoa (*C. canis*)—0.57 %, as well as *Ct. canis, Ct. felis*, cestoda (*D. caninum*), nematoda (*T. vulpis, U. stenocephala*), and ectoparasites (*Tr. canis*)—0.13 %. The mixed infestations with seven-members were characterized by associated parasitizing of *Ct. canis*, *Ct. Felis*, nematoda (*T. canis, T. vulpis*), cestoda (*D. caninum*), protozoa (*C. canis*), and ectoparasites (*Tr. canis*)—0.32 % and *Ct. canis*, cestoda (*D. caninum*), nematoda (*T. vulpis, U. stenocephala, T. canis*), ectoparasites (*Tr. canis*), and protozoa (*C. canis*)—0.41 %.

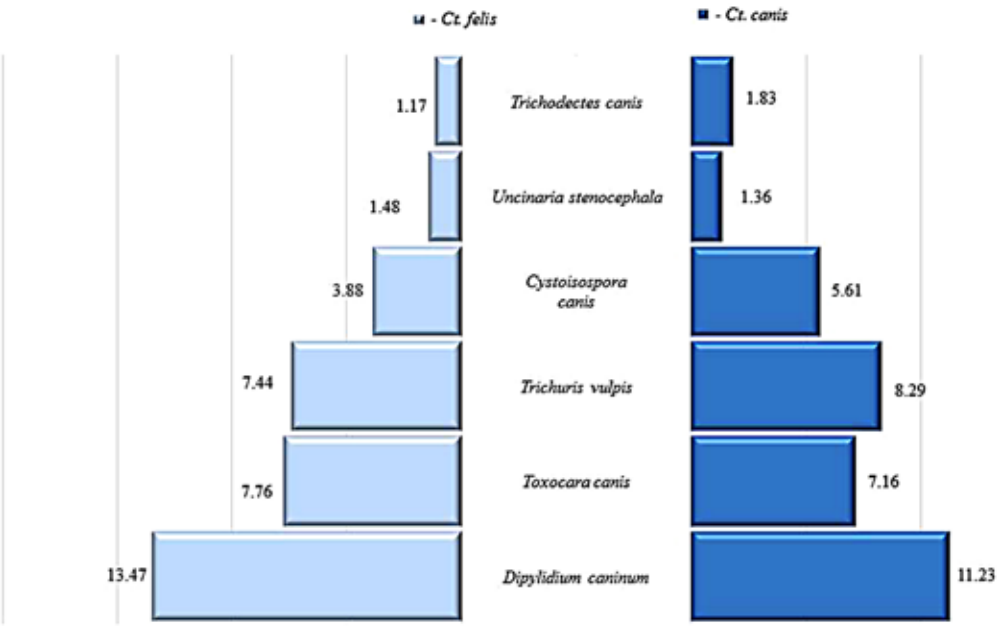

Fig. 3. Species composition of the fleas *Ctenocephalides* genus co-members in mixed infestations of domestic dogs

Cestodes *D. caninum* were the main co-member of the *Ct. felis* fleas in mixed infestations. A lower percentage was the characteristic of other associations (Fig. 3).

Thus, haematophagous ectoparasites of the *Ctenocephalides* genus were common pathogens of invasive diseases among the population of domestic dogs in Ukraine, where the fleas were represented by two species, i. e., *Ct. canis* and *Ct. felis*; the dominant species was *Ct. felis*. However, fleas of the identified species were more likely to parasitize in dogs simultaneously with the causative agents of nematode, cestode, coccidian and entomotic infestations. The most common co-members of fleas' *Ct. canis*, as well as *Ct. felis*, were the cestodes *D. caninum*.

DISCUSSION

It is known that fleas are a group of blood-sucking parasitic insects that plays an important role in the transmission of focal disease pathogens of both humans and domestic animals around them including dogs on most continents of the world [22, 29]. According to the literature, *ctenocephalidosis* of domestic dogs is a very common infestation worldwide, caused by ectoparasites of *Ctenocephalides* genus. Thus, scientists have found that in France, 89 % of the domestic dogs were infested with *Ct. felis* fleas. Dog infestations with *Ct. canis* were detected in only 10 % of the cases [3]. Other authors reported that the prevalence of *Ct. felis* in dogs was 90 % in the USA and 57 % in Germany [18]. In Korean climatic conditions, parasitism of the one species (*Ct. canis*) was found in 28.4 % of the dogs [1]. In Erzurum region of Turkey the prevalence of *Ct. canis* was 31.25 % on dogs, and 4.17 % with *Ct. felis* [2]. At the same time, in Mexico, *Ct. felis* is the most common species of fleas that parasitize dogs in 17 states, where the prevalences ranged from 3.75 to 100 %. However, *Ct. canis* was found in only 9 states of Mexico, mainly in areas with high or low temperatures; the prevalence ranged from 1.40 to 100 % [19, 20]. According to scientists in the metropolis of Moscow, the prevalence of *Ct. felis* in dogs was 26.64 %. And the stray dogs were the most infested where the prevalence reached 100 % [24]. In the Ukraine, questions about the epizootiological features of the spread of fleas have been studied in fragments and were described in only a few works [26].

Therefore, a study was conducted to determine the prevalence of fleas among the population of domestic dogs in Ukraine, Poltava city; their species composition and features of flea parasitism as part of mixed infestations was also investigated. The studies have shown that *Ctenocephalides* spp. fleas are common and adapted to the climatic conditions of the north-eastern part of Ukraine (Poltava) among the population of domestic dogs. This is evidenced by the high prevalence rates, reaching 49.48 %, with an in-

tensity of up to 72 specimens per animal. It was found that the flea environment was represented by two main species, where *Ctenocephalides felis* was dominant, the prevalence was 36.05 %, and the intensity was 15.87 ± 0.34 specimens per animal. The second species (*Ct. canis*) was diagnosed slightly less; the prevalence was 23.98 %, and the intensity was 13.63 ± 0.35 specimens per animal. This dominance of *Ct. felis* over other species of fleas has been established by other authors, who noted that this was due to their greater adaptability to environmental conditions, as well as their high resistance to insecticides [8, 15, 25]. At the same time, other scientists pointed out that in Korean climates, most *Ct. canis* fleas have been found on dogs. According to them, keeping dogs in rooms with underfloor heating affects the development cycle of parasitic insects [1].

Also, we determined that *Ctenocephalides* spp. were parasitizing domestic dogs as mixed infestations more often (P = 31.18 %) together with cestodes (*Dipylidium caninum*), nematodes (*Toxocara canis, Trichuris vulpis, Uncinaria stenocephala*), coccidia (*Cystoisospora canis*) and ectoparasites (*Trichodectes canis*). Overall, 33 types of mixed infestations were detected, where the fleas of the *Ctenocephalides* order were parasitizing in associations with 2—7 other agents. The most often we determined 2-member mixed infestations, and cestodes *D. caninum* were dominant co-member of the fleas in dogs (P = 13.47 %). This relationship has been explained by the development cycle of the cestode of this species, where fleas are intermediate hosts. Reports from scientists suggest that the level of infestation of *Ctenocephalides* spp. fleas by cysticercoides of dipilidia can reached 100 % [4]. Also, there are reports of the specificity of different flea species in terms of the infestation by *D. caninum*, which may indicate the existence of two clear genotypes from infected *Ct. felis* and *Ct. canis* [5].

Our data on the associative parasitism of fleas in domestic dogs can increase the effectiveness of therapeutic and preventive measures; to conduct comprehensive treatment of animals, as well as to take into account the species composition of mixed infestations when prescribing drugs.

CONCLUSIONS

Two species of fleas, *Ctenocephalides felis* and *Ctenocephalides canis*, were determined on domestic dogs in the territory of Poltava city, Ukraine. The prevalence rates were 36.05 and 23.98 %, respectively, with an average prevalence of *Ctenocephalides* spp. 49.48 %. Features of parasitising of isolated fleas in dogs were characterized by the formation of mixed infestations, where helminths *T. canis*, *T. vulpis*, *U. stenocephala*, *D. caninum*, coccidia *C. canis* and chewing lice *Tr. canis* were their co-members. Moreover, the most often was determined the parasitizing of *Ct. felis* together with *D. caninum*, prevalence reached 13.47 %. *Ct. canis* fleas were detected mostly together with *D. caninum* (11.23 %) and *T. vulpis*, and the prevalence was 11.23 and 8.29 % respectively.

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TETANUS IN ANIMALS — SUMMARY OF KNOWLEDGE

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ABSTRACT

Tetanus is a neurologic non-transmissible disease (often fatal) of humans and other animals with a worldwide occurrence. *Clostridium tetani* is the spore producing bacillus which causes the bacterial disease. In deep penetrating wounds the spores germinate and produce a toxin called tetanospasmin. The main characteristic sign of tetanus is a spastic paralysis. A diagnosis is usually based on the clinical signs because the detection in the wound and the cultivation of *C. tetani* is very difficult. Between animal species there is considerable variability in the susceptibility to the bacillus. The most sensitive animal species to the neurotoxin are horses. Sheep and cattle are less sensitive and tetanus in these animal species are less common. Tetanus in cats and dogs are rare and dogs are less sensitive than cats. Clinically two forms of tetanus have been recognized, i. e. localized and generalized. The available treatment is not specific because the toxin in neuronal cells cannot be accessed by antitoxin antibodies. The aim of the therapy is to: neutralise the unbound neurotoxin, inhibit *C. tetani* growth in the wound, and provide supportive care to mitigate the effects of the neurotoxin. The treatment is difficult with an unclear prognosis.

Key words: animal; *Clostridium tetani*; toxin; spasm; treatment

INTRODUCTION

Of the clostridial neurotoxins, botulinum neurotoxin and tetanus neurotoxin are the most potent toxins [15]. Their extraordinary toxicity is caused by neurospecificity and metalloprotease activity, which results in the paralysis. Tetanus is potentially a fatal disease and in some countries it is still very active. Tetanus is a traumatic clostridiosis, an infection in humans and other animals with worldwide distribution. It is a neurologic, toxic, non-transmissible disease characterised by the spastic paralysis caused by the neurotoxin which has been identified as tetanospasmin. Tetanospasmin is produced by an anaerobic bacteria *Clostridium tetani* in a deep wound under anaerobic conditions. Various animal species are differently susceptible to this infection [22, 30, 38, 35].

Clostridium tetani

Clostridium tetani is an anaerobic, spore-forming bacillus. The vegetative form of *C. tetani* is a thin motile rod 0.3—0.6 μm wide and 3—12 μm long. The cell is typically a peritrichous flagella, however some strains are non-motile and non-flagellated. Young cultures are gram-positive, however older cultures can lose the Gram coloration. *C. tetani* can be cultured on blood agar, media with peptones or tissue extracts mostly under anaerobic conditions and at an optimum temperature of 37 °C. Some strains are non-toxigenic and do not produce the toxin [35, 41]. Under adverse conditions, the spores are produced in the process of sporulation; these are—in contrast with the vegetative forms of *C. tetani*—very durable. The vegetative forms are sensitive to disposal and they are vulnerable to disinfection, heat and changes in the environmental pH. The spores are able to survive exposure to various environmental conditions and resistant to disinfectants, such as ethanol and phenols. They can be destroyed by autoclaving at 120 °C for 15—20 minutes. The spores are round and have a terminal with the characteristic shape of a „tennis racket" (0.7 μm × 0.5 μm) and can survive in the soil for 10 years or more [22, 23, 40].

The formation of the spores is variable and depends on the strain type. *C. tetani* spores occur worldwide and they are most often found in soil rich in organic matter in hot and humid environments, but also in the dust, on plants, in contaminated feed, in animal digestive system and the faeces. These bacteria occur in the intestine of animals but they are not the part of the normal digestive flora and do not result in infections. *C. tetani* is not an invasive bacterium and it is not able to enter healthy cells but a deep wound with damaged and necrotizing tissue is a favourable environment for *C. tetani* growth [10, 22, 42].

Toxins

When the spores reach an appropriate anaerobic conditions in the organism, they happen to become pathogenic; then they germinate and multiply in the contaminated wound. Deep wounds without air access and with the presence of necrotic tissue favour spore germination. Deep penetrating injuries (injury to paw or limb, dog bite, biting of contaminated objects in replacement of deciduous tooth), castration, puncture and surgical wounds, and umbilical wounds in neonates are among the most common cases of tetanus. The contaminated wound can be very small and difficult to find. Locally, exotoxins are produced at the site of the wound. *C. tetani* produces two main toxins, neurotoxin (tetanospasmin) and hemolysin (tetanolysin). The others are tetanolepsin and nonspasmogenic toxins. The role of tetanolepsin in the pathogenesis of tetanus is not clinically significant. The influence of the nonspasmogenic toxin is not completely known; it may be involved in blocking transmission in peripheral neuromuscular junctions and it may be the cause of peripheral nervous system paralysis [1, 4, 22, 35]. Tetanolysin causes changes in the permeability of liposomes and biological membranes and can affect a variety of cells including: erythrocytes, neutrophils, macrophages, fibroblasts and others. The effect of this oxygen-sensitive hemolysin results in local tissue necrosis [4, 35].

Tetanospasmin (Tetanus neurotoxin—TeNT) is synthesized as a single-chain polypeptide with a molecular weight of 150 000 Da, encoding with the gene localized on the plasmid. In the form of a single-chain it is weakly active. The toxin must be activated by clostridial or exogenous host proteases to a form consisting of the light (~50 kDa) and heavy chains (~100 kDa), which are bound by a disulphide bridge [20, 33, 38]. One serotype of tetanospasmin is known, but small variations in the amino acid sequence of TeNT proteins without a significant effect on their antigenicity has been reported [12, 27].

Tetanospasmin ascends the axons of motor nerves, which are located adjoining to the contaminated wound and after reaching the cell bodies within the spinal cord, it climbs to the brain. Short nerves are affected before longer nerves and that is the reason why cranial nerves are typically involved before limb nerves [4, 36]. Tetanospasmin permeate also by the lymph to the blood stream. There is a possibility that the circulating toxin might be able to cross the blood-brain barrier and it could bind to axon terminals at distant sites. Tetanospasmin inhibits the release of the inhibitory neurotransmitters glycine and γ-aminobutyric acid (GABA) at interneurons in the spinal cord and brain. This process results in the classical sign of tetanus—continual excitation of the nerves. The high toxicity of TeNT is due to very fast binding to neurons and the activity of enzymes responsible for the cleavage of the necessary proteins to block neurotransmitter release with ensuing neuroparalysis [13, 22, 35, 37].

Tetanus in animals

Different animal species differ in their sensitivity to neurotoxins. The most susceptible species are the horse,

guinea pig, monkey, then sheep, mouse, goat, and human; whereas carnivores such as cats and dogs are less sensitive and birds and poultry are the most resistant species [41]. The interspecies difference in sensitivity and resistance is related to the relative difficulty for the toxins to penetrate and bind to the nerves in different species [1]. The tetanus wound can be very small and sometimes it is difficult to find it. Tetanus may develop even after the wound has completely healed. The parts of the body with a deep wound in contact with soil have the greatest risk for tetanus formation. Accidental wounds and punctures with iron, wooden object or prickly plants at the end of the limbs, the lower side of the trunk and the abdomen are most dangerous. The umbilical infections in new-borns, mainly in lambs and foals are a common predisposing factor for the formation of tetanus. Puerperal tetanus arises after the contamination of the vaginal and uterus mucosa following a complicated delivery. Another risk factor for tetanus is the biting of various objects during replacement of deciduous teeth in dogs. The contamination with *C. tetani* can also occur during castration, injection, ear tagging, shearing and many other traumatic interventions in the body [6, 9, 11, 16, 31].

