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A DIAGNOSTIC ALGORITHM FOR THORACOLUMBAR SPINAL CORD PATHOLOGIES IN DOGS: A SYSTEMATIC REVIEW

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Summary

The most common neurological spinal cord injury in dogs is between the third thoracic and third lumbar vertebrae (T3-L3). Using the PubMed database, a systematic review was designed to develop a diagnostic algorithm for the most frequent pathologies in this segment. The algorithm was created by following criteria: the way of evolution - acute and progressive, acute and non-progressive and chronic; the patient's age (young, adult, geriatric); presence or absence of pain; symmetry or asymmetry of lesions; best imagistic protocol (radiography, mielography, C.T., M.R.I.). Every data was materialized in an algorithmic tabel. The acute and progressive evolution is present in intervertebral disc extrusion (IVDE type 1) and meningoencephalitis of unknown origin (MUE). Acute non-progressive nucleus pulposum extrusion (ANNPE), compressive hydrated nucleus pulposus extrusion (HNPE) and ischemic mielopathy (IM) evolution is acute and non-progressive. Intervertebral disc protrusion (IVDP) type 2, neoplasia, degenerative myelopathy (DM), spondylosis deformans (SD) and discospondylitis are chronic diseases. Canine patients were grouped by age in: young (IVDE type 1), adult (over 7 years – ANNPE, HNPE, SD, DM) and geriatrics (IVDP type 2, neoplasia). Pain is always present in IVDE type 1 and SEE. The asimmetry of lesions is constant in ANNPE, HNPE and IM but also can be found in neoplasia. Native radiography is a useful diagnostic method for SD, neoplasia, vertebral fractures or luxations and discospondylitis, but it is not recommended for MUE, SEE, ischemic and degenerative myelopathy diagnosis. Advanced imaging like myelography and computed tomography is a more reliable method of diagnostic for IVDE (type 1), ANNPE, HNPE, IVDP (type 2), ischemic myelopathy, neoplasia, DM, SEE, discospondylitis and spondylosis deformans. However, MRI is the golden standard for every spinal cord pathology. This algorithm is a useful guideline for everyday veterinary medical practice concerning the thoracolumbar spinal cord diseases in dogs.

Keywords: dog, spinal cord, T3-L3, algorithm, diagnostic

The most common neurological spinal cord injury in dogs is located between the third thoracic and third lumbar vertebrae (T3-L3). Using the PubMed database, a systematic review was designed to achieve a summary of all spinal cord pathologies including the most suitable and objective investigation methods to establish the diagnosis (22, 35, 48).

The aim of this study was to develop a diagnosis algorithm for the most frequent pathologies in the T3-L3 segment which can be used by every veterinary practitioner.

Materials and methods

PubMed database was used to find the most updated articles about spinal cord pathologies in dogs.

The algorithm was created by the following criteria: the type of evolution as an acute and progressive, acute and non-progressive or chronic disease; the patient's age (young – age <6 years, adult - aged between 7-10 years or geriatric – age >10 years); presence or absence of paraspinal hyperesthesia on the physical examination; the symmetry or asymmetry of clinical signs and lesions; best imagistic protocol to approach (radiography, myelography, C.T. or M.R.I.). Every data extracted from selected published papers was materialized in an algorithmic table designed to follow a plan of diagnosis step by step for every spinal pathology.

Results and discussions

Fifty-five articles describing the latest methods of diagnosing spinal cord pathologies in dogs were selected from PubMed database. The most common spinal cord diseases were taken under review: intervertebral disc extrusion (Type 1, IVDE), meningoencephalitis of unknown origin (MUE), injury of spinal cord and vertebral column, acute non-progressive nucleus pulposum extrusion (ANNPE), compressive hydrated nucleus pulposus extrusion (HNPE) and ischemic myelopathy (IM), intervertebral disc protrusion (type 2, IVDP), spinal neoplasia, degenerative myelopathy (DM), spinal epidural empyema (SEE), spondylosis deformans (SD) and discospondylitis.

The acute and progressive evolution is characteristic for IVDE type 1 and MUE. ANNPE, HNPE and IM have an acute and non-progressive evolution. IVDP type 2, neoplasia, DM, SEE, SD and discospondylitis are chronic diseases. Canine patients were grouped by age in: young (under 6 years of age), adult (aged between 7 and 10) and geriatrics (over 10 years of age). The most common spinal cord injury in young dogs was represented by IVDE type 1 and MUE, while in adult dogs ANNPE, HNPE, SD, ischemic or degenerative myelopathy were more oftenly found. Geriatric dogs were more prone to develop IVDP type 2 and neoplasia (6).

Paraspinal hyperestesia on physical examination is always prersent in IVDE type 1 and SEE. The asimmetry of lesions is constant in ANNPE, HNPE and IM but can also be found in neoplasia. Native radiography is a useful diagnostic method for SD, neoplasia, vertebral fractures or luxation and discospondylitis, but it is not recommended for MUE, SEE, ischemic and degenerative myelopathy diagnosis. Advanced imaging like myelography and computed tomography is a more reliable method of diagnostic for IVDE (type 1), ANNPE, HNPE, IVDP (type 2), ischemic myelopathy, neoplasia, DM, SEE, discosponylitis and spondylosis

deformans. However, MRI is the golden standard for every spinal cord pathology (6, 9, 27, 34, 42, 44, 52, 53, 57).

In the category of acute progressive spinal cord pathologies, IVDE is a painful disease which typically occurs in dogs under 6 years of age. The clinical signs are usually symmetric, but sometimes if the extrusion is lateralized, the neurologic deficits may be present only on the affected side. Radiography may reveal intervertebral disk mineralization in the affected region, but it is not enough to establish the diagnosis. Advanced imaging (CT or MRI) is the most appropriate method for detecting the extrusion of the disc. CSF analysis showing a dissociation between albumin and globulin ratio might be a complementary exam, but it is not necessarily relevant (9, 27, 44).

Meningomyelitis evolve with painful neurological signs and may have a multifactorial etiology, being classified in infectious or non-infectious meningomyelitis. The age is not relevant in this disease but in MM with unknown origin breed predisposition may be an important factor in developing the disease. Lesions are symmetrical and MRI is the most proper diagnostic tool to distinguish this type of inflammation from other spinal cord diseases. CT may also be performed but the sensitivity and details are lower than in MRI. CSF puncture and the cytological exam may reveal an increase of total nucleated cell count (TNCC) a high proteins level and number of inflammatory cells, being an important criterion for the final diagnosis (14, 15, 25, 46, 50, 55).

Traumatic injuries of the spinal cord or vertebral column are produced by different kind of accidents. This type of pathology is not correlated with age, the neurologic deficits may be symmetric or lateralized, pain is usually present but it may disappear depending on the severity of lesions. Common findings on radiography are fractures or luxation of vertebral bodies but it should be taken into consideration the fact the microlesions may release small bone fragments in the medullar canal, which are not observable. In this light, CT is the best approach of imagistic protocol because it generates high quality resolution of bone imaging (24, 31, 36, 41).

Spinal epidural empyema represents another acute and progressive pathology. The disease is not correlated with the patient’s age and evolves with dramatic paraspinal pain to the affected region. Radiologic exam may be performed but if the pathology does not affect the bone structure, the lesion is not observable. However SEE may also evolve as a result of diskospondylitis, case in which radiography may play an important role for diagnosis. CT is a good alternative when vertebra are affected but the golden standard for imagistic protocol is MRI in which extradural lesions may be noticed. CSF puncture and analysis are very suitable investigation methods for SEE diagnosis, revealing an elevation of protein concentration and neutrophilic pleocytosis. CSF microbiologic exam and antibiogram are necessary for establishing the etiology and proper treatment protocol of SEE (18, 33, 43).

The acute non-progressive nucleus pulposum extrusion, compressive hydrated nucleus pulposus extrusion and ischemic myelopathy which is most often

represented by fibrocartilaginous embolic myelopathy (FEM) have an acute, non-progressive evolution (21).

ANNPE has a characteristic clinical set presentation with a peracute onset of severe neurological deficits. All the clinical signs are lateralized and pain is present in most of the cases. Age represents an important predisposing factor, dogs under 7 years of age being more prone to develop ANNPE. Advanced imaging like CT scan reveals the spinal cord compression, but it is not the most suitable method to differentiate ANNPE from other compressive pathologies. MRI offers the final diagnosis in this type of lesion (8, 17, 26, 39).

On top of differential diagnosis, the most important disease is fibrocartilaginous embolic myelopathy. FEM is considered as a non-painful pathology and lesions are lateralized. Age is not related to the evolution of disease but in some cases, younger dogs are predisposed. MRI should be the best approach to differentiate FEM from ANNPE and to exclude other extramedullary spinal lesions. CSF can be taken into consideration for evaluation. Abnormal findings like increased protein level and elevated cell counts may help, but the final diagnosis can only be established by the histopathological assessment of the spinal cord but this method is not entirely necessary because of the patient's good prognosis and fast recovery (1, 21).

Compressive Hydrated Nucleus Pulposus Extrusion evolves with an acute onset of clinical signs which are typically symmetric. Patients under 7 years of age are predisposed to develop this disease due to some genetic factors. Compared with the ANNPE and FEM, in HNPE pain may be present. MRI is the most reliable diagnostic method in HNPE. Several studies reported a pathognomonic MRI finding on the extruded material which has the typical bilobed or seagull appearance. CT can be used but only contrast-enhanced which is increasing the sensitivity up to 90% and specificity to 100% to differentiate HNPE from Hansen type 1 IVD extrusion (3, 4, 17, 19, 20, 38).

In the chronic category, the most frequent pathologies are: intervertebral disc protrusion (IVDP) type 2, neoplasia, degenerative myelopathy (DM), spondylosis deformans (SD) and discospondylitis.

Intervertebral disc protrusion represents one of the most common spinal pathologies with a chronic evolution. The disease can be painful and it mostly affects dogs over 8 years of age. Lesions are always symmetrical and minor neurological deficits may be expressed. Radiography may reveal intervertebral disk mineralization in the affected region, but this single exam is not enough to establish the diagnosis. Myelography may be performed but the method's sensitivity is only at 35% of cases, compared to the contrast enhanced CT which showed a 80% sensitivity, fact which makes it the most valuable diagnostic tool in intervertebral disc protrusion. CSF analysis has no diagnostic value in this spinal pathology (2, 6, 9, 27, 30, 53).

Diskospondylitis represents a chronic condition produced by a microbial factor. It evolves with an acute set of signs including paraspinal hyperestesia and symmetric neurological deficits. The age is not a predisposing factor. The

radiologic exam may reveal different stages of osteolysis of the affected vertebrae. The proliferative bone tissue it is not symmetrical, which is an important aspect on the differential diagnostic with spondylosis deformans (SD). In the SD, lesions are produced by a proliferative process of bone tissue only on the ventral side of the vertebra. CT scan can offer information about the exact region of medular compresion, does not conclude the diagnosis. Anyway, for diskospondylitis, the final stage of the diagnosis protocol is biopsic puncture from the affected region. It is imporant to complete this stage to differentiate diskospondylitis from bone neoplasia on the cytological exam (5, 10, 12, 23, 51, 54).

Spinal neoplasia is classified by the type of tissue affected. Bone cancer is frequent in geriatric patients and the protocol of diagnosis is the same as in diskospondylitis. When neoplasia is present in the spinal cord, MRI is the golden standard for a certain diagnosis. A study including 53 dogs with spinal tumors, in 50% of cases neoplasia was located in the extradural region, 35% intradural but extramedullary and only 15% intramedullary. To establish the type of tumor, biopsic puncture and citologic exam must be performed (7, 29, 32, 37, 45, 49).

Canine degenerative myelopathy it is an age-related disease which affects dogs with over 8 years of age, breed predisposition being an important factor. Lesions are constantly symmetric and the pain is absent. Radiologic exam and advanced imaging are not suitable methods of diagnostic, the certain diagnosis of DM is based only on histopatological examination, which shows degeneration and nerve fibre, loss of ascending sensory and descending motor pathway (13, 16, 28, 40, 41, 47, 56).

Conclusions

The first important steps in establishing the diagnosis of thoracolumbar spinal cord injuries in dogs include the patient's history, pathology evolution, presence or absence of pain and neurologic deficits' symmetry or asymmetry. Paraclinical diagnostic methods like advanced imaging and CSF analysis are necessary to obtain the most accurate diagnosis in spinal cord pathologies, and the algorythm presented in this paper may offer a fast, objective guidance for every veterinary practitioner.

ALGORITHM OF DIAGNOSIS FOR THORACOLUMBAR SPINAL CORD PATHOLOGIES IN DOGS								
	DISEASE	**AGE**	**PAIN**	**SYMMETRY OF LESIONS**	**RX/ MYELO**	**CT**	**MRI**	**OTHER EXAMS**
Acute progressive	IVDH type 1	<6	+	-/+	-/+	+	+	-/+ CSF
	Meningomyeloencephalitis of unknown origin (MUO) SRMA, EME, GME, NE	-	+	-	-	+/-	+	CSF/ Histology
	Meningomyeloencephalitis of infectious origin	-	+/-	-	-	+/-	+	CSF
	Injury of the spinal cord	-	+	-	+/-	+	+/-	-
	Spinal epidural empyema	-	+	-	-/+	-/+	+	CSF / Histoloy
Acute non-progressive	Acute non-progressive nucleus pulposum extrusion	>+7	+/-	+	-	-	+	-
	Compressive hydrated nucleus pulposus extrusion	>+7	-/+	-/+	-/+	-/+	+	-
	Ischemic myelopathy	-	-	+	-	+/-	+	-/+ Histology
Chronic	IVDP type 2	+8-12	-/+	-	-/+	+	+	-/+ CSF
	Neoplasia	>10	+/-	+/-	+	+	+	+/- CSF
	Degenerative myelopathy	+/- 8	-	-	-	-	-/+	Histology/Biopsy
	Diskospondylitis	-	+/-	-	+/-	+	+	Biopsy /Histology

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COMPARATIVE RESEARCH ON THE EFFICACY OF OCLACITINIB AND OF METHYLPREDNISOLONE IN DOGS WITH ALLERGIC DERMATITIS

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Summary

In recent years, the incidence of allergic dermatitis in dogs has increased greatly, and the causes that favor these type of dermatitis are multiple. We mention that in these animals the therapeutic course is laborious, the animals in many cases needing treatment throughout the rest of their lives. In our study we aimed to evaluate the effectiveness of two products that have the role of reducing the severity of the clinical signs, respectively the reduction of pruritus in dogs with allergic dermatitis. The research was performed on 20 dogs, which were divided into two groups: group 1 received methylprednisolone 1-2 mg / kg (Medrol®; Zoetis Inc.), and group 2 received oclacitinib 0.4-0.6 mg / kg (Apoquel®; Zoetis Inc.) for 28 days. Subsequently, pruritus was monitored using an analogue scale from 0 to 3 (0 - absent pruritus, 1 - mild, occasional pruritus, 2 - moderate, constant or intermittent pruritus that does not disturb sleep, 3 - severe pruritus, annoying which disturbs the sleep) both by the owner and the veterinarian, at the beginning of the experiment, 4-6 hours after the treatment, then the dogs were reassessed on day 7, 14 and 28, after the treatment. Also, the evolution of the status of skin lesions was monitored during the 28 days. Significant improvement was observed in both groups of animals after treatment, both in terms of pruritus intensity and a reduction in clinical signs. It should be noted that in dogs treated with oclacitinib, showed an improvement in the otitis media associated with *Malassezia*. Also, at the half-dose of treatment, better results were observed for the oclacitinib-treated group. Thus, oclacitinib can be considered a very good alternative, instead of glucocorticoids, in reducing the clinical signs associated with allergic dermatitis.

Key words: canine, allergic dermatitis, oclacitinib, methylprednisolon

Canine atopic dermatitis is a disease that in recent years has continued to affect a high number of dogs. If in 2001 the prevalence of this disease was 15% (6), in 2017 the prevalence of this disease reached 20-30% (8).

Clinical signs of this disease include dermatitis with locations in the head, limbs and abdomen and moderate or intense pruritus. In an attempt to keep this disease under control, the use of glucocorticoids, cyclosporines and antihistamines are used.Oral corticosteroids are widely used to reduce the pruritus associated with various diseases. Oclacitinib has recently been placed on the market and a substance that may represent a safe alternative in the fight against pruritus in allergic dermatitis (8).

In this study, we tried to evaluate the efficacy and safety of oclacitinib (Apoquel®; Zoetis Inc.) in comparison to methylprednisolone (Medrol®; Zoetis Inc.) in the control of pruritus and reduction of associated clinical signs.

To evaluate the efficacy of the drug treatment, the CADESI test was used in a simplified version (13).

The CADESI test was adapted in veterinary medicine as an objective method for assessing the severity of atopic dermatitis. It is based on the test used in human medicine-SCORAD (Scoring for severity of atopic dermatitis) which evaluates the severity of dermatitis on a scale of 0 to 3 and includes six clinical parameters (erythema, erosion, lichenification, papules / edema, crusts and skin drying) evaluated in 11 body regions (5, 7). Currently, several versions of the CADESI test are used in veterinary dermatology. The test proposed by Germain et al. is based on the evaluation of three parameters (erythema, excoriation, lichenification) on a scale from 0 to 3 in 4 areas of the body (muzzle area, limbs, axils and groin area) (4). Olivry et al. suggested the introduction of the CADESI-03 variant evaluating 4 parameters on a scale from 0 to 5 in 62 body regions (11).

Twenty dogs diagnosed with allergic dermatitis participated in the study.The twenty dogs were divided into two groups: group 1 received methylprednisolone, and group 2 received oclacitinib. The study period was 28 days for both groups.

Methylprednisolone is a synthetic steroid with very good anti-inflammatory effects, it is a derivative of prednisolone (6-methyl prednisolone). It has a stronger anti-inflammatory power than prednisolone and a lower tendency to induce water and sodium retention. The advantage it has over older corticosteroids is its ability to achieve anti-inflammatory effects equal to corticosteroids but at much lower doses, at the same time separating the anti-inflammatory effects from other mineralocorticoid activities. The chemical formula of methylprednisolone is C22H30O5 (Fig. 1), the molecular mass is 374.47 g / mol, the percentage of protein binding is 78%, the metabolism of the substance is carried out in the liver, kidneys and tissues, elimination by the kidney, the half-life of 18 -26 hours (15).

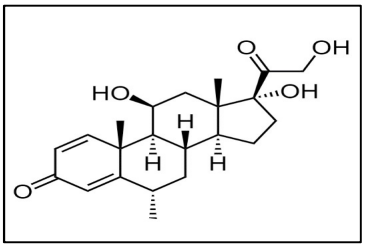

Fig. 1. The chemical formula of methylprednisolone (15)

Oclacitinib is a synthetic cyclohexylamino pyrolopyrimidine with Janus kinase inhibitory effect, being selective for Jak1. It inhibits signal transduction when the kinase is activated and thus helps regulate the expression of inflammatory

cytokines. The chemical formula is C15H23N5O2S (Fig. 2), molecular weight 337.44 g/mol, product bioavailability is 89%, protein binding rate is 66.3-69.7%, metabolized in liver with half-life of 3.1-5.2 hours (16).

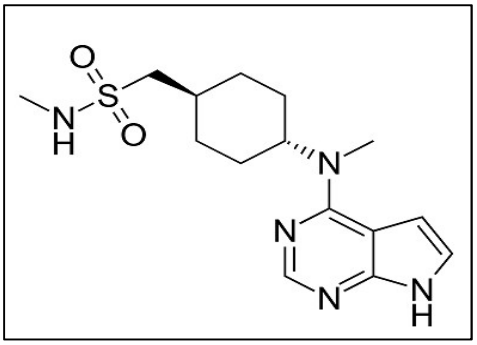

Fig. 2. The chemical formula of oclacitinib (16)

Oclacitinib is well absorbed in the form of PO, it takes less than an hour to reach the maximum plasma concentration. In most dogs, itching begins to subside four hours after ingestion of the compound, and at 24 hours it completely disappears (16).

Materials and methods

The dogs were diagnosed with the Polycheck® test and dogs with high pollen reactivity were selected.

Sampling for allergy tests was performed by puncture of the cephalic vein and blood collection in vacutainers without anticoagulant with or without separating gel. The samples were centrifuged 30 minutes after collection for 3-5 minutes at 3000 rpm, then the expressed serum was transferred into Eppendorf tubes and kept in the refrigerator at 2-8°C until the Polychek® test was performed.

Dogs with other common diseases, or suffering from neoplasms, were not included in this study. The age of the dogs that participate in the study was between 3-6 years. 75% (15/75; 95% CI 53-88) of dogs participating in the study were sterilized.

The clinical signs found in the participating dogs were: mild or intense pruritus, erythema, excoriation, scratching lesions, otitis, alopecia. Clinical signs were evaluated using the CADESI test at the beginning of the study on day 1 and then on day 7, 14 and 28 after starting the drug treatment. The maximum value that can be obtained by an individual is 240 points (13).

For the CADESI test, 15 body regions were inspected: the ears, the eye and the eyelid area, the muzzle area, the neck area, the interdigital area of the anterior and posterior limbs, the axillary area, the thoracoabdominal area and the flank area.

Four clinical signs were evaluated on a scale from 0 to 4 (erythema, primary efflorescence, secondary efflorescence and lichenification):
Erythema was evaluated according to the following classification:

- 0 - absent erythema
- 1 - erythematous macula without visible edema
- 2 - visible erythema without edema and high temperature in the neighboring tissues
- 3 - erythema with visible inflammatory edema
- 4 - erythema with inflammatory edema and efflorescence

Primary and secondary efflorescence were classified into:

- 0 - absent
- 1 - a single type of efflorescence, except for erythema: papules as primary efflorescence or scales as secondary efflorescence
- 2 - two types of efflorescence except the erythema present on the skin: papules and pustules as primary efflorescences or scales and scabs as secondary efflorescences
- 3 - three types of efflorescence except the erythema present on the skin: papules, pustules and nodules as primary efflorescences or scales, crusts and erosions as secondary efflorescences
- 4 - four types of efflorescence except the erythema present on the skin: papules, pustules, nodules and boils as primary efflorescences or scales, crusts, erosions and ulcerations as secondary efflorescences

The presence of lichenification was classified as follows:

- 0 - absent
- 1 - accentuated marks of the skin
- 2 - accentuated markings of the skin and hyperpigmentation
- 3 - accentuated skin markings, hyperpigmentation and hyperplasia
- 4 – accentuated skin markings, hyperpigmentation and hyperplasia, with visible loss of skin flexibility, coexisting ruptures and cracks.

The dogs were divided into two equal groups: group 1 who received methylprednisolone and group 2 who received oclacitinib.

At the beginning of the study, all dogs had moderate or severe pruritus, which was monitored by the dog owners during the study using an analogue scale from 0 to 3 (0 – absent pruritus; 1 - mild, occasionally, pruritus; 2 - constant or intermittent moderate pruritus that does not disturb sleep; 3 - severe pruritus, annoying disturbing sleep). The dogs were treated against ectoparasites before the study began and during it. There were no drug treatments other than those proposed, changes in diets or protocols for desensitization of animals. None of the dogs underwent allergen-specific immunotherapy prior to this study.

The dogs were randomly divided into two treatment groups with methylprednisolone or oclacitinib. The study period was 28 days for both groups.

Oclacitinib treatment was performed with oral tablets, the dosage being 0.4-0.6 mg / kg twice daily at 12 hours for 14 days. After 14 days treatment continued at the same dosage once daily until day 28.

Treatment with methylprednisolone was performed with oral tablets, the dosage being 1-2 mg/kg divided into two halves every 10 hours. From day 7, the tablet administration interval was reduced to once every two days, divided into two halves every 10 hours. Both treatments could be administered by the owners along with the food.

After the beginning of the study, the dogs were scheduled for control after 7, 14 and 28 days, monitoring the skin condition and the evolution of the clinical signs.

On day 0 of the study, the owners monitored the pruritus evolution before administering the treatment and after 2-6 hours after the start of the treatment, they continued to monitor the pruritus on days 7, 14 and 28. At the end of the study, all the visual analog scales performed by the owner and the veterinarian were taken in consideration.

The statistical data were processed with Excel 2016 and STATISTICA 8.0 Program.

Results and discussions

Dogs from the following breeds participated in this study: Bichon Maltez (n = 2), Bichon Frize (n = 1), Akita Inu (n = 2), Poodle (n = 3), Mops (n = 1), French Bulldog (n = 2), German Shepherd (n = 2), Bernese Mountain Dog (n = 1), Labrador Retriver (n = 1), Golden Retriver (n = 1), Pekingese (n = 1), English Bulldog (n = 1), West Highland White Terrier (n = 2). The number of dogs was made up of 10 males and 10 females. Among males 30% (3/10; 95% CI 10-60) were sterilized, and among females 60% (6/10; 95% CI 31-83). The age of the dogs included in the study ranged from 3-6 years with a mean age of 4.3 ± 1.5 years.

The reactivity of dogs evaluated using the Polychek® test revealed a 90% sensitization (18/20; 95% CI 69-97) in mites. The reactivity of each dog is shown in Table 1.

Of the dogs subjected to the allergic test 85% (17/20; 95% CI 61-96) reacted to Dermatophagoides farinae; 70% (14/20; 95% CI 48-85) reacted to the mixture of herbs; 45% (9/20; 95% CI 25-66) reacted with D. pteronyssinus and the mixture of birch / alder / hazelnut in dogs; 35% (7/20; 95% CI 18-56) reacted to rye pollen; 30% (6/20; 95% CI 14-51) reacted to Acarus siro; 15% (3/20; 95% CI 5-36) reacted with Lepidoglyphus destructor; 10% (2/20; 95% 2-30) reacted with Tyrophagus putrescentiae, Malassezia sp. and nettle and 5% (1/20; 95% 1-23) of dogs reacted to ambrosia, patlagin and mackerel.

