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ANIMALS´ LOGISTICS AND PROTECTION WITH A FOCUS ON SLAUGHTER ANIMALS DURING TRANSPORT IN ACCORDANCE WITH THE RULES

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ABSTRACT

This paper is devoted to the transport of slaughter animals. There are obstacles that needs to be pointed out, removed, and resolved. General requirements for the movement of slaughter animals within the Union have proven to be an unjustified administrative burden and costly. It also presents how important is to keep and follow all the rules provided by European Union to prevent any issues and keep the animals in good conditions and welfare during the transport. Bad conditions and not keeping the rules during animal transport can cause the stress of the animals and have a huge effect on their health. The stress can cause injuries and even a death of the animals that were healthy and had no signs of any sickness before the transfer. The main goal of the paper is to present the circumstances under which safe transport depends on choosing the correct logistical management, starting with the proper vehicles. Specific rules apply depending on the type and quantity of animals transported. The paper explains and summarizes the special establishment for road transport, including all mandatory actions, documents, and data centralisation (COMEX, TRACES, IMSOC).

Key words: animals; data; slaughter; transport; vehicles

INTRODUCTION

In the European Union, billions of animals, mainly cattle, sheep, pigs, and birds, are slaughtered for meat consumption. Meat and meat products are considered excellent sources of zinc, haem iron, bioavailable B vitamins, and essential amino acids. The consumption of meat is, however, inherently related to animal transport and slaughter. For thousands of years, ethical questions have arisen relative to the killing of animals for consumption [32].

The lives of people and living organisms in an advanced technical society are based on the transport of mass or a signal from one place to another. Transport is overcoming the space, i.e., the change of location, using traffic funds. Every transport system consists of transported cargo, vehicles, transport logistics, and workers in transport.

The transportation of animals to slaughterhouses is a major welfare concern. The number of slaughterhouses has decreased over time in Europe due to centralisation. This is expected to increase transport time for animals and consequently negatively affect animal welfare [20].

Transportation is an activity that directly involves moving people and things (cargo) with vehicles. It is the commercial (or non-commercial) part of the transport process and represents the transport performance. The task of transport is the right choice of vehicle and the most suitable transport process, during which various transport problems must be solved. The transport logistics refer to the organization and management during the transport itself.

To control and assess of the current situation across the Europe how the rules are followed, the European Parliament has set up a "Committee of Inquiry for Animal Protection During Transport", which can cause the resolution for calling stricter rules. The European Union law 1/2005 (EC 1/2005) [33], which addresses animal transportation safety, is not followed. The controls during the animal's transfer consistently fail. To prevent and minimalize bad conditions it is essential to provide continuous education not only for the drivers/carriers but also for all involved staff that are participating in whole process of logistics.

WHAT IS THE MEANING OF TRANSPORT?

Transport is the intentional movement (driving, sailing, and flight) on the transport routes or the operation of the transport facilities. It is also a branch of the national economy that ensures the movement of people, things, and animals. Animal transport is the movement of animals by one or more transport resources. It includes all related actions from administration, continuing with loading, unloading, transfer, and resting until the animal reaches its final destination and the transfer is completed (EC 1/2005) [8].

There are a lot of ways the animal can be transported. Under the transportation resources, we include road motor vehicles, railway wagons, ships, aircraft used for loading and transporting animals, as well as containers for land transport, ship transport, or air transport. The transporter can be any person or legal entity transporting the animals. It can be at their own expense or at the expense of a third party. The transported can also be a person or entity that provides resources for transport to a third-party for-profit purpose. The final destination place is the place where all transported animals are unloaded from the transport resources, except for resting and transhipment points. The final place of transport can be a new owner, relocation, or slaughterhouse (EC 1/2005) [8].

Vehicles used for animal transportation

Driving any vehicle involves great risk and responsibility. This responsibility is higher for drivers who transport animals. Drivers need to have professional knowledge and skills. They must have knowledge not only about their vehicle and the rules of the road but also about how to protect and take care of transported animals. This will help them not only in handling animals but also in identifying and distinguishing sick, hungry, and frightened animals that are behaving abnormally. Every vehicle and all the equipment for loading and unloading that is used to transport the animals must meet the basic design requirements (Fig. 1). Any motor vehicle or trailer used for the transport of animals must be constructed, maintained, and operated so as not to endanger the animals during loading, transport, and unloading. Furthermore, it must protect animals from injuries, unnecessary suffering, bad weather, excessive noise, and vibrations. At the same time, it must not have any sharp edges, protrusions, unnecessary gaps, or spaces that could cause injuries to animals, it must ensure the safety of the animals [22].

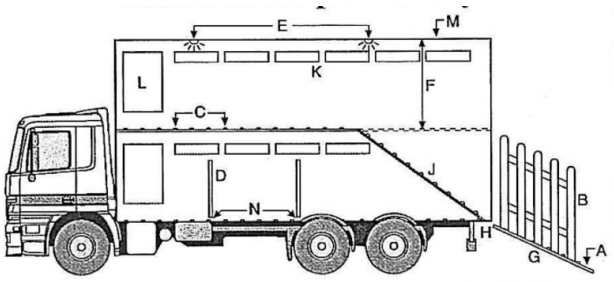

Fig. 1. Requirements for vehicles used for animal transport [22]

A – Loading ramp with steps; B – Sidewalls, minimal height 1.3 m; C – Steps or anti-slip flooring on each floor; D – Barriers: minimum height 1.27 for cattle, 76 cm for pigs, sheep and calves; E – Sufficient lighting for inspection; F – The height between the individual floors must be large enough for the animals to stand naturally and must provide adequate ventilation; G – Drop of the loading ramp no more than 29 degrees; H – The step at the upper end of the ramp must not exceed 21cm and the gap must not be wider than 6 cm; J – Drop of internal ramp no more than 33 degrees; K – Sufficient ventilation on all floors; L – Access to all floors; M – Roof; N – No larger gaps between the internal partitions and the floor.

When transporting animals, it is important to pay attention to:

a) Space and department – limitations of movement; b) Ventilation; c) Temperature and humidity – can cause heat stress; d) Noise; e) Movement of the vehicle and vibration; f) Water, feed and rest; g) Handling of animals; h) Loading and unloading.

Vehicles and their fittings that are used for livestock must be strong enough to contain the animals and prevent their escape. The design of the crate must be such that cattle cannot jump out of the crate under normal circumstances [18].

Vehicle identification

Vehicle that would be used for animal transport is identified based on a physical inspection and technical license of a motor-trailer vehicle (technical license) or a similar document, while checking in particular the vehicle registration number, type of means of transport (car, car with superstructure, semi-trailer, trailer, container, ...), brand and type designation of the means of transport [30].

Special establishment for road transport [8]

1. Animals must not be allowed to escape from vehicles, and those that do the transport must be built safely. Vehicles must be equipped with a roof that ensures effective protection against adverse weather.

2. The availability of tethering gear in large animal transport vehicles, should tethering of these animals be required. If it is required to divide the vehicle into compartments, the partitions must be strong enough.

3. Vehicles must have suitable equipment for loading and unloading animals. According to the applicable legal regulations, during the transport, the animal must be marked and registered, accompanied by a veterinary certificate and a companion document (EC 1/2005) [8] containing the following:

a) Owner and origin of animals,

b) Place of dispatch and place of destination,

c) Date and time of departure.

Based on the new rules of the European Union (1/2005/EC) it is required that all new vehicles transporting animals for more than eight hours have the appropriate equipment, including a satellite navigation system (GPS), a water security system for transported animals, a microclimate assurance system, and a warning system that alerts the driver to potential problems. Such vehicles must be officially approved in advance by the authorities. All older vehicles (after 2009) must have a satellite navigation system installed as well [11].

Modern technologies play an important role in the safety of animal transport. More modern transporters have, for example a recording camera which is directly displaying in the driver's cabin. The driver can have a full control and an overview of what is happening in the transporter, and whether the animal is trying to escape or could be injured. Thanks to the camera, he can react immediately [36].

Carrier registration

Every transporter that has its establishment or places of business in the Slovak Republic must be registered in the register of transporters maintained by the State Veterinary and Food Administration of the Slovak Republic and must be issued a certificate of ability to transport animals to the territory of the Slovak Republic and the territory of the European Union, which is numbered in a way that excludes confusion. Anyone who wants to provide the transportation of animals is obliged to apply for registration with the State Veterinary and Food Administration before starting the activity itself [28]. There are 2 types of the permit: Type 1 – the carrier's permit for short trips (up to 8 hours); Type 2 – the carrier's permit for long journeys (over 8 hours). Every Slovak transporter that transports animals and their transport is no longer than eight hours, but the distance is more than 65 km, must have a document on animal transporter registration (carrier registration). When animals are transported for more than eight hours, SVFA SR (State Veterinary and Food Administration of the Slovak Republic) requires that a copy be attached to the registered carrier's application for a registration document certificate of approval of a road vehicle intended for long journeys. This certificate is issued by the relevant regional veterinary and food authority. The request for this document must be sent to the SVFA SR within 24 hours at the latest before transporting live animals to another member state of the European Union [31].

Other necessary actions included

- To register at SVFA SR (State Veterinary and Food Administration of Slovakia), an application and an honourable statement are submitted, along with a copy of the business certificate or an extract from the commercial register.

- SVFA SR (State Veterinary and Food Administration of Slovakia) will issue the transporter a certificate of com-

petence to transport animals (transporter's certificate) and enter it in the list of transporters.

- The validity of the carrier's certificate may be suspended for 1 year.

- Withdrawal of the transporter's certificate and removal from the register of transporters may take 5 years.

- The ban on the registration of the transporter can last for 5 years.

Main general points that need to be complied in animal's transport

- According to EU legislation and directives for animal protection, no person shall transport or cause animals to be carried in a manner likely to cause injury or excessive suffering to them [4].

- Transportation arrangements must be made in advance to minimize the length of the journey and provide for the needs of the animals. Animals must be fit for transport.

- Workers who handle animals must be properly trained and competent.

- Whole transportation till the end destination must be carried out without delay and must include regular animal welfare checks.

- Animals must have sufficient height and floor space available. Water, feed and rest must be provided when needed [8].

Obligations of the carrier

- The means of transport must be cleaned and disinfected

- At the end of the transport, the transporter must ensure the cleaning and disinfection of used vehicles for the transport.

- The transporter places the animals appropriately so as not to cause them injury or unnecessary suffering.

- Ensures that the transport of animals is as fast as possible, without unnecessary delay to the destination.

- Must carry out loading and unloading of animals in the presence of the breeder.

- Do not leave the means of transport unattended during road transport of animals under supervision.

- The number of animals must correspond to the specified maximum number of animals according to the vehicle surface.

- Animals must be in special cages and containers during transport with suitable bedding according to the type of animal and fixed in a way that prevents it shifting or overturning during travel.

- The animals must have a sufficient supply of water during the journey.

- The animal transporter ensures sufficient air exchange without drafts and maintains the optimal temperature of the vehicle.

- The animal transporter must study the current legislation regarding the protection of animals during transport.

- The animal transporter must follow all established work legislation regarding the transport of animals, violation of the internal directive associated with damage to the animal at fault by the carrier is considered a misdemeanour, and the authority of the veterinary administration can cancel the decision on the animal transporter's permit (carrier's registration) [2].

To receive the authorization for journeys over eight hours, the transporter must provide:

- valid certificates of approval vehicles and containers,

- details of procedures enabling transporters to trace and record the movement of road vehicles under their responsibility and to contact the drivers at any time,

- contingency plans in the event of emergencies,

- all new vehicles used for the transport of farmed animals or horses (except registered horses) have a satellite navigation system. This requirement will apply to all vehicles (old and new) used for the transport of farmed animals or horses (except registered horses) from 2009 [27].

Training and competence certification for drivers of animal transport vehicles

- There are strict requirements laid down in the relevant regulation that drivers must first complete a training course before they can be tested to establish whether they are suitable to transport animals. It is not possible to test whether a person is a suitable driver to transport animals before they are sent on the training course. There are no requirements for two-stage training or for drivers to gain experience before they start to drive large, multi-decker trailers.

- By the Article 6 (5) and Article 17 of Council Regulation (EC) No 1/2005 on the protection of animals during transport it is mandatory for persons driving a road vehicle transporting animals to hold a certificate of competence. Based on this regulation, training is a prerequisite for an-

yone who want to provide the transport of animals. This certificate of competence confirms that the road drivers and attendants have completed a training on the technical and administrative aspects of this regulation, caring for the animals during transport and the impact of driving behaviour on the animals. But this training is not including the road safety aspects when driving certain vehicles. Therefor the attendants must undergo an additional separate training required by the directive on the training of professional drivers [10].

If the drivers want to transport animals, they must get an 'animal transportation certificate of competence' to be able to transport pigs, horses, cattle, goats, sheep, or poultry: for commercial reasons or journeys of 65 km and over.

There are many ways how to obtain the certificates (online courses, seminars, workshops, …). There are also different certification levels, based on the animal's species and type of transport (pet taxi, animal ambulance drivers, long distances drivers, …). But they all must to fulfil EU regulations requirements.

Plan of transportation

Transportation is a multicomponent phenomenon starting with the entrance of animals into the vehicle through its gate and ending with their unloading at the slaughter plant. It consists of the effects of multiple factors, such as vehicle design, handling at loading and unloading, among others, which have a great impact on the welfare [17].

