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CONTENTS

BOVINE AND SWINE PARTHENOTS GENERATING THROUGH ELECTRICAL STIMULATION OF THE OOCYTES

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Abstract

Electrical stimulation is an alternative to chemical activation to induce $Ca2^{+}$ influx, responsible for the formation of pores in the cellular membrane. In order to activate the oocytes, electrical stimulation (E.S.) was performed on 30 oocytes derived from gilts (L1), sows (L2), heifers (L3) and cows (L4). We considered that the stage of development of four cells is eloquent for certifying the ES's division triggering and the results we are considering only refer to these parthenots. Following application of ES, oocyte activation occurred as follows: 6.6% at L1, 16.6% at L2, 20% at L3 and 46.6% at L4. It is obvious the higher maturation rate of oocytes from adult females as compared to young females (16.6% in sows versus 6.6% in gilts and 46.6% in cows versus 20% in heifers). The method of electrical stimulation of oocytes in the fusion chamber used in this paper is effective for activating the division in both bovine and swine oocytes. Activation of oocyte division following electrical stimulation is clearly superior when using oocytes from adult females. The electrical stimulation method used generated the upper division activation in cattle compared with the results obtained using swine oocytes.

Keywords*: oocytes electrical activation, ART technology*

Introduction

Activation of the oocyte during fertilization is caused by intracellular calcium oscillations whitch is triggered by the spermatozoa entry, after this, a series of events makes fertilization complete such as: inactivation of maturation promoting factor (MPF) and of mitogen activated protein kinase that leads to resumption and completion of meiosis, DNA synthesis and pronuclei formation (Paffoni et al., 2008).

Intracellular calcium increase in the oocyte can be induce without a spermatozoa, by using activating agents such as: ethanol, Ca^{++} ionophores and electroporation (Paffoni et al., 2008).

Artificial oocyte activation is used in assisted reproduction laboratories as a step in obtaining parthenogenetic embryos - source of parthenogenetic embryonic stem (pES) cells. These pES cells might serve as a source of tissue for transplantation (Kim et al., 2007). Parthenogenesis consists in the growth and development of embryos from oocytes that have not been fertilized by spermatozoa (Bevacqua et al., 2011). Also during some protocols of nuclear transfer (NT) enucleated *in vitro* maturated metaphase II oocytes and microinjected with the donor cell into the perivitelline space, are activated by electrical pulses. As donor cell can be used cumulus, oviduct, skin, liver cells, blastomeres, fibroblast, adipocytes and other types of cells (Lai and Prather, 2003).

By comparing the degradation rate following parthenogenetic activation with that obtainded after IVF, Cevik et al. (2009) did not notice significant differences, indicating that the activation of bovine oocytes by electrical stimulation and chemical agents yields good results, and the culture medium can support parthenogenetic development. In swine, oocyte activation as measured by the presence of pronucleus varies from 22% to 74%. The DC voltage field pulses cause temporary formation of pores in the plasma membrane, thus allowing extracellular and

intracellular exchange of ions and molecules. The number of blastocyst cells is a good indicator of embryo quality.

The purpose of this paper was to observe how oocytes from cattle and swine at different reproductive stages respond to the electrical stimuli used to activate cell division.

Materials and methods

Oocytes were obtained from ovaries from heifer, cows, gilt and sows. They were transported in 0.9% NaCl solution in isothermal bags at a temperature of 25-38°C. The ovaries were brought from the Smithfield slaughterhouse in Timisoara and from Macea, Arad County, which is 80.5 km (1h and 9 min) to the assisted reproduction lab of CLC-HC. For harvesting oocytes, the method of suction and cultivation of category I oocytes was chosen.

In vitro maturation was performed in 400 µl of TCM 199 medium supplemented with 10% ECS and coated with mineral oil at 38.5°C, 5% CO_2 for 44 hours for sow oocytes and 24 hours for bovine. For denudation, 0.1% hyaluronidase was used.

The extraction of the second polar body was performed using the Axiovert 40 CFL Narishige micromanipulation system, equipped with a NIKON reversed phase contrast microscope. Enucleated oocytes were placed in the culture medium in the incubator immediately after enucleation to allow the membrane and the cytoskeleton to recover rapidly after enucleation procedures.

The next step is the transfer of cumulus cells into the enucleated oocytes. Cumulus cells used as donor cells were not treated to reach a stage of the cell cycle, after some authors were G0 or G2/ M after other authors (Lai and Prather, 2003). For balancing, they are left for 10 seconds in the electrofusion medium.

The electrofusion process was performed using the Electro Cellfusion CFA 500 (Krüss, GmbH).

The device was set as follows: AC voltage: 0, AC duration: 0, AC post fusion time: 0, DC voltage: 160 V, DC pulse lenght: 30 µsec, number of pulses: 3, field power: 1.6 kV/ cm, fusion camera: BTX Microslide model 450-1 (1mm gap).

The electrofusion medium is composed of a 0.3 M medium consisting of 0.5 mM HEPES, 0.01% BSA, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$.

The reconstitued oocytes are then placed in the fusion chamber and covered with fusion medium. Using a pipette, the reconstitued oocytes were manually arranged so that the contact surface between the cytoplasm and the donor cell became parallel with the electrodes. Then the electric shock was applied.

The reconstitued oocytes were transferred to the maturation medium with TCM 199 and 15% ECS and incubated at 38.5°C, 5% CO_2 and constant humidity. Production of the division was checked every four hours, and the medium was changed every 24 hours.

Results and discussions

The results obtained following the use of electroactivative technique of bovine and swine oocytes are inserted in table 1 and shown in figure 1 and 2.

Table 1.

Results obtained after oocyte activation following electrostimulation

	Oocytes that started the cellular division	
	n	**%**
Gilts	2	6.6
Sows	5	16.6
Heifers	6	20
Cows	14	46.6

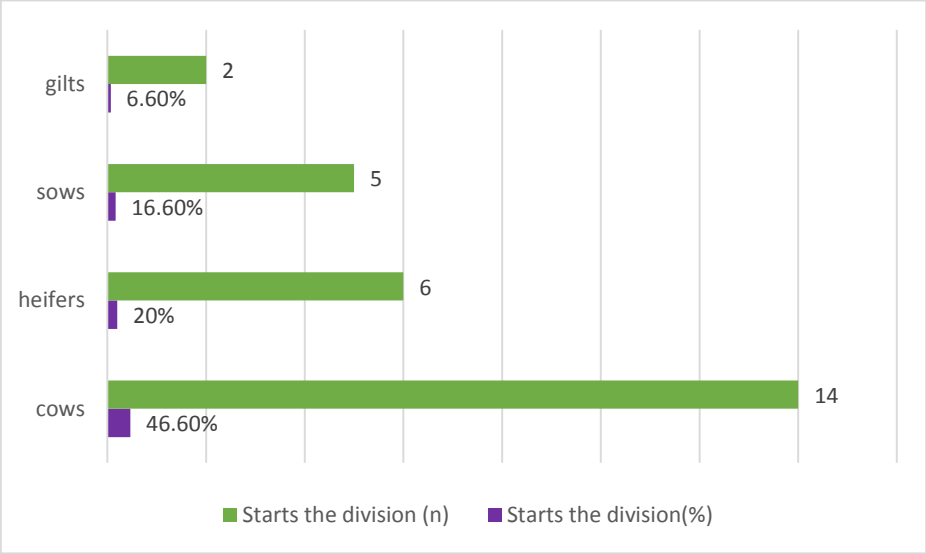

Figure 1. Cattle and swine oocytes activation following electrostimulation

For oocyte activation, electric stimulation was performed on 30 *in vitro* maturated oocytes, from gilts (L1), sows (L2), heifers (L3) and cows (L4). We considered that the stage of four cells is eloquent for certifying the ES's division triggering and the results we are considering only refer to these parthenots.

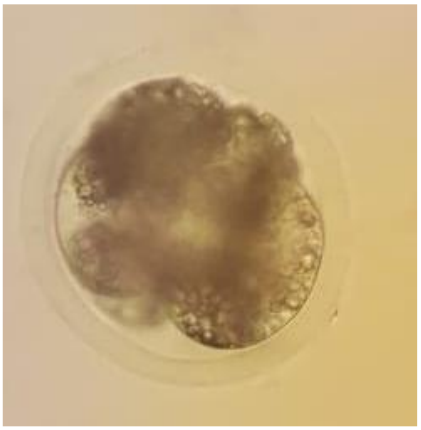

Figure 2. Swine parthenot in the four-cell stage

Observations were made at regular four-hour intervarls, the divisions emerging after 24 hours.

Following application of ES, oocyte activation occured as follows: 6.6% at L1, 16.6% at L2, 20% at L3 and 46.6% at L4. It is evident the higher maturation rate of oocytes from adult females as compared to young females (16.6% in sows versus 6.6% in gilts and 46.6% in cows versus 20% in heifers). It is supposed that this may be due to the increased availability of oocytes from adult females to go through consecutive SE divisions, probably through the existence of already mature cell cycle paths in correlation with the generative stimuli of the division. It is widely accepted that the cytoplasm of oocytes contains the necessary information for nuclear reprogramming, thus enabling the cell division to be triggered following electrostimulation.

Different biology of sexual cycles between bovine and swine species has an impact on the superior percentage of bovine oocytes that starts the division (both in adult and young female). Figure 1 clearly reveals these differences and allows us to observe the division of oocytes.

In 2017, Keller et al. obtain 14.63% zygotes in the four cells stage consecutive ICSI in cattle and in 2016, Godja et al. signals ICSI generation of 30% divisions attested by expressing the two pronuclei.

Consistent with the present results, previous studies have confirmed that parthenogenetic bovine blastocyst have a total number of cells significantly lower than IVF blastocyst.

Milazzotto et al. (2008) have demonstrated that despite the fact that there is no difference between electrical stimulation and chemical activation on blastocysts production rates, electrical activation has determined blastocysts with a higher percentage of viable cells.

During fertilization, oocyte activation is induced by the release of intracellular calcium after binding of sperm to the plasma membrane of oocytes. The meiotic resumption occurs because of the transient calcium oscillations. Thus, differences in blastocyst rates in this study can be explained by different calcium oscillation models that have been promoted through various treatments and by the addition of BSA as an activating attenuation agent because albumin has important calcium binding properties. Intracellular calcium oscillations are known to mediate cellular functions, such as gene expression and cell cycle regulation.

Rizos et al. (2002) demonstrated that the embryo culture medium plays a crucial role in determining the quality of the blastocyst. The number of blastocyst cells was higher when the

activated oocytes were co-cultured in TCM 199 with bovine oviduct epithelial cells or grown in SOF with BSA. When the number of viable cells was evaluated, the first group had better results.

Ozil and Huneau (2001) reported changes in calcium influx during oocyte activation affecting the development of post-implant embryo in rabbits due to interference in the epigenetic reprogramming of the zygote genome. These epigenetic abnormalities are transmitted through blastomere divisions and lead to changes in gene expression patterns.

It is widely accepted that this influx of calcium during oocyte activation may interfere with the expression of antiapoptotic and proapoptotic genes in preimplantated embryos.

Wu et al. (2017) investigating the survival and activation of the oocytes vitrified before and after electrostimulation, have demonstrated that the oocytes vitrified at 4-5 hours after electrostimulation generated a satisfactory survival rate as well as the pronuclei formation.

Li et al. (2017) studied th *in vitro* growth of oocytes activated by gene stimulation and then treated with various concentrations of AZD5438, inhibitor of cyclin-dependent kinases 1,2 and 9. The results obtained demonstrate that the electrical activation of swine oocytes in combination with AZD5438 treatment, lead to an increased rate of blastocyst formation in parthenogenetic activation as well as in somatic cell nuclear transfer experiments.

Following the ability to develop oocytes activated by an electric stimulus and treated with anisomicin, Zhang et al. (2017) have shown that using this method results in an increased percentage of blastocysts.

Conclusions

The method of electrical stimulation of oocytes in the fusion chamber used in this paper is effective for activating the division in both bovine and swine oocytes.

Activation of oocyte division following electrical stimulation is clearly superior when using oocytes from adult females.

The electrical stimulation method used generated the upper division activation in cattle compared with the results obtained using swine oocytes.

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APPLICATION OF INFRARED THERMOGRAPHY IN RABBIT ORTHOPAEDIC MODELS

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Abstract

The rectal or internal temperature (BT) is a reference method for body temperature. BT and ear temperature (BT_{ear}) were recorded in rabbit orthopaedic experimental model - White New Zeeland rabbits (N = 14), for a six day post-surgery period. Ear (BT_{ear}) temperature measured with infrared thermography (IRT) camera was compared with rectal body temperature (BT) measured with digital thermometer. Each BT_{ear} and BT methods were studied by analysis of variance and for BT classes such as: hypothermia ($BT_h \geq$ than 38,5°C), normothermia (BT_n) and hyperthermia or fever ($BT_f \geq$ 40,0 °C). Mean differences, linear regression and Pearson correlation were analysed. BT_{ear} was positively correlated with rectal temperature (BT); r=+0.579 at p <0.001. The regression equation model was statistically acceptable (p<0.001) and value of internal body temperature can be estimated on ITR measurements by relation: BT (°C) = 25.498 + BT_{ear} x 0.361 with R^2=0.336. This study demonstrates that IRT technology, a passive and non-contact technology can be effectively used for estimating BT changes in rabbits.

Keywords: *infrared thermography, body temperature, ear temperature, rabbit.*

Introduction

Experimental infrastructure of *Horia Cernescu* Research Unit is a research infrastructure which is running projects under Authorization no. 535 / 19th of May 2016. Animal orthopaedic models are considered to produce high levels of pain, suffering or distress and any manipulation can increase those. Even the rectal temperature measurement is a simple intervention which involves manipulation and restrain – and can produce stress and pain for the animals.

The infrared thermography (IRT) is enabling sensitive and alternative methods of measuring body temperatures (BTs) and it can be used to identify both hypothermia and hyperthermia or fever (8). Non-contact IRT is a promising technology that recently has been reviewed for its use in veterinary applications (6,7). Among other applications, non-contact IRT has been examined to successfully detect inflammatory conditions or fever in horses, cows or ponies (1,2 & 5).

Specific target of the study was to establish associations between *internal (rectal) body temperature (BT)* measured by digital thermometer and ear temperature (BT_{ear}) assessed by non-contact infrared thermography (FLIR infrared camera) in order to reduce the manipulation of animal in future projects (e.g. developed with orthopaedic rabbit models).

Materials and methods *Animals and data collection* - Two groups out of five[1], which forms the sample, (14 out of 35 White New Zeeland rabbits) were used for both external and internal temperature monitoring six days after surgery intervention.

[1]*The animal are coming from a study of the regenerative potential of mesenchymal stem cells at the level of meniscal lesion. The approved APS divided the rabbits into 5 groups (BMAC - Bone Marrow Aspirate Concentrate, PRP - Platelet-Rich Plasma, AC - Agili C, CD - Chondrotissue and C - Control), each group had 7 animals.*

For refinement reason, the telemetry was used for identification - ID microchip was implanted s.c. to each rabbit. The rabbits had 6 months of age and 3,562.07±68.16 g weight (X ± sx), measured ~~h~~ one day before creating animal orthopaedic models with knee trauma (causing meniscus and cartilage lesions). All animals were clinically healthy and normotherms - 38.5-40.0 ^{o}C.

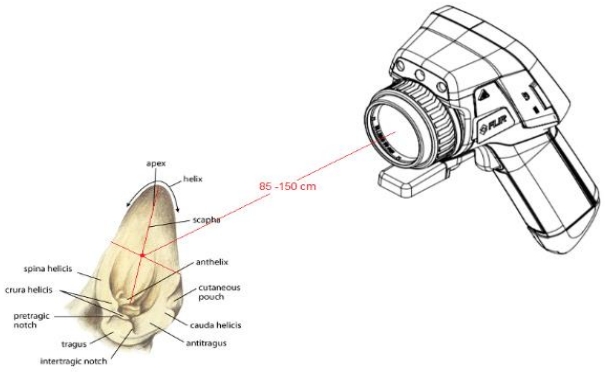


Figure 1: Election point (spot) for measuring rabbit ear temperature by infrared camera.
Surce of imagines: Anatomy of rabbit ear, ilustration by Gheoghe Constantinescu for Merk, (11) & FLIR® (10).

Body temperature was determined using two measurement locations. Internal body temperature (BT) was measured by insertion of a digital thermometer (*Flex Temp Smart, OMRON Healthcare Co., Ltd., Kyoto, Japan* with accuracy ± 0,1°C between 32,0°C to 42,0°C) approximately 1 cm into the rectum to acquire an automated reading upon pressing the measurement button. Ear temperature (BT_{ear}) was detected by high resolution infrared detection camera *(FLIR E50 Multi-Spectral Dynamic Imaging, MSX®, Wilsonville, Oregon, USA)* with 50mK (0.05°C) thermal sensitivity by spot and thermal imagines. The infrared images were obtained by manually focus, upon pressing the laser for guidance of the spot and recording into camera. The election point was in the ear scapha, on the axis of auricular pavilion at the half distance between apex and tragus (see figure no. 1 and 2). The infrared image was automatically adjusted.

Housing and feeding. The rabbits were kept in a three level *Techniplast® X-type cage, L x l x h* = 784 x 820 x 1830 mm and 4.264 cm^2 space. The walls and floors are made of transparent (side panels) or opaque polycarbonate (rear panels, discontinuous floor and trash and purine trays). In the rabbits' compartment, the environment temperature and humidity were continuously monitored (every half an hour) by multi-functional wireless digital device *Weather Station PCE-FWS 20*. The environmental temperature was 21.07±0.2 and the study do not sustain significant differences between days of measurements. The value of air speed in the room of rabbit was 0.01 m/s (one time per day measurements).

Figure 2: Measuring the rabbit ear temperature by IRT.
The position of the technician involved in IRT non-contact temperature prelevation (left) and laser point of the FLIR camera on the ear scapha, the election point. The door of the cage close (on the left) or open (in the right). In the right picture the animal went to the back of the cage to avoid human manipulation or restrain.
Surce: UEX Media, Experimental Unit, 2018.

Adult rabbit consumed daily 160-180 g of pelleted feed with ~~the~~ digestibility up to 65%. The metabolic energy density of feed was 1980±50 kcal / kg. The calories were coming from protein (23%), fat (10%) and carbohydrates (67%).

Statistical Analysis: *Paired t tests* and *Bland–Altman plot* analysis were used to assess differences in mean values for BT's measured by IRT versus the reference method (rectal temperature). Analysis of associations with several factors or variables (BT classes by BT_{ear}, weight, environmental temperature) were performed based on *Variance Analysis* (ANOVA), *Pearson correlation* and *regression* with *IBM® SPSS® Statistics* software, product of IBM Corporation, 2015. Significance was determined at a value of $\alpha = 0.05$.

Results

Body internal or rectal temperature (BT) was measured on 14 animals for seven consecutively days at the same hour - 22:00 p.m. The average and standard error (X±sx) of internal body temperature measured by digital thermometry was 39.03±0.7 ^{o}C. During the monitoring period, 10 rabbits were in hypothermia, in the next several hours or in first day after surgical procedures – the temperature was 37.93±0.16 and 5 rabbits had fever – 41.04±0.29 ^{o}C. The study could not associated the classes of BT with body weight (F=1,789 at p=0.173), day of study or room temperature.

Ear temperature (BT_{ear} – figure no. 3) for the same 14 rabbits was 37.50±0.12 ^{o}C, with 1.53±0.10^{o}C less then BT – the difference between internal and BT_{ear} temperature was significant (t=10.20, at p<0.001). The values of BT_{ear} (X±sx) for the rabbits with hypothermia BT class was: 35.73±0.40 ^{o}C (with 95% CI 34.78 ÷ 36.69 ^{o}C). For normothermic BT rabbits the ear temperature was 37.72±0.10 ^{o}C, with 95% CI between 37.51 to 37.92 ^{o}C.

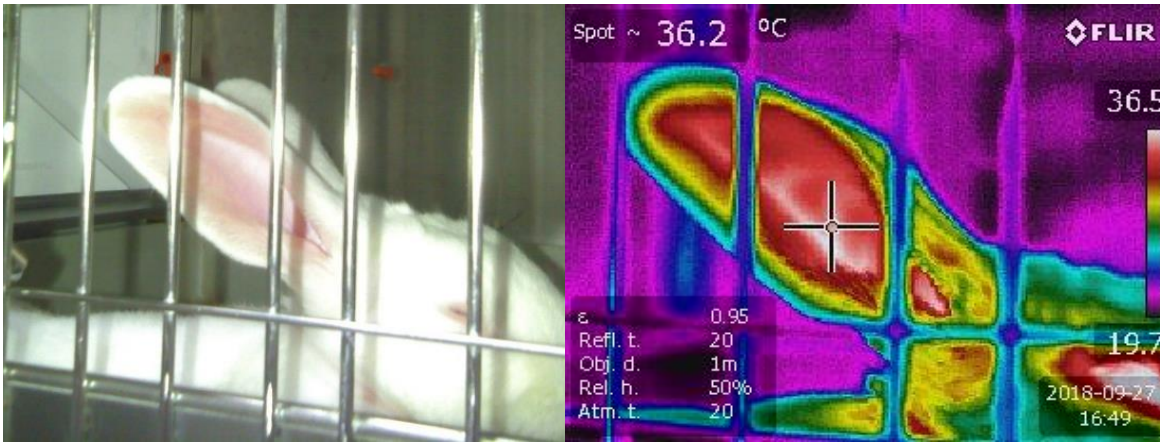
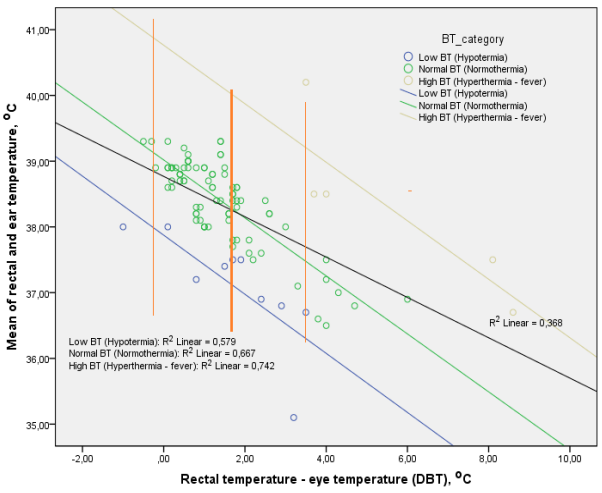


Figure 3: Clasical and thermal imagins of ear captured by thermocamera of the rabbit.
The clasical pictures of FLIR camera (left) and the thermal imaging of the same animal (right). The spot temperature of the election point was considered the ear temperature (BT_{ear}). The WNZ rabbit was in the cage – no contact between technician and animal and no door open was performed. There is a difference between a spot temperature (36.2ºC) and maximum temperature of termograpy image (36.5ºC).
Surce of imagines: UEX Media, Experimental Unit, 2018.