Tetanus can be observed in non-immunized horses quite often and various forms have been recorded [46]. Cattle are less susceptible than horses and sheep, but outbreaks of tetanus have been reported. General risk factors are accidental wounds, surgical castration, dehorning, and drug injections [11, 16]. In swine, tetanus is observed more often in piglets and is fatal. In cats and dogs, tetanus is rare. Cats are approximately 10 times more resistant than dogs and dogs are 600 times more resistant to tetanus than horses [22].

Signs of tetanus

Tetanus signs are characterized by spastic paralysis and by hyperactivity of voluntary muscles. With tetanus, there are two situations affecting the muscles reported, i. e. rigidity and tetanic spasms. Muscle rigidity are the tonic, involuntary, prolonged contractions. Tetanic spasms are shorter lasting muscle contractions, which can be triggered and amplified by sensory stimulations (light, touch, noise and other) [8, 45].

The incubation period of tetanus lasts 3 to 18 days and depends on the infective dose, local anaerobic conditions and the distance from the tetanic lesion to the central nervous system [28, 36]. Neuro-toxins are absorbed at the neuromuscular endplate and disseminate via retrograde axonal transport. Short nerves are involved before longer nerves. This is why the cranial nerves are preferentially affected over the nerves responsible for the extremities. Clinically, two forms of tetanus have been observed, i. e. localized and generalized [8, 36].

Localized tetanus is rare and may precede generalized tetanus. It causes painful spasm of muscles adjacent to the wound site. Next to the site of infection, one muscle or limb becomes stiff. Subsequently, the stiffness spreads to the opposite limb. If the localized tetanus is not treated, it can affect the entire central nervous system. It may occur in relatively resistant animals, most often in feline patients [1, 46].

Generalized tetanus has a difficult course and involves a large number of symptoms (Table 1). Untreated tetanus ends in the death due to paralysis of the diaphragm and respiratory muscles [8, 24].

Table 1. Clinical signs of tetanus [1, 3, 9, 34, 35, 44, 45]

CLINICAL SIGNS OF TETANUS			
Muscle rigidity	Trismus	Dyspnoe	Contracted facial musculature
Hyperextension of the limbs	Dysphagia	Hyperaesthezia	Erect ears
Stiff gait	Anorexia	Hypersensitivity to auditory or tactile stimulation	Miosis, enophthalmos, prolaps 3rd eyelid
Inability to rise or stand	Regurgitation	Urethral and anal sphincter hypertonicity	Wrinkled forehead
Tetraplegia	Vomiting	Opisthotonos	Risus sardonicus
Extension of the tail	Ptyalism	Torticollis	Swelling of the face

Fig. 1. Characteristic ear position—typical sign of a tetanic dog

Dogs with generalized tetanus are characterised by rigidity of the face muscles that quickly progresses to severe muscle rigidity, a stiff gait and the inability to rise or stand, hyperextension of the limbs and the tail. Typical representative signs of tetanus in dogs include contracted facial musculature, unambiguous ear attitude (Fig. 1) and wrinkled forehead. Dogs commonly have reflex muscle spasms associated with auditory or tactile stimulation and may vocalize during spasms because of pain [1, 3, 9, 22, 34, 35, 44]. They may appear to have some complications associated with: respiratory compromise (laryngospasm, aspiration pneumonia, and central respiratory arrest), autonomic dysfunction (dysuria, urine retention, constipation, and hyperthermia) and protracted paralysis (coxofemoral luxation, and decubitus ulcers). Failure in these different systems may result in some infections; for example, infection of the urinary or respiratory tract and others. Common complications are megaoesophagus and hiatal hernia [1, 2, 6, 9, 14, 21, 28, 32, 47].

Diagnosis

The diagnosis of tetanus in animals is usually based on: the clinical signs, case history, presence of the wound, and progression of the disorder. The overall picture of tetanus disease involves marked muscle rigidity and spastic paralysis. In some cases, a wound may not be detected due to a long incubation period. The detection of *C. tetani* in contaminated wounds is very problematic because of specific culture conditions and low bacterial counts. *C. tetani* must be cultured on specific media in compliance with strict anaerobic culture conditions [13, 22]. The detection of tetanospasmin can be used for diagnostics. It can be detected in the culture supernatant by a mouse bioassay and the typical spastic paralysis observed in mice. Tetanospasmin can be detected by ELISA with specific polyclonal or monoclonal antibodies [29]. In the serum it is possible to identify the circulating antibodies against the tetanus toxin. The minimal level of antibodies in humans considered as protective is equal to > 0.01 $IU.l^{-1}$, but tetanus cases have been reported also at this concentration. The determination of antibody titres in dog serum may be unreliable [17, 22]. Detection of *C. tetani* is possible by the PCR methods; the limit was estimated to be 10 *C. tetani* in the sample [26]. Localized tetanus can be diagnosed by electromyography. Changes in the values of haematological and biochemical parameters are nonspecific for the diagnosis. [13, 18].

One must rule out toxicities in a differential diagnosis (included poisoning with strychnine, organophosphates, metaldehyde and drugs), immune-mediated polymyositis, spinal trauma, meningoencephalitis, subarachnoid haemorrhage or cyst, acute cerebellar disease, and disorders of calcium homeostasis. The determination of diagnosis is the most difficult at the onset of the disease and in the apparent absence of the wound [22, 35, 44].

Management of tetanus treatment

Treatment of animals with tetanus consists of three main steps (Table 2): neutralising the circulating tetanospasmin, inhibiting the growth of *Clostridium tetani* and alleviating the symptoms of the neurotoxin effects. All three phases of therapy happen simultaneously. The treatment of tetanus is complicated and protracted; it must be complex and the prognosis is unclear. For neutralising the tetanus, antitoxin is administered which is used only to bind the circulating toxin. But tetanospasmin is traveling within axons and it is not known what role the circulating toxin plays in the progression of tetanus. It is possible, that antibiotics have a greater effect than antitoxins and the possibility of a cure is assumed even without the administration of antitoxins. One hundred units of antitoxin per 1 kg of body weight are recommended (maximum dose of 20 000 IU) given by a slow, over ten minutes intravenous administration. Repeated dose of antitoxin is not recommended because of

Table 2. Therapy of tetanus [1, 28, 35, 39, 43, 45]

Management of antitetanic therapy	Group of drugs	Drugs
Neutralising the circulating tetanospasmin	Antitoxin	Tetanus antitoxin
Inhibiting the growth of *Clostridium tetani*	Antimicrobial drugs	Penicillin G Amoxicillin-Clavulanate Clindamycin Tetracycline Metronidazole
Alleviating the symptoms of the neurotoxin effect	Sedatives and muscle relaxants	Diazepam Methocarbamol Phenothiazines Magnesium sulphate

the risk of anaphylactic and anaphylactoid-type reactions. An intradermal testing dose of antitoxin (0.1—0.2 ml) is needed to predict hypersensitivity, however, it does not guarantee complete certainty. In case of anaphylaxis, doses of adrenalin, corticosteroids and antihistamines should be given immediately [6, 9, 22, 35].

Antibiotics arrest the growth and reproduction of *C. tetani* and thus also multiplication of the neurotoxin [30]. At the same time, antibiotics suppress secondary infections. The first administration of antimicrobials should be injected intravenously to ensure the necessary level of antibiotics in the blood as soon as possible. At the same time, the wound should be cleaned of any foreign material such as devitalised and necrotic tissue. This action may also help reduced the production of toxins. At surgical wound debridement, veterinarians must consider the risk of patient anaesthesia. The antibiotic of first option is procaine-penicillin G at a dose of 20—100,000 IU.kg^{-1} administered intravenously, intramuscularly or subcutaneously every 6—12 hours. Other antibiotics, which may also be effective are: tetracycline at a dose of 22 mg.kg^{-1} orally every eight hours, clindamycin at a dose of 3—10 mg.kg^{-1} every eight to twelve hours intravenously, intramuscularly or orally, and amoxicillin-clavulanate at a dose of 12 mg.kg^{-1} orally or subcutaneously every twelve hours [22, 28, 35, 45]. In human patients, metronidazole is used and that has achieved good results in dogs also. Metronidazole rapidly achieves therapeutic concentrations in all body fluids and tissues, including abscess cavities. It has a strict anaerobic spectrum of activity (bactericidal against anaerobes) and is not affected by enzyme activity or the local pH in the organism. In dog patients, a dose of 10 mg.kg^{-1} every eight to twelve hours orally or intravenously is preferred. The disadvantage of metronidazole is a higher risk of toxicity [3, 5].

In the case of severe generalised tetanus, the patient must be sedated for muscle relaxation. A combination of acepromazine with buprenorphine and imidazolam or diazepam is used to control painful convulsions and muscle spasms. Diazepam is a muscle-relaxant and combines both anticonvulsant and anxiolytic activities. As a supportive treatment in the suppression of muscle spasms, magnesium sulphate ($MgSO_4$) has been proven successful, as a calcium channel blocker providing muscle relaxation [1, 28, 39, 43].

The animal patient is hypersensitive to auditory or tactile stimulus, and therefore it should be kept in a quiet, dark room. In order to reduce the risk of possible associated health problems, the patient must be placed on a soft pad and turned frequently to prevent pressure sores. It is necessary to ensure basic life functions, such as control of urination and defecation, complement fluid therapy and nutritional support [18, 32].

The recovery from tetanus is slow. Untreated tetanus is usually fatal due to respiratory failure. Cured tetanus infection and low concentration of tetanospasmin in blood does not usually induce an immune response. Vaccination is the only way to protect against tetanus. For prevention of disease, the tetanus-toxoid-containing vaccine is used; it contains tetanus toxoid, a non-toxic form of tetanospasmin. The first *C. tetani* vaccine strain was derived from a laboratory strain collected by G. W. McCoy around 1917—1919 at the US Public Health Service, Hygienic Laboratory [7, 17, 19, 25].

CONCLUSIONS

Tetanus is a neurological disease caused by the action of the neurotoxin tetanospasmin which is responsible for spastic paralysis. The bacterial agent *C. tetani* produces the neurotoxin in the wound under anaerobic conditions. Between animal species, there is considerable variability in the susceptibility to tetanospasmin. Out of all animal species, the horses are the most sensitive to the neurotoxin. Tetanus in cats and dogs is rare and they are the least susceptible of mammals.

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EPIDEMIOLOGICAL AND COMPARATIVE DIAGNOSTIC STUDY OF *ANAPLASMA* SPP. INFECTION IN GOATS FROM NORTH-EASTERN ALGERIA

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ABSTRACT

Anaplasmosis is an infectious disease affecting goats and other ruminants. Our goal was to estimate the prevalence of *Anaplasma* spp. infections, and to identify different tick species in goa ts along with their associated risk factors. The epidemiology of *Anaplasma* spp. (*A. ovis*/*A. marginale*) was investigated from April to September 2016 in dairy goats from three different agro-ecological areas in the northeast region of Algeria (Guelma and El-Taref). We tested 182 goat sera using a MSP5 competitive ELISA (cELISA) test for IgG antibody screening, and by microscopic examination using the Giemsa (May-Greunwald-Giemsa) stain of 128 blood smears to detect intraerythrocytic inclusions bodies. The results demonstrated a total infection rate of 78.02 % and 42.18 % using cELISA and MGG, respectively. Only two species of ticks collected were identified, i. e. *Rhipicephalus sanguineus* (85.39 %) and *Rhipicephalus bursa* (14.60 %). Our study revealed that factors, such as the season, the type of farming, the hygienic status and the climatic conditions of the studied areas were found to be significantly associated with the tick infestation of goats ($P < 0.05$). The *Anaplasma* spp. infection prevalence was found to be highly dependent on the climatic conditions, the season ($P < 0.05$) and most closely related to the type of breeding and herd management ($P = 0.000$).

The evaluation of the Giemsa technique showed specificity and sensitivity of 60.71 % and 45.16 % respectively. Calculating the concordance between the two techniques revealed Cohen's Kappa value of 0.038 in the range 0.21—0.40, which corresponded to a very

low agreement. The McNemar test results showed that both tests gave significantly different results ($P < 0.05$). This work provides evidence for *Anaplasma* spp. in goats from north-eastern Algeria.

Key words: caprine anaplasmosis; cELISA; epidemiology; MGG staining; ticks

INTRODUCTION

Anaplasmoses are common tick-borne zoonotic bacterial diseases of domestic animals. Goats and sheep on pastures are often infested with ticks which carry the anaplasmoses that constitute important diseases of livestock that is endemic in tropical and subtropical regions of the world [11, 33]. Anaplasmoses have been reported as diseases of major economic importance, as they cause heavy losses due to mortality, decreased production and lowered working efficiency of the affected animals [16, 62]. The causative agents are intracellular gram-negative bacteria that belong to the family *Anaplasmataceae* which is divided into six recognized *Anaplasma* species: *A. ovis, A. marginale, A. centrale, A. platys, A. bovis,* and *A. phagocytophilum* [62]. Small ruminants host several *Anaplasma* species, including *A. ovis, A. marginale* and the zoonotic agent *A. phagocytophilum* causing human granulocytic anaplasmosis [58]. Clinical manifestation depends on the immune status, as well as the welfare of the animals. *A. ovis* infects primarily sheep and goats causing a subclinical infection, while severe infections may involve anaemia, abortion, and mortality when combined with other stress factors, such as co-infection, hot weather, vaccination, and heavy tick infestation [57]. The acute disease caused by the intraerythrocytic *Anaplasma marginale* consists of: anaemia, icterus, fever, anorexia, and lethargy which they are most notable in cattle [52]. The transmission to the hosts (numerous species of mammals including humans) occurs via vectors, such as the hard ticks of the Ixodidae family. *A. marginale* and *A. ovis* are mainly transmitted to mammalian hosts by *Rhipicephalus* spp. and *Dermacentor* spp. [30].

Anaplasmosis diagnosis is usually based on the microscopic examination of Giemsa-stained blood smears, or serological and molecular diagnostic procedures. Microscopic diagnosis may be difficult in carrier animals, thus various serological techniques have been used for the detection of *Anaplasma* specific antibodies such as indirect immunofluorescence antibody (IFA), enzyme-linked immunosorbent assay (ELISA), and complement fixation tests (CF) [20]. The competitive ELISA (cELISA) is dependent on the use of a monoclonal antibody ANAF16C1 that recognizes the conserved antigen MSP-5 of different *Anaplasma* species, and it has high sensitivity and specificity values [41].