It is the responsibility of the transporter to assemble a transport plan for the animal relocation if they transport animals intended for exchange or export to third countries or if the estimated duration of the transport will exceed eight hours. The carrier must have it attached throughout the journey to the health certificate. The transport plan must further determine all resting places and transfer points. Good planning of transportation can eliminate its negative effects on the animals. It is also very important to take into the account the weather.

The regulatory timeline is basically the chronological order and timeframe in which the travel prerequisites need to be completed. Timing may be critical. All needed appointments must be met and kept ensuring that the animals have the best quality circumstances for travel [1].

Some countries require that certain steps related to identification, vaccinations, tests, and treatments be done in a precise order and within a given timeline.

Requirements must be followed or else the animal(s) may not be allowed entry into the destination location or may be quarantined (possibly for an extended period of time) upon arrival.

The following steps are advised

- Schedule an appointment with veterinarian for animal's exam: Most states, territories, and countries require certificates of veterinary inspection to be completed by accredited veterinarians.

- Sharing information with veterinarian: The destination authorities (consulate or embassy of the country, state, or territory animal health department, etc.)

- Do not ask veterinarian to do anything illegal: Such actions would jeopardize a veterinarian's licenses, accreditation, and career, and may also result in prosecution by state or federal government.

- Arrangements for animal care in transit and at arrival.

- Keep a copy of veterinary inspection (certification) and all supporting documents [1].

Mandatory documents for transportation

Animal Welfare is a priority for the European Union. The EU first introduced legislation on animal protection during transport more than 40 years ago. The current legislation (the Transport Regulation) defines the responsibilities of the various parties in the transport chain and regulates the transport of live vertebrate animals within the EU, including checks on animals when entering or leaving the EU [15].

Each animal transported to the slaughterhouse must have a „passport". Accompanying document: a passport with the exact registered document number. Where is recorded the date of transportation, date of execution, date of birth of the animal, number of the animal, name and address of the animal owner, colour, sex, and origin of the animal. In addition to this passport, they also record all veterinary procedures. In addition to this passport, a document on the relocation of individual species must also be issued, for each species separately, where it is recorded from where and where the animal is relocated.

Other mandatory documents are health certification for live animals transported to slaughterhouses within the EU, all the modules are included in Commission Implementing Regulation (EU) 2020/2235 [25].

Centralised data

Each year, millions of live animals are transported by road, sea, rail, and air within, and to and from, the European Union, for a number of reasons, such as slaughter, fattening or breeding (Fig 2). To protect their welfare during those journeys, the EU adopted Council Regulation (EC) No. 1/2005 on the protection of animals during transport. An evaluation of the regulation showed that, when correctly implemented and enforced, it had a positive impact on animal welfare. However, in some area's weaknesses persist, largely due to insufficient implementation. On June 19th, 2020, the European Parliament set up the Committee of Inquiry on the Protection of Animals during Transport (ANIT). The work of the committee focused on investigating how EU rules laid down in Regulation 1/2005 are being implemented by Member States and enforced by the European Commission [35].

There exist national databases created for animal identification, farm registration and disease control:

Central registration of farm animals – it fulfills the tasks of the state in the field of registration of farms and animals of cattle, pigs, sheep and goats. It issues animal passports (currently for cattle) in accordance with Government Regulation No. 305/2003 Coll. and relevant legislation aimed at marking, registration and keeping of the Central Register of cattle, pigs, sheep and goats in connection with relevant EU regulations. Creates an electronic database of animal movements with a link to the IACS (the Integrated Administration and Control System) [26].

EU has two databases which provide the information on animal transport:

1. COMEXT – It is Eurostat's reference database for detailed statistics on international trade in goods [16].

2. TRACES – (Trade Control Expert System) is the European Commission's online platform for sanitary and phytosanitary certification required for the importation of animals, animal products, food and feed of non-animal origin and plants into the European Union, and the intra-EU trade and EU exports of animals and certain animal products [14].

It is important to mention IMSOC (Information Management System for Official Controls) which is a part of TRACES database. It implements electronically most of the provisions of the new OCR (Official Controls Regulation (EU) 2017/625). Based on that there is exchanging between Member States & Commission of all data-documents needed to perform Official Controls (Certification and eCertification). It also implements Administrative Assistance and Cooperation and unify the systems in relation to risk to human health, plant health, animal health and welfare. IMSOC provide tools to collect and manage the reports on official controls provided by Member States to the Commission. It gives us the opportunity to streamline communication to/from non-EU Countries and international organisations [24]. The rules for the operation of IMSOC are established in Regulation Implementing of Commission (EU) 2019/1715 [12].

Even if we have those databases there are no comprehensive centralised data on EU level. The main reason for this is that the EU legislation does not require member states to collect and report data on the transport of live animals. The Transport Regulation only requires member states to report on the inspections carried out. In the context of preventing transmissible animal diseases, the Animal health law requires member states to record animal movements in national identification and registration databases. These national databases were created for the purpose of animal identification, farm registration, and disease control. The Commission's view is that these databases are not appropriate for the extraction and analysis of data on animal transport [15].

TRANSPORT OF LIVESTOCK

Transport of livestock is undoubtedly the most stressful and injurious stage in the chain of operations between farm and slaughterhouse and contributes significantly to poor animal welfare and loss of production. When the rules are not followed, the effects of transportation and movement might include stress, bruises, trampling, suffocation, heart failure, heat stroke, sunburn, bloat, poisoning, predation, thirst, tiredness, injuries, and fights [9].

Livestock animals are homeothermic, it means that they must maintain a constant body temperature. A drop in temperature of 7 °C to 8 °C is undesirable, but animals are able to fully recover from this state under suitable conditions. If the body temperature of the animals increases, they enter a state of hyperthermia. An increase in temperature of only 5 °C is fatal in many cases. Animals constantly produce heat as a result of metabolism. However, they also must get rid of it in order to maintain a constant body

temperature. The combination of high temperature and high humidity can cause severe heat stress to animals in the vehicle. Since animals are constantly producing heat, the risk of heat stress is much greater than the risk of cold stress, especially when the vehicle is fully loaded. The risk of cold stress increases in cold weather and in a cold environment, with a sparsely loaded vehicle, and very young or shaved animals are more susceptible. Heat stress can be reduced by ventilation, reducing load density, traveling in the cooler part of the day. Animals must be checked regularly.

Water, feeding, and resting are the basis of animal well-being. The requirements depend on the species, previous feeding and consumption and the length of the planned trip. Animals should have access to fresh and clean water.

It is very important carefully place livestock into the vehicle. If animals are placed too tightly, there is a high risk of being trampled or suffocated. If they have too much free space around them, any sudden change in movement can cause a fall and injury [37].

Segregation during transport

It is preferable that the following classes of livestock be segregated and transported in separate groups: horned cattle, hornless cattle, adult bulls, cattle greatly different in size (cows and calves may preferably be transported together under some circumstances), and weak cattle, which should be segregated from strong cattle [18].

Preparing livestock for slaughter

Enhancing the conditions for livestock headed for slaughter has many benefits. Productivity, animal welfare, and employee safety will all increase as a result [5].

At the time of slaughter, animals should be healthy and physiologically normal. Slaughter animals should be adequately rested. They should be rested, preferably overnight, particularly if they have travelled for some time over long distances. However, pigs and poultry are usually slaughtered on arrival as the time and distances travelled are relatively short and holding them in pens is stressful for them. Animals should be watered during holding and can be fed if required. The holding period allows for injured and victimized animals to be identified and for sick animals to be quarantined. Millions of animals are transported every day around the world. Most animals farmed for human consumption are transported at least once, if not many more times, during their lifetime. It is important that these journeys are kept as stress- and injury-free as possible. Animals should be slaughtered as close to the point of production as possible. Transport should be minimized by traveling directly from the farm to the slaughterhouse [21].

Each slaughterhouse must have appropriate equipment and facilities for unloading animals from vehicles. Animals must be unloaded as soon as possible after arrival; if delay cannot be avoided, they must be protected against inclement weather and provided with sufficient air exchange. All the conditions and requirements for animal transport and housing in a slaughterhouse are included in Requirements for the protection of animals at the time of their slaughter 315/2003 [38].

Other important advice to carriers

- First, it is general welfare. Animals must always have the priority over merchandise.

- Animals of different species should not be housed in the same container, but there are exceptions to this. Compatibility depends on several factors, such as sex, state of maturity, physical size and the nature of the animals concerned.

- To avoid cross-infection, and for health and hygiene reasons, human contact with animals should be avoided. Animals, therefore, should not be housed near foodstuffs or in places to which unauthorized persons have access.

- No animal should be transported with radioactive material or other substances dangerous to health.

- Containers should be secured to the aircraft, rail wagon, lorry or ship to avoid any possible movement and, when being handled, it is important that every care should be taken to ensure that the containers are kept in a horizontal position.

- Advance preparation should be made for any necessary quarantine measures or other animal health regulations at the ports of intermediate stops or final destination [7].

During the pandemic situation of COVID–19, we were facing many issues and obstacles for the animal transport as well as the transfer of food and products made from raw materials and all related products. There was a significant risk of disease spread due to significant problems caused by transport delays that could last several hours. To prevent this emergency, the State Veterinary and Food Administration of the Slovak Republic has strongly rec-

ommended marking all the vehicles in visible places that had been used for those purposes

TRANSPORTING DURING THE GLOBAL PANDEMIC

To prevent the spread of COVID-19, the European Union has allowed countries in Europe to carry out health checks of people in all vehicles that cross borders on entry and exit to ensure prevention of the spread of the virus and provide medical assistance to those who may be ill. However, despite these checks, the EU has also stated that "the free movement of goods must also be guaranteed, and this is particularly important for essential goods such as food, including livestock" [3].

There are many agencies and organizations that are cooperating with the EU and its parts to control, report, and make suggestions for improving the conditions of animal welfare during animal transport, as follows:

FOUR PAWS – is the global animal welfare organisation for animals under direct human influence, which reveals suffering, rescues animals in need and protects them [22].

WOAH – World Organisation for Animal Health – provides technical support to Member Countries requesting assistance with animal disease control and eradication operations, including diseases transmissible to humans [23].

ECA – Court of Auditors – audits the accounts and oversees the implementation of the budgets of the institutions of the European Union [6].

Eurogroup for animals, Eurogroup for farming – As the pan-European animal protection organisation, the primary focus of Eurogroup for Animals is to work in the interest of animals towards a society in which they are valued and respected as sentient beings. Thanks to a united network, we protect animals by achieving better legislation, highest standards, enforcement and by driving societal change [13].

Council of Europe – It is the continent's leading human rights organisation. It includes 46 member states, 27 of which are members of the European Union [34].

HATS – Handling of animals at transport and slaughter: It is internationally recognized as one of the most forefront research centres in the area of animal welfare in connection with handling at transport and slaughter. They carry out research on Animal transport logistics; Herding and driving techniques; Overnight lairage; Stunning and stun quality; and Slaughterhouse work environment. Main focus is on farm animals. Members of the group serve as experts for the European Food Safety Authority (EFSA), national and international authorities, the industry and NGOs (non-governmental organisations). They have published many articles, books, chapters and held congress presentations. HATS also coordinates or participates in research projects [29].

CONCLUSIONS

The basic idea of all companies is to increase productivity and reduce costs with the goal of achieving the highest possible profits. The treatment of animals prior to slaughter has a significant impact on both the quality of the meat and its processing. Because of this, it is crucial to cut down on distance travelled and to do everything in your power to avoid having items in poor condition while being transported. Despite the greater distance, farmers choose to purchase their meat from cheaper slaughterhouses in order to profit.

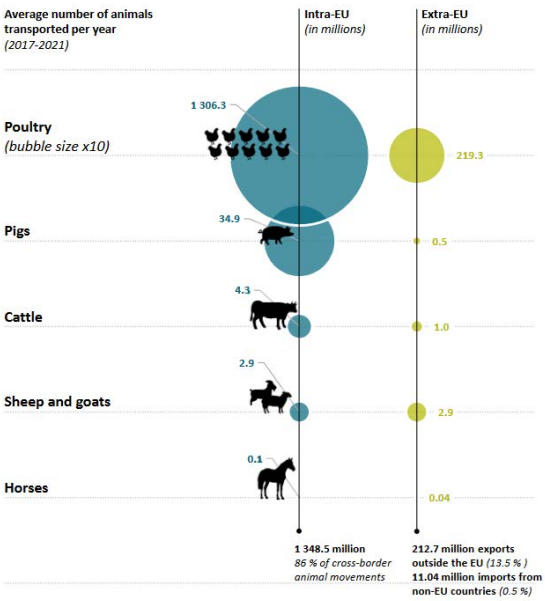

Fig. 2. Composition of live animal transport between member states and to or from non-EU countries in 2017–2021 (Data on domestic transport are not included in Comext) [2]
Source: ECA team, 2023.