The difference (D_{BT}) and average (X_{BT}) between BT and BT_{ear} was performed for each animal and for all measurements. The average and standard deviation values X_{BT} 38.28±0.76ºC and the average of difference was D_{BT} = 1.53±0.10ºC (Graph no. 1). The Pearson correlation was found between BT and BT_{ear} (r = + 0.579, at p <0.001), BT and X_{BT} (r = + 0.831, at p <0.001), BT_{ear} and X_{BT} (r = + 0.934, at p <0.001), BT_{ear} and D_{BT} (r = - 0.783, at p <0.001).



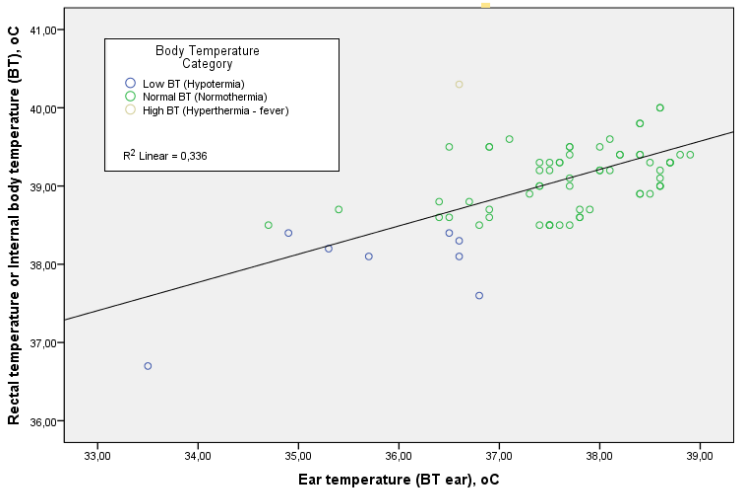
In the graph, the oblique thin lines are the lines of best fit for the data, the thick solid vertical line is the mean difference (bias), and the vertical thin lines are the 95% limits of agreement (mean difference ± 1.96 SD, respectively 1.53±1,96x0.86ºC). Points located on the left to -0.16 or on the right to +3.22 on the x-axis represent an underestimation of temperature by the IFR method in comparison to rectal core body temperature as measured by digital thermometer.
The value for regression of DBT for XBT has a determination coefficient R^2 = 0.368; the values of R^2 for each classes of BT are show in the graph.

Graph 1. Bland-Altman plots of body temperature in rabbits comparing rectal (BT) and ear temperature (BT_{ear})

The linear regression (Graph no. 2) of body temperature in rabbits (BT) by spot ear temperature (BT_{ear}) is written in a relation *(1)*:

$$\boldsymbol{BT = 25.498 + 0.361 \times BT_{ear}} \qquad (1)$$

($R^2 = 0.336$, t value = 11.36 at $p < 0.001$)



Graph 2. Body temperature (BT) regression by ear temperature (BT_{ear}).

Discussion

Using telemetry for ID identification (microchip) and temperature measurement (thermal camera) represent both responsibility of researcher and refinement of experimental techniques. Ear, tympanic membrane and hypothalamus share blood supply from the carotid arteries (3); past research has reported that tegument surface temperatures are correlated with body temperature (4,9). However this non-contact, fast and flexible approach gave resulted in too low, non-physiological temperatures, probably due to the isolating fur at the measuring spot. Furthermore, analysis of a single defined spot (in our case: middle or ear) on the auricular surface seems questionable for reproducible temperature measurements, since it can be strongly influenced by the isolating fur and heat accumulation in resuscitation cage used in intensive therapy, after anesthesia. A more sophisticated telemetry device - microchip implanted s.c. - permit both animal identification and body temperature and give more accurate values of temperatures measurement.

Compared to a single spot infrared thermometer, thermography generates images that can be analyzed on a per pixel basis as if thousands of infrared spot thermometers are used simultaneously. Using thermal imaging we were quickly able to identify the ear areas as the most prominent and warmest surface of the rabbit ear scapha (Fig. 1). Thus, the IRT can be used for measurement of temperature of orthopedic rabbit model even if the coefficient of multiple determination is not very high (R^2 was 0.336) because this method reduce the restrain and animal fixation time resulted. If the value of thermal spot imaging is abnormal there can be used classical measurements. Because there is no direct contact, this fast, flexible technology contributes to decrease the risk of pain, suffering or distress in orthopedic rabbit models.

Conclusions

- The ITR, a non-contact, fast and flexible technology eliminate the restrain and manipulation of rabbit orthopaedic models. As a result, the levels of pain, suffering or distress are reduced.
- The spots of IRT imaging taken from ear (BT_{ear}) can be used to estimate the body temperature by a regression equation.
- BT_{ear} can be useful to identify both hypothermia and fever rabbits.
- Using a more sophisticated microchip for both temperature measurement and animal identification have to by experiment in a future research projects.

Ethic statement

Study protocol was designed and followed in strict accordance with the guidelines of Experimental Units under Veterinarian Authority authorization no. 002 / 2018 and was a supporting activity for the project ORTO STEM REGEN & SIHFALCAG.

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THE DIAGNOSIS OF BOVINE TUBERCULOSIS IN BISTRIŢA-NĂSĂUD COUNTY DURING 2013-2017

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Abstract

Bovine tuberculosis is still a problem, both in terms of economic losses, animal health and the increased risk of human infections. Limited possibilities of veterinarians to diagnose and control tuberculosis could result in a rapid spread of this disease in cattle herds and making the eradication procedures longer and less efficient. Correct and accurate diagnosis of positive animals in the database and their culling could lead to the eradication of bovine tuberculosis from Romania. The aim of this study was the epidemiological and microbiological evaluation of bovine tuberculosis status on a 5 year interval (2013-2017) in Bistriţa-Năsăud County. Laboratory techniques have highlighted the presence of Mycobacterium bovis on microscopic fields, tuberculosis lesions in the organs using histological preparations and the growth on Löwenstein-Jensen medium. The most important screening technique for the diagnosis is the tuberculin test. From the total number of 375644 cattle tested over the 5-year interval, 364558 (97.04%) have been the subject of this tuberculin intradermal reaction. A total of 757 animals have shown an inconclusive result to the initial single test, all these have been retested 42 days later with the comparative simultaneous test, and just 27 came positive. From the total of 225 cattle diagnosed as positive, just 19 have been confirmed by pathological, cultural and experimental infection on guinea pigs.

Keywords: *catlle, tuberculosis, intradermal test, eradication.*

Introduction

Bovine tuberculosis (bTB) is one of the most serious economic animal health problems affecting the cattle industry worldwide, with incidence in cattle herds increasing since the mid-1980s. The single intradermal comparative cervical tuberculin (SICCT) test is the primary screening test in the bTB surveillance and control programme in most countries (Karolemeas, 2012). The official technique for diagnosis of bovine tuberculosis (bTB) worldwide is the tuberculin skin test, based on the evaluation of the skin thickness increase after the intradermal inoculation of a purified protein derivative (PPD) in cattle. (Casal, 2007).

Bovine TB in infected herd may occur due to the persistence of the microorganism in the environment or because of its introduction in a previously free herd. Furthermore, indirect transmission due to the presence of infected goats in the farm could contribute to the recirculation of bovine TB within the cattle herd (O'Hagan, 2018). The purchase of infected animals and the interaction with infected cattle or goats at common pastures could be the external sources of bovine TB (Filia, 2016).

In Romania, in the previous years, the single test was carried out on cattle over 6 months of age twice a year by intradermal inoculation of 0.1 ml bovine tuberculin in the neck area in a square with the 5 cm side. Currently testing is represented by the simultaneous comparative test, which is performed once a year for all cattle and buffaloes over 6 weeks of age, prior to vaccination procedures and involves inoculation of bovine and avian tuberculin at a dose of 0.1 ml administered strictly intradermally in two squares with the 5 cm side.

Field surveillance of British cattle using the single intradermal comparative cervical tuberculin (SICCT) test shows a higher incidence rate of bovine tuberculosis (bTB) in dairy

compared to beef herds, but a lower probability of post-mortem examination confirmed (PMC) Mycobacterium bovis infection in dairy herds (Downs, 2016).

The aim of this study was the epidemiological and microbiological evaluation of bovine tuberculosis status on a 5 year interval (2013-2017) in Bistriţa-Năsăud County.

Materials and methods

The study was conducted in Bistriţa-Năsăud County, is a retrospective observational study, and a total number of 364,558 cattle have been the subject of intradermal tuberculin test. The procedure involved the administration of strictly intradermal of avian and bovine tuberculin in two distinct squares on the side of the neck. The two areas were previously prepared, skin fold was measured on both areas and avian tuberculin was injected in the upper square while bovine tuberculin in the lower square. The administered amount was 0.1 ml in both cases.

The interpretation was performed 72 hours after the administration by measuring the skin fold in both squares. Cattles with positive results were culled while inconclusive results were retested 42 days after the first tuberculin test and 21 days after deworming. If both these tests are positive, animals are considered positive and culled. The disease was confirmed using microscopic examination using Ziehl-Neelsen staining method, cultivation on Lőwenstein-Jensen medium, histopathological examination of the tuberculous granuloma and experimental infection on guinea pigs.

Results and discussions

Laboratory techniques have highlighted the presence of *Mycobacterium bovis* on microscopic fields, tuberculosis lesions in the organs using histological preparations and the growth on Löwenstein-Jensen medium. The most important screening technique for the diagnosis is the tuberculin test. From the total number of 375644 cattle tested over the 5-year interval, 364558 (97.04%) have been the subject of this tuberculin intradermal reaction. A total of 757 animals have shown an inconclusive result to the initial single test, all these have been retested 42 days later with the comparative simultaneous test, and just 27 came positive. From the total of 225 cattle diagnosed as positive, just 19 have been confirmed by pathological, cultural and experimental infection on guinea pigs.

Table 1

Positive cattle to the tuberculin test

Interval	Total number of cattle	Total number of cattle tested	Positive
2013	74439	70887	119
2014	75542	72115	18
2015	78758	77656	18
2016	71226	71919	20
2017	75679	72900	50
Total	357644	365477	225

The confirmation of the positive cases revealed by the tuberculin test only validated an average of 8% (19 confirmed from 225).

Table 2

Confirmed cases of positive cattle to the tuberculin test

Interval	Positive to the tuberculin test	Confirmed	% confirmed
2013	119	12	10%
2014	18	5	28%
2015	18	0	0
2016	20	0	0
2017	50	2	4%
Total	225	19	8%

Conclusions

The study concerning the diagnostic of bovine tuberculosis in Bistriţa-Năsăud County during 2013-2017 concluded that:

- the incidence of tuberculosis evaluated by both positive and confirmed cases dropped over a five year period;
- mandatory screening of bovine tuberculosis is an important measure that will have an important contribution to the eradication of the disease;

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CLINICAL AND EVOLUTIVE ASPECTS IN DERMATOLOGICAL DISEASE THERAPY IN DOGS

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Abstract

In veterinary pathology, dermatopathies represent a challenge for the veterinarian due to the complex etiology and pathogenesis. More and more common, dermatological conditions have a diversity of clinical and evolutive aspects, which is why it is difficult to make a diagnosis of certainty and to establish a proper treatment. The aim of this study is to highlight the evolution of some dermatopathies by making a complete allergic investigation with an epidemiological investigation and a complex clinical examination, with an emphasis on the topical or systemic treatment used that has a favorable influence on the evolution of the disease. The clinical trial was conducted in a veterinary clinic, on 17 dogs in which 27 dermatopathies with different etiologies were diagnosed, treated and monitored. The materials and methods used consisted of: allergy investigation that provides information about the patient's situation; clinical examination consisting of evaluation of apparent mucous membranes, facies, attitudes, abnormal behavior, maintenance status, temperament, body temperature, pulse, cardiac and respiratory rate of the patient; complementary examinations: brushing, scotch-test, trichogram, cutaneous scarring, cytological exam, cutaneous biopsy, ultraviolet light exam, mycological examination, bacteriological examination, allergic tests, immunological tests, endocrine tests. Dermatopathies revealed a clinico-lesional pleiomorphism with the following manifestations: pruritus, alopecia, pyodermatitis, erythema, papules, crusts; which required a differential diagnosis and after performing the complementary examinations it was allowed the diagnosis of certainty. Of the 27 dermatopathies examined, 40.74% had bacterial etiology, 25.92% had micotic etiology, 18.51% had parasitic etiology, 11.11% had allergic etiology, 3.7% had other causes. Bacterial dermatitis has the highest occurrences with both superficial and deep pyoderma. To relieve pruritus it was used therapeutic baths with antiseboric and chlorhexidine shampoos, which provided body hygiene and completed the systemic treatment of superficial and deep pyodermatitis with bacterial and micotic etiology. The most effective treatment approach was achieved by combining both antibiotherapy, antipruritic therapy, topical antiparasitic drugs and dietary food, according to the established certainty diagnosis, the evolution being favorable.

Keywords: *dermatological diseases, dogs, evolution, therapy*

Introduction

In veterinary pathology, dermatopathies represent a challenge for the veterinarian due to the complex etiology and pathogenesis. More and more common, dermatological conditions have a diversity of clinical and evolutive aspects, which is why it is difficult to make a diagnosis of certainty and to establish a proper treatment[1, 3].

The aim of this study is to highlight the evolution of some dermatopathies by making a complete allergic investigation with an epidemiological investigation and a complex clinical examination, with an emphasis on the topical or systemic treatment used that has a favorable influence on the evolution of the disease [4, 6].

Material and methods

The clinical trial was conducted in a veterinary clinic, on 17 dogs in which 27 dermatopathies with different etiologies were diagnosed, treated and monitored.

The materials and methods used consisted of:

- allergy investigation that provides information about the patient's situation: knowledge of the age and the breed of the patient is very important, as some breeds are more prone to sensitivity to certain environmental allergens; knowledge of the diet used because many

dogs develop food intolerances; the presence or absence of parasites, as well as the presence of flea debris reveals important information for a diagnosis; absence or presence of pruritus; local or systemic therapy used before presentation at a veterinarian [5];

- Clinical examination consisting of evaluation of apparent mucous membranes, facies, attitudes, abnormal behavior, maintenance status, temperament, body temperature, pulse, cardiac and respiratory rate of the patient [9];
- Complementary examinations: brushing, scotch-test, trichogram, cutaneous scarring, cytological exam, cutaneous biopsy, ultraviolet light exam, mycological examination, bacteriological examination, allergic tests, immunological tests, endocrine tests [2];

For systemic therapy, 6 classes of therapeutic agents were used: glucocorticoids, H1 antihistamines, antibiotics, antimycotics, antiparasitics and immunomodulators. Cutaneous agents used in the local therapy have been represented by topical antipruritic, antiparasitic, antibacterial, antifungal, astringents, emollients and moisturizers [7, 8].

Results and discussions

The results concerning the etiology, treatment and healing time of the 17 investigated patients are presented in the following tables and graphs.

Table 1.

Classification of canine dermatopathies according to etiology

Etiology	Number of cases	%
Bacterial dermatitis	11	40,74
Parasitic dermatitis	5	18,51
Allergic dermatitis	3	11,11
Mycotic dermatitis	7	25,92
Another cause	1	3,70

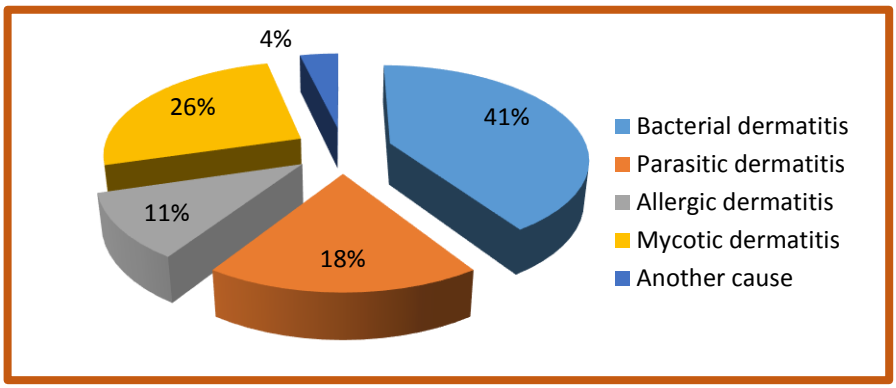

Figure 1 Canine dermatopathies according to etiology

The results presented in Table 1 and Figure 1 show that in the 27 evaluated dermatitis, 11 were diagnosed as dermatitis with bacterial etiology, 7 with dermatitis with mycotic etiology, 5 with dermatitis with parasitic etiology, 3 with dermatitis with allergic etiology and 1 patient with dermatitis with traumatic etiology.

Table 2.

Diagnosis and recovery time of evaluated dogs in this study

No. of case	Breed	Age, sex	Diagnosis	Recovery and re-evaluation time
1	Akita Inu	1 year 8 months, ♂	Superficial pyoderma	2 months
2	Mixed-breed	1 year 3 months, ♂	Superficial pyoderma, Demodicosis	1 month
3	Mixed-breed	6 years, ♀	Demodicosis	4 months
4	Akita Inu	5 years, ♀	Superficial pyoderma, Atopic dermatitis	6 months
5	Shitzu	2 years, ♀	Otitis media, Demodicosis	5 months
6	Boxer	1 year 6 months, ♂	Demodicosis, Otitis media	2 months
7	English Bulldog	2 years 3 months, ♂	Superficial pyoderma	6 months
8	Caniche	9 years, ♂	Superficial pyoderma, Otitis media	4 months
9	Alaskan Malamute	3 years, ♂	Mycotic dermatitis	1 month
10	Amstaff	5 years 6 months, ♀	Superficial pyoderma	3 months
11	Shitzu	3 months, ♂	Superficial pyoderma,	1 month
12	Labrador Retrievers	3 years, ♂	Traumatic pyoderma	1 month
13	Shar-pei	4 years, ♂	Superficial pyoderma, Otitis media	1 month
14	Beagle	3 years, ♂	Superficial pyoderma, Mycotic dermatitis	1 month
15	Dwarf Schnauzer	2 years, ♀	Atopic dermatitis	3 months
16	Pug	3 years, ♂	Atopic dermatitis	2 months
17	French Bulldog	1 year, ♂	Demodicosis, Atopic dermatitis, Furunculosis, Superficial pyoderma	2 months

In dermatopathies with bacterial etiology, antibiotic therapy was administered for at least 14 days, in some cases it was necessary a prolonged administration, reaching a period of 2-3 months. Treatment continued for another week after the lesions disappeared. Antibiotic therapy was represented by the following preparations: Xiclav, Synulox, Kessium, associated with a probiotic (Eubiotic).

Figure 2 Bacterial dermatitis

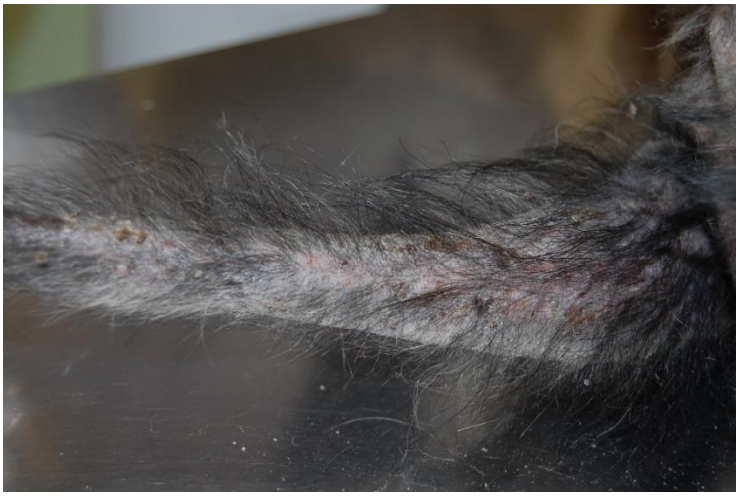

Figure 3 Parasitic dermatitis

For parasitic dermatopathies, external solutions or antiparasitic chewable tablets such as Advocate spot-on solution, Bravecto, have been successfully used. It has been noticed that periodic use of antiparasitic agents plays an important role in the treatment of dermatopathies.

In the case of allergic dermatopathies, the intradermal test revealed allergens, in which two patients were diagnosed positively and immunotherapy was administered for at least 2 years. Local therapy with promising results such as Allerderm Spot-on has been applied helping to restore skin integrity.

