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Article

Hoop structure for horses during winter season: controlling the low critical temperature

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Abstract: Hoop barns, the low input housing structures, can be used in housing horses during fall and winter seasons. One of the hoop structures issue is the level of temperature, which is close to environmental temperature. The aim of this study is to show some technological measures which can overcome the weaknesses of hoop structures such as: feeding strategy, water system and body warming of horses using IR film. The control variable was the skin temperature of six horses, repeatedly measured with FLIR® thermo camera during several levels of environmental temperature. In the study, by using IR heating film at outside temperatures such as 0, -5 and -10°C, the body temperature measured in three body regions (neck, shoulder point and internal angle of eyes) did not differed significant (F=0.167 at p =0.847): without IR heating system, differences were observed (F= 8.905 at p =0.000). Moreover, in low BCS's animals, below -10°C environmental temperature, in absence of IR heating and if less fiber was present in the diet, low critical temperature signs were observed. In conclusion the hoop structure can be used successfully in horses even when outside temperatures are below low critical temperature of horses if certain conditions are assigned such as: water at 10-15°C, additional hay for fiber, with or without infrared heating.

Keywords: hoop structures, low temperature, horse

1. Introduction

Hoop structures have been used as effective alternative housing for grow-finish swine in the United States, Canada and Australia for over 30 years [4]. In Romania a hoop structure was studied [6,7,9,10,11, 13] and has been operable at Banat University – *Horia Cernescu* Research Unit since 2012. Hoop structures offer a distinct advantage for animal production due to the substantially smaller capital investment, relative to a conventional confinement building along with substantial reductions in energy operating cost.

Energy use is reduced because these structures are not heated or mechanically ventilated. In cold seasons, horses utilize the low energy consumption systems - IR heat panels to increase the thermal comfort. During warm seasons, structures with a north/south long axis orientation in open areas, will experience substantial natural air flow for ventilation. In addition, the high arch-shape of the structure creates a "chimney effect" that facilitates natural air flow. Furthermore, hoop structures are also versatile buildings that are easily converted to facilities for other types of livestock or for feed or equipment storage if a farmer decide to discontinue swine production and focus on other enterprises [9].

Finnish researchers investigated the respiratory health effects of loose indoor/outdoor group housing on weanling foals in cold Scandinavian winters (below −20°C in Finland for several consecutive weeks during the winter season**)** and they found that, generally, the foals did very well in this environment. The general conclusion was that keeping weanling horses in cold loose housing systems does not seem to increase the occurrence

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of respiratory diseases, but special attention should be focused on ventilation, air quality and feeding-practices [12].

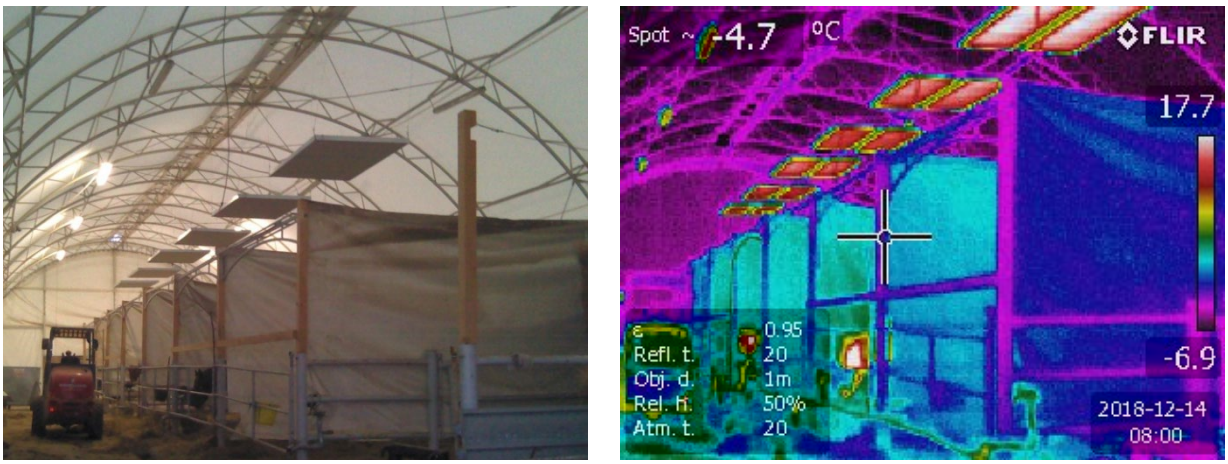
The specific objective of this report was to establish baseline conditions and expectations for keeping horses in hoop systems in Romania during the winter season of the year by using IR heating film panels.

2. Materials and Methods

Animals and data collection: six horses (Thoroughbreds, Romanian sport horse and Arabian breeds) weighing 580.83±17.14 kg were obtained from private owners on November 2018 by Research Contract no. 6944 from 25.09.2013 and placed in the ruminants hoop structure at the *Horia Cernescu Research Unit*, infrastructure of Life Science University "*King Michael I*" Timisoara, RO. The group consisted of 4 females and 2 males which were placed in individual pens inside the hoop structure after deworming based on fecal analysis. The horses were acclimated to their new location for 15 days. No haircuts were performed during the trial, but one physiological change was observed - an increase in density and length of the horse's coat. On the fifteenth day, horses were weighed and scored for body condition scoring. Subsequently, horses were weighed and scored for BCS every month considering the Henneke's scale [5] for jumping horses. Inside and outside temperature and humidity of hoop were continuously monitored using a multi-functional wireless digital device *Weather Station PCE-FWS 20.* The *FLIR (E50 Multi-Spectral Imaging Dynamic, MSX®, Wilsonville, Oregon, USA). A* thermocamera was used for collecting the external body temperature of horses several times, when environmental temperature was in the limits established by the study. The body temperature was taken in three points (neck, head – internal angle of eyes and point of shoulder – *figure 2*) during the trial period (December and January) depending on the inside temperature inside the hoop: at 0.0±2.0°C, at -5.0±2.0°C, and -10.0±2.0°C.

Feed: Feed consisted of two intakes of 2.0 kg of washed oats and three intakes of 3.0 kg of grasses hay (less than 2% of their body weight in hay per day), 40 ml of corn oil daily, salt, vitamins A, D, E and 2:1 calcium-phosphorus mixture. For medical reason, during January one horse received just 1.25 kg of grasses hay per intake. Composition of the feed was analyzed by *Foss InfraXact*® (Hillerød 3400, DK), NIR equipment. Feed was stored in bags in the hoop structure and feeders were filled manually after weighing using *Ranger Mate* (American Calan, Northwood, New Hampshire, USA). Each pen was equipped with one nipple/cup water fountain and one feeder. In order to prevent freezing of pipes, a heating cable was used – the temperature was set to 10-15°C.

Housing: The *Experimental Unit* – hoop structure used for this trial has a total exterior dimension of 12 X 36 m and has concrete flooring, although it was primary designed for heifers and redesigned for horses' during the trial (*figure 1*). The primary design feature of these types of structures consists of uniformly spaced metal arches which are covered with a tightly woven plastic tarpaulin which is stretched out over the arches. The arches are attached to the top of vertical posts. These posts serve as the foundation of the structure. The tarp is stretched by means of small winches attached to the exterior surface of the posts. The interior of the posts is faced with wooden boards or sheet material to create a wall of 1.75 meters in height. The arched ends of the structure are typically covered with similar plastic canvass material with some type of roll-up doorway. The end-walls are often partially or completely opened during warm weather to increase air flow and reduce internal structure temperature and totally closed during the cold season. During winter, the one side inlet (15 cm x 36m) and top outlet 30cm x 36 m) were completely open in order to stimulate the air flow and to avoid the formation of condensation drops. The six horses were housed in the hoop structure, one per pen. Each of the six pens in which horses were housed measured 3.6 X 5.50 m. This offered 23.76 m^2 per animal which is more than the recommendation on horse housing for research purposes – [4,8]. Each of the 6 pens were equipped with one heating panel (handmade - two rows of 2 m^2 of heating film, power 200W/m^2, with long-wave infrared radiation, of 4 μm ÷14 μm anchored in arch ceiling structure, parallel with floor, at 3.5 meters high (*figure 1*).