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EVALUATION OF NUTRITION ACCORDING TO MILK METABOLITES AND COMPONENTS IN SEASONAL DEPENDENCE IN DAIRY COWS

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ABSTRACT

The objective of this study was to evaluate the nutrition according to the milk metabolites and components during the seasonal dependence of the dairy cows. In the summer time, it was confirmed that significantly lower content of milk protein and fat ($P < 0.001$) and lower milk, protein, and fat yields ($P < 0.001$) occurred, compared to the autumn period. The content of milk lactose was not statistically significant ($P > 0.5$). The evaluation of nutrition according to protein in milk and milk urea in the summer period was confirmed in the 2nd group of milk samples which represented 53 % of the group's higher content of milk urea, higher content of urinary nitrogen excretion, and a lower nitrogen utilization efficiency compared to the 1st group of milk samples. The autumn period was confirmed in the 2nd group of milk samples which represented 53 % of the group with higher milk yield and lower content of milk proteins and milk fat compared to the 1st group of milk samples. The evaluation of nutrition using the analysed content of milk urea and proteins in milk represents a suitable tool for evaluating the impact of malnutrition or overfeeding of energy and protein on milk production and other components.

Key words: Holstein dairy cow; milk urea; milk yield; season

INTRODUCTION

Dairy cow's milk contains 12–14 % dry matter composed of proteins (2.5–4 %), fat (3–5 %), and lactose (~5 %) [18]. To assess the balance between the intake of proteins and energy in the feed ration, the evaluation of the content of milk urea (MU) is used, which is part of the non-protein nitrogen in milk dairy cows [9]. According to M a r e n j a k et al. [14] the optimal concentration of MU in cow's milk is 10–30 $mg.dl^{-1}$. Climate change poses a major threat to the sustainability of livestock production and the preservation of the ecosystem [7]. Dairy cows optimally tolerate an environmental temperature in the range of 5–25 °C, which represents their thermoneutral zone, when they do not have an increased need for energy to create or get rid of body temperature [10]. A large amount of metabolic heat is generated in dairy cows during lactation and they also accumulate heat from radiant energy. As a result of the higher temperature of the environment and the limited cooling capacity, there is an increase in body temperature [30]. At a temperature above 25 °C, dairy cows enter heat

stress, when the compensation of the metabolic load due to heat stress affects feed intake with an impact on the quantity and quality of production, deterioration of reproduction, and the health status of dairy cows, together with increased energy consumption to regulate body temperature and its release from the body by skin and inhalation. The result is increased respiratory and heart rates, impacting feed intake, physiological and metabolic functions, reproduction, and the immune system [7, 12, 29].

The aim of our study was to evaluate the nutrition according to analysed milk urea and protein content in milk Holstein dairy cows during the seasonal dependence.

MATERIALS AND METHODS

Data collection

This study included 140–160 Holstein cows in the first lactation phase from a Slovakian dairy farm from January 2018 to December 2018. The cows were selected according to season into two groups: summer (June – August) and autumn (September – November). Feed rations of dairy cows were predominantly based on corn and alfalfa silage, supplemented with different carbohydrate feeds (cereal grains and cereal grain by-products) and protein supplements (soybean and rapeseed meal) provided as the TMR (total mixed ration) *ad libitum*. The nutritional composition of the TMR and daily feed intake were intensively monitored and the dairy cows were kept in a free-stall housing system.

Analysis of total mixed rations

The samples of the TMR were taken from the feed manager and were analysed once per month for dry matter (DM), which consisted of crude protein (CP), fat, acid and neutral detergent fibre (ADF, NDF), and starch analysed by conventional methods according to the Commission Regulation (EC) No. 691/2013 [5]. The DM was determined by weight upon drying the sample at 105 °C under the prescribed conditions. The CP content was determined by the Kjeldahl method using a 2300 Kjeltec Analyzer Unit (Foss Tecator AB, Sweden). The fat was determined by the device Det-Gras (JP SELECTA, Spain). The ADF and NDF were determined using a Dosi-Fiber Analyser (JP SELECTA, Spain), and the content of starch was determined polarimetrically. The net energy for lactation (NEL) and non-fibre carbohydrates (NFC) were calculated according to the nutrient requirements of dairy cattle [17].

Analysis of production parameters

The farm was evaluated by the Breeding Services of the Slovak Republic using the Breeding Information System [20]. The cows were milked twice a day and individual milk samples were analyzed once per month for protein, fat, lactose content, and MU (milk urea) by spectrophotometric tests in the near-infrared region using MilkoScan FT+ (Foss Electric, Denmark) and BENTLEY FTS (Bentley Instruments Inc., USA) in the Central analytical laboratory for milk with accreditation under registration number 096/5878/2015/2. The total number of analysed samples of milk in one month was 140–160. Daily yields of protein, fat, and lactose were calculated according to S t o o p et al. [26]. Conversion of MU to milk urea nitrogen (MUN) was calculated according to O u d a h [19]. The nitrogen utilization efficiency (NUE) was calculated according to H u h t a n e n et al. [8] and the urinary nitrogen excretion (UNE) according to K a u f f m a n and S t - P i e r r e [11].

Environmental conditions during the summer and autumn periods

The influence of climatic conditions by measuring air temperatures during the summer (June – August) and autumn periods (September – November) were evaluated according to daily measurements by a climatological station located 13 km from the farm. Maximum and minimum daily temperatures were recorded in a climatological booth at a standard height of 2 m above the ground for 24 hours. The maximum daily temperature was usually recorded 2 hours after the culmination using a mercury thermometer, and the minimum temperature usually before sunrise was recorded using an alcohol thermometer. Information on the average, maximum, and minimum temperatures during the day was provided by the Slovak Hydrometeorological Institute of the Slovak Republic [23].

Ethical considerations

In this study we used data obtained with the consent of the farm in collaboration with The Breeding Services of Slovakia, using the Breeding Information System.

Statistical analysis

The data analysis was carried out via the Graph Pad Prism 8.3 (GraphPad Software, San Diego, CA, USA). The results of each variable were expressed as the means and

standard deviations (SD). One-way analysis of variance (ANOVA) was used to evaluate the statistical significance with Tukey's multiple comparisons between summer and autumn groups with a significance level set at $P < 0.001$.

RESULTS

The mean concentration of nutrients in the TMR and the production parameters of dairy cows are listed in Table 1. The nutrient content of TMR in the summer and autumn periods fluctuated according to the recommendation for the first lactation phase by standardized nutrient requirements [17]. In the summer, the dry matter intake (DMI) was reduced (23.8 ± 0.4 kg.d^{-1}) with the feed residues in the feed trough at 5–10 % compared to the calculated DMI (25.1 ± 0.4 kg.d^{-1}).

Table 1. Nutritional composition of the TMR, production and composition of milk in the summer and autumn periods

	Summer period	Autumn period
Nutrients in TMR (% Dry Matter)		
CP	14.6 ± 0.4	15.4 ± 0.3
Starch	24.1 ± 0.7	25.5 ± 0.4
Fat	4.3 ± 0.2	4.1 ± 0.1
NDF	34.4 ± 0.3	33.9 ± 0.9
ADF	21.6 ± 0.5	20.2 ± 0.3
NFC	37.4 ± 0.1	38.7 ± 0.7
NEL	6.7 ± 0.2	6.7 ± 0.2
Milk production and composition		
Milk yield (kg.d^{-1})	36.00 ± 5.3***	42.20 ± 6.6
Protein (%)	2.70 ± 0.1***	3.40 ± 0.2***
Fat (%)	3.20 ± 0.5***	3.48 ± 0.4***
Lactose (%)	4.85 ± 0.1	4.85 ± 0.1
Protein yield (kg.d^{-1})	0.95 ± 0.2***	1.43 ± 0.2***
Fat yield (kg.d-1)	1.12 ± 0.3***	1.69 ± 0.3***
Lactose yield (kg.d^{-1})	1.70 ± 0.4	2.04 ± 0.4

CP – crude protein; NDF – neutral detergent fibre; ADF – acid detergent fibre; NFC – non-fibre carbohydrates; NEL – net energy of lactation; TMR – total mixed ration; ***P < 0.001

Climatic summaries in the summer and autumn periods are shown in Table 2. In the summer period, the average daily temperatures were 20.4 ± 3.0 °C, with an average maximum temperature of 33.5 °C and a minimum value of 14.2 °C. In the evaluated period, the daily temperature above 25 °C with an average of 29.5 ± 2.8 °C (maximum 35.5 °C) was confirmed on 64 days out of a total of 92, which means that 70 % of the days during the summer period were dairy cows exposed to heat stress. In the autumn period, the daily average, maximum, and minimum ambient temperatures were 9.3 ± 5.0 °C, respectively, 21.0 °C, and -1.5 °C without the influence of heat stress during this period.

Table 2. Climate summaries in the summer and autumn

	Period	
	Summer	Autumn
Temperature, maximum (°C)	35.5	21.0
Average temperature (°C)	20.4 ± 3.0	9.3 ± 5.0
Days temperatures > 25 °C	64	-

During the summer period, it was confirmed that significantly lower content of milk protein and fat ($P < 0.001$) and lower milk, protein, and fat yields ($P < 0.001$) compared to the autumn period. The content of milk lactose was not statistically significant ($P > 0.5$).

The graphic representation proposed by S p o h r and W i e s n e r [24] is successfully used to diagnose of nutrition dairy cows according to the analysed content of protein and urea in the milk. Graphic representation of protein and urea content when inserting individual data of analysed protein samples (%) on the Y axis and MU (mg.dl^{-1}) on the X axis evaluates the breakdown of the analysed set of samples per group of dairy cows into 9 evaluated areas according to the reference, reduced or increased analysed urea and protein in milk.

The evaluation of nutrition according to protein in milk and MU in summer is presented in Fig. 1 and in Table 3. The 1st group consisted of milk samples with 15–30 mg.dl^{-1} MU and < 3.2 % proteins (represented 36.0 % of the group) and the 2nd group consisted of milk samples with MU > 30 mg.dl^{-1} and proteins < 3.2 % in milk (represented 53.0 % of the group). Milk production in both groups was very close with reduced protein content and fat in milk. In the 2nd group of milk samples were confirmed higher content of MU, higher content of UNE, and a lower NUE compared to the 1st group of the milk samples.

The evaluation of nutrition according to protein in milk and MU in the autumn period is presented in Fig. 2 and in Table 4. The 1st group consisted of milk samples with MU 15–30 mg.dl^{-1} and optimal proteins 3.2–3.8 % (represented 34 % of the group) and the 2nd group consisted of milk samples with MU 15–30 mg.dl^{-1} and low

proteins (< 3.2 %) in the milk (represented 53.0 % of the group). The 2nd group of milk samples confirmed higher milk yield and lower content of milk proteins and milk fat compared to the 1st group of the milk samples.

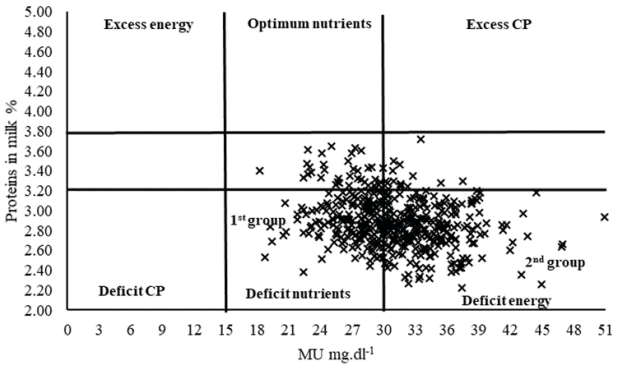

Fig. 1. Relationship of protein in milk and milk urea (MU) in the summer period

Table 3. Evaluation of milk in the summer period according to protein and urea in milk

	1st group Deficit nutrients	2nd group Deficit energy
	MU 15–30 mg.dl^{-1}	MU > 30 mg.dl^{-1}
	Proteins < 3.2 %	Proteins < 3.2 %
% of the group	36.0	53.0
Milk yield (kg.d^{-1})	39.5 ± 5.5	39.8 ± 7.3
Protein (%)	2.87 ± 0.2	2.80 ± 0.2
Fat (%)	3.12 ± 0.5	3.21 ± 0.8
Protein yield (kg.d^{-1})	1.13 ± 0.2	1.11 ± 0.2
Fat yield (kg.d^{-1})	1.23 ± 0.2	1.28 ± 0.3
MU (mg.dl^{-1})	27.00 ± 2.5	34.60 ± 4.0
UNE (%)	35.6	45.6
NUE (%)	31.5	30.6

MU – milk urea; UNE – urinary nitrogen excretion; NUE – nitrogen utilization efficiency

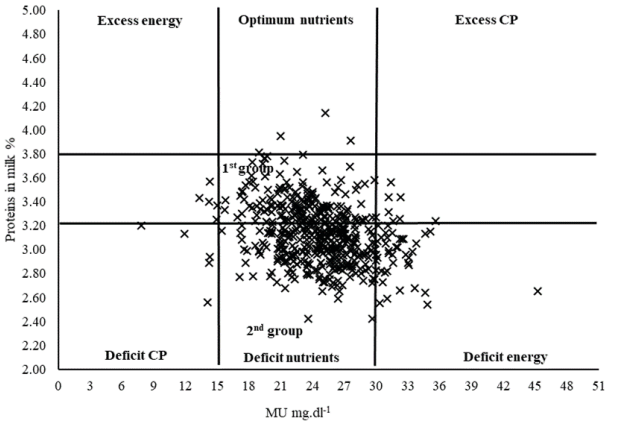

Fig. 2. Relationship of protein in milk and milk urea (MU) in the autumn period

Table 4. Evaluation of milk in the autumn period according to protein and urea in milk

	1st group Optimum nutrients	2nd group Deficit nutrients
	MU 15–30 mg.dl^{-1} Proteins 3.2-3.8 %	MU 15–30 mg.dl^{-1} Proteins < 3.2 %
% of the group	34.0	53.0
Milk yield (kg.d^{-1})	39.8 ± 6.9	42.6± 6.4
Protein (%)	3.38 ± 0.1	2.98± 0.1
Fat (%)	3.56 ± 0.6	3.28 ± 0.6
Protein yield (kg.d^{-1})	1.34 ± 0.2	1.27 ± 0.2
Fat yield (kg.d^{-1})	1.41 ± 0.3	1.40 ± 0.3
MU (mg.dl^{-1})	23.04 ± 3.2	24.65 ± 3.0
UNE (%)	29.0	31.0
NUE (%)	32.1	32.4

MU – milk urea; UNE – urinary nitrogen excretion; NUE – nitrogen utilization efficiency

DISCUSSION

In the summer, the influence of heat stress was confirmed by finding a high number of days with measured temperatures above 25 °C and with an impact on the decrease in DMI (5–10 % increase of residues on the feed trough compared to the calculated DMI).