The study of *Anaplasma* spp. infection in goats was the main topic of this survey. Currently, limited data is available concerning this bacterial infection in Algeria. The aims of our work were as follows: (i) the estimation of *Anaplasma* spp. prevalence in goats based on microscopy and serological screening; (ii) the analysis of some risk factors for the *Anaplasma* infection; (iii) the identification of ticks infesting goats; and (iv) the performance evaluation, including the sensitivity and specificity determination of the different diagnostic tools such as microscopy and serologic methods used for detecting caprine anaplasmosis.

MATERIALS AND METHODS

Presentation of the study area

We carried out our study in two provinces in north-eastern Algeria (Guelma and El-Taref). The territory of Guelma is characterized by a sub-humid climate in the centre and the North, and semi-arid towards the South. This climate is mild and rainy in the winter and warm in the summer. The temperature varies from 4 °C in the winter to more than 35 °C in the summer. The geography of the district is characterized by a diversified relief (mountains, plains and plateaus, hills, and foothills) which mainly retains significant forest cover and the passage of wadi Seybouse which constitutes the main watercourse.

The El Tarf province is located in the far northeast of Algeria along the Tunisian border. The climate is generally humid. The humidity decreases from North to South in the following way: a coastal zone that presents a hot and humid climate; a mountainous area that occupies most of the region and has a mild humid climate in the north and cool in the south. The annual precipitation rate is 900 ml to 1200 ml. The study area has a total of 112,740 goats (69,200 in Guelma and 43,540 in El-Taref). The farming system is generally extensive. Animals are fed hay, bran, and grass during the grazing season; this goes from March to December with variations depending on weather conditions.

Study design and the target population

The choice of regions consisted of an appropriate sample that was governed by the objective to include the different agro-ecological strata of Algeria and regions representative of the variety of different indigenous goat breeds.

Blood samples were collected from goats from the different local farms in Guelma and El-Taref provinces of Algeria. Eligibility criteria involved a suspicion of anaplasmosis among goats in areas with sub-humid, humid and sub-arid climates. The animals were surveyed for anaplasmosis from April to September 2016, during the dry and wet seasons.

A questionnaire was developed and administered at the household level to collect individual animal and flock-level data for risk factor evaluation. A standardized questionnaire was developed for each sample in order to document the: age, sex, goat breeding, health status (tick infestation, agalactia, abortion, and sterility) of the animal, characteristic of the farm and climatic conditions. The goats were kept on pastures from spring to autumn and were stabled during the winter months. Animals from the farms had a history of frequent abortions.

The farms were mainly made up of goats of local breeds (100 %). The samples included 128 females (64.64 %) and 70 males (34.34 %). The animals were divided into three age classes: < 6 months (4 %), 6 to 12 months (30.8 %) and more than 12 months (65.6 %). Our samples concerned 15 municipalities distributed in the 3 agroecological zones of the northeast region of Algeria. The choice of goats sampled from each farm was made at random. This process allowed us to carry out a random sampling of 198 blood samples from goats aged between 4 months and 7 years old coming from 15 municipalities located in the provinces of Guelma and El-Taref and from 23 goat farms (14 from Guelma and 9 from El-Taref).

The cELISA test is the most commonly used serological method to detect antibodies against *Anaplasma* spp. and used as a reference diagnosis among microscopic examination of blood smears.

The required sample size was calculated according to the following formula with an expected prevalence of 10 % and a 95 % confidence interval (CI) [56]:

$$N = [Z^2 \times P(1 - P)]/d^2$$

Where:

N—is the number of samples to be collected in the study,

Z—is the value of the normal distribution for the confidence interval of 95 % [$Z^2 = (1.96)^2$],

P—is the expected prevalence,

d—is the absolute error of 10 %

A minimum of 100 samples was required. To increase the power of the statistical analysis, the sample size was multiplied by 2. Twenty-three herds were randomly selected and the herd sizes ranged from 5 to 120 heads.

At the individual level, the sample size was determined for each flock to detect the existence of the disease. The calculations were performed according to the formula commonly used in veterinary epidemiological surveys by Thrusfield [56]:

$$n = [1 - (1 - p)^{1/d}] \times [N - (d/2)] + 1$$

Where;

n—is the size of the sample in each flock,

p—is the detection probability of at least one seropositive goat,

N—is the size of the flock,

d—is the number of seropositive goats in the herd.

The probability of detecting at least one seropositive goat in a flock was 95 % (p = 0.95), while the number of seropositive goats in each flock (d) was calculated assuming that the herd was equal to 10 %. Finally, 10 to 30 blood samples were collected from each flock, making a total of 210 goats.

Sample collection

Blood sampling

A total of 198 blood samples were collected from goats of different gender and age groups (4 months to 7 years). Jugular venous blood samples were collected from each individual animal into two 5 ml vacutainer tubes (BD vacutainer, BD-Plymouth, UK), one containing EDTA and one additive-free. The blood smears were prepared and examined immediately. To separate the sera, the additive-free blood was allowed to clot for about 15—30 min at room

temperature. The tubes were then centrifuged at 1000—2000 rpm for 10 min and the serum was collected. The sera specimens were stored at –20 °C for further use.

Tick sampling

Once the blood was collected, the entire body of each of the 198 animals was inspected for ticks. In order to reduce the time of restraint and handling of the animals, we focused particularly on the ears, neck, udders and external genitalia (predilection sites of the tick's attachment). A minimal of three ticks were collected from the infested animals selected for blood sampling using entomological blunted clockmakers forceps through the examination of the perineum surface according to Baker and Ducasse [11]. All collected specimens were immediately placed in 70 % ethanol inside tubes labelled with the identification number and the date of collection and stored at 4 °C. In the Laboratory of parasitology of ENSV, Algeria, each tick was observed under a binocular magnifier for morphological identification and classified up to the species, sex, and life stage according to the taxonomic keys developed by Walker et al. [60], Meddour-Bouderda and Meddour [42].

Laboratory analysis

Microscopic examination of blood smears

Among the 198 goats sampled, only 128 smears were subjected to microscopic examination.

May-Greunwald-Giemsa (MGG) was chosen for staining and microscopic examination of blood smears accordingly the standard protocol [16]. The slides were allowed to air-dry before being fixed with absolute methanol. Fixed smears were stained with 10 % Giemsa and examined using a compound microscope under oil immersion lens. About 2 fields were examined from each slide for the presence of *Anaplasma* spp. by the same operator. Inclusion bodies consistent with anaplasmosis (due to *A. marginale* or *A. ovis*) were identified based on morphology and intra-erythrocytic localization [19].

Competitive ELISA (cELISA) assay

Sera (from 182 goats) were screened for the presence of *Anaplasma* spp. antibodies by using the *Anaplasma* Antibody Test Kit, cELISA from VMRD Inc. (Pullman, WA, USA) following the manufacturer's instructions.

The optical density (OD) was measured at 620 nm with an ELISA microplate reader (Immunoskan ELISA, BDSL). The results were calculated according to the formula:

100 [1 — (Sample OD ÷ Negative Control OD)]. The positive sample OD must be > 30 %. The optical densities (OD) obtained were converted into percentages of inhibition, calculated according to a formula indicated by the manufacturer. The cut off threshold was determined at 30 %. Samples with a percentage below 30 % were negative and those with a percentage above 30 % were positive.

Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences version 20.0 (SPSS Inc. Chicago, USA). Data were categorized according to the region and used statistical methods such as chi-square and odds ratios. The calculated prevalence was estimated at a 95 % confidence interval (95 % CI). The impact of the defined risk factors on absence or presence of disease was examined using the Chi2 test, or in some cases the exact Fisher test (age, gender, breed, livestock production system, presence of ticks, history of abortion, pregnant status, climate condition, and hygienic quality). The differences were considered statistically significant when $P < 0.05$. The kappa statistic was undertaken to assess concordance between blood smear microscopic examinations and the serologic method with the calculation of specificity, sensitivity, accuracy, positive predictive value, negative predictive value, McNemar and Cohen's Kappa values. The agreement with kappa values of 0.00 to 0.20 was considered light, 0.21 to 0.40 fair, 0.41 to 0.60 moderate, 0.61 to 0.80 substantial and 0.81 to 1.00 almost perfect.

RESULTS

Tick species identification and associated risk factors

A total of 102 ticks were collected from 198 goats in the provinces of Guelma and El-Taref. 89 ticks were identified as adults (29 male and 60 female) and 13 ticks in the nymphal stage. Two tick species were identified among the overall collected ticks into one genus: *Rhipicephalus*. Seventy-six specimens 76 (85.39 %) were classified as *Rhipicephalus sanguineus*, and 13 (14.60 %) as *Rhipicephalus bursa*. All of the nymphs collected were identified as *R. bursa*. Of the 198 goats examined, 55 were infested with at least one tick species, an overall prevalence of 27.77 % (15.9—39.6 %, 95 %

Table 1. Risk factors analysis of exposure to a goat infestation by ticks

Variables	Number sampled	Positive number [%]	95 % CI on prevalence	○	95 % CI on OR	P-value
Age						
≤ 6 months	8	0 (0)	00.00—37.22			
6—12 months	61	9 (14.75)	07.73—25.95	0.73	0.24—4.42	0.610
>12 months	129	17 (13.18)	08.30—20.19			
Sex						
Male	71	11 (42.3)	23.3—61.3	1.36	0.53—3.41	0.463
Female	127	15 (57.7)	38.7—76.7			
Season						
Autumn	26	0 (0)	0.00—11.53			
Spring	91	8 (8.79)	04.30—16.62	0.13	0.07—2.07	0.009**
Summer	81	18 (22.22)	14.46—32.50			
Dress Colour						
Black	88	13 (14.77)	8.70—23.79			
White	42	5 (11.9)	4.73—25.46			
Black and White[a]	29	2 (6.90)	0.85—23.03			
White and Black[a]	21	4 (19.05)	7.08—40.59	1.13	0.06—2.02	0.402
Black and Brown	1	0 (0)	0.00—100.00			
Grey	1	0 (0)	0.00—100.00			
Wheel	16	0 (0)	0.00—18.75			
Type of breeding						
Extensive	126	11 (8.73)	4.80—15.10			
Semi-extensive	62	15 (24.19)	15.14—36.25	3.57	0.25—62.27	0.008**
Intensive	10	0 (0)	0.00—30.00			
Hygienic quality						
Good	112	12 (10.71)	06.10—17.94			
Moderate	69	14 (20.29)	12.37—31.35	0.47	0.20—1.09	0.047*
Bad/ poor	17	0 (0)	00.00—17.64			
Climate						
Humid	71	13 (18.31)	10.89—28.99			
Sub-humid	83	7 (08.43)	03.89—16.66	1.50	0.54—4.17	0.019**
Semi-arid	44	6 (13.64)	06.02—27.09			

OR—Odds Ratio; CI—Confidence Interval at 95 %; *—significant at P ≤ 0.05; **—significant at P ≤ 0.01; [a]—the difference between the terms of coat color «Black and White» and «White and Black» lies in the intensity and dominance of the colour of which the first colour is the most dominant

CI). The evaluation of the infestation rate by tick species identified showed a large predominance of *R. sanguineus*, of which 15 goats were infested, followed by *R. bursa* with an infestation rate of 7.27 % (4 goats). Thus, 65.46 % of the goats were infested with ticks in the larval stage.

We have studied some risk factors that may influence the rate of goat tick infestation. The factors considered were: sex, age, breed, farm hygiene, season and climate of the area. The results recorded in Table 1 revealed that factors such as: the season, the type of farming, the state of the hygiene of the farm, as well as the climatic conditions of the studied areas were found to be significantly associated with tick infestation of the goats ($P < 0.05$). For the tick harvest season, the prevalence of infestation was very high in the summer (69.2 %) compared to the spring (30.8 %), compared to 0 % in the fall.

If we now take into account the climatic conditions of the study areas, the prevalence of tick infestation was higher in the humid areas (50.0 %), followed by the sub-humid and semi-arid areas (26.9 % and 22.2 % respectively). Factors such as gender, age, and breed or coat colour do not significantly affect the tick infestation of goats.

Microscopic examination using MGG

A total of 128 blood smears were examined for the presence of intraerythrocytic inclusion bodies (considered as positive for *A. ovis* and/or *A. marginale*) using the Giemsa stain. *Anaplasma* spp. appeared as small spherical

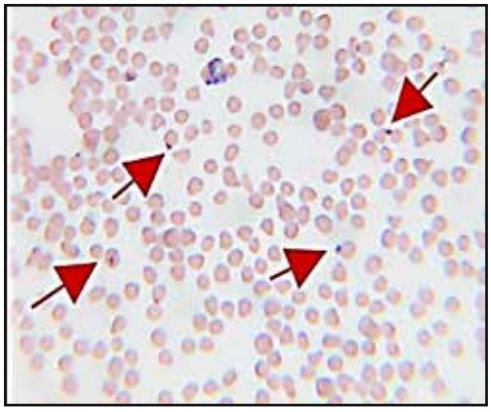

Fig. 1. *Anaplasma* spp. identified on blood smears stained with Giemsa, × 8 eyepiece and × 100 objective

Table 2. Risk factors analysis of exposure to *Anaplasma* spp. infection by cELISA

Variables	Number sampled	Positive number (%)	95 % CI on prevalence	Odds Ratio (OR)	95 % CI on OR	P—value
Age						
≤ 6 months	8	4 (50.00)	15.35—84.64			
6—12 months	55	40 (72.73)	59.68—82.81	0.57	0.26—1.21	0.146
> 12 months	119	98 (82.35)	74.45—88.23			
Sex						
Male	66	46 (69.70)	57.73—79.50	1.10	0.58—2.07	0.041*
Female	116	96 (82.76)	74.79—88.63			
Season						
Autumn	26	21 (80.77)	61.67—91.95			
Spring	38	13 (34.21)	21.15—50.17	1.35	0.45—4.06	0.588
Summer	82	62 (75.61)	65.24—83.69			
Dress Colour						
Black	87	66 (75.86)	65.84—83.70			
White	38	32 (84.21)	69.20—92.94			
Black and White[a]	25	17 (68.0)	48.27—82.94	0.96	0.53—1.34	0.298
White and Black[a]	18	15 (83.33)	59.95—94.99			
Black and Brown	1	1 (100)	0.00—100.00			
Grey	13	10 (76.92)	49.06—92.50			
Type of breeding						
Extensive	115	98 (85.22)	77.50—90.66			
Semi-extensive	58	42 (72.41)	59.71—82.33	9.18	1.72—48.98	0.000**
Intensive	09	02 (22.22)	26.90—80.27			
Hygienic quality						
Good	103	81 (78.64)	69.71—85.52			
Moderate	65	49 (75.38)	63.60—84.33	0.51	0.10—2.52	0.406
Bad/poor	14	12 (85.71)	58.81—97.24			
Climate						
Humid	74	56 (75.68)	64.71—84.10			
Sub-humid	66	54 (81.82)	70.70—89.44	1.40	0.54—3.62	0.480
Semi-arid	42	32 (76.19)	61.30—86.69			
Pregnant status						
Yes	26	23 (88.46)	70.20—96.82	1.70	0.45—6.37	0.426
No	88	72 (81.82)	72.38—88.59			
Tick infestation						
Yes	158	122 (77.22)	70.04—83.09	2.37	1.09—5.16	0.501
No	24	20 (83.33)	63.53—93.93			

OR—Odds Ratio; CI—Confidence Interval at 95 %; *—significant at P ≤ 0.05; **—significant at P ≤ 0.01; [a]—the difference between the terms of coat color «Black and White» and «White and Black» lies in the intensity and dominance of the colour of which the first colour is the most dominant

deep purple intraerythrocytic inclusions, measuring about 0.2 um to 0.5 µm (Fig. 1). Among the specimens, 54 were found positive with an overall prevalence of 42.18 %.