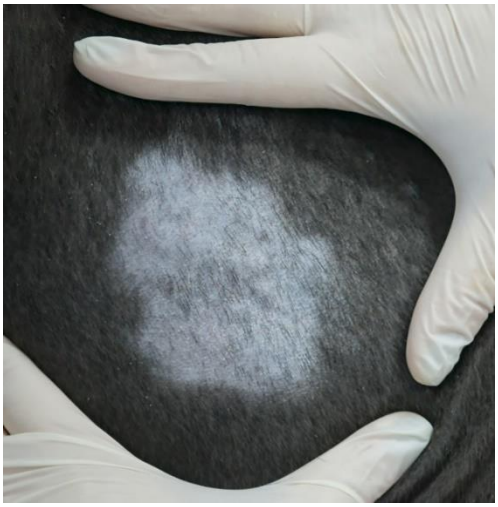

Figure 4 Allergic dermatitis

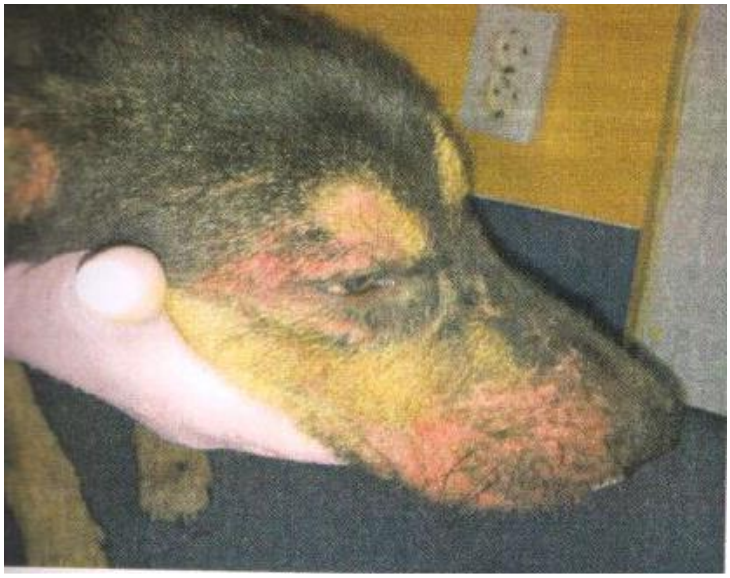

Figure 5 Mycotic dermatitis

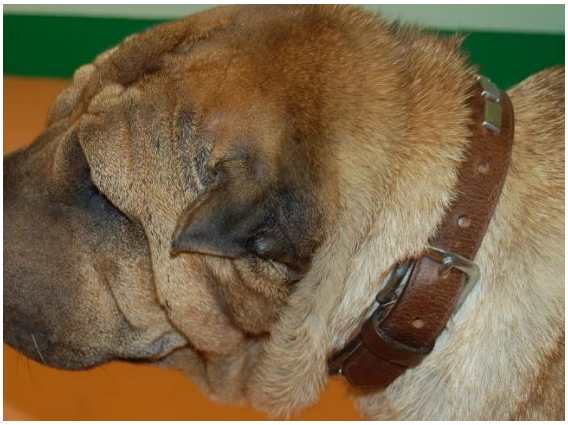

Figure 6 Mycotic dermatitis

For fungal dermatitis, it was successfully used ointments and drugs with antimycotic substances such as Fungiconazol, Micodermin.

In addition to specific systemic and local treatments, good results have been seen when it was used the hypoallergenic food diet. Also an important role in relieving the lesions was the treatment baths with antiseboric shampoo or Clorexiderm shampoo 4% based on chlorhexidine digluconate.

Table 3.

The distribution of cases according to evolution

No of cases	Evolution		
	favorable	stationary	unfavorable
27	24	2	1

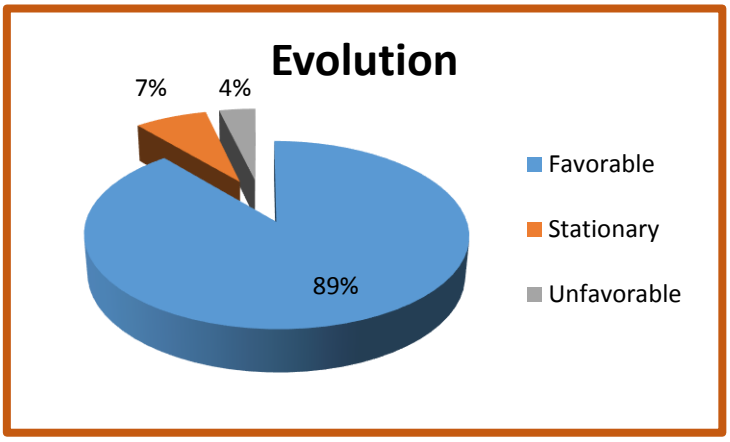

Figure 7 Percent representation of cases according to evolution

Conclusions

Following the examination of the 27 cases of dog dermatitis we can conclude:

1. Dermatopathies revealed a clinico-lesional pleiomorphism with the following manifestations: pruritus, alopecia, pyodermatitis, erythema, papules, crusts, which required a differential diagnosis and after performing the complementary examinations it was allowed the diagnosis of certainty.
2. Of the 27 dermatopathies examined, 40.74% had bacterial etiology, 25.92% had micotic etiology, 18.51% had parasitic etiology, 11.11% had allergic etiology, 3.7% had other causes. Bacterial dermatitis has the highest occurrences with both superficial and deep pyoderma.
3. Allergic dermatitis required a difficult differential diagnosis, requiring an intradermmatic test to reveal the allergens found in the two studied cases.
4. Complementary examinations such as trichography and skin rash have highlighted parasites such as *Demodex spp*. For this parasitic dermatitis, a favorable result has been the use of spot-on pipettes, such as Advocate (which contains Imidacloprid and Moxidectin).
5. To relieve pruritus it was used therapeutic baths with antiseboric and chlorhexidine shampoos, which provided body hygiene and completed the systemic treatment of superficial and deep pyodermatitis with bacterial and micotic etiology.
6. The most effective treatment approach was achieved by combining both antibiotherapy, antipruritic therapy, topical antiparasitic drugs and dietary food, according to the established certainty diagnosis, the evolution being favorable (Table 3 and Fig. 7).

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THE THERAPEUTIC MANAGEMENT OF PARASITIC DISEASES IN GOATS

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Abstract

Parasitic diseases occupy a major place in goat farms, antiparasitic preparations are numerous, with different efficacy and costs, and if the cost is too high it becomes an inconvenience for goat breeders. The aim of this study was to know the main parasitoses that can be encountered in a goat farm as well as to test the efficacy of the antiparasitic preparations used, depending on the active substance contained, the doses used and the route of administration. This study was conducted on goats with different breed: Saanen, French Alpine, Alba de Banat and Carpatina. From each age and sex category we chose 10 animals from which we harvested the necessary samples and whose treatment was monitored up to the final results. The groups have been numbered as follows: females-group 1, males-group 2, kids-group 3. The crust harvesting was performed depending on the suspected acariosis: for psoroptic mites, the scraping was superficial and from the edge of the lesions; for sarcoptic mites, deep scraping was performed, mites were found in the deep layers of the epidermis; to identify Demodex spp. it was necessary deep scraping, these mites being located at the base of the hair follicle and sebaceous glands; for the detection of pulmonary strongyles a larvhelmintoscopic method was used; for the detection of heavy trematode eggs the centrifugation method was used. Following the examinations, we initiated the treatment for gastrointestinal strongylosis, eimeriosis and scabies using Evomec (ivermectin), Ascacid (albendazole), Sulfaquinoxaline and Diazinol (diazinon). Mild infestations with gastrointestinal Strongyles in adults (100%), mild infestations with Scabies mites (3.3%) and massive infestations with Eimeria spp. in youngsters (100%) were encountered in the study. The therapy resulted in the insignificant elimination of the strongyl eggs, the total elimination of the mites and the elimination of less than half of the oocysts of Eimeria spp. In addition to deworming, prevention must be done, prophylaxis being achieved by maintaining farm hygiene, providing natural lighting, quality feed, avoiding weaning stress and restricting access of other species in accommodation.

Keywords: *goats, parasitic diseases, therapy*

Introduction

It is known that goat farms have grown in recent years, with more and more breeders opening farms of different types and sizes, exploiting a growing variety of goat breeds, both locally and imported[3, 8].

Parasitic diseases occupy a major place in goat farms, antiparasitic preparations are numerous, with different efficacy and costs, and if the cost is too high it becomes an inconvenience for goat breeders[7].

The aim of this study was to know the main parasitoses that can be encountered in a goat farm as well as to test the efficacy of the antiparasitic preparations used, depending on the active substance contained, the doses used and the route of administration. At the same time, it was intended to optimize the approach of antiparasitic therapy in a goat farm depending on the parasitic diseases encountered[5, 6].

Materials and methods

This study was conducted on goats with different breeds, like Saanen, French Alpine, Alba de Banat and Carpatina. From each age and sex category we chose 10 animals from which we harvested the necessary samples and whose treatment was monitored up to the final results. The groups have been numbered as follows: females-group 1, males-group 2, kids-group 3. The goats had good maintenance, but half of the kids had diarrhea.

To identify gastrointestinal strongyle eggs, samples of feces from adult animals, from groups 1 and 2 were collected. These samples were also used to highlight the pulmonary strongyles as well as the trematodes. In order to identify coccidia oocysts, samples from group 3 were collected[1, 4].

In order to reveal the presence of scabies mites, hair and cutaneous samples were harvested from the animals in the study groups witch presented pruritus and hyperkeratosis.

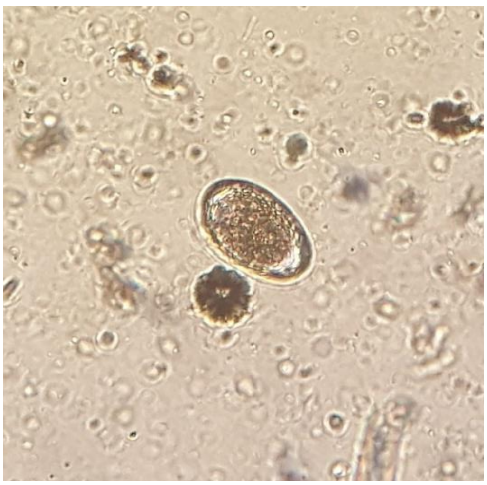
To identify gastrointestinal Strongyles and Eimeria oocysts, the coproparazitologic examination was performed by the Willis method[2]. For the detection of the mites, the microscopic examination of the skin scrapings and superficial crusts was performed[9].

The crust harvesting was performed depending on the suspected acariosis:

- in the case of psoroptic mites, the scraping was superficial and from the edge of the lesions;
- in the case of sarcoptic mites, deep scraping was performed, mites were found in the deep layers of the epidermis;
- to identify *Demodex spp*. it was necessary deep scraping, these mites being located at the base of the hair follicle and sebaceous glands;
- for the detection of pulmonary strongyles a larvhelmintoscopic method was used, namely the Vajda method;
- for the detection of heavy trematode eggs the centrifugation method was used.

Results and discussions

Following the microscopic examination of the preparations from the feces samples from group 1 and 2, were observed Strongyles eggs in small quantities, all the samples were positive (Fig. 1 and 2).



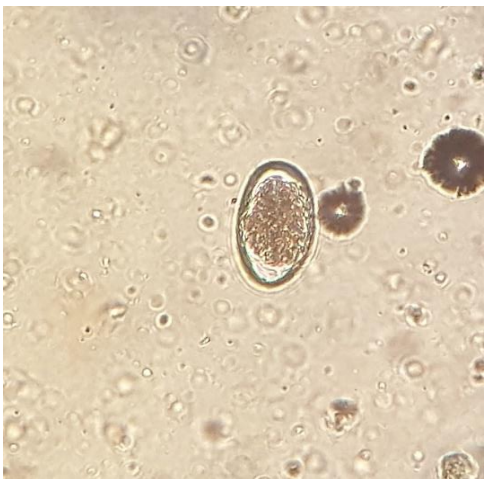
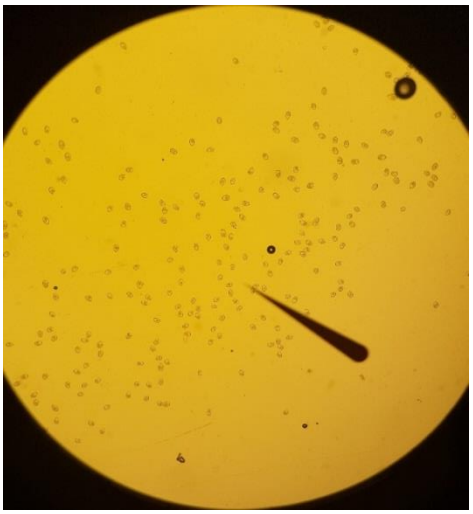


Figure 1 and 2 Strongyles eggs

Following the coproparazitological examination of the faeces collected from group 3, the presence of oocysts of *Eimeria spp*. was observed in a very large number, all samples being positive, as shown in Figures 3 and 4.



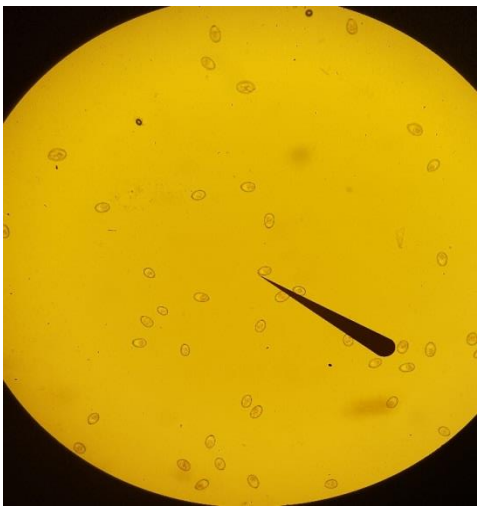


Figure 3 and 4 *Eimeria spp,* observed in a very large number

Following the parasitological examination of the skin, one of the samples was positive for scabies (Figure 5). Samples for pulmonary strongyles and trematode were negative.

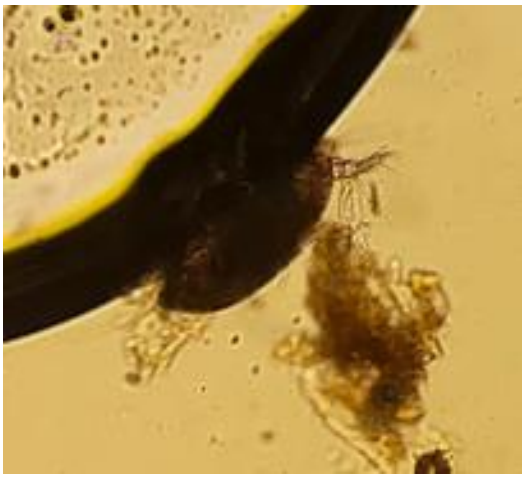


Figure 5 Positive sample - the presence of a mite

Following the examinations, we initiated the treatment for gastrointestinal strongylosis, eimeriosis and scabies using Evomec (ivermectin), Ascacid (albendazole), Sulfaquinoxaline and Diazinol (diazinon).

Goat farm deworming were made as follows:

- deworming against gastrointestinal strongylosis was performed before mating (2-3 weeks), 2 months before calving and during lactation only by necessity;
- against coccidiosis, kids were dewormed at one month's age;

- external solutions against parasites were used between may and june.

Evomec was administered at dose of 1.5 ml per animal subcutaneously and Ascacid at a dose of 20 ml per animal orally, in groups 1 and 2. After 10 days, the coproparasitological examination was repeated.

Against scabies were made sprays with DIAZINOL at dose of 20 ml/20 liters of water for the three batches and after 10 days the dermato-parazitologic examination was repeated. In order to combat coccidiosis, in group 3, SULFAQUINOXALINA was used in drinking water at a dose of 5 ml/ 4 liters of water, daily for one week, and after 10 days the coproparasitic examination was restored.

After the treatment, for groups 1 and 2, two samples went positive with infestation but insignificant (Fig. 6).

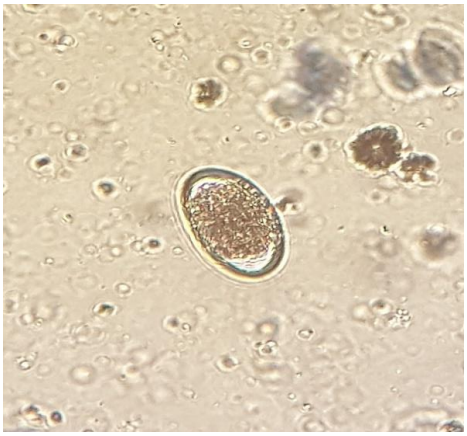


Figure 6 Strongyles eggs

After using Diazinol, the samples were negative with no scabies mites on any of the analyzed samples.

After 10 days from the end of the treatment for coccidiosis in kids, the samples continued to be positive, as can be seen in Figure 7.

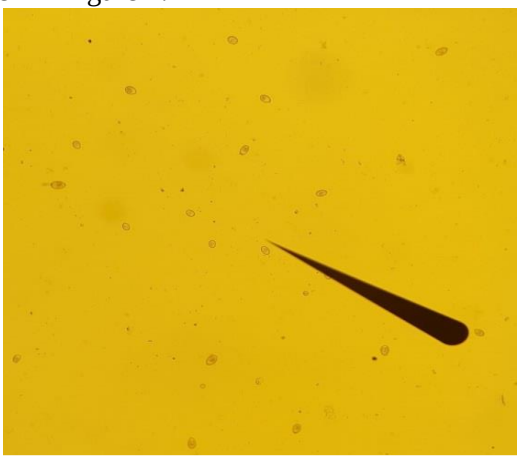


Figure 7 *Eimeria spp*., positive sample after the treatment

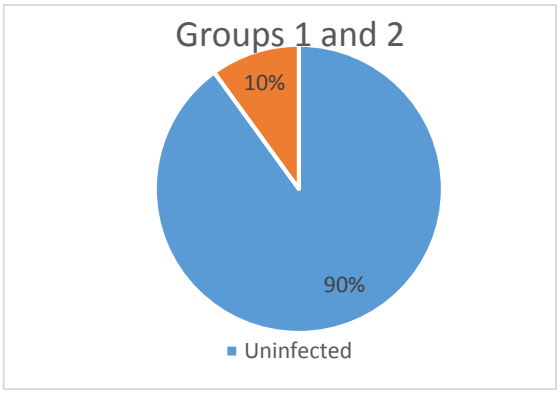


Figure 8 The percentage results after anthelmintics deworming in groups 1 and 2

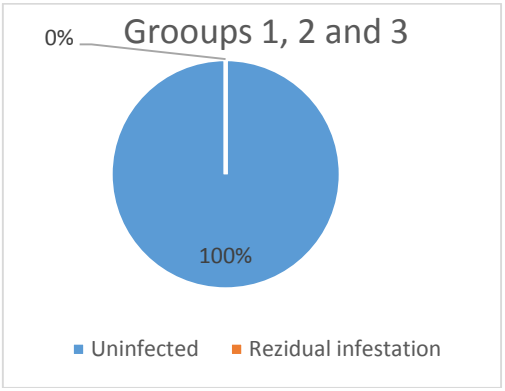


Figure 9 The percentage results after the treatment against scabies in groups 1, 2 and 3

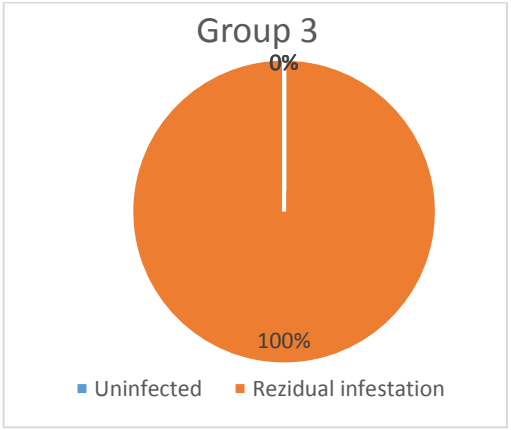


Figure 10 The percentage results after the treatment against coccidiosis in group 3

Conclusions

In this study, 30 animals were examined and divided into 3 groups (1,2 and 3). The information obtained from the clinical examination, laboratory examinations, deworming and post-therapeutic monitoring allowed the following conclusions to be drawn:

1. Mild infestations with gastrointestinal Strongyles in adults (100%), mild infestations with Scabies mites (3.3%) and massive infestations with *Eimeria spp*. in youngsters (100%) were encountered in the study.
2. The anthelmintic therapy consisted of administration of ivermectin and albendazole to adult populations and the results consisted in the elimination to insignificantly the strongyl eggs (10% residual infestation of the animals in groups 1 and 2)(Fig. 8).
3. The therapy against scabies mites consisted of diazinon sprays in all three lots, the results being represented by the total elimination of the mites (100%)(Fig. 9).
4. The therapy against coccidiosis was achieved by administering Sulfaquinoxaline to the kids group, the results being constituted by the elimination of less than half of the oocysts of *Eimeria spp* (100% residual infestation)(Fig. 10).
5. In addition to deworming, prevention must be done, prophylaxis being achieved by maintaining farm hygiene, providing natural lighting, quality feed, avoiding weaning stress and restricting access of other species in accommodation.