(**a**) (**b**)

Figure 1. Normal *(a)* and FLIR thermocamera *(b)* imagines of trial place – six pens in hoop structure each with one IR heating panel suspended in arches of ceiling (Hutu, 2018).

Statistical Analysis: Analysis of external body temperature of horses were performed using one-way *Analysis of Variance (ANOVA)* for temperature of three body regions in presence or absence of IR heating panels. All data comparing body temperatures in case of using/not using IR heating was analyzed using two-sample *Student's* t-tests. For lower number of data, the Wilcoxon Signed Ranks Test was used.

3. Results and Discussion

Body weight was in average 580.83±17.14 kg at the beginning of the study (November 2018) and 585.83±18.09 kg at the end, on 31th of January 2019. The easy training activities were done during the study. In those conditions, even if the temperature in the hoop was lower, horses became heavier - but the difference between the start and finish of the trial cannot be statistically sustained (Z=-0.843 at p = 0.339 - *Wilcoxon Signed Ranks Test*).

Body condition scoring was in average adequate for jumping horse (BCS = 5.54±0.12). BSC was assessed three times: during the accommodation period, at the end of December and at the end of January. Major differences were not observed during the study period (p=0.317, *Wilcoxon test*): in average BCS was 5.41±0.24 at the beginning of the study (November 2018), 5.58±0.20 in December and 5.62±0.22 at the end of the study period (January 2019).

Temperature and Humidity: The inside temperature was higher (+1.56 °C at $p< 0.001$) and humidity index was lower (-0.49% at $p< 0.001$) than outside measurements. There was a strong correlation between inside and outside temperature ($r = 0.925$ at $p < 0.01$) and humidity index ($r = 0.829$ at $p< 0.01$). There were no clinical signs of low critical temperature among horses even though daily minimum temperatures often exceeded -10.0° C during January, with one exception. One horse that scored 4.5 in BCS had horripilation, muscle contractions, reduced blood flow at the level of the extremities such as ears, muzzle and legs as a sign of cold stress. As a preventive measure, horses that are body clipped or with low BCS will benefit from a blanket. Blankets are also beneficial for short term, in extremely cold, wet weather [3].

External body temperature of horses was measured in three body regions, with and without IR heating film, at outside temperatures such as 0, -5 and -10°C. *Without IR heating panels,* at previously mentioned environmental temperature, the body temperature measured was 18.28±0.74°C, 18.90±0.72°C and 22.19±0.65°C – in the study, the differences were significant ($F= 8.905$ at $p =0.000$). When measured in each body region (middle of neck, shoulder point and internal angle of eyes) the temperature was well differentiated (F=38.34, p=0.000): 17.79±0.38°C for neck region, 18.09±0.63°C for shoulder point and 23.49±0.50°C for internal angle of eyes. The variability between several body regions is normal and was reported by several authors [1].

With IR heating panels on, the body temperature measured was 19.99±0.85°C, 19.34±0.76°C and 19.61 ±0.78°C – in the study, the differences did not differ significantly (F=0.167 at p =0.847). It appears clearly that IR body heating panels reduce the variability of body part temperature and the influence of external negative temperature of the environment. Measured in each body region (neck, shoulder point and internal angle of eyes) the temperature was well differentiated (F=36.46, p=0.000): 17.79±0.37°C for neck region, 17.92±0.65°C for shoulder point and 23.23±0.46°C for internal angle of eyes). Because the aim of using IR heating film is body's warming and not to heat the air of the stable, IR panel heating film had a lower electricity power consumption - for one horse the electrical power consumption is 0.4 kWh (1.44 MJ). For all six pens the electricity consumption was 2.4 kWh (8.64 MJ) which is similar with a domestic air heater.

The *FLIR* thermocamera used for collecting the external body temperature worked pretty well in cold climate, like other authors suggested [2].

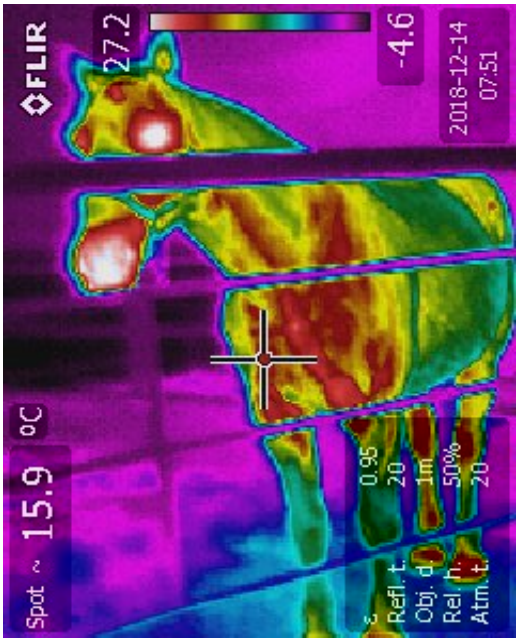


Figure 2. **FLIR® IR image with temperature spot on "point of shoulder".**

The low critical temperature's signs were observed at environmental temperature below -10°C in one horse– it was the case of a horse that had a lower BCS's, in absence of IR heating and with less fiber in the diet – because of colic prevention measure. Horses increase body metabolism through various physiological mechanisms. Bacterial fermentation of forage in the hind gut of the horse is one of them – by this, horses can generate a tremendous amount of heat. As a result, horses can tolerate much colder weather than humans. Practically, the addition of fiber to the diet will increase heat from fermentation.

In conclusion of the study, the hoop structure can be used successfully in horses, even when outside temperatures are below the low critical temperature of horses, if certain conditions are fulfilled such as: water at 10-15°C, feed rich in fiber and BSC in optimum ranges.

5. Conclusions

Hoop structures can be used for housing horses during the winter season, if an increased diet of grass hay is provided. Increasing the external temperature by using panels with long-wave infrared radiation of 4 μm ÷14 μm does increase thermal comfort and will facilitate the housing of horses in extremely cold environments. Because the aim of body warming by IR heating film is not to heat the air of the stable, IR panels had a lower electricity power consumption - for one horse the electrical power consumption is 0.4 KW/hour. Low body condition score imposes the increase of grass hay intake, use of a blanket, or IR heating systems in order to avoid the clinical signs of low critical temperature in the hoop structure.

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Institutional Review Board Statement:

The study was conducted according to the guidelines of the Low 43 11.04.2014 and approved by the Ethics Committee of *Horia Cernescu* Researh Unit, protocol code POUEX1.1 from 27.05.2016.

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Conflicts of Interest: "The authors declare no conflict of interest."

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Article

Histopathological and macroscopical considerations of induced experimental periodontitis in rats

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Abstract: Ten Wistar male rats were used to induce experimental periodontitis by placing a 5-0 cotton thread ligature at the base of the first superior molar on the left side. Before this phase, the molar went through the process of scaling, rooting and planning. Soft movements of the molar were realized for creating an accumulation of plaque by flattening and resulting in the displacement of the gingival tissue, thus provoking an inflammatory response. After seven days, the ligatures were removed in all ten rats. After 14 days, results obtained showed gross aspects of periodontitis and microscopical lesions as well, installed in the periodontium. In addition, an inflammatory response with bone necrosis and alveolar bone loss was observed microscopically. This study aims to test an experimental protocol of periodontitis confirming the presence of this pathology by gross aspects and histopathological aspects. In conclusion, the tested procedure can provide all the critical biological elements in periodontal disease, representing good features for the biomaterial testing domain.

Keywords: chronic inflammation; dental ligature; bone necrosis.

1. Introduction

Many investigations have been done in the last years trying to search for an appropriate and efficient treatment for periodontal disease, and all the ways of therapy were very developed.

Periodontitis is spread worldwide and can affect a significant majority of both human and animal populations, so pathology needed to be experimentally induced in some species in order to assess all the factors involved in its development accurately and also to be able to test the effectiveness of different methods of therapy, some being experimental or ongoing implementation. Therefore, this experimental study was implemented to test a novel therapy represented by a hydrogel enriched with a photosensitizer and natural essential oils extract (i.e., oregano, frankincense and thieves blend) that could potentially treat and reverse the associated clinical and pathological symptoms.