Prevalence of *Anaplasma* spp. antibodies using cELISA

The overall prevalence of *Anaplasma* spp. antibodies in the goats were determined using the MSP5 cELISA for *Anaplasma* spp. Of the 182 animals tested, 142 (78.02 %) were found to be positive for antibodies against the *Anaplasma* spp. antigen (*A. ovis* et/ou *A. marginale*). The prevalence of *Anaplasma* spp. infection in the herd goats was 47.4 % (n = 148). However, the infection rates were higher among females (67.6 %), animals of the age group superior to 12 months (61.1 %), and in infested animals by ticks (85.9 %) with no significant correlation (P > 0.05) (Table 2).

There was a highly significant association (P = 0.000) between the seroprevalence of *Anaplasma* spp. and the type of breeding and herd management (Table 2). However, no significant association was found with other factors such as: sex, age, pregnant status, abortion, tick infestation, season, coat colour, hygienic and climatic conditions (P > 0.05) (Table 2). The highest seropositivity in goats was observed in the extensive herd type (50 %). The infection rate was higher but not significantly among the humid climate (39.4 %) compared to the sub-humid and semi-arid climate (P > 0.05). Indeed, no significant relationship was observed between the *Anaplasma* spp. seroprevalence and the presence of *R. Sanguineus* and *R. bursa* (P > 0.05).

Comparative study of the two methods (MGG and cELISA)

The performance of the blood smear method was evaluated using the cELISA as a reference test (Table 3). For the blood smear method, sensitivity, specificity, relative accuracy, Cohen's kappa coefficient, Mc Nemar test, positive predictive value (VPP) and negative predictive value (VPN) were calculated.

Comparing the data obtained from the microscopic examination with cELISA, it was observed that the blood smear method had a lower sensitivity (45.16 %) and specificity (60.71 %) than cELISA. The results showed an accuracy of 48.46 %, a VPP of 79.24 % and a VPN of 75 %. The value of Kappa as 0.038, which means that there was a very weak agreement. The Mc Nemar test result showed that the two methods gave significantly different values (P < 0.05).

Table 3. Comparison of the MGG with the cELISA test as a reference test for the diagnosis of *Anaplasma* spp.

Anaplasma spp.	cELISA		MGG	
			+	–
Goats	+	93	42	51
	–	28	11	17
	Total	**121**	**53**	**68**
Intrinsic values	Se = 45.16 %		k = 0.038	
	Sp = 60.71 %		Mc Nemar test (P < 0.05)	
	Ra = 48.46 %			

Se—sensitivity; Sp—specificity; Ra—relative accuracy
K—Kappa value

The Gold standard of truth for the diagnosis of *Anaplasma* spp. relies on the combination of the microscopic examination and cELISA.

DISCUSSION

No previous study on anaplasmosis had ever been conducted in the Guelma and El-Taref regions before. Therefore, the present study was part of a large survey initiated to address the lack of data on the epidemiological features of anaplasmosis in the Guelma and El-Taref regions. The plan was to study all of the epidemiological links, i.e. goats and ticks; along with the risk factors associated with anaplasmosis. In Algeria, there is a lack of information about the *Anaplasmataceae* infections in animals and mainly in ruminants, where only *A. phagocytophilum*, *A. platys* and *Anaplasma* spp. were found in cattle [23].

Our study described a survey of ticks and tick-borne bacteria such as *Anaplasma* spp. circulating in different areas of North-eastern Algeria. *R. sanguineus* and *R. bursa* were the only tick species detected in the studied regions and they can serve as vectors of *A. marginale* and/or *A. ovis* as reviewed by K o c a n et al. [33].

Accordingly to our findings, *Rhipicephalus* spp., the most incriminated tick in the transmission of ovine and caprine anaplasmosis [30], has frequently been detected and abundant in the Gharb and the Middle Atlas regions of Morocco [35]. Nevertheless, the role of other biting hematophagous insects, such as the *Hippoboscidae*, flies (e. g. horseflies) and

some species of flea, should not be underestimated with regard to the transmission of anaplasmosis [32].

The North African ecosystem offers a favourable ecology for several tick species that can be competent vectors of several pathogens [17]. In Tunisia, the most dominant tick species collected from sheep was *R. turanicus* followed by *R. sanguineus* and then *R. annulatus* [24]. *R. anguineus* have been proposed previously as vectors of *A. ovis* in Mediterranean countries [4, 58]. Recently, A k t a s et al. [4] reported the presence of *A. ovis* DNA in the salivary glands of *R. sanguineus* collected from Turkey. L u et al. [38] showed that *Dermacentor nuttalli*, *Hyalomma asiaticum* and *Rhipicephalus pumilio* were the main vectors of *A. ovis* in China and *R. turanicus* was one of the main vectors of *A. ovis* in Sicily (Italy) [58]. Recently, using quantitative real-time PCR, S a d e d i n e et al. [53] has detected *A. ovis* in the blood of small ruminants, *Rh. sanguineus* s. l. and *Rh. bursa*. A previous study had already reported the presence of *A. ovis* in these ticks [10]. In Tunisia, B e l k a h i a et al. [14] indicated that ticks collected from goats were infected by *A. ovis*, such as *R. turanicus* and *R. sanguineus* s. l. with an absence of infection in *R. bursa* and *R. annulatus*. In addition, *Anaplasma ovis* has previously been reported in small ruminants and ticks from Algeria and Tunisia [9, 13, 15] and in ticks from Ethiopia [55]. However, further studies are needed to be done to identify the main vectors of *A. ovis* in our country.

Moreover, in our current study, we did not detect any case of *A. phagocytophilum* infection and no hard tick vector such as *Ixodes ricinus* was identified. Other tick species like those of the genus *Rhipicephalus* and *Hyalomma* have been suspected of transmitting this bacteria [21] and have been known to be widely distributed in Morocco [35] and probably in Algeria.

In contrast to our finding, B e l k a h i a et al. [12] and B e n S a i d et al. [15] showed that ruminants (cattle, goats, and sheep) infested by ticks were statistically more infected with *Anaplasma* spp. than those free from ticks. No statistically significant differences were found in the prevalence of *Anaplasma* spp. in animals or humans exposed or not to ticks [28, 29]; the same results were found in the current study. According to A i t L b a c h a et al. [3], it seems that the prevalence of *Anaplasma* spp. was very significantly influenced by the tick infestation and by tick-borne diseases prophylactic measures. Our results are in disagreement with the results of recent studies showing the strong relationship between the density of tick infestations and the occurrence of anaplasmosis [39].

In various previous serosurveys on the occurrence of *Anaplasma* spp. in animals, the serologic methods, the cut-off points, the study sample, and the study group population have been variable. Although a comparison of the present data with those reported in previous seroepidemiological studies may be difficult, it is worthwhile comparing our data with the seropositivity rates reported from other Mediterranean countries. In this longitudinal investigation, results clearly indicated evidence of *A. marginale/A. ovis* infection in goats sampled in all investigated bioclimatic areas and during the three seasons (autumn, spring, and summer). The average prevalence rate of *A. ovis/A. marginale* infection in goats was 78.02 % (minimum 30.7 % in spring and maximum 43.6 % in autumn). It was similar to those reported in Iraq (75.22 %) [44], higher than that observed in Turkey (66.4 %) [6], in Tunisia (65.3 %) [15], in Egypt (48 %) [31] and in Italy (31.7 %) [57], and lower than that estimated in Angola (100 %) [34], in Kenya (89 %) [40] and in China (27.5 %) [63], and it was much higher than the level observed in Korea (6.6 %) [36].

In this study, goats of all ages were susceptible to anaplasmosis, but the severity of the infection was directly related to the age, but the relationship was not statistically significant. The relatively high seroprevalence was observed in the age group above 1 year old as compared to other age groups. It has been reported that goats of all age groups are susceptible to *A. ovis/A. marginale* infection, but older animals may suffer from a greater reduction in haematocrit values [51]. The results of our study were completely in line with the findings of N a q i d, Z a n g a n a [44], and R a z m i et al. [52], who reported that adult animals were more susceptible to anaplasmosis infection than younger animals. This finding indicated that adult goat may have more opportunities for exposure to ticks carrying the pathogen than younger animals. This could be explained by the fact that adult goats were more exposed to tick infestation carrying *Anaplasma* spp. because they went through more tick seasons.

A significant relationship in the prevalence was revealed between male and female goats. This is in contrast with what has been reported elsewhere concerning goat anaplasmosis [15, 50]. Similar to our current findings, N a q i d and Z a n g a n a [44] have reported that females were more susceptible to *A. ovis/A. marginale* infec-

tion than males, but Zhou et al. [64] showed contrasting results. This could be related to the proportion of the populations sampled. Furthermore, most of the farmers keep a larger number of females than males especially for breeding purposes which affected the proportion of the sex ratio. In others, females were kept for a comparatively longer period within the breeding herd and this increased their chance of exposure to infections, but male animals were usually sold off at younger ages than females.

The current study showed that goat farmers use open lands for grazing with minimal grazing in the highlands regions. Similarly to the findings by Jordan [47], the difference in herd management could expose small ruminants to greater tick numbers and could explain the higher seroprevalence in goats. Grazing goats showed higher seropositivity compared to those who remain in permanent housing and this can be explained by the fact that grazing systems may increase the risk of getting vector ticks and enhance the risk of anaplasma infections [31]. A higher seroprevalence was demonstrated for the extensive system (69.0 %) when compared to the semi-extensive and intensive systems (29.6 % and 1.4 % respectively). Consistent with our current results, Stoltsz [54], Torina and Caracappa [57] showed that the grazing system and the type of breeding influenced potentially the seroprevalence of *Anaplasma* spp. infections in goats. The relatively high seroprevalence of *Anaplasma* spp. observed in our study could be due to the favourable environmental conditions, especially in spring, for the survival and proliferation of the tick vectors, since the goats are reared under extensive and semi-intensive systems.

Goats are usually vaccinated for certain diseases like brucellosis as part of the national vaccination campaigns in Algeria. These vaccination campaigns, already applied at the same period of the tick infestations, might explain the high seroprevalence as there is the possibility of iatrogenic transmission among goats by contaminated needles. Such stressful conditions, scarcity of water and pasture during summer, might exacerbate clinical diseases associated with *Anaplasma* spp. infections. In addition, the high prevalence of other diseases in dairy ruminants in Algeria, such as mastitis and respiratory diseases, might predispose animals to clinical disease associated with *Anaplasma* spp. infection such as described in Jordan [47, 48]. *Anaplasma* spp. prevalence was not affected by farm locations or the hygienic statute of farms. These findings corroborate with a previous study that did not find a significant difference in Iran [61].

In our study, the microscopic examination of goat blood smears obtained from Guelma and El-Taref provinces, demonstrated that 42.18 % of the goats were infected with *A. ovis/A. marginale*. This method has been commonly used in previous studies on ovine and caprine anaplasmosis surveys [2, 52]. The highest prevalence rate was found in the Ain La-assel region (50 %), followed by Ain-Makhlouf (44.8 %) and Ain-Arbi (37.9 %), but no statistically significant differences among regions were found ($P > 0.05$). Our prevalence is close to that found in the northeast region of Iran (Mashhad) (47.53 %) [52]. A lower positivity rates were reported in Tamil Nadu in India (26.15 %) [50], in Iraq (Baghdad) (28.8 %) [5], and in Iran (Ahvaz) (19 %) [31]. A previous study in the Duhok region of Iraq [44] reported a higher prevalence of *A. ovis* in local breeding goats (55.86 %) than that observed in our study.

One of the previous studies performed in Sicily (Italy) came to the conclusion that animals under poor health conditions may express a higher infection rate and also contribute to multiple *Anaplasma* infections [58]. The distribution and abundance of tick vectors can be affected by climatic conditions, including humidity and temperature [37]. These climatic conditions may be favourable for the survival of the ticks that transmit *Anaplasma* spp. and could increase the possibility of other tick-borne disease being spread to livestock and wild animals; therefore resulting in a higher prevalence rate as was observed in our study. The prevalence rate of *Anaplasma* spp. varied significantly in different agro-ecological areas considered in this study. However, it was noticed that goats in the sub-humid climate were more affected than in the humid and semi-arid climate. In fact, *Anaplasma* infections exhibited a certain seasonality linked in particular to the abundance of vectors; however, the preferential seasons vary according to region and bioclimatic conditions during the year [8, 37]. The humid and sub-humid stages constitute a very favourable biotopes for *R. Sanguineus* and *R. Bursa* [18] which are species recognized as vectors of *A. ovis* in goat in the Mediterranean region [13]. Our results do not correspond to those mentioned in the literature and this deference can be linked to the origin of the animals, which are in commercial exchange (sale and purchase) almost all year and they are transported between the different agro-ecological areas. Velusamy et al. [59] indicated

that there was no seasonal effect on goat anaplasmosis in India. F r i e d h o f f [30] has reported that many factors affect the prevalence of *Anaplasma* spp. infections including: age, sex, breed, and the general conditions of the animals.

We had shown that *Anaplasma* infections were related to pregnancy disorders, such as abortion in goats. A history of abortion was associated with *Anaplasma* spp. seropositivity in small ruminants from Jordan [47]. It is interesting to declare that animals or flocks showing high seropositivity and/or tested positive by MGG against the pathogen did not show any clinical signs of the disease; other surveys have reported similar findings [51].

Nevertheless, serological diagnostic assay tests could be more practical for the diagnosis of a large number of animals than the conventional microscopic test including Giemsa stained blood smears. Also, serological diagnostics are more sensitive and specific diagnostic tools to detect and differentiate *Anaplasma* spp. in carrier animals. Several studies have specified that cELISA test has a very high sensitivity and specificity in the diagnosis of antibodies against *Anaplasma* species such as *A. marginale*, as well as *A. centrale*, *A. ovis* and *A. phagocytophilum* [26].

In our study, cELISA detected a higher number of infected animals with *A. ovis/A. marginale* (142/182; 78.02 %) than Giemsa stained blood smear (54/128; 42.18 %). Overall, the blood smear method showed average specificity, low sensitivity and accuracy with high VPP and VPN values. Calculating the concordance between the two methods using Cohen's Kappa test showed values from 0.00—0.20, which corresponds to a very weak concordance. The result of McNemar's test showed that the method gave significantly different values ($P < 0.05$). The result obtained in this study is comparable with those found in Egypt by A b o u - E l n a g a et al. [1] where they recorded a prevalence of 37 % and 67 % in cattle using the Giemsa staining and cELISA respectively. M o u l o u d i [43] obtained a frequency of 25.4 % and 63.9 % by MGG and cELISA respectively. These discrepancies could be explained by differences in the stage of infection and the antibody responses. During the chronic infections, antibodies remained in the blood circulation for a longer period (up to 10 years after the infection) even with low levels of bacteraemia [50]. The low sensitivity of MGG compared to serological tests could be explained by the low bacteraemia which characterizes the chronic carriage of this infection and that *Anaplasma* cannot be easily detected in blood smears after clinical manifestation [49].