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CLINICAL AND BIOCHEMICAL STUDIES ON CONTAGIOUS CAPRINE PLEUROPNEUMONIA WITH SPECIAL REFERENCE TO LIPOPROTEINS PROFILE AND FIBRINOGEN LEVELS

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Abstract

Contagious caprine pleuropneumonia (CCPP) is a worldwide disease that lead to great economic losses. The goal of this investigation was to investigate the hematological picture, lipid profile and fibrinogen levels in goats with CCPP. A total of 355 goats were selected for this study. CapriLAT Latex Agglutination test was used to detect antibodies rapidly against Mycoplasam capricolum subspecies capripneumoniae (Mccp) from serum samples ((N=355). Then paired blood samples were collected from 65 Mycoplasma capricolum subsp. capripneumoniae (Mccp)-infected goats (positive cases), together with 20 healthy controls. Lipid profile, blood hematology picture and some biochemical blood values were determined. The examined hematological parameters revealed a significant increase in total leucocytic counts, neutrophil and monocytes with a significant decrease in PCV value, hemoglobin content, RBC count in the goats with Mccp compared with the control group. Biochemical parameters examination revealed increased in the diseased goats, except high-density lipoprotein cholesterol (HDL-c), and total cholesterol that found significantly decreased than control group. Fibrinogen value was consider higher in the diseased goats when compared to the control ones. This study will open avenue on the possible pathophysiological role of hematological parameters, lipid profile and fibrinogen in goats that affected with CCPP.

Keywords*: Goats, HDL-c, Latex Agglutination, , LDL-c, Mycoplasma,*

Introduction

Mycoplasmosis is a disease caused by different Mycoplasma species. The disease affect different livestock including sheep and goats. Infection by *Mycoplasma* has an economic impact due to indirect losses like delayed market weight, emaciation, and infertility, that caused by subacute or chronic pneumonia in small ruminants. In goats, contagious caprine pleuropneumonia (CCPP) is considered to be with high fatality. The causative agent of the disease is *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp). The disease has a mortality rate that reach 100% and mortality rates that reach 60–80% (Kumar et al. 2011; Ibrahim et al. 2016; Larios-Hernández et al., 2017).

The disease in goats in the Kingdom of Saudi Arabia is locally known as Abou Romh and is well known by the herder in different parts of country. The disease cause serious problem and was reported in goats in Saudi Arabia. (2012, Ministry of Agriculture report; Radwan, et al, 1985, Tharwat and Al-Sobayil, 2017, El-Deeb et al., 2017).

Clinico-pathological profile of CCPP showed that the disease mainly affect respiratory system. The clinical picture of the disease presented in different forms in endemic areas. These forms can be Peracute, acute and chronic forms. In the affected goats with peracute form, the animal death may occur in a period of 1 - 3 days showing minimal clinical signs profile. On the other hand, the acute form show signs of high fever (41-43°C), then the animal be anorexic and lethargy, after that cough commence with difficulty in respiration. The cough usually is frequent

but violent and productive. At terminal stage of the disease, the goat become with abnormal gait and specific posture. The affected animal show continuous mouth salivation pain and grunting. Terminally nasal discharge of frothy nature may seen. Within 7 to 10 days in the acute form, the death of affected animal may occur. In the Subacute forms, the sign and severity are mild and the cough is the only sign. In the chronic form of CCPP, the signs are nasal discharge, chronic cough, and debilitation (Samiullah, 2013; El-Manakhly and Tharwat, 2016, Tharwat and Al-Sobayil, 2017; El-Deeb et al., 2017). Haematologically, the infected animals were anemic, with leukocytosis followed by leucopoenia, the total erythrocyte count, hemoglobin and hematocrit values were significantly decreased. While the mean corpuscular hemoglobin and mean corpuscular volume values were significantly increased. Blood biochemistry characterized usually by increase in albumin and total serum protein while serum glutamic pyruvate transaminase (SGPT), globulin fraction of serum, serum calcium and blood sugar were significantly increased (Shah et al, 2017)

The difficult definitive diagnosis is characteristic because the organism (Mycoplasma) is most fastidious which lead to the missing of diagnosis when routine bacteriological examinations. The isolation of the Mycoplasma (Mccp) from different clinical samples (in live or dead animal) is considered to be the golden method for confirmatory diagnosis of CCPP (Samiullah, 2013; Nicholas and Churchward. 2012; OIE 2009). Latex Agglutination Test (LAT) is a serological test that used in detection of Mccp antigen in goats infected with CCPP. The microspheres were coated with anti-Mycoplasma capricolum subsp. Capripneumoniae polyclonal immunoglobulin G (IgG) antiserum and purified *M. capricolum* subsp. *capripneumoniae* capsular polysaccharide (CPS). The LAT is sensitivite and have low cost and easily be applied in the field, without the need for trained personal and sophisticated equipment (March et al. 2000, Bahir et al., 2017).

The main objectives of this study were to investigate the hematological and biochemical profiles (especially lipoprotein profiles and fibrinogen) of pneumonic mycoplasmosis caused by *M. capricolum* subsp. *capripneumoniae* in goats.

Materials and methods

Animals

Three hundred and fifty five goats were investigated in this study. Sixty-five were with clinical signs of CCPP (diseased group) while 20 healthy goats considered as control group (that were negative to CCPP using CapriLAT Latex Agglutination test.). All animals were from Al-Ahssa region, Saudi Arabia. All Animals from the two groups were 1-5 years age and with free grazing husbandry. A full clinical examination was done to all goats. Detailed clinical signs were observed and then recorded. Accordingly, goats that showed clinical signs of pneumonia and positive for CapriLAT Latex Agglutination test for *Mycoplasama capricolum* subsp. *Capripneumoniae* (MCSC) were selected for further investigations.

Sampling

Paired blood samples were collected from the healthy goats and those infected with Mccp (positive for CapriLAT Latex Agglutination test). For hematological investigations, the blood samples were taken in heparinized tubes. While the other samples were taken in plain tubes for chemical investigations. The second samples were centrifuged at 3,000 rpm for 10 min, to obtain clear serum that stored in Eppendorf tubes at −20 °C until used.

CapriLAT Latex Agglutination test.

This test was used on serum samples (N=355) for rapid detection of antibodies against Mccp. In this test, the latex beads were coated with a capsular polysaccharide (CPS) purified from

Mccp cells. When added to the serum from infected animal antibodies recognized the CPS and bind and cross-link the latex particles causing agglutination.

Test procedure

1-The kit was removed from the refrigerator and allowed to cool to room temperature. 20 µl of serum were spot onto a black reaction slide using a pipette.
2-The latex reagent was well shacked and one drop of the reagent was added next to the spot of the serum.
3-Serum and reagent were mixed together using a pipette and the mixture was spread out in the reaction cell.
4-The reaction side was rocked from left to right for three minutes and any agglutination occur was recorded.

Hematology and Biochemical profile

Hematology

Hematological investigations were conducted using an automatic cell counter (VetScan HM5 Haematology system, ABAXIS, USA).). The parameters determined were total erythrocytic count (RBCs), Hemoglobin concentration (Hb), Packed cell volume (PCV- HCT), Total leucocytic count (WBCs), Erythrocytic indices including (MCV, MCH, MCHC), Differential leucocytic count (monocytes, lymphocytes, granulocytes).

Lipoprotein profile and liver enzyme

The serum lipoproteins mainly cholesterol, low density lipoprotein (LDL) cholesterol high-density lipoprotein (HDL) cholesterol and triglyceride were measured using the methods described by Nazifi et al. (2002). Serum Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) concentrations were measured using an automated biochemical analyzer (VetScan VS2, Abaxis, NC, USA). Fibrinogen concentration was determined using commercial ELISA kit (Bicompare, South San Francisco, CA 94080 USA cat No. ABIN1053650). Procedures were done following the manufacturer's instructions.

Statistical analysis

Data analysis was carried out using statistical software program Minitab 16 (Minitab Inc. Quality Plaza, 1829 Pine Hall Rd State College, PA 16801-3210, USA). Because of the small size of the control group and non-normally distributed parameters in CCPP cases, each blood parameter was assessed using non-parametric analysis (Mann-Whitney) to compare the data between cases and controls. The differences of the values between CCPP cases and control group were accepted as statistically significant when *P* value was lower than 0.05 ($p<0.05$).

Results

Clinical picture

The clinical examination of affected animals under investigation revealed, cough, nasal discharge (mucopurulent discharge), fever, decreased in appetite and growth, with diminished milk production among affected dams.

Hematology

Table 1, showed that there were significant differences in hematological parameters between infected and non-infected animals. There was a significant increase ($P<0.05$) in the level of Total Leucocyte Count (TLC), lymphocytes, neutrophils and monocytes between infected and

non-infected goats, with a significant decrease in hematocrit percent, hemoglobin content, RBC count in the goats with Mccp compared with the control group.

Table 1

Summary statistics of the level of hematological parameters in control goats and those with contagious caprine pleuropneumonia (CCPP)

Parameter	Control (N=20)				CCPP Cases (N=65)				
	Mean	Median	Min	Max	Mean	Median	Min	Max	*P*Value*
Hb (gm/dl)	12.35	12.84	10.35	14.33	8.22	8.33	7.5	9.33	<0.001
PCV (%)	30.11	29.88	29.2	32.33	19.55	19.66	17.44	20.42	<0.001
RBC (×106/µl)	11.85	12.11	10.44	13.33	7.33	7.22	6.43	6.44	<0.001
WBCs per µl	8877	8775	8100	9400	14200	14100	13000	16000	<0.001
Mature neutrophil per µl	3,100.6	3120.0	2900	3300	8661	8,675.4	8510	8,868.0	<0.001
Band neutrophil per µl	0	0	0	0	774.2	796.1	532.3	922.4	<0.001
Lymphocyte per µl	5,345.3	5237.4	5122.3	5490.3	5,192.4	5,102.0	4830	5880.4	<0.0411
Monocyte per µl	177.71	180.0	147	203	321.1	305.0	239	390.0	<0.001
Eosinophil per µl	336.36	345.22	279.1	381.1	341.9	352.11	279.2	381.53	<0.841

**p* Value resulting from non-parametric Wilcoxon Mann-Whitney test

Lipoprotein profile and liver enzyme

Table 2, showed an increase in the biochemical values in the diseased goats, except for high-density lipoprotein cholesterol (HDL-c), and cholesterol in which significant decrease was observed when compared to control group. Fibrinogen levels was considerably high in the diseased goats when compared to the control ones.

Table 2

Descriptive results of selected biochemical parameters in goats with contagious caprine pleuropneumonia (CCPP) and in healthy ones

Parameter	Control (N=20)				CCPP Cases (N=65)				
	Mean	Median	Min	Max	Mean	Median	Min	Max	*P*Value*
ALT (IU/l)	25.39	25.55	20.25	28.35	53.26	58.36	24.22	71.23	<0.001
AST (IU/l)	62.33	63.22	57.44	68.22	173.88	196.31	64.26	201.23	<0.001
Triglyceride (mg/dl)	55.44	56.22	54.12	58.54	94.45	99.15	57.23	100.33	<0.001
LDL-c (mg/dl)	25.88	26.43	25.22	27.44	31.55	32.30	27.44	34.54	<0.001
HDL-c (mg/dl)	51.55	52.52	48.77	52.66	17.22	15.44	11.11	42.22	<0.001
Cholesterol (mg/dl)	65.44	65.95	58.55	68.87	42.33	41.47	38.32	67.33	<0.001
Fibrinogen (mg/dl)	251.25	250.26	220.15	277.15	366.25	355.15	330.5	388.25	<0.001
Total proteins (mg/dl)	7.88	7.77	7.64	7.94	6.22	6.11	5.56	6.55	<0.001
Albumen (mg/dl)	3.46	3.36	3.31	3.51	2.62	2.52	2.44	2.84	<0.001
Globulin (mg/dl)	4.55	4.42	4.43	4.66	5.43	5.33	5.24	5.61	<0.001

**p* Value resulting from non-parametric Wilcoxon Mann-Whitney test

Discussion

The clinical signs described for mycoplasamas infection worldwide were varied. This is because of different types of Mycoplasmas cause the disease. The presence of viruses and other bacteria may complicate the infection giving different clinical picture when there is field outbreak (pasteurellosis) as part of the causative picture (Maré, 2004). The clinical examination of affected animals under investigation revealed, cough, nasal discharge (mucopurulent discharge), fever, decreased appetite and poor growth, with diminished milk production among affected dams. The detected clinical picture is in accordance with those reported by Ayling and Nicholas, (2007); Gonçalves et al., (2010); Dezfouli et al. (2011); Rifatbegovic et al., (2011).

High WBCs count levels and neutrophils percentage in diseased animals may be a reflection of immunity reflexes (El-Ghmati et al., 1996). Former investigations (Civelek et al., 2007; El-Bahr & El-Deeb, 2013) reported increase in total leucocytic count (WBCs) in calves that

affected with bacterial pneumonia. Furthermore, the increase in WBCs was reported in many infections (LaMonica et al., 1981; Civelek et al., 2007).

The decreased levels of Hb and RBCs indicate the anemic state of animals with Mycoplasma pneumonia, which may be attributed to loss of appetite and desire to eat. On the other hand, the infection produces hyperactive radicals i.e. hydrogen peroxide in the body, which lead to destruction of erythrocytes in the body that ultimately leads to anemia (Mondal et al, 2004).

In the inflammatory process usually secretion of inflammatory cytokines (IL-1, IL-6, and TNF) occur. These cytokines change the vascular levels of a multiplicity of proteins that are produced mostly in the liver (El-Bahr and El-Deeb, 2013). Usually in healthy animals these proteins level is little or even, undetectable so when there is elevation in these level may indicate animal diseases (Glass et al., 2003; El-Deeb and Iacop, 2012). The precise category of APPs and the time sequence for variations in these proteins fluctuate by species based on the starting signal or causal inflammatory procedure**.** In the current investigation, it was observed that a significant increase in the values of fibrinogen (negative acute phase protein) in Mycoplasma-infected animals when compared with healthy ones (El-Deeb and Iacop, 2012).

Due to septicemic nature of disease caused by Mccp, different visceral organs are affected with different degree of toxicity. The liver is the most common organ targeted by this species of Mycoplasma that was evident by necrotic foci on its surface on gross examination (Mondal et al., 2004). The hepatotoxicity ultimately increases the enzyme levels in the liver function tests (Gutierrez et al., 1999, Mondal et al., 2004, Sadique et al., 2012). Due to the involvement of the liver, there is a significant increase in SGPT. Similar findings were also reported by Gutierrez et al. (1999) and Mondal et al. (2004). Due to the liver damage, protein synthesis is also affected, which leads to decrease in total serum protein. In the present study, there was a significant reduction in total serum protein and albumin while globulin increased significantly. This reduction may also be due to the fact that Mycoplasma spp. also consumes protein for their proliferation. Increased globulin levels might be due to the production of antibodies in response to infection. Normal ratio of albumin and globulin, which is slightly above 1 is also disturbed in the present study. There is a significant decrease in the Albumin/Globulin Ratio. These findings are in accordance with the findings of Kaneko and Cornelius (1970) and Kumar et al. (1994).

Conclusively, this study shed the light on the possible pathophysiological role of hematological parameters, lipid profile and fibrinogen in goats with CCPP.

Conflict of interest

Authors disclose no conflict of interest in this study.

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MICROBIAL PROBIOTICS – THE ACTION MECHANISM AND THE USE OF THEM

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Abstract

The scientific paper presents important aspects of the mechanism of action and use of probiotic microorganisms, which administered in adequate quantities has beneficial effects on the host. The investigation has the main objective to present an analysis of the importance of probiotics performance and functionality, which are available in food or as nutritional supplements, most frequently represented by strains of Lactobacillus, Bifidobacterium and Streptococcus. The research focused on the activity of the probiotic action as enhanced or concentrated cultures of lactic acid bacteria, which serve not only to prevent or reestablish a malfunctioning of the digestive tract, but have a positive influence on the immune system.

Key words: *Probiotics, Bacteriocins, Immune system, Metabolism, Probiotic bacteria.*

Introduction

Probiotics are benefic bacteria having an important role for our health. They are present everywhere: inside and on the body surface, making good things happen, but mostly probiotics are found in bowels. Some of these microorganisms Lactobacillus acidophilus, Bifidobacteria, Bifidobacterium longum, Escherichia coli etc are very important [3],[1].

Although it seems hard to believe, inside of the human and animal body there is a heavy fight every day. Probiotics form a psyhical protective layer along the walls of the intestines in order to protect from the invasion of harmful bacteria [2].

Probiotics constitute 60% from the immune function secreting substances which combat pathogens like: bacteria, viruses and fungi maintaining the body healthy. Besides contributing to the immune system, probiotics have also other important functions which influence positively the organism [4], [6].

The lack of a sufficient quantity of benefic bacteria weakens the immune system and we are exposed to microbes, harmful bacteria and viruses which can cause different diseases. When the digestive tract is healthy, the positive bacteria filter and eliminate everything that can harm – toxins, harmful substances and other residual products. In the same time, tey absorb everything the body needs (nutrients from food and water), assimilates them and distributes them at the cell level [5].

Most probiotics colonize in the intestine. These organisms form a neutral network, sometimes being called as the „second brain". This neutral network from the intestines captures information from the external environment and maintain a constant communication with the first brain through the central nervous system contributing in decision making based on so called intuituin [7,8].

From this point of view, the main objective of this research is represented by the study of some microbiological aspects on how to act and to use probiotics, which are important products in immune potentiation of the animal and human organism.

Material and method

For the realization of this study there were performed bacteriological investigations of some probiotic species of bacteria: Lactococcus cremoris, Lactobacillus acidophilus, Streptococcus lactis etc.

The investigations have been subjected to microbiological conduct of investigation which consisted in visualization of development characters of microbial cultures and studying the microbial colonies (edges, color, consistency), the development character of cultures in the liquid medium through visualization the development characteristics (turbidity, sediment, consistency, smell, etc)

Results and discussions

The detailed analysis of the performed researches gave us the possibility to state and analyze the probiotics effects based on the bacterial studied species through the activity of the action mode and other characteristics which are very complex.

According to the performed studies on probiotic microbial species traded on the world market, these species present a special interest and perform their activity through a complex of benefic effects on the organism (table 1).

Table 1.

Probiotic products produced from microorganisms traded on the world market

Product	**Contains**	**Animal species**	**Effects**
All-Lacc	Lactobacili	Piglets	Reduces mortality, increases daily average gain
Lacto-Sacc	Lactobacili Saccharomyces	Pigs	Reduces the incidence of diarrhea and mortality
Fralac-Lbc	Streptococcus faecium	Allspecies	Prophylactic for diarrhea, growth promoter
Cocbactin	Lactobacillus acidophilus	Calves	Increases daily average growth
Oralin	Entrococcus faecium	Taurine Swine Poultry	Improves productive performance
Protexin	Streptococcus faecium, Steptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus casei.	Horses, sheep, pigs	ÎImproves health and productive performance
Probios	Lactobacillus acidophilus, Lactobacillus pantarum, Lactobacillus casei	Calves Swine	Increases appetite, milk production, reduces mortality

Many researches studied the action of probiotics as selected cultures or concentrated of lactic bacteria, which serve not only for prevention or restore a malfunction of the digestive system, but also have a positive influence on the immune system.

Thus the production of inteferon and immunoglobuline may increase very significantly by using probiotics regularly, this fact increases the resistance to diseases and promise the establishement of a normal state.

Therefore, in order to exercise the role of maintaining the microbiota balance, protecting the host organism against various diseases, in order to improve the nutritional status of the individuals

who have consumed probiotics, it is necessary to consider the following: the microbiota strain must withstand (salivary, stomach, intestinal), have good resistance to acid pH of the stomach, survive in large numbers when passing through the stomach, have resistance to action of bile acids, organic acids and lysosome, produce a sufficient amount of organic acids and decreased intestinal pH so as to prevent the development of pathogens and their toxinogenesis respectively.

Important aspects reveal colon colonization and adhesion to intestinal tract epithelial cells, the ability to assimilate cholesterol and hydrolyze lactose, proliferate in vivo in conditions of antagonism with putrefactive and pathogenic bacteria, resistance in technology process to obtain products harvested as probiotics

The current researches on the mode of action of probiotics has focused on the importance of bacteriocins characterized as low molecular weight peptides produced by some bacteria that exert a bactericidal effect on other bacterial species. Bacteriocins have an important practical applicability in preserving food, but also in preventing bacterial infections. They have a restricted inhibition spectrum, acting especially on Gram-positive bacteria, but many bacteriocins produced by lactic bacteria are active against food pathogens such as; Bacillus cereus, Clostridium botulinum, Clostridium perfrin ges, Listeria monocytogenes, Staphylococcus aureus etc.

Most of the bacteriocins produced by lactic acid bacteria are thermoset, thereby maintaining the activity after the heat storage of food processes. Until now, the niche produced by Lactococcus lactis subsp. lactis is the only bacteriocin approved for use in the food industry.

Table 2.

Characteristics of the main probiotic bacteria used in fermented milk products

Genus	**Species**	**Optimum temperature (°C)**	**Main final products**	**Secondary final products**
Streptococcus	S. thermophillus	40-44	L (+) lactic acid	Acetaldehyde, acetone, acetone, diacetyl
Lactobacillus	L. bulgaricus	40-44	D (-) lactic acid	Acetaldehyde, acetone, acetone, diacetyl
	L. helveticus		DL lactic acid	Acetaldehyde, acetic acid, diacetyl
	L. lactis		D (-)lactic acid	Acetaldehyde, acetic acid, diacetyl
	L.acidophillus		DL lactic acid	Acetaldehyde
	L. casei	25-30	L (+)lactic acid	Acetic acid
	L. kefir		DL lactic acid	Acetic acid, acetaldehyde, ethanol, CO2
Bifidobacterium	B. breve	35-38	L (+)lactic acid,acetic acid	Formic acid, succinic acid, acetaldehyde, acetone, acetoine, diacetyl
	B. bifidum			
	B. longum			
	B. infantis			

Of a particular interest for researchers is the anti-inflammatory action of lactobacilli on immunomodulatory effects. Therefore, the most studied strains of lactobacilli in terms of supporting the immune system are of particular interest (by stimulating the production of antibodies

in the case of infections) and non-specific (by stimulation of phagocytosis, one of the most important mechanisms of defense of the body, the most important phagocytic cells being leucocytes).