The evaluation of production parameters (Table 1) confirmed reduced milk yield and synthesis of milk components with a decrease in fat and protein yield in the milk. Our conclusions agree with L i u et al. [13] who confirmed the effect of heat stress with a subsequent decrease in feed intake on reduced milk production and components. However, heat stress can also affect milk production directly through mechanisms that are independent of reduced DMI. The decrease in feed intake only contributed to approximately 35 % of the heat stress-induced decrease in milk production [21]. Rather, other feed intake-independent alterations induced by heat stress, particularly those related to nutrient partitioning, may be associated with changes in post-absorptive glucose and lipid metabolism changes [31]. The main precursor for the synthesis of lactose in the mammary gland is glucose, and the production of lactose is the main osmoregulator and therefore affects the production. During heat stress, the body tries to generate less metabolic heat (especially the skeletal muscles) and uses glucose to an increased extent. Subsequently, there is an insufficient supply of glucose to the mammary gland with a decrease in milk production [1]. Also, as a result of

the redirection of blood flow to peripheral tissues for heat removal, the supply of substrates to the mammary gland is limited [30].

The lactose content in the milk did not change due to heat stress, which agrees with the conclusions of Zheng et al. [34] because milk sugar is the most stable component of milk.

Heat stress reduces rumen motility, which affects the passage of digested in the gastrointestinal tract and reduces the production of volatile fatty acids [10, 15, 16, 32]. Heat stress with the impact of a decrease in feed intake (decreased roughage and increased concentrate) increases lactate production and decreases rumen acetate production with a decrease in rumen pH and milk fat [27, 33].

The confirmed reduced milk protein content during heat stress agrees with previous results [10, 22] when heat stress (rather than reduced amino acid intake) affects the synthesis process itself, especially α- and β-casein synthesis [2].

By evaluating the nutrition of dairy cows according to the analysed content of protein and MU using a graphic representation (Fig.1, Fig. 2), it points to the impact of disruption of the supply of nutrients and energy from feed ration on the parameters of protein metabolism to influence the components of milk. In the summer season, the feed ration was formed to produce 39.6 ± 1.1 kg.day^{-1} with a calculated protein content of 3.2 % and a fat content of 3.6 %. The production values in the divided groups (Table 3) confirm the same milk production as with the calculated feed ration, which was close in both groups but with a significantly reduced proportion of protein and fat in the milk. There was a significant difference in the composition of milk in terms of MU and the estimated daily amount of UNE and NUE. The Impact of heat stress on protein metabolism and amino acid balance such that the absorptive function of the rumen epithelium along with the amount of urea reabsorbed from the blood, causes urea to accumulate in the blood and transport to milk [4]. The confirmed higher content of MU in the second group points to the metabolic stimulation of body reserves in dairy cows due to thermal stress, when amino acids released by proteolysis from skeletal muscle are used for the synthesis of urea, which passes from the blood into the milk after deamination in the liver [3, 6]. The urea is filtered from the blood by the kidney and is excreted from the body in urine. Blood enters the kidneys through the renal artery and is filtered through the nephrons. This process concentrates the urea for excretion in the urine. Therefore, urea excretion is proportional to blood urea concentration. Because it is a small molecule, urea readily diffuses across cellular membranes. As milk is secreted in the mammary gland, urea diffuses into and out of the mammary gland, equilibrating with urea in the blood. Because of this process, MU equilibrates with and is proportional to blood urea. This process allows MU to be an excellent predictor of urea in urine. According to Huhtanen et al. [8] increases in MU were associated with a decrease in NUE.

In the autumn period, the feed ration was formed to produce 38.9 ± 1.7 kg.d^{-1} with a calculated protein content of 3.2 % and a fat content of 3.6 %. The production values in the divided groups (Table 4) confirm the reduced content of protein (2.98 ± 0.1 %) and fat (3.28 ± 0.6 %) in the 2nd group, which represented 53.0 % of the group with increased milk yield (42.6 ± 6.4 kg.d^{-1}) compared to the estimated daily production for the calculation of feed ration. The limited supply of nutrients and increased daily production of milk for the 1st stage of lactation in accordance with the genetic potential of the herd will not cover the needs for the synthesis of milk components (protein and fat), where the reduced supply of amino genic and lipogenic nutrients causes a decrease in milk components. The recommended procedure for feeding dairy cows with high genetic potential requires, when calculating the feed ration, to calculate the norm of the need for nutrients according to weight, production, and components in milk. The milk yield for individual dairy cows in the group according to the current milk yield for the 83rd percentile and according to the average production using the recalculation of the production stimulating factor (PSF) [25, 28].

CONCLUSIONS

The results from this study show a statistically significant ($P < 0.001$) impact of the season on milk yield and milk components. Our results confirmed that nutritional evaluation using the analysed milk urea and protein content in milk represents a suitable tool for evaluating the impact of malnutrition or overfeeding of dairy cows on farms. The assessment of milk urea, urinary nitrogen excretion, and nitrogen utilization efficiency is a useful

tool for the environment as well. Changing technologies through milk urea analysis, farms help to reduce the release of N into the environment.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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OCCURRENCE, ACTIVITY AND CONTROL OPTIONS AGAINST BITING MIDGES (DIPTERA: *CULICOIDES*) IN HORSES

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ABSTRACT

Biting midges are insects which annoy horses as they cause discomfort and by the sucking of blood, they cause painful lesions that, in certain cases, can trigger acute allergic reactions. They also act as potential vectors of many pathogens of bacterial, viral and parasitic origin. The aim of this study was to investigate seasonal dynamics, abundance and species composition of biting midges, and implementing protection options at the Equestrian centre of the University of Veterinary Medicine and Pharmacy (UVMP) in Košice in the years 2021 and 2022. During this time period (of 2021 and 2022), we captured 4797 biting midges. Our results showed that in 2021 the most frequently captured species were *C. obsoletus/C. scoticus* (prevalence 86.6 %), followed by *C. punctatus* and *C. pulicaris* (prevalence 5.37 % and 3.86 %, resp.). In addition, *C. lupicaris*, *C. newsteadi*, *C. furcillatus*, *C. festivipennis*, *C. slovacus*, *C. tauricus*, *C. clastrieri*, *C. dewulfi*, *C. fagineus* and *C. circumscriptus* were identified as the minority species. In this year, the highest abundance of these insects (1758 biting midges) was observed at the end of June. In 2022, we detected a clear dominance of *C. obsoletus/C. scoticus* (prevalence 97.0 %) and the highest number of biting midges (655) was captured in mid-May). In this year, the minority species included, *C. pulicaris*, *C. punctatus* and *C. lupicaris*. The identification of the host blood showed that man *(Homo sapiens)* was the dominant host, namely of 80 % of the examined biting midges of species *C. obsoletus/C. scoticus*, *C. dewulfi*, *C. punctatus* and *C. festivipennis*. In one female of species *C. fagineus* we identified the host blood from horses *(Equus caballusi)*. In 2022, we detected the blood of a domestic rabbit *(Oryctolagus cuniculus)* in the species of *C. obsoletus/C. scoticus*.

Key words: *Culicoides*; host preference; prevalence; species composition

INTRODUCTION

Biting midges are tiny, blood-sucking insects that annoy not only animals, but also humans. Raids of their large swarms irritate people involved in outdoor activities, affect productivity and work usability of farm and pet animals and induce various forms of hypersensitive responses, particularly in horses. Equine Insect Bite Hypersensitivity (IBH) is the most frequent allergic skin disease in horses

[17] manifested as chronic recurrent seasonal allergic dermatitis. The sensitive reaction of horses is the response to proteins found in the saliva of the biting midges that are considered strong allergens. The animals react to them by increased production of serum IgE antibodies as a result of hypersensitive type I and IV reactions [20]. This involves a hereditary disease that appears usually in the second and third year of life and tends to grow worse with the increasing age of the affected individuals. It was confirmed that particularly the saliva of *Culicoides pulicaris* [21] is considered the causative agent of the above mentioned allergic dermatitis although other biting midge species parasitise on horses, such as *C. obsoletus/C. scoticus*, *C. lupicaris*, *C. punctatus* and other [19]. Horses affected by IBH show seasonal manifestations with gradual progression from spring to autumn when the *Culicoides* are active, and usually regress during the winter period, however, clinical signs can persist in winter in serious chronical cases. The initial clinical signs can be characterised as numerous papillae, hyperaesthesia and sensitisation of skin with intense pruritus resulting in local loss of hair and excoriations evident along the dorsal medial line, at the basis of the mane and tail [4] and, occasionally, beside ears [11]. However, the most important reason for permanent studies of biting midges, their species composition, occurrence and activity is their ability to transmit pathogens that induce many diseases included in the list of diseases defined by the World Organisation for Animal Health (WOAH) that result in serious economic losses related to disturbances of the health state, emergency measures and restrictions concerning the international trade of animals and animal products [22].

The aim of this study was to investigate the seasonal dynamics, abundance, species composition of biting midges and the potential prevention options that are comprehensively affected by many biotic and abiotic factors. The study was carried out at the Equestrian centre of the University of Veterinary Medicine and Pharmacy (UVMP) in Košice in the years 2021 and 2022, where equine patients with signs of IBH occurred recently.

MATERIALS AND METHODS

The entomological investigations were carried out in the period of May to October of the years 2021 and 2022 in the Equestrian centre of UVMP in Košice. This is a specialised facility of the University of Veterinary Medicine and Pharmacy in Košice with the capacity of housing approximately 60 horses. In addition to the housing area, there is available also an indoor riding arena with special surface, covered and outdoor sand riding area, as well as grass runs for horses, grass parkour and social facilities for personnel.

Capture of the biting midges

Biting midges were collected using miniature light traps CDC 1212 (John W. Hock, USA) with UV light that were situated at two sites, inside the house for horses and outside at the runs. The light was turned on at dusk (between 6:00 and 7:00 p.m.) when the activity of biting midges gradually increases. The catch was collected early in the morning (between 6:00 and 7: 00 a.m) on the following day.

Storage, processing and conservation of samples

In the laboratory, the biting midges were separated from other insects, rinsed in 50 % ethanol, conserved in the vessel containing 70 % ethanol and placed in refrigerated apparatus prepared for determination of species.

Morphological diagnosis of biting midges

Using a binocular magnifying lens ZEISS-STEVI DV-4 and an entomological tweezers we sorted the biting midges on a Petri dish into basic complexes and determined the species on the basic typical morphological characteristics according to the morphological key [14]. Subsequently, the females were divided to four groups according to their physiological state: nulliparous (female that never sucked blood – has empty abdomen); parous (female that sucked blood – blood fragments in abdomen are visible under binocular lens); engorged (visibly filled-up abdomen that acquires a more oval shape); gravid (engorged abdomen full of eggs) [9]. Such separation of females is necessary as for detection of host blood and pathogens the biting midges with fresh blood in the abdomen are used.

PCR species diagnosis of biting midges and the host blood

In the cases of morphological similarity of species, we used a molecular diagnostic method for their identification. Genomic DNA was isolated employing the commercial kit Dneasy® Blood and Tissue Kit (QIAGEN, GmbH.,

Hilden, Germany). It was isolated from the female that previously sucked blood (engorged) according to the protocol in the information leaflet of the kit manufacturer. DNA samples were stored in a freezer at -20 °C. The concentration of the obtained DNA was measured by means of a Qubit 4 Fluorometer (Thermo Fisher Scientific, USA) and diluted to the final concentration of 20–50 ng.μl^{-1}.

Fragment of mitochondrial gene of cytochrome b (cyt b) of size approximately 350 bp was used to identify the host blood. The total volume of each sample destined for PCR analysis reached 47 µl and contained the following: 21 µl Mastermix Emerald AmpGT, 21 µl nuclease free water, 1 µl primer cyt bb1, 1 µl primer cyt bb2 a 3 µl DNA. The PCR reaction took place in Mastercycler nexus X2 (Eppendorf, Germany), using universal primers for vertebrates, namely forward primer cyt bb1 (5´-CCA TCM AAC ATY TCA DCA TGA AA-3´) and reverse one cyt bb2 (5´-GCH CCT CAG AAT GAY) ATT TGK CCT CA-3´). The amplification proces s consisted of the initial denaturation step at 94 °C for 5 min, followed by 35x (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) and extension at 72 °C for 7 min [18]. Altogether 24 samples were subjected to isolation and PCR analysis for identification of host blood.