However, a limitation of the microscopic examinations (Giemsa stain blood smear) has been their inability to differentiate the *Anaplasma* spp. organism and other similar structures like Heinz bodies, Howell-Jolly bodies, or staining artefacts. An additional complaint is the fact is that this method need special experiences, particularly in animals with a very low level of bacteraemia [45]. Giemsa stained blood smears can be used as a suitable method to detect *Anaplasma* spp. in clinically suspected animals for the acute diseases [22]. This makes microscopic analysis unreliable for the detection of persistent *Anaplasma* spp. infections in animals [46]. To circumvent this problem, an alternative diagnostic technique, such as serological diagnostic tests [45] and nucleic acid-based assays [46] can be used for the detection of tick-borne parasites.

The observed prevalence was lower when estimated by cELISA tests than with MGG method. Similar differences have been found in other studies and may reflect the absence of detectable levels of bacteraemia in some animals [25]. The use of serological tests for antibodies detection against these pathogens is the most suitable tool for epidemiological studies to assess the importance of these infections in the goat populations [7]. The "Gold standard" method for the diagnosis of *Anaplasma* spp. relies on the combination of the microscopic examination and the cELISA [27].

The difference observed in the prevalence rates of *Anaplasma* species among different countries and areas of the same country may be due to several factors including: environmental and climatic conditions (such as weather, soil, and types of vegetation), genetic (the susceptibility of each host species and breeds), ecological (such as composition and interactions of the tick populations, the presence of other animal species including wildlife reservoirs), and husbandry practices [13, 15, 58]. In our study, the high prevalence of *Anaplasma* spp. infections in goats is attributable to environmental and climatic factors that make the northern regions a very suitable one for tick vector life.

CONCLUSIONS

In this study, we reported a high prevalence of *Anaplasma* spp. infections in goats using two different methods

such as the Giemsa blood smear and serologic screening in North-eastern Algeria. Although we did contribute towards broadening the epidemiological knowledge about *Anaplasma* spp. infection even though it is impaired by the absence of a correct diagnosis, often based solely on clinical manifestations rather than on a laboratory investigation. Our results do not show the infection status of the region due to the small amount of material collected. Also, vector biology, host preference and zoonotic potential of different *Anaplasma* species need to be further studied. The results of our work did allow us to glimpse a series of studies in perspective aimed at further improving the knowledge of caprine anaplasmosis in Algeria.

The methods used in this investigation made it possible to detect with certainty this infection which threaten the reproduction/production capabilities of goats. The choice between these two techniques will depend on their performance, as well as the availability of the equipment, laboratory conditions and the number of samples to be tested. The evaluation of such techniques are becoming more and more important to be able to use sensitive and specific tests dedicated to epidemiological surveys, which conditions the measures to be taken for good control of such infections unnoticed in breeding. However, further molecular screening of different *Anaplasma* species in blood and ticks collected from goats with molecular characterization are needed to estimate the real prevalence and clarify the genetic variability of *Anaplasma* spp. in goats from North-eastern Algeria.

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CANINE DIROFILARIASIS: A CASE REPORT AND REVIEW OF THE LITERATURE

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ABSTRACT

Dirofilariasis is a disease of clinical importance in dogs. It is to this end that a case of a seven-year-old mongrel with dirofilariasis was presented to examine the unique features and presentation in the canine patient in question. The dog had clinical signs consistent with the disease. Further diagnostic tests were performed to establish the presence and severity of the disease and make an appropriate treatment plan. The Knott's test revealed *Dirofilaria* species in the circulating blood and radiography showed right ventricular hypertrophy with pulmonary arterial enlargement and increased bronchial opacification. The treatment instituted was ivermectin therapy by subcutaneous injection every two weeks for six months, cardiac glycoside and antibiotics for 14 days. The clinical signs resolved after completion of the treatment. A general overview of heartworm infection in dogs was also done to update current knowledge of the disease. Though the risk of significant propagation of *Dirofilaria immitis* is considered low, with the climate change and international pet travel regulations, this emerging zoonosis remains a threat.

Key words: case report; *Dirofilaria immitis*; dog; Nigeria; zoonosis

INTRODUCTION

Etiology and Epidemiology

Canine dirofilariasis, also called heartworm disease, is a non-contagious, parasitic disease caused by a filarial/small thread-like worm, *Dirofilaria immitis* of the family *Onchocercidae* [20]. The parasite is one of the most pathogenic nematodes distributed worldwide, primarily affecting dogs and cats [34]. Mosquitoes (*Aedes, Culex, Anopheles* species) are the vectors which transmit this parasite from host to host [18]. Alongside *D. immitis* is another filarial worm, *D. repens*, and both are considered to have great clinical importance. They are relatively widespread in many parts of the world and have significant zoonotic potential [35].

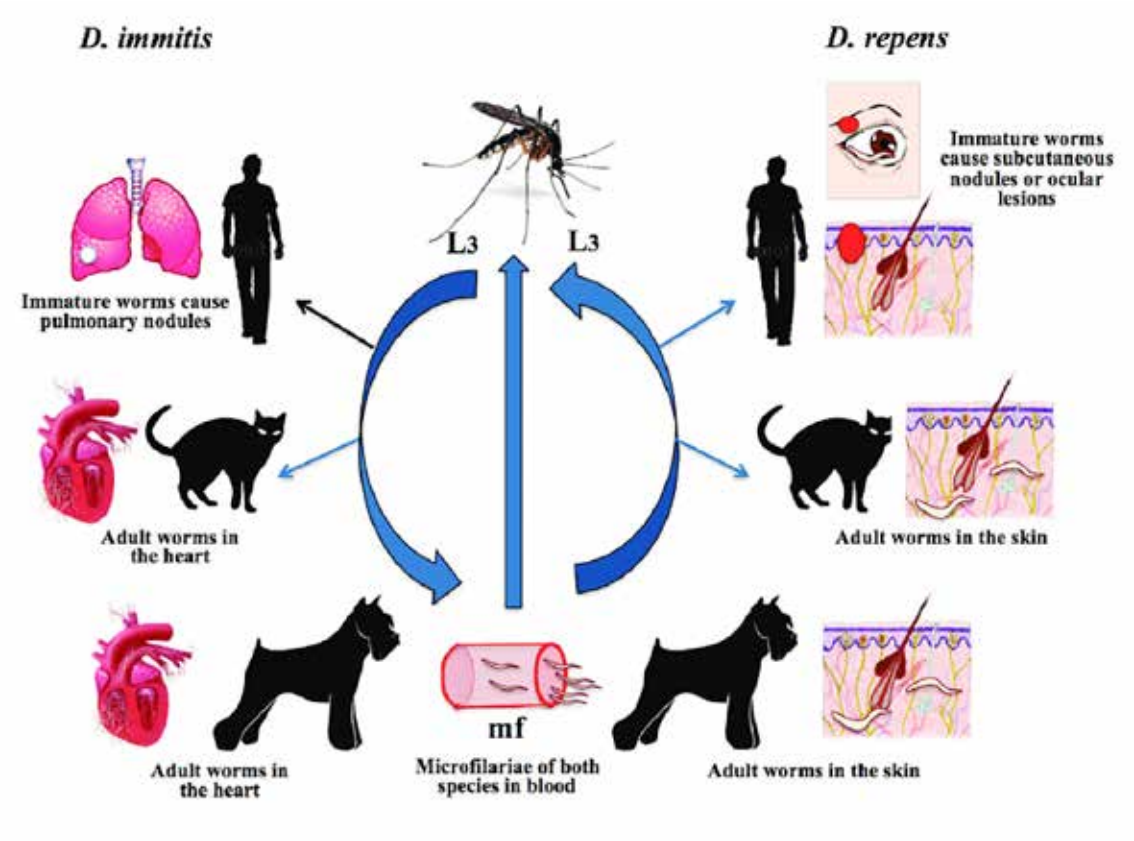

Fig. 1. Biological life cycle for *Dirofilaria immitis* and *Dirofilaria repens* [35]

Dirofilaria immitis establishes itself in the cardiovascular system of the primary host, while *D. repens* invades the subcutaneous tissues of the primary host (Fig. 1) [35].

Pathophysiology

Dogs are considered the definitive host for *D. immitis*, though, more than 30 animal species (e. g. coyotes, foxes, wolves, domestic and wild felids, ferrets) and humans (accidental hosts) may be infected [2, 17]. Adult worms mostly dwell in the right ventricle and pulmonary artery, but occasionally can be found in the epidural space, brain, anterior chamber of the eye, lungs or the arterial system [5].

Microfilariae, larvae in the first larval stage (L1), are produced by the female adult worm and released into the bloodstream of the primary host [31]. Further development of the microfilariae must take place in the vector after taking a blood meal from the host [35]. The development from L1 to L3 can occur in different mosquito species, requiring an average temperature of about 14—18 °C over a period of 30—60 days [8, 16]. The infective larval stage L3, is transmitted through inoculation into the skin of another primary host by the vector and undergoes development from L3 to L4 in the host's subcutaneous tissue in about 10—12 days [35]. Development from L4 to L5 takes place in the muscles of the host 50 to 70 days after injection [16]. Juvenile worms (L5) penetrate the systemic veins and are transported to the pulmonary arteries where they continue to develop into adult worms. In severe cases, worms may also enter the right heart chamber and caudal *vena cava*, but in most cases, they are retained in the pulmonary artery and its branches [14].

The maturation of the female worms in the pulmonary arteries of the primary host cause inflammatory reactions in the pulmonary microvasculature and larger arteries of the hosts [16]. The pulmonary artery expands in diameter, the endothelium and tunica media thicken, and the blood vessels may become obstructed by formation of thrombi causing pulmonary hypertension [35]. The presenting lesions of right ventricular enlargement, pulmonary vasculature dilation and parenchymal lung infiltrates are consistent with heartworm disease [3] (Fig. 2). As the disease progresses, the clinical signs seen will depend on the duration of the infection, the worm burden in the host and the host-parasite interactions [1]. This eventually culminates into a multisystemic disorder affecting the heart, lungs, liver and kidneys [30].

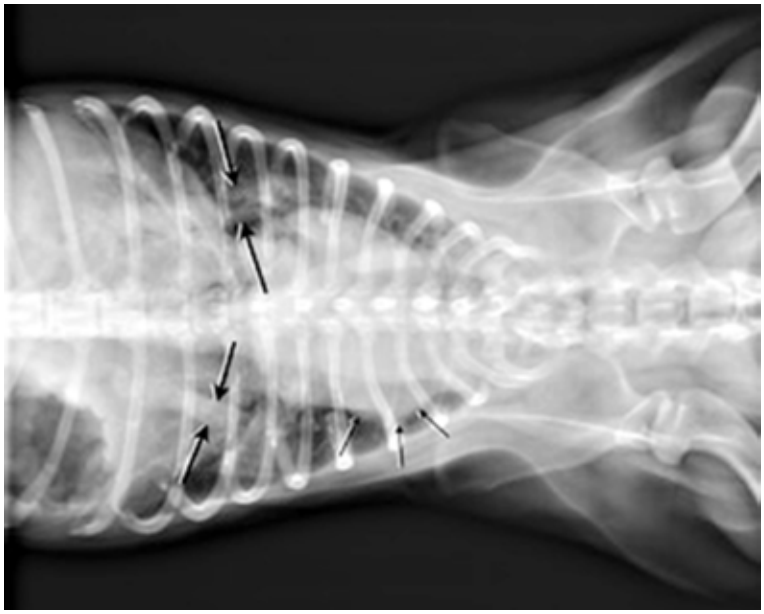

Fig. 2. Ventro-dorsal thoracic radiograph showing blunted, enlarged and tortuous pulmonary arteries (large black arrows) and an enlarged pulmonary trunk (small black arrows) [3]

Prevalence and risk factors

The prevalence depends on many factors, such as the methods performed and preselection of the samples; therefore, different prevalence reports exist within and between countries. The prevalence of *D. immitis* is reported to be 1.0 % in South Australia [10], 5.5 % in Brazil [25], 19.0 % in Spain [19], 20.9 % in South Korea [29] and 30.8 % in South Africa [26]. In Nigeria, reports of the prevalence of dirofilariasis vary from 4.8 % as reported in Northeast Nigeria [11], 3.36 % in Eastern Nigeria [34] and 2.15 % in the Middle belt of Nigeria [22]. This has been proposed to be due to an increase in dog ownership, effects of stray dogs, international pet travel etc.

Factors determining the prevalence of *D. immitis* in an area include: climate, mosquito species/subspecies and the density of the primary host population [16]. Risk factors for infection of the primary host include: animal species (dogs are the natural primary host), sex (male dogs are more vulnerable), habitat (dogs who live outdoors are more exposed to infection), the size of the primary host (large dogs are more likely to become infected than small dogs), and age due to the longer period of exposure to mosquito bites [12].

Clinical signs

The clinical signs of heartworm disease are due to the damage caused to the organs in which they are established e.g. the pulmonary arteries, the right heart chamber, eyes, kidneys, central nervous system and subcutaneous tissue [17]. The signs can be mild, moderate or severe. The mild signs include: cough, chest pain, hemoptysis, low-grade fever, chills and malaise. The moderate signs are: dyspnoea, coughing, weight loss, syncope, and other signs of right-sided heart failure [17]. Caval syndrome can be seen in severely affected dogs, where worms invade the right ventricle and caudal *vena cava*. This interferes with the closure of the tricuspid valve impeding normal blood flow, leading to cardiovascular collapse. Dogs may also present with intravascular haemolysis, hemoglobinuria and acute general malaise [15].

In some cases, there could be occult infections in which the microfilariae in the circulating blood of infected dogs are not seen. This may be due to: single sex worm infection (male or female), the presence of immature females during the prepatent period of development, geriatric infection or ectopic infection [6].

Diagnosis

Direct diagnosis of heartworm infection can be done by microscopic identification of microfilariae in the blood, while indirect infection can be diagnosed using several blood tests to determine the presence of antigens and/or microfilariae in the host. The presence of worms in the heart or subcutaneous tissues during surgery or post-

mortem is definitive [27]. Laboratory examinations using parasitological (Buffy coat, wet mount, modified Knott's test) and serological (ELISA) techniques may aid the diagnosis. Molecular testing, such as the polymerase chain reaction (PCR) and tissue histochemistry, can help differentiate microfilaria species [16]. Other diagnostic procedures such as general blood tests, radiography, echocardiography and electrocardiography should be performed to determine the degree of severity and serve as a guide in the treatment regimen.