As a general definition, periodontitis is an inflammatory chronic infectious oral disease caused by specific pathogen agents which lead to the destruction of supporting tissue that supports the teeth; respectively, it causes the loss of the periodontal ligament and, in the end, the loss of the alveolar bone. Clinically this pathology causes symptoms such as gum bleeding, dental laxity and plaque on teeth and could also develop a local inflammation as gingivitis [1,2]

All these symptoms are considered to result from the response of a capable host to exist as a microbial biofilm represented by bacterial pathogens [3]. Serum levels of inflammatory cytokines, such as interleukin-1beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor (TNF), are increased in patients with severe chronic periodontitis [1,4]. There is a great variety of pathogens in the composition of the oral bacterial flora from one subject to another, depending on the species, the age and the cleaning possibilities, as well as the

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fluctuation of the host response to the interaction of bacterial species, are some of the main reasons why the particular etiology of periodontal disease could not be acknowledged [5–7]. Bacteria are known to be the first etiological agent of periodontal disease, and it has been considered that more than 500 different bacterial species are involved, like *Streptococcus mutans, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, etc.* [8,9].

A big part of the data published on periodontal disease etiology comes from human medicine research[10]. Periodontitis in domestic animals is almost identical to that found in humans in terms of progression and clinical presentation [11]. The accelerated rate of evolution of this affection reported in pets compared to humans may be caused by poor oral hygiene and the absence of routine dental care [12]. This pathology can suffer some changes depending on the animal's habitat and the genetic predisposition of each organism [13,14].

The aim of this study is: 1) to test the experimental induction of local inflammatory response in males rats by placing a braided ligature on the first superior molar in order to induce periodontitis lesions; 2) to prove the success of inducing the periodontal disease by histopathological aspects, by the presence of neutrophils, demineralization, necrosis and alveolar bone loss [15]; 3) to test the specific effects of biomaterials as hydrogels, also combined with natural extracts and laser therapy, implementing new ways of alternative therapy in the management of periodontal disease in animals in the following studies; 4) creating the possibility of avoidance of the analgesic medications because of their side effects defined by gastrointestinal affections and the emergence of antibiotic resistance when using an antibacterial medication because all these represent ways of conventional methods of treating periodontitis [2,16,17].

2. Materials and Methods

Ten medium weighted males, Wistar rats were used for this experiment. After the clinical exam, consisting of a short evaluation of their general status (i.e., checking the grimace, checking their degree of hydration, checking their appetite, and inspecting their skin), the rats were weighed in order to determine the weight loss that rats may suffer after the step of placing the ligature. The body weight variation was evaluated and monitored every day after the ligature placement for ten consecutive days. This evaluation was realized in order to establish whether there is necessary the implementation of a supportive therapy or adjust this therapy.

Optimal accommodation habitat was ensured throughout the experiment; the rats were housed in the Establishment for Breeding and Use of Laboratory Animals of USAMV (Cluj-Napoca, România) in standard conditions, at a temperature of 22–23 C, humidity 55%, and 12-h light/dark cycle. The rats were kept in plastic cages with free access to standard rodent granular food (Cantacuzino Institute, Bucharest, Romania) and freshwater ad libitum. In addition, an extra hyper lipidic diet (Cantacuzino Institute, Bucharest, Romania) with a smooth consistency for the first days after the surgery procedure was provided.

The rats were allowed to acclimate to the laboratory environment for two weeks. All procedures involving laboratory animals' use followed the European guidelines and rules 337, as established by the EU Directive 2010/63/EU and the Romanian law 43/2014 and were performed by an experienced practitioner. The study protocol was approved by the Research Ethics Committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania, and they were authorized by the State Veterinary Authority (aut. No. 306/24.03.2022).

The surgical procedures were performed under the effect of general injectable anesthesia; this was an injectable type for the application of the ligature and was performed by administering the following anesthetic substances: Xylazine (Xilazin Bio 2%; Bioveta, Czech Republic), injectable solution 7,5 mg/kg IM and Ketamine injectable solution (Narkoman Bio 10%; Bioveta, Czech Republic), 75 mg/kg IM. The experimental protocol for inducing periodontitis consisted of the application of a surgical technique in order to position the 5-0 cotton thread ligature (BioSintex; Ilfov, Romania) at the base of the molar. To extract the ligature, seven days later, the following were administered: Midazolam (Dormicum 0.1%; F. Hoffmann-LA Roche Ltd., Switzerland), injectable solution 0.02 mg/kg SC and Ketamine, injectable solution 70 mg/kg IM. The animals were euthanized three weeks post-induction, according to the procedures recommended by Law no. 43/2014, through profound narcosis with Isoflurane (Isoflutek 1000 mg/g; Laboratorios Karizoo

S.A., Spain). The animal was considered dead at the moment of absence of cardiac and respiratory activity. Then the axo-atloid dislocation was performed to ensure the phenomenon's irreversibility if the inducing protocol's side effects significantly weakened the animals; according to the bioethical protocol, they were euthanized before the end of the study period.

As we already mentioned, all rats were weighed to have an effective body condition scoring tool for laboratory animal welfare in the first step.

The second step involved the application of a 5-0 cotton thread ligature (BioSintex; Ilfov, Romania) at the base of the first left superior molar (Fig. 1), previously performing a slight looseness of the gum in the submarginal position and secondary performing a dislocation of the periodontal ligaments (Fig. 2).

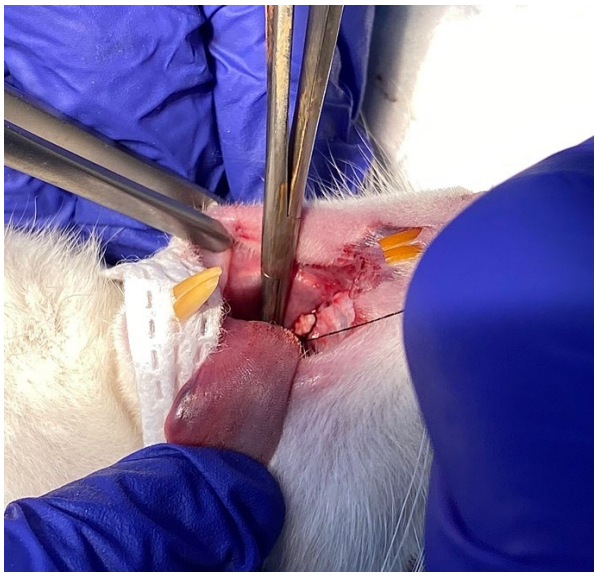

Figure 1. Placing the ligature at the base of the first left upper molar.

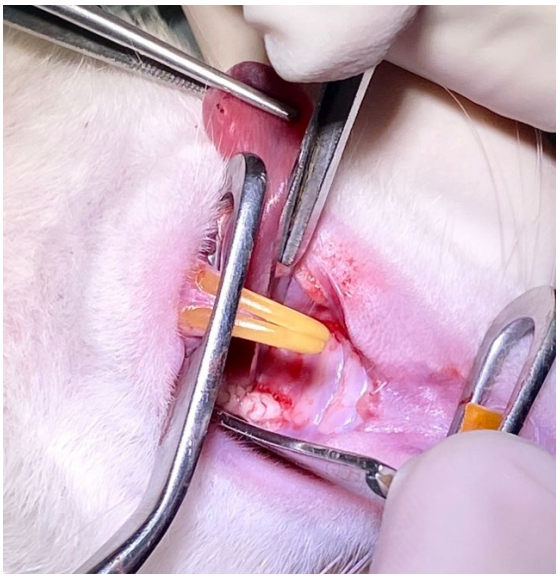

Figure 2. Creating the detachment of the gingiva by performing a slight looseness of the gum.

After the ligature placement (Fig. 3), the rats were evaluated daily, and a pre-determined quantity of specific food consisting of softer consistency hyper lipidic diet (Cantacuzino Institute; Bucharest, Romania) was offered in order to check their post-surgery appetite.