Visualisation, purification and sequencing of PCR products for identification of biting midge species, diagnosis of host blood and pathogen species are identical. Electrophoresis was performed in agarose gel (1 %) stained with GoodView TM Nucleic Acid Stain into which we loaded 5 µl of the PCR product and visualisation took place under UV light. The positive PCR products were sent to a qualified workplace in the Czech Republic for purification and sequencing. The sequencing was performed in both directions with the same primers as those used for PCR analysis.

The result of sequencing is a symbolic linear code referred to as the section that represents arrangement of the sequenced molecule. Immediately after analysis of chromatograms by means of software MEGA X [12], we conducted nucleotide sequence alignment by means of software GeneTool Lite 1.0 (BioTools Inc., California, USA).

RESULTS

Altogether, we captured 4797 biting midges. The most numerous were the species *C. obsoletus/C. scoticus* with a prevalence of 86.6 % in 2021 and 97 % in 2022. Minority species included *C. punctatus*, *C. pulicaris*, *C. lupicaris*, *C. newsteadi*, *C. furcillatus*, *C. festivipennis*, *C. slovacus/C. tauricus*, *C. clastrieri*, *C. dewulfi*, *C. fagineus* and *C. circumscriptus* (Table 1). In 2021, the highest abundance of biting midges was recorded at the end of June (1758) (Fig. 1), while in 2022, the highest number of them were captured in mid-May (655) (Fig. 2).

Table 1. Species composition of biting midges captured in the Equestrian centre of UVMP in 2021–2022

Species	2021	2022	Total	Prevalence 2021	Prevalence 2022
C. obsoletus/ C. scoticus	3116	1165	4281	86.6 %	97.0 %
C. lupicaris	28	1	29	0.78 %	0.08 %
C. newsteadi	25	0	25	0.70 %	0.00 %
C. pulicaris	139	22	161	3.86 %	0.02 %
C. punctatus	193	12	205	5.37 %	0.01 %
C. furcillatus	61	0	61	1.70 %	0.00 %
C. festivipennis	3	0	3	0.08 %	0.00 %
C. clastieri	3	0	3	0.08 %	0.00 %
C. dewulfi	1	0	1	0.03 %	0.00 %
C. tauricus/ C. slovacus	21	0	21	0.58 %	0.00 %
C. fagineus	2	0	2	0.06 %	0.00 %
C. circumscriptus	1	0	1	0.03 %	0.00 %
Culicoides spp.	4	0	4	0.11 %	0.00 %
Total	3597	1200	4797	100 %	100 %

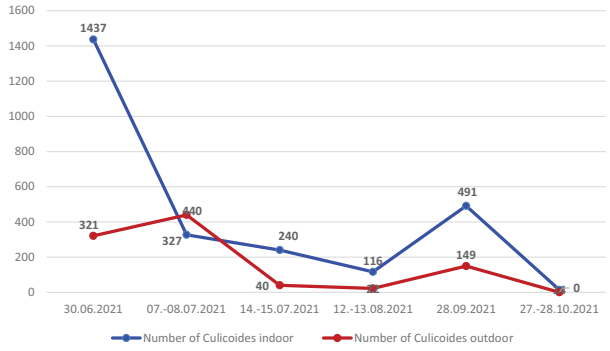

Fig. 1. The seasonal dynamics of *Culicoides* in the Equestrian centre of UVMP in 2021

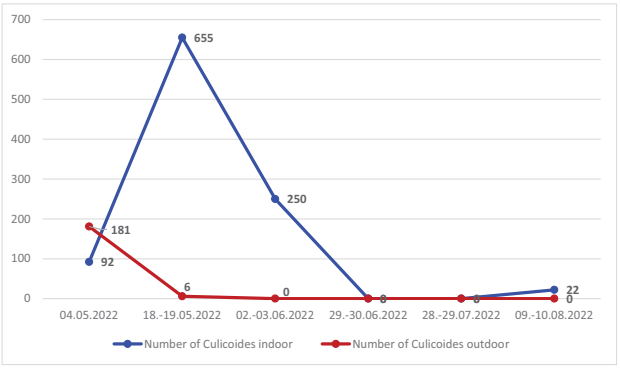

Fig. 2. The seasonal dynamics of *Culicoides* in the Equestrian centre of UVMP in 2022

Table 2. Identification of host blood in the captured biting midges

Species	Host	Sequence of cytb host gene
C. fagineus	*Equus caballus*	catgatgaaacttcggctccctcctaggaatctgcctaatcctccaaatcttaacaggcctattcctagc catacactacacatcagatacgacaactgccttctcatccgtcactcacatctgccgagacgttaactac ggatgaattattcgctacctccatgccaacggagcatcaatatttttatctgcctcttcattcacgtag gacgcggcctctactacggctcttacacattcctagagacatgaaacattggaatcatcctacttttcac agttatagctacagcattcatgggctatgtcctaccatgaggccaaatatc
C. obsoletus/C. scoticus	Homo sapiens	tcagcatgatgaaacttcggctcactccttggcgcctgcctgatcctccaaatcaccacaggactattcc tagccatgcactactcaccagacgcctcaaccgcctttcatcaatcgcccacatcactcgagacgtaaa ttatggctgaatcatccgctaccttcacgccaatggcgcctcaatattctttatctgcctcttcctacac atcgggcgaggcctatattacggatcatttctctactcagaaacctgaaacatcggcattatcctcctgc ttgcaactatagcaacaggcttcataggctatgtcctcccgtgagg
C. dewulfi	Homo sapiens	tcagcatgatgaaacttcggctcactccttggcgcctgcctgatcctccaaatcaccacaggactattcc tagccatgcactactcaccagacgcctcaaccgcctttcatcaatcgcccacatcactcgagacgtaaa ttatggatgaatcatccgctaccttcacgccaatggcgcctcaatattctttatctgcctcttcctacac atcgggcgaggcctatattacggatcatttctctactcagaaacctgaaacatcggaattatcctcctgc ttgcaactatagcaacagccttcataggctatgtcctcccgtgagg
C. punctatus	*Homo sapiens*	tcagcatgatgaaacttcggctcactccttggcgcctgcctgatcctccaaatcaccacaggactattcc tagccatgcactactcaccagacgcctcaaccgcctttcatcaatcgcccacatcactcgagacgtaaa ttatggctgaatcatccgctaccttcacgccaatggcgcctcaatattctttatctgcctcttcctacac atcgggcgaggcctatattacggatcatttctctactcagaaacctgaaacatcggcattatcctcctgc ttgcaactatagcaacagccttcataggctatgtcctcccgtgagg
C. punctatus	*Homo sapiens*	tcagcatgatgaaacttcggctcactccttggcgcctgcctgatcctccaaatcaccacaggactattcc tagccatgcactactcaccagacgcctcaaccgcctttcatcaatcgcccacatcactcgagacgtaaa ttatggctgaatcatccgctaccttcacgccaatggcgcctcaatattctttatctgcctcttcctacac atcgggcgaggcctatattacggatcatttctctactcagaaacctgaaacatcggcattatcctcctgc ttgcaactatagcaacagccttcataggctatgtcctcccgtgagg
C. festivipennis	*Homo sapiens*	tcagcatgatgaaacttcggctcactccttggcgcctgcctgatcctccaaatcaccacaggactattcc tagccatgcactactcaccagacgcctcaaccgcctttcatcaatcgcccacatcactcgagacgtaaa ttatggctgaatcatccgctaccttcacgccaatggcgcctcaatattctttatctgcctcttcctacac atcgggcgaggcctatattacggatcatttctctactcagaaacctgaaacatcggcattatcctcctgc ttgcaactatagcaacagccttcataggctatgtcctcccgtgagg
C. obsoletus /C. scoticus	*Homo sapiens*	tcagcatgatgaaacttcggctcactccttggcgcctgcctgatcctccaaatcaccacaggactat-tcctagccatgcactactcaccagacgcctcaaccgcctttcatcaatcgcccacatcactcga-gacgtaaattatggctgaatcatccgctaccttcacgccaatggcgcctcaatattctttatctgcctct-tcctacacatcgggcgaggcctatattacggatcatttctctactcagaaacctgaaacatcggcat-tatcctcctgcttgcaactatagcaacagccttcataggctatgtcctcccgtgagg
C. tauricus /C. slovacus	*Homo sapiens*	tcagcatgatgaaacttcggctcactccttggcgcctgcctgatcctccaaatcaccacaggactattcc tagccatgcactactcaccagacgcctcaaccgcctttcatcaatcgcccacatcactcgagacgtaaa ttatggctgaatcatccgctaccttcacgccaatggcgcctcaatattctttatctgcctcttcctacac atcgggcgaggcctatattacggatcatttctctactcagaaacctgaaacatcggcattatcctcctgc ttgcaactatagcaacagccttcataggctatgtcctcccgtgagg
C. obsoletus /C. scoticus	*Homo sapiens*	catgatgaaacttcggctcactccttggcgcctgcctgatcctccaaatcaccacaggactattcctagc catgcactactcaccagacgcctcaaccgccttttcatcaatcgcccacatcactcgagacgtaaattat ggctgaatcatccgctaccttcacgccaatggcgcctcaatattctttatctgcctcttcctacacatcg ggcgaggcctatattacggatcatttctctactcagaaacctgaaacatcggcattatcctcctgcttgc aactatagcaacagccttcataggctatgtcctcccgtgagg
C. obsoletus /C. scoticus	*Oryctolagus cuniculus*	tgatgaaactttggctctctactaggcctgtgccttataattcaaattttcactggcctattcttagcca tacactacacctctgacacaacaacagcattctcatcagtaacccatatttgccgagatgttaactatgg ctgacttatccgatacctccacgctaacggagcatctatattctttatttgcctctacatacacgtaggc cgcggaatctactatggatcatacacatacctagagacctgaaacattggcatcatcctcctattcgcag taatagccacagcatttatgggttatgtcctcccatgagg

Identification of host blood in biting midges in our study showed that the dominant host of these insects were humans (*Homo sapiens*), namely in as many as 80 % of the investigated biting midges, specifically in *C. obsoletus/C. scoticus*, *C. dewulfi*, *C. punctatus* and *C. festivipennis*. In a female of *C. fagineus*, we identified blood from a horse *(Equus caballusi)*. In 2022 we identified blood of a domestic rabbit *(Oryctolagus cuniculus)* in *C. obsoletus/C. scoticus* (Table 2).

DISCUSSION

In Slovakia, biting midges show a pronounced seasonal activity. Our study was conducted in the years 2021 to 2022, when we captured altogether 4797 biting midges of 14 species, the dominant species being *C. obsoletus/C. scoticus* with prevalence of 86.6 % in 2021 and 97 % in 2022. The total number captured in 2021 was 3597, most of them (73 %) captured inside in the house for horses. In 2022, the catch totalled 1200 biting midges and again most of them (85 %) in the interior. Interesting were the differences in abundance and species composition determined in the same place in the period of 2018 to 2020 [10] and the results obtained in this study. In the years 2018 to 2020 there were captured as many as 35 463 biting midges of 17 species. The highest abundance and species diversity was observed in 2019. Of the minority species there were detected *C. bysta*, *C. picturatus*, *C. clastieri*, *C. dewulfi* and *C. deltus* [10]. The differences in an abundance and species composition were probably related to the construction changes in the past two years. Both the internal and external facilities underwent reconstruction that resulted in the elimination of biotopes suitable for propagation of larvae of biting midges requiring an abundance of decomposing organic matter and humid environment. The highest abundance of biting midges was recorded in species of subgenus *Avaritia* that includes biting midges *C. obsoletus*, *C. scoticus* and *C. dewulfi*. These species also show morphological similarity. According to Z i t r a et al. [24] morphological diagnosis of their females is impossible, thus it is necessary to use molecular methods for better identification of the species. The mentioned species are the main viral vectors in the palearctic realm and at the same time belong among the most numerous in Europe [3]. Their high prevalence was recorded also in Germany [2], Ireland [7], the Netherlands, Italy and Sweden [15].