Prophylaxis, treatment and control

Wolbachia pipientis, an intracellular bacterium, lives in symbiosis with *D. immitis*. Studies have shown that the inflammatory reaction occurring during heartworm infection is partly due to *W. pipientis* [32]. Therefore, the use of an anthelminthic agent in combination with an antibacterial agent is strongly recommended [24, 33]. Melarsomine dihydrochloride, an organic arsenical, is the most used anthelminthic agent. It is administered at a dosage of 2.5 mg.kg^{-1}, deep intramuscular injection in the lumbar muscles, twice within a 24-hour interval. Ivermectin, a macrocyclic lactone, at 200 µg.kg^{-1} can be given as a single dose subcutaneously, four to six weeks after treatment with melarsomine dihydrochloride [8]. Doxycycline, an antibacterial agent, will reduce the burden of *W. pipientis* in all stages of *D. immitis* infections.

Prophylactic treatment of all dogs living in areas exposed to infections is recommended to reduce the number of microfilariae in the bloodstream, and thereby curtail transmission. The treatment consists of a monthly dose of orally administered milbemycin or topically applied ivermectin.

CASE PRESENTATION

On the 17th of September, 2018, a seven-year-old, female mongrel was presented to the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta, Nigeria, with complaints of frequent episodes of coughing for about two years. The coughing became persistent and severe about two weeks before presentation. The pet had been placed on multivitamins and a teaspoon of cod-liver oil given four times daily in food orally before presentation. Different antibiotics such as oxytetracycline, penicillin-streptomycin and tylosin had been prescribed for the pet at different times by different veterinarians.

On clinical examination, the pet weighed 17 kg, rectal temperature was 40.2 °C, and the heart rate was 81 beats per min. The ocular mucous membranes were pinkish and there was no enlargement of the superficial lymph nodes. Heart auscultation revealed arrhythmia, although the femoral pulse was strong and regular. Capillary refill time was one second and the jugular vein appeared normal without visible pulsations. The dog was panting and coughing to some extent in the examination room and auscultation of the lungs revealed severe crackles on both sides of the chest.

A tentative diagnosis of bronchopneumonia and dirofilariasis was made. The differential diagnoses included: congestive heart failure, endocarditis and pericarditis. The blood was obtained for haematology and parasitological analysis. The pet was also scheduled for chest x-ray (lateral and ventro-dorsal views).

The haematology was within normal limits (Packed Cell Volume, PCV—50 %; total white blood cell count, WBC—$10.8 \times 10^{9}.l^{-1}$), no parasite was found in the blood film using the wet mount and thin smear techniques. On day two of the presentation, Knott's test was carried out on the blood sample and *Dirofilaria* species was found microscopically (Fig. 3).

The radiography results showed right ventricular hypertrophy with pulmonary arterial enlargement, and increased bronchial opacification (Fig. 4). Based on the medical history, clinical signs and the presence of the worms, a confirmatory diagnosis of canine dirofilariasis was made. The dog was classified as moderately affected.

Management and outcome

Treatment included 300 µg.kg^{-1} ivermectin (Ivomec®, Merial) administered subcutaneously every two weeks. In addition, 2.5 mg (1/2 tab.) enalapril (Lotrial®, ATOZ Pharmaceuticals, India) orally once daily and 0.125 mg (1/2 tab.) digoxin orally once daily was administered for 14 days. After treatment daily, the dog was admitted to the veterinary clinic for observation for six hours. The dog was released from the clinic in the evenings, for observation by the owners. Exercise was limited the first few weeks after each treatment. No side effects were observed following the treatments.

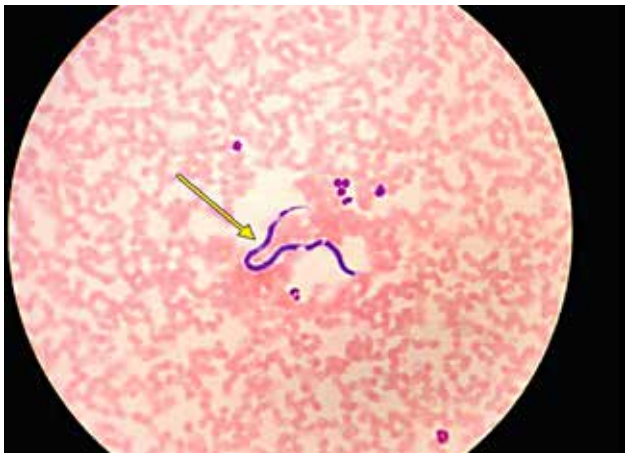

Fig. 3. *Dirofilaria immitis* (Arrow). Magnification ×100 of the microscope with a phone (Infinix) camera from top. The anterior end of *Dirofilaria immitis* tapers anteriorly as seen with the Knott's method which is the standard for diagnosis of canine dirofilariasis [7]

Fig. 4. The lateral thoracic radiographic view of the animal showing right ventricular hypertrophy with pulmonary arterial enlargement and increased bronchial opacification consistent with canine dirofilariasis

By day 14, the dog's clinical signs had improved and there was no microfilaria detection in the blood after the first shot of ivermectin. The dog continued to have a mild cough at this stage, and was treated with doxycycline (Ronaxan vet®, Merial) 10 mg.kg^{-1} once daily for 14 days which was initiated to take care of the potential concurrent infection with *W. pipientis*. Ivermectin therapy at 300 µg.kg^{-1} was continued every two weeks for six months.

Three months later, a serological antigen test was carried out (immunoassay, Laboklin Laboratories, Germany) and the test was negative. Six months later, the dog was presented to the clinic in very good physical condition and had stopped coughing. The breathing pattern and respiratory rate were normal.

DISCUSSION

This case of canine dirofilariasis in a seven-year-old patient was diagnosed based on the history, clinical signs observed, the laboratory investigations (PCV, CBC, Knott's test) and radiographic findings. This agrees with V a t n e [35] who reported canine dirofilariasis in a three-year-old dog. With a prepatent period of about six months, where female *D. immitis* worms grow between 13.5 to 30.5 cm long and can start producing microfilariae, it is unlikely to see clinical signs in dogs younger than one-year-old [8].

The long duration of coughing episodes in the bitch coupled with the previous administration of tylosin without remission aided in the diagnosis of this case. An adult worm can survive for up to seven years in the primary host, while microfilariae have a lifespan of up to two years, hence the long duration [27]. Clinical signs seen are consistent with other cases of moderate canine dirofilariasis reported [4, 13, 23]. Eosinophilia is most often seen in dirofilariasis which is caused by the body's cellular response to the circulating microfilaria in the blood vessels.

The modified Knott's method [28] was used for the diagnosis of the blood parasite in this case, a method that is more sensitive than a thin smear in diagnosing microfilariae and it helps in concentrating microfilariae for easy identification. The parasitological technique is a type of concentration test used in screening parasites in blood of low parasitaemia and this technique is used to distinguish between *Dirofilaria* and *Dipetalonema* species. A stationary rather than a migratory pattern of movement is indicative of *Dirofilaria* species when viewed under the microscope. Another diagnostic technique used to aid in diagnosing this case was radiography. The lateral view of the thoracic region of the pet showed enlargement of the right chamber of the heart and pulmonary artery, as previously reported by B a r r et al. [3].

Ivermectin, a macrocyclic lactone, was administered as an endectocide, subcutaneously once every two weeks. It works by the activation of glutamate-gated chloride channels, preventing the secretion of immunomodulatory proteins by the parasite, resulting in increased vulnerability of the worm to attack by the host's immune system [36]. According to N e l s o n et al. [21], the use of ivermectin can be used to treat the adult stages of the worm. Enalapril

and digoxin were dispensed as a supportive treatment in this case to reduce and alleviate the cardiopulmonary signs in the pet. Enalapril, an angiotensin converting enzyme inhibitor, is indicated for the treatment of congestive heart failure and hypertension. This drug, whose use in dogs in well tolerated, acts by preventing the formation of angiotensin-11 (a potent vasoconstrictor) by competing with angiotensin-1 for the enzyme angiotensin-converting enzyme. [9]. Digoxin, a cardio-tonic glycoside, also indicated for use in the treatment of congestive heart failure in dogs was administered to the dog. The drug acts by increasing myocardial contractility with increased cardiac output.

CONCLUSIONS

A case of canine dirofilariasis based on the history, clinical findings, laboratory investigations and radiography were presented. Veterinarians, physicians, pathologists and parasitologists should have an increased awareness of this entity. Various epidemiological and diagnostic means for the identification and molecular characterization of the species, and natural hosts can help to establish low prevalence rates of this emerging zoonosis and devise control measures.

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INDIVIDUAL WATER SOURCES AND THEIR POTENTIAL EFFECT ON HUMAN AND ANIMAL HEALTH IN ENVIRONMENTALLY BURDENED REGION

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ABSTRACT

The quality of water in a well that serves as an individual drinking water source, located in the Slovakian region previously burdened with mining activities, was investigated in relation to the quality of surface water in the same region. Selected microbiological and physicochemical parameters were determined in samples of ground water (well) and surface water (brook and river). Plate counts of coliform bacteria, *E. coli*, enterococci and bacteria cultivated at 22 °C and 37 °C were determined. Microbiological quality of samples collected from the well was generally satisfactory. Examination of the samples of surface water (river) showed a significant organic pollution indicated by fluorescence spectra. In all water samples the values of: pH, electrical conductivity, dissolved oxygen, ammonium ions, nitrites, nitrates, chlorides and chemical oxygen demand (COD_{Mn}) were below the limits set by the state legislation. In the surface water, high levels of arsenic were found. The sum of calcium and magnesium in the well water was close to or below the recommended minimum level. In this well water, the level of antimony exceeded 10-fold the maximum limit for drinking water and was of the largest concern as this well water has been used for drinking, cooking, and watering of animals and vegetables for a long period of time. This poses a risk of accumulation of this metalloid in the food chain.

Key words: antimony; arsenic; drinking water; ground water; health risk, surface water

INTRODUCTION

According to the data published in 2018, approximately 89.25 % of the Slovak population was connected to public water supply [30]. However, the number of inhabitants who disconnect from this public water supply network, primarily for economic reasons, continues to increase. They tend to use water from their own wells as a supplementary source, or prefer bottled water for drinking. In villages not connected to public water supply, people use their own wells, often with water of uncontrolled or unknown quality. Water from

these individual sources frequently does not meet the quality specified for drinking water by Regulation of the Government of the SR No. 496/2010 Coll., amending and supplementing the SR governmental regulation No. 354/2006 Coll., which determines the requirements on water intended for human consumption and control of water intended for human consumption, hereinafter referred to as the Regulation No. 496/2010 Coll. [38], especially in terms of microbiological indicators which indicate general or faecal pollution. Also, the levels of physico-chemical parameters including heavy metals can exceed the limits set by legislation.

Free-of-charge analysis performed occasionally throughout the Slovak Republic (SR), for example on the occasion of the World Water Day (22 March), is only informative as it focuses mostly on nitrates or nitrites, and does not provide a complex picture about water quality or potential sources of its contamination.

Section 2 of the Regulation No. 496/2010 Coll. [38], defines individual drinking water sources for the purpose of this regulation as sources with daily capacity of less than 10 m^3 of drinking water, or supplying water to less than 50 persons. Natural persons or legal entities owning such water sources, pursuant to Section 1, paragraph b), are not classified as persons obliged to fulfil the obligations listed in the Section 3, including the obligation to maintain the prescribed quality of water, ensure disinfection and also inspection thereof.

In our study we focused on the monitoring of the quality of water from a well located near the confluence of a brook and the Ida River in the region of Zlatá Idka village, situated in the Spis-Gemer Ore Mountain (SGOM) area. There are additional wells in this area used as individual drinking water wells. In the region, mining activities were carried out in the past that exploited deposits mainly of heavy metals, such as arsenic (As) and antimony (Sb) [8, 22, 24, 44].

The Ida River, which flows through the location, is one of the tributaries of the Bukovec water-supply reservoir, which serves as one of the drinking water sources for Košice, the regional capital and the second largest city in the SR (population almost 240 000). For this reason, long-term monitoring and regular inspection of the quality of water in this region is performed [5, 22].

Levels exceeding the Sb upper limit value (ULV) (Sb = 0.005 $mg.l^{-1}$) were observed, for example, in the Zlatá Idka region, in the Bukovec water-supply reservoir and at other sites [24].

Antimony and arsenic (with atomic numbers 51 for Sb and 33 for As) are considered metalloids (chemical elements with properties intermediate between those of typical metals and non-metals), elements in the group 15 of the periodic table, and both occur naturally in the environment at trace levels.

Arsenic occurs in nature in approximately 200 rocks, in concentrations below 1—15 $mg.kg^{-1}$, up to concentrations of 900 $mg.kg^{-1}$ in case of sedimentary rocks. As levels in surface water in Europe range between 0.1 and 1.7 $\mu g.l^{-1}$, in ground water they are in the range of 0.1—2 $\mu g.l^{-1}$, while in volcanic rocks the concentrations rise to 3,400 $\mu g.l^{-1}$, and in the mining areas they reach up to 48,000 $\mu g.l^{-1}$ in ground water sources [39].

As a recognized human carcinogen causing skin cancer, As^{III} is more toxic than As^{V}. It inhibits biochemical oxidation [1]. Under reduction conditions, it prevails in As^{III} form; As^{V} is an inorganic form predominant under the oxidation conditions in water and soil. Elementary arsenic or As^{3-} occurs in water very rarely. Organic forms of arsenic are less prevalent in nature than the inorganic ones. Arsenic compounds most frequently occurring in fresh water systems are monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) [27].

Arsenic is the only contaminant that following exposure through drinking water causes cancer of human skin, lung and bladder, probably also liver; it is also responsible for a range of adverse effects, including hyperkeratosis and peripheral vascular disease [1, 26].

Whereas arsenic, a popular poison in ancient history, has absolutely been known as a potentially dangerous element, less is known about antimony; the mechanism of its impact on the environment is not entirely known [53]. Sb can be released into the environment by natural discharges such as volcanic eruptions, forest fires, or wind-blown dust. The antimony concentration in air ranges from a nanogram per cubic meter ($ng.m^{-3}$) to about 170 $ng.m^{-3}$ and less than 5 ppb dissolves in lakes or rivers. Antimony is essentially present as pentavalent in fresh and sea water, and as trivalent under anaerobic conditions, as well as a result of anthropogenic activities [47].

Antimony released to the environment will eventually end up in either of the two compartments, soil or sediment, depending on: the release, form of antimony, meteorological conditions, etc. As a constituent of soil, antimony will also be transported into streams and waterways due to

weathering and run-off from soils; much of this antimony is associated with particulate matter. The absorption of the water soluble compound antimony potassium tartrate following oral intake is approximately 5 % in humans and in experimental animals. The less soluble inorganic antimony compounds are only absorbed to a little extent. Both the absorption of antimony trioxide and the elimination of antimony from blood is a slow process. Antimony is distributed to most tissues with the highest concentrations found in the bone marrow and thyroid gland, followed by spleen, lung, liver, ovaries, heart, kidney, femur and skin. Antimony can also be detected in the testes and brain. The minimal lethal dose for oral intoxication by antimony potassium tartrate has been reported to be 300 mg for a child and 1200 mg for an adult. According to the EU-RAR, no reliable human information regarding the acute toxicity after single oral intake of antimony trioxide is available, and no human data after single dermal exposure to antimony trioxide could be located [6].