Following the administration of oclacitinib the pruritus began to decrease after four hours in 30% of the dogs, and after six hours the pruritus was reduced to 40% in the dogs reaching the value of 1 on the analogue scale. On day 7 all dogs presented with pruritus condition that was maintained until day 28. At dose change, on day 14, at half the dose, pruritus continued to be inhibited.

Table 1

Allergen reactivity to Polycheck® test

	Breed	Age (Years)	Allergen reactivity
1	Maltese	5	RP, U
2	Maltese	4	Mix 3, As
3	Bichon Frise	4.5	Df, RP, U, As
4	Akita Inu	5	Df, Mix 1, Mix 3
5	Akita Inu	3	Df, Mix 1
6	Caniche	6	Df, Dp, M, Lp, As, Mix 3
7	Caniche	4	Df, Dp, M, Mix 1, Mix 3
8	Caniche	5	Df, Rg, Mix 1, Mix 2, Pt, So
9	Mops	3.5	Df, As, Tp, Mix 3
10	French Bulldog	4	Df, Dp, Lp, As.Tp, Mix 1, Mix 3
11	French Bulldog	5	Df, Mix 1
12	German Shepherd	6	Df, Dp, Mix 3
13	German Shepherd	5	RP, Mix 3
14	Bernese Mountain Dog	3	Df, Dp, Mix 3
15	Labrador Retriver	4	Df,Dp, Mix 1, RP, Mix 3.
16	Golden Retriver	5	Df, Dp, Lp, RP, As, Mix 1
17	Pekingese	5	Df, Mix 3
18	English Bulldog	4.5	Df, Dp, RP, Mix 3
19	West Highland White Terrier	4	Df, Dp, Mix 1, Mix 3
20	West Highland White Terrier	5	Df, RP, Mix 3

Df: *D. farinae*; Dp: *D. pteronyssinus*; As: *Acarus siro*, Tp: *Tyrophagus putrescentiae*; Ld: *Lepidoglyphus destructor*; Rg: ambrosia; Mix 1: birch, alder, hazelnut mix; RP: rye pollen; Mix 2: plantain, willow, poplar; Mix 3: Grass-mix; M: *Malassezia* sp.,, U: stinging netle, Sp: lamb's quarters, Pt: plantain, So: sorel

In dogs who received methylprednisolone the pruritus started to subside after four hours in 20% of the dogs, and after six hours the pruritus was reduced to 30% of the dogs, on day 7, 60% of the dogs did not show pruritus and 40% showed mild pruritus. After reducing the dosage on day 14, 50% of the dogs showed mild pruritus. On day 28, 60% of the dogs showed mild pruritus.

The most significant difference between the two products in the fight against pruritus was on day 14 when drug doses were reduced, the dogs receiving oclacitinib did not show pruritus even after the dose reduction in the dogs who received methylprednisolone showed mild pruritus. The evolution of pruritus in the two groups is shown in tables 2 and 3.

Table 2

Pruritus evolution in group 1 who received methylprednisolone

Group1	Initial Pruritus	After 4 hours	After 6 hours	Day 7	Day 14	Day 28
French Bulldog	3	3	3	0	1	1
German Shepherd	2	1	1	0	0	0
German Shepherd	3	3	3	1	1	1
Bernese Mountain Dog	2	1	1	0	0	0
Labrador Retriver	2	2	2	0	0	0
Golden Retriver	3	3	3	1	1	1
Pekingese	3	3	3	1	1	1
English Bulldog	3	3	3	1	1	1
West Highland White Terrier	2	2	1	0	0	0
West Highland White Terrier	2	2	2	0	0	1

Table 3

Evolution of pruritus in group 2 who received oclacitinib

Group1	Initial Pruritus	After 4 hours	After 6 hours	Day 7	Day 14	Day 28
Maltese	2	2	1	0	0	0
Maltese	3	3	3	0	0	0
Bichon Frise	2	1	1	0	0	0
Akita Inu	3	3	3	0	0	0
Akita Inu	3	3	3	0	0	0
Caniche	2	2	1	0	0	0
Caniche	2	2	2	0	0	0
Caniche	2	1	2	0	0	0
Mops	2	2	2	0	0	0
French Bulldog	2	1	1	0	0	0

In similar studies, the researchers observed a 55% reduction in pruritus intensity on day 6, compared to day 0 in the case of oclacitinib (2) and a 33% reduction in pruritus when using methylprednisolone (12).

After discontinuation of treatment on day 28, dogs in group 1 began to show pruritus after 6 ± 2 days, group 2 began to show pruritus after 7 ± 2 days.

Side effects seen after oclacitinib administration: polydipsia, increased appetite, vomiting, diarrhea.

Polydipsia was reported in 80% of cases, increased appetite appeared in 50% of cases, vomiting was present in 40% of cases, diarrhea was present in 40% of cases.

Some researchers have observed other negative effects following the administration of oclacitinib for longer periods: bone marrow suppression, otitis,

urinary tract infections, weight gain, pyodermitis, folliculitis, demodicosis, dermatomycosis, hematuria (3, 14)

Side effects observed after methylprednisolone administration: vomiting, lethargy, polyuria, polydyspsis, increased appetite, aggressiveness. Vomiting was present in 30% of cases, lethargy 60% of cases, polyuria / polydipsia 100% of cases, increased appetite 30% of cases, aggression 20% of cases. These side effects have also been observed by other researchers following the administration of glucocorticoids (10) .

In the CADESI test on day 1 the dogs in group 1 obtained a total score of 583 points, and group 2 a total score of 594 points (Table 4). The highest score was recorded when evaluating the presence of erythema, group 1 recorded 249 points (40.7% of the total amount of the lot), and group 2 recorded 220 points (37.03% of the total amount of the group).

Table 4

Results of CADESI test in dogs with atopic dermatitis

Day of ex.	Group	E	P1	P2	L	Total (max=2400)
Day 1	L 1	249 (40,7%)	123 (21%)	115 (19,7%)	96 (16,4%)	583
	L 2	220 (37%)	118 (19,8%)	133 (22,3%)	123 (20,7%)	594
Day 7	L 1	200 (42,7%)	15 (3,2%)	135 (28,8%)	118 (25,2%)	468
	L 2	118 (28,4%)	13 (3,1%)	159 (38,3%)	125 (30%)	415
Day 14	L 1	94 (48,7%)	0 (0%)	56 (29%)	43 (22,2%)	193
	L 2	64 (40,2%)	0 (0%)	43 (27%)	52 (32,7%)	159
Day 28	L 1	53 (51,9%)	0 (0%)	28 (27,4%)	21 (20,5%)	102
	L 2	4 (4%)	0 (0%)	25 (25%)	27 (27%)	100

E (Erytema), P1 (Primary efflorescences), P2 (Secundary efflorescences), L (Lichenification). The results are expresed as total points/group(%)

After treatment, the score of the group decreased from 583 to 102 points, the CADESI score decreasing by 82.5%. The score for group 2 decreased from 594 to 100 points, registering a decrease of the CADESI score of 83.16%.

One week after the start of the treatment (day 7) the reduction of primary efflorescences and erythema was observed and the increase of secondary efflorescences and lichenification. In group 1 the score of primary efflorescences decreased from 123 to 15 points, a reduction by 87.8%, and in group 2 the score of

efflorescences decreased from 118 to 13 points, a reduction by 88.98%. Erythema was reduced by 19.67% in group 1, and in group 2 by 46.36%.

Two weeks after the start of treatment (day 14) with prednisolone and oclacitinib, the disappearance of primary efflorescences and the reduction of erythema, secondary efflorescence and lichenification in both groups were observed. In group 1, the CADESI score decreased by 66.89% compared to day 1 and by 73.23% in group 2. Erythema decreased by 62.24% compared to day 1, secondary efflorescence by 51.3% and lichenification by 55 ,2%.

After 5 weeks (day 28) of treatment, there was a CADESI score of 102 points for group 1 and of 100 points for group 2. The erythema decreased from 249 points to 53 points in group 1, a decrease of 78.71%, and in group 2 it decreased from 220 points to 48, a decrease of 78.18%. Secondary efflorescences decreased by 75.65% in group 1, respectively by 81.2% in group 2, compared to day 1. Lichenification decreased by 78.12% in group 1, respectively by 78.04% in group 2.

Statistical analysis of the data obtained revealed a significant difference between the two treatments, the group receiving prednisolone had better results in terms of erythema on day seven and 14 ($p < 0.001$), primary efflorescence on day seven and lichenification on day 28 ($p < 0.05$) (Table 5). Statistical differences were observed regarding the CADESI test in the two groups, which means that both treatments were able to reduce the clinical signs ($p < 0.05$).

Table 5

Statistical analysis of the differences in group 1 and 2 for the CADESI test

	Prednisolon (n=10)		Oclacitinib (n=10)		The difference and the level of significance
	x±Sx	SD	x±Sx	SD	
Erytema Day 1	24,9±2,58	8,18	22±2,26	7,14	2,9
Erytema Day 7	20±1,49	4,71	11,8±0,77	2,44	8,2***
Erytema Day 14	9,6±0,71	2,27	6,4±0,54	1,71	3,2***
Erytema Day 28	5,3±0,39	1,25	4,8±0,2	0,63	0,5
Primary EF. Day 1	12,3±0,36	1,15	11,8±0,67	2,14	0,5
Primary EF. Day 7	1,5±0,42	1,35	1,3±0,44	1,41	0,2
Primary EF. Day 1	11,5±0,5	1,58	13,3±0,36	1,15	1,8**
Primary EF. Day 7	13,5±0,4	1,26	15,9±0,65	2,07	2,4**
Secundary EF Day 14	12,3±0,3	1,15	4,3±0,21	0,67	8
Secundary EF Day 28	2,8±0,29	0,91	2,5±0,4	1,26	0,3
Lichenification Day 1	9,6±0,71	2,27	11,8±0,67	2,14	2,2**
Lichenification Day 7	12,3±0,36	1,5	12,5±0,3	0,7	0,2
Lichenification Day 14	4,3±0,21	0,67	5,2±0,51	1,61	0,9
Lichenification Day 28	2,1±0,1	0,31	2,8±0,2	0,91	0,7**

EF(efflorescences), x ± Sx (mean ± standard error), SD (standard deviation), *** very significant correlation ($p < 0.001$), ** significant correlation ($p < 0.05$)

The score obtained on the CADESI test on day 1 according to the location of the lesions is as follows:

In group 1 on the right ear auricula there were recorded 70/583 (12%) points, the left ear auricula 63/583 (10.8%) points, on the left eyelid and left eye 22/583 (3.7%) points, on the right eyelid and the right eye 27/583 (4.6%) points, on the muzzle 96/583 (16%) points, on the neck 30/583 (5.1%) points, in the right/left anterior interdigital area 44/583 (7.5%) points, respectively 43/583 (7.3) points, in the right / left posterior interdigital area 23/583 (3.9%) points, respectively 25/583 (4.2%), in the left / right axillary area 37/583 (6.3%), respectively 40/583 (6.8%) points, on the abdomen 23/583 (3.9%) points were registered, and on the left / right flank 25/583 (4.2%) of points respectively 15/583 (2.5%) points.

In group 2 on the right/left ear auricles registered 45/594 (7.5%) points, respectively 46/594 (7.7%) points, at the level of the left eyelid and left eye 33/594 (5,5%) points, at the level of the right eyelid and the right eye 15/594 (2.5%) points, on the muzzle there were 123/594 (20.7%), on the neck there were 50/594 (8.4%) of points in the previous right/left anterior interdigital area were registered 60/594 (10.1%) points and 59/594 (9.9%) respectively, in the posterior interdigital area right /left 14/594 (2.3%) points, respectively 13/594 (2.1%), in the left/right axillary area 40/594 (6.7%), respectively 33/594 (5.5%) points, on the abdomen were registered 15/594 (2.5%) points, and on the left/right flank 22/594 (3.7%) points respectively 26/594 (4.3%) (Fig. 3).

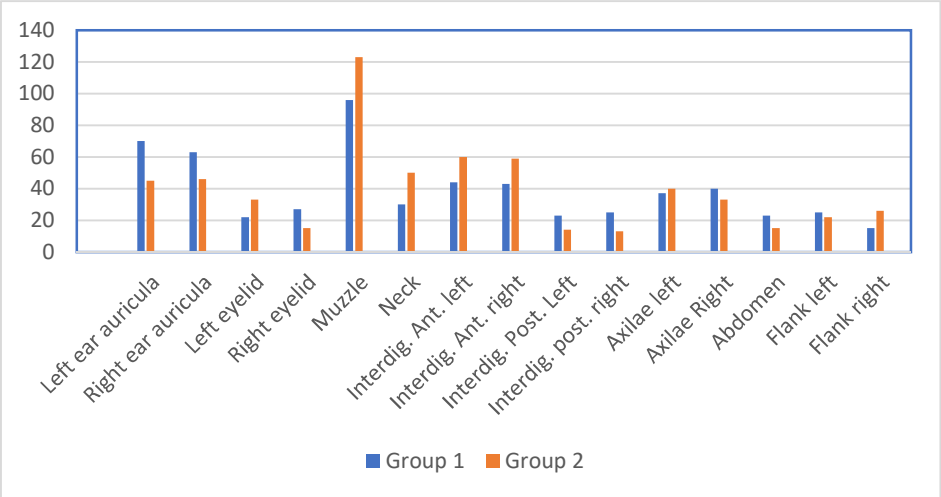

Fig. 3. Results of CADESI test in fixed localisation of skin lesions
Interdig. Ant. left/right – interdigital spaces of front feet left/right; Interdig. Post. left/right - interdigital spaces of hind feet left/right

On day 28, at the clinical examination, a higher proportion of lesions were observed on the muzzle (14.7%), the ear auricular, on the flank area (9.8%), the

anterior interdigital area (7.8%) and the axillary area. (7.8%) in group 1, and for group 2 the highest number of lesions was on the ear auricula (15%), on the muzzle (13%), the anterior and posterior interdigital area (10%), the flanks and abdomen (8%) (Fig. 4). In dogs treated with oclacitinib, an improvement in otitis media associated with *Malassezia* sp was observed.

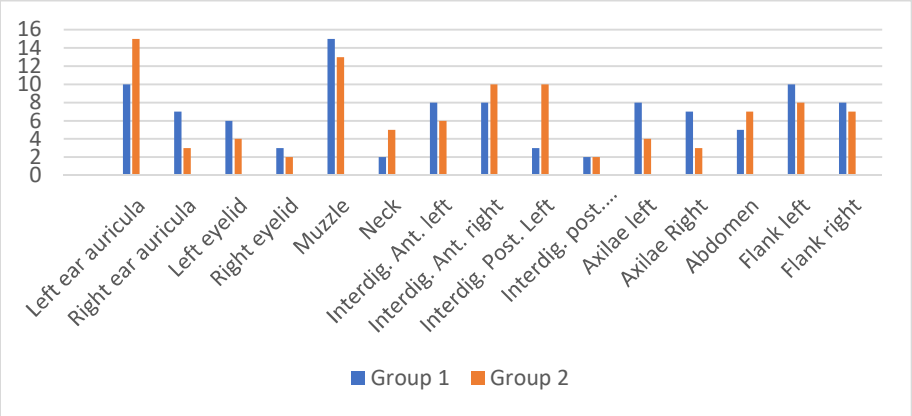

Fig. 4. Results of CADESI test in fixed localisation of skin lesions in day 28 of the study
Interdig. Ant. left/right – interdigital spaces of front feet left/right; Interdig. Post. left/right - interdigital spaces of hind feet right/left

Other researchers confirmed the beneficial effects of using oclacitinib at a dose of 0.4-0.6 mg / kg for 14 days, then once daily for up to 630 days, with pruritus reduction of up to 63.9% on the day 90 of treatment and clinical signs with 66.4% (1).

A review of pharmacotherapy in atopic dermatitis published by Olivry et al., revealed that oral administration of glucocorticosteroids (prednisone, prednisolone, methylprednisolone) reduces skin lesions by 50% to 58-86 % of treated dogs (9).

Conclusions

In dogs with allergic dermatitis, both tested products were effective in reducing pruritus.

Oclacitinib was more effective from this point of view both in the first part of the study until day 7 and after the dose reduction on day 14.

The dogs treated with methylprednisolone showed slight pruritus intensification after dose reduction on day 14.

After discontinuation of treatment on day 28, both groups began to show pruritus, which indicates the need to extend the therapeutic protocol at low doses, or trying a desensitization therapy.

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THE RELEVANCE OF ULTRASOUND EXAMINATION IN FORESTOMACH DISESES IN CATTLE

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Summary

In veterinary medicine, abdominal ultrasound in cattle for gastrointestinal diseases is not widely used in Romania. The limitation of this investigation technique is a determined by the co-operation of the animal, the time required for a complete examination, the impossibility of carrying out prior preparation for the ultrasound examination, the presence of large amounts of adipose tissue and abundant hairline.

Clinical and ultrasound investigations in this study were conducted in Ilfov county and Bucharest during March 2019 - July 2019 on a number of 7 cattle for full evaluation.

The ultrasound was performed using the Aquila (Pie Medical) ultrasound with a convex probe with a frequency of 5-7.5 MHz

The result included various conditions as biochemical indigestion (n=1), rumenal acidosis (n=2), rumenal tympany (n=1), reticulitis (n=2) and traumatic reticuloperitonitis (n=1).

The increased relevance and specificity of the ultrasound changes, recommends this imaging technique, as an alternative of choice (complementary to the other clinical, hematological and biochemical investigations), in the diagnosis of diseases in cattle.

Keywords – forestomach, ultrasound, cattle

In veterinary medicine, abdominal ultrasound in cattle for gastrointestinal diseases is not widely used in Romania.

The limitation of this investigation technique is a determined by the co-operation of the animal, the time required for a complete examination, the impossibility of carrying out prior preparation for the ultrasound examination, the presence of large amounts of adipose tissue and abundant hairline.

In order to confirm the diagnosis and to differentiate between other diseases with similar manifestations, the ultrasound examination can be used (Fig. 1, Fig. 2, Fig. 3, Fig. 4) (11, 12).

In order to detail the methods used in carrying out these investigations, we considered it appropriate to present further the ultrasound examination of the forestomachs in healthy cattle.

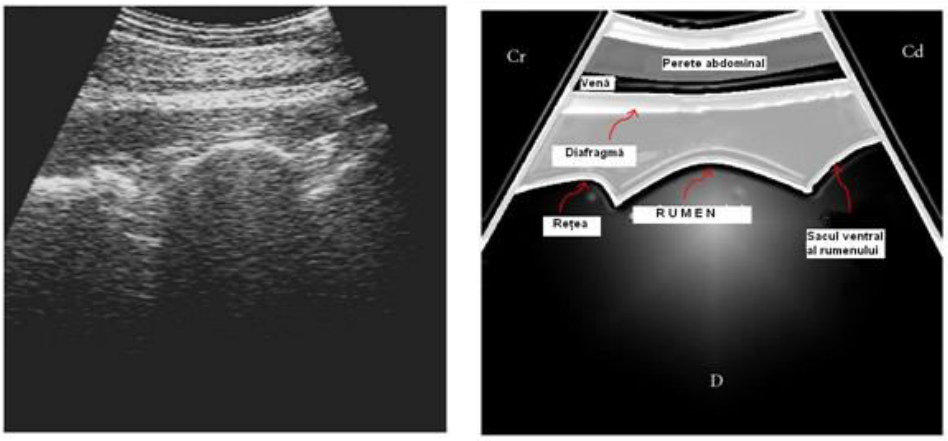

Fig. 1. Ultrasonogram of the ventral sac of the rumen, reticulum, imaged from the left paramedian side, by placing the transducer parallel to the longitudinal axis of the body (C r- Cranial, Cd- caudal, and D- dorsal) (11)

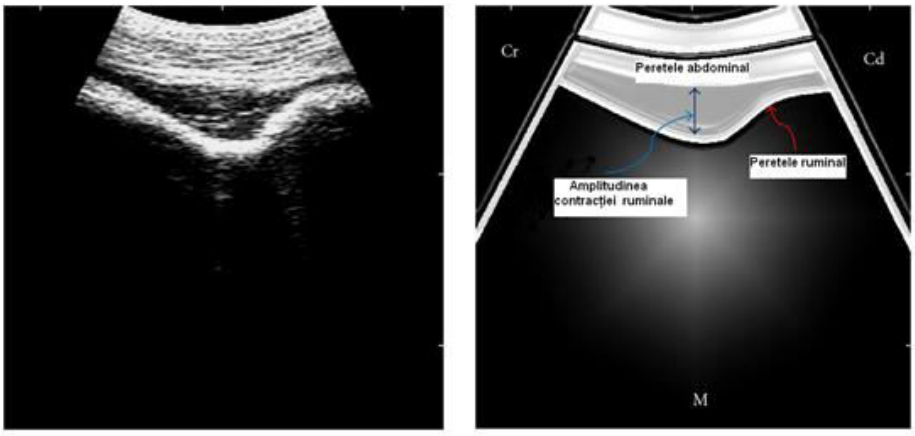

Fig. 2. Ultrasonogram of the rumen obtained from left mid-paralumbar fossa at the peak of its contraction, by placing the transducer parallel to the longitudinal axis of the cow. Cr - cranial, Cd - caudal, and M - medial (11*)*

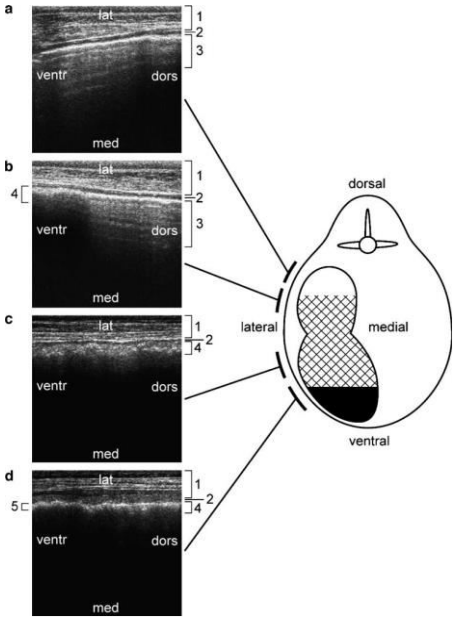

Fig. 3. Ultrasonographic findings and interpretation with respect to the stratification of the rumen in cattle; 1- abdominal wall, 2- rumen wall: a - reverberation lines (3) indicative of a gas-filled space (gas dome); b -abrupt transition from gas dome (3) to fibre mat (4); c -ingesta with gaseous inclusions (fibremat; 4) at the rumen wall; d -transition from fibre mat (4) to a comparatively sharp demarcating ruminal wall with no signs of gaseous ingesta fluid layer- 5 (12)

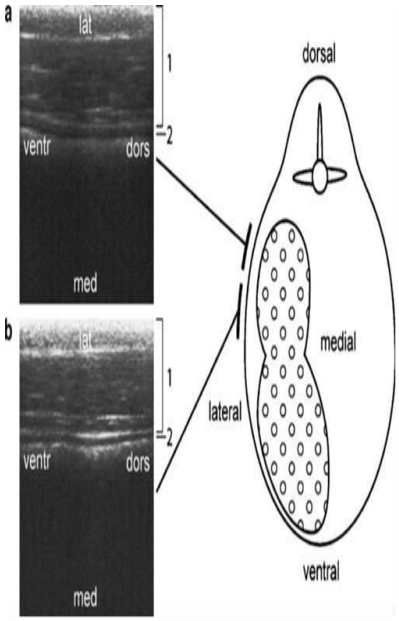

Fig. 4. Ultrasonographic findings and interpretation with respect to the stratification of the dorsal rumen contents (in two levels, a and b); 1 - abdominal wall; 2 -rumen wall; reverberation lines indicative of a gas-filled space (gas dome) (12)

Materials and methods

Clinical and ultrasound investigations in this study were conducted in Ilfov county and Bucharest during march 2019 - july 2019 on a number of 7 cattle for full evaluation.

The ultrasound was performed using the Aquila (Pie Medical) ultrasound with a convex probe with a frequency of 5-7.5 MHz.

Results and discussions

The result included various conditions as biochemical indigestion (n=1), rumenal acidosis (n=2), rumenal tympany (n=1), reticulitis (n=2) and traumatic reticuloperitonitis (n=1).In simple biochemical indigestion, the ultrasound changes of the rumenal wall are reduced, and the content type is easily appreciated through the characteristics of the of echostructure and echogenicity (Fig. 5).

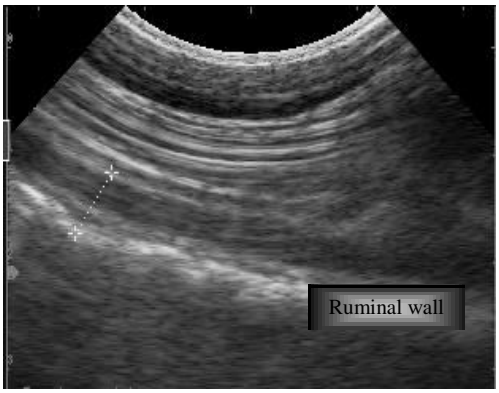

Fig. 5. The rumenal wall (delimited by the cursor ends) is distinguished, without changes of the specific architecture. Rumen content is delimited and indicated by numerous reverberation artifacts - image obtained by placing the probe at the level of the dorsal rumen sac.

In rumenal acidosis, changes in rumenal and systemic biochemistry lead to both inflammatory / edematous parietal reactions at the organ wall, as well as changes in rumen content, associated with loss of characteristic echogenicit (Fig. 6).

The ultrasound evaluation of the rumen wall allows to highlight the organ damage (both at the level of the dorsal and ventral rumen sac), as well as changes of rumen content - in the medial and ventral area. It can be appreciated that the parietal changes of edematous / infiltrative type (at the dorsal / median / inferior level), the hypomotility (the reduction of the frequency and the amplitude of the rumen contractions), together with the reduction of the parietal tone (the evident hypotonic / apparently anfractuous aspect) are ultrasound criteria useful in the diagnosis (1, 3).