A laboratory study (Argentina, 2011) demonstrated the action of lactobacilli on the mucosal immune system and its contribution to the prevention of intestinal and respiratory infections. Administration of these lactobacilli in both children and adults reduces both the frequency and duration of infectious diarrheal episodes, particularly those caused by Rotavirus, as well as in various enterococcal gastrointestinal infections. The mechanisms of action of bacteriocins are diverse and complex due to their particular chemical structure, in most cases acting on the cell membrane through pore formation or at the level of essential cell processes (transcription, translation, replication, biosynthesis of cell wall components).

In this context, based on the presented analyzes, we mention the importance of the performance and functionality of probiotics - mixed or individual cultures of live and non-pathogenic microorganisms, available in food or as nutritional supplements most frequently represented by strains of Lactobacillus, Bifidobacterium and Streptococcus. Simultaneously, starting from the idea that dairy products are an ideal carrier of live bacteria in the organism, we appreciate the correlation between the probiotics functionality and activity with the functionality of dairy products and the maintenance of the probiotics viability.

Conclusions

The activity of probiotics as selected or concentrated lactic bacteria cultures serve to prevent or restore digestive dysfunction and have a positive influence on the immune system.

1. Evaluation of the use of probiotics in various human and animal diseases are relevant characteristic effects by inhibiting the growth of pathogenic microorganisms and increasing the immune response.
2. The use of probiotics represents an additional alternative for increasing and maintaining the health of the human and animal body, implicitly the quality of life.

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THE CORRELATION OF THE MORPHOLOGICAL PECULIARITIES OF THE HINDLIMB IN MAMMALS, CONCERNING THE AUTOPODIUM, DEPENDING ON THE TYPE OF GROUND SUPPORT

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Abstract
In digitigrade and plantigrade species, the head of the talus distinctly appears which together with the glenoid cavity provided by the central bone also favor lateral movements, offering better adaptability. The essential differences are observed at the level of the metatarsal head, where carnivores and plantigrade animals have a dorsal hemispherical joint surface, completed by a real crest in the plantar side, delimiting two condyles separated by an intermediate crest. This is essential for the digitigrade and plantigrade type because when the limb takes contact with the ground, the joints hyperextension also occurs and a finger abduction movement, widening the support area.
Keywords*: autopodium, ground support, hindlimb.*

Introduction

A crucial role in development and adaptation of species to the environmental conditions and survival strategies is the way the animal's body takes contact with the ground. The autopodium of mammals consists of three large regions, the basipodium, represented by the carpal and tarsal bones, the metapodium, consisting of metacarpal and metatarsal bones and the phalanges.

If we talk about support and locomotion of them, in mammals there are three types of displacement: unguligrade, digitigrade and plantigrade types.

In solipeds, ruminants and pigs, species which travel long distances, the contact with the ground is reduced to a small surface of the hoof, having an essential role in the efficient fast start and speeding up.

Carnivores take contact on the sole faces of the phalanges, the contact surface with the ground is larger, but provides more accurate control of the movement.

In primates, bears and the other plantigrade, the support is done throughout the entire autopodium, including phalanges, metapodium and basipodium.

Materials and methods

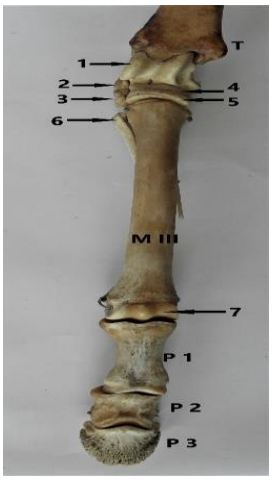
To identify the conformational features, each skeleton from the studied mammals were processed (horses, cows, pigs, dogs, cats, rabbits, nutrias and brown bears). After boiling and scraping off the adherent tissues, the bones were treated with hydrogen peroxide for about one hour. Description, identifying and highlighting the bones in those studied species were done by measuring and comparing the results with references to our own existing findings. In order to highlight these results pictures were taken, using an OLYMPUS 25 megapixels and

Results and discussions

The purpose of this research work is to point out the number of bones, bones disposition, articular surface together with their joint angles, resulting as an adaptation to the three types of displacement of the body support and locomotion.

In the three types of support on the ground in mammals, the autopodium appears to be varied in length, shape and structure, depending on the influence of each segment that offers support of the body weight.

The basipodium, placed approximately mid-length at the unguligrade and far distal to the other categories, has the primary role of cushioning the pressure force that occurs during the leaps and extending the range of the limb movements in the plantigrade type. Therefore, both the thoracic and pelvic basipodium consist of two rows of short bones between which a central bone is place (Gheție et al. 1971, Coțofan et al. 1999, Spătaru 2002 and Spătaru 2009).



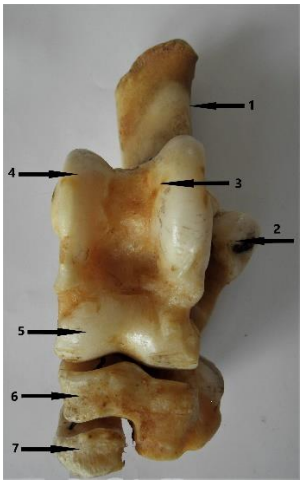


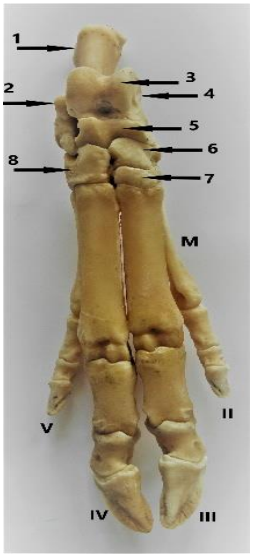
Figure 1. The dorsal aspect of the pelvic autopodium in horse (left)
T- tibia, M III– the third metatarsal bone, P1- first phalanx, P2 – second phalanx, P3- third phalanx,
1-talus, 2 -calcaneus, 3-os cuboideum, 4 –os naviculare, 5 – os cuneiforme laterale,
6 – os metatarsale IV, 7- caput os metatarsale III.

Figure 2. The dorsal aspect of the tarsum in cattle (right)
1-calcaneus, 2 - facies articularis malleolaris, 3- trochlea tali proximalis, 4- talus, 5- trochlea tali distalis, 6 – os navicuboideum, 7- os cuneiforme intermediolaterale.

In ruminants, the highest numerical reduction is found, five tarsal bones, the cuboid being welded with the central bone and the lateral cuneiform with the intermediate one (Figure 2). In solipeds, the number of tarsal bones is six by welding the intermediate cuneiform with the medial (Figure 1). Six we also encounter in leporids, the numerical reduction is due to the welding of the medial cuneiform at the proximal end of the second metatarsal bone, which thus presents a proximally directed prolongation (Figure 6).

There are seven in the swine and in carnivores (which are digitigrade type) (Gheție et al. 1971, Coțofan et al. 1999, Spătaru 2002 and Spătaru 2009) (Figure 3,4 and 5). In plantigrades of this research work, meaning the brown bear and the nutria, they have eight tarsal bones, because of the presence of four cuneiform bones (Hrițcu et al. 2000, Spătaru et al. 2008, Spătaru et al. 2014 and Spătaru 2016) (Figure 7 and 8). A higher number of tarsal bones reflects the adaptation of the plantigrade support towards a better mobility (Riga et al. 2008 and Spătaru et al. 2010). The tarsal joint, in all species is specialized for predominantly monoaxial, flexion and extension movements has adapted for this function the talus bone which has a trochlear articular surface with which it

joins the tibial cochlea. Talus is also involved in jointing between calcaneus and the central bone (*Articulation talocalcaneocentralis*) with the articular head which corresponds to the glenoid cavity provided by the central bone. In horses, the trochlea of the talus is obliquely dorsolaterally, the articular head of it being flattened, results a reduced mobility at the level of the *talocalcaneocentralis* joint. In ruminants and pigs, the talus provides two other trochlea (Figure 2 and 3), the articular surfaces for the calcaneus and for the central bone which favours a type of motion produced predominantly as flexion and extension of the joint. In ruminants, for strengthening the region, the central and cuboid (fourth tarsal bone) bones merge into a massive *centroquadrale* bone (Gheție et al. 1971, Coțofan et al. 1999, Spătaru 2002 and Spătaru 2009).



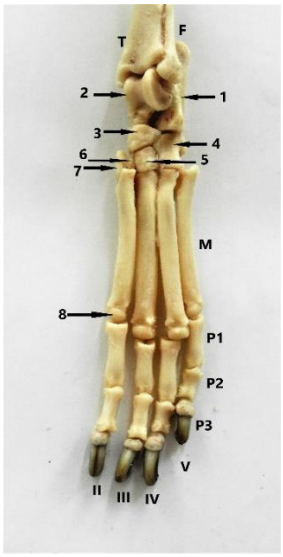


Figure 3. The dorsal aspect of the pelvic autopodium in pig (left)
M – ossa metatarsalia, II-V- from the second to the fifth finger, 1-calcaneus, 2 - facies articularis malleolaris, 3- trochlea tali proximalis, 4 –talus, 5- trochlea tali distalis, 6 - os naviculare, 7- os cuneiforme laterale, 8- os cuboideum.

Figure 4. The dorsal aspect of the pelvic autopodium in dog (right)
F- fibula, T- tibia, M – osmetatarsale,II-V- from the second to the fifth finger, P1- first phalanx, P2 – second phalanx,P3- third phalanx,1-calcaneus, 2 –talus, 3- osnaviculare, 4 – oscuboideum, 5 – oscuneiformelaterale, 6 – oscuneiforme intermedium, 7- osmetatarsale I, 8- caput osmetatarsale

In digitigrade and plantigrade species, the head of the talus distinctly appears from the body through a true neck (*Collum tali*), which together with the glenoid cavity provided by the central bone also favor lateral movements, offering easy adaptability to the locomotor type of movements: flexion, extension, abduction and adduction (Figure 4, 5, 6, 7 and 8) (Gheție et al. 1971, Coțofan et al. 1999, Spătaru 2002 and Spătaru 2009).

In the case of the plantigrade species studied, the bear and the nutria, there was a reduction in length of the calcaneus, and a tendency towards a third surface in nutria. The *Sustentaculum tali* is prominent, and the coracoid process is flattened. If in unguligrades and digitigrade types the position of the calcaneus is oblique cranio-distally, in plantigrades, the

calcaneus has a horizontal position during the ground support. Talus in plantigrade species has a shorter neck compared to digitigrades, with a slightly dorso-ventral obliquely trochlea from medially to laterally (Hrițcu et al. 2000, Spătaru et al. 2008, Spătaru et al. 2014 and Spătaru 2016).

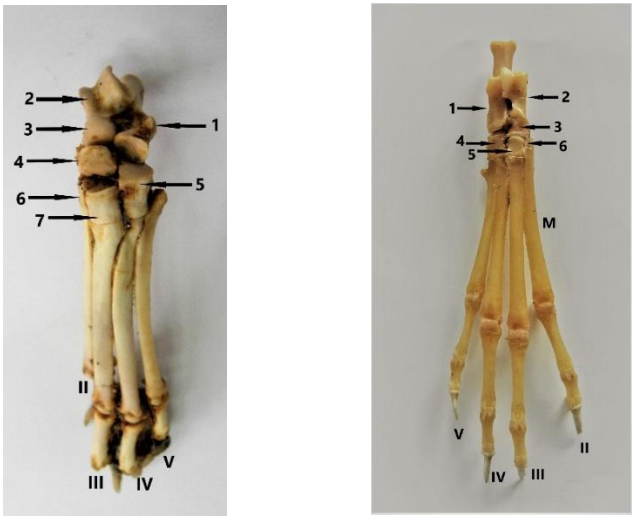


Figure 5. The dorsal aspect of the pelvic autopodium in cat (left)
II-V- from the second to the fifth finger, 1-calcaneus, 2 –talus, 3- caput articulare os talus, 4 – os naviculare, 5 – os cuboideum, 6 – os cuneiforme intermedium, 7- os cuneiforme laterale.

Figure 6. The dorsal aspect of the pelvic autopodium in rabbit (right)
M - metatarsal bone, II-V- from the second to the fifth finger,
1- calcaneus, 2 –talus, 3- os naviculare, 4 – os cuboideum, 5 – os cuneiforme laterale,
6- os cuneifome intermedium.

In the case of unguligrades and digitigrades, if compare the thoracic bazipodium jointing angle which is about 180°, with the tarsal articular angle that is about 150 ° -160 °, the last one favors the propulsion. In plantigrade, the angle is reduced to 90° in relation with the ground, favoring the control increasing of the autopodium movements (Gheție et al. 1971, Coțofan et al. 1999, Spătaru 2002, Riga et al. 2008 and Spătaru 2009).

In pentadactyl archetype, the pelvic metapodium is represented by five metatarsal bones, each of them being approximately equal in length. The digitigrades show a variation of number of metatarsal bones compared to the number of metacarpals. Thus, in carnivores there are four equal metatarsal bones in size and a rudimentary one for the first finger (Figure 4 and 5). In rabbits there are only four (Figure 6). Among the unguligrades, pigs have two large metatarsals (III and IV) and two small ones (II and V), the ruminant metatarsals III and IV are welded and the equines have only one, the third (cannon bone) with additional two rudimentary ones, II and IV (Figure 1 and 3).

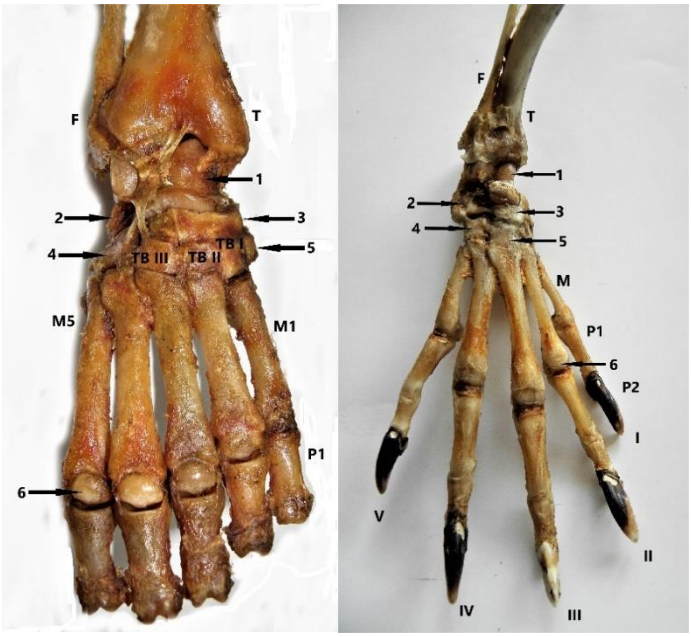


Figure 7. The dorsal aspect of the tarsal and the metatarsal region together with the first phalanges of the brown bear (left)

T- tibia, F- fibula, TB I -first tarsal bone, TB II – second tarsal bone, TB III – third tarsal bone, M1 – metatarsal bone 1, M5 – metatarsal bone 5, P1 – first phalanx, 1- talus, 2 – calcaneus, 3- os naviculare, 4 – os cuboideum, 5 – the fourth cuneiforme bone, 6- caput os metatarsale

Figure 8. The dorsal aspect of the pelvic autopodium in nutria (right)

F- fibula, T- tibia, M - metatarsal bone, I-V- from the first to the fifth finger, P1- first phalanx, P2 –second phalanx, 1- talus, 2 –calcaneus, 3- os naviculare, 4 – os cuboideum, 5 – os cuneiforme laterale, 6- caput os metatarsale.

The pentadactyl archetype of the pelvic metapodium is preserved only in plantigrade that need a wider support base. There is a decreasing in length of the metapodium which is correlated with the type of support and locomotion in order starting with unguligrade, digitigrade and plantigrade (Gheție et al. 1971, Coțofan et al. 1999, Spătaru 2002 and Spătaru 2009).

In the matter of metatarsal bones, the proximal joint surfaces are slightly convex and elongated dorsoplantar in digitigrades and plantigrades. In the latter case, the metatarsal bones describe an arch with a plantar-oriented concavity. They are compressed and attached to the proximal third of the region to become divergent distally, amplifying the support base of the body weight.

The essential differences are observed at the level of the metatarsal head, where carnivores and plantigrade animals have a dorsal hemispherical joint surface, completed by a real crest in the plantar side, delimiting two condyles separated by an intermediate crest. This is essential for the digitigrades and plantigrades because of when the limb takes contact with the ground, the joints hyperextension also occurs and a finger abduction movement, widening the support area. Also, the abduction is only possible in the extension of the fingers when the proximal phalanx joint surface, represented by a large glenoid cavity separated in two by a medial groove

takes contact with the congruent surface of the articular head of the metatarsal bone, the joint allows for large movements, flexion-extension, laterality, but also with slight rotation capability (for adaptation to the ground irregularity) (Figure 4, 7 and 8) (Hrițcu et al. 2000, Spătaru et al. 2008, Spătaru et al. 2014 and Spătaru 2016).

In ungulates, the joint surface consists of two condyles, with the convexity directed dorsoplantar, being separated by a median crest. The higher the width of the condyles and the more prominent is the crest, the possibilities for movement within the joint are limited to one axis (flexion and extension). This conformation gives to the movements at this level a high degree of precision, necessary in mammals for sprinting (especially equines, Figure 1) movements (Gheție et al. 1971, Coțofan et al. 1999, Spătaru 2002, Riga et al. 2008 and Spătaru 2009).

The most important element in description and the interpretation of the autopodium is the number of fingers on which the body weight support is done. The pentadactyl archetype encountered in primates and other plantigrades is represented by five fingers. The only finger to which the regression is observed is the shortest first finger (I), and only two phalanges are encountered. The rest of fingers, named from medially to laterally II-V, are approximately equally developed, independent, very mobile and they can perform a large abduction, which increases the feet support of the body weight (Figure 7 and 8) (Gheție et al. 1971, Coțofan et al. 1999, Spătaru 2002 and Spătaru 2009). For abduction of fingers in nutria, indispensable in aquatic locomotion is the presence of the interdigital membrane (Hrițcu et al. 2000).

In unguligrades, the differences between the body weight, speed and distances travelled, determine slightly large differences between one species and another.

In pigs, the support is done by on the two main axial fingers, the third and the fourth, the second and fifth fingers are shorter, taking only the support on the ground if is necessary, that is why they were called secondary fingers. The first finger is not present, losing its bone representation over the entire length of the autopodium (Figure 3).

In ruminants, support is on the two main fingers, the third (III) and the fourth (IV), the first and second fingers disappear altogether. The vestigial of the second (II) and fifth (V) fingers appear in the form of two horn like capsule in the pastern region.

In horses, the support is only on the distal end of the third finger (III), the second (II) and fourth fingers (IV) appear in rudimentary form, as spurs in the fetlock joint region, and fingers I and V no longer appear (Figure 1).

The first (I) and the middle (II) phalanges are similar in form and conformation in all species, with obvious differences in length and anterior-posterior flattening at the forelimb and hindlimb.

The third phalanx is the most morphologically distinct from one support type to another. Thus, in solipeds, the distal phalanx takes the form of a vertical half of cone, which presents a sole and a parietal surface. In the other unguligrade, ruminants and pigs, the third phalanx, takes the halved aspect of the one described in solipeds, with three sides, axial, abaxial and solar, with one dorsal edge (Figure 1 and 3). In digitigrades and plantigrades, the third phalanx becomes transversally flattened, taking the shape of the claw (Figure 4, 5, 6 and 8), the required supporting surface supplemented by the greater number of fingers placed in abduction movement position. On the sole surface (*Facies solearis)*, all phalanges are covered by a protective tissue, the plantar cushion, which differs in structure and appearance from digitigrade to plantigrade and unguligrade. The distal phalanx is wholly covert in unguligrade by the hoof or partly by the claw in digitigrade and plantigrade, their role being in protection of the extremities of legs in movement, climbing or

in hunting prey (Gheție et al. 1971, Coțofan et al. 1999, Spătaru 2002, Riga et al. 2008 and Spătaru 2009).

Conclusion

The presence of the jointing head of the talus, in digitigrades and plantigrades, leads to better adaptability and increases the mobility of the entire tarsal articular complex.

The pentadactyl archetypes preserved only in plantigrades that need a more extensive support base at both metapodium and acropodium levels.

In plantigrades, the metatarsals bones are compressed, being in close contact in the proximal third, become divergent distally, amplifying the support base of the body weight.

The greater the width of the condyles from the distal extremity of the metatarsum and the more prominent the crest which separates the condyles, the more movement possibilities within the joint are limited to the one axis (flexion and extension).

The dorsal presence of the articular surface which resembles with an articular head at the distal extremity of the metatarsus in the plantigrade and digitigrade types, leads simultaneously to hyperextension and abduction of the fingers when taking contact with the ground, which means a widening of the support area and better motion control.