Identification of host blood is inevitable for the control of spreading of new species of biting midges and the associated transmission of pathogens. Sucking blood of host by the insect depends on its ability to remain active when the suitable hosts are available. Blood of the host parasitized by the vector affects its intestinal microbiota. During blood sucking the microbicidal effector molecules such as antimicrobial peptides are expressed [16]. Last but not least, the blood is a source of proteins necessary for the females before laying the eggs. Some studies implied that the selection of a suitable host for biting midges *Culicoides* is limited by the availability of hosts in their surroundings [23]. On the other hand, anthropogenic activities provide suitable opportunities for the transmission of novel pathogens among people. In the study by C a l v o at al. [5], conducted in the National park Picos de Europa in northern Spain, the scientists focused on identification of host blood in biting midges and ascertained that 95.8 % of host blood originated from mammals and 6.00 % from the domestic fowl *(Gallus gallus)*. The most frequent hosts were sheep (87.7 %), people (6.5 %), cattle (3.7 %) and small rodents *Microtus savii* (2.1 %). This study indicated that ruminants were the preferred source of blood subsistence for *Culicoides* in the investigated territory. However, there were observed many situations in which the biting midges exhibited opportunistic behaviour related to the abundance of specific potential hosts as the sources of blood. In the case of low abundance of the preferred hosts, the biting midges are capable of adapting. This can explain the fact that in 80 % of samples examined in this study, we detected in human blood, namely in those from *C. obsoletus/C. scoticus*, *C. dewulfi*, *C. punctatus*, *C. Taurica's/C. slovacus* and *C. festiveness*. In only one sample obtained from *C. fagineus*, we identified horse *(Equus caballus)* as the source of blood. Rabbit blood *(Oryctolagus cuniculus)* was detected in *C. obsoletus/C. scoticus*.

The above statements indicates that the necessity of protecting people and other animals from midge baits. From the entomological aspect, we could suggest the use of insecticide or repellents to protect susceptible animals from bites, however, one study conducted in Switzerland showed that the common insecticides and repellents failed to produce the required effect [13]. With respect to protection and well-being of animals, they should be kept in housings protected against insects (e.g. by treatment of windows and doors by contact insecticides) and regular use

of light traps. Regrettably, this provokes some criticism as some of such measures appear to be impractical, expensive and financially infeasible and have none or only minimal effect. For people temporarily exposed to biting midges the use of repellents appears to be a suitable measure of protection against insect bite; N,N-dietyl-m-toluamid (DEET) is considered the gold standard for this purpose [8]. With respect to protection of animals, C a r p e n t e r et al. [6] recommended to disrupt the development of biting midges by elimination of suitable biotopes for larvae which was supported also by our study that showed improvement after reconstruction of the Equestrian centre. In horses it is possible to use allergen-specific immunotherapy (allergen-SIT), consisting of subcutaneous administration of an allergen extract from biting midges. Although the mechanism by which the allergen-SIT mediates its anti-inflammatory effects has not yet been completely explained, various studies revealed that allergen-SIT affects the response of APC, cells, T cells, B cells and effector cells [1]. Allergen-SIT results in early induction of Treg buniek supressing Th2-Th1 cells and other effector cells which can result in early protection against midge baits.

CONCLUSIONS

There are many reasons that justify the need for entomological monitoring of species composition, abundance, seasonal activity and host preference of *Culicoides* species. Global changes (anthropogenic, climatic and social) that occurred in the past two decades resulted in spreading and emergence of new serious diseases of various aetiology. Biting midges are also involved in transmission of their causative agents. The methods of molecular biology allowed us to determine the host activity of biting midges in the Equestrian centre of the UVMP in Košice and to characterise biological specialties of parasite-host relationships that are affected by local adaptation to the available hosts as well as the environmental factors.

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OCCURRENCE OF TET GENE-BEARING ANTIMICROBIAL-RESISTANT *ESCHERICHIA COLI* FROM DAIRY FARMS IN NIGERIA

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ABSTRACT

This study focused on antibiotic resistance genes as emerging contaminants with potential global human health implications. Intensive livestock farming has been identified as a major contributor to the spread of resistant bacteria and genes. The study examined antimicrobial-resistant *Escherichia coli* and tetracycline-resistant genes in raw milk from commercial dairy farms in Kano State. Out of 300 registered farms, 54 (18 %) were purposively sampled for the study. A total of 313 milk samples were collected and processed through enrichment and inoculation on selective media for *Escherichia coli* isolation. The antibiogram pattern of the isolated *Escherichia coli* strains was assessed using the disk diffusion method. The results revealed resistance to various antimicrobial agents, with no resistance to quinolones but high resistance to ampicillin (100 %), erythromycin (73.3 %), and tetracycline (46.7 %), among others. The multiplex polymerase chain reaction was conducted on all *Escherichia coli* isolates to detect tet genes (tet A, B, C, D, and M), and one isolate carried the tet M resistance gene, while six (40 %) others carried the tet A resistance gene. The study concludes that a significant proportion of the cultured *Escherichia coli* strains were resistant to one or more tested antibiotics, indicating a potential public health threat associated with *Escherichia coli* contamination in raw milk. We recommend implementing robust regulatory policies governing the use and sales of antimicrobials in animal production. Furthermore, we suggest further investigation into other resistant genes that these isolates might carry to better understand the extent of antibiotic resistance in the region.

Key words: antimicrobial resistance; dairy cows; *Escherichia coli*; raw milk

INTRODUCTION

Nigeria ranks amongst the fastest-growing populations in the world, with estimates indicating that the country's population will be above 350 million by the year 2050 [15]. Currently, the country is the most populous in Africa [30]. Meeting the food demand of such a large population requires accelerating food production including intensification of both arable and food animal production [7]. Intensive livestock and fish production favour the highest level of use of chemical and veterinary drugs to combat

and prevent diseases, improve factors of production, and in the management of stress [2]. Several studies have shown a close association between intensive veterinary drug use and the emergence and spread of antimicrobial-resistant pathogens and the occurrence of their residues in edible animal products [13]. Antimicrobial Resistance (AMR) is a major global health challenge of the 21st century [3]. Antibiotic resistance genes (ARGs) are emerging contaminants posing a potential worldwide human health risk [31]. Intensive livestock farming has been identified as a major factor in the dissemination of resistant bacteria and antimicrobial resistance genes (ARGs) [8].

Many interventions in the livestock and food production sector in developing countries like Nigeria sought to transform smallholder operations into intensive ventures [18]. One of the intervention programs that were able to boost local productivity and farmer's capacity, is the Federal Government of Nigeria Commercial Dairy Project, evidently increasing the output of milk and milk products in regions where the program operates [12]. The operations programs were characterized by inappropriate and indiscriminate use of veterinary drugs, in addition to poor drug use regulations and control in the country [10]. These factors are well-recognized enablers of the emergence and spread of resistant pathogens and the dissemination of ARGs coding for resistance amongst animals, potentially zoonotic, and environmental bacteria [28]. The contributions of these enhanced dairy production initiatives in the spread of resistant pathogens and ARGs in Nigeria have not been studied [29]. However, local literature abounds with reports of contamination of milk and milk products with pathogenic and often zoonotic [25]. Milk and milk products can be contaminated with antimicrobial-resistant bacteria or resistant genes from the environment, and during processing, handling, transportation, and storage [20].

The occurrence of antimicrobial-resistant bacteria and antimicrobial-resistant genes in milk compromises the quality of the product, a situation that will affect the marketability of the product and its wholesomeness [32]. The incidence of antimicrobial-resistant bacteria and ARGs in the dairy supply chain is of great concern [6]. This will defeat the aim of the intervention given that infection with the resistant pathogen is bound to increase the cost of production and can make it difficult for the farmers to repay their loans and access further grants, in addition to other socio-economic costs.

Recently in 2019, the country began implementing a National Action Plan against AMR modelled along the recommendations of the Global Action Plan that offers a One Health platform for combating AMR in human and animal health. Sustained surveillance at all fronts of livestock, poultry, and fish production is essential for detecting the emergence and trend of resistance in priority zoonotic pathogens. Most of the information on AMR from animal food production systems is from poultry production, and a limited number of reports [1, 23] described the occurrence of important reservoirs of antibiotic-resistant genes in Nigerian poultry production environments. They have also provided evidence of the spread of such genes from poultry farms to human populations via manure and water [21]. This study provides insight into the problem of antimicrobial resistance to *Escherichia coli* in dairy production in one of the major milk-producing regions of Nigeria [5].

MATERIALS AND METHODS

Sampling procedure/sample collection

This was a cross-sectional study and purposive sampling was conducted. Three hundred commercial dairy farms were registered under the commercial dairy project by the Kano State Agricultural and Rural Development Authority (KNARDA). Eighteen percent (18 %) of the total dairy-registered farms were sampled giving a total of fifty-four (54) farms. This was based on a pilot study that was earlier conducted. A systematic random sample was employed in each farm visited, whereby every 3rd cow in the milking parlour or milking arena was sampled. The animals were sampled according to how they were arranged in the milking parlour sometimes the milking sequence of the farmers was followed. Hand milking procedure was used to extract milk from all four quarters of the udder with the exception of two farms that employed the machine milking process. The udder was thoroughly cleaned and decontaminated with clean water and soap. Also, the hands of the milker were washed with chlorhexidine soap. The udder and milker's hands were then dried properly with a sanitary towel and each cow was restrained properly in order to collect the milk sample. The milk was collected from all four quarters of the mammary glands. Approximately 25 ml of raw milk was collected from each quarter

in a sterile sample bottle giving a total of 100 ml per cow. Twenty-five percent of the milking cows were sampled from each farm. The milk samples collected were stored on ice packs and transported to the Department of Veterinary Public Health and Preventive Medicine Laboratory, Ahmadu Bello University Zaria, where they were analysed 4–6 hours after collection.

Isolation and identification of *E. coli* from milk

Enrichment of sample

Lauryl sulphate tryptose (LST) broth containing MUG (4-methylumbelliferyl-B-D-glucuronide) was used for the enrichment. It was done according to Manafi [22]. One millilitre (1 ml) of the milk sample was dispensed into 5 ml of the broth (LST) in test tubes containing Durham's tubes and incubated for 24 hours at 37 °C and observed for fermentation/gas formation in the test tubes. This was evident by the gas that was trapped at the top of the Durham's tubes which pushed the media down.

Inoculation and incubation of samples

Exactly 0.1 ml of the incubated sample in broth was streaked on eosin methylene blue (EMB) agar, with a sterile inoculating loop, and incubated at 37 °C for 24 hours. Greenish metallic sheen growth was suggestive of *E. coli* which was inoculated on nutrient slants for further biochemical tests.

Conventional biochemical test

Suspected *E. coli* isolates on EMB were further screened by means of five biochemical tests, namely: Simmon citrate, urea, triple iron sugar (TSI), sulphate, indole, motility (SIM), methyl red (MR), and Voges-Proskeur (VP). Various reactions of the tests such as colour change, motility, and gas formation were used to interpret results as either positive or negative after a 24-hour incubation. These tests were carried out as described by Gbarakoro et al. [16].

Identification of *E. coli* by Microbact GNB Kit 12E

Commercially available biochemical test strip Microbact GNB 12E (Oxoid, UK) was used according to the manufacturer's instructions to confirm isolates as *E. coli* based on the result of the biochemical tests. Using the software, the results of individual tests were automatically evaluated and the resulting identity of the organism and its percentage probability were recorded.

Determination of antibiotic susceptibility of *E. coli* isolates

The antibiotic susceptibility profile of each isolate was determined using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) protocol [14]. Colonies (4–5) of the test isolates from overnight cultures on EMB plates were picked and emulsified in sterile normal saline. The turbidity of the suspension was adjusted to match 0.5 MacFarland's standard. Ten (10) µl of the suspension was then dispensed and spread on Mueller-Hinton agar plates to create a uniform lawn. The pre-inoculated plates were used for the antibiotics disc diffusion test.

Extraction of DNA

DNA extraction was done as described by Higgins et al. [17]. Briefly, the tubes containing the cultures were centrifuged at a medium speed (1,000 × g) on a tabletop centrifuge to pellet the cells. All but 100 µl of the supernatant was discarded, and the pellet was re-suspended. Aliquots were removed and processed as described below. Colonies of *E. coli* on EMB plates were removed with a sterile plastic loop and vortexed in 100-µl volumes of sterile water in a microcentrifuge tube to dislodge the bacteria. The sample was then centrifuged, the supernatant was discarded, and the pellet was re-suspended in 30 µl of sterile water and processed. A 30-µl sample was suspended in 200 µl of Instagene matrix and vortexed, followed by heating at 56 °C for 15 min. The samples were vortexed again and heated at 100 °C for 8 min and then centrifuged to pellet the matrix. Aliquots of 10 and 20 µl (the recommended amount) were used as templates for PCR.

Extraction with the Isocode paper was done according to the manufacturer's instructions. Briefly, 10-µl aliquots of bacterial cultures were spotted directly onto 8-mm-diameter disks of the paper and DNA was eluted in a 100-µl volume of sterile water, with 10 to 20 µl used as the template for PCR.

1. PCR analysis of 16S r RNA genes for *E. coli* identification

After the extraction of DNA, *E. coli* was identified by PCR using gene 16S r RNA. A control strain was used: *E. coli* ATCC 25922, accession number x80724. The detection of gene 16S r RNA in *E. coli* was done using the following primers:

Primers: F: TGA CGTTA CCCGCA GAAGAA, R: CTCCAA TCC GGACT ACGACG (Size of the product 832bp).

2. Detection of tetracycline (tet) resistance genes

For those *E. coli* isolates found to be resistant to tetracycline, polymerase chain reaction (Multiplex PCR) was used to detect five different types of tet genes – tet A, tet B, tet C, tet D, and tet M commonly reported in *E. coli* [9, 26].