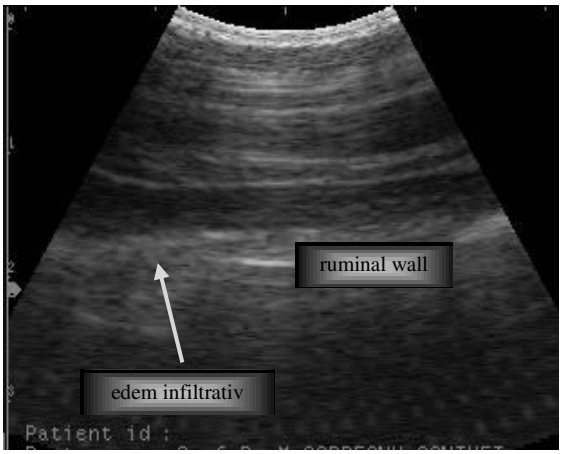

Fig. 6. Important parietal edema at the rumen, with hypoecogenic infiltrate at the level of superficial parietal components (mucosa). Rumen content is inhomogeneous with the presence of reverberation artifacts (specific to gaseous accumulations)

The delimitation of the specific parietal components is easy, the lumen / content interface is highlighted by the hyperecogenic (high reflectogenic) area, and the dorsal gas dome is indicated by the reverberation artifacts (hyperecogenic lines parallel to the skin plane of the abdomen) characteristic of gas accumulations, accompanied by diffuse posterior shadowing.

There are also situations in which the cause of the recurrent rumenal meteorism can be highlighted, by the existence of changes at parietal level - structural inhomogeneities (adhesions, expansive processes), accompanied by the alteration of the specific architecture (Fig. 7, Fig. 8, Fig. 9) (7).

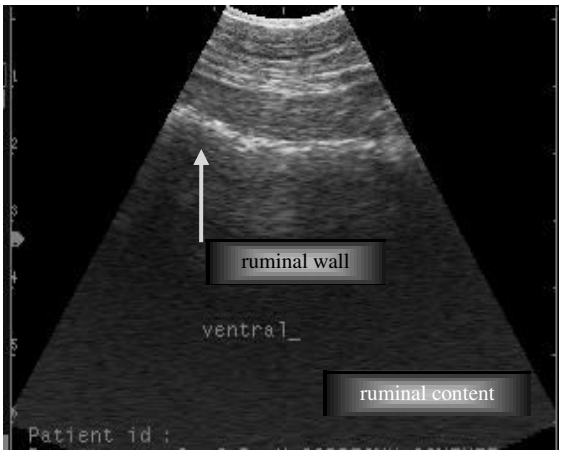

Fig. 7. The wall of the dorsal rumen sac - obvious parietal reaction, the mucosa is strongly thickened. The mucosal lumen interface is marked by the reflective strip, represented by the accumulation of gas

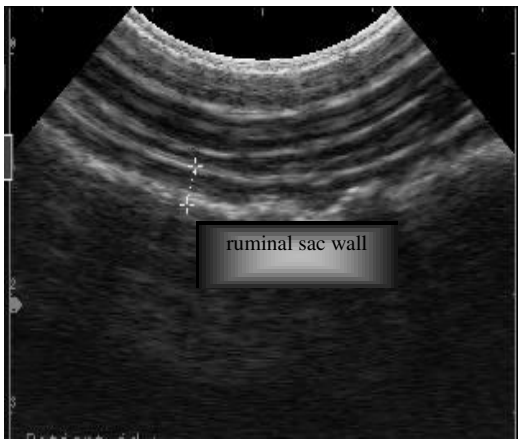

Fig. 8. The rumenal distension (without parietal modifications), without modifications of the specific architecture is noted. The gas content is highlighted by the numerous reverberation artifacts

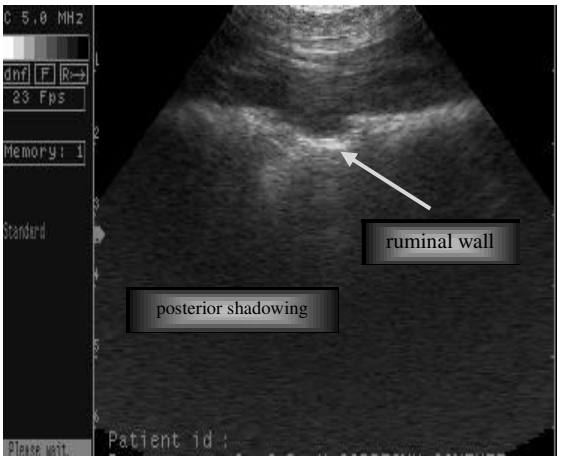

Fig. 9. Structural inhomogeneity at the level of the rumen wall (with the loss of the specific architecture). Dorsal gas dome, with diffuse posterior shadowing, characteristic

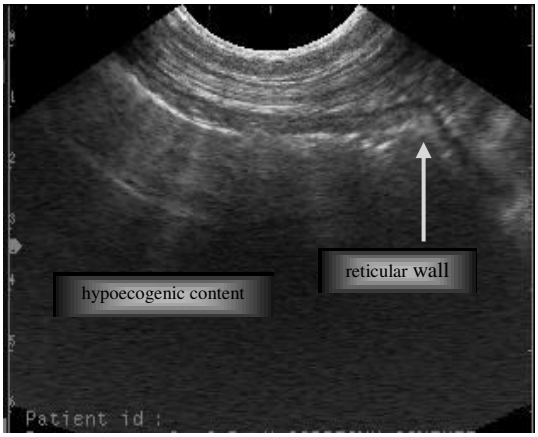

Fig. 10. Reticulitis - parietal inflammation, with edema-infiltrative appearance. The reticular content is slightly abundant, hypotonic wall

The ultrasound approach of the reticulum and the adjacent structures is relatively easy, allowing the identification of the parietal components, the tonus and the contractility, but also the type and quantity of intralumenal content (Fig. 10, Fig. 11). Inflammatory type is dominated by a hyperrepresentation of the wall, with the preservation of the specific architecture, together with the changes recorded by the neighboring structures and by the alterations of tonus and reticular motility (4).

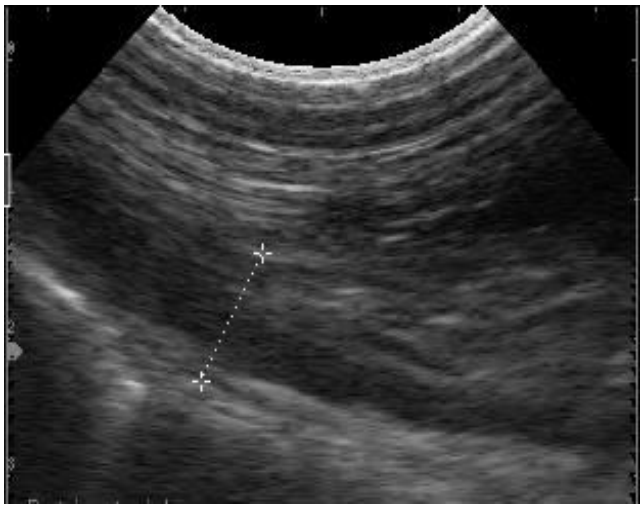

Fig. 11. Diffuse reticuloperitonitis. Clear parietal (diffuse) and periparietal reaction with peripheral transsonic collection

Conclusions

The increased relevance and specificity of the ultrasound changes, recommends this imaging technique, as an alternative of choice (complementary to the other clinical, hematological and biochemical investigations), in the diagnosis of diseases in cattle.

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INTERACTIVE TEACHING METHOD WITH APPLICABILITY IN VETERINARY MEDICINE

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Summary

Technology has invaded most domains through various methods, becoming necessary in certain situations. Whether it replaces repetitive steps, performs complicated calculations, or helps to expose ideas more clearly by displaying complex schemes in a simpler way, technology will always be a very useful tool. In education this is found, for example, in PowerPoint presentations using projectors and special laser remote controls. Many teachers have been looking for new ways to increase classroom activity, so researchers are studying ways to improve teaching-learning methods. A useful technique for this purpose was defined by the periodic interruption of the presentation using electronic voting technologies. These technologies have been studied in different countries on different subjects, having several names such as SRS (Student Response System) or CCS (Classroom Communication System) and have been proven as practical methods that have improved teaching methods involving students directly. This paper presents some alternatives, their advantages and disadvantages. The electronic voting technologies can be classified, from the point of view of the used equipment, in those that use only the computer or the laptop from which it is presented, together with the smartphones of the audience (which we will call simple technologies), or those that require special equipment. The special equipment consists of remote controls (called "clickers") assigned to each auditorium, a device that will receive the remote control signal (infrared, radio, wireless) and a computer that will process the data and display the response on the display (either the screen or the projector). In case of using the simple technologies, the presenter (the teacher) only has to install a software specially developed for the PowerPoint application, or can open a web page specially designed for presentations that will contain the online system for electronic voting, and the auditor (students) will use their smartphone to answer questions by accessing a web page, by opening a mobile application or by SMS. Both options will require technical support, noting that a simple system will benefit from online support. Every technology benefits from its own advantages or disadvantages, and they are usually connected to the costs, ease of use, number of devices needed, and speed of the process.

Keywords: electronic voting devices, e-conference, teaching, SRS, ARS

There are countless studies proving that deep, durable learning is encouraged when students actively engage in acquiring the concepts they study and build their own understanding (8, 16, 20, 35), but achieving that level of interactivity is not at all easy (34, 48), especially when dealing with large classrooms, hence research has been conducted to make classes more interactive (4, 6, 15, 24, 31, 33, 38, 42, 50).

One of the methods used to increase student engagement, named "*Peer Instruction*" (10) would present students with questions, then they would formulate individual answers and report their answers to the instructor, after which they would discuss their answers with those around them and at the end of the discussions the students would be polled for their answers, which might have changed. The author noticed that the student mastery of both conceptual reasoning and problem solving had increased; also the students were more motivated, so overall the reactions were positive. However there were always some students which were shy or plain reluctant to being taught in a non-traditional manner.

Taking the results of implementing "*Peer Instruction*" in consideration and, with the apparition of electronic voting systems such as the one used in the famous TV Show "*Who Wants to Be a Millionaire?*" marked the beginning of electronic classroom interaction systems. They were based on the various technologies and were given names such as Personal Response Systems (PRS) or Student Response Systems (SRS) or Classroom Communication Systems (CCS) or Group Response Systems (GRS) etc. and they had one common purpose: to increase student engagement (2, 7, 13, 14, 23, 25, 27, 29, 30, 51, 52). To avoid confusion with electronic voting which refers to elections, the term "*Audience Response Systems*" will be used from now on.

Early stages of "Audience Response Systems" (ARSs)

Sources mention a meaningful connection during the late 1960s (40): William Simmons, an IBM executive who was fed up with unproductive corporate meetings, and Theodore Gordon of The Futures Group, who conceived and partially developed what would today be an "ARS" (21, 46). Simmons saw that Gordon's system had practical applications for large corporate meetings, allowing people to state their true opinions anonymously. Soon enough Simmons and Gordon, in 1972, form a startup called "Applied Futures Inc." and they develop and market an ARS system called "*Consensor*" all the while in meantime the same type of system was used in biology and chemistry teaching (3). The first patent was filed in 1972 and granted in 1973 with Gordon and his assistant, Harold S. Becker as inventors (18). A second patent was filed by Simmons in 1974 and granted in 1976 (41).

The "*Consensor*" was a system which allowed the audience to turn a dial selecting any number from zero to ten showing their level of agreement to a verbally transmitted question. If the majority would agree, a green lamp would light, else a yellow or red lamp would light depending on the level of disagreement.

Skipping to 1991, a very early computer based ARS was used in education, allowing students to rate how well they understood portions of lectures, answer multiple choice questions and answer short essay questions. The system was called "The Networked Classroom". A key issue was pointed out in the article: when the teacher would want to type the answers during the class, it was proven to be too slow, so all questions were eventually answered verbally (26).

Using computers to set up was extensive work – due to the fact that a computer was necessary for every two students, it required proper care for heating, lighting and other conditions, but, again, anonymity is key. The author writes that responses become more diverse, more alive, due to anonymity (39).

Categories of ARSs

In more recent years it can be observed that the amount of studies conducted regarding ARS has increased beyond expectation, scientists even developing their own systems (17, 22, 28, 43, 52, 54). As today, they can be separated in two main types: systems that require additional hardware other than the PC or laptop on which the lecture is presented and those that only require specific software to be installed on the machine used during presentations or, in case of on-line ARSs, internet access is required.

The first category, when the systems eventually became somewhat mobile, required remotes (7, 14, 21, 44), named "*clickers*" (Fig. 1) through which the auditorium would interact (5, 37, 45, 52, 53). They would transmit the answer through either infrared or radio frequency to a receiver (or more, in case of infrared which, in large auditoriums would cause issues due to overlapping signals) which would be connected to the machine used to present the lecture (Fig. 2). Answers would be interpreted by the software and, in a short time after closing the poll, a graph could be displayed with the outcome.

Fig. 1. One variation of a "*clicker*". This example works with questions which have up to ten multiple choice answers

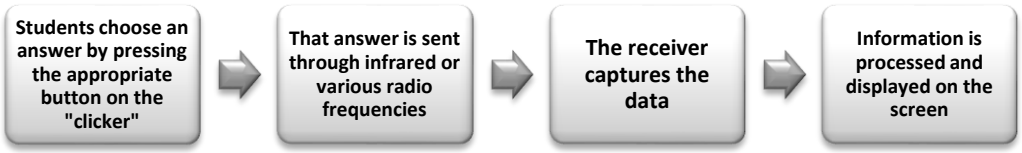

Fig. 2. The process of using "*clickers*"

The second category can be used online or offline. It requires the auditorium to use their own phones, laptops or PDAs (in some cases PDAs or computers were provided by the university) and the connection can be done over the internet, through SMS or using a local area network (1, 2, 11, 12, 27, 29, 53, 54).

More recently, some slightly different online ARSs appeared and their utility was researched during classes: game-based online ARSs (47). At least one study was conducted comparing classic online ARSs with game-based online ARSs (49).

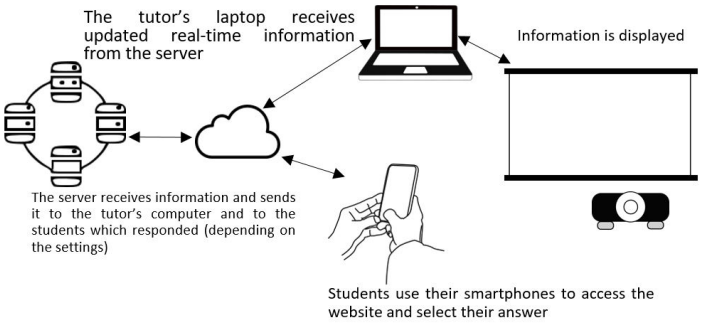

Fig. 3. The process of using online ARSs

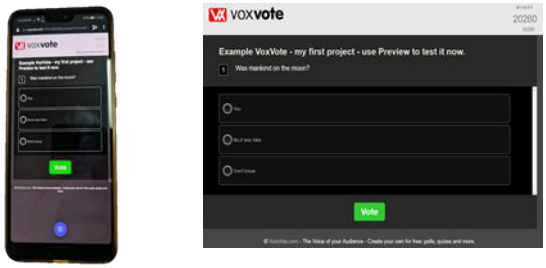

Fig. 4. An example of online ARSs. Voxvote™. Shown as seen by students on a smartphone (left) or laptop (right)

The way connected ARSs work varies, but can be generalized for online ARSs: the tutor uses a computer (PC or laptop) with access to the internet to which a type of display is connected (it may be a projector or large screen etc.), the students will either connect online (using personal smartphone or laptop or PDA) to a specific web or send the answer through SMS to a specific number in a pre-set format, all according to the software used. Then the server will collect the information and process it, presenting in real time (Fig. 3). There are many online

ARSs which offer a free limited access, one of them is Voxvote™ (Fig. 4). Most of them provide a way to merge themselves with Microsoft PowerPoint®, the usual software used to present lectures. They do so by using of an Add-in which needs to be installed and it adds a specific tab in the usual toolbar (Fig. 5).

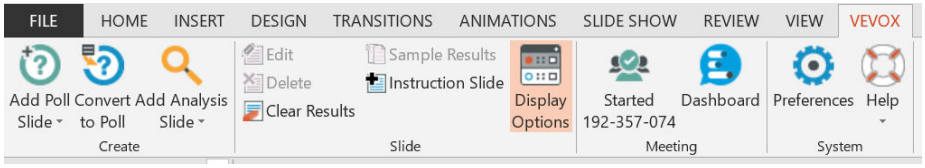

Fig. 5. Vevox™ Add-In application shown in MS PowerPoint® toolbar

Benefits and struggles of ARSs throughout the years

In its early stages, the studies show improvements in exam scores regarding the lower half (7), and, when used in tandem with "*Peer Instruction*" teaching method, it generated large and statistically significant improvements in standardized test results (10).

Even in 2004 some very positive responses were obtained from students after using an ARS: notably lectures became more interactive and/or interesting, involving the whole class, the students also enjoyed contributing opinions to the lecture and allowing them to see what others think about it; as well as the key point, anonymity, which allows students to answer without embarrassing themselves (13).

In a study conducted in 2007 comparing the use of PDAs with personal phones and with "*clickers*" the author practically predicts the future saying that, even though current phones are lacking from the computing, networking and interface capabilities, they are our best bet regarding ARS (29). At that time, a study regarding the use of SMS in ARSs, had shown the following results: positive outcome on student motivation, anonymity; slow typing of text and cost concerns being the downsides (32). ARSs were used in fields such as Medicine, Dentistry, Veterinary Science, Biology, Psychology, Computing Science, Statistics, and Philosophy, seemingly there was a total lack of constraints on the subject it could be used in (13).

In the vast literature published across 80 years we can observe common benefits of using an ARS in education, especially when the classroom has many students in attendance. Upon asking a question, or posting a poll during class, the results are immediately available to the tutor (21), thus usual mistakes can be discovered and corrected during class using additional explanations. The obtained results can be displayed in various ways and are much easier to interpret. He or she may present the data to the students through the preferred type of multimedia presentation.

Anonymity was the key word (2, 13, 32, 39, 51, 53) ever since the 1960s (40). Combining it with the advantages mentioned above proved to be a success in many domains (2, 45).

Most students claimed that they were able to better assimilate the topics taught compared to a regular class, that ARS helped them to understand the concepts behind problems, that they became more involved in class, that they were required to think more compared to a regular class (52). Students also noticed that they were more motivated to attend classes and they felt the overall experience was improved, that they could express themselves much easier effects, they were more likely to answer personal questions, they enjoyed using an ARS and that it had a positive influence on their learning experience (2, 45). Valle and Douglas mention that the positive outcome may be influenced by the students desire to be engaged and that it is due to their poor view on traditional lecture styles (5, 45). Other studies mentioned increased student cooperation and discussions (11, 13, 54).

Automatic grading (class assessment can be done instantly once students answer quiz questions using ARSs) is another beneficial trait of ARS usage, although it breaks the anonymity and forces students to register in the online platform or select numbered "*clickers*" which are specifically assigned to them (53).

Some downsides and negative comments can be found in the results of existing literature, but those mostly refer to the costs of mobile phone tariffs in case of SMS based ARS (29), or "*clicker*" costs (12), or the stress induced by quick questions in the gamification style of online ARSs (49). Dervan states that sometimes lecturers could seem more focused on technology than with teaching (12).

From numerous studies a clear view on the trajectory of ARSs can be deducted: Online ARSs with gamification. There were several researchers which predicted the decline of "*clickers*", PDAs, laptops and that in the end mobile phones, and later, smartphones will be the main tools (2, 29) due to their reduced size and importance in everyday lives and increased capabilities (53).

Final thoughts

In 2014 ARSs were thought as increasingly popular solutions for education (45). In 2015 scientists were referring to ARS as a standard educational tool (27). Abdullah cites three articles stating that an ARS system named Socrative is an active learning tool that is widely used in teaching (1).

Ever since 2007, the presence of mobile phones on university campuses was referred to as ubiquitous (29). More recent studies show that students prefer to use their personal smartphones (53).

Going forward to present day, a trend can be observed on using Online based ARSs. It is mostly due to the fact that smartphones have become vital in people's lives, especially the young, hence we can presume that most, or perhaps all, students have at least one smartphone to use during classrooms. We can go

even further and presume that most, or perhaps all, students have at least a consistent mobile internet, although unlimited internet plans are affordable in Romania. Reports show an increase in smartphones and mobile internet connectivity (9, 19, 36). Acknowledging previous information, adding the idea of gamification and Wang's research which has had success (48), it can be concluded that implementing a similar system in Romanian faculties is possible and it would be an improvement in our actual education system.

Conclusions

Technology will continue to grow and envelop our daily lives, making its usage more and more comfortable. Every domain is trying to use it as much as possible as a mean of improvement. Teaching shouldn't be an exception. We believe there is enough information for a similar study using Online ARSs with gamification to be conducted in Romania. There are, still, some points we must not forget: pedagogy is above technology and using ARSs blindly will not be sufficient.

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DNA BARCODING METHOD USED FOR RUPICAPRA SPECIES IDENTIFICATION

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Summary

The identification of the areas populated by the species protected by law is essential for the establishment of their protected areas. The classical methods of identification are time consuming requires highly specialised human resources; sometimes they are invasive and can easily fail in providing accurate results. Molecular biology forensic methods have been developed to overcome all the issues that may appear when applying those classical methods. The most used DNA based methods is the species identification through DNA barcoding. In the study presented here, the mitochondrial COI gene was sequenced to determine the species using dried feces, collected from *Rupicapra rupicapra* specie protected areas from Retezat Mountains. The feces were harvested from different four locations and feces of domestic goat were used as negative control in the study. The DNA sequencing results reveled that all the collected samples were belonging to *Rupicapra rupicapra* species. The results of this study represents a first step in establishing a protocol for wild species identification and also it study highlights the need for accurate wildlife reference material from each country in order to convict wildlife cases.

Keywords: Wildlife forensics, COI-like sequences, Cytb, DNA barcoding

In several countries in Europe, genetic methods that use non-invasively collected DNA samples (feces and hair) are an important component in monitoring black goat populations (11). In Croatia, Poland, Slovakia and Spain genetic tests are used to supplement or confirm the results obtained by other methods such as tracking populations at feeding sites, tracking traces left in the snow, counting females with chicks, telemetry. In countries that do not use genetic or telemetry methods, absolute estimates are based on much more relative measures. Small populations are generally subject to more intensive and expensive monitoring methods for accurate estimates.

Recent advances in molecular genetics techniques have created a number of new and surprising possibilities in the field of research, conservation and management of chamois populations. This advancement in the field has led to a plethora of genetic research on bear populations and increased interest in genetic technology in the number of researchers in other scientific disciplines (12, 14).

The *Rupicapra rupicapra* (chamois) is a species of goat-antelope native to mountains in Europe, including the European Alps, the Pyrenees, the Balkans, parts of Turkey, the Caucasus and the Apennines (2).

The chamois is a very small bovid. Female chamois are their young live herds of up to 100 individuals, adults males tend to live solitarily for most of the year. Primarily diurnal in activity, they often rest around mid- day and may actively forage during moonlit nights. Chamois can reach an age of 22 years in captivity, although the maximum recorded in the wild is from 15-17 years of age (11).

The illegal hunting of animals can seriously affect the dynamics of populations, threating the viability of many species worldwide.

The Rupicapra sp. lives in well-defined areas. These areas are sometimes invaded by sheep (transhumant) and domestic goats (5).

DNA Barcoding is a method of species identification using a short section of DNA from a specific gene or genes. Barcodes are used to identify unknown species, parts of organism (Fig. 1).

DNA can be isolated from various sources such as: tissue of calf, blood, animal matrix, vegetal matrix, bones. The quality and quantity of nucleic acids are essential factors in the success of any following analyses.

DNA is a molecule that encodes the genetic instructions of an organism, and it is unique to each species. DNA Barcoding is a method that uses this unicity. All animals and plants, and many microorganisms, use the instability of oxygen to power the processes of life. The molecules in food are oxidized and the energy is used to build new molecules, to swim or crawl, and to reproduce (9).

Cytochrome C Oxidase is a membrane protein. Most of the surface atoms are carbon and sulfur. In the cell, these atoms are buried inside a membrane (9). These regions, which prefer a watery environment, stick out on opposite faces of the membrane. This arrangement is perfect for the job performed by cytochrome C oxidase, which uses the reaction of oxygen to water to power a molecular pump. As oxygen is consumed, the energy is stored by pumping hydrogen ions from one side of membrane to the other. Later, the energy can be used to build ATP or power a motor by letting the hydrogen ions seep back across the membrane.

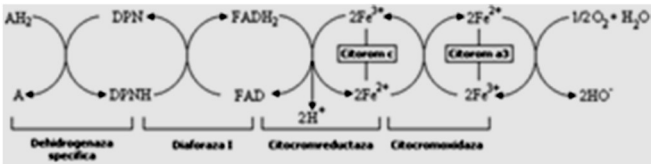

Fig. 1. Cytochrome C oxidase I molecular pathway (9)

Cytochrome C oxidase, the terminal enzyme in the respiratory chain, catalyzes the four electron reduction of molecular oxygen and couples the reaction to the generation of a proton gradient across the mitochondrial inner membrane.

The most commonly used barcode region for animals and some protist is a portion of the cytochrome C oxidase I (COI or COX 1) (Fig. 1) gene, found in

Mitochondrial DNA. Other genes suitable for DNA Barcoding are the Interval transcribed spacer (ITS) rRNA often used for fungi and RuBisCO used for plants (5).

Applications include identifying plants leaves due to absences of flowers or fruit, helps to identifying larvae stages of insects, which not leave significant characters than adults or are less known, identifying the nutrition level of animals, identifying products of herbal supplements, wood, skin and other parts.