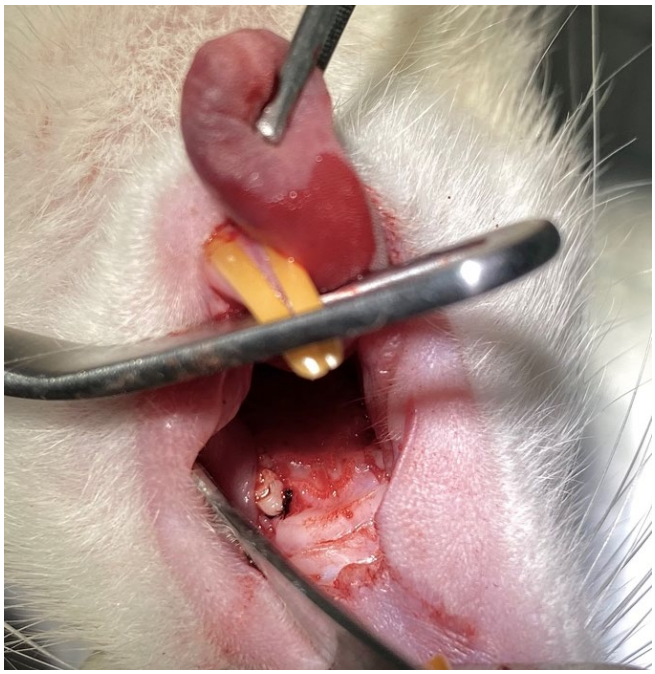

Figure 3. Aspect after the placement of the ligature

In the first two days post-op, a 10% solution of Glucose (B Braun Pharmaceuticals; Melsungen;, Germania) injection was administered if they did not eat any food. The analgesia was also managed with an injectable solution of Tramadol (Tramadolum; 50 mg/1 ml Krka D.D., Slovenia). Finally, the third stage took place one week after the ligature was placed, when the rats were anesthetized again with the same anesthetic protocol, and the ligature was removed (Fig. 4).

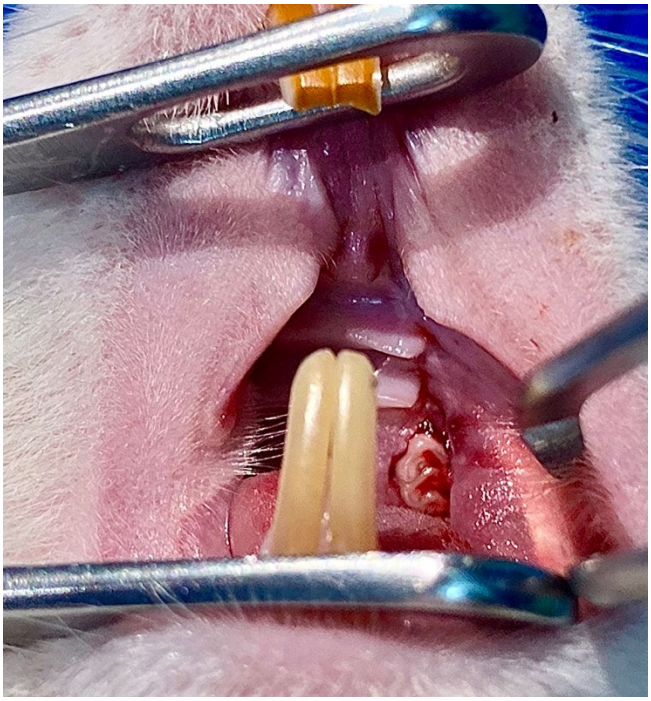

Figure 4. The aspect of the molar after the ligature was removed

This stage was followed by scaling, rooting and planning the affected molar. Next, easy and slight movements of the molar were applied to create an accumulation of bacteria by flattening, resulting in the detachment of the gingival tissue with the help of the dental curettes. The movements were repeated ten times by tractioning the molar in all the lateral planes, thus provoking an inflammatory response.

One week after removing the ligature, the rats were anesthetized again because the computed tomography analysis was performed with a Siemens Somatom Scope machine (SOMATOM Scope, Siemens, Germany) before euthanasia. The anatomical region of interest contained the maxilla of the rats; the targeted region is represented by the soft tissues around the upper left molar, the attachment tissues of the molar and its corresponding dental alveolus. The molars were scanned axially with a thickness of 1 mm, and the recorded images were saved in DICOM (Digital Imaging and Communications in Medicine) format on the Siemens workstation in the PACS server. The analysis of bone density in order to confirm the successful induction of periodontitis at the level of the upper left molar was carried out with the help of the Syngo Somaris 5 CT VC 28 program (Syngo VC 28; Siemens Health Care Sector, Forchheim, Germany).

So the rats were euthanized one week after the ligature was removed, after which the left maxilla was sampled for histopathological analysis to confirm the onset of periodontitis. In addition, gum and bone sampled from the injured site have been submitted for histological examination. After fixation in 10% buffered neutral formalin, the mandibular samples were decalcified using a mix of 1:1 (formic acid and clorhidric acid) for 24 hours and embedded in paraffin. Five-micron thickness sections were stained by the hematoxylin-eosin method (HE). The slides were examined under a BX51 Olympus microscope (Olympus Life Science Europa; Hamburg, Germany), and images were taken with an Olympus UC 30 digital camera (Olympus Life Science Europa; Hamburg, Germany) and processed using Olympus essential stream software. Sections were examined by an independent observer blinded to the experimental protocol.

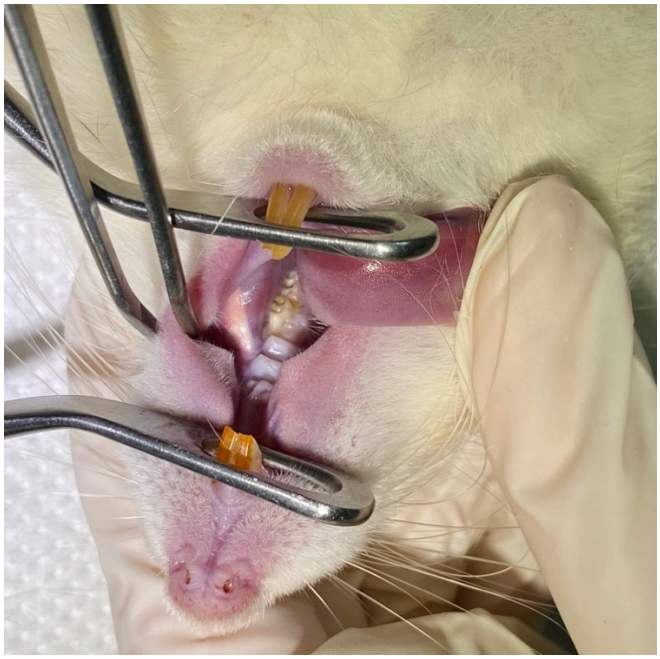

Figure 5. Gross aspects of installed periodontitis **-** accumulation of the plaque is present, and also discoloration of the molar is visible after seven days

3. Results

Ten rats were subjected to this procedure after the presentation of the protocol described above. Seven days after the experimental protocol, we could see the onset of periodontitis. Gingivitis was observed in five subjects, more moderate, in four others more acute, and in one subject, gingivitis was reduced, so dental laxity was recorded in only nine out of ten rats. This evaluation was performed with the help of the clinical scoring of periodontitis, which implies the mobility scoring and the gingival bleeding performed for each individual [19,20].

Six of ten rats lost weight, with variations ranging from 25 to 60 g, considered a moderate amount. When the rats lose more than 10% of their body weight, euthanasia is considered because this parameter is considered a pain assessment recommendation.

After the euthanasia of all ten rats, a necropsy was performed. Grossly, yellowish discoloration of the molar, mobility within the alveolus and gum reactivity have been observed in nine rats (Fig. 6).