Natural organic matter or NOM is a broad term for the complex mixture of thousands of organic compounds found in all surface, ground and soil waters. These compounds are derived from decaying plant and animal matter. NOM is highly variable and relative concentrations of individual compounds can vary significantly from source to source. For several practical and hygienic reasons, the presence of NOM is undesirable in drinking water [32].

Organic matter in water is composed of two major fractions: dissolved and non-dissolved; defined on the basis of the isolation technique using filters (0.1—0.7 μm). Dissolved organic matter (DOM) is the fraction of organic substances that passes the filter. DOM is a heterogeneous mixture of carbon-containing molecules present in all aquatic ecosystems. Globally, DOM plays a key role in carbon and nutrient cycling, and as a substrate for microbial growth, it is one of the main risk factors promoting microbiological growth in distribution networks [7]. DOM optical properties (absorbance and fluorescence) are widely used for studying changes in DOM composition and concentration [33]. Although spectroscopic techniques do not necessarily measure directly the small bioavailable molecules consumed by heterotrophic bacteria, numerous studies have shown that optical measurements are nevertheless sensitive proxies of the wider DOM pool and track subtle changes in water quality. DOM fluorescence is a sensitive tracer of sewage contamination, correlating with *E. coli* abundances [3] and nutrients [2] across systems. Excitation-emission matrix (EEM) fluorescence spectroscopy has been widely used to characterize DOM in water and soil [9].

In this study the quality of well water used as an individual drinking water source, located in the Slovakian region previously burdened with mining activities, was investigated together with the quality of surface water in the close vicinity of the well.

MATERIALS AND METHODS

Sampling sites

Samples of the ground and surface waters were collected from the region of the village Zlatá Idka, situated in the Spis-Gemer Ore Mountain (SGOM) area in Eastern Slovakia where mining activities were carried out in the past. It is a small village with close to 400 inhabitants. It is connected to the public water supply but not to the public sewer system (installation planned in the years 2021—2027).

Samples of ground water were obtained from a well about 6 m deep, serving as a drinking water source for two households (W). Water from this well is used for drinking, cooking, personal hygiene, watering livestock and crops.

Samples of surface water were collected at the following sites (Fig. 1):

- from an Idčiansky brook—site A—running at a distance of about 20 meters from the investigated well,
- from the confluence of the brook and the Ida river—site B,
- from the Ida river below Zlatá Idka village—site C.

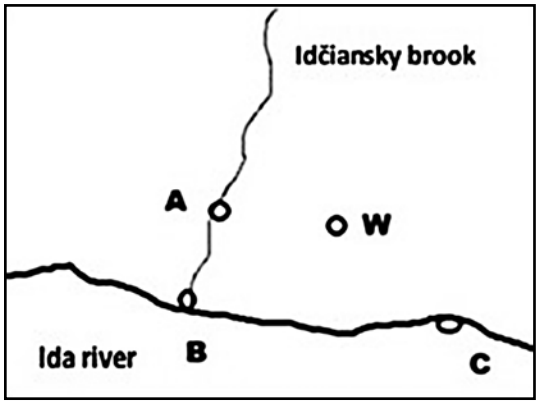

Fig. 1. Ground and surface water sampling sites at Zlatá Idka village A—Idčiansky brook; B—confluence of the Ida river and the brook; C—Ida river downstream below the village; W—individual water well

Investigations of the quality of ground and surface water were carried out from October 11, 2016, to March 7 (except February 2017), 2017 in monthly intervals (altogether 5 samplings).

In the samples we determined the selected physico-chemical and microbiological parameters indicating potential and existing contamination of water with faeces. The samples collected at the last sampling in March were examined for arsenic and antimony levels and for obtaining the fluorescence excitation-emission spectra. The results were compared with the limits stipulated by relevant legislation.

Microbiological examination

Microbiological examinations were carried out according to the Regulation No. 496/2010 Coll. [38]. They included the determination of colony forming units (CFU) of bacteria cultivated at 22 °C (BC22) and 37 °C (BC37), according to the STN EN ISO 6222 [41], coliform bacteria (CB), and *E. coli* according to the STN EN ISO 9308-1 [46], and enterococci (EC) according to the STN EN ISO 7899-2 [42].

According to the WHO [52], *E. coli* or thermotolerant coliform bacteria must not be detected in any 100-ml sample. Also, total coliform bacteria must not be detectable in any 100-ml sample [46].

Physico-chemical examination

Sensorial evaluation of water (colour, odour, turbidity) was carried out on site and verified in a laboratory after transfer of the samples.

The chemical examination included the determination of electrical conductivity, pH, and saturation with dissolved oxygen. Preliminary qualitative analysis involved the detection of ammonium ions (NH_4^+), nitrites (NO_2^-), nitrates (NO_3^-) and chlorides (Cl^-) using colour reactions. If the presence of the tested substance was confirmed, the respective parameter was determined quantitatively. Also, chemical oxygen demand (COD_{Mn}), the sum of the calcium and magnesium ($Ca^{2+} + Mg^{2+}$) and the levels of arsenic and antimony were determined.

The pH was determined according to the STN ISO 10523 [45] (pH-meter HACH, WATERPROF pH Tester 30, HACH). The conductivity was measured using a conductometer WTW (InoLab Cond 720, Mettler Toledo), according to the manufacturer's instructions.

The quantitative determination of nitrates was carried out directly in samples using an ion-selective nitrate electrode WTW (InoLab pH/ION 735P, Mettler Toledo), according to the manufacturer's instructions. The chlorides were determined by titration according to the STN ISO 9297 [43] by titration. The determinations of $Ca^{2+} + Mg^{2+}$ were performed also by titration, according to Horáková [19]. The dissolved oxygen was determined electrochemically using a HACH oxygen probe LDO HQ Series Portable Meters (HACH), according to the manufacturer's instructions and the chemical oxygen demand by oxidation with $KMnO_4$, according to the STN EN ISO 8467 [40].

Arsenic and antimony were determined in samples collected in March by a specialized water laboratory of the East Slovak Water Company, workplace Kokšov-Bakša [44].

Fluorescence Excitation Emission Matrix (EEM) spectroscopy

Fluorescence spectroscopy is a highly sensitive technique that can be used to characterize organic matter in water sources through three-dimensional spectra called excitation-emission matrices (EEM). This method was used to examine samples collected in March. To obtain the EEM spectra the samples were examined under a luminescence spectrophotometer Perkin Elmer LS 55 (Medical and Clinical Biochemistry Institute and LABMED, Inc., USA) at the Faculty of Medicine, Pavol Jozef Šafárik University in Košice (Slovakia), using the following settings: excitation wavelength 250—450 nm with gradual 10 nm increment increase, range λ = 250—600 nm (excitation/emission slit: 5/10 nm, quartz cuvette of width 1 cm, emission monochromator scanning rate: 20 $nm.s^{-1}$). The EEM were obtained using a FIW Inlab software [11].

Statistical analysis

The results of the physico-chemical examinations are presented as the means ± SD of five samplings at the following sites: Idčiansky brook (A), confluence of river and brook (B), end of the village (C) and individual water well (W). The statistical analysis was carried out by the one-way analysis of variance (ANOVA) using post hoc Tukey multiple comparison test (the Prism 3 software). The differences at the level of $P < 0.05$ were considered significant.

RESULTS

Physico-chemical parameters

Ground water

The results of the physical examinations complied with the legislative requirements. The presence of NH_4^+ ions was detected qualitatively only in one sample of well water collected on October 11, 2016. The quantitative determinations showed that its level at this sampling reached 1.9 mg.l^{-1} (limit 0.5 mg.l^{-1}). All other samples provided negative results. The presence of NO_2^- ions was not detected at any sampling. The upper limits for NO_3^-, COD-Mn and Cl^- were not exceeded. The contents of calcium and magnesium were close to or below the recommended range of 0.55—1.55 samplings (Table 1). The level of arsenic in the well was below the statutory limit. However, it was alarming that the value of the antimony concentration was 10-fold higher than the permitted value for drinking water (Table 2).

Surface water

The chemical examinations of the surface water indicated good quality of water at all sampling sites with the exception of arsenic and antimony. The levels of arsenic in surface water samples ranged between 17 and 28.5 μg.l^{-1}, which classified them as the A2 category of water quali-

Table 1. Results of physico-chemical examination of surface and ground (well) water

Parameters	A	B	C	Limits[2] Surface water (OH-MH)	W	Limits[3] Ground water
pH	6.8 ± 1.2	6.6 ± 1.3	6.5 ± 1.3	6.5—8.5 (A1)	7.2 ± 0.8	6.5—9.5
O_2 saturation [%]	88.8 ± 6.8	93.6 ± 5.8	93.9 ± 4.9	< 80 (A1)	74.3 ± 11.6	>50
Conductivity [mS.m^{-1}]	19.9 ± 5.6	14.8 ± 3.6	14.7 ± 3.0	100 (A1)	25.2 ± 2.2	125
Cl^- [mg.l^{-1}]	7.8 ± 5.2	8.2 ± 3.7	8.2 ± 4.5	100 A1	7.6 ± 3.4	250
NO_3^- [mg.l^{-1}]	11.8 ± 9.6	13.5 ± 10.4	14.7 ± 11.3	7—11[1] (A3)	11.8 ± 3.8	50
COD_{Mn} [mg.l^{-1}]	0.7 ± 0.6	1.9 ± 1.9	1.4 ± 0.8	2.0 (A1)	0.4 ± 0.1	3
Ca^{2+} + Mg^{2+} [mmol.l^{-1}]	0.8 ± 0.4	0.8 ± 0.3	0.8 ± 0.3	-	0.9 ± 0.4	1.1—5

Results are expressed as means ± standard deviation (n = 5); differences in the levels of parameters A, B and C were insignificant; Sampling sites: A—Idčiansky brook; B—river and brook confluence; C—Ida river below the village; W—individual source of drinking water (well); OH —recommended limit value of the indicator; MH—limit value of the indicator; [1]The concentration values for these characteristics need not to be complied with if justified by geographical or climatic conditions; [2]Limits stipulated by the Regulation No. 269/2010 Coll. [37] Category A1—water requiring simple physical treatment and disinfection, or rapid filtration and disinfection; Category A3—water requiring intensive physico-chemical treatment and disinfection such as coagulation, flocculation, filtration, activated carbon adsorption, chlorine or ozone disinfection, critical point chlorination and decantation; [3]Limits stipulated by the Regulation No. 496/2010 Coll. [38]

Table 2. Arsenic and antimony levels determined in surface and ground (well) water

Parameters	A	B	C	Regulation No. 269/2010 A2 category	Regulation No. 269/2010 A3 category	W	Regulation No. 496/2010
As [μg.l^{-1}]	28.5	21.8	17	20—50	50—100	0.005 mg.l^{-1}	0.01 mg.l^{-1}
Sb [mg.l^{-1}]	0.0827	0.0657	0.0517	0.01—0.025	> 0.025	0.05	0.005

Sampling sites: A—Idčiansky brook; B—river and brook confluence; C—Ida river below the village; W—individual source of drinking water (well); 269/2010—limits for A1 and A2 categories of surface water stipulated by the Regulation No. 269/2010 Coll. [37]; 496/2010—limits for ground water stipulated by the Regulation No. 496/2010 Coll. [38]; Category A2—water requiring physico-chemical treatment and disinfection such as coagulation, flocculation, filtration, chlorine disinfection, pre-chlorination and decantation; Category A3—water requiring intensive physico-chemical treatment and disinfection such as coagulation, flocculation, filtration, activated carbon adsorption, chlorine or ozone disinfection, critical point chlorination and decantation

ty, according to the Annex No. 2, section A: Surface Water Intended for the Supply of Drinking Water, of the Regulation of the Government of the SR No. 269/2010 Coll., hereinafter referred to as Regulation No. 269/2010 Coll. [37]. Due to antimony, determined in March in concentrations ranging between 0.0517 and 0.0827 mg.l^{-1}, the water quality was classified as the worst A3 category of the aforesaid standard. In both cases, the highest concentrations of the monitored metalloids were observed at the confluence of the river and the brook, the sampling site B (Table 2).

Microbiological parameters

Microbiological results obtained by examination of samples of water from the well and the surface water differed considerably.

Ground water

The microbiological quality of water in the well (W) was very good as no *E. coli* or enterococci were detected in the 10 ml samples. The total coliform bacteria were presented in this water only at the fourth sampling in January (3 CFU.10 ml^{-1}). According to the Regulation No. 496/2010 Coll. they should not be detected in any 10 ml of well water intended for individual supply (max. 50 people). Also, the levels of bacteria cultivated at 22 °C (0—52 CFU.1 ml^{-1}; limit 500 CFU.1 ml^{-1}) and 37 °C (5—20 CFU.1 ml^{-1}; limit 100 CFU.1 ml^{-1}) complied with the statutory limits for drinking water.

Surface water

Results obtained by the microbiological examinations of the surface water in the investigated area (A, B, C) are presented in Table 3.

Investigations of water from the Idčiansky brook (A), river and brook confluence (B), and the river below the village (C), revealed considerable variations between the sampling sites, individual parameters, and samples collected at the individual samplings. With regard to the CB, *E. coli* and EC which represented the indicators of faecal pollution of water, the highest contamination was detected at site B. Most of the results indicated that at this site the levels of these three parameters exceeded the limits or recommendations of A2, even of the A3 categories. Especially the counts of enterococci (faecal streptococci) at this site were extremely high at all samplings indicating not only potential risk, but the real presence of animal or human faeces. Overall, samples collected at all three sampling sites classified the water in the worst quality category of A3, as specified in the Annex No. 2 of the Regulation No. 269/2010 Coll., section A [37].

Table 3. Results of microbiological examination of surface water

Parameter	Limits for categories [37]			Samplings				
	A1	A2	A3	1	2	3	4	5
CB CFU/100 ml	50	5000	50 000	A: 17 000 B: 67 000 C: 28 000	A: 4 000 B: 12 000 C: 3 000	A: 8 000 B: 8 000 C: 22 000	A: 6 000 B: 0 C: 9 000	A: 2 000 B: 10 000 C: 5 000
E. coli CFU/100 ml	0	10*	100*	A: 1 000 B: 5 000 C: 2 000	A: 0 B: 0 C: 0	A: 0 B: 7 000 C: 5 000	A: 2 000 B: 0 C: 5 000	A: 0 B: 7 000 C: 2 000
EC CFU/100 ml	300	1000*	1000*	A: 12 000 B: 19 000 C: 3 000	A: 0 B: 48 000 C: 58 000	A: 2 000 B: 7 000 C: 11 000	A: 0 B: 13 000 C: 45 000	A: 2 000 B: 7 000 C: 2 000
CB22 CFU/1 ml	100	100	100	A: 0 B: 0 C: 0	A: 580 B: 650 C: 18 800	A: 0 B: 0 C: 0	A: 0 B: 0 C: 100	A: 0 B: 0 C: 0
CB36 CFU/1ml	20	200*	1000*	A: 340 B: NC C: 3 400	A: 70 B: 450 C: 6 600	A: 60 B: 80 C: 10 400	A: 140 B: 10 C: 4 800	A: 20 B: 80 C: 60

CB—coliform bacteria: EC—enterococci; CB22—bacteria cultivated at 22 °C; CB36—bacteria cultivated at 36 °C; NC—non-countable; Sampling sites: A—Idčiansky brook; B—confluence of Ida river and the brook; C—Ida river below the village; Limits and categories are specified in the Regulation No. 496/2010 Coll.; *—levels recommended by [37] for parameters for which no limits are stipulated

EEM spectroscopy

The unfavourable microbiological results indicated considerable organic pollution of the examined surface water that was also confirmed by the fluorescence spectra (Fig. 3).