DNA barcoding has many applications in various fields like preserving natural resources, protecting endangered species, controlling agriculture pets, identifying disease vectors, monitoring water quality, authentication of natural health products and identification of medicinal plants (2, 4, 7).

Materials and methods

As biological material, feces of *Rupicapra sp.* collected from Retezat Mountains, and feces collected from a local farm of domestic goat. Total genomic DNA was isolated and purified from collected feces.

The primers used in this study are listed in the Table 1, and were proposed by Inanova et al. (7).

Table 1

Primer pairs used in this study

Primer code	FOREWARD sequence	Expected amplicon size
VF 1d F	TCTAACCACCACAARGAYATYGG	700
VR 1 d R	TAGACTTCTGGGTGGCCRAARAARAARAAYCA	
dgLCO- 1490 F	GGTCAACAAATCATAAAGAYATYGG	750
dgHCO – 2198 R	TAAACTTCAGGGTGACCAAARAAYCA	

For DNA extraction the The FastDNA SPIN Kit for Feces (MP Biomedicals, Irvine, CA) was used. Further pufification of DNA was achived using the NucleoSpin gDNA Clean‑up Kit (Macherey-Nagel, Düren, Germany). The obtained purified DNA suspension was used as in the following PCR reactions.

The quality and quantity of extracted DNA was assessed by spectrophotometric method using a NanoDrop 8000 spectrophotometer, (ThermoScientific, USA).

PCR was carried out in final reaction volumes of 50 µl containing 50 ng of DNA template. The composition of amplification mixture was carried out according to instructions for GoTaq Green PCR Master Mix (2X) (Promega, USA) commercial kit.

The enzymatic reaction was performed on a Corbett Thermal Cycler (Corbett CGI-96 Palm-Cycler) with the program: first denaturing step for 3 min at 94°C, followed by 45 cycles of: denaturation at 94°C for 45 sec, annealing at 60°C for 30 sec and extension at 72°C for 2 min, the final step of extension at 72°C for 5 min.

The resulting PCR products were run on 1.7 % agarose gels in TAE buffer at 100 V for 30 minutes. The PCR products were purified from the agarose gel using the Monarch DNA Gel Extraction Kit (New England BioLabs, USA). Purified DNA amplicons were sent for sequencing at the Macrogen Laboratory, Amsterdam, Netherlands (1). Obtained sequences were uploaded in the NCBI database and aligned using the T-Coffee on line DNA sequences alignment tool (10).

Results and discussions

Lately, achievements in the field of establishing a DNA barcode for most species have encounting more and more recordings. Ward et al in 2005 (13) barcoded 207 species of Australian Marine fishes and were able to differentiate one from the other by cox I barcoding. Their results showed some phylogenetic relation that species invariably clustered within genera and genera clustered within families (13). Hogg and Herbert (6) evaluated the sequence diversity in the mitochondrial cytochrome-C oxidase 1 gene as a tool for resolving differences species of Arctic springtails. Foottit (3) using DNA barcoding were able to discriminate nearly 300 species of aphids and concluded that 96% were well differentiated though sequence variation was low. However, with regard to wild species and especially those with areas of population and small numbers of individuals, one can observe a lack of development of the barcoding DNA system and a lack of registration in the databases of reference sequences. In order to identify the chamois after the DNA barcode only two reference partial sequences are available. In 2017, Perez et al (12), summited to NCBI Database, the cytochrome oxidase subunit I (CO I) pseudogene, partial sequence of 276 base pairs, for *Rupicapra sp*. having the accession number MF383366. The other sequence was made available by Bounas et al in 2018 (2). In the NCBI Database the accession number for this sequence is KY771089.1, and it was uploaded as a 533 base pairs of cytochrome oxidase subunit 1 (COI) pseudogene, partial sequence. This record resulted from a case study of a case of poaching identification against *Rupicapra sp*. here the DNA was extracted from hair samples. In this framework, no recordings were made with cases of identify the specie using DNA extracted from feces.

In this study, the biological material consisted of samples of fecal material from the chamois and as control for the experiment the feces of domestic goat were used. Each sample was analyzed in duplicate. In all 4 samples in both cases, it was found quantifiable DNA (in smaller or larger quantities). The PCR reactions were carried out with both primers pairs, the pair VF 1d/ Vf 1d that was proposed for barcoding the mammals and the pair dgLCO- 1490/ dgHCO – 2198 that were developed to be used on a wide range of animals (Fig. 2).

Very good results were obtained in both reactions, and further analysis was made with both molecular markers. The amplicons were migrated in agarose gel and the identified DNA band was extracted and purified from the migration support.

The purified DNA sequences were send for sequencing to a specialized laboratory and the sequencing data were received on electronical support. The

alignment of the obtained sequences were performed using the on line analysis tool T- Coffee (Fig. 3).

The presence of DNA in the 4 samples, especially those in the chamois, has shown that the delimitation of the area based on biological evidence (in this case: fecal material) is possible. Besides the delimitation of the area, the identification of the species can be achieved.

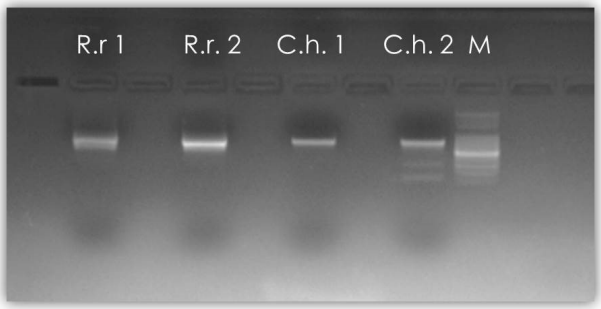

Fig. 2. Amplicons for CO I molecular markers. R.r. 1 – R. rupicapra, mammals primers; R.r. 2 – R. rupicapra, animal diverse primers; C.h. 1 – C. hircus, mammals primers; C.h. 1 – C. hircus, mammals primers; M – DNA Ladder PCR Marker (Promega)

The results obtained show that the Co I molecular marker of chamois has a addition of a DNA sequence when compared to the domestic goat. This result was somehow expected since the fragment that was obtained for the chamois, of 1180 base pairs was significant higher than the sequence obtained in the case of domestic goat, of 720 base pairs and also significant larger when compared with the sequences that were found in the NCBI database.

The alignment of the two sequences revealed that the CO I sequence for the chamois has a gain in the interior part of the molecular marker when compared with the same marker sequence obtained for domestic goat. However, further studies are necessary since the lack of a proper and valid reference sequence makes any interpretation of own data very difficult.

Even if this study began as an attempt to identify the chamois populated restricted areas by developing a method to differentiate between biological traces, respectively fecal materials, from chamois and domestic goat, whose populations can invade their territories. Although poaching is the main threat to the safety of chamois populations, the penetration of domestic animals into their areas can also harm them by competing for resources and also a potential source of infection with pathogens that can become very dangerous in wild populations.

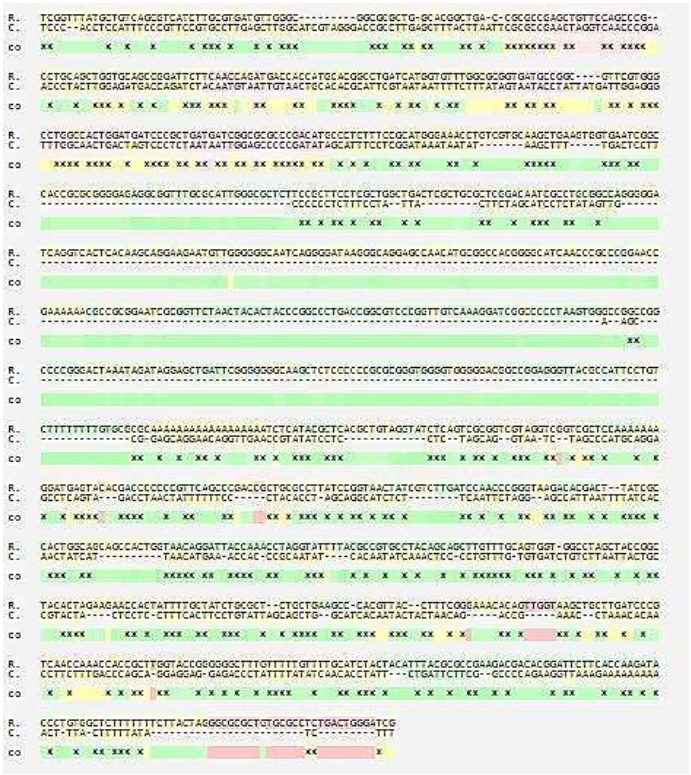

Fig. 3. The multiple sequence alignment result as produced by T-coffee software. R – *Rupicapra sp*. CO I DNA sequence; C – Capra sp. CO I DNA sequence; CO – asterix marks the consensus in the both sequences

Conclusions

The results obtained revealed that the collected samples belonged to the *Rupicapra rupicapra* species. With this study it was proven that the wild animals population can be very easy and accurate monitored by using molecular techniques, namely the DNA barcoding method. In the future, a more concrete sample (hair, blood) is needed for the development of a *Rupicapra sp*. molecular marker that is to be used in DNA barcoding method. By using this molecular tool a better hierarchy of certain categories of animals can be achieved, identification of more details about the specie development and the clear delimitation of a common traits of some close related species.

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SMALL RUMINANTS PIROPLASMOSIS IN THE BALKAN AREA: REVIEW FOCUSED ON *BABESIA* AND *THEILERIA* SPECIES

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Summary

Haemosporidiosis are non-contagious parasitic diseases, produced by piroplasms, protozoa of the blood or of the lymphoid system with endoglobular localization. This category includes babesiosis, parasites that infect red blood cells and theileriosis which affects the erythrocyte and lymphatic system.

The disease is transmitted through the *Ixodidae* ticks. The systematic classification is the *Protozoa* branch, *Sporozoa* class, *Haemosporidia* subclass and *Achromatorida* order that includes two important families: *Babesiidae* and *Theileriidae.*

The etiology of small ruminants' piroplasmosis in the Balkan area includes *Babesia* and *Theileria* species. The etiological agents of babesiosis in small ruminants are: *Babesia ovis*, *B. motasi*, *B. crassa* and *B. spp.*; while theileriosis in sheep and goats is produced by: *Theileria ovis, T.* sp. *MK*, *T.* sp. *TO3, T. annulata*, *T. luwenshuni*, *T. uilenbergi*, *T.* sp. *TO1*.

The prevalence of piroplasmosis is between 21.5% and 86.12%. The most prevalent species are *T. ovis* (17.0-67.7%) and *B. ovis* (0.44-70.81%). In the case of coinfections with 2 or more species of haemoparasites the prevalence registered was 30.6 -52.24%. The most common association in mixed infection was between *Babesia ovis* and *Anaplasma ovis* or *Theileria ovis* and *Anaplasma ovis*.

Regarding the susceptibility of the host it was observed that sheep are more receptive to develop piroplasmosis than goats.

The vectors of babesiosis and theileriosis are ticks which belong to the *Ixodidae* family. Its species are included in the following genera: *Rhipicephalus* (*R. bursa*, *R. turanicus*, *R.sanguines*), *Ixodes* (*I. ricinus, I. gibbosus*), *Hyalomma* (*H. marginatum*, *H. anatolicum, H. detritum, H. excavatum, H. plumbeum, H. dromedarii, H. rufipes, H. impeltatum), Haemaphysalis (H. sulcata, H. conccina, H. parva, H. punctata*). The most common species of ticks identified as vectors of piroplasmosis in small ruminants were *Rhipicephalus bursa* and *Ixodes ricinus*.

The clinical signs describe: fever, inappetence, icterus, haemoglobinuria and the modifications of the haematological parameters such as anaemia, pancytopenia, thrombocytopenia, leukopenia, correlated to the parasitaemia level. In coinfections the severity of the clinical signs increases.

Diagnosis in babesiosis and theileriosis is made by the following methods: microscopical techniques - microscopic examination of thin blood and lymph smears, serological methods - indirect immunofluorescence test (I.F.A.T.) and molecular assay - polymerase chain reaction (P.C.R.) methods by conventional and nested PCR form, reverse line blotting (R.L.B.)

Treatment of diagnosed animals is made with imidocarb dipropionate 1mg/kg body weight dose, administered by intramuscular injection.

The current review processing publications retrieved from the PubMed and Google Academic database regarding sheep and goat babesiosis and theileriosis. A lot of evidence is needed regarding the current situation of small ruminant piroplasmosis in the Balkan area to know the distribution of the involved pathogens in limiting the spread.
Key words: *Babesia*, *Theileria*, sheep, goats, Balkan area

Haemosporidiosis are non-contagious parasitic diseases, produced by piroplasms, protozoa of the blood or of the lymphoid system with endoglobular localization. This category includes babesiosis, parasites that infect red blood cells and theileriosis which affects the erythrocyte and lymphatic system. The disease is transmitted through the *Ixodidae ticks*. Piroplasmosis infect a large number of animal species including wild and domestic ruminants (14, 22). *Theileria* species that affect the small ruminants are *T. lestoquardi*, *T. hirci*, *T. ovis* and *T. separata* (16, 34) while babesiosis in small ruminants it is produced by *B. motasi* and *B. ovis* (35). Theileriosis and babesiosis have an economic importance and significantly affect the international trade of animals (42).

The aim of this review is to present the collected data regarding small ruminants piroplasmosis in the Balkan area especially babesiosis and theileriosis.

Etiology

The etiology of small ruminants' piroplasmosis in the Balkan area includes *Babesia* and *Theileria* species.

The systematic classification is the *Protozoa* branch, the *Sporozoa* class, the *Haemosporidia* subclass, the *Achromatorida* order that includes two important families: *Babesidae* and *Theileridae* (20).

The etiological agents of babesiosis in small ruminants are: *Babesia ovis*, *B. motasi*, *B. crassa* and *Babesia* sp. (15, 27, 38, 41, 44, 45); while theileriosis in sheep and goats is produced by: *Theileria ovis*, *T.* sp. *MK*, *T.* sp. *TO3*, *T. annulata*, *T. luwenshuni*, *T. uilenbergi*, *T.* sp. *TO1* (9, 15, 23, 39, 41).

The *Theileria* forms detected in the erythrocytes are polymorphic: round, oval, annular and anaplasmoid (2). Also, fusiform and club shaped forms were observed (41).

Babesia has been identified in pair form (double pyriform) and single ring form (intra-erythrocyte and extraglobular) (6, 38). Most of the parasite pair form were double pear shaped while the single elements were round or oval. Paired items were more frequently encountered than single forms (41).

Life cycle

Babesia spp. life cycle

While the tick feeds with blood from the sheep / goat it inoculates sporozoites in the host's body. Sporozoites invade the erythrocytes, inside which they become trophozoites. The trophozoites are divided by binary division resulting merozoites

that will parasitize new erythrocytes. The asexual multiplication process described above is merogony.

Gametogonia (sexual multiplication) begins with the intra-erythrocyte evolution of merozoites in gametocytes, a parasitic stage that will infect the tick (invertebrate host) within a new meal. With the ingested blood, gametocytes reach the intestine of the vector, where they evolve into gametes (bodies with rays). The gamets form a motile zygote called the ookinet. It penetrates the intestinal wall, at the level of the epithelium and undergoes a meiotic division resulting the kinets. Through hemolymph the kinets disseminates in the peripheral tissues of the invertebrate host, including the ovaries.

Ovarian localization of the parasite makes possible the transovarian transmission of babesiosis. The kinets located in the other tissues multiply by forming secondary kinets that will invade the salivary glands of the tick.

Due to this localization, it is possible the transstadial transmission of the parasite. Inside the salivary glands the kinets form a multinucleated syncytium (sporoblast) that will be activated during a new blood intake and will produce potentially infectious sporozoites that will be released into the bloodstream of the small ruminant (30).

***Theileria* spp. life cycle**

The infesting element for the vertebrate host is the sporozoites inoculated by the tick during the blood intake. Sporozoites invade lymphocytes. In the host cells cytosol, sporozoites undergo schizogonic transformation (asexual multiplication) and form multinucleated schizonts that implements an uncontrolled proliferation process in the lymphocytes.

The daughter cells will produce uninucleated merozoites that will invade the erythrocytes. Merozoite-parasitic erythrocytes will be taken up with blood by the tick during feeding. In the intestine they form micro and macrogamets that merge into the zygote. The zygote enters the intestinal wall where it evolves in the kinet stage.

Through the hemolymph kinets reach the salivary glands of the vector, making possible the transstadial transmission of theileriosis. Here, multinucleate sporoblasts are formed which will produce uninucleated sporozoites.

During the feeding process, the ixodids will inoculate the sporozoites into the circulating stream of the vertebrate host so the life cycle is resumed (31).

Prevalence

The prevalence of piroplasmosis in small ruminants in the Balkan area varies between 21.5% (38) and 86.12% (44).

Depending on the area, the prevalence differs: In Turkey, in the central area of Anatolia, the prevalence registered through Reverse line blotting (RLB) was 34.9% (28); in the east of the country: 38.36% by PCR (6); in the south: 40.5% through RLB (39); in the southwest: 84% by PCR and 74.43% by RLB (15).

Mixed infections with two or more species of haemoparasites registered a prevalence between 30.6% (46) and 52.24% (15) established through PCR and RLB analysis). The most common association in co-infections was between *Babesia ovis* and *Anaplasma ovis* (44) and between *Theileria ovis* and *Anaplasma ovis* (46).

The most prevalent species of piroplasma are *T. ovis* and *B. ovis*. The prevalence of *T. ovis* is 17.0-67.7% (19, 38) and 0.44-70.81% for *B. ovis* (12, 44).

Depending on the area, the prevalence registered for *T. ovis* by PCR was: 21.05% (44), 35.9% (46), 35.56% (6), 54.03% (4), 58.79% (7). RLB recorded prevalences: 33.9% (28), 18.90% (9), 28.99% (12), 35.4% (39). The prevalence by IFAT was 24.6-63.2% (19, 41).

Other identified *Theileria* species are: *Theileria* sp. *MK*: 0.3-0.99% (9, 39), *T.* sp. *OT3*: 0.43% (9), *T. annulata*: 3.9% (39), *T. luwenshuni*: 1.3%, *T.* sp. *OT1*: 0.3%, *T. uilenbergi*: 0.6% (15).

The prevalences recorded for *B. ovis* by PCR are: 5.2% (46), 21.42% (1), 5.43% (6), 15.0% (45). By RLB the prevalence was: 2.6% (28), 0.44% (12). The prevalence established by the IFAT method was: 42.15% (25), 52.1% (41), 38.1% (43).

The prevalence of the other species of *Babesia* was *B. motasi*: 0.1%, 6.38%, 10.5% (15, 27, 41); *B. crassa*: 0.7%, 12.6% (15, 41); *B.* sp. 3.5% (38).

The prevalence of piroplasmosis is higher in sheep than in goats (7, 12, 41).

Vectors

In Romania, the species of ticks identified in sheep and goats are: *Ixodes ricinus, Hyalomma marginatum*, *H. inermis*, *H. sulcata, H. parva, H. concina, H. annulatus, Rhipicephalus sanguines, R. bursa, Dermacentor marginatus, Haemaphysalis punctata and Haemaphysalis parva.* (18, 24, 29, 32). *Ixodes ricinus* is the most widespread hard tick in Romania (33). The main host of *Haemaphysalis punctata* in Romania are wild and domestic ruminants (33). By PCR method *Babesia ovis* was detected in *R. bursa* collected from sheep (10).

In Bulgaria, seven species of ticks that parasitize sheep and goats were identified: *R. bursa*, *R. sanguineus*, *I. ricinus*, *Dermacentor marginatus*, *Haemaphysalis sulcata*, *H. punctata*, *Hyalomma plumbeum*. *Rhipicephalus bursa* is the dominant species. The most often encountered species of *Ixodidae* differs by season: *I. ricinus*, *D. marginatus* are found in the spring, *R. bursa*, *R. sanguineus* in the summer and *H. sulcata*, *H. punctata* in the autumn season (11).

In Greece, the identified vector species are: *R. sanguineus*, *R. bursa*, *I. ricinus*, *I. gibbosus*, *Hyalomma marginatum*, *H. excavatum*, *H. dromedarii*, *H. rufipes*, *H. impeltatum*, *H. anatolicum*, *Haemaphysalis parva*, *H. sulcata*, *H. punctata*, *D. marginatus*. The most frequently identified species differ depending on the area: *R. sanguineus* (4.47-64.8%) and *D. marginatus* (4.1-15%) (17, 21).

In Bosnia and Herzegovina, *Ixodes ricinus* was the most prevalent species found in sheep and goats followed by *D. marginatum*, *R. bursa*, *Hyalomma marginatum*, *R. sanguineus*, *Haemaphysalis punctata* (36).

In Turkey, the most common ticks that parasitize small ruminants belong to *Rhipicephalus* genus. Most of the ticks collected from sheep and goats have been identified as *R. bursa* and *R. turanicus* (3, 13, 39).

The population of ticks is influenced by the geographical-climatic conditions and differs from one area to another. Thus, in the Mediterranean part of Turkey, *Rhipicephalus* and *Hyalomma* genus predominate, while in the temperate climate region of the Black Sea, *Ixodes* genus is more popular (9).

The tick species identified in Turkey are: *Rhipicephalus bursa*, *R. turanicus, R. sanguineus*, *Ixodes ricinus*, *Hyalomma marginatum*, *H. anatolicum*, *H. detritum*, *H. excavatum*, *H. plumbeum*, *H. dromedarii*, *H. rufipes*, *H. impeltatum*, *Haemaphysalis sulcata*, *H. conccina*, *H. parva*, *H. punctata* (13, 15, 37, 39, 46).

Rhipicephalus genus contains the most important vectors which transmit babesiosis and theileriosis to small ruminants (13, 46). By PCR, the presence of *T. ovis* was detected in *R. bursa*, *R. turanicus*, *R. sanguineus* while *B. ovis* was identified in *R. bursa* and *R. turanicus* (13, 15, 44). *R. sanguineus* parasitizes usually in dogs but has also been found in sheep and goats (41). Following the analysis of the ovaries collected from female *R. bursa*, the presence of *B. ovis* was concluded (38), and in the salivary glands of the ticks, both *B. ovis* and *T. ovis* were identified (3).

Other species of *Babesia* such as *B. microti* and *B. bigeminata* have as vector *R. bursa* tick (13) while *B. motasi* and *B. crassa* are transmitted by external parasites of the *Haemaphysalis* genus (15).

R. bursa next to *Hyalomma excavatum* were also identified in the Anatolian wild goats, being the cause of paralysis (37).

The ticks can be infected with only one parasite species or they can have mixed infections. The highest frequency of piroplasmosis was found in *R. bursa*, *R. turanicus*, *Ixodes ricinus* (13). By PCR, it was found that the prevalence of *B. ovis* was 16.37% and that of *T. ovis* 19.27% in *Rhipicephalus bursa* (3, 5).

In Croatia, the ticks collected from both healthy and symptomatological positive sheep belong to the following species: *Haemaphysalis sulcata*, *H. punctata*, *Rhipicephalus bursa*, *R. turanicus*. By the means of PCR method, the piroplasmoses found in the vectors were *T. ovis* and *T.* sp. *OT3*. *Theileria ovis* was most often identified in *R. bursa* and in the blood of clinically healthy sheep while *T. sp. OT3* was detected in animals with clinical signs (23).

Clinical examination and symptomatology

Babesia ovis and *Theileria lestoquardi* are highly pathogenic especially for sheep and cause severe clinical infections. The other species are less pathogenic or non-pathogenic for small ruminants (6, 46). The pathogenicity of *B. motasi* is not reduced and appears to be moderately virulent. In contrast, *B. crassa* is considered as being non-pathogenic for small ruminants (1).

Although, the patogenity of each species is different: non-pathogenic (*Theileria ovis*) and highly pathogenic (*Babesia ovis*) in the case of coinfections the

evolution of the disease is more severe and mortality is increased (15). Coinfections with two or three different pathogens may increase the severity of tick-borne disease, especially when the infected animal is stressed (46).

Observable clinical signs in small ruminants were anaemia, anorexia, depression, weakness, lethargy, recumbency, and fever (39). The primary clinical sign shown by the infected sheep was fever (>40-41°C) followed by bristling of the head hairs, respiratory insufficiency, icterus, loss of appetite, diarrhoea and lethargy. Haemoglobinuria, jaundice and coughing were also observed (43, 44). Anaemia, anorexia and hyperthermia are characteristically for ruminant theileriosis (39, 26).

In the examined sheep acute febrile illness characterized by the temperature of 41–42°C, apathy, mild anaemia, lethargy, anorexia and slightly icteric sclerae were observed (23).

The haematological parameters of infected animals changed correlated with the level of parasitaemia. Pancytopenia is characterized by anaemia, leukopenia and thrombocytopenia and occurs in animals with a high level of parasitaemia. The platelet count (PLT), plateletcrit (PCT) are low while the mean platelet volume (MPV) is increased. In the biochemical profile, infection with *B. ovis* causes blood urea nitrogen and total bilirubin increase (43).

Haematology analyses revealed anaemia (haematocrit was 15.6 -17 % for two sheep and haemoglobin concentration was 6.4 and 6.9 g/dl). A slight leukocytosis was observed (white blood cell was 15.780/µl and 15.600/µl). Urine and blood sample were collected from both animals for further analysis. Anisocytosis, polychromasia, Heinz and Howell-Jolly bodies were observed on the blood smears. In addition, parasitic forms similar to *Babesia* spp. were detected in the erythrocytes (26).

Thrombocytopenia was one of the most prominent results in this study. All infected animals had very low PLT counts. This result shows that there is a problem in the production of platelets or excessive platelet consumption during the course of the disease. Thrombocytopenia in the infected animals can be resolved after treatment, except in the cases with severe anaemia. Normally the haematological values return to normal levels after treatment if the anaemia isn't very severe (43).