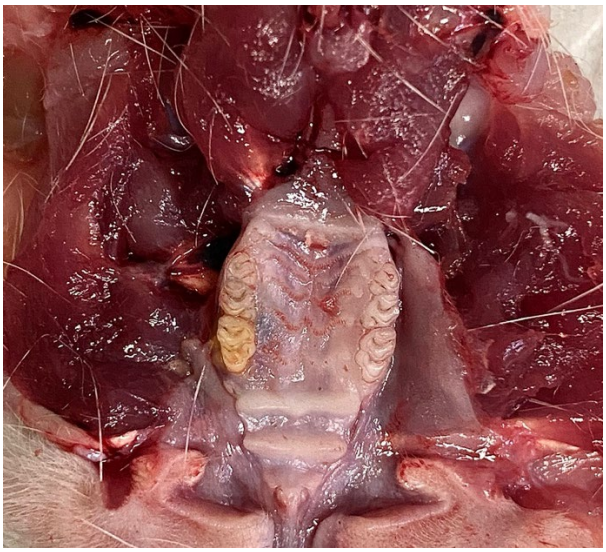

Figure 6. Gross features of periodontitis after euthanasia, represented by yellowish discoloration, gum retraction and reactivity, mobility within the alveolus

Following the histological aspects after the microscopical analysis, the tooth and the periodontal ligament (the periodontium), respectively, the dental alveolar bone, showed a normal appearance for one rat. However, microscopically, lesions such as inflammation, demineralization, thinning, and bone resorption could be seen in nine rats at different stages.A moderate gingival retraction was observed in the cervix of the teeth and the interdental space, and chronic and superficial local gingivitis were completed in eight rats (Fig. 7).

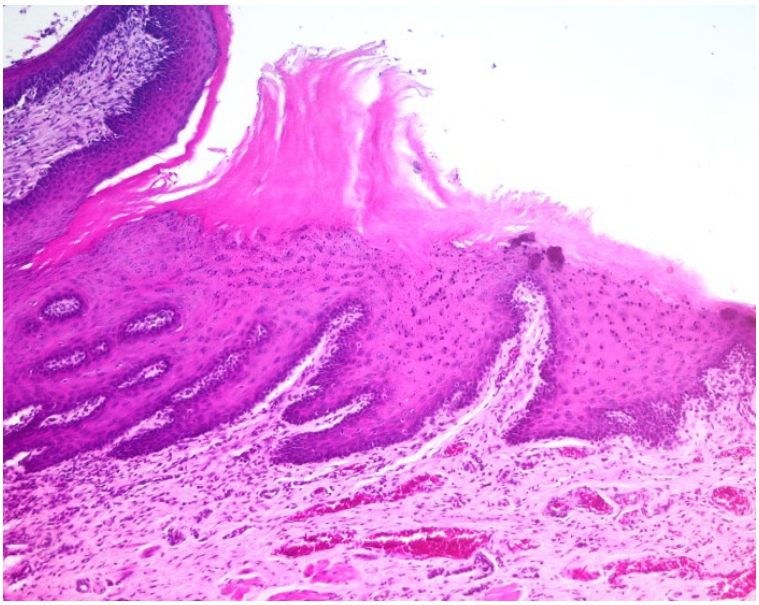

Figure 7. Hyperplasia and severe hyperkeratosis of the gingival epithelium, the subepithelial lamina propria is infiltrated with rare mononuclear cells; HE staining X20

The superficial part of the inflammatory process is covered by a layer represented by bacterially infected tissue and fodder debris. Furthermore, some segmental osteoclastic resorption of the alveolar bone was seen as also suppurative process represented by focal periodontitis extending from the previously described gingival defect (Fig. 8).

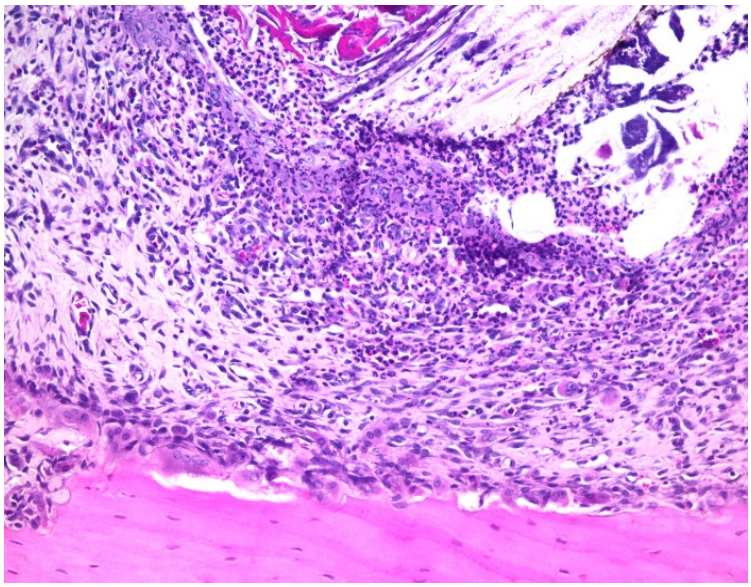

Figure 8. Chronic suppurative gingivitis, moderate-segmental osteoclastic resorption of the alveolar bone; HE staining X10

On the site of the subgingival region, abundant granulation tissue was seen, which could split the septic point and replace the dental ligament. In addition, hyperplasia and hyperkeratosis of the gingival layer with the organization of the anatomic epithelial papillae, divided by a fibro-vascular inflammatory stroma, moderate-segmental osteoclastic resorption of the alveolar bone tissue and periodontitis were observed. The inflammatory process was expressed by multiple bands and degenerated neutrophils combined with mononuclear cells and reactive fibroblasts (Fig. 9).

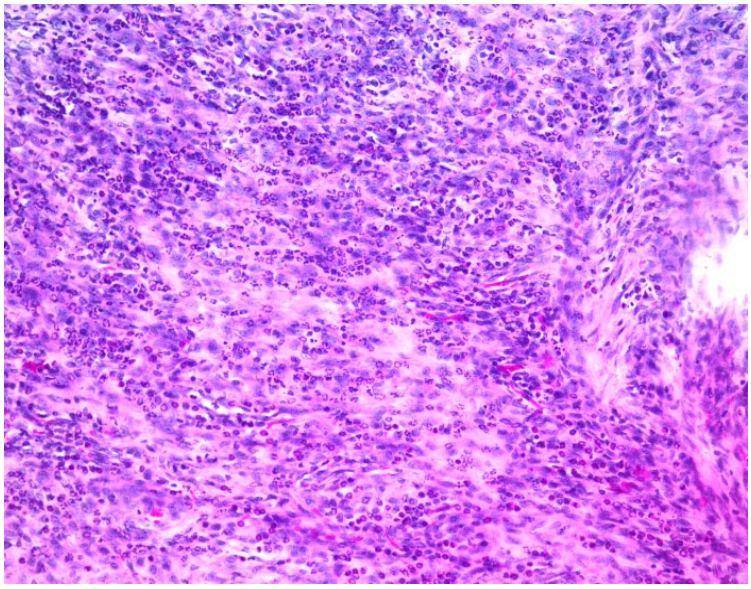

Figure 9. Granulation tissue, inflammatory infiltrate consisting primarily of neutrophils, rare mononuclear cells and reactive fibroblasts; HE staining x20

Also, the presence of hyperplasia and hyperkeratosis in the gingival epithelium layer was observed. Thus, the production of irregular epithelial papillae, divided by abundant inflammatory granulation tissue with neutrophils first and then macrophages, was observed. Furthermore, the superficial gingival area presented a minimal ulcer covered by a cellular layer mixed with cellular debris and nutrients. Fig. 9 also represented aspects of inflammatory granulation tissue with invasive degenerated neutrophils mixed with mononuclear cells and reactive fibroblasts. Gingival epithelial hyperplasia was also noted with hyperkeratosis and a partial replacement of the dental ligament with fibro-vascular connective tissue.

The CT scan showed changes in bone and periodontal tissue (Fig. 10) observed in 8 rats.

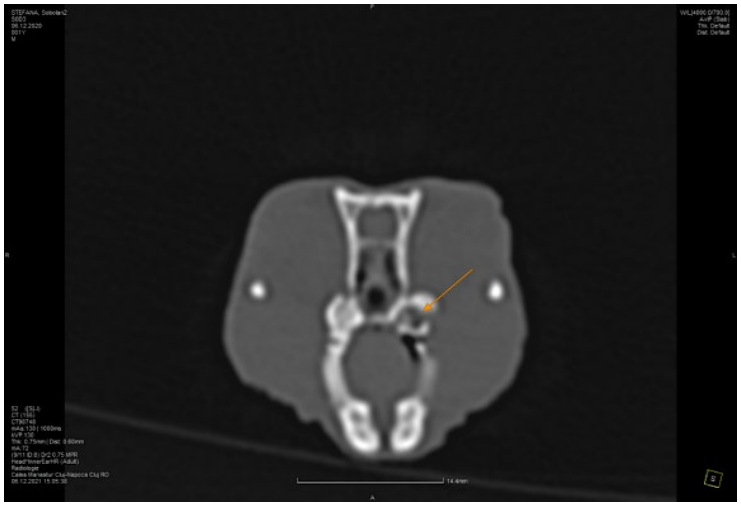

Figure 10. CT images consistent with changes in bone and periodontal tissue, bone necrosis, rarefaction and alveolar bone loss

4. Discussion

The tested procedure can provide all the critical biological factors in periodontal disease, representing good features for the biomaterial testing domain. Currently, ligature-induced periodontitis in rats is the primary model used in periodontal research, and alveolar bone loss is the main parameter evaluated by radiographic, morphometric, and histological techniques [22].