Water from the well and from the brook (A) did not show any significant differences; in addition to humic substances, there were also detected bioorganic degradable substances (proteins).

The samples of water from the Ida River at both sampling sites, B and C, showed a high content of humic substances and pollution by bioorganic products, proteins and sewage residuals.

All four samples were qualitatively similar and differed only in the intensity of pollution or the concentration of organic substances (from the lowest to the highest):

- Humic substances and humic acids: the well and the brook provided almost identical results; the Ida River at the confluence and the Ida River behind the village showed the highest values;
- Proteins: brook; well, the Ida River behind the village; the Ida River at the confluence showed the highest values;
- Complexes of proteins and humic substances: well, brook values were almost identical; the Ida River behind the village; the Ida River at the confluence showed the highest values;
- Sewage: the Ida River at the confluence.

All samples showed significant similarities as they came from the same, significantly polluted location.

DISCUSSION

Our investigations of ground water in the region polluted by previous mining activities exploiting deposits mainly of heavy metals showed that with regard to selected chemical parameters (Table 1), excluding As and Sb, the water corresponded to drinking water quality with the exception of NH_4^+ ions at one sampling (October). The presence of these ions that indicate fresh pollution with animal or human wastes can be related to a single pollution incident, for example intensive precipitation and/or application of manure. The good quality of surface water, for example high O_2 saturation and low levels of chlorides and nitrates may be related to the colder season of the year with limited agri-

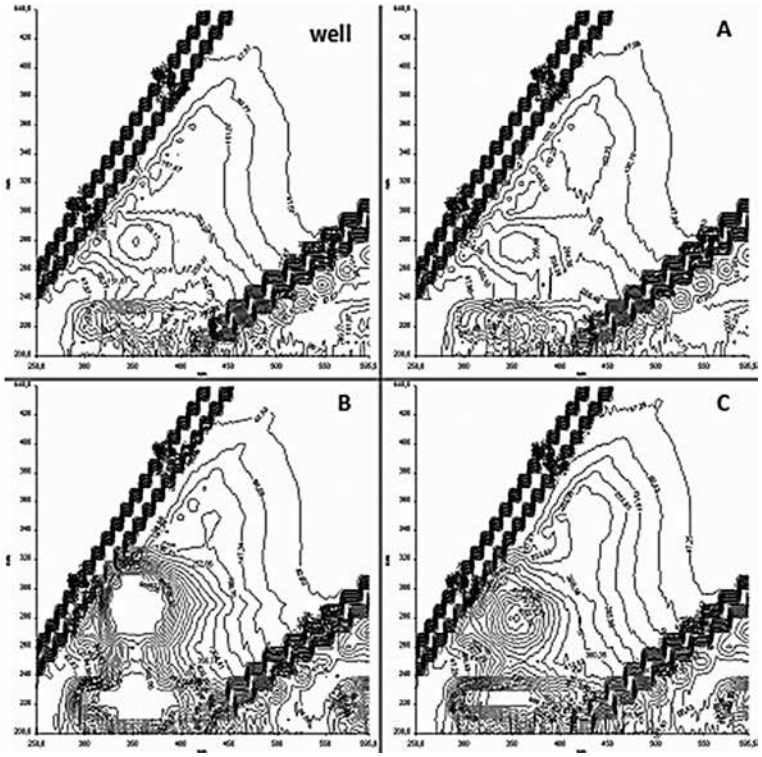

Fig. 3. Fluorescence spectra of the well and surface waters A, B, C
A—Idčiansky brook; B—river and brook confluence; C—Ida river below the village

cultural activities that could affect the investigated parameters. Although the content of Ca and Mg was very close to the lowest acceptable limit, even a little below it, no significant health effects of such levels have been confirmed.

Our microbiological examinations of the ground water provided very good results as no *E. coli* or enterococci were detected in 10 ml samples. The total coliform bacteria were detected only at the fourth sampling in January (3 CFU.10 ml^{-1}) indicating the possibility of some accidental contamination. Also, the levels of bacteria cultivated at 22 °C (0—52 CFU.1 ml^{-1}; limit 500 CFU.1 ml^{-1}) and 37 °C (5—20 CFU.1 ml^{-1}; limit 100 CFU.1 ml^{-1}) indicated that the general pollution complied with the statutory limits for drinking water. The well was covered and no immediate source of pollution was present, except for the Idčiansky brook at about 20 m distance from the well. Although the well was not very deep (6 m), evidently the soil filtration capacity was sufficient to cope with most of the potential contamination.

However, the limit for antimony in the drinking water was exceeded 10-fold in this well which poses a health risk to humans and other animals, especially at long-term consumption of such water as discussed below.

The determined chemical parameters indicated a good quality of surface water at all sampling sites with the exception of arsenic and antimony. The level of arsenic in surface water (17—28.5 $\mu g.l^{-1}$) classified it as the A2 category of water quality. However, antimony concentrations ranging between 0.0517 and 0.0827 $mg.l^{-1}$, which classified this water as the worst A3 category. The highest concentrations of the metalloids were detected at the confluence of the Ida River and the Idčiansky brook at the sampling site B (Table 2).

The samples collected at the site B showed also the highest contamination with CB, *E. coli* and EC that represent the indicators of faecal pollution. Most of the results at this site exceeded the limits or recommendations of A2; even of A3 categories. Especially the counts of enterococci (faecal streptococci) at this site were extremely high at all samplings indicating not only the potential risk but the real presence of animal or human faeces. Overall, samples collected at all three sampling sites classified the water in the worst quality category A3. The unfavourable microbiological results indicated considerable organic pollution of the examined surface water that was also confirmed by the fluorescence spectra (Fig. 3). The Idčiansky brook and the Ida river flow through the village of Zlatá Idka and the absence of public sewer system evidently contributes to the poor quality of water in this region.

The humic acids and proteins in the water confirmed by the fluorescence spectra may play a role also in the content and interactions of metalloids in the water.

The interaction with humic acids can be described as the adsorption of Sb^{III} by humic acids and it is governed by the Langmuir isotherm. The extent of the Sb^{V} bond to organic mass has not been clarified. However, it was proved that the antimonic acid forms complexes with functional groups containing oxygen, where the complexation takes place in humic substances. The interactions of Sb with a solid stage indicate that antimony is present in the solution [51].

Binding is significant at acidic pH for Sb^{III} and Sb^{V}, but in the case of Sb^{V}, it decreases above pH 6—8. The adsorption of antimony by iron oxides in acidic media takes the shape of forming Fe-O-Sb bonds. Natural organic matter (NOM) can bind metal ions and accelerate antimony redox reactions in aqueous systems [13, 14, 18].

There are 9 deposits of antimony ores in the Slovak territory (Little Carpathians, Low Tatras, Spiš-Gemer Ore Mountains), which served as important antimony sources in Europe, until the mining was discontinued in 1991.

The research project "Estimation of Health Risk from Arsenic and Antimony Presented in the Environment of Zlatá Idka Village", sponsored by the State Health Institute in Košice, carried out in the period 2000—2004 in the surrounding area of Zlatá Idka (SGOM) focused on health risks of the long-term impact of As and Sb accumulation, revealed that the contents of these metalloids observed in mining and surface waters exceeded the statutory limits. In the surface flow of the Ida River below the Zlatá Idka village, the content of As reached 32 to 34 $\mu g.l^{-1}$ and levels of Sb were 0.055 to 0.065 $mg.l^{-1}$, exceeding the limits permitted by the Regulation No. 269/2010 Coll. [37]. Human samples (urine, blood, hair) showed excessive values of As and Sb concentrations, especially in inhabitants from the area of Zlatá Idka village (SGOM) [35, 36]. The As and Sb concentrations determined in the blood (16.3 $\mu g.l^{-1}$ and 3.8 $\mu g.l^{-1}$), urine (15.8 $\mu g.l^{-1}$ and 18.8 $\mu g.l^{-1}$), nails (3.179 $\mu g.kg^{-1}$ and 1.140 $\mu g.kg^{-1}$) and hair (379 $\mu g.kg^{-1}$ and 357 $\mu g.kg^{-1}$) were higher than values of people from non-contaminated areas. Health risk calculations for the ingestion of soil, water, and vegetables indicate a very high carcinogenic

risk (> 1/1.000), as for the content in soil and water [34]. The studies by Rapant et al. [35, 36] revealed different causes of death in Zlatá Idka than in other villages of the region. People from this village suffered more frequently from different diseases, mainly diabetes, due to the negative impact of Sb on the glucose level in blood. Also there was observed an increased number of mental disorders and cancer, the latter attributed mostly to As.

The Council of the European Communities established the value of 5.0 $\mu g.l^{-1}$ [10] as the highest permitted level for antimony in water intended for human consumption (this Directive has been incorporated into the respective regulations of the EU member states). On the other hand, the U. S. Environmental Protection Agency (USEPA) suggested a maximum antimony concentration of 6 $\mu g.l^{-1}$ in drinking water [49].

For a long periods of time, a drinking water supplier operating in the region has been dealing with the increased concentration of As and especially Sb, applying modern technologies based on iron-based sorption materials, to achieve the required quality of water [24, 48]. Despite the availability of the existing drinking water source, the public water-supply network, many households have not connected thereto and use their own wells as a drinking water source, without having the quality of water from their source inspected.

In Slovakia, there are a number of locations of antimony mineralization with possible occurrence of gold ore, for example in the Spiš-Gemer Ore Mountain (Betliar, Čučma and Poproč). However, according to the results of the survey carried out in 1977—1983, they did not reach the deposit parameters. Similar economic insignificance was observed for antimony occurrence in the regions of Kremnica and Zlatá Baňa [4, 20].

The high concentrations of antimony and arsenic in the environment have been detected not only in Slovakia, but all over the world. The impact of these individual metalloids on the environment, as well as their presence in the food chain and impact on human and other animal health present a global problem without frontiers.

In Greece, the results of groundwater analyses from the region of Eastern Thessaly indicated elevated concentrations of As and Sb due to the presence of the arsenopyrite and stibnite in the Melivoia, Sotiritsa and Ano Polydendri areas. Both of them, with similar geochemical behaviour in the environment, are noted for formation of complexes. According to Kelepertsis et al. [25] about 5,000 people in the mentioned area drink water containing As and Sb above the values defined by the EC and US EPA guidelines.

Antimony, with the most common forms of Sb ore in nature, such as Sb_2S_3 (stibnite, antimonite), is the only form of Sb ore mineral in the world´s largest Sb ore deposit—Xikuangshan, China [16, 31]. In the same area, the heavy contamination (Sb, As, Cd, and Hg) in the soil profiles was mostly located in the uppermost soil layers enriched in organic matter (depth 40 cm) and exhibited downward migration in the soil profiles. Sb and As showed significant mobility in the profiles [21, 50, 55].

The results of Li et al. [28, 29] established the importance of considering the bioaccessible Sb and As data rather than the total content for health risk calculations, because it could provide more realistic estimations of the health risks posed by the Sb and As contamination.

Fu et al. [17] and Yang et al. [56] studied the impact of bioaccumulation of As, Sb and Hg in a large Sb mining/smelting area in Hunan, China, that had been seriously polluted by Sb. Water from the reservoirs and river near the Sb mine area was not suitable for use as drinking water because of high Sb concentration, seriously exceeding the Chinese guideline for drinking water. However, it has not been proved so far, whether the co-exposure to As and Sb has a synergistic effect on human health [54].

In the past decades, it was observed that the cancer risk was higher among people who were regularly exposed to high environmental arsenic concentrations. UNICEF estimates that in Vietnam there are approximately 10—15 million of the people (about 13.5 % of the population) using drinking water from tube wells, the arsenic concentrations of which ranged from 8—579 ppb (mean 301 ppb) [23].

According to Etim [12] there is a health risk due to increased occurrence of arsenic, antimony and selenium in shallow groundwater systems of Ibadan Metropolis (Southwestern Nigeria) caused by its significantly higher levels with 100 % of examined samples exceeding the WHO safe limits.

While the Environmental Protection Agency (EPA) allows 0.006 parts of antimony per million parts of drinking water, the guidelines for Drinking-water Quality [49], WHO established a tolerable daily intake (TDI) of 6 $\mu g.kg^{-1}$ body weight/day for antimony [52, 53], and the limit for Sb in drinking water, pursuant to the applicable laws in the SR, is 0.05 $mg.l^{-1}$.

With regard to constantly rising concentration of Sb in the environment, it is inevitable to pay urgent attention to the research of its bioaccumulation and the examination of Sb migration and integration in the food chain and, subsequently, biological effects thereof. So far, the information about antimony, with regard to the risk to human health from the accumulation thereof, is insufficient and the existing studies indicate that increased concentrations of antimony present a global problem, as it evidently causes diseases of the liver, skin, and the cardiovascular system [6, 12, 13, 15, 17, 26, 54].

The fluorescence spectra obtained in our study indicated high organic pollution (DOM) of the river, while those of the well and brook water showed almost identical level of humic substances and bioorganic degradable substances but lower than those found in the river water.

Zhang et al. [57] used UV-vis and fluorescence (EEM) spectroscopy to investigate the characteristics of DOM in river flowing into a key drinking water source in China and reported that they had a profound influence on the quality and safety of water. They detected spatiotemporal variations of spectral characteristics on DOM. Their concentration was much higher in the wet season and the data provided technical support for improving the quality and comprehensive treatment of drinking water sources.

In addition to the above discussed and historically known possibilities of geogenic burden, that affects the quality of drinking water sources, there are also sources primarily anthropogenic, for which it is difficult to estimate their impact on the quality of water, and they definitely require regular inspections. Every source of drinking water intended for long-term use regardless of the daily consumption or the source size must not represent any hazard to health. On the contrary, its composition should have a positive impact on health and represent a safe choice how to adhere to the drinking regime as an essential part of a healthy lifestyle of each individual.

In addition to the discussed area of the Zlatá Idka village, there are many other locations where wells are used as sources of drinking water, with quality of water not necessarily complying with the drinking water criteria.

CONCLUSIONS

In many areas burdened with industrial and agricultural production, the individual water sources, serving primarily as sources of drinking water as well as water for watering of animals and plants, can be significantly contaminated by secondary products or biological waste. According to the results of our study concerning the quality of ground water in the investigated area, the level of antimony was of the greatest concern. It was also very high in the surface water. As the drinking water used on a daily basis must not represent any hazard to health due to long-term consumption of xenobiotics or other life endangering contaminants, it is very important to ensure adequate protection and carry out regular inspection and disinfection of all individual sources of drinking water. It would be appropriate to consider potential modifications of the legislation with the aim to ensure verification of the quality of each individual source intended for the long-term use as a source of drinking water for humans and farm animals.

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