In babesiosis the anaemia was categorized as normocytic–normochromic. This type of anaemia indicates that the red blood cell production in the bone marrow is insufficient. There was no correlation between the level of parasitaemia and the degree of anaemia. This fact shows that measurement of the haematological parameters is important in understanding the severity of infection in parasitaemic animals (43).

Following acute infections, recovered animals frequently retain subclinical infections. In the case of late-stage treatment, death may occur (6). In the post-mortem examination lesions of haemolytic crisis with jaundice, gun-metal coloured kidneys and discoloured liver are observed (26).

Diagnostic methods

For the identification of babesiosis and theileriosis in small ruminants different diagnostic methods are used such as: microscopic methods, serological methods and molecular methods (8, 12, 19).

Microscopic methods. The diagnosis of babesiosis and theileriosis in the acute phase of the disease is based on the microscopic examination of the blood smear correlated with the present clinical signs (2, 6, 9).

To perform the smear, the blood is collected from the jugular vein or from the peripheral auricular vein (37, 43, 41, 44). The collected blood is used immediately for preparing smears or is stored in vacutainers with EDTA for later use (2, 27, 37, 41).

The sample is dried at room temperature, fixed with absolute methanol for 5 minutes, then stained with 5%-10% Giemsa solution for 30-45 minutes or by Diff Quick staining method. The smear is examined with the x100 immersion objective to identify intracellular piroplasms. (1, 2, 12, 23, 27, 38, 41, 43, 44).

The smears were considered negative for piroplasmosis if no parasite was identified in 20-50 microscopically analysed fields (1, 2, 9, 39, 44).

Serological methods. Serological methods are used to establish the diagnosis in subclinical infections (4, 6).

The most commonly used serological method for identifying piroplasmosis is indirect immunofluorescence - IFAT (25, 41, 43).

The disadvantage of the IFAT method in establishing the diagnosis is the presence of cross-reactions between species of the same kind of parasites, which creates false positive / false negative results (6, 40).

The highest seropositivity was encountered for ruminants over 12 months old (25). The recorded antibody titter was not directly proportional to the level of parasitaemia, however, piroplasmosis was observed in a higher percentage in seropositive animals than in seronegative animals (41). The level of seropositivity increased directly in proportion to the degree of anaemia, from 12.5% to 100% (43).

Molecular methods. Polymerase chain reaction (PCR) can detect piroplasmosis in both symptomatic and asymptomatic animals.

A PCR method was developed for detecting *B. ovis* in small ruminants using 2 oligonucleotide primers (Bbo-F 50-TGGGCAGGACCTTGGTTCTTCT-30 and Bbo-R50CCGCGTAGCGCCGGC-TAAATA-30) targeting fragment 549-bp of the parasite gene ssu rRNA. By PCR, *B. ovis* could be detected when the parasitaemia was 0.00001% resulting an increased sensitivity of the test (more sensitive than examining 200 fields under the electronic microscope) (1).

By reverse line blooting (RLB) technique, primers RLB-F2 (50-GACACAGGGAGGTAGTGACAAG-30) and RLB-R2 (Biotin-50-CTAAGAATTTCACCT-CTGACAGT-30) were used to amplify the bands ~ 390 and

~ 430 that correspond to the hypervariable region V4 of *Theileria* spp. and *Babesia* spp. (6).

The sensitivity of PCR and RLB methods were compared for the detection of *Theileria* sp. *MK*. Primers RLB-F (GAGGTAGTGACAAGAAATAACAATA) and RLB-R (TCTTCGATCCCCTAACTTTC) were used in the RLB method to amplify the variable fragments ~ 460 and ~ 520 bp for *Babesia* and *Theileria* species. RLB is a good diagnostic option for epidemiological study. By PCR method *Theileria* sp. *MK* was detected using the primers Tmk-F (CATTGTTTCTTCTCATGTC) and 990 (TTGCCTTAAACTTCCTTG) to amplify the 757 bp fragment of the 18S ssu rRNA gene. PCR is superior to RLB because it is easy to use and does not require much time. By both methods the lowest concentration in which piroplasms could be identified in the blood corresponded to a 0.0001% parasitaemia (8).

To identify *Babesia* spp. and *Theileria* spp., the PCR method was used within the primers RLB F2 (5` GAC ACA GGG AGG TAG TGA CAA G`3) and RLB R2 (5`TCT TCG ATC CCC TAA CTT TC`3) which amplify the 460 and 520-bp fragments of the ssu rRNA gene. For the RLB method, the primers RLB F2 and RLB R2 were used, which amplified the region of the hypervariable V4 of the 18 S rRNA gene (28).

Catchall PCR was used to detect *Babesia* spp. and *Theileria* spp. The used primers were RLB-F2 (5 '-GACACAGGGAGGTAGTGACAAG) and RLB-R2 (biotin-5' -CTAAGAATTTCACCTCTGACAGT) which amplify the 390-430 bp fragment of the V4 hypervariable region of the 18S gene (9).

For *Babesia* and *Theileria* species identification the primers RLB F2 (5'-GACACAGGGAGGTAGTGACAAG-3') and RLB R2 (biotin-5'-CTAAGAATTTCACCTCTGACAGT-3') were used to amplify the 3604-4p fragments of the hypervariable 18 S4 rRNA gene (12).

The primers RLB-R2 (Biotin-5'-CTAAGAATTTCACCTCTGACAGT-3 ') and RLB-F2 (5'GACACAGGGAGGTAGTGACAAG-3') were used to amplify the 360-430 bp fragments of V4 hypervariable region for *Theileria* and *Babesia* 18s rRNA gene from vectors. Molecular techniques allow the pathogen detection at a low level of parasitaemia and makes possible the identification of coinfected hosts (13).

In a study developed to detect the prevalence of piroplasmosis in wild goats in Turkey, PCR method was performed. The primers used for *Babesia* spp. were BJ1 (5'-GTC TTG TAA TTG GAA TGA TGG-3 ') and BN2 (5'-TAG TTT ATG GTT AGG ACT ACG-3') to amplify the 18s rRNA gene fragment. For *Theileria* spp. the nested PCR method was used with Thei-F1 plus Thei-R1 * and Thei-F2 plus Thei-R2 * primers to amplify the 18s rRNA fragment (37).

Specific species PCR is recommended to estimate the prevalence of the pathogen and to identify the coinfected hosts. RLB can detect the coinfections but not all of them, so the specific species PCR method is recommended for establishing the prevalence (15).

Primers Bbo-F5 '(Bbo-F 50-TGGGCAGGACCTTGGTTCTTCT-30) and Bbo-R3' (Bbo-R50CCGCGTAGCGCCGGC-TAAATA-30) were used to amplify the 549-

bp portion of the *B. ovis* ssu rRNA gene and the 520 bp portion of *T. ovis* ssu rRNA within the species-specific PCR method (44).

The prevalence for *T. ovis* was determined by nested PCR method. The outer primers used were TS sr 170F (5′-TCGAGACCTTCGGGT-3 ′) and TS sr 670R (5′-TCCGGACATTGTAAAACAAA-3 ′). Internal primers were TS sr 250FN (5′-CGCGTCTTCGGATG-3′) and TS sr 630 RN (5′-AAAGACTCGTAAAGGAGCAA-3 ′) to amplify the 398-bp fragment of the *T. ovis* ssu rRNA gene. By nested PCR method, it was possible to detect a parasite cell from 107 sheep erythrocytes, which is the equivalent of a 0.00001% parasitaemia. The nested PCR method has higher sensitivity and specificity than single round PCR, being able to detect parasitic DNA in blood with 0.00001% parasitaemia while single round PCR detects it in the case of 0.0001% parasitaemia (4).

For detecting *Babesia* spp. and *Theileria* spp. the RLB method was used; the primers RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3 ') and RLB-R2 (Biotin-5'-CTAAGAATTTCACCTCTGACAGT-3') were used to target the 460 -540 bp fragment of V4 hypervariable region for 18S rRNA gene. By comparing the diagnostic methods the rate of piroplasms identification was found to be higher for RLB than for the other two methods (PCR and microscopical examination). This method has the advantage of detecting piroplasms at a very low concentration of parasitaemia (10-8%) and to identify simultaneously *B. ovis* and *T. ovis* by using specific oligonucleotides (38).

In a study developed in Greece, the RLB method was performed to identify the species of *Theileria* and *Babesia* in ticks collected from small ruminants. The primers used were those of the catch all variant (5'-TAATGGTTAATAGGAGCAGTTG-3 ') targeting the hypervariable region V4 of the 18S rRNA gene (17).

Treatment

Ruminants diagnosed positively following examination of blood smear and clinical signs were treated with imidocarb dipropionate at a dose of 1-1.2 mg / kg by intramuscular injection (26, 39, 43).

The second dose is given after 10 days (26). In severe cases, vitamin B12 20μg / kg was also administered by intramuscular injection (44).

Conclusion

Piroplasmosis of small ruminants is a problem of economic and veterinary interest in the Balkan area, especially in the countries dealing with sheep and goat farming. The species with the highest prevalence in small ruminants are *Theileria ovis* and *Babesia ovis*. Depending on the geographical region and the method of diagnosis used the prevalence recorded differs between 21.5-86.12%. Piroplasmosis is diagnosed more often in sheep than in goats.

The most common vectors identified are *R. bursa* and *I. ricinus*. The primary method of diagnosis consists in examining the stained blood smear, but the most accurate and efficient method of diagnosis is PCR.

A lot of evidence is needed regarding the current situation of small ruminant piroplasmosis in the Balkan area to know the distribution of the involved pathogens in limiting the spread.

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USE OF THE BURSAL INDEX FOR DISCRIMINATION OF THE AVIAN BURSAL INFECTION DISEASE VIRUS STRAINS

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Summary

Infectious avian bursal disease or Gumboro's disease, is an infectious-contagious disease, produced by a specific virus, which affects chickens in the first 3-6 weeks of life, being characterized anatomically-clinically by altering the general condition, diarrhea with cretaceous white feces, decubitus, immunosuppression, catarrhal or catarrhal-hemorrhagic inflammation of the Fabricius bursa, hemorrhagic myositis and nephrosis-nephritis.
Keywords: Infectious avian bursal disease, bursal index, very virulent strains plus

The avian infectious bursal disease virus, from an antigenic point of view, contains two serotypes, in serotype 1 being included strains classified in 6 antigenic subtypes called variants. Depending on the pathogenicity there are a few pathotypes: classic, very virulent, very virulent plus and attenuated vaccine strains (2, 4).

VP2 protein is present in the structure of viral RNA, which plays an important role in inducing neutralizing antibody synthesis. This protein is encoded by a hypervariable region gene that includes fragments present in the highly virulent pathotype (sequence vv) and fragments present in attenuated strains (sequence att) (1, 9).

For the discrimination of strains of the infectious avian bursal disease virus, the bursal index can be used. Bursal index represents the ratio between the weight of the Fabricius bursa and the body weight, having 3 groups of limit values. This index was initially used to assess the intensity of the immune response induced by vaccines for infectious avian bursal disease immunoprophylaxis (9, 10).

The researches were carried out in order to demonstrate the importance of calculating the bursal index for discriminating pathogenic strains from attenuated vaccine strains, in a primary outbreak of infectious bursitis from a broiler flock and for confirming the results it was used real-time Polymerase Chain Reaction (rPCR).

Materials and methods

The researches that are the subject of this work were carried out in a flock of 24000 broiler chickens, existing in two shelters from a farm in the western part of

the country where the infectious avian bursal disease evolved. This disease started as a primary outbreak at the age of 3 weeks.

This outbreak was observed by clinical and pathological anatomy examination, the necropsies being performed on fresh bodies for the detection of specific lesions and for the collection of Fabricius bursals.

For calculating the Bursal index were used 10 broiler corpses from each shelter. After the autopsy, each bursal and the corresponding corpse were weighed separately and the values obtained were recorded in the table.

The bursal index was calculated according to the formula: the weight of the Fabricius bursa multiplied by 100 and the result obtained was divided by the weight of the corpse. The data obtained were written in the table and after the arithmetic mean was calculated (7, 8).

To confirm the infectious avian bursal disease, the real-time Polymerase Chain Reaction technique was used, which was performed in the molecular biology laboratory of the SN Pasteur institute in Bucharest (1, 5).

Results and discussions

In the respective farm infectious avian bursal disease debuted in two shelters of broiler chickens at the age of 3 weeks in the form of a primary outbreak because the disease was not reported in the previous series of broiler chickens.
The characteristic symptoms evolved between the 21 and 27 days of the life of the chickens being represented by depress, decubitus, loss of appetite and diarrhea with white cretaceous feces. The morbidity increased progressively from the first day until the 5th day, after which it started to decrease, and the mortality was maximum also during the first 3 days of evolution (Fig. 1).

Fig. 1. Bursal infectious disease – clinic aspect

The following lesions were noted by the daily autopsy from broiler corpses: catarrhal or hemorrhagic catarrhal, nephrosis, deposits of urate in ureter and hemorrhages in the musculature of the leg. The Fabricius bursas were increased in volume with the inflamed lamellae and a yellowish white fibrin content (Fig. 2 and Fig. 3).

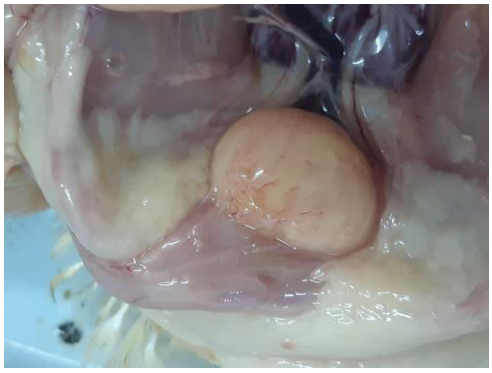

Fig. 2. Fabricius bursa with catarrhal evolution

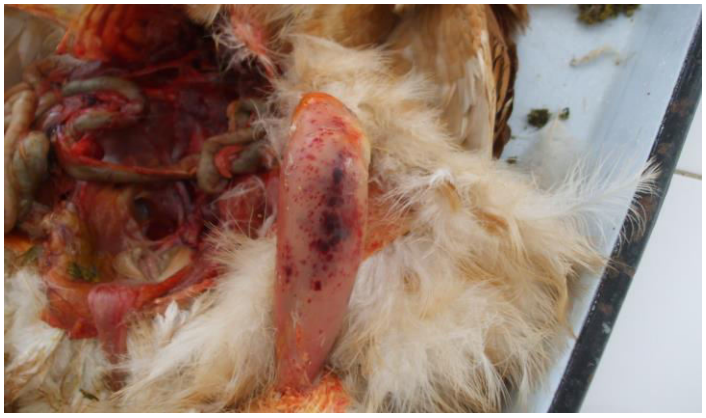

Fig. 3. Hemorrhages in the muscles of the leg

The above mentioned laboratory technique (rPCR) confirmed the disease and the strains of the highly virulent plus pathotype were detected, with the amplicons weighing 141 bp.

In order to discriminate the strains of the infectious avian bursal disease virus who produced the primary outbreak in the mentioned farm, the bursal index calculated at 20 bodies was used and the values obtained are shown in Table 1.

This index allows to assess the virulence of infectious avian bursal disease virus strains that produce outbreaks of disease and their differentiation from intermediate and vaccine strains.

The characteristic values of each group of strains are: over 0.9 attenuated vaccine strains, 0.7-0.9 intermediate strains and below 0.7 virulent strains. Frequently the values of 0.2-0.3 indicate the presence of very virulent plus strains (7, 8).

The results obtained regarding the calculation of the bursal index for the corpses studied are shown in Table 1. Analyzing these results were found we can see that the values of this index were different as follows: at 8 corpses the bursal index was below 0.2, at 9 corpses the values of the bursal index were between 0.2 and 0.3, at 2 corpses the values were between 0.3 and 0.4, and for a single corpse the value of the bursal index was between 0.5 and 0.6.

Comparing the results obtained with the limits of the values of the 3 groups it turns out that in 17 corpses, the bursal index, through the obtained values, shows that the strains responsible for producing the outbreak are very virulent plus. Only at 3 corpses the values of this index are characteristic of virulent strains. The arithmetic mean of bursal index values is between 0.2 and 0.3, indicating that infectious avian bursal disease virus strains as a whole were highly virulent plus.

The results provided by the evaluation of the bursal index were confirmed by the rPCR technique which detected very virulent plus strains and a vaccine strain, the results being shown in Fig. 4.

The highly virulent infectious avian bursal disease virus strains circulate frequently in broiler farms, are responsible for the breakdown of the specific immunity of the chicks and for the outbreak of infectious avian bursal disease outbreaks. These strains frequently occur as a result of an escalation phenomenon generated by the new vaccines used in the prophylaxis of the disease associated with the new vaccination pathways. This phenomenon induces the appearance of the mutant stems that through repeated passages gain in virulence, finally being classified in the very virulent pathotype (3, 4, 6).

Table 1

Corpse weight, bursal weight and bursal index

Age 21 days			
No. Corpse	**Corpse weight (kg)**	**Bursal weight (g)**	**Bursal index**
1	1.362	3	0.220
2	1.550	8	0.516
3	1,379	2	0.145
4	1,256	4	0.318
5	1.490	4,42	0.296
6	1,278	4	0.312
7	1,172	3	0.255
8	1,169	2,39	0.204
9	1,235	3	0.242
10	1,170	2,32	0.198
11	1,323	2,22	0.167
12	1,189	3,26	0.274
13	1,408	2,49	0.176
14	1,556	3	0.192
15	1,362	2,02	0.148
16	1,189	3	0.252
17	1,434	2	0.139
18	1,306	2,26	0.173
19	1,309	3	0.229
20	1,487	3	0.201
		Bursal index (average)	0.23285

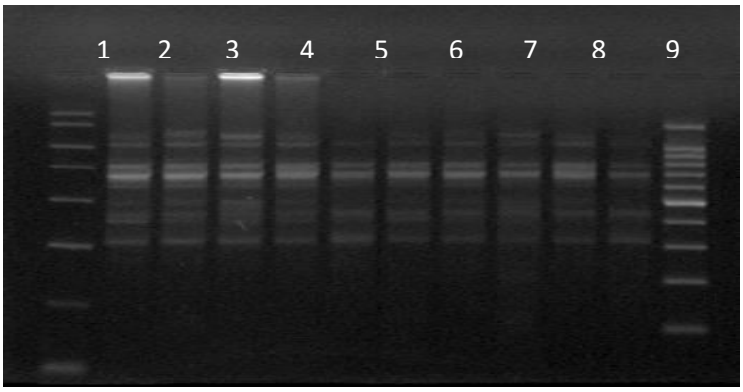

Fig. 4. Gel-electrophoresis post RAPD-P1. Line 1 – standard ADN, PCR Marker (Sigma, P2993); line 2 – 2022-1 vv; line 3 – 2022-2 vv; line 4 – 2024-1 vv; line 5 – 2024-2 vv; line 6 – IPIplus, positive control IBDV-vv; line 7 – 2022-1 att; line 8 – 2022-2 att; line 9 – 2024-1 att; line 10 – 2024-2 att; line 11 – IPI, positive control IBDV-att; line 12 – standard ADN, 100bp

Conclusions

After the bursal index was made, it was concluded that the viral strains are very virulent.

Confirmation of the disease was performed using the polymerase chain reaction technique with reverse transcriptase.

The use of the bursal index established that the outbreak of the monitored infectious avian bursal disease was triggered by some very virulent strains because the obtained values corresponded to the index limits specific to these strains.

This index can be used in routine research on monitoring infectious avian bursal disease outbreaks, produced by very virulent strains.

The molecular biology technique real-time Polymerase Chain Reaction detected the highly virulent strains, confirming the results obtained by calculating the bursal index.

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INCIDENCE OF HEMATURIA IN SMALL COMPANION ANIMALS

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Summary

Hematuria can be undetectable on gross examination of urine samples, or the red blood cells (RBCs) can impart a variety of colors to urine and must be distinguished from other components that discolor urine.

This study was conducted in the Universita Degli Studi Di Perugia, Dipartimento Di Patologia, Diagonstica E Clinica Veterinaria, in the period between 23.09.2019 – 25.11.2019, using IDEXX UA dipstick ad IDEXX VetLab UA.

The study recorded 34 animals with gross and microscopic hematuria. In case of hematuria prevalence by species, felines obtained the precentage of 67.65% and canine of 32.35%. Based on gender, hematuria prevalence registered the precentage of 29,41 % for females and 70,59 % for males.

Of the 34 samples analyzed 47,06 % were diagonsed with macroscopic hematuria and 52,94 % with microscopic hematuria.

Hematuria is a common problem encountered in feline and canine medicine. There are many possible causes, ranging from infections to infiltrative cancers. A logical and thorough diagnostic investigation is needed to ensure an accurate diagnosis and the most appropriate therapies are initiated.

Keywords: hematuria, incidence,dogs, cats

Hematuria can be macroscopic or microscopic. Macroscopic hematuria is grossly visible in the urine, while microscopic hematuria is not apparent with the naked eye and is variably defined (3, 4). There are numerous causes of hematuria, including pathology of the urinary and genital tracts, as well as more generalised bleeding disorders (1, 5). Although hematuria can be asymptomatic, specific clinical signs may provide clues about the underlying problem (8, 10).

Hematuria can be undetectable on gross examination of urine samples, or the red blood cells (RBCs) can impart a variety of colors to urine and must be distinguished from other components that discolor urine (2, 6, 8).

Materials and methods

This study was conducted in the Universita Degli Studi Di Perugia, Dipartimento Di Patologia, Diagonstica E Clinica Veterinaria, in the period between 23.09.2019 – 25.11.2019, using IDEXX UA dipstick ad IDEXX VetLab UA.

In order to establish the incidence of hematuria in dogs and cats, fresh midstream urine samples (5-10 mL) were obtained from animals with urologic signs.

We evaluated 34 samples, 23 from feline and 11 from canine. The urine samples were first evaluated macroscopically, and its color and clarity were recorded; than we used IDEXX UA dipstick for detecting the blood in IDEXX VetLab UA Analyzer and Clinical Refractometer RHCN-200ATC for measuring the urine specific gravity.

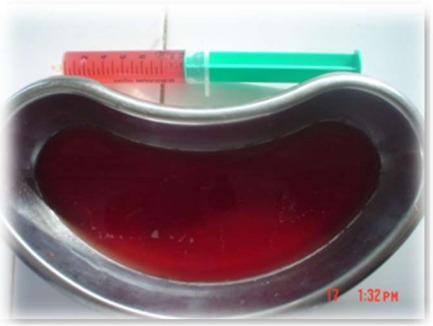

Fig.1. Gross hematuria

Fig.2. Gross hematuria

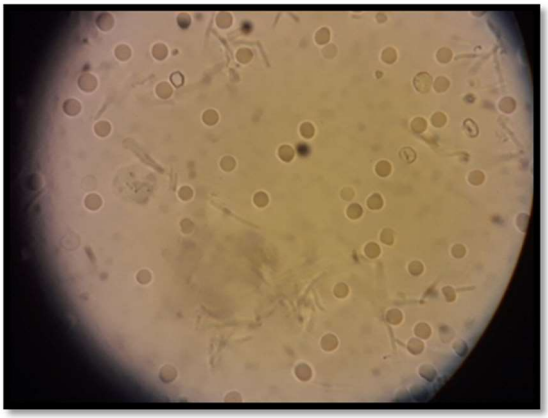

Fig.3. Microscopic hematuria

Results and discussions

The study recorded 34 animals with gross and microscopic hematuria. In case of hematuria prevalence by species, felines obtained the precentage of 67.65% and canine of 32.35% (Fig. 4). Based on gender, hematuria prevalence registered the precentage of 29,41 % for females and 70,59 % for males (Fig. 5).

Based on this analysis and on the diagnosis established, the felines are more susceptible to manifest hematuria, being liable to develop FUS (feline urological syndrome), urinary lithiasis and cystitis (9).

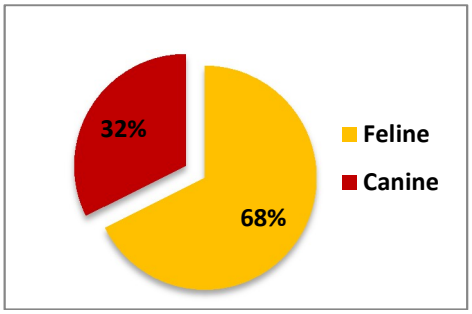

Fig. 4. Hematuria prevalence by species

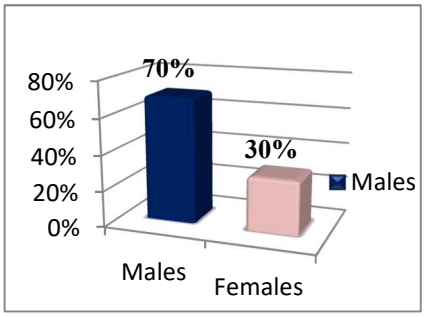

Fig. 5. Hematuria prevalence by gender

Of the 34 samples analyzed 47,06 % were diagonsed with macroscopic hematuria(Fig. 1, Fig. 2) and 52,94 % with microscopic hematuria (Fig. 3, Fig. 6).

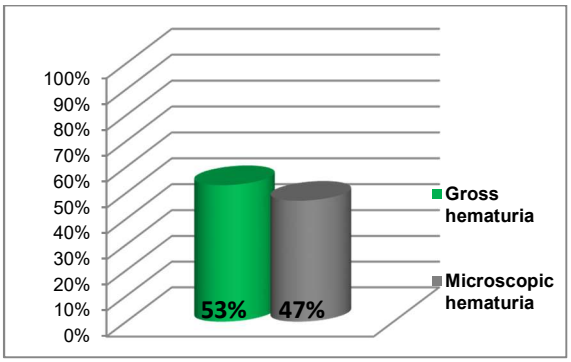

Fig. 6. Gross hematuria and microhematuria

Conclusions

Hematuria is a common problem encountered in feline and canine medicine. There are many possible causes, ranging from infections to infiltrative cancers. A logical and thorough diagnostic investigation is needed to ensure an accurate diagnosis and the most appropriate therapies are initiated.