Because of the positioning of the ligature on the superior molar, as an advantage, there was a better resistance of the ligature during the induction of the periodontitis, also because the saliva is accumulated by the gravity reason on the mandibular sides, not on the maxillary ones. The main problem with the experimental techniques of inducing periodontitis consists in the resistance of the ligature on the molar, which may require another intervention of replacing the ligature or adding an extra ligature placed tight. Nevertheless, we succeeded in inducing periodontitis lesions after seven days, demonstrated by gross aspects and microscopical investigations, even if some authors reported that the ligature should be kept in position from 15 to 60 days to induce periodontal destruction [23].

Although we did not investigate the specific parameters of inflammation and necrosis by paraclinical investigations, the macroscopical aspects and the clinical signs were enough to prove and confirm the presence of periodontitis.

Comparative to other techniques described for inducing experimental periodontitis, such as placing a ligature on one of the incisors or on a group of incisors with a ligature on eight, the technique we chose was more stable, more resistant and much safer. Also, compared with another method of inducing experimental periodontitis by creating a lesion on the gum and inoculating an extract of specific periodontitis pathogens [24] [25] on the lesion created, the technique we used did not affect the general health and condition of the rats [26]. The body mass is considered a clinical endpoint, especially in periodontal experimental protocols where the prehension capacity of the animal is intensely affected, and the appetite could be poor. Other clinical endpoints for laboratory animals are their behavior, reluctance to move, dehydration and pain [18]. Alongside food consumption, monitoring these parameters is usually realized once a week and is considered a good practice as part of standard husbandry care [21]. We chose to evaluate and monitor these parameters every day after the ligature placement for ten consecutive days precisely so that we can closely observe the changes that occur during the installation of this pathology.

Another main aim of this research is to demonstrate in the following studies the effectiveness of regenerative therapy with biomaterials, photosensitizing agents and photodynamic therapy, reversing all the effects of periodontitis induced by the initially placed ligature.

5. Conclusions

This study showed that placing a cotton or silk thread around the cervical region of the upper left molar causes gingival inflammation, and the first symptoms of periodontitis developed from the seventh day of the experiment onwards. In the present study, it has been demonstrated that experimental periodontitis has general systemic biological implications; poor body conditions, weight loss secondary to loss of prehension and increased oral pain, and the correlation between periodontal disease and general health. This experimental protocol followed the exact surgical steps by performing ligatures, rooting and scaling and demonstrated gross and microscopic lesions. This article has been created to prove all steps necessary to achieve successful experimental periodontitis explicitly.

Author Contributions: Conceptualization, S.M, A.D. and R.C.; methodology, A.D and S.M.; software, R.P..; validation, A.D., A.N. and A.R.P.; formal analysis, A.N.; investigation, A.R.P. and S.P.; resources, S.M., A.R.P. and S.P.; data curation, R.P.; writing—original draft preparation, S.M. and A.D.; writing—review and editing, S.M and A.D..; visualization, A.N.; supervision, L.O.; project administration, L.O. All authors have read and agreed to the published version of the manuscript".

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Conflicts of Interest: All procedures involving laboratory animals' use followed the European guidelines and rules 337, as established by the EU Directive 2010/63/EU and the Romanian law 43/2014 and were performed by an experienced

practitioner. The study protocol was approved by the Research Ethics Committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania, and they were authorized by the State Veterinary Authority (aut. No. 306/24.03.2022).

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CVJ CLUJ VETERINARY JOURNAL

Review

Diagnostic methods used for the detection of *Theileria equi*: review of the last decade

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Abstract: *Theileria equi* is one of the aetiological agents responsible for EP and is transmitted by ticks to horses, mules, donkeys and zebras. Clinical signs are often nonspecific and can easily be confused with other pathologies. Although acute, sub-acute and chronic forms have been described, the most common situation in equines is that of asymptomatic carrier, characterized by undetectable or extremely low parasitaemia and lack of clinical signs. Identification of the parasitic agent, as well as the immunity acquired as a result of infection can be done by direct and indirect methods such as molecular and serological methods. This study aims to identify the most commonly used diagnostic methods of EP with the highest specificity and sensitivity and the fewest limitations. In order to achieve the aim of this study, a systematic database search was carried out, resulting, after a preliminary selection, in a total of 97 publications considered eligible. It was concluded that molecular diagnostic methods can overcome many of the limitations of traditional methods and are essential to identify and distinguish genotypes of *T. equi*. Nonmolecular diagnostic methods may lack sensitivity and specificity, but they are still widely used and useful to support clinical and epidemiological research.

Keywords: *Theileria equi*, equine piroplasmosis, PCR, cELISA, blood smear.

1. Introduction

Equine piroplasmosis (EP) is a disease of Equidae caused by *Theileria equi*, *Theileria haneyi* and *Babesia caballi* [1,2] transmitted by ticks to horses, mules, donkeys and zebras. Infected animals can remain carriers for long periods of time and act as sources of infection for tick vectors. Introducing carrier animals into an area where tick vectors exist can lead to an epizootic spread of the disease. Transplacental transmission of *T. equi* from carrier mares to their foetuses has also been shown [3]. Although acute, sub-acute and chronic forms have been described, the most common situation in equines is that of asymptomatic carriers. In the chronic form, animals show a dry symptomatology such as decreased exercise tolerance, while the carrier stage is characterized by undetectable or extremely low parasitaemia and lack of clinical signs [4].

Identification of the parasitic agent can be done by direct methods, blood or stained organ smears during the acute phase of the disease and by molecular and serological methods in carrier animals, low parasite burden makes detection extremely difficult [5].

The sensitivity of microscopic examination of blood smears and smears of lymph node needle aspirates is low, so that false negative results are regularly observed [6]. The most important feature is that this method is only useful in detecting infected erythrocytes in the acute phase of the disease.

Several serological tests have been developed to increase the sensitivity of the diagnosis, especially in those carrier horses that show no clinical signs. These tests include the complement fixation test (CFT), indirect immunofluorescence assay (IFA), Western blot (WB) and competitive enzyme-linked immunosorbent assay (cELISA) [5].

The CFT test depends on complement activation during the specific antibody-antigen interaction. Infected horses seroconvert on CFT approximately 8 to 11 days after infection, and titters begin to decline at 2 to 3 months [7,8]. CFT is a very specific test, but is

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not sensitive in chronic or inapparent phases of infection, mainly because some antibodies produced during these phases of infection do not bind complement [9].

IFAT is thought to be more sensitive than CFT during chronic infection. However, the need to dilute serum to improve specificity in IFAT performance reduces sensitivity. IFAT is often used as an adjuvant test to help analyse CFT results [5]. Sensitivity and specificity of CFT and IFAT for *T. equi* were reported differently, thus a sensitivity between 47% and 63% was reported for CFT and 89-96.6% for IFAT, and specificity was between 94-96% for both [10].

A method recommended and approved by the OIE for international testing for equine piroplasmosis is cELISA, which is considered the most sensitive test for the detection of chronic or inapparent *T. equi* [11,12].

The cELISA technique described by commercial kit manufacturers involves binding of primary monoclonal antibodies to antigen-coated plate, binding detected using horseradish secondary peroxidase (HRP). The presence of the HRP marker of the secondary antibody is quantified by the addition of an enzyme substrate and subsequent development of the color product. A poorly developed colour is due to inhibition of binding of the primary monoclonal antibody to the solid phase antigen and indicates the presence of *T. equi* antibody in the serum sample. [12].