The PCR assay was performed in 50 µl volume containing 0.5 µg of extracted DNA as template, 2.5 µl PCR buffer mix, 300 µM deoxynucleoside triphosphate (dNTPs), primers (see primers sequences in Table 1) reported by N g et al. [24] and $MgCl_2$ were optimized for each multiplexed primer group. The reaction was carried out with an amplification thermal cycler (Applied Biosystem 9700) in repeated cycles of initial denaturation at 94 °C for 5 minutes followed by 35 cycles of 94 °C for 1 minute, primer annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute 30 seconds, and final extension/elongation at 72 °C for 10 minutes.

Agarose gel electrophoresis

The PCR products were analysed by gel electrophoresis using 1.5 % agarose (1st base agarose biotechnology grade) in TAE buffer, which was subjected to 80 volts for 50 minutes. The bands were stained with Ethidium bromide (1 $\mu g.ml^{-1}$) for 5 minutes and visualized under translumination. Product sizes were determined by comparing them with the 100 bp ladder.

RESULTS

The isolation of *E. coli* from milk samples

Escherichia coli was isolated from 55 (17.57 %) out of the 313 milk samples collected and tested. Out of the 55 *E. coli* isolates identified using the conventional biochemical test, only 15 (27.27 %) were confirmed to be *E. coli* using the Microbact 12 E kit (Fig. 1).

Antibiotic resistance pattern of *Escherichia coli* isolates

The antibiogram of 15 isolates confirmed by the Microbact 12 E kit is shown in Table 2 along with their respective resistance pattern. None of the *E. coli* isolates was resistant to the quinolones (nalidixic acid and ciprofloxacin). All 15 *E. coli* isolates showed resistance to ampicillin (100 %), while 11 (73.3 %) were resistant to the macrolide (erythromycin) and 2 (13.3 %) of the *E. coli* isolates were resistant to nitrofuran (nitrofurantoin) and cefixime (cephalosporin). Also, 5 (33.3 %) of the *E. coli* isolates were resistant to amoxycillin+clavulanic acid (synthetic penicillin) and co-trimoxazole (sulpha drug). Also, 4 (26.7 %) of *E. coli* isolates were resistant to gentamicin and 6 (40 %) were resistant to kanamycin (aminoglycosides), 7 (46.7 %) of the *E. coli* isolates were resistant to tetracycline (Fig. 2).

Table 1. Tetracycline resistance PCR primers

Plasmid	Tetracycline resistance genes	PCR Primer sequence 5'–3'	Expected amplicon size (bp)
pSl18	tet(A)	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210
pRT11	tet(B)	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659
pBR322	tet(C)	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	418
pSl106	tet(D)	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	787
pJ13	tet(M)	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	406

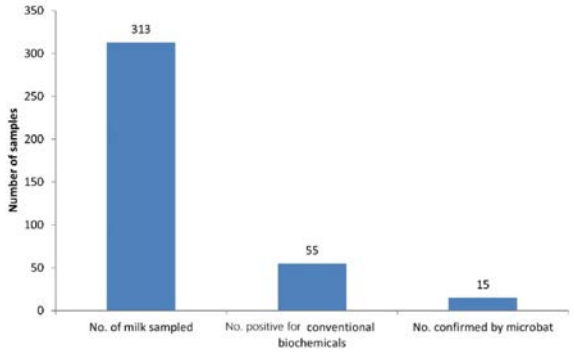

Fig. 1. Screening of *E. coli* isolates from milk samples

Table 2. Antibiotic resistance patterns of *E. coli* isolates from milk of dairy cows in Kano state

Isolate ID	Resistance profile
AA	AMP, TE, K, E
AB	AMP, AMC
AC	AMP,TE, E
BA	AMP, AMC, SXT, K, E, C
CA	AMP, AMC, TE, K, CN, E
DA	AMP, AMC, CFM, TE, SXT, K, E, F
DB	AMP, K, CN, E
DC	AMP, CN, E
DE	AMP
EA	AMP, AMC, SXT, E
EB	AMP, SXT, E
EC	AMP, AMC, TE, E
FA	AMP, CN
FB	AMP, TE
GA	AMP, CFM, TE, SXT, K, E, C

AMP – Ampicilin; TE – Tetracycline; K – Kanamycin; E – Erythromycin; AMC – Amoxycillin + Clavulanic acid; SXT – Co-trimoxazole; C – Chloramphenicol; CN – Gentamicin; CFM – Cefixime–F–Nitrofurantoin

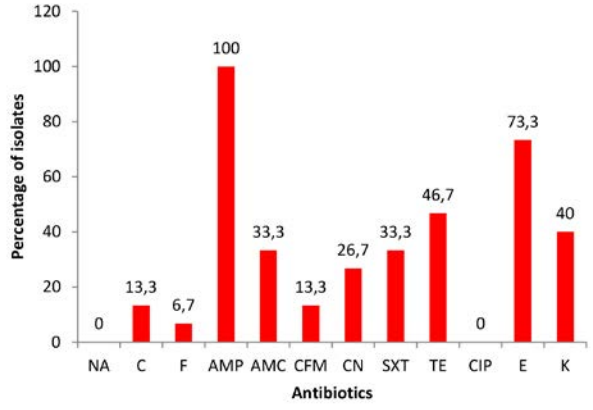

Fig. 2. Percentage resistance of *E. coli* isolates to various antibiotics

AMP – Ampicilin; TE – Tetracycline; K – Kanamycin; E – Erythromycin; AMC – Amoxycillin + Clavulanic acid; SXT – Cotrimoxazole; C – Chloramphenicol; CN – Gentamicin; CFM – Cefixime–F–Nitrofurantoin

Escherichia coli isolates and multiple antibiotic resistance indices

The antibiograms were used to calculate the multiple antibiotic resistance indices, 73.3 % of the *E. coli* isolates had MAR (multiple antibiotic resistance) values greater than 0.2. The MAR indices of the bacterial isolates are displayed in Figure 3; MAR was calculated by dividing the total number of antibiotics to which an isolate was resistant by the total number of antibiotics tested (12).

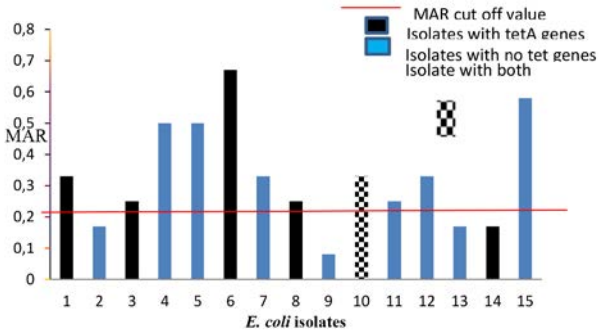

Fig. 3. Multiple antibiotic resistance index of the *E. coli* isolates.

Multiplex PCR for determination of tetracycline resistance genes

The agarose gel electrophoresis of PCR products from the 15 *E. coli* isolated by Microbact 12 E kit is shown in Fig. 4. The PCR result showed that 6 (40 %) of the isolates (AA, BA, CA, DA, EC, GA) were carrying *tet* A gene and the isolate EC carried both *tet* M and *tet* A genes.

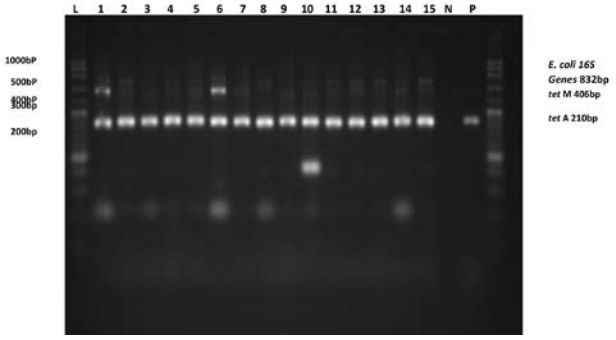

Fig. 4. Multiplex PCR result displaying *tet* M (406 bp) at lane 10, *tet* A (210 bp) at lanes 1, 3, 6, 8, 10, and 14, and *E. coli* 16S RNA genes.

L represents the 100 bp ladder; N = Negative control; P = Positive control.

Note: Lane1 = AA; 2 = AB; 3 = AC; 4 = BA; 5 = CA; 6 = DA; 7 = DB; 8 = DC; 9 = DE; 10 = EA; 11 = EB; 12 = EC; 13 = FA; 14 = FB; 15 = GA

DISCUSSION

This study revealed a low occurrence of *E. coli* in milk samples from dairy farms in Kano state, Nigeria. All the *E. coli* isolates from Microbact 12E displayed resistance to more than one type of antibiotics on the antibiotic disc diffusion test.

Most of the *E. coli* isolates had Multiple Antibiotic Resistance Index (MAR) greater than 0.2, this suggests that they were isolated from an environment where antibiotics are probably abused or frequently used. The resistance pattern varied between and within different classes of antibiotics. None of the *E. coli* isolates were resistant to any of the quinolones tested (nalidixic acid and ciprofloxacin). Colignon and McEwen [11] attributed low-level resistance of quinolones to low usage in livestock therapy in Nigeria as due to their relatively high cost they are not readily available compared to other classes of antibiotics. Testing showed that 13.3 % of the isolates were resistant to chloramphenicol (a synthetic antibiotic) and cefixime (a cephalosporin), which implied resistance spread to broad-spectrum antibiotics and a new generation antibiotic which will make the treatment difficult. Thirty-three point three percent (33.3 %) of the isolates were resistant to amoxicillin+clavulanic acid (a synthetic penicillin) and co-trimoxazole (a sulpha drug), this resistance pattern most likely was due to the integron system of a multidrug resistance mechanism. In most cases of sulpha drug resistance, integron systems are involved [19]. Furthermore, 26.7 % and 40 % of the isolates were resistant to gentamicin and kanamycin (aminoglycosides) respectively. Resistance to tetracycline was 46.7 % and all of the isolates were resistant to ampicillin (a synthetic penicillin) in this case there is the likelihood that the efflux pump system of drug resistance is involved as it gives room for resistance to different classes of drugs at the same time. Similar high-level resistance to ampicillin by *E.coli* isolated from milk was reported by [4].

The PCR result showed that 6 (40 %) of the isolates carried *tet* A gene which codes for efflux pump and EC isolate carried *tet* M gene which codes for both efflux pump and ribosomal protection resistance mechanisms (see Fig. I) and this supports the multidrug resistance displayed by most of the *E. coli* isolates as these two resistance mechanisms give room for the multidrug resistance. Isolate EC displayed multiple resistances on the disc diffusion test. The *tet* A and *tet* M genes are the most common resistance mechanisms of *E. coli* to tetracyclines as reported by Sheykhsaran et al. [27] and the findings of this research suggest that *tet* A and *tet* M are the predominant resistance genes of the *E.coli* isolates in the study area.

CONCLUSIONS

This study shows that raw milk from commercial dairy farms in Kano State had an occurrence of multi-drug resistant *E. coli* from 15 milk samples (4.79 %) and this is a major public health threat. Out of the 15 *E. coli* that were isolated and identified, 14 were resistant to more than one antibiotic from the disc diffusion test, and 73.3 % of the *E. coli* isolates had MAR index greater than 0.2.

The study further revealed that some of the *E. coli* isolates carried genes that code for both efflux pumps and ribosomal protection resistance mechanisms.

We recommend that the farmers should be enlightened on the proper hygienic practices of milking and handling milk. Also, there should be a good regulatory policy by the government on the use and sales of antimicrobials in animal production in the country. There is a need to further detect other resistant genes apart from *tet* genes as the isolates could be carrying them.

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RADIOGRAPHIC PELVIMETRY IN RELATION TO DYSTOCIA IN BULLDOGS

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ABSTRACT

Dystocia is a common complication of parturition in the breed of English Bulldogs, where most females are not able to have a natural parturition and many litters are delivered by caesarean section. The aim of this study was to evaluate the diameter of the pelvis of 11 females of English Bulldogs through radiographic pelvimetry and do proper measurements to observe if there are any correlations between a narrow pelvic canal and dystocia detected in the breed. The external parameters were also assessed to look for any differences between weight, height, and length in relation to the dystocia in the breed. The mean weight of the dogs with natural parturition was 21.25 ± 0.91 kg, whereas the females which had undergone caesarean section was 25.16 ± 1.44 kg (P = 0.0004). A P-value shows strong evidence for the hypothesis of this study as a possible factor of dystocia of the breed. The values of height and length in the category of dams which had undergone natural parturition were in cm 44.8 ± 2.48 (length) and 36.1 ± 0.98 (height). In the category of dogs which had undergone caesarean section, the parameters were in cm 45.4 ± 2.60 and 36.0 ± 1.58, respectively. The P-value for length were 0.72 for length and P-value for height were 0.83. Difference between the diameter of the pelvis in the two groups of English Bulldog females was P = 0.12, so there is no significant evidence. However, it could be significant for a possible future study with a higher number of animals for measuring pelvic diameter.

Key words: bitch; caesarean section; dystocia; parturition; radiographic pelvimetry

INTRODUCTION

English Bulldogs have a high incidence of dystocia, a complication of parturition where the dam has difficulty in passing the foetus through the pelvic canal [16]. It is commonly presumed that the English Bulldog is predisposed to dystocia due to foetal-pelvic disproportion caused by a small, narrow pelvic canal and deep position of the pregnant uterus in combination with large foetal head and shoulders [2]. This has led to a higher prevalence of emergency caesareans sections in veterinary clinics.