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THE EVALUATION OF THE THERAPEUTIC RESULTS APPLIED IN CASE OF DIFFERENT DERMATOPHYTOSIS EPISODES

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Summary

Dermatophytosis is a superficial, contagious skin infection produced by the genera *Microsporum* and *Trichophyton*. It is an important skin disease because it is contagious, infectious and can be transmitted to people. In the therapeutic protocol the offer is generous, treatment being possible topically with itraconazole 1% gel, clotrimazole, miconazole, lime sulfur, enilconazole and systemic with griseofulvin, ketoconazole, itraconazole. 22 dogs (12 out of 22 dogs with clinical signs), 45 cats (34 out of 45 cats with clinical signs) and 2 goats (only one goat with clinical signs) diagnosed with dermatophytosis were treated topically twice weekly with eniconazole 2% solution. The environment was disinfected weekly with antibacterial and antifungal solution. Despite the enilconazole recommendation for cattle, horse and dog, the skin lesions of cat and goat were cured after treatment with enilconazole without side effects.

Keywords: dermatophytosis, cat, dog, goat, therapy

Superficial mycosis is represented by fungal infections which affect the superficial layer of the skin, hair and claw, can be produced by dermatophytes and non-dermatophytes. The first one produce dermatophytosis and the second one dermatomycosis (5, 11).

Dermatophytosis is a superficial, contagious skin infection that is spread most commonly via direct contact with another infected animal but can be spread by traumatic fomite inoculation (4, 7, 10).

The most common pathogens of small animals belong to the genera *Microsporum* and *Trichophyton*. It is an important skin disease because it is contagious, infectious and can be transmitted to people (5, 7, 9).

The infection is always follicular, the lesions usually start with the appearance of agglutinated hair strands at the base of a crust of several millimeters in diameter, after which the hair falls, leading to the appearance of a circular shape depilated area, well delimited, characteristic ring lesion (11).

In most dermatophytosis cases, *Microsporum canis* is the identifiable causative agent. Although typically self-limiting by 4 months after the onset of clinical lesions, treatment of affected cats is recommended to shorten the clinical course of disease and reduce the risk of spreading infection (2, 6).

Current treatment recommendations include the use of systemic antifungal drugs to eradicate the infection within the intrafollicular portion of the hair and

concurrent topical therapy to disinfect the haircoat and environmental cleaning to remove infective material (3).

Itraconazole has been shown to be effective when administered as pulse-dose therapy (week on–week off) for systemic treatment of human dermatophytosis because it binds tightly to keratin and persists in the stratum corneum for up to 4 weeks after discontinuation of oral administration. Concentrations above minimum inhibitory concentration levels (0.1 µg/ml) were detected in cat hair after cats received 5 mg/kg q24h for three alternating weeks. This finding, and a favorable safety profile, made it an attractive alternative to griseofulvin and ketoconazole. Between 1995 and 2016, there were 12 reports on itraconazole use to treat feline dermatophytosis (10).

In therapeutically protocol the offer is generous, treatment being possible topically with itraconazole 1% gel, clotrimazole, miconazole, lime sulfur, enilconazole, and systemic with griseofulvin, ketoconazole, itraconazole (11).

The important thing is to take into consideration every epidemiological situation and the treatment of animals with clinical lesions, asymptomatic ones, which are the main reservoir of *Microsporum canis* (5,7).

At the same time is essential the environmental deep cleaning, due to the increased spores resistance (months, years), owners treatment has to be considered because of the zoonotic character of the disease (1).

In this context the research purpose was to evaluate the therapeutical results applied in case of animals with/without clinical symptoms, environment and owners diagnosed in different dermatophytosis episodes.

Materials and methods

In this study were treated 22 dogs (12 out of 22 dogs with clinical signs), 45 cats (34 out of 45 cats with clinical signs) and 2 goats (only one goat with clinical signs).

The diagnostic was based on anamnesis, clinical signs and culture media microscopic examination. The samples were incubated at 25°C used Dermatophyte Test Medium plate (DTM) (3) (Fig. 1).

The therapeutic protocol was isolation of the animals with clinical signs, application of the topic treatment on the symptomatic and asymptomatic animals, cleaning the environment with antifungal disinfectant and the recommendation for the owners to consult a specialist.

The treatment was performed used enilconazole 0.2% twice weekly, on the whole cats and dogs haircoat and only on the mammary goat lesions.

The environment was disinfected weekly with antibacterial and antifungal solution (Fig 2).

The therapeutic protocol stopped when the DTM results indicated the absence of dermatophytes macroconidia.

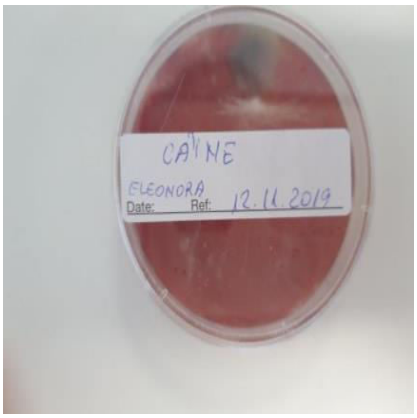

Fig.1. Dermatophyte Test Medium

Fig. 2. Antifungal solution

Results and discussions

The anamnesis, clinical signs and microscopic examination of DTM macroconidia established the dermatophytosis diagnostic produced by *Microsporum spp*. (Fig. 3-6).

Fig. 3. Skin lesions - cat

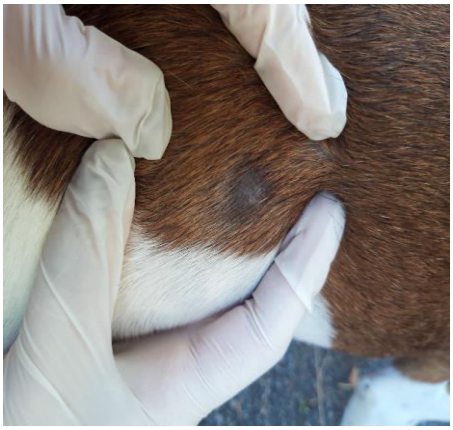

Fig. 4. Skin lesions - dog

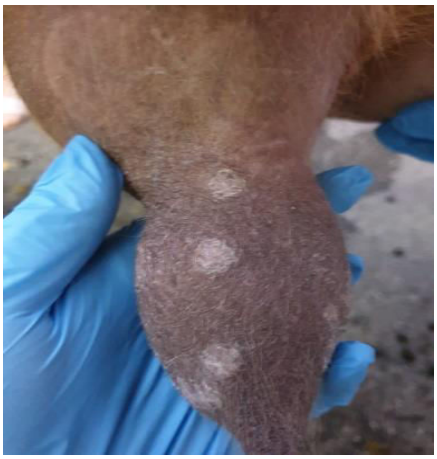

Fig. 5. Skin lesions – goat

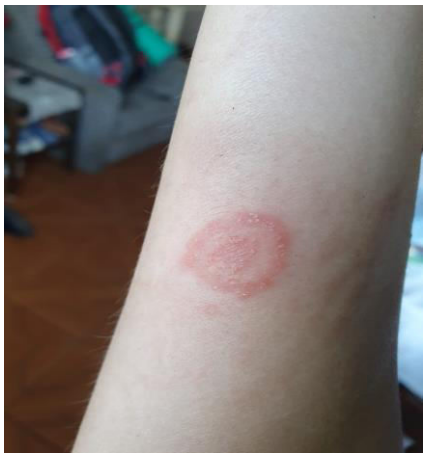

Fig. 6. Skin lesions - owner

The results of the study revealed more rapid clinical healing in young animals (1.5 month) compared with adult animals (2.5 months) and in cats (1.5 month) compared with dogs (2 month).

The absence of dermatophytes macroconidia in DTM was observed after 6 months of treatment and environment disinfection.

The goat skin lesions have been recovered after 1 month of treatment. The negativ results of DTM was performed after 4 months of treatment and environment disinfection. No side effects in cats, dogs and goat after enilconazole therapy (Fig. 7-12).

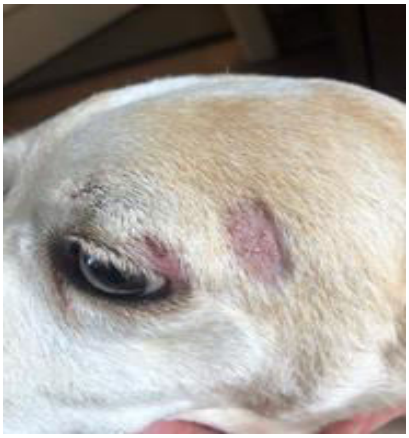

Fig. 7. Before treatment

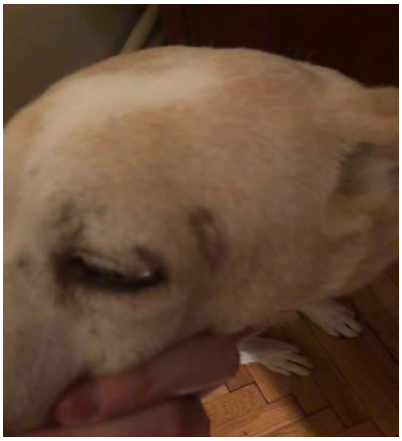

Fig. 8. Ater 2 weeks treatment

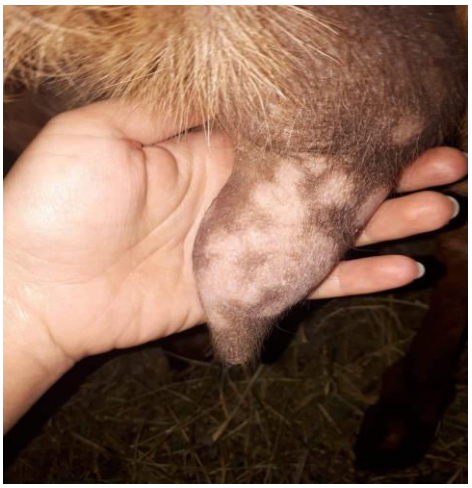

Fig. 9. Before treatment

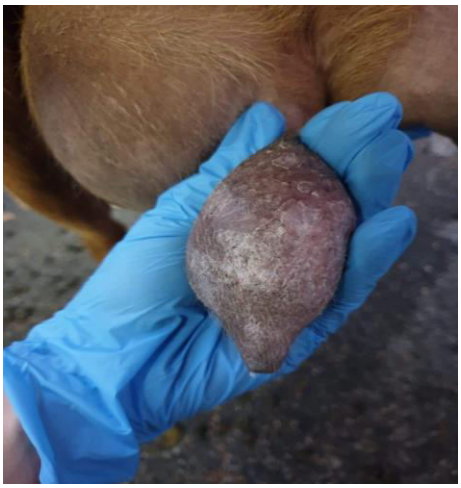

Fig. 10. After 3 weeks treatment

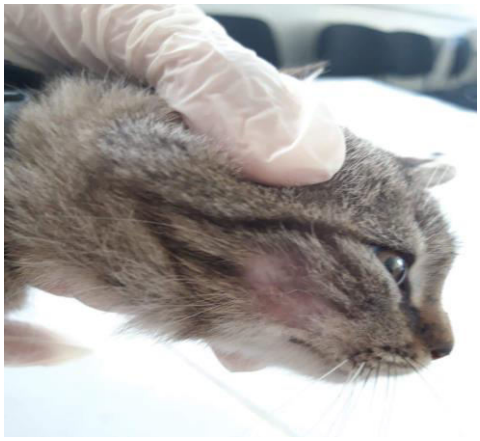

Fig. 11. Before treatment

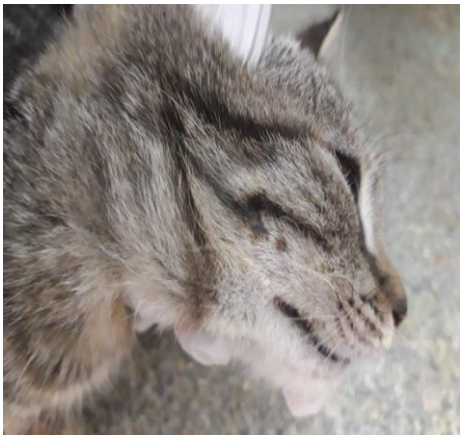

Fig. 12. After 2 weeks treatment

The therapeutic results of our study could be compared with the results of other authors. For an efficient treatment, all animals should be treated, even humans if it is the case, while treatment should not be interrupted untill the DTM culture will be negative, because hair grow and disappearance of the clinical signs are not indicators of complete healing. Prevention is the solution to avoid spreading. Immunosuppression and stress are detrimental.

Current industry treatment protocols for feline dermatophytosis all recognize the need for concurrent systemic and topical antifungal therapy. Systemic therapy eradicates the infection from within the hair follicle where topical applications are unlikely to penetrate. Topical therapy kills viable fungal spores adhered to the haircoat that could potentially result in positive fungal culture scores and act as a potential source of infection for in-contact animals and people (10).

The first controlled study to closely monitor the effects of oral itraconazole administered at 5 mg/kg using a pulse-dose (week on–week off) regimen, in the absence of topical antifungal therapy, for the treatment of dermatophytosis was performed in 2017. The results demonstrate that itraconazole produced clinical cure and facilitated and reduced the time to mycological cure compared with untreated controls. The results demonstrate the potential for systemic itraconazole use as a cure for *M. canis* dermatophytosis and underscore the need for adjunctive topical therapy to eliminate infection and reduce the risk of exposure of in-contact animals and people (10).

Even if the enilconazol is recommended only for bird, cattle, horse and dog, the latest findings support twice weekly application of enilconazole and lime sulfur, and application of adjuvant focal topical therapy daily or every other day (8, 12).

Conclusions

We recommend the use of topic enilconazol 2% solution, twice weekly, in the treatment of dermatophytosis until clinical recovery and negative results of Dermatophyte Test Medium plate (DTM).

The treatment of asymptomatic animals, the antifungal environment disinfection and the owner`s information about the zoonotic risk of infestation are the most important chains of the parasitological control.

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HEART RHYTHM DISORDERS IN DOGS WITH RENAL FAILURE MONITORED BY THE HOLTER METHOD

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Summary

Holter monitoring is useful to correlate arrhythmias with clinical signs, given that renal failure is frequently associated with electrolyte imbalances that have direct correlations with changes in heart rhythm. In patients undergoing hemodialysis, monitoring the electrical changes are associated with the electrolytic disorders. Holter monitoring also tracks changes in cardiac output to correlate intensive therapy with the hemodynamic response. Holter monitor records heart rate through electrodes.

Keywords: hemodialysis, dogs, rhytm disorders, holter

Holter monitoring is useful to correlate arrhythmias with clinical signs, given that renal failure is frequently associated with electrolyte imbalances that have direct correlations with changes in heart rhythm. In the renal patient monitoring, the electrical changes are associated with the electrolytic disorders (4). Holter monitoring also tracks changes in cardiac output to correlate intensive therapy with the hemodynamic response (2). Holter monitor records heart rate through electrodes (1, 4).

Materials and methods

The Holter ECG TLC5000 system (one of the smallest ECG recording devices in the world) (Fig. 1), is composed of a recorder that displays the shape of the ECG waveform in real time, which has as an analysis function the R point, ST segment, T wave, QT and the ability to graphically review their trends. It presents analysis functions for heart rate variability that can be set for 5 minutes, 1 hour or 24 hours (3).

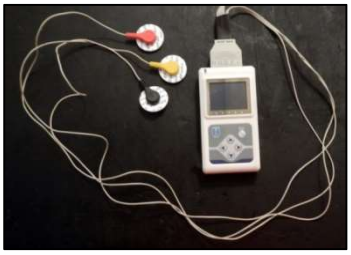

Fig. 1. Holter ECG TLC5000 (orig.)

The researches of the present study were carried out in the Clinic of the Faculty of Veterinary Medicine of Bucharest, at the Dialysis Center, between January 2015 - June 2017. During the research, over 15 cases of dogs with renal failure were taken into the study. All dogs were monitored by the Holter electrocardiographic method for a standard 24-hour period, during which they were under hydro-electrolytic support therapy. The interpretation of the electrocardiogram results was done by Dr. Florin Leca, who provided us with the Holter ECG TLC5000 System.

The Holter monitoring started by clipping the areas where the electrodes and adjacent cables were to be located. Before being applied to the patient, the electrodes were connected to the adhesive applicators. Adhesive applicators are impregnated with contact gel for ECG. They are not irritating to the skin of the patient. We used 3 electrodes connected to the adhesive applicators (Fig. 2, Fig. 3).

Fig. 2. Electrode attached to the adhesive applicator (orig.)

Fig. 3. Electrodes connected to the adhesive applicators (orig.)

The electrodes were applied, with the adhesive part of the applicators on the clipped areas of the patient's body in the following order: the red electrode (R), on

the right side, at the level of the intercostal space 3; the yellow electrode (L) on the left side at the level of the intercostal space 4 and the black electrode (N) at the sternal level.

It is important that the electrodes connected to the adhesive applicators to be in excellent contact with the skin of the patient, in order to avoid the artifacts that will interfere with the Holter registration (Fig. 4).

Fig. 4. Application of electrodes on patient (orig.)

All electrodes and conducting wires were protected by applying an elastic band and wrapping it around the patient (Fig. 5). Then, the Holter monitor was attached, turned on and set. The monitor was left on for 24 hours. Applied in this way, the monitor does not affect the patient's activity in any way.

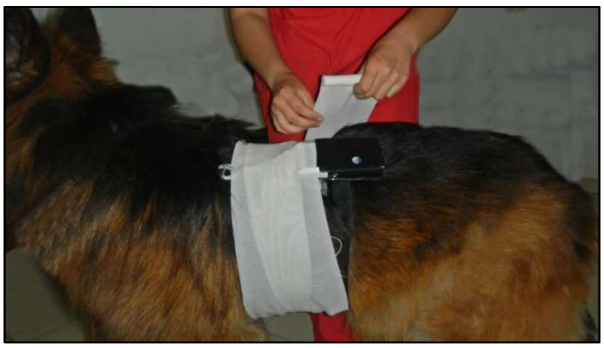

Fig. 5. Application of an elastic band to protect the conductive wires (orig.)

The heart rate was recorded digitally on a memory card, which was downloaded later, in the electronic system equipped with a special program, for analysis.

To remove the monitor, it was stopped and carefully removed from the elastic tape and the adhesive electrodes.

The recordings were analyzed using the special program for analyzing ECG recordings in real time.

During the research, 15 patients with renal impairment belonging to different breeds, of different ages and sexes, who underwent electrolytic therapies (specific to renal disorders) and Holter monitoring were taken into account. Of these, 6 representative cases were evaluated in this study, which can be subjected to the rigors of a scientific study. The excluded cases had several reasons for exclusion (imminent death at the time of the Holter system installation or immediately thereafter, detachment of electrodes during monitoring or data recorded without statistical character).

The study was conducted on 3 males and 3 females, of different breeds and aged between 2 years and 8 years. Each patient was given a clinical observation record in which the data observed at the patient inspection, anamnesis, the results of the paraclinical examinations (biochemical examination, complete blood count, ultrasound examination, etc.) were passed. Patients submitted to hemodialysis were provided with hemodialysis sheets, for each hemodialysis session, in which all information regarding the parameters used to establish the therapy was noted.

Results and discussions

Holter monitoring is an integrated and important part of the renal impairment therapy in patients submitted to hemodialysis. Also, the corroboration of the hemodynamic data obtained by other methods (echocardiography, tensiometry, hematological examinations etc.) with the Holter electrocardiogram helps to establish in real time the positive or negative evolution of the treatment in renal failure in dogs. All the pathologies identified in this study are presented and described in the images below (Fig. 6-10).

The 60-minute heart rate variability is an indirect indicator for evaluating the hemodynamic response in renal disease, by quantifying the cardiac response to pre-pregnancy and post-pregnancy changes.

During this study, we have selected 6 cases and we observed the most often occurring pathologies: from the total of 6 cases taken into the study, hyperkalemia was found in only one patient, hypokalemia in 4 cases, hypernatremia in 3 cases, hyponatremia in only one case, hyperchloremia in only one case and hypochloremia in one case.

Of all the patients studied, 3 had only one electrolytic imbalance (hyperkalemia = n. 1; hypernatremia = n. 1, hypokalemia = n. 1) and 3 cases had multiple electrolytic imbalaces (hyponatremia, hypokalemia and hypochloremia = n.

1; hypernatremia and hypokalemia = n. 1; hypernatremia, hyperchloremia and hypokalemia = n. 1) (Table 1).

During our study, we have observed at the EKG: sinus rhythm with negative P wave, with terminal segment sub-leveling (Fig. 6) and the disappearance of the P wave, the widening of the QRS interval, the reduction of the heart rate (Fig. 7), or the installation of ventricular fibrillation followed by asystole (Fig. 8).

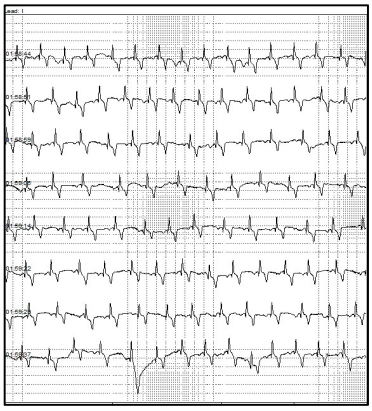

Fig. 6. EKG during dialysis (orig.)

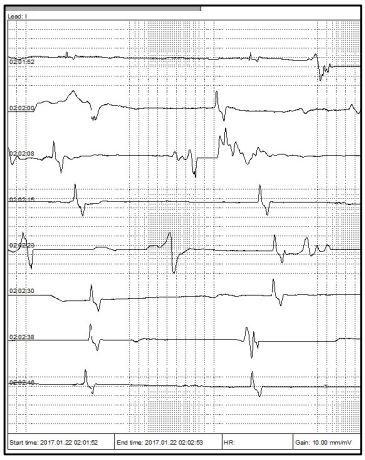

Fig. 7. EKG during dialysis (orig.)

We have also accomplished the graphical representation of the calculated heart rate variability indices by the Time-Domaine method with the Holter TLC5000

electrocardiograph for 60 minutes (Fig. 9) and at the time of installing hyperkalemia (Fig. 10).

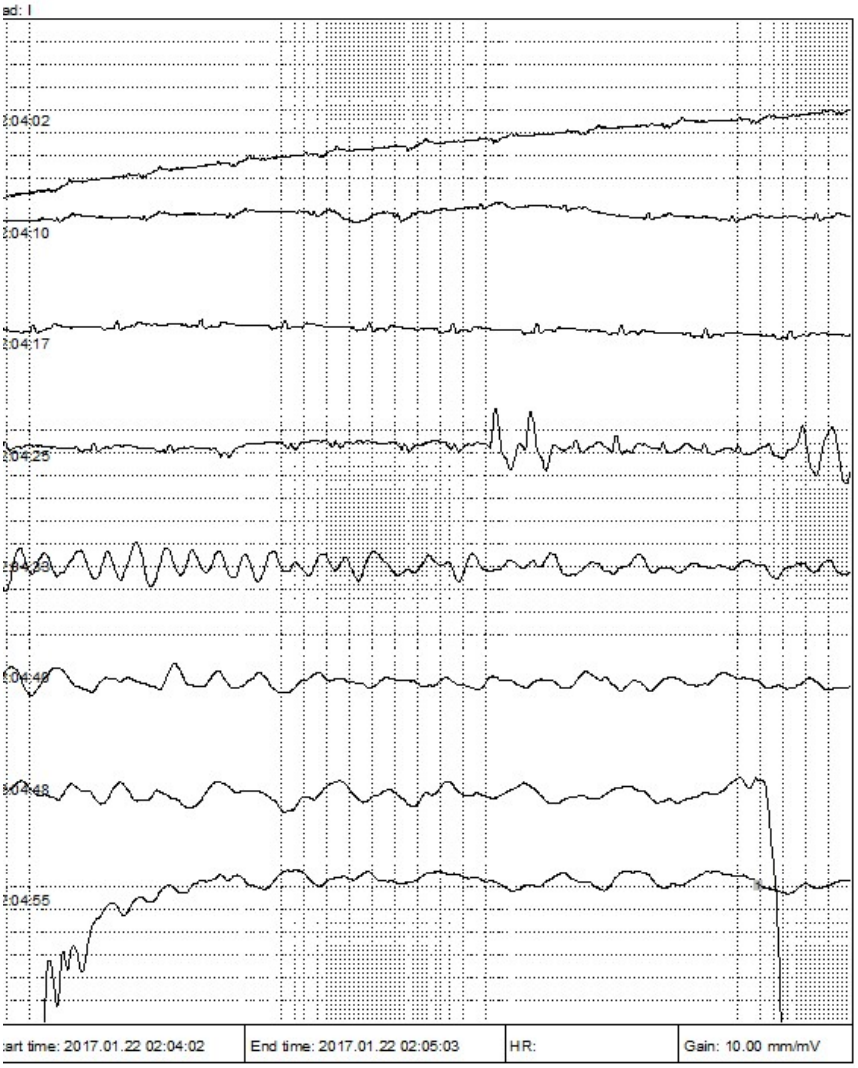

Fig. 8. EKG after dialysis (orig.)