Polymerase Chain Reaction (PCR) is a widely used method to rapidly make millions or billions of copies (full or partial copies) of a given DNA sample. In conventional PCR, after amplification, PCR products or amplicons are run on agarose or PAGE gels to detect the presence or absence of DNA amplification. But in Real Time PCR, amplification is monitored after each PCR cycle. Nested PCR is a modification of PCR that was designed to improve sensitivity and specificity and involves the use of two primer sets and two successive PCR reactions [13,14]. The basic principle of multiplex PCR is the same as that of conventional PCR, except that multiple primer pairs are required in the same reaction. Primers can be specifically combined with the corresponding DNA template, and more than one DNA fragment will be amplified simultaneously in a single reaction [15].

The PCR technique is one of the OIE recommended methods for the diagnosis ofEP, suitable for: infection-free equine population, infection-free individual animal, contribution to eradication measures, confirmation of clinical cases, prevalence of infection-surveillance. Several PCR diagnostic protocols are currently available, some of which are recommended by the World Organisation for Animal Health [5].

Unlike molecular diagnostic methods, serological tests have limited sensitivity and specificity. PCR help to identify asymptomatic carriers and can identify a low parasitaemia of up to 0.017 % for *T. equi* [16,17]. Application of PCR assays, targeting EMA-1 gene, BC-48 gene and 18S ribosomal RNA (rRNA) gene, demonstrated a higher level of analytical sensitivity and specificity than serological and microscopic detection.

2. Materials and Methods

To achieve the aim of this study, a systematic multi-stage search of Pubmed and Science Direct databases was conducted to identify all eligible studies.

The keywords "equine piroplasmosis", "PCR", "molecular diagnosis", "Theileria equi", "blood smear", "cELISA" were entered. Articles were selected from the period 2012 to 2022 and had as subjects the diagnosis by molecular methods and description of new protocols for molecular diagnosis of *T. equi*, identification of parasites by direct microscopy and serological methods.

The key terms "equine" and "equine piroplasmosis" allowed the identification of studies in both horses and donkeys.

After selecting papers based on titles and abstracts, studies were further analysed by detailed examination of the full text. Articles that were included in the study had to meet all of the following criteria:

(i) original research articles based on molecular diagnostic techniques, direct microscopy and serological methods;

(ii) study conducted between 2012 and 2022;

(iii) the diagnostic method must be clearly specified.

The research resulted in 478 articles, which were subsequently checked to determine whether they met all the proposed criteria as well as to eliminate duplicates. After a preliminary screening of the studies performed a total of 97 publications were considered eligible.

From 266 articles results following the introduction of the keywords molecular diagnostic and PCR, 32 were aimed at the determination of EP by molecular methods and 4 articles presenting the development and validation of a new molecular diagnostic protocol for EP (fig. 1).

Regarding the identification of parasite species by direct microscopy, a total of 7 articles were identified.

Serological diagnostic methods are frequently used in EP diagnosis, thus following the primary search in the two databases a total of 202 published articles were identified and based on the selection criteria 54 articles were considered eligible (fig. 2).

3. Results

One of the main objectives of this analysis was to identify the most commonly used diagnostic method with the highest specificity and sensitivity and the fewest limitations.

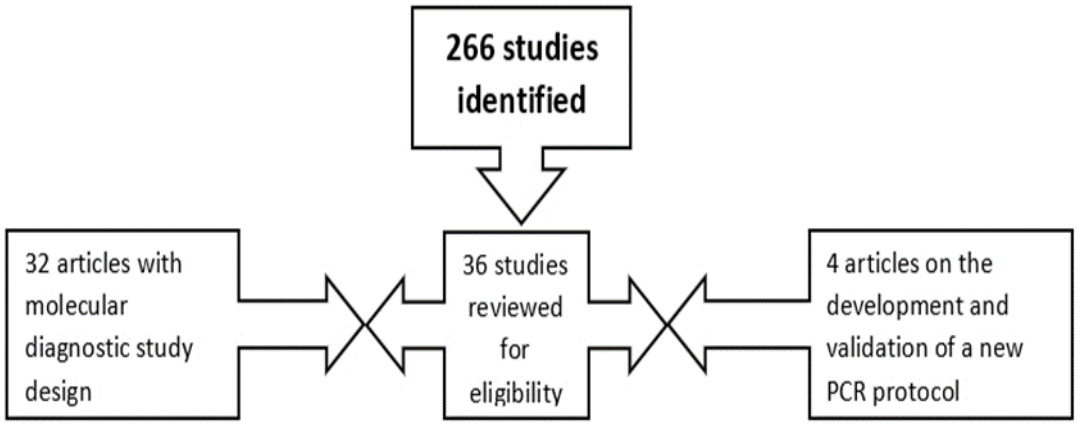

Figure 1. Selection process of studies using molecular diagnostic methods

Of the 36 articles studied, 14 authors used nested PCR, 11 conventional PCR, 6 Real Time PCR and 5 Multiplex PCR as molecular diagnostic methods.

Following the analysis of the 4 articles based on the development and validation of a new molecular di-agnostic protocol, it was concluded that 2 articles aimed to develop a new Real Time PCR protocol, one conventional PCR and one nested PCR.

The primers and probes, in the case of Real Time PCR, used were selected by the authors according to the targeted genes, namely 18S rRNA as well as BC48 (*B. caballi*) and EMA-1 (*T. equi*) genes.

Table 1. Primers and probe used in the articles studied

No	Primers	Probe	Reference
1	GTAATTCCAGCTCCAATAG AAAGTCCCTCTAAGAAGC	TTCGTTGACTGCGCTTGGCG CTAAGAAGCGGAAATGAAA	[18]
2	TCGAAGACGATCAGATACCGTCG	TGCCTTAAACTTCCTTGCGAT	[19]
3	GAAAYTGCGAATGGCTCATTAM CACCGGATCACTCGATCGGTAGG	GGATAACCGTGSTAATTSTAGGGC GTGTGTACAAAGGGCAGGGACG	[19]
4	5′- TCGAAGACGATCAGATACCGTCG-3′	5′- TGCCTTAAACTTCCTTGCGAT-3′	[20]

5	GCATCCATTGCCATTTCGAG TGCGCCATAGACGGAGAAGC AATGTTGAGCAAGTCCTTCG TTAGTAGAACAAAGCAACGGC		[21]
6	5' GGT TGA TCC TGC CAG TAG T-3′ 5' TTG CGA CCA TAC TCC CCC CA-3′		[22]
7	5′- GTCTTGTAATTGGAATGATGG-3′ 5′-TAGTTTATGGTTAGGACTACG-3′		[23]
8	5′-TCGACTTCCAGTTGGAGTCC-3′ 5′-AGCTCGACCCACTTAT CACC-3′ 5′- ATTGACCACGTCACCAT CGA-3′ 5′-GTCCTTCTTGAGAACGAGGT-3′		[24]
9	5′- CTGACTACAAGGTYGTATAC-3′ 5′-TGTCGTCACTTAGTAAAATAGA -3′	TeEMA1-P 6-FAM- TTCTCCGTCTATGGCGCA- MGBNFQ	[25]
10	5'- AAGCCATGCATGTCTAAGTATAAGCTTTT-3' 5'- GAATAATTCACCGGATCACTCG-3'		[26]
11	5‟- TTCGTTGACTGCGCTTGGCG-3‟ 5‟-CTAAGAAGCGGAAATGAAA-3‟		[27]
12	5′- TCGAAGACGATCAGATACCGTCG-3′, 5′-CTCGTT CATGATTTAGAATTGCT-3′ 5′-TGCCTTAAAC TTCCTTGCGAT-3′		[28]
13	5′ -CGA TCC CCT ATC AGC C-3′ 5′ -TCC TTA GAT AGA TGG TGT TGG-3′	5′ -TTC TGG TGT TGA CAA CAT GAC TAC TG-3′	[29]
14	5′ -GCG GTG TTT CGG TGA TTC ATA-3′ 5′ -TGA TAG GTC AGA AAC TTG AAT GAT ACA TC-3′	5′ -AAA TTA GCG AAT CGC ATG GCT T-3′	[29]
15	TCG ACT TCC AGT TGG AGT CC AGC TCG ACC CAC TTA TCA C ATT GAC CAC GTC ACC ATC GA GTC CTT CTT GAG AAC GAG GT		[30]
16	5′ – CCG TGC TAA TTG TAG GGC TAA TAC A-3′ 5′ -GCT TGA AAC ACT CTA RTT TTC TCA AAG -3′		[31]
17	5′ CCA TACAACCCACTAGAG 3′, 5′ CTGTCATTTGGGTTTGATAG 3′, 5′ GACAACAGAGAGGTGATT 3′, 5′ CGTTGAATGTA ATGGGAAC 3′		[32]