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THE HEPATOPROTECTIVE EFFECT OF SOME HERBAL AND MINERAL PREPARATIONS IN THE TREATMENT OF VARIOUS HEPATOPATHIES IN DOGS AND CATS

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Abstract

A series of herbal medicines and minerals such as milk thistle, curcumin, sea buckthorn, artichoke and zeolite can be used as alternative solutions in scavenging oxygen free radicals incriminated in triggering of liver disease. The aim of this study was the therapeutic application of different herbal and mineral preparations with antioxidant properties and evaluation of their hepatoprotective potential in the complex therapy management of various types of liver disorders diagnosed in dogs and cats. The study was performed between October 2016 – September 2017 on 16 dogs and 4 cats of different breeds, sexes and ages which were presented and diagnosed with different degrees of liver failure in Medical Clinic of the Faculty of Veterinary Medicine from Iasi. Anamnesis and physical examination have highlighted that the clinical onset of liver disease was nonspecific, in most cases, the diagnosis of a liver disorder could be deduced from the results of the biochemical tests corroborated with the ultrasound findings. The therapeutic management of liver disease has depended on the etiopathology, the degree of biochemical resonance of the cytolytic, cholestatic process and the synthesis imbalance in the liver as well as the presence or absence of complications of the liver pathology. Thus, according to the established protocol, patients were divided in three groups and they received the basic drug treatment in combination with hepatoprotective preparations of vegetal and mineral origin, respectively, milk thistle (silymarin), curcumin, sea buckthorn and zeolite. The inclusion of phytotherapy in the complex treatment of liver disease has contributed to the normalization of hepatic transaminases and cholestasis parameters. A positive therapeutic result was obtained after therapy with antioxidants from vegetable origin in mild and average forms of liver disease. In the final stage of the liver disease, treatment with natural hepatoprotective agents had a palliative character with transient reduction of clinical signs and temporary improvement of quality of life.

Keywords*:antioxidant, cytoprotection, hepatoprotective activity, herbal medicines, liver disease*

Introduction

In the last decade, a multitude of pharmacotoxicological studies have been carried out in animal models and human clinical trials in order to find natural solutions for decontracing adverse reactions of synthetic drugs, restoring unbalanced hepatic functions and increasing hepatocyte resistance to the action of various harmful factors. The association of allopathic therapies with plant supplements is gaining ground to potentiate the therapeutic strategies and prevent adverse effects. A series of plant compounds with proven antioxidant principles, such as flavonoids, polyphenols, carotenoids complexes, tocopherols, terpene, phytosterols, ascorbic acid, can be used in scavenging oxygen free radicals incriminated in triggering liver pathology (Stănescu and al., 2002). Thus, numerous studies have concluded that biologically active substances found in certain plants such as milk thistle, sea buckthorn, artichoke, curcumin have the role of rebuilding the hepatic cell and increasing the ability of the liver to defend against oxidative stress.

Material and methods

The study was conducted between October 2016 and September 2017 at the Medical Clinic of the Faculty of Veterinary Medicine in Iaşi and included 16 patients with clinical signs and biochemical results associated with a hepatobiliary disorder, of which 12 were dogs and 4 cats of

different ages, sexes and breeds. Patients underwent a clinical, biochemical and ultrasound examination. The evaluation of each patient's condition involved the development of an individual clinical diagnosis card that included data from the history of the disease (early signs of the disease, preexisting or coexisting conditions, potential hepatotoxic treatments, vaccine status, diet) and data on the current state of the animal at the time of consultation obtained by general semiological methods. Functional liver status was assessed according to basic biochemical syndromes: cytolytic (ALT / TGP, AST / TGO), cholestasis (GGT, alkaline phosphatase / ALP) and insufficient synthesis function (total protein, urea and serum glucose).

The ultrasound examination of abdominal organs was performed using Aquila Pro Vet with a convex probe of 5 and 7.5 MHz to highlight the changes in size, shape and echogenity of the liver, canals and gall bladder, spleen and stomach before and after treatment.

The therapeutic management of liver disease depended on the etiopatology, the degree of biochemical resonance of the cytolytic, cholestatic process and the synthesis imbalance in the liver as well as the presence or absence of complications of the liver pathology. Thus, according to the established protocol, patients received basic drug therapy in combination with hepatoprotective preparations from herbal and mineral origin, respectively, silymarin (milk thistle), curcumin, sea buckthorn, artichoke and zeolite (Table 1).

Table 1

Herbal and mineral medication used in outpatient / hospital treatment of hepatopathies in dogs and cats

Herbal / mineral product Active ingredient	**Indications**	**Dose, duration and frequency of treatment**
Milk thistle Silymarin / silybin	Different types of liver disorders, Cholangitis Cholecystitis	20-50 mg/kg/day/p.o., 1-2 months, 2-3 times/year
Curcumin		350 mg/day/p.o., 1-3 months
Artichoke		3.25 mg/p.o., 30 days
Sea buckthorn		300 mg/animal/p.o., 1-2 months, 2 times/year
Zeolite		600 mg/animal/day/p.o, 3 months

The duration of treatment was between 1 - 3 months, 2-3 times/year. Regarding the classic hepatic therapy strategies, it has been used IV fluid therapy with isotonic solutions (0.9% Saline, Ringer, glucose 5%), antioxidants (ascorbic acid - vitamin C, tocopherol - vitamin E, N - acetylcysteine), vitamins (thiamine, pyridoxine), anti-hemorragic agents (phytomenadione, ethamsylate), diuretics (spironolactone and furosemide), antisecretory drugs (H2 antagonists: ranitidine, famotidine and proton pump inhibitors: pantoprazole), choleretic agents (dry artichoke extract, ursodeoxycholic acid), antibiotics (amoxicillin and clavulanic acid, metronidazole, enrofloxacin, third generation cephalosporin: ceftriaxone,), glucocorticoids (prednisone, methylprednisolone, dexamethasone), antipyretic (metamizol).

Depending on the treatment applied, the patients were divided into 3 groups:

● Patients with jaundice secundary to an acute toxic hepatitis or parasitic hepatitis (babesiosis, dirofilariosis) who received silymarin + sea buckthorn (4 dogs) and silymarin + curcumin (2 dogs) for 30 days;

● Patients with ascitic syndrome as a result of a straight heart failure or a neoplastic process (2 dogs), which has been prescribed the combination of silymarin + sea buckthorn + curcumin + zeolite for 3 months, 2-3 times/year;

● Patients with cholestasis syndrome in angiocolites, colangitis, triadites (2 dogs and 4 cats) who received silymarin + sea buckthorn + artichokes for 30 days.

Results and discussions

Of the 16 patients diagnosed with different hepatopathies, 14, respectively 10 dogs and 4 cats of different breeds, sexes and ages responded positively clinically and biochemically to therapy with hepatoprotective plant products (Table 2).

Anamnesis and clinical examination have highlighted that the clinical onset of liver disease was nonspecific and included the appearance of a dyspeptic syndrome such as loss of appetite, nausea and vomiting, intestinal metheorism, diarrhea or constipation, abdominal colic, various degrees of dehydration, discrete to severe weight loss within a few weeks, polyuria / polydipsia, hyperkeroma urine, apathy to coma, multiple organ failure, and symptoms of advanced hepatic disease such as ascites, jaundice and coagulopathy (bruising, petechiae). In most cases, the diagnosis of a liver disorder could be deduced from the paraclinical results corroborated with the ultrasound findings. In terms of hepatobiliary pathology in patients following adjuvant therapy with herbal active principles have prevailed toxic hepatitis (7/10 dogs), ascites secondary to neoplasia or heart failure (2/10 dogs); and in cats have predominated cholangiohepatitis (2/4 cats) and triadites (2/4) (Table 2).

Table 2

Hepatic biochemical changes (values before and after treatment) in dogs and cats diagnosed with different hepatopathies

ALT alanine aminotransferase, AST aspartate aminotransferase, ALP alkaline phosphatase, GGT –glutamyl transferase, BUN - Blood urea nitrogen

No.	Patient	Diagnosis	Previous treatment	Phytohepato-protective products	Biochemical changes
1.	**2.**	**3.**	**4.**	**5.**	**6.**
1.	Dog Shit-Tzu, ♀, 2 y, 3.6 kg	Gastroenteritis Pyloric spasm Toxic hepatitis	Imizol	Silymarin 150 mg/day, 1 month Sea buckthorn, 2X300mg /day, 1 month	AST 317→30 ALT 215→105 ALP 255→181 BUN 57→25
2.	Dog, Mixed breed, ♀, 12 y,3 kg	Hepatic failure Jaundice Polyradiculo-neuritis	Antibiotics Imizol Physiotherapy	Silymarin 150 mg/day, Curcumin 350mg/day, 1 month	ALT 77→39 AST 333→119 GGT 23→12 ALP 184→110

3.	Dog, Bichon, ♂, 5 y, 8 kg	Hemorrhagic gastroenteritis Toxic hepatitis Acute kidney injury (AKI) Urinary lithiasis Prostatitis	Dental scaling Uretrostoma	Silymarin 2X150 mg /day, Curcumin, 350mg/ day, 3 months	AST 219→29 ALT 284→48 BUN 34→33.5
4.	Dog, Mixed breed, ♂, 10 y	Babesiosis Heartworm	Imizol Doxycycline	Silymarin, 250 mg/day Sea buckthorn, 2X300 mg /day	ALT 123→87 AST 56→25
5.	Dog, Bichon, ♀, 8 y, 8kg	Congestive heart failure Hepatomegaly Ascites	Paracentesis	Silymarin, 2X150 mg/day; Sea buckthorn, 300 mg/day, Curcumin, 500 mg/day, 3 months; Zeolit clinoptilolit, 600 mg/day, 6 months	No changes
6.	Dog, Boxer, ♀, 14 y, 10 kg	Hepatobiliary carcinoma Ascites	Paracentesis	Silymarin, 2X150 mg/day; Sea buckthorn, 300 mg/day, Curcumin, 350 mg/day, 3 months; Zeolit clinoptilolit, 600 mg/day, 3 months	ALT 192→100 AST 76→55 ALP 1345→1200 GGT 31→24
7.	Dog, Akita Inu, ♂, 9 y, 25 kg	Babesiosis Acute liver failure Jaundice Acute kidney injury (AKI)	Imizol	Silymarin, 2X150 mg/day; Sea buckthorn, 2X300 mg/day 1 month	ALT 87→44 AST 329→117 ALP 157→103 BT 4,57→0,2
8.	Dog, Caniche, ♂, 7 y, 20 kg	Cholangiohepatitis Jaundice Babesiosis	Imizol	Silymarin, 2X150 mg/day; Sea buckthorn, 2X300 mg/day 1 month	ALT 92 →45 AST 58 →11 ALP 98→97 GGT 4→no changes
9.	Dog, Westie, ♀, 15 y, 6 kg	Cholangiohepatitis Polyradiculo-neuritis	Ursofalk 250 mg/day	Curcumin 350 mg/day, Silymarin, 100mg/day, Artichoke 3.25 mg/day, 6 months	ALT 167→30.3 AST 47.7→23.5 ALP 353→69.1 GGT 8.93→2.80

10.	Dog, Terrier, ♂, 7 y	Arthritis Cholestasis	AINS	Silymarin, 150mg/day, Sea buckthorn, 2X300 mg/day Artichoke 3.25 mg/day, 21 days	ALP 1056→165 AST 365→102
11.	Cat, Mixed breed, ♀, 6 y, 3,6 kg	Dyspepsia Triadites	Antibiotics	Silymarin, 150mg/day, Sea buckthorn, 300 mg/day 3 months	ALT 105→64 AST 174→19
12.	Cat, European Shorthair, ♂ 10 y, 3 kg	Chronic kidney injury Hepatic failure Triadites	IV fluid therapy	Silymarin, 150mg/day, Sea buckthorn, 300 mg/day 1 month	ALT 136→46 ALP 97→44
13.	Cat, British Shorthair, ♀, 4 y	Cholangitis/ Cholangiohepatitis syndrome (CCHS)	Antibiotics	Silymarin, 150 mg/daily, 3 weeks, Artichoke, 3.25 mg/daily, 2 weeks	ALP 86→61 ALT 57→45 GGT 7 →no changes
14.	Cat, European Shorthair, ♀, 6 y	Cholangitis/ Cholangiohepatitis syndrome (CCHS)	Antibiotics	mg/daily, 3 weeks, Artichoke, 3.25 mg/daily, 2 weeks Sea buckthorn, 2 X 300 mg/day 1 month	No biochemical changes

All patients had a positive clinic evolution after administration of different combinations of herbal active substances. Thus, asthenic, dyspepsic or algic syndrome have improved in 7-14 days after administration of hepatoprotective plant products, and the jaundice has relieved after approximately one month beyond treatment initiation. Also, in patients who developed a weight loss syndrome, the association of sea buckthorn oil with silymarin contributed to the improvement in body weight decline as a result of loss of appetite.

In a patient (Bichon female, 8 years old) with ascites syndrome as a result of a neoplastic process associated with congestive heart failure, 2 months after following hepatoprotective herbal and mineral products therapy (silymarin + sea buckthorn + curcumin + zeolite) associated with weekly therapeutic paracentesis, it seen a marked improvement in the state of health and quality of life, with decreasing intensity to disappearance of ascites (Figure 1). In another patient diagnosed with hepatobiliary carcinoma and secondary ascites, being at the final stage of the disease, therapy with silymarin + sea buckthorn+ curcumin + zeolite was palliative, with transient decrease of

clinical signs, temporary improvement of quality of life and biochemical results, corresponded by a slight decrease in liver transaminases and cholestatic parameters.

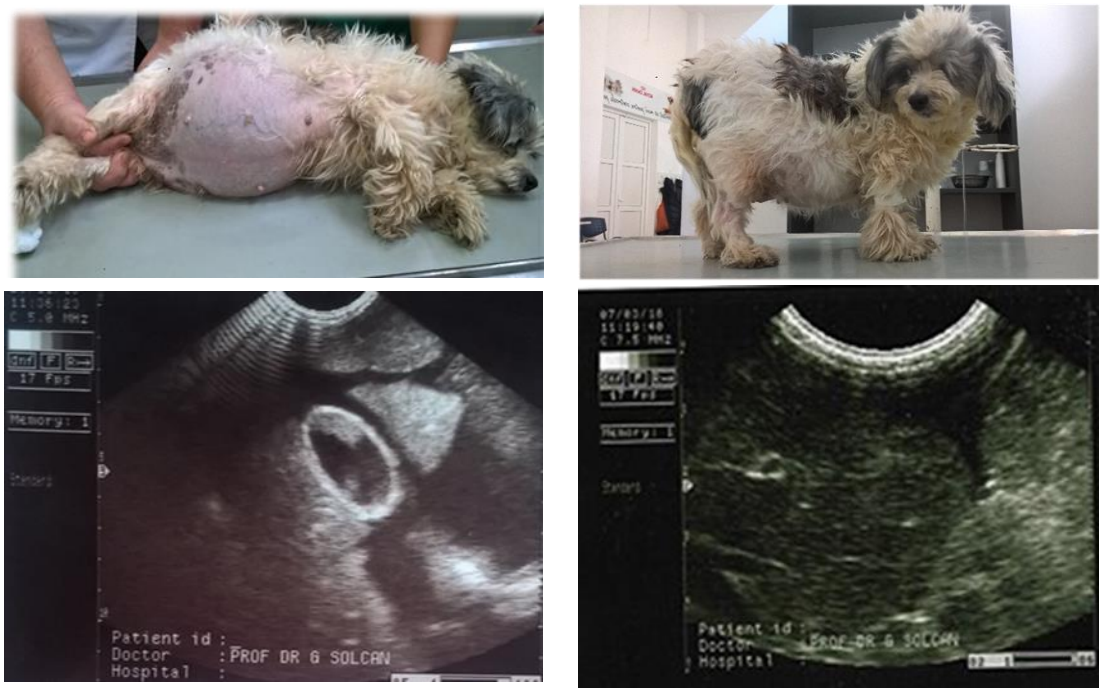

Figure 1

Dog, Bichon, ♀, 8 years old, diagnosed with mixed ascitic syndrome, clinical and ultrasound aspects before (left pictures) and after treatment (right pictures)

In the majority of patients from the three groups diagnosed with different hepatopathies, administration of various herbal drugs combinations has been beneficial in the evolution of biochemical indices. Thus, in patients diagnosed with cholangiohepatitis or cholestasis who received artichoke extract in addition to silymarin and sea buckthorn oil, an improvement of liver enzymes was observed and the alkaline phosphatase activity decreased to normal values.

The reports from medical literature shows that silymarin, sea buckthorn, curcumin and artichoke exhibit antioxidant, anti-inflammatory, antifibrotic, cytoprotective, membrane-stabilizing, detoxifying and improving liver function synthesis properties (Webster & Cooper, 2009; Loguercio et al. 2011; He et al., 2015, Colak et al., 2016). Interesting data on the antitumor action of these hepatoprotective agents have been reported due to the ability to regulates hepatocyte apoptosis and down-regulate gene products involved in profilation of tumor cells, angiogenesis and metastasis (Agarwal et al., 2006; López-Lázaro, 2008; Ramos et al., 2014). These effects confirm that the active principles of these plants can be used in the complex treatment of different kinds of hepatic diseases.

Conclusions

1. The association of various medical plants with allopathic therapy in the complex management of liver disease has helped to normalize the activities of hepatic transaminases and cholestasis indices, with a high degree of cytoprotection.

2. A positive therapeutic result was obtained after therapy with antioxidants from herbal origin in the mild and average forms of liver disease.

3. The administration of silymarin + sea buckthorn, silymarin + curcumin or artichoke for a period of 14 -30 days in patients with toxic, infectious or gall bladder disease lead to improvement of liver function and cytolytic indices, as well as reducing the activity of cholestasis markers.

4. For end-stage diseases, natural hepatoprotective therapy was palliative, with transient diminution of clinical signs and temporary improvement in quality of life.

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IN VITRO STUDY OF DIMINAZENE ACETURATE COMPLEX WITH B-CYCLODEXTRIN FOR *ICHTHYOPHTHIRIUS MULTIFILIIS*

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Abstract

The histophagous ciliate Ichthyophthyrius multifiliis can cause lethality in farmed carp brood (Cyprinus carpio) as well as other representatives. In the present study, an antiparasitic substance (diminazene aceturate) and its complex with a cyclodextrin were tested for its activity against this pathogen in vitro. The purpose of this paper is to highlight the therapeutic potential of diminazene and the enhancement by the β-cyclodextrin. Of these, the complex proved to be more effective (i.e., killed all parasites in a test period of 6-8 hours). Administration in filtered water suggests that these compounds can not be effective in bathing. In view of these findings, we will discuss the potential utility of chemotherapy as a strategy for controlling ciliatosis in farmed fish.

Keywords*: DSC, β-ciclodextrine, diminazene aceturate, complex*

Introduction

Diminazene is an aromatic diamidine of synthetic origin linked to a triazene bridge. The experimental procedure for the synthesis of this compound and other diazoaminobenzenes has been reported by Hochst (Hochst, 1954) with the primary objective of controlling diseases caused by blood transmissible protozoan parasites such as *Trypanosoma sp*. and *Babesia sp*. Diminazene is also known as diminazene aceturate when it is a pharmaceutical formulation containing two salt acetates (Atsriku et al., 2002). The experimental procedure for the synthesis of diminazene aceturate derives from the physicochemical characteristics of diminazene, such as instability and insolubility in aqueous solutions.

Since diminazene aceturate has been used for decades in the treatment of animal tripanosomiasis, some species of the genus *Trypanosoma*, such as *T. congolense*, developped tolerance and resistance (Moti et al., 2015). For this reason, several studies have investigated alternatives to improve success in tripanosomal treatment using new drugs in combination with the chemotherapy agent diminazene aceturate (Mbaya, 2009; Tonin, 2011), as well as the search for new pharmacological applications.

Despite the existing scientific literature that has been addressing this topic for over 50 years, several review articles have discussed in detail the chemical/pharmacological activities of diminazene aceturate. Two articles of comprehensive review (Peregrine, 1993) highlighted its pharmacological functions, the most recent being that of Kuriakose et al. (Kuriakose, 2014), which reports the pharmacological importance for modulation of the immune system.

Therefore, diminazene aceturate is still the target of studies on its therapeutic potential and therefore attracted great interest in the development of new research. Thus, given the pharmacological potential of diminazene, the aim of this study was to develop the systemic and pharmacological effects by including it in a cyclodextrin (β-cyclodextrin) and the use of the complex against aquatic protozoans such as ciliate *Ichthyophthirius multifiliis*.

Ichthyophthirius multifiliis is a protozoan that causes "white spot" disease and is a major burden for farmers and aquarists around the world. The infected stage of the parasite invades the

skin and gills of the fish, penetrates into the epidermis and is located above the basal lamae (Ventura, 1985). Here it turns into trophont stage feeding on fish tissues until it reaches a size of 0.5-1.0 mm and is macroscopically visible as a white spot (Buchmann, 2001). The mature trophont comes out of the fish and turns into a tomont, looking for bottom surfaces for encysting in a tomocyst where asexuous reproduction occurs. When the trophont leaves the fish host, it disrupts the epidermis and the epithelium, which can perturb osmoreglation and may leave fish sensitive to secondary infections (Matthews, 2005).

Various treatments have been used to combat the parasite with treatment regimens that change according to the new legislation on toxicity and carcinogenicity of the applied substances. The trophont status of the parasite, which is protected from the epidermis of the fish, is generally more resistant to treatments than the previous stage but requires intensive effort and repetitive treatments to eliminate the infection by targeting the frontal phase. A search for new effective and safe compounds for the treatment of the disease is in progress and some new candidate drugs seem promising.

In this article we proposed to include diminazene aceturate in β-cyclodextrin to obtain a favorable *in vitro* effect against the protozoan *Ichthyophthirius multifiliis*.

Material and method

The substances used, diminazene aceturate (purity> 97%) and β-Cyclodextrin (purity> 98%) were purchased from Sigma-Aldrich. All other reagents were of analytical quality. The water used was double distilled and deionized.

All experiments and handling steps were performed at 22°C. The fish were taken from a breeder in Iasi who owned carp fish, carp "mirror" and Japanese carp (koi) recently purchased from Israel. Carp were infested naturally with *I. multifiliis* and anesthetized in 100 mg / L MS-222, and mucus samples were collected by scraping the skin. The mucus was dispersed in a Petri dish containing dechlorinated water. Under a stereomicroscope, live trophonts (Figure 1) were taken up with a Pasteur pipette and placed in another Petri dish containing dechlorinated water. This step was repeated twice to separate the trophonts from the surrounding mucus.