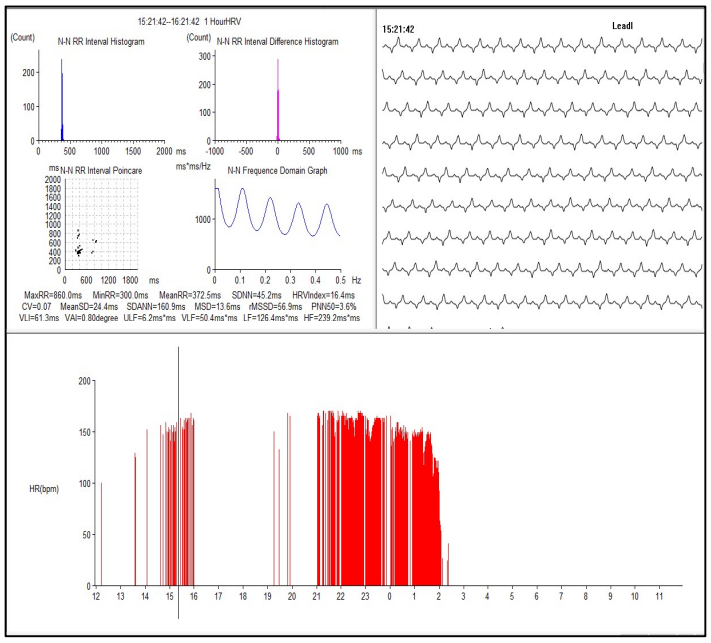

Fig. 9. Holter monitoring for 60 minutes (orig.)

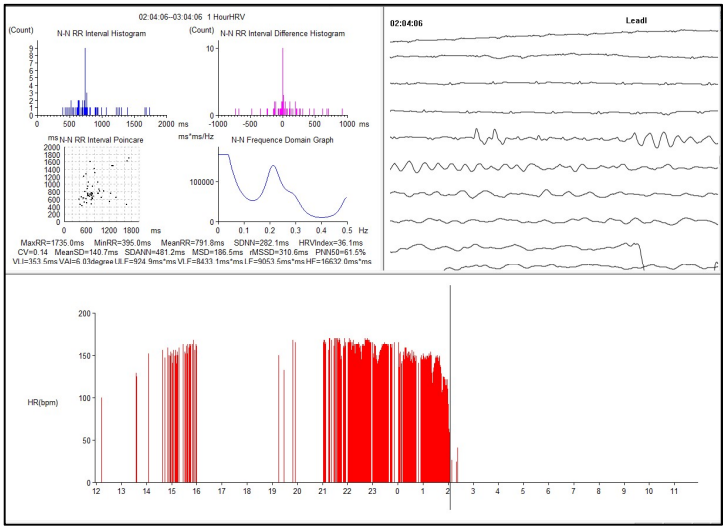

Fig. 10. Holter monitoring for 60 minutes (orig.)

Table 1

Synthetic presentation of the studied cases

Nr. crt	Species	Breed	Age	Sex	Therapy	Primary affection	Electrolytic imbalance	Case completion
1	Canine	Labrador	6	M	Hemodialysis + Hydro-electrolytic support	Ehrlichiosis	Hyperkalemia	Non-exitus
2	Canine	Mixed breed	8	M	Hydro-electrolytic support	Renal failure	Hyponatremia, Hypokalemia, Hypochloremia	Exitus
3	Canine	German shepherd	2	F	Hemodialysis + Hydro-electrolytic support	Renal failure	Hypernatremia	Non-exitus
4	Canine	Tosa – Inu	8	M	Hemodialysis + Hydro-electrolytic support	Dirofilariosis	Hypernatremia, Hypokalemia	Non-exitus
5	Canine	German shepherd	8	F	Hemodialysis + Hydro-electrolytic support	Renal failure	Hypokalemia	Exitus
6	Canine	Mixed breed	3	F	Hemodialysis + Hydro-electrolytic support	Babesiosis	Hypernatremia, Hyperchloremia, Hypokalemia	Non-exitus

Conclusions

Following the analysis of the above data, the following conclusions can easily be drawn:

1. Holter monitoring is an integrated and important part of the renal impairment therapy in patients submitted to hemodialysis.
2. The corroboration of the hemodynamic data obtained by other methods (echocardiography, tensiometry, hematological examinations etc.) with the Holter electrocardiogram helps to establish in real time the positive or negative evolution of the treatment in renal failure in dogs.
3. Holter monitoring is an extremely useful way of correcting the hemodynamic parameters of the patient with renal impairment depending on the reactivity of the cardio vascular system to the treatment, but also in treating the primary pathology.
4. The 60-minute heart rate variability is an indirect indicator for evaluating the hemodynamic response in renal disease, by quantifying the cardiac response to pre-pregnancy and post-pregnancy changes.

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PERFORMANCE CHARACTERISTIC EVALUATION OF FOLIN-CIOCALTEU MICRO-METHOD FOR TOTAL POLYPHENOLS DETERMINATION FROM PLANT EXTRACTS

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Summary

This paper aims to describe the standard operation procedure for performance characteristic evaluation of Folin-Ciocalteu micro-method for total polyphenols analysis from vegetal matrices. The key of this method consists in the use of a microplate reader due to multiple advantages: using small amounts of extract and reagent as well, assuring a good repeatability and a considerable reduction of the total analysis time. Besides that, it is an accurate and easy to accomplish method. Standard calibration curve was performed using concentrations of 0, 0.25, 0.50, 0.75, 1 mg/ml of gallic acid and a microplate reader TECAN Infinite M1000 Pro at 750 nm was used. Statistical evaluation of linear calibration function was performed through following parameters: the standard deviation and coefficient of variation with simple and repeated analysis, the repeatability and reproducibility, the limit of detection (LOD) and the limit of limit of quantification (LLOQ).

Keywords: polyphenols, micro-method, method validation, UV/VIS, Folin-Ciocalteu

Polyphenols are a category of plant compounds that offer various health benefits. They have antioxidant, anti-inflammatory and biological effects that are manifested in the prevention of pathological conditions. Polyphenols can act as antioxidants, meaning they can neutralize harmful free radicals that would otherwise damage the cells and increase the risk of conditions like cancer, diabetes, and heart disease (10).

Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins. These compounds are among the most widely occurring secondary metabolites in the plant kingdom, acting mainly as phytoalexins, attractants for pollinators, contributors to plant pigmentation, antioxidants, and protective agents against UV light, among others (5).

Although quantitative determination of polyphenols is hampered by their structural complexity and diversity, several methods have used to determine polyphenols in plant extracts (1).

Polyphenols in plant extracts react with specific redox reagents as the Folin-Ciocalteu reagent to form a blue complex that can be quantified by visible-light

spectrophotometry (8).

The reaction forms a blue chromophore constituted by a phosphotungstic-phosphomolybdenum complex (6, 8) where the maximum absorption of the chromophores depends on the concentration of the phenolic compounds (8).

Materials and methods

For the quantification of the phenolic compounds in some dried plants, Folin-Ciocalteu method was used following the protocol described by TAMAS-KRUMPE Octavia Maria *et al*. (9). This method consisted in several steps: 25 µl of the final solution (alcoholic extract of dried plant material), 125 µl of the Folin-Ciocalteu reagent and 100 µl Sodium carbonate (Na_2CO_3) were pipetted into a 96-well plate.

The standardized method, with a reaction time of 30 min, wavelength of 760 nm and Gallic acid as the standard was used to validate the method for the determination of total polyphenols. Analyses were performed in triplicate. The absorbance of the samples was read with a microplate reader TECAN Infinite M1000 Pro.

Analytical method validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use (7).

According SR ISO CEI 17025:2018 method validation analytical laboratory is „confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled".

The clause 5.4.5.2 of ISO/IEC 17025 presents that validation must be performed in the following cases: „non-standard methods; laboratory-designed/developed methods, standard methods used outside their intended scope, amplifications and modifications of standard methods''.

Verification and validation are independent procedures that are used together for checking that a product, service, or system meets requirements and specifications and that it fulfils its intended purpose (11). These are critical components of a quality management system according ISO 17025:2018.

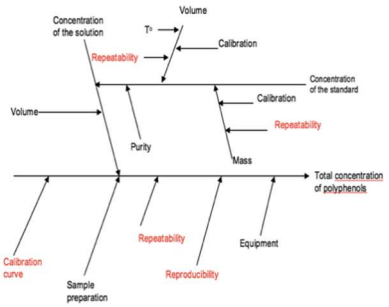

Fig. 1. Ishikawa diagram for the determination of the concentration of polyphenols

For the validation report several factors have to be taken into consideration such as: linearity, accuracy, precision, specificity, selectivity, sensitivity, stability, uncertainty matrix effect, repeatability and reproducibility, limit of detection and quantification.

To create an overview with all those factors we managed to introduce them into the Ishikawa diagram: a causal diagram

used to identify potential factors that could cause an overall effect. The factors that have been taken into consideration and analysed were: the calibration curve, the repeatability and reproducibility and the limit of detection and quantification.

Calibration curve

The calibration curve of the Gallic acid was determined from five concentration points over the range of concentrations: 3.90, 7.80, 15.62, 31.25, 62.50, 125.00, 250.00 mg/ml, following the Lambert-Beer law (2). Statistical analysis was performed according LGC Guide for calibration curve preparation. Interpretation of the statistical results was performed according ISO14502-1:2005.

Repeatability and reproducibility

According to the standard ISO 5725-1:1994, the *repeatability conditions* are „Conditions where independent test results are obtained by the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short time intervals" and *the reproducibility conditions,* according to the same standard are "Conditions where the results are obtained with the same method, on identical test items, in different laboratories, with different operators, with using different equipment".

For this paper, repeatability and reproducibility have been calculated according the Standard *ISO 14502-1:2005.*

Limit of detection and quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value (3). For the validation of this method, the limit of detection was calculated with the following formula: **LOD = 3.3 × (S Y / a)** – where SY is SD of blank response "a" for the slope of a linear calibration line.

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices (3). For the determination of this parameter, the following formula was used: **LLOQ = 10 × (S Y / a)** – where SY is SD of blank response "a" for the slope of a linear calibration line.

Results and discussion

Molecular absorption spectrophotometry in ultraviolet/visible light (UV/VIS) is an analytical method based on the property of an ion or molecular species to absorb at certain wavelengths of UV/VIS radiation. Thus, the absorption can be considered as a specific process related to the structure of the absorbing species, which determines the energy involved in the electron transition. However, to make the method more selective, normally reagents are used to convert the species of interest into a form that allows the absorption of the radiation to be measured with greater sensitivity and/or selectivity (2).

The European Pharmacopoeia indicates a single general method for determination of total polyphenols in all herbal drugs: the Folin-Ciocalteu method (4) witch we managed to transform into a micro-method because of the number of advantages that is confers.

During the process of developing this method, we intended to achieve a repeated number of readings to make a correlation between them regarding *repetability* and *reproducibility.*

At the first reading the following results were obtained (Table 1, Fig. 2)

Table 1

The results obtained at the first reading

Concentration of standards	Instrument readings
3,9000	0,0358
7,8000	0,0566
15,6200	0,1121
31,2500	0,2266
62,5000	0,3782
125,0000	0,8317

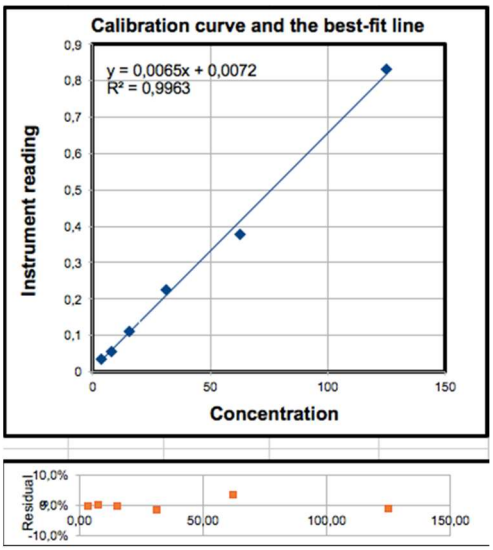

Fig. 2. The Correlation between absorbance and concentration on the first calibration curve

After a period of 5 days the same procedure was reassessed in the same laboratory conditions by a different co-worker, resulting the following data (Table 2, Fig. 3):

Table 2

The results obtained at the second reading

Concentration of standards	Instrument readings
3,9000	0,0433
7,8000	0,0913
15,6200	0,1398
31,2500	0,2595
62,5000	0,4598
125,0000	0,8233

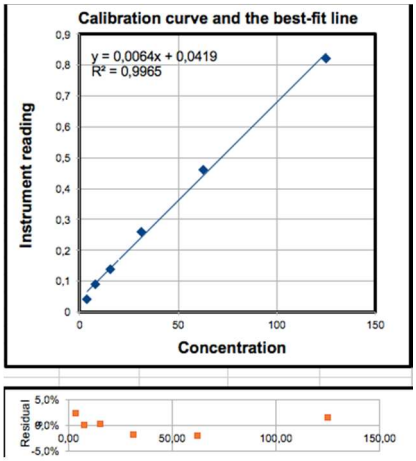

Fig. 3. The Correlation between absorbance and concentration on the second calibration curve

In order to validate the method, we determined: repeatability, reproducibility limit of detection and limit of quantification using the formulas mentioned above and compared the results.

As one can observe in the Table 3 the results are related from a determination to another.

Table 3

Correlation between the parameters calculated with the results obtained at the first and second reading

Calibration curve	Reproducibility	Repetability	LOD	LLOQ
1.	1,0081	0,0529	0,0624	0,1891
2.		0,0033	0,0039	0,0119

Conclusions

This paper's purpose was to develop and validate a micro-method for the determination of the total polyphenols in some dried plants according to Folin-Ciocalteu technique. This method grants a significant number of benefits that should be considered.

Firstly, by the use of the micro-method, the reagent consumption is reduced up to ten times in comparation with the classical macro-method. Secondly, the incertitude budget is drastically reduced by assuring homogenous sample preparation of the samples which takes only a few minutes (with the use of a multichannel pipette) and a very good quality of the reading which is done in only a few seconds.

Regarding repeatability and reproducibility, because the micro-method is so fast and easy to perform, with conditions that do not change over time, the measurement results will not be affected, proving a highly method robustness.

Concerning the evaluated parameters the method is verified and it can be used in the working conditions of the laboratory and is suitable for the proposed purpose.

Aknowledgment

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WILDLIFE REHABILITATION: TRIAGE AND COMMON PATHOLOGIES

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Summary

More than ever, wild animals are adapting to live in cities or close to human settlements. Therefore, the number of casualties affected by anthropic factors has become a real issue. Wildlife rehabilitation is defined as the professional treatment and care of sick, injured or orphaned wild animals with the final goal of releasing them back into their natural habitat. Whether an animal can be rehabilitated or not is the first decision that needs to be made on admission, to prevent further suffering. The most common pathologies, the recommended medical approaches and the expected recovery rates will be presented in the forthcoming article. Wildlife casualties all over Romania suffer from lack of veterinary care and rehabilitation facilities. Communication between finders, local small animal practices and the existing rehabilitation centers is essential in order to provide the animals with specialized care and the best chances of returning to the wild.

Keywords: wildlife rehabilitation, euthanasia, trauma, pathology

Generally, when a wild animal is found by a member of the public, it's going to be presented to the local veterinarian for medical assessment and further treatment. Considering that training of veterinarians doesn't routinely include the pathologies found in wildlife, the medical professional can find himself in a difficult position making the best decision while keeping the welfare of the casualty in mind as a primary concern (9,13).

The welfare of wild animals has to be viewed very differently than that of domestic animals. Captivity for life is never a good option for a wild-born animal, as two of its primary liberties cannot be met: the liberty from stress and the liberty to express its normal patterns of behavior. If the lesion or pathology that the animal presents is going to incapacitate its ability to survive in the wild at the end of the rehabilitation process – the decision of humanely euthanizing the casualty has to be made in order to prevent further suffering (9). Even if the pathology is treatable in theory, the questions of access to appropriate facilities (stress-free environment and cages, flight aviaries for training, suitable food etc.) and the commitment (economical, time and staff related) still remain to be asked before deciding on rehabilitating the animal.

Materials and methods

The approaches and treatments presented in the present article are based on the cases admitted at a wildlife rehabilitation center near Bucharest, Romania. Cases have been treated here from 2016 until present time and during this period the facilities, training of the staff and medical protocols have been improved to better suit the needs of the animals. The number of animals has increased each year, reaching 1705 cases in 2019.

Most common protected species admitted at the center are represented by white-breasted hedgehogs (*Erinaceus roumanicus*), bats (*Nyctalus noctula, Pipistrellus sp., Vespertilio murinus),* daytime raptors (*Buteo buteo, Buteo rufinus, Falco tinnunculus, Falco subbuteo, Accipiter nisus, Accipiter gentilis* etc.), owls (*Athene noctua, Asio otus, Strix aluco, Otus scops* etc.), members of the Corvidae family, european turtles (*Emys orbicularis*), tortoises (*Testudo hermanni, Testudo graeca*), seagulls (*Larus sp., Chroicocephalus ridibundus*), members of the Turdidae family (*Turdus merula, Turdus philomelos, Turdus pilaris*), foxes (*Vulpes vulpes*), red deer (*Capreolus capreolus*), red squirrels (*Sciurus vulgaris)* and other species. Considering that the center is situated in a peri-urban area, most species admitted are synanthropic and they also include some animals considered invasive in nature, for example common pigeons (*Columba livia domestica*), exotic turtles (*Trachemys sp*.) and pheasants (*Phasianus colchicus*) (Fig. 1).

The protocols and procedures used and described in this paper are based on the BSAVA Manual of Wildlife Casualties (2016), personal adaptations and cross-experience internships with other rehabilitation centers. Recommended dosages for medications in different species, along with some physiological references are to be found in the **Exotic Animal Formulary** (3).

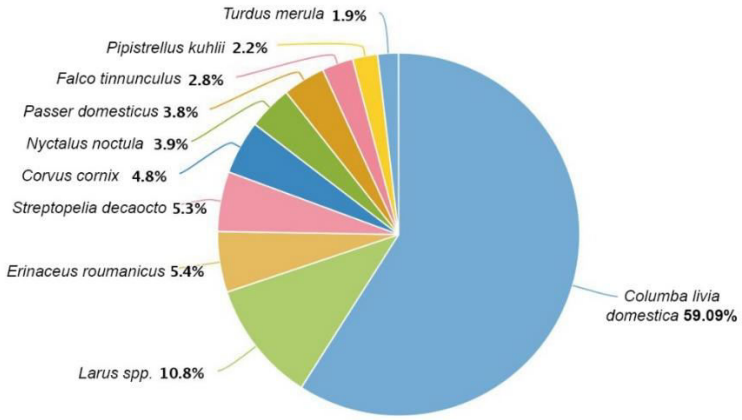

Fig. 1. Animal species prevalence in a peri-urban wildlife center in Romania

Results and discussions

Whether an animal can be rehabilitated or not is the first decision that needs to be made on admission, to prevent further suffering. This is where triage comes in, as a first step in the assessment of a wild casualty.

Animals that present lesions such as open necrotic fractures, spinal fractures, missing front limbs or pathologies like advanced trichomoniasis, paramyxoviruses and poxviruses are triaged directly as euthanasia cases (Fig. 2). As a general statistic from most wildlife centers, an ideal release rate would be somewhere between 35% and 40%.

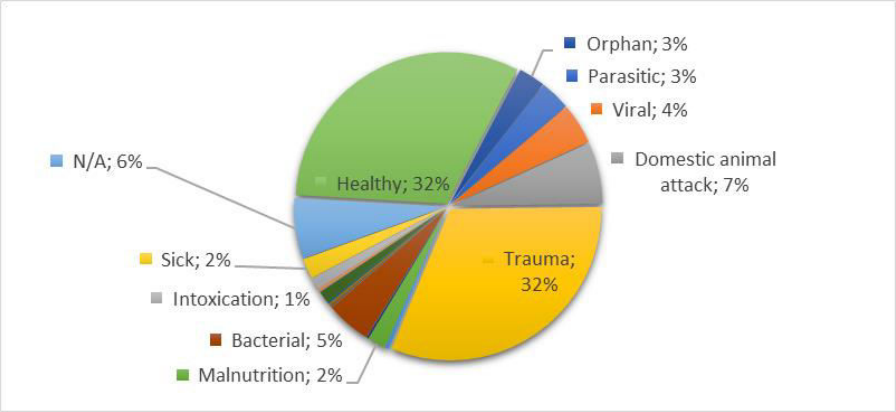

Fig. 2. Most prevalent causes of animals' admission in a peri-urban wildlife center

Fractures and dislocations

As emergency treatment, immobilization and analgesia are required. *An "8-figure"* bandage is best used for birds (Fig. 3). Strapping the wing to the body is only necessary for shoulder and humeral lesions. Analgesia is a primary concern, as pain will increase the levels of stress of the animal. Meloxicam and carprofen are some of the first choices, but for animals with a low tolerance to pain (like lagomorphs and rodents) the drug of choice is generally an opioid (tramadol, buprenorphine, butorphanol).

In order for a fracture to be a candidate for surgical fixation, it would have to be closed, the vascular and nervous structures would have to be intact and should not be close to a joint. Before attempting to operate on a fracture, both deep pain sensation and bleeding have to be present in the area distal to the lesion.

In birds, fractures need to be fixed less than 48 hours after the trauma to prevent the appearance of a periosteal callus which would prevent the lesion from healing in a physiological position. Fractures involving the coracoid, scapula, clavicle or proximal humerus are considered inoperable, due to the restricted movement of

the shoulder joint after healing. Fractures of the medial humerus and fractures of the ulna and radius are routinely fixed, generally using a combined method, with one intramedullary pin and two external fixators and have a good prognosis. Tibial fractures are easily fixed with intramedullary pins or even just immobilization of the limb in aligned lesions (10).

In both mammals and birds, there are ethical considerations in rehabilitating pelvic fractures. Therefore, in such a case, the decision has to be based on the identification of the sex, as females of all species would be prone to dystocia/ egg binding after release.

Fig. 3. An *"8-figure"* bandage, applied as first aid for a fracture in a bird

Cat attacks

Recent studies state that over 14% of wildlife admissions are caused by domestic cat attacks, 78% of which do not survive or have to be euthanized due to the severity of the injuries (8). Any trauma can be suspected of being caused by a domestic animal, even if not witnessed by the finder. Also, cats carry *Pasteurella* spp. in their saliva and infection can lead to acute septicemia (1). If the injuries are sustainable, the administration of a large-spectrum antibiotic is recommended for a minimum of 4 days (ex. amoxicillin, ceftazidime, enrofloxacin). The antibiotic course should be started even if the lesion is not apparent, but the finder rescued the animal from a cat attack, as small animals can be affected even by scratches. Dosages can be found in Table 1.

Head trauma

Head trauma is most common in birds due to hitting windows or moving cars. Many wildlife centers still use corticosteroids, but recommended treatment is mainly supportive and symptomatic. Most cases recover well after a 24-hour period of fluids, stable temperature and a quiet environment. In raptors, it's especially important to perform an ophthalmological exam, because pathologies such as

retinal detachment and bleedings in the posterior chamber are commonly found following head injuries.

Infectious diseases

Pigeons commonly carry paramyxovirus (PPMV-1) and Avipoxvirus. Quarantine is highly recommended for new birds and vaccination should be done in clinically healthy individuals. Sick birds should be euthanized as both viruses are airborne (12).

Paramyxovirus is easily suspected in individuals that present torticollis (pathognomonic symptom), ataxia, nystagmus and other neurological signs (Fig. 4). Affected individuals should be euthanized, as the disease is easily transmitted to other birds and the recovery rate is low. Most surviving individuals have neurologic sequelae that prevent them from being released.

Pigeon pox is easily recognizable due to the small nodules that appear near the eyes and mouth, on the feet and wings and even in the cloacal region. These animals can recover with supportive treatment. However, most centers choose to euthanize due to the high risk of transmission to other birds.

Fig. 4. Pigeon (left) and sparrowhawk (right) displaying torticollis, a common symptom of *Paramyxovirus* infection

Parasites

High parasite burdens almost always indicate a concurrent pathology. A natural balance exists between the wild host and its parasites, so treatment should only be applied in the case of clinical disease and after the laboratory diagnostic confirmation. Prophylactic treatment is not recommended in wildlife.

In birds, the most prevalent parasitic pathology is trichomonosis, very common in pigeons and doves, but also in raptors, which become infected by preying on pigeons. It can be suspected in weak, anorexic birds, with white granulomatous deposits in the mouth and esophagus. Diarrhea might also occur. If not treated, the lesions extend to the bone tissue and cause necrosis. Metronidazole

is the drug of choice, this pathology having a positive response to treatment. General and liver function support is also recommended (11).

Ectoparasites like feather lice and mites are present on most birds, but they shouldn't be treated unless they present heavy infestations. Ticks can sometimes be found around the eyes and they should be removed.

In corvids and seagulls with respiratory problems, *Syngamus trachea* has been found to be the cause quite often (eggs can be found by rubbing a cotton swab in the tracheal region or in the faeces) (Fig. 6). Blackbirds and starlings with digestive problems should be checked for intestinal *Capillaria* spp. Most commonly used drug in the treatment of nematodes in birds is fenbendazole, but if injectable treatment is preferred, ivermectin is a viable option (7) (Table 1).

In mammals, most prevalent parasitic pathologies are caused by: respiratory nematodes in hedgehogs (*Crenosoma striatum, Capillaria aerophila*) (Fig. 5) and coccidia organisms in young hares (*Lepus europaeus*) (2, 5, 11). For hedgehogs, which present high metabolic rates, there are specific protocols that have been proven effective. In the case of *Crenosoma striatum*, levamisole (27 mg/kg SC q24h, 3 days – repeated from day 13). *Capillaria aerophila* is easily treated with ivermectin (3 mg/kg SC, 3 treatments – repeated every 7 days). Young hares can develop clinical intestinal coccidiosis due to captivity stress and die in less than 48 hours. Toltrazuril (2,5-5 mg/kg PO) can be administered after coprological diagnosis (Table 1).

Coproparasitological examination is a straightforward, fast and cheap method of diagnosis in wildlife centers (6). Concurrent pathologies are usually found in animals with high parasite burdens (11).