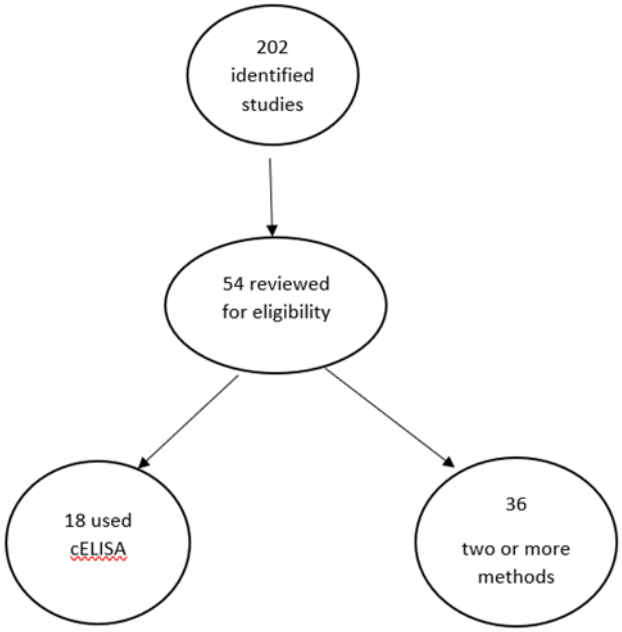

Figure 2. Selection process of studies using serological diagnostic methods.

Of the 54 articles identified based on serological diagnostic methods, 18 used cELISA as the diagnostic method and 36 achieved the aim of the study by at least two diagnostic methods, one of which was cELISA.

Identification of parasite species by direct microscopy was identified as one of the diagnostic methods used in 7 articles. In one study it was used as the only method, while in 6 articles it was used together with ELISA or PCR.

4. Discussion

T. equi, one of the main pathogens causing EPhas previously been subclassified into a number of clades based on sequence diversity of the 18S SSU rRNA gene. Current methods for clade-level genotyping of *T. equi* are laborious, PCR products must be generated, purified and sent for Sanger sequencing, and the presence of multiple allelic types in samples requires an additional molecular cloning step [22]. The 18S rRNA gene is a widespread target because nucleotide substitution rates are low and there is no evidence of lateral gene transfer between genetic lines [31]. Despite these facts, it can be observed that variable regions of this gene are often used for phylogenetic studies, in particular the 18S rRNA gene of *T. equi*, which has shown a high degree of sequence heterogeneity in different regions of the world [34].

After a primary infection with *T. equi* animals remain infected and become asymptomatic carriers with fluctuating levels of parasitaemia, a lifelong stage. Because parasitaemia levels fluctuate throughout the lifetime of the animal, the sensitivity of the duplex qPCR assay could be further improved by serial testing of initial cELISA positive/ qPCR negative tests [25].

In Romania, the first study using PCR on EP prevalence was conducted by Gallusová et al. in 2010-2012, which resulted in a prevalence of 25.4% for both piroplasma species from 18 localities inside and outside the Danube Delta [35].

It is important to note that different genotypes of *T. equi* (referred to as A-E) circulate in Europe, which may ultimately explain some differences in prevalence between countries, even though no link between genotype and virulence has been established so far [36].

In a study by Ribeiro et al. (2013), a 52% prevalence for *T. equi* infection was detected following PCR examination of 25 blood samples collected by jugular vein puncture and splenic puncture, respectively. The results of the study showed that 20% of the animals examined were positive in splenic puncture but negative in venous blood, while 28% were positive in jugular vein blood but negative in splenic puncture. Asymptomatic horses did not show parasitaemia but had infected erythrocytes in the spleen [37].

Development and validation of a new qPCR diagnostic protocol targeting the EMA-1 gene for *T. equi* and18S RNA for *B. caballi* was performed by Lobanov et al. (2018) demonstrating 100% specificity. In comparison, the samples under study were examined by both duplex qPCR and ELISA. Different results were

obtained for *B. caballi* by the two methods respectively 7.9% by qPCR and 58.6% by ELISA, which can be explained by the fact that *B. caballi* is eliminated after a period of time [25].

A study by Vieira et al. (2017) showed that 13.33% of seronegative tested animals were positive by PCR and 7.8% with negative PCR result were positive by ELISA. In this study, 7 horses were positive for *T. equi* by ELISA and negative for *T. equi* by nPCR. These are likely to be chronically infected carrier animals in which parasitaemia is below the detection threshold of molecular diagnostic techniques. A low and long-lasting parasitaemia could stimulate the immune system in animals that maintain serum antibodies at detectable levels [26].

Camino et al. (2019) performed a comparison between results obtained by several diagnostic methods namely cELISA, Real Time PCR, microscopic examination and haematological and biochemical screening. The study was carried out on 140 equines with specific clinical signs of EP and reported a prevalence of 9% by microscopic examination, while by cELISA and PCR the prevalence was 50.7% and 42.9% respectively [38].

Another study conducted in Iran by Abedi et al. (2019) on 106 apparently healthy horses resulted in a prevalence of 3.77% by direct microscopy and 50.94% by PCR.

Salinas-Estrella et al. (2022) compared in a study the results obtained by nPCR and duplex qPCR concluding that there was a relatively low concordance between nPCR and duplex qPCR for both piroplasma species and that it is also important to repeat the tests in serologically positive and molecularly negative animals and vice versa [39].

Ybañez et al. (2018) used blood smear, immunochromatographic test (ICT) and PCR as diagnostic methods for 105 Philippine horses, resulting in 23 animals positive for *T. equi* by ICT, 26 by PCR and no positive animals after examination of blood smears [24].

The cELISA technique is one of the most commonly used diagnostic methods in the diagnosis of EP being able to identify both carrier stage and acute infections, it is also simpler and less expensive than molecular techniques, but can still give false negative or false positive reactions, having a sensitivity of 95% and specificity of 99.5% [10].

Because seropositive animals in an asymptomatic population are not an indicator of recent or active infection, several authors have also tested seropositive samples by molecular methods to confirm or refute the presence of the piroplasm genome [40-46].

The use of direct microscopy as one of the diagnostic methods was identified in 7 of the articles reviewed. Positive results were presented in 4 studies [47,48,49] while for 3 articles the authors reported that no parasites were identified by this method [50,51,52].

The smear, from blood or lymph node, is a traditional method of agent identification in infected animals, but it is increasingly less used due to low specificity. The percentage of erythrocytes and leukocytes infected, in the clinical phase of the disease, with clinically expressed *T. equi* is between 1 and 5%, making identification on smear difficult [47].

Microscopic examination of smears is classified by the OIE as not suitable for testing the infection-free equine population and for use in contributing to eradication measures. This method is suitable in very limited circumstances for testing an individual infection-free animal, but is recommended with limitations for clinical confirmation of cases of EP [5].

5. Conclusions

Identification of the parasitic agent or infection can be done by direct methods, blood or lymph node smears during the acute phase of the disease, and by molecular and serological methods when in carrier animals the low parasite load makes detection extremely difficult.

Although some diagnostic methods may lack sensitivity and specificity, they are still widely used and useful to support clinical and epidemiological research.

Of all available serological methods, ELISA is the technique with the highest sensitivity and specificity, suitable for studying the prevalence of *T. equi* infections in equine populations. Serological methods are more sensitive compared to other diagnostic methods (clinical examination and direct microscopy) used, but even these techniques have limitations, e.g. they are not able to differentiate between current and previous infections.

Molecular diagnostic methods can overcome many of the limitations of other techniques and are essential to identify and distinguish genotypes of *T. equi*.

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