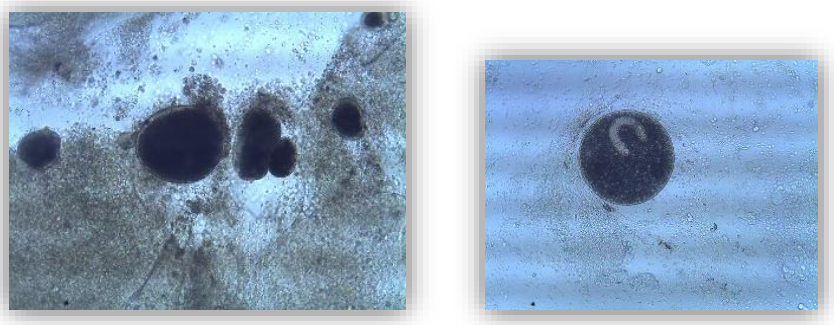

Figure 1 Trophonts of *I. multifiliis* (x20)

Using diminazene aceturate and β-CD-Diminazene aceturate, we prepared a stock solution of 100 mg/ml in dimethyl sulfoxide (DMSO) and diluted to the required concentrations using dechlorinated water. The pH of the dechlorinated water was not affected by the addition of the substances and was in the range of 7.5-8.0. For *in vitro* tests, 2 ml of the test solution was placed

in the wells of the microtiter plates, and a total of 100 trophonts was added. The controls were made in dechlorinated water containing appropriate concentrations of DMSO. After 6 hours, viable and non-viable trophonts were counted under a stereomicroscope, and movement and intact structure were used as viability criteria. The microtiter plates were re-incubated for a further 12 hours. Trophonts were counted on the MOTIC 40X microscope in the Bürker Türk counting room.

Compounds or suspensions of powder compounds (the tablets were previously sprayed) in distilled water or dimethylsulfoxide (DMSO) were prepared from the compounds. The resulting stocks or commercially purchased solutions were diluted in phosphate-based physiological saline solution (PBS, pH 7.2) or in water at the final concentrations used in the screening. Tests in filtered water allow automatic exclusion of the possibility of partial or total inactivation of the test substance under the given conditions. A substance considered to be effective *in vitro* by this procedure can be expected to be effective in administrating in the bath or medicated feed to infected fish.

The ciliates in the late exponential phase or in the early plateau phase of culture were concentrated by centrifugation at 650 x g for 5 minutes and then resuspended in PBS or filtered water. After counting into a hemocytometer, 10 µl of ciliate suspension containing ~100 ciliates in each well of 96-well microtiter plates containing 90 µl of the candidate antiprotozoal at the required dose in PBS or filtered water. The final doses tested were: 100, 50, 25, 12.5, 6.2, 3.1, 1.5 and 0.8 ppm for the remaining test substances. Each determination was carried out in duplicates. To exclude the possible effects of the solvent in the compounds dissolved in DMSO, duplicate wells with PBS or filtered water containing the highest DMSO concentration used (up to 2.5%) were included. Plates were incubated at 18°C for 24 hours. Ciliary motility after incubation was checked using a phase contrast illumination microscope. Prior to scanning, each culture plate was gently rotated to ensure a uniform distribution of the ciliate in the medium. The minimal lethal concentration (CML) for each drug was defined as the highest drug dilution at which 100% of the ciliate were lysed or non-motile (there was no evidence of ciliary motion using a 40x objective).

Thermogravimetric analysis was determined by thermal desorption of diethylamine on a DuPont Instruments Thermal Analyst 2000/2100 coupled with a 951 thermogravimetric analyzer. Differential Scanning Calorimetry (DSC) measurements were performed using a DSC 823 (Mettler Toledo). The sample was packed in aluminum cans placed in the DSC cell and then heated at a rate of 10°C/min from room temperature to 200°C, maintained for 2 minutes at 200°C, then cooled to ambient temperature.

Results and discussion

Although the effects on cell morphology were different, most active substances induced cell rounding and vacuolization changes before the eventual lysis. In all β-CD-diminazene aceturate tests, all ciliates died after 6 hours at doses of 100 and, respectively 80 ppm. In similar *in vitro* studies, diminazene aceturate have been shown to be effective against other fish pathogens, such as *Gyrodactylus spp*. This compound is not commonly used in aquaculture to control ectoparasite diseases. However, we consider that diminazene is suitable for the treatment of this disease, given the ectoparasite location of *Ichthyophthirius multifiliis*. When a ciliatosis outbreak due to *Ichthyophthirius multifiliis* is diagnosed for the first time in a large farm, many fish in the affected reservoir already have an infection characterized by the presence of numerous ciliate in the tegument and in the gills. In such cases, the respiratory capacity of fish will be seriously compromised, and treatment will not only be ineffective but may even accelerate death by reducing

oxygen availability. However, given its efficacy for killing free ciliate forms, we may consider future chemotherapeutic use in aquaculture that could be used for prophylactic purposes.

The chosen substance was diminazene aceturate or Berenil. This was a drug widely used in tripanosomiasis and babesiosis. Although the compound is on the market since 1955, the mechanisms of action are poorly understood. While early reports show that Berenil possesses tripanolytic and tripanostatic properties, some studies show that it can affect the immune system of the host. Recent studies show that treatment with Berenil reduces the production of proinflammatory cytokines (IL-6, IL-12 and TNF) *in vivo* and *in vitro*. Berenil's ability to disrupt major intracellular signaling pathways leading to the production of proinflammatory cytokines indicates that it can be used in the treatment of diseases that produce excess proinflammatory cytokines (Shiby K., 2014).

Thermogravimetric analysis of the complex

The analysis of diminazene aceturate with β-cyclodextrin was carried out at the National Research and Development Institute for Chemistry and Petrochemistry (ICECHIM) in Bucharest.

After confirming and characterizing complex formation in solution, we continued with the preparation of the complex in solid state. For this purpose, we used the lyophilization method, which usually offers good yields over other methods, but which has industrial scale applications, given its simplicity.

In addition, the characteristics of the mixture by lyophilization means that it could easily be incorporated during the manufacture of the feed.

As stated, the β-Cyclodextrin inclusion complex with diminazene aceturate was obtained by the lyophilization method (coevaporation). To determine the efficacy of complexing with diminazene aceturate, they were mixed in various proportions using a mixture of water and 50:50 glacial acetic acid as a wetting agent. Preliminary studies have indicated the need to reduce the pH of the wetting agent to facilitate partial dissolution of the drug and thus improve complex formation; Indeed, formation of the complex does not occur if the wetting agent is only water. Acetic acid has been chosen in view of its high volatility, so it is rapidly eliminated from the complex, minimizing toxicity problems.

For the preparation of inclusion complexes, diminazene aceturate and β-cyclodextrin were mixed in appropriate proportions and then milled. Subsequently, the wetting agent was added. The obtained paste was dried in an oven at 40°C for 24 hours and the 200-500 μm fraction was obtained and used for subsequent tests. Using this method, mixtures of diminazene aceturate and β-cyclodextrin were prepared in molar ratios of 1:1.

The stability of the inclusion complex was determined both in the presence of inert matter and in the presence of air. Fig. 2 shows both thermogravimetric analysis for diminazene/β-cyclodextrin complex. The air and inert mass loss curves are similar, demonstrating a high stability to the oxidation complex inclusion. There is a mass loss of about 4% at temperatures up to 160°C, which corresponds to evaporation of the solvents, a mass loss of about 15% at temperatures up to 250°C and a weight loss of over 40% at 350°C. Mass loss occurring in the temperature range of 200-250°C is probably due to dehydration of cyclodextrin and at temperatures of 250-350°C, probably due to dextrin degradation.

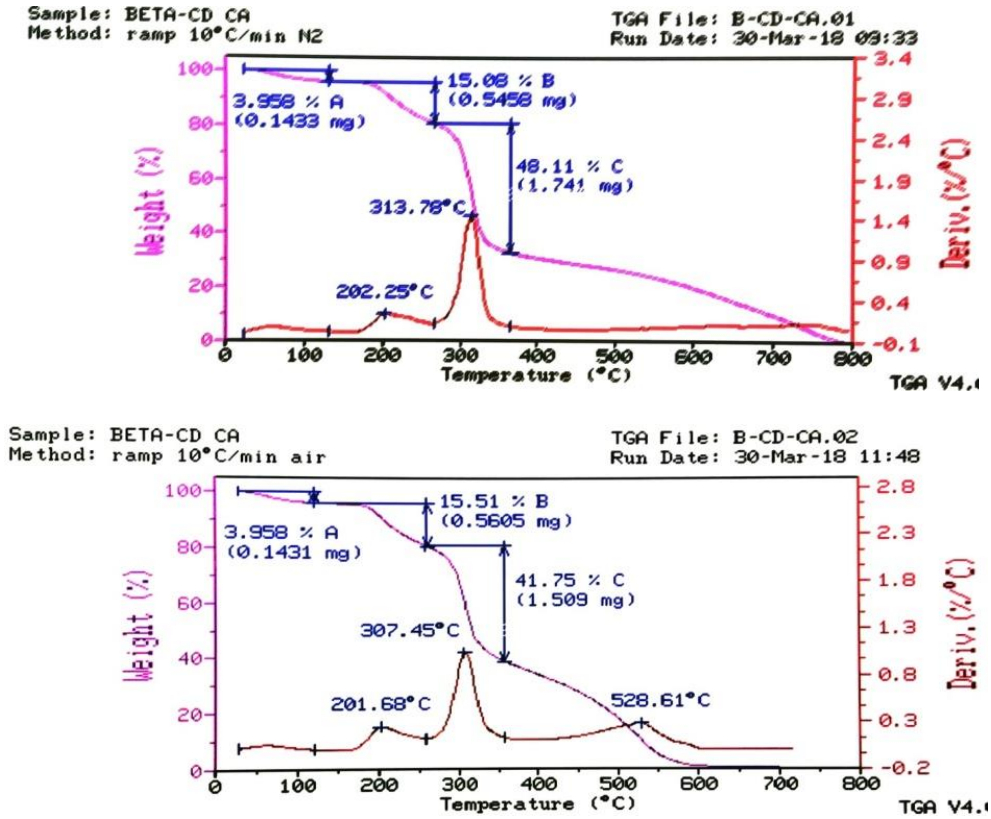

Figure 2 Termogravimetric analysis in inert gas and in synthetic air for diminazene aceturate/ β-cyclodextrin complex

Differential Scanning Calorimetry analysis of the complex

Thermal curves obtained by DSC provide information on the stability of the complex and, implicitly, the degradation temperature of one component (Figure 3). We noticed the presence of exothermic phenomena at temperatures up to 146.4°C as well as the occurrence of stronger interactions between the two components of the inclusion compound, whereas from about 160°C, endothermic processes such as phase changes or even degradation occur.

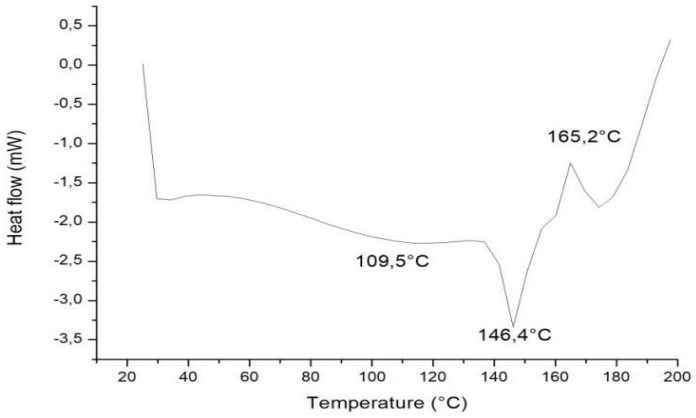

Figure 3 DSC analysis of diminazene aceturate/ β-cyclodextrin complex

In vitro analysis of the complex for I. multifiliis

The β-CD-diminazene aceturate complex was the one that showed activity against *Ichthyophthirius multifiliis* (MLC = 80 ppm). This product inhibits the transport of electrons associated with oxidative phosphorylation and thus deprives the cell of its energy source. β-CD-diminazene aceturate also has lethal *in vitro* activity against *Tetrahymena pyriformis*, another pathogenic ciliate. However, despite its *in vitro* efficacy, a number of factors should be considered in conducting tests to determine *in vivo* therapeutic capacity. First, bathing should be done cautiously, as this compound is very toxic in this way (Schmahl et al., 1989). Secondly, diminazene aceturate exhibit a lower intestinal absorption compared to β-CD-Diminazene aceturate (Swan, 1999), so that oral tolerance in food can be greater than the tolerance of bathing administration.

Table 1.

Efficacy of the complexed drug in different doses (mortalities in 6 h)

Dosage(ppm)	**Control**	**DA**	***β*-CD-DA**
1,5	0	12	1
3,1	1	19	5
6,2	1	25	8
12,5	1	32	17
25	1	35	30
50	1	34	80
100	32	87	100

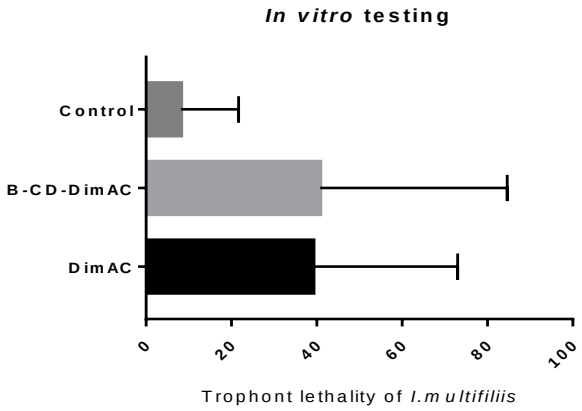

Figure 5 The effectiveness of the substances in 6h

Furthermore, β-CD-diminazene aceturate can be a good candidate for controlling ciliatoses caused not only by *Ichthyophthirius multifiliis* but also against other protozoans living in tissues (Schmahl et al., 1989). The mechanism of action against these parasites is not clear, although it has been suggested that they can act on enzymes, affecting the synthesis of pyrimidine.

Despite the fact that it has been shown that bathing is highly effective against certain intracellular or tissular parasites of freshwater fish, we can assume that its efficacy would be more significant in medicated feed. Therefore, oral administration should not be excluded in future evaluations of *in vivo* efficacy. In the present study, high efficacy when administered in water argues its future use *in vivo* for the treatment of ciliatoses.

Conclusions

We chose only diminazene aceturate and complex β-CD-diminazene aceturate for their proven antiprotozoan activity that killed the ciliates in 6-8 hours at quite high doses (CLM = 80 ppm in both cases). Numerous authors have reported the effectiveness of the bath for the treatment of ectoparasites and subepidermic protozoan infections of fish. Moreover, continued administration of diminazene in fish feed may be effective in eliminating the *Ichthyophthirius multifiliis* ciliate trophonts that parasite into the skin of the fish. In this case, the uncomplexed compound causes rupture of the external alveolar membrane and alteration of intracellular digestion. The mechanism of the antiprotozoan substance is not precisely known and appears to differ from one organism to another. However, in other protozoa, diminazene aceturate due to its chemical structure containing two identical cationic groups (dicyclic diamidine) has a high affinity for the adenine-thymine base pair sequence in cDNA, resulting in non-covalent interactions (electrostatic interactions and hydrogen bonds). In the mitochondrial genome, diminazene aceturate interacts strongly with the minor helix of the double helix so that this diamidine compromises essential replication processes and induces changes in ribosomes, mitochondrial membranes and amino acid transport (Kuriakose et al 2012, Sow et al., 2012, Caramelo-Nunes C., 2011). Due to the ability of this compound to produce *in vitro* mortality for *Ichthyophthirius multifiliis*, the possible effects on cell division in similar protozoa suggest that their efficacy *in vivo* should be analyzed independently.

The stability of the inclusion complex was determined both in the presence of the inert matter as in the presence of air. The thermogravimetric analysis for the diminazene aceturate-β-

cyclodextrin complex, in air and inert matter, is similar, demonstrating a high stability to the oxidation of the inclusion complex.

In conclusion, the candidate inclusion complex tested proved to be effective against *Ichthyophthirius multifiliis in vitro*. In view of these results, the efficacy of this compound *in vivo* clearly deserves attention. However, it should be kept in mind that various factors can be expected to influence the success of chemotherapeutic measures on ciliate in farmed carp or other fish. First of all, *Ichthyophthirius multifiliis* is a highly virulent species that breaks rapidly (by binary division) and migrates through tissues as well. Secondly, only a few chemotherapeutic agents have been accepted for use in aquaculture by legislative organs in different countries, making selection of medicines difficult for the treatment of infectious fish diseases, including ciliatosis.

Finally, the fact that this inclusion complex from the current study showed increased *in vitro* activity could mean that it can also be effective enough in oral administration (in a medicated feed), but very difficult to achieve in fish at an advanced stage of disease.

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IN VIVO STUDY OF CONJUGATED DIMINAZENE ACETURATE FOR ICHTHYOPHTHIRIOSIS OF FARMED CARP

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Abstract

The aim of this study was to explore the efficacy of a veterinary drug, Diminazene aceturate (4,4'-(1-Triazene-1,3-dyil)-bis-(benzenecarboximidamide), in an inclusion complex with β-cyclodextrin as a suitable treatment for parasitic diseases caused by Ichthyophthirius multifiliis in farmed carp. The efficacy was determined by the reduction in the infection intensity. The complexes were prepared by the coevaporation method and were characterized by DSC and FTIR. The selected stoichoimetry for the chosen drug was 1:1. Administration of Diminazene aceturate and complex was carried out by including appropriate doses in animal feed. Our studies suggest that the Diminazene–β-cyclodextrin complex results in a reduction in the infection degree and decrease in the trophont size in the treated fish. The oral treatment of Diminazene aceturate in inclusion complexes may be an alternative to bath treatments in carp farming.

Keywords: *inclusion, β-cyclodextrin, diminazene aceturate*

Introduction

In recent years, the inclusion of cyclodextrins in drugs has been used as a mean to improve drug properties, such as low solubility and slow dissolution rate, in order to improve bioavailability and to reduce adverse effects. A large number of studies have been published on the inclusion complexes of cyclodextrin, many of them in order to establish appropriate preparation procedures. Thus, previous studies in our laboratory and those of another group in this field have been aimed at assessing the usefulness of the complexes formed with regard to aspects such as stability(Szejtli, 1988), bioavailability(Vila-Jato și colab., 1986) or reduction of unwanted properties, e.g., Gastrointestinal ulcer(Otero Espinar et al., 1991) or undesirable organoleptic properties (Szejtli, 1988; Anguiano-Igea și colab., 1996).

Most studies aim to improve medicines for people. In view of the public health problems that have recently occurred in livestock farms and in aquaculture, increasing importance is attached to the control and formulation of medicinal products used in the treatment of animals for human consumption. The high propagation density characteristic of aquaculture promotes the emergence of infectious diseases of all types (viral, bacterial, fungal and parasitic). Parasitic diseases are particularly important, especially those caused by life-cycle species, including fast-spread ectoparasites. Such infections can have serious economic consequences, mainly due to outbreaks that have caused high mortality rates. For many such diseases, effective vaccines and treatments are not available.

One of the most representative species in freshwater aquaculture is carp - *Cyprinus carpio*. Parasites that affect this cyprinid include *Gyrodactylus sp.*, *Ichthyobodo necator* and *Hexamita salmonis* and histophagus ciliate *Ichthyophthirius multifiliis*. In the next study we evaluated the efficacy of a complex drug used in the treatment of parasitic infections in other animals, predominantly by oral administration (Tojo et al., 1994). Administration of oral drugs is generally preferred, because administration by immersion often leads to environmental contamination.

In the oral treatment study, we evaluated the efficacy of Diminazene aceturate and diminazene acetaturate complex, which were mainly administered by incorporating them into animal feed. Diminazene, like other antiparasitic drugs, is poorly soluble in water, so it is poorly absorbed in the intestine, greatly reducing its efficacy. Medicines such as diminazen have poor organoleptic properties and some animals reject the food they contain. In addition, besides decreasing treatment efficiency, poor absorption and low solubility, water contamination problems and the waste container also occur. In the present study, we evaluated the use of cyclodextrin inclusion complexes for the delivery of active substance to *I. multifiliis* infected farmed carp (*Cyprinus carpio*). We also examined the taste of feed containing β-cyclodextrin inclusion complexes.

The purpose of this article is to explore the efficacy of a veterinary medicinal product diminazene aceturate in inclusion complex with β-cyclodextrin as appropriate treatment in some parasitic diseases caused by *Ichthyophthirius multifiliis* in farmed carp. Efficacy is determined by the reduction in infection intensity. The complexes were prepared by the co-evaporation method (lyophilization) and were characterized by FT-IR, DSC. The selected stoichiometry was 1:1 for the drug and β-cyclodextrin. Administration of the complex was performed by including the appropriate dose in animal feed. Our estimates suggest that these Medicated-Cyclodextrin complexes will result in a reduction in infestation, but also a decrease in trophont intensity in the treated animals (fish). Oral and complex drug treatment may be an alternative to carp curing treatments.

Material and methods

Solubility studies

Solubility studies of substances alone or in the presence of β-cyclodextrin will be performed by the Higuchi and Connors method (1965). The excess of drugs will be added to tubes containing 10 ml of water or an aqueous solution of β-cyclodextrin (0-16 mM). The tubes are shaken at 80 cycles / min for 5 days in a water bath at 20 ° C ± 0.5 ° C. When equilibrium is reached, the tubes are centrifuged for 5 minutes at 5000 rpm, then the supernatant is filtered through cellulose nitrate membranes (0.45 Amp Pore size) to remove the suspension of the materials. The amount of drug in solution is determined by UV spectrophotometry at 306 nm. The stability constant is calculated from the initial linear region of the phase solubility diagram as described by Higuchi and Connors (1965).