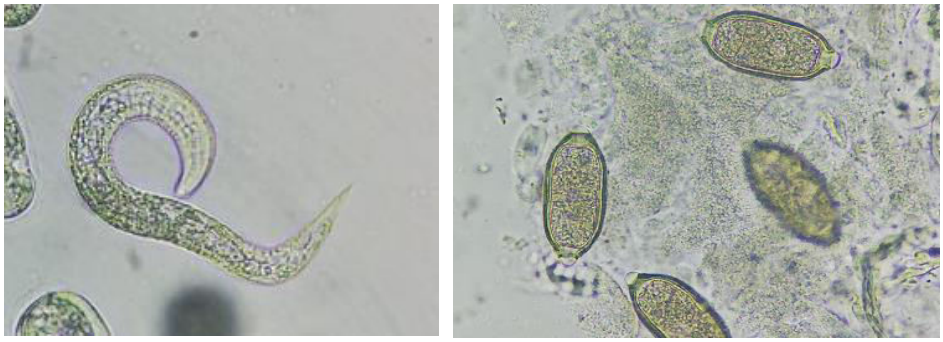

Fig.5. *Crenosoma striatum* larvae (left) and *Capillaria aerophila* eggs (right), identified in the faeces of a white breasted hedgehog (*Erinaceus roumanicus*)

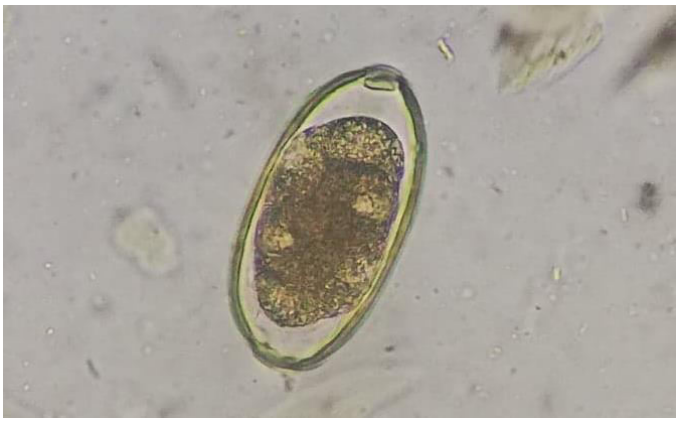

Fig. 6. *Syngamus trachea* egg in the faeces of a seagull (*Larus* sp.)

Orphans

Orphans are often found by members of the public. It's important to establish with the finder if the animal is a true orphan or if the baby is alone as a part of the natural behavior of the species. Knowledge on the natural behavior of the species is required to distinguish between orphaned babies/juveniles and animals left alone by their parents. All babies found, with the exception of those which are obviously injured/ in distress, should be observed from a distance to determine whether an intervention is necessary.

For example, hares normally leave the nest for several hours before returning to nurse the babies and hedgehog females leave the nest on a normal basis and might try to destroy it if they sense a new smell when try return.

Hypothermia, hypoglycemia and dehydration are the main causes of death in neonates and should be attended to before attempting to feed the animals. *"The five golden rules of orphan care are: dry, clean, warm, quiet, and well-fed"* (4).

Energy requirement for small mammals is $2 \times [70 \times (kg)^{0,75}] = kcal/day$. Energy requirement / energy content of the formula is the amount of formula per day divided by the natural capacity of the stomach in multiple feedings. The amount given at each feed should not exceed the natural capacity of the stomach, it is appropriate to feed up to 5 - 7% of the body weight per meal. The total amount per day, as well as the frequency should reach the species energy requirement. Never feed baby *ad libitum*, as commercial formulas are highly palatable and overfeeding may lead to gastric distention/ torsion, atony, vomiting, diarrhea.

Table 1.

Common drugs used in the treatment of wild animals (according to the Exotic Animal Formulary and Vale Wildlife Rehabilitation Center, United Kingdom)

Drug	Species	Dosage	Usage
Meloxicam	Reptiles	0,1-0,5 mg/kg PO, SC q24-48h	Non-steroidal anti-inflammatory, analgesia
	Birds	2 mg/kg PO, SC q24h	
	Mammals	0,2 mg/kg PO, SC q24h	
Carprofen	Reptiles	1-4 mg/kg PO, SC, IM, IV q24h	Non-steroidal anti-inflammatory, analgesia
Butorphanol	Most species	0,4-1 mg/kg SC, IM	Analgesia
Amoxicillin trihydrate	Reptiles	22 mg/kg PO q24h	Large spectrum antibiotic; can be used in animals that present cat-inflicted injuries
	Birds	150 mg/kg PO, SC q12h	
	Mammals	15 mg/kg PO, SC q12h	
Enrofloxacin	Reptiles	5-10 mg/kg PO, SC q24h	Antibiotic; injectable form may cause local necrosis
	Birds	15 mg/kg PO, Sc q12h	
Dexamethasone	Most species	2 – 4 mg/kg SC, IV	Steroidal anti-inflammatory; may use in shock/ head trauma
Levamisole	Hedgehogs	27 mg/kg SC q24h, 3 days, repeat from day 13	Antiparasitic, drug of choice in hedgehog lungworm infestation
Ivermectin	Most species	0,2 mg/kg PO, SC	Antiparasitic for nematodes and ectoparasites
	Hedgehogs	3 mg/kg SC, 3 treatments, 7 days apart	Drug of choice for *Capillaria aerophila* infestations
Fenbendazole	Most species	50-100 mg/kg PO, repeat in 3 weeks	Antiparasitic for nematodes
Metronidazole	Birds	50 mg/kg PO q24h, 5-7 days	Antiparasitic; drug of choice in avian trichomoniasis
Toltrazuril	Lagomorphs	10 mg/kg PO	Antiparasitic for coccidia

Lagomorphs may eat up to 10-25% of their body weight per one meal.

Introducing a formula is done gradually, by starting with oral rehydration solution and mixing in the milk, increasing the formula's quantity after each feeding (4). Choosing a formula from the existing ones on the market might be the most decisive factor for the survival of the animal. No milk replacer will ever meet the quality of the mother's milk. The goal is to have a positive growth rate and choose a formula that the animal is able to digest properly.

Birds have different nutritional needs depending on the species and age. The natural diet of the animal should be substituted as closely as possible.

Malnutrition

Malnutrition is often seen if the finder has attempted to care for the animal. Treatment should address the resulting pathology:

- Gastrointestinal symptoms following administration of a wrong diet (eg. cow's milk in hedgehogs, meat in insectivorous birds);
- Feather damage is common and appears as a consequence of metabolic pathologies and sometimes unsuitable accommodation;
- Vitamin A deficiency is often seen in turtles and tortoises;
- Metabolic bone disease (especially in raptors, hedgehogs and tortoises) if wrong diet is administered in young animals (generally, diets high in phosphorus should be avoided);
- Obesity – especially in wild hedgehogs that have been kept as pets.

Imprinting

Imprinting is an ethological issue that in wildlife rehabilitation is considered the gravest of captivity's issues. This happens when the animal starts to associate humans with its own species. Imprinting is not 100% reversible and dramatically lowers the animal's chances of survival after release. Interactions with the animals, especially during hand-rearing, should be limited to feeding. Talking both to and near the animal should be restricted.

Conclusions

Wildlife rehabilitation has become increasingly necessary as species adapted to urban habitats. While, most casualties end up in small animal practices, this article sums up the most prevalent pathologies and how they should be managed. The key to giving wild casualties the best chance of returning to the wild is a better communication between practices and the existing wildlife professionals.

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ASSESSMENT OF THE RESISTANCE OF SUPERFICIAL AND DEEP TOTAL CRACKS TREATMENT THROUGH COMPRESSIVE FORCES

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Summary

There are many types of hoof affections that involve chipping, tearing, and cracking of the hoof wall. Among these the cracks require regular treatment and evaluation. Depending on the depth the cracks are classified into deep and superficial one and can present different position on the hoof wall: solear area, coronary band, total, heel and quarter crack. The treatment is done according to the location and depth and involves stabilization of the cracks margins for providing time to growing a healthy wall from the coronary region. In the study was compared two methods of treatment for superficial and deep total cracks to determine the resistance by using the compression test. The results that we obtain help us for choosing the best method of treatment.

Keywords: horse, hoof cracks, treatment, compressive test

When the humans discovered the utility of horses, they immediately understood the need to protect the horse's feet.

The earliest forms of horseshoes can be found as early as 400 BC. Materials was from plants, rawhide and leather strap gears referred to as "hipposandals" by the Romans. In Ancient Asia, the people used for their horses protection made from woven plants. The shoes were not just for protection but also to calm the existing injuries that horse might have sustained in its activities. In several parts of Northern Europe known for its cold and wet climate, many protection for hoof have been tried and only from the 6th century AD, the metal ones were used (4, 5).

After the appearance of the metal shoes, several application techniques have been tried, including fixing the heated horseshoe to the hooves, practiced by the horsemen from Great Britain and France in the 16th century AD called the "hot shoeing technique" (1).

Use of nails for fixing the horseshoe to the hoof can lead to destruction of the hoof wall by the appearance of cracks (2).

Hoof cracks can occur at any time and are not depending by the breed or competition. Horizontal cracks, are usually due to injury or the result of an abscess that destroy the growth at the coronary band. The cracks can be complete, affect

from the coronary band to the sole or incomplete and they can affect at the toe, quarter, heel or bar (6, 10).

A crack can affect the external wall to involving its full thickness and the underlying sensitive tissue. The sensitive tissue likely cause pain and lameness and can bleed or develop an infection in deeper structures (1).

Treatment of the cracks involve stabilizing the margins and blocking the advancement until the new portion of hoof will grow from the coronary region. The materials used to treat the cracks include horseshoes, metal wires, screws, plates, epoxy compounds or associations between them (7).

In present exist other alternatives to fix the margins of the cracks or to create an artificial horseshoes by using special adhesives like epoxy materials ones. The material will be applied directly into the wall defect or on the sole or to taking the shape of the hoof or for therapeutic purposes (8, 11).

To apply the best method of treatment, we have to evaluate the depth of the crack and the loss of the hoof tissue and if necessary use the local treatment.

The study was carried out to determine the resistance of the epoxy material and to compare two methods of treatment for deep total cracks.

Material and methods

The cases that were included in the study were examined regarding the integrity of the hoof and gait for establishing the diagnosis of lameness, and those with acropodial disorders were selected.

For testing the resistance of the treatment, pieces of the hoof wall that were cracked or in which flaws were created, were taken (after the horses were sacrificed), reproducing a total wallcracks. The samples were collected from the hoof having a thickness of 2 cm and a length of 8 cm, a total area of 16 cm^2.

A control group consisting of 5 samples was tested for the superficial total cracks without using any means of fixation.

Lots of 5 samples were set up. The following treatment methods have been tested for:

Superficial total cracks:

- Lot 1: simple used of epoxy material;
- Lot 2: use of epoxy material and screw and metal wire;

Deep total cracks:

- Lot 1: simple use of epoxy material;
- Lot 2: use of epoxy material and screw and metal wire;

The material used for fixing the edges of the cracks was represented by the Isoform Durofloor epoxy (component A / component B, 1: 2), being an epoxy material. The metallic wire is represented by the cerclage wire and the screws had a length of 25 mm.

Working technique:

By using the epoxy material like a single method of fixing, the material was inserted into the crack after it has been initially processed by removing the debris from the wall and the manure to give a secure attachment to the substrate. The epoxy material was applied between the edges of the crack.

In the second technique when for fixing we used epoxy material, cerclage and screw, the epoxy material was applied after screw was inserted in the wall at a distance of 0,3-0,5 mm of the margins and the cerclage surrounded all the screw in a zig-zag shape. The distance between two screws on the same part of the wall is 10 mm.

The tests of resistance for each type of treatment was performed by applying compressive forces with the help of the Instron 8874 Materials Testing Machine (Fig. 1) in the research laboratory of the Polytechnic University of Timisoara.

Fig. 1. Instron 8874 Materials Testing Machine

The force released by the machine was identical for each sample. The sensors that are fixed in the arm of the machine have the ability to detect the value of the force at which the gear used to secure the edges of the crack is lost. The support surfaces were straight, thus being eliminated an uneven distribution of the compression force.

Results and discussions

Results obtained from testing the control group through compression tests Table 1.

Table 1

Results obtained for the control group

Sample	Superficial total crack
Sample 1	3550 N – 22,53 kg/cm^2
Sample 2	2800 N – 17,84 kg/cm^2
Sample 3	3200 N – 20,39 kg/cm^2
Sample 4	2500 N – 15,90kg/cm^2
Sample 5	3870 N – 24,57 kg/cm^2

The minimum value at which the loss of resistance was found was 2500 N - 15.90 kg/cm^2 in the case of superficial total cracks without any method of treatment.

The results obtained after the compression force test in the case of treatments for superficial total cracks:

Lot 1. Testing the strength of the epoxy material in the case of a superficial total crack through dynamic ex vivo compression forces - Table 2, Fig. 2.

Fig. 2. Testing the resistance of the epoxy material in a superficial total crack

Table 2

Results obtained after testing the resistance of the epoxy material in a superficial total crack

Sample	Newton force
Sample 1	5200 N / 33.14 kg/cm^2
Sample 2	4820 N / 30.69 kg/cm^2
Sample 3	3840 N / 24.47 kg/cm^2
Sample 4	5540 N / 35.28 kg/cm^2
Sample 5	4800 N / 30.59 kg/cm^2

On a value of 3840 N / 24.47 kg/cm^2 the epoxy material cracked. The crack had a horizontal line and the epoxy material was detached over the entire length of the crack. In the case of 3 samples cracks were observed on the surface of the epoxy material and then it was detached from the substrate.

Lot 2. Testing the strength of the epoxy material in the case of a deep total crack through dynamic ex vivo compression forces - Table 3.

Table 3

Results obtained after testing the resistance of the epoxy material in a deep total crack

Sample	Newton force
Sample 1	3550 N / 22.53 kg/cm^2
Sample 2	4220 N / 24.06 kg/cm^2
Sample 3	3450 N / 21.92 kg/cm^2
Sample 4	2840 N / 18.04 kg/cm^2
Sample 5	3200 N / 20.39 kg/cm^2

The strength of the epoxy material in case of a total crack is reduced. The obtained values are close and the minimum value was 2840 N / 18.04 kg/cm^2. There was a crack between the epoxy material and the edge of the seed in most samples. The epoxy material was detached from the substrate with no cracks in its structure.

Lot 3. Testing the strength of the epoxy material, screw and cerclage in case of a superficial total crack through dynamic ex vivo compression forces - Table 4.

Table 4

Results obtained after testing the resistance of the epoxy material, screw, cerclage in a superficial total crack

Sample	Newton force
Sample 1	8008.03 / 51.03 kg/cm^2
Sample 2	8007.24 / 51.02 kg/cm^2
Sample 3	9030.45 / 57.51 kg/cm^2
Sample 4	12014.93 / 76.52 kg/cm^2
Sample 5	8245.57 / 52.51 kg/cm^2

The obtained results indicate a very good resistance of the gear using the epoxy material, the metallic wires and screws inserted in the margins of the crack. We observe the appearance of cracks in the gear structure, these cracks being located near the metallic wires. In case of two samples, there was a tendency to bend the screws anchored in the wall.

Lot 4. Testing the strength of the epoxy material, screw and cerclage in case of deep total crack through dynamic ex vivo compression forces - Table 5, Fig. 3.

Table 5

Results obtained after testing the resistance of the epoxy material, screw, cerclage in a deep total crack

Sample	Newton
Sample 1	7500.03 / 47.72 kg/cm^2
Sample 2	8020.46 / 51.08 kg/cm^2
Sample 3	7080.45 / 45.07 kg/cm^2
Sample 4	9002.20 / 57.30 kg/cm^2
Sample 5	8600.57 / 54.75 kg/cm^2

Fig. 3. Testing the resistance of the epoxy material, screw, cerclage in a deep total crack

The changes occurred consisted in bending of the screw and loss of adhesion between the epoxy material and the edge. Also was observe advancing of the crack in depth.

The treatment of the cracks envolve using of the horseshoes to balance the hoof and apply different material for stabilise the cracks margin.

Veterinarians and farriers commonly use such polymer, epoxy and acrylic patching materials or “glue” to stabilize and repair superficial hoof cracks (9).

After Smith SJ., using screw, cerclage and epoxy material as a method for treating the hoof cracks increase the resistance and ensures a superior treatment compared to the simple use of one of the methods (3).

Conclusions

Using the epoxy material as a stabilization method in superficial and deep cracks has increased the resistance to the compression force

The simple use of the epoxy material does not offer enough resistance to stabilize the crack edges

Better results were obtained by using epoxy material, cerclage wire and small screws inserted in the cracks margin.

The limitations of this study are represented by the non-participation of the structures inside the hoof to the exerted compression forces.

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EPIDEMIOLOGIC AND THERAPEUTIC CONSIDERATIONS IN CANINE PARVOVIROSIS

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Summary

Canine parvovirus (CPV) infection is a contagious viral disease and an important cause of serious and often fatal gastrointestinal disease in puppies worldwide. The disease is caused by a virus CPV-2, belonging to the group of parvoviruses from which two distinct parvoviruses are now known to infect dogs, namely CPV-2 and CPV-1. For this study we surveyed 132 dogs, treated at University Veterinary Clinics (57 cases /15 +CPV / 3 dead) and one veterinary private clinic from Timisoara (75 cases / 50 +CPV / 15 dead) during March to November 2018. CPV disease was suspected in young (7 weeks – 8 months), unvaccinated, or incompletely vaccinated dogs with relevant clinical signs (haemorrhagic gastroenteritis). Confirmation of diagnosis was performed by CPV Ag, based on a chromatographic immunological method for qualitative detection of parvovirus antigen. Of the 132 dogs, 65 (49.24%) were positive and 67 were negative for CPV-2. The overall reported death rate was 27.69% (18/65). Geospatial analysis revealed large numbers of CPV-2 cases in urban areas comparatively with rural areas near Timisoara. Therapy consists in the administration of antibiotics, antiemetics, antidiarrheics, intravenous fluids, enema and hyperimmune serum. The therapy lasts between 7 and 14 days, and in some cases remission of clinical signs can be observed after 3 days of treatment. For a comprehensive epidemiological investigation of canine parvoviral disease is necessary for the study to be continued on a larger number of cases.

Keywords: canine, parvovirus, prevalence, treatment, gastroenteritis

Canine parvovirus (CPV) infection is a contagious viral disease and an important cause of serious and often fatal gastrointestinal disease in puppies worldwide. The disease is caused by a virus belonging to the genus *Protoparvovirus* and the family *Parvoviridae*, a group of parvoviruses from which two distinct parvoviruses are now known to infect dogs, namely the pathogenic CPV-2 and CPV-1. Canine parvovirus type 2 (CPV-2) is one of the most important enteric pathogenic viruses of dogs. CPV-2, the causative agent of acute haemorrhagic enteritis and myocarditis in dogs, is extremely contagious, causing high morbidity and mortality in shelters and breeding kennels (13, 11).

CPV-2 has three antigenic variants known as types 2a, 2b, and 2c, the first two antigenic variants, CPV-2a and CPV-2b, are now distributed worldwide (20).

Severity of clinical evolution depends on virulence of the strain, age, breed, host's defences and size of inoculum. The disease is characterized by a rapid

clinical course with enteritis characterized by foul smelling bloody diarrhoea and vomiting, fever, disseminated intravascular coagulation, brain or spinal cord haemorrhage finishing with death. Canine parvovirosis can affect dogs at any age, but severe infection is most common in puppies between 6 weeks and 6 months of age, but the most severe evolution is in puppies less than 12 weeks that do not have prior immunity. All breeds are susceptible to the disease, although the mixed breeds are described to be less susceptible than many pure-breds. (19). Rottweiler, Doberman pinscher, American pit bull terrier, Labrador retriever, and German shepherd are more susceptible than other, but reasons for breed susceptibility are unclear (4).

No agent-specific treatment has proven effective, therefore treatment for canine parvovirosis is largely supportive and symptomatic. The main components of treatment include fluid therapy, antibiotics, antiemetics, nutritional support, and not least, antiviral treatments and pain management (7, 9, 12, 19, 2).

Materials and methods

For this study we surveyed 132 dogs, treated at University Veterinary Clinics (57 cases / 15 +CPV / 3 dead) and one veterinary private clinic from Timisoara (75 cases / 50 +CPV / 15 dead) (Fig. 1) during March to November 2018.

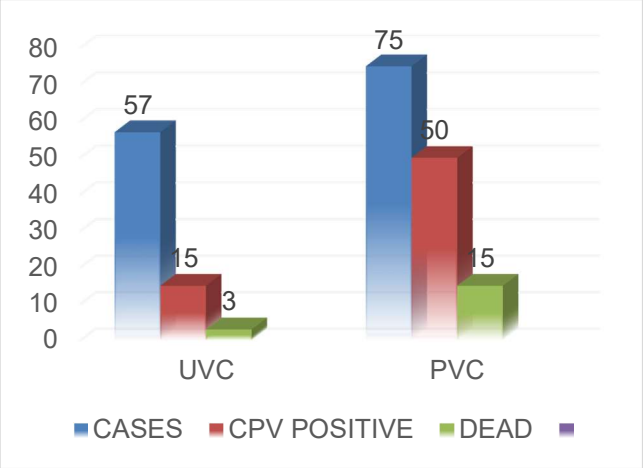

Fig. 1. Surveyed dogs in the study
UVC – University Veterinary Clinics; PVC – Private Veterinary Clinic

CPV disease was suspected in young dogs, between seven weeks and eight months age. Different breeds and half breeds of dogs were studied.

Were studied unvaccinated or incompletely vaccinated dogs with relevant clinical signs, specific for haemorrhagic gastroenteritis, with diarrhoea or haemorrhagic diarrhoea, anorexia with or without vomiting (Fig. 2).

Fig. 2. Young dogs, unvaccinated with haemorrhagic gastroenteritis

The animals subjected to the study came from both rural and urban areas, having owners or coming from nurseries or from different associations.

Confirmation of diagnosis was performed by CPV Ag, based on a chromatographic immunological method for qualitative detection of parvovirus antigen (Fig. 3).

Fig. 3. CPV Ag, based on a chromatographic immunological method for qualitative detection of parvovirus antigen

For each dog included in the study, data was collected regarding age, sex, breed, clinical signs and CPV vaccination history, area of origin, rural or urban and the evolution of the disease recorded accordingly. The data was analysed using Office Excel.

Results and discussions

Of the 132 dogs, 65 (49.24%) were positive and 67 were negative for CPV-2. The overall reported death rate was 27.69% (18/65) (Fig. 4).

The positive dogs belonged to several breeds, including the half breeds, being no significant differences between the pure breeds and the half breeds, regarding the presence of CPV.

Geospatial analysis, regarding area of origin, revealed large numbers of CPV cases in urban areas comparatively with rural areas near Timisoara.

Therapy consists in the administration of antibiotics, antiemetics, antidiarrheics, intravenous fluids, enema and hyperimmune serum.

The therapy lasts between 7 and 14 days, and in some cases remission of clinical signs can be observed after 3 days of treatment.

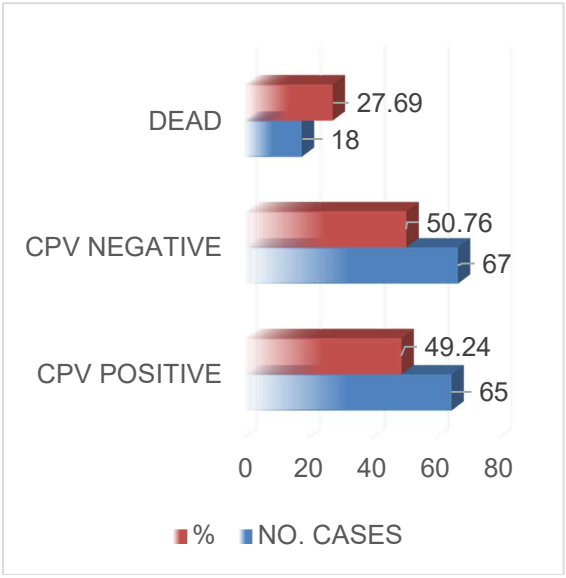

Fig. 4. Results obtained in the study regarding positivity and disease outcome

A study by Zourkas et al., (2015), (21) in Australia, out of a total of 4870 CPV cases revealed that most of them were in dogs under 12 months, unvaccinated and twice as many came from the urban area compared to those from the rural area. Non-vaccination of dogs can be attributed to poor socioeconomic status and lack of proper veterinary education.

The mortality rate in our study is slightly above the average reported in Europe, in other studies, of 24% (3), but under Australia, 42-47% (10, 21) and North America, 36% (8).

Moreover, CPV can manifest in dogs of any breed, age or sex (16), however certain predispositions have been reported and are widely accepted (21).

Some studies show that survival rate may be under 9% if no treatment is undertaken but may exceed 80% in tertiary care facilities (12).

Treatment for CPV infection is largely supportive and symptomatic. The main components of treatment include: fluid therapy, antibiotic treatment, antiemetic treatment, and nutritional support, antiviral treatments and pain management (12).

In the situation where the dog survives the first 4 days, they will usually recover rapidly and become immune to the virus for life (13).

Regarding sex, our study showed that it has no influence on the occurrence of CPV-2 infection in dogs. This agrees with similar findings by Folitse et al., (2017) (1), Singh et al., (2013) (17), Gombac et al., (2008) (5) and Ogbu et.al., (2016) (14). Different results have been obtained by Houston et al., (1996) who found males to be more susceptible and Umar et al., (2015) (18) reported females to be more susceptible.

With regards to age distribution of CPV cases is in generally accepted and supports the somewhat popular view that parvovirosis commonly affects puppies under 12-month age (10, 15).

Conclusions

For a comprehensive epidemiological and therapeutically investigation of canine parvoviral disease is necessary for the study to be continued on a larger number of cases.

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AUTHORS INDEX

CONTENT