Host used for complexation

The host is represented by the molecule of β-Cyclodextrin (Fig. 1).

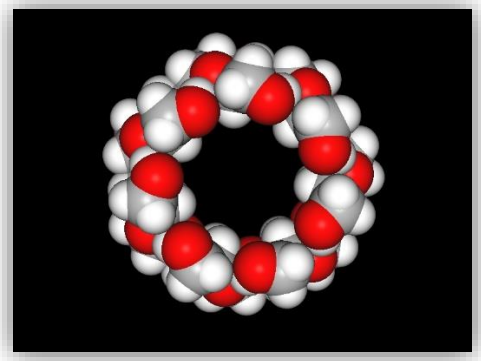


Figure 1 β-Cyclodextrin. Picture made with MarvinSketch

Cyclodextrins are substances composed of sugar molecules (-d-glucopyranose) linked together in a ring form, namely cyclic oligosaccharides. They are also called cyclo-amylose, cyclomaltose or Schardinger's dextrins and are non-reductive in nature. The CD's nomenclature is based on the number of units of glucose in its structure, so that the CD has 6 units of glucose called α-CD, the 7-linkage CD is called β-CD and the one with 8 units is called γ-CD. The CD-e glucopyranose units are linked via the 1-4 bonds. The formation of glucopyranose unit linkages gives the CDs a conical shape (Arun et al., 2008). There are hydrogen bonds between the 2-OH and 3-OH groups around the outer edge. These links are the weakest in α-CD and the strongest in γ-CD. Around the bottom edge, the 6-OH groups can also form hydrogen bonds, but the bonds are destabilized by dipolar effects and are not normally present in the CD crystals. In α-CD, the hydrogen bond is pure 3-OH (donor), 2-OH (acceptor). But in β and γ-CD, the bond changes between it and 3-OH (acceptor), 2-OH (donor). The CDs are amphipathic structures in which the 3-OH and 2-OH groups are exposed on the broader edge and on the outer edge of the 6-OH group. The outer cavity is lined with these hydrophilic groups and the inner surface is etched by the ether as anomeric oxygen atoms. Thus CDs have a hydrophobic inner cavity and an outer hydrophilic surface (Zhou and Ritter, 2010).
For our study, we considered working with β-cyclodextrin.

Prepararation of solid drug-β-CD complexes

After confirming and characterizing the formation of the complex in the solution, we proceeded to prepare the complex in a solid state. For this purpose, we used the co-evaporation method (lyophilization), which usually offers good inclusion rates (Blanco et al., 1991), which is attractive for industrial scale applications, given its simplicity. In addition, the characteristics of the lyophilization mixture provide the possibility of being easily incorporated into the animal feed at the time of manufacture (typically based on granulation and extrusion procedures).

As stated, inclusion complexes with β-Cyclodextrin were obtained by the lyophilization method (Szejtli, 1988). To determine the complexity efficacy, drug mixtures and β-cyclodextrin at different proportions were lyophilized using a 50:50 v/v mixture of water and glacial acetic acid as a wetting agent. Preliminary studies have indicated the need to reduce the pH of the wetting agent to facilitate partial dissolution of the drug and thus to improve complex formation; Indeed,

formation of complexes does not occur if the wetting agent is only water. Acetic acid was chosen in view of its high volatility so that it was rapidly removed from the complex, minimizing toxicity problems.

For the preparation of inclusion complexes, the drug and β-cyclodextrin are mixed in suitable proportions and then milled in a mortar. Subsequently, the wetting agent (Blanco et al., 1991) was added. The thus obtained paste was oven dried at 40°C for 24 hours and the 200-500 μm fraction was obtained and used for subsequent tests. Using this method, drug and cyclodextrin mixtures were prepared at a molar ratio of 1:1.

Complex characterization in solid state

Complexes obtained by the lyophilization method were characterized by, FT-IR, differential scanning calorimetry (DSC).


A


B

Figure 2 Diminazene (B) and complexed diminazene (A) in solid state

Differential scanning calorimetry

Aluminum tubes containing 1-2 mg of product were placed in a Shimadzu DSC50 DSC (gas air, temperature range 50-250°C, heating rate 10°C / min). DSC is a useful technique that allows us to determine temperature transitions, such as melting, boiling, dehydration or crystallisation, which may occur in the sample material, resulting in an endothermic or exothermic reaction.

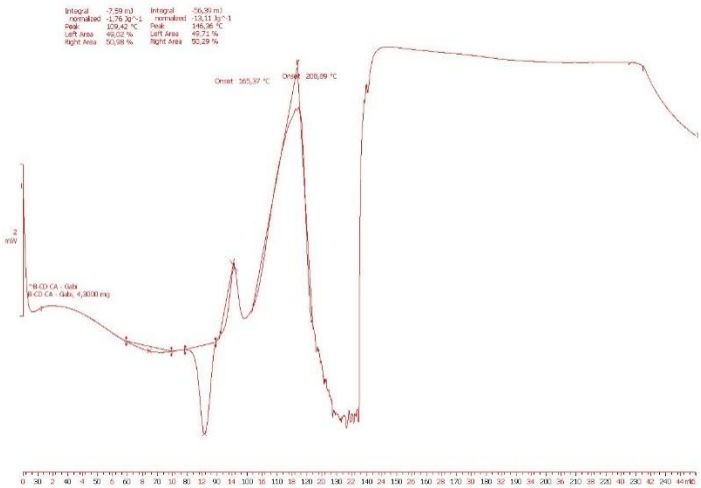
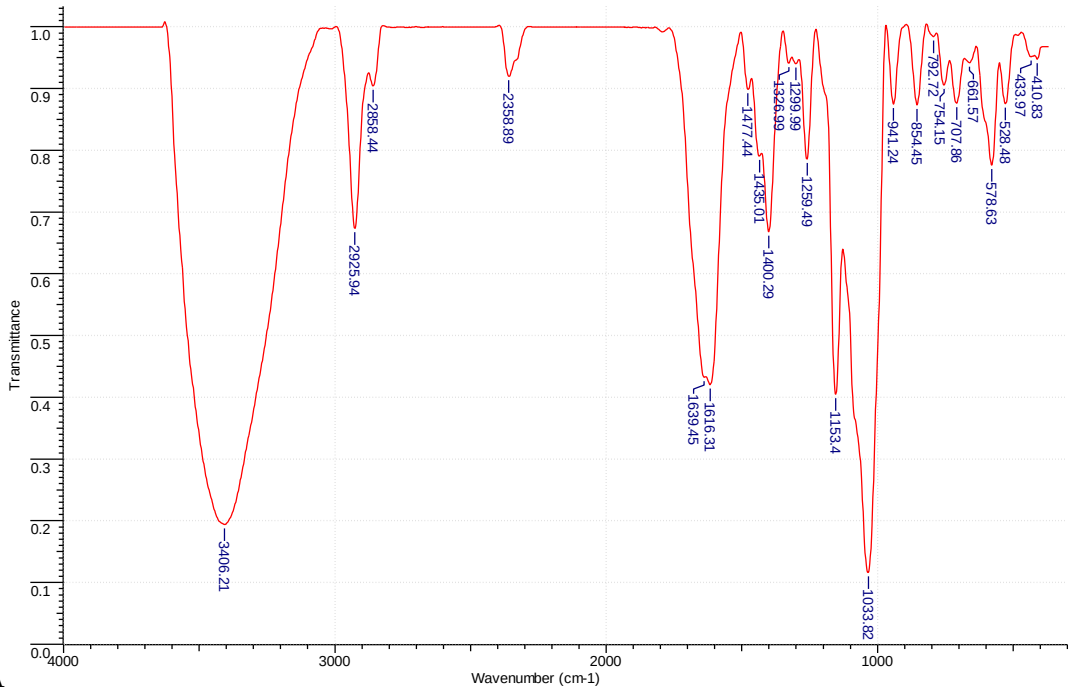


Figure 3. Thermogravimetric analysis of complex β-CD-Diminazene aceturate

Thermal curves obtained by DSC can provide information about complexing the drug with cyclodextrins and about their crystalline state.

FT-IR analysis (Fourier Transform Infrared Spectroscopy)



A

Figure 4. FT-IR analysis forthe complex Diminazen-β-CD

Variation of the shape, passage, and intensity of the IR absorption peaks of guests or hosts may provide sufficient information for the occurrence of inclusion. The graph showed the IR spectra of Diminazene, β-CD and their inclusion complex. The IR spectrum of Diminazene showed its characteristic bands. There was a very strong absorption band at 1616 cm^{-1} for C=O stretching vibrations.

The absorption band at 1400 cm^{-1} was indicated for the stretching vibration of C-C in the hexatomic ring. 941 cm^{-1} was for the C-H absorption band in the C-conjugated system. The IR spectra of the inclusion complex are similar to β-CD because of the reduced amount of diminazene in the system. However, some variations in the spectra were found. The absorption band at 1639 cm^{-1} disappeared or was shifted to the small wave numbers in the diminazene/β-CD inclusion complex, indicating that the C=O stretch vibration was restricted after the formation of the inclusion complex. 1400 (1477) cm^{-1} was strongly weakened, indicating that a majority of the diminazenhexametal ring was included by β-CD, but perhaps only part of diminazene was included, only one hexatomic ring of the two. In the present article, diminazen inclusion complexes with β-CD have been prepared and complex structures have been investigated by FT-IR. The experimental results showed that the module of the complex was the part of the diminazene molecules were included in the β-CD cavities.

Preparation of the feed containing the drug

Firstly prepare a homogeneous mixture with mortar and pestle, the drug complex and the commercial feed.


A

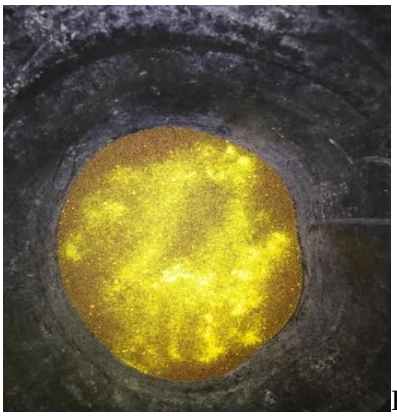

B

Figure 5. The feed used for the inclusion of the drug

The feed is previously ground in a blade mill. The mixture is moistened with water to obtain a suitable consistency for subsequent extrusion, then extruded (into an extruder) using the cylinder with a 2 mm orifice. The obtained pellets are dried in a furnace for 24 hours. They have an average weight of 0.2g and one kilo of feed contains about 5000 pellets.

Characterization of animal feed

The drug content in food can be determined by spectrophotometry at 306 nm. 15 minute release tests (15 minutes being the estimated maximum time required for fish ingestion) were performed to determine if significant amounts of drug can be lost after release into water before ingestion.

Determination of drug content

The drug-containing feed (15 mg) was weighed, milled and maintained for 24 hours in 500 ml of 1:50 v / v acetic acid / acetic acid (to ensure complete dissolution of the drug). The samples are then centrifuged at 2000 rpm for 10 min at room temperature and filtered through cellulose nitrate membranes (pore size 0.45 Am). The drug content was determined in the filtrate by UV spectrophotometry at 306 nm.

Release studies

The feed (15 mg) containing the drug was placed in a beaker containing 500 ml of water and subjected to slow magnetic stirring. After 15 minutes, a sub-sample was taken and centrifuged as in the previous paragraph. After filtration to remove the supernatant, the drug was quantified by UV spectrophotometry.

In vivo studies

Fish stock

The fish (*Cyprinus carpio*) is obtained from a local fish farm (Research and Development Center for Aquaculture and Ecology Iaşi from Al. I. Cuza University) and has been acclimated for at least 36 hours in a 250 l reservoir, with aeration and constant temperature (19 ± 1 ° C, pH 6,5).

The natural light-dark cycle is simulated (14-16 light hours, 8-10 hours dark). The fish will be fed on a daily basis with commercial food.

Infection

The fish used for the analysis are naturally infected with *I. multifiliis*, but also with other parasites. They show clinical signs, which are then confirmed by examining dermal microscope (Motic x20).

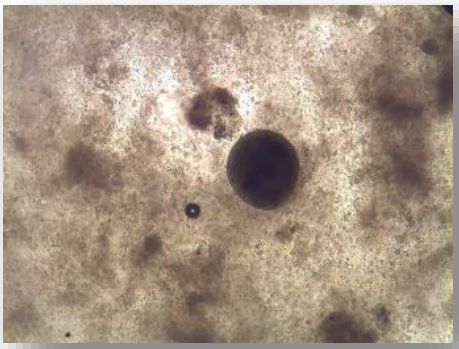


Fig. 6. Trophont of *I. multifiliis* collected from the skin of an infested carp (20x)

Drugs and analisys

The treatment trials were performed on groups of 7 infected and maintained in 180L tanks. Simultaneous control tests (also 7 fish: identical but not medicated feeds) were also performed. The tank conditions (water source, flow rate, aeration, pH, temperature, light/dark cycle) are the same as those of the acclimatization period. The treated fish received medicated feed (5g / kg feed) daily throughout the experiment. In all cases, the feeds provided will be 1.7-2% of body weight per day. Throughout the test period, fish have been regularly monitored to ensure that they eat food and to check for signs of toxicity.

The harvested fish are saplings of the *Cyprinus carpio* species with body mass between 78 - 101 g.

Table 1.

Body weight of the fish selected for the study

Nr. Crt.	**FOR CONTROL**	**FOR DIMINAZENE ACETURATE**	**FORβ-CD-DIMINAZENE COMPLEX**
1.	82,32 g	86,35 g	87,19 g
2.	78,83 g	91,02 g	101, 95 g
3.	93,24 g	88,33 g	87,95 g
4.	82,22 g	81,48 g	88, 04 g
5.	88, 37 g	95,66 g	95,15 g
6.	97, 82 g	99,07 g	94,22 g
7.	79,35 g	82,77 g	97,11 g

After the 24 hours completion of the test, the fish will be anesthetized to determine the intensity of the infection.

In the first series of analisys, we compared the efficacy of uncomplexed feed and feed containing the β-cyclodextrin-drug complex (in both cases with 5 g of drug/kg of feed) to treat *I. multifilius* infection. For these tests, we used naturally infected fish, allowing us to evaluate the effectiveness of the treatment.

For these tests, I started the treatment immediately and continued it for 10 days. Duration of *I. multifiliis* infection is variable and temperature dependent: duration is approximately 9-10 days (Tojo et al., 1994b, Tojo and Santamarina, 2001). These tests thus, test efficacy against the various stages of the parasite life cycle.

Determining intensity of the infection

The fish were anesthetized by immersion in water with clove oil (0.3 ml/l) in a 150 l separable basin until the breathing became weak. A mucus sample will be taken by gently scraping a part of the body surface of the fish (skin and fins). Mucus samples will then be examined under a microscope (100x). In the case of naturally infected fish, the intensity of the infection will be recorded on a five-point scale after examination of the entire field.

Results and discussions

Solution interactions between drug and β-Cyclodextrin were investigated by examining the diagram in the solubility phase. As can be seen, solubility increases with increasing β-cyclodextrin concentration, indicating that these inclusion complexes have limited aqueous solubility. However, the complexity solubility of the complex will be greater than that of the drug alone.

Compared to the values obtained for cyclodextrin inclusion complexes of other drugs, the stability constant in our case is high. This indicates that these complexes have a high stability in solution due to strong interactions between cyclodextrin and drug. Although high, this stability constant is within the optimal range to improve the bioavailability of poorly soluble drugs: Values below this range involve excessive dissociation in the solution leading to precipitation of the drug, while very high levels imply inappropriate dissociation, so that the free medication remain insufficient for effective absorption.

Analysis of the flat region of the diagram in the solubility phase indicated that the drug-β-cyclodextrin ratio in the complex in solution was 1:1.

To determine the characteristics of lyophilized products, I will use the differential scanning calorimetry. It shows DSC traces for Diminazene, β-cyclodextrin and various lyophilized mixtures. DSC traces for β-cyclodextrin show the thermal events characteristic of this excipient, resulting in the loss of water molecules inside the cavity (wide endotherm which occurs between ambient temperature and 140°C) and the reversible transition to 220°C. Cyclodextrin melts and decomposes at temperatures above 250°C. The DSC route for Diminazene presented an endotherm at different temperature degrees, corresponding to their melting.

All the mixtures tested (1:1) showed the drug fusion endotherm, indicating the presence of the free crystalline drug. In fact, the product is probably a 1:1 complex mixture and excess free cyclodextrin. Furthermore, the examination of the results indicates that the melting point decreases with the increase in the proportion of cyclodextrin, suggesting that a solid dispersion is formed between the two components. The presence of excess cyclodextrin can thus increase the dissolution rate, even if the drug is not complexed.

Based on these results, we selected the 1: 1 ratio for *in vitro* studies because this proportion assured that the drug was in complex form. In addition, the use of excess cyclodextrin is common in the preparation of inclusion complexes.

The selected dosage form (food containing the drug) requires that the feed pellets remain in the water until ingested. Under these circumstances, it is very important to have minimal medication loss from the dosage form in the first few minutes in contact with water: this would lead not only to drug loss but also to water contamination. The main feed components (flour, proteins, oils, etc.) and the procedures used to make it (typical extrusion) favor negligible or very slow release. Once ingested, the gastrointestinal environment favors disintegration and digestion, which results in the release of the soluble components contained in the pellet. This will confirm the amount of medicine released in the first 15 minutes in contact with water at a temperature close to that of the growth tanks (20°C) with a slight stirring. Our results indicate that neither drug-only pellets nor pellets with the complex have released significant amounts of drug during this period. It is important that the fish consume all the pellets within 15 minutes.

After confirming that the drug is not lost from pellets in water, we have conducted tests in which the infected carp was given food containing a drug or complex. The food that contained the complex was quickly eaten and I did not see any evidence of low palatability; by contrast, feed containing only the medicine (Diminazene) was sometimes rejected. This is, of course, an important aspect of a treatment given in animal feed. The ability of cyclodextrin to hide the unwanted taste of drugs such as bitterness, such as diminazen, is known.

Table.2.

Treatment results of carp with feed containing Diminazene aceturate (DIMA) or DIMA-β-Cyclodextrin (in both cases 5g drug/kg feed, after 10 days)

Carp no.	*DIMA-β-Cyclodextrin*	*DIMA*	*Control*
1	+	+++	+++
2	+	+++	+++
3	±	+++	+++
4	±	+++	+++
5	-	++	++
6	-	+++	**M**
7	-	++	**M**

Fish stock used for this test (7fish per group) were naturally infested with*I. multifiliis*. Legend: zero (-) no trophond detected; very low (±), a single trophont; low (+) 2-10 trophonts; moderate (++) 11-50 trophonts; high (+++) >50 trophonts; (M) dead fish.

Results of carp treatment with feed containing the drug or complex. As can be seen, untreated fish have maintained high infections, and 2 out of 7 fish died (Table 2) during the 10-day test period. In fish treated with uncomplexed Diminases, no mortality occurred, but the intensity of infections remained high in all fish throughout the test period. In fish treated with Diminazenecomplex, by contrast, the intensity of infections dramatically decreased to zero in 3 out of 7 fish and at very low levels in the other four fish.

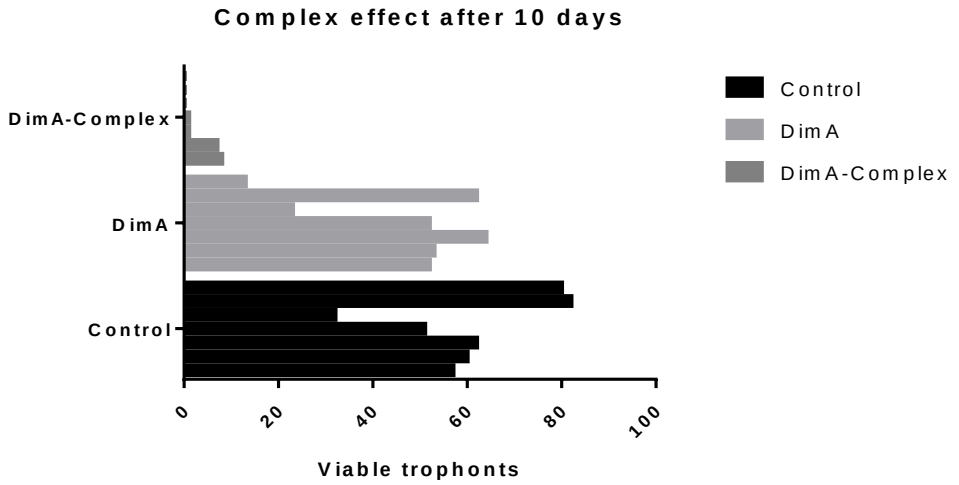


Figure 7. Viability analysis of the trophonts after 10 days of treatment with medicated feed

Figure 7 shows the very high intensity of the parasite load in the first two groups (of the 7 fish/group) than the group in which we used the Diminazene-β-Cyclodextrin complex in the feed.

Currently, our laboratory is conducting studies to investigate this phenomenon and to determine the doses and time needed for the treatment to completely eliminate the parasite.

In conclusion, preliminary results will suggest that these inclusion complexes of cyclodextrin with an antiparasitic are a promising option for the treatment of ichthyophtiriosis in farmed carp. Because *I. multifiliis* is a localized parasite in the skin, it is not easily accessed by drugs. Cyclodextrin inclusion complexes seem to improve accessibility and avoid the need to use immersion treatments, which often can not be authorized from the perspective of public health and environmental issues.

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