The literature in the field of dystocia of brachycephalic breeds are limited, with only a few articles having been written about the subject. Few articles have been written about other breeds with similar difficulties, however none to date have compared the pelvic diameter of natural whelping females, versus a category of females that had undergone dystocia in the English Bulldogs.

The causes of dystocia in English Bulldog are not completely determined, but there are several reasons why brachycephalic dogs like the English Bulldog might have an increased risk of dystocia and puppy mortality. Brachycephalic dogs are typically susceptible to obstructive dystocia because of the foetal head size or the dam's narrow pelvic canal. Bulldogs, in addition, are specifically reported to have slack abdominal musculature that inhibits positioning of the foetus in the pelvic inlet [11]. Further, the reproduction problems are seen as many dams cannot give birth naturally. It is pointed out in multiple causes, the gene pool of the breed is limited and with limited number of individuals there is often used same male for multiple females. The size of the foetal heads and shoulders can also be a complicating factor, together with a narrow and light pelvic diameter of the dam will give negative expectations [12].

Radiographic pelvimetry is a method performed through radiography, where the capacity and the diameters of the pelvis are measured. It is a valuable method for morphometric evaluation of the pelvis in dogs. However, it permits only two-dimensional evaluation of the pelvis and has a limited accuracy, based on distortion of the pelvis due to its angle relative to the detector. Pelvimetry has been found to be a useful technique for assessing the pelvic diameter and the correlation to dystocia in dogs. To the author's knowledge, radiographic pelvimetry has mainly been used retrospectively in dogs and cats to look for correlations between dystocia and the measurements of the pelvis [9, 10]. It is not known if there have been any scientific studies in comparable pelvic assessments to observe differences in the pelvic diameter in English Bulldogs.

Computed tomography (CT) is a new way of assessing the pelvis of dogs. It is a high-resolution method, which enables a three-dimensional evaluation of the pelvis. In the latest study, computed tomographic pelvimetry was used to study the pelvis of English Bulldogs compared to other non-brachycephalic breeds [2].

MATERIALS AND METHODS

In this study, there were included 11 females, aging 2 to 7 years, recruited from the population of English Bulldogs by the arrangement of the Norwegian Bulldog Club to undergo a radiographic examination of the pelvis. External parameters (height, length, and weight) were measured. The study was performed with the informed consent of the owners and the physical experiment was completed in the small animal veterinary clinic "Vetsentrum" in Norway. The dogs were divided into two groups: bitches which had given birth *per via naturales* (n=6) and bitches which had given birth to puppies by caesarean section (n = 5).

The reproduction history of the females was collected orally through an interview on the day of the experiment. Height, weight, length of the dog are external parameters that were measured. A clinical examination was also done to assess the overall health of the dog before sedation and the radiographical examination.

The dogs followed the identical medical procedure based on their weight to acquire correct doses. The radiographs were taken with sedation and anaesthesia to get the optimal accuracy of the radiographs and to limit the movement of the dog during the radiography. All premedications and anaesthesia solutions were given through intravenous catheter taken on both front legs in the cephalic vein. Sedation used for the study were 0.01 $mg.kg^{-1}$ dexmedetomidine hydrochloride (Dexdomitor Vet, 0.1 $mg.kg^{-1}$, ORION PHARMA, Corporation Espoo, Finland) and methadone hydrochloride (Synthadon Vet 10 $mg.kg^{-1}$, Le Vet Beheer BV, Netherlands) with dose 0.2 $mg.kg^{-1}$ before injectable anaesthesia. For the injectable anaesthesia was used propofol (Propolipid 10 $mg.kg^{-1}$, Fresensius Kabi Ab, Finland) intravenous injection with a dose 10 $mg.kg^{-1}$ for induction of anaesthesia. For optimal breathing for the dog, an endotracheal tube where applied, with lidocaine 1 $mg.kg^{-1}$ (Xylocain 100 mg/spray, Aspen Pharma Trading Ltd., Ireland) in the larynx before application. The total intravenous anaesthesia (TIVA) Syringe Pump SK-500I with propofol (Propolipid, Fresensius Kabi, Finland) 10 $mg.kg^{-1}$ were used for maintaining anaesthesia through the radiography, where it was set on 22.5 ml per-hour. Fluids were given through the whole procedure, 5 $ml.kg^{-1}.hour^{-1}$ IV Sodium chloride (Natriumklorid, Fresensius Kabi 9 $mg.ml^{-1}$, Finland) through an infusion pump from KruTech VIP 2000 (KRUUSE, Denmark). An injection of atipamezolhydrochloride (Antisedan vet, Orion, Finland) were given intramuscularly 15 minutes after the radiographic the examination as done, with a dose of 5 $mg.kg^{-1}$. This procedure was replicated identically for the next 10 dogs in the radiographic procedure with different doses depending on the weight of the dams examined.

The X-ray apparatus that was used in all experiments during this diploma thesis were GER Int. (Cuy, France). The program used for the X-ray apparatus is Vet-Tech 300HF.

The focus-film distance is the distance between the centre of the anode of the X-ray tube and the film in the cassette and were 73 cm for all the dogs. The computer imaging software for reading, displaying and measurements of the X-ray images is Vet-Exam plus 9.6.0. Cassette reader and cassette is from CR43VET. The collimator used in this experiment was a Ralco, model R302/A. Kilovoltage (kV) used in all the dogs were 76, while milliampere per second was 13.5. Slight adjustments could be made on kV depending on the size of the dogs to get optimal radiographs.

The radiographs were taken in left lateral and dorsal recumbency [3]. In dorsal recumbency, the radiographs were obtained in ventrodorsal projection, and the dog was placed in a flexed ventrodorsal (frog-legged) position, and the central beam was focused on the midline between the *trochanter major* on the two femurs [9]. This projection is typically preferred in sedated animals, especially those that dislike extension of the hindlimbs due to the pain in the hip [8]. The lateral (LL) view would be obtained initially before ventrodorsal (VD) view, and the animal was placed in lateral recumbency with the legs in a natural position on the table. To prevent rotation in axial skeleton of the pelvis, padding should be placed between the femurs, and the femurs should be parallel to each other. The radiographic beam was centred on the hip joints where the trochanter major was used as landmarks [8].

After radiography, the radiographs of the pelvis were measured directly with a digital ruler in the radiography program and the measurements were done as follows: *conjugata vera* (CV) = distance between *promontorium* (cranial end of *sacrum*) and *pecten ossis pubis* (cranial end of *symphysis*), *conjugata diagonalis* (CD) = distance between *promontorium* and caudal end of *symphysis*, *diameter verticalis* (DV) = vertical distance between *pecten ossis pubis* and *sacrum* orthogonal to the *symphysis pelvis*, and *diameter transversa* (DT) = the horizontal distance between *corpus ossis ilii* (Fig. 1, 2) [3, 9].

In the study by Johansen [9] there were used fewer measurements to assess the pelvic proportions. Johansen stated: "To make sure that the horizontal distance was found when measuring DT a line was drawn between two distinct bony landmarks on the pelvis, namely the two lateral *tuber ischiadica*. The distance DT was measured parallel to the green line." (Fig. 2) [9].

The area of the pelvic inlet was determined by using the formula $\pi \times CV/2 \times DT/2$ [9, 17] with the measurements seen in the Figure 1 and 2.

Statistical analysis

Statistical tests were performed using Microsoft Excel with Anova: Single Factor data analysis with T-test. A P-value of < 0.05 was considered significant. Mean and Standard Error (SE) were calculated using Microsoft Excel.

Ethical approval

We confirm that the owners of patients in this study approved the medical process. The study was conducted according to the regulations of the local Institutional Animal Care and Use Committee.

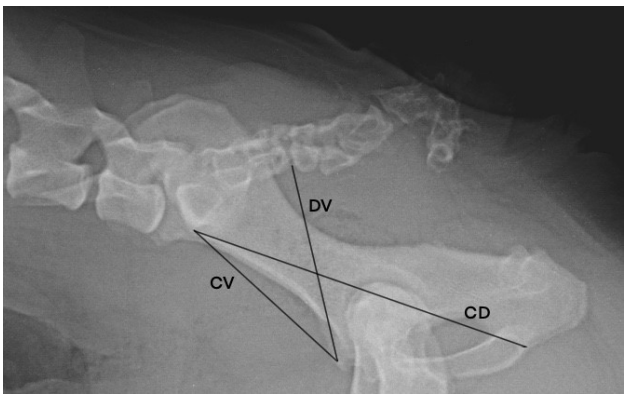

Fig. 1. Measurements on the lateral radiographs: CV = *conjugata vera;* DV = *diameter verticalis*; CD = *conjugata diagonalis*
Source: Self-made figure.

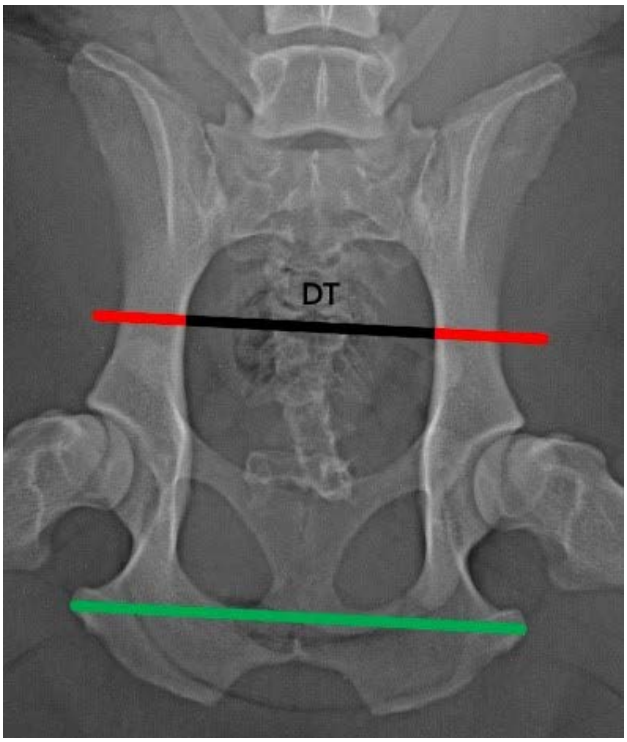

Fig. 2. Measurements on the ventrodorsal radiographs: DT = *Diameter transversa*
Source: Self-made figure.

RESULTS

The results are divided into two central components: the external parameter component where the external measurements of the female dogs in this project are listed, and the radiographic pelvimetry component where the internal measurements of the pelvic canal of the female dogs are listed.

To get an idea of the size of the female English Bulldogs, all dogs were weighted and measured to acquire external parameters of the dams. The two groups of the dogs were compared by length, height and weight with P-values. The results are seen in Table 1.

Table 1. The mean number with Standard Error (SE) of external measurements of naturally birthing dams and dams that has given birth through caesarean section and a comparison by P-value.

Measurement	Natural birth (n = 6) Mean + SE	Caesarean section (n = 5) Mean + SE	P – value
Length (cm)	44.8 ± 2.48	45.4 ± 2.60	0.721
Height (cm)	36.1 ± 0.98	36.0 ± 1.58	0.8350
Weight (kg)	21.25 ± 0.91	25.16 ± 1.44	0.0004

n – number of dogs

Pelvic diameter was measured and assessed through radiography and the results with comparisons of the two groups of dams are found in Table 2. All dams contributing to the radiographic study is included, both females that had a natural birth and the females that gave birth through caesarean section. The pelvic measures were compared of the two groups of females.

Table 2. Comparison of pelvic measurements of bitches that had given birth naturally and bitches that had given birth through caesarean section by means of P-values, mean and SE

Measurement	Natural birth (n = 6) Mean + SE	Caesarean section (n = 5) Mean + SE	P-value
CV (cm)	6.50 ± 0.3	6.48 ± 0.3	0.8937
DV (cm)	6.32 ± 0.3	6.15 ± 0.3	0.4291
CD (cm)	10.23 ± 0.4	10.15 ± 0.56	0.8068
DT (cm)	5.05 ± 1.15	5.31 ± 0.21	0.6240
CV/DT	1.46 ± 0.48	1.22 ± 0.08	0.2991
Area of pelvic inlet (cm)	28.29	27.01	0.1202

CV = Conjugata vera; DV = Diameter verticalis; CD = conjugata diagonalis; DT = Diameter transversa. CV/DT = relation between the vertical and horizontal diameter of the pelvic inlet. Calculated with the formula π×CV/2×DT/2 to get the results of the area of pelvic inlet; n = number of dogs.

DISCUSSION

The purpose of this study was to use the external measurements of all the females of the study and look if there is any correlation between the height, weight or length to dystocia and narrow pelvis diameter. The values of height and length in the category of dams which had undergone parturition *per via naturales* were in cm 44.8 ± 2.48 (length) and 36.1 ± 0.98 (height). In the category of dogs which had undergone caesarean section, the parameters were in cm 45.4 ± 2.60 and 36.0 ± 1.58, respectively. The values show that there were only minor differences in height and length. The P-value for length was 0.721 for length and P-value for height was 0.835. In the thesis of Johansen [9], it was seen a significant difference in height of the dams and the correlation with caesarean section, where the dams had generally shorter height than the dams which had given birth naturally. In this study with the two categories of dogs, there was not seen any significant difference between the height and the length.

The mean weight and SE of the dogs with natural parturition was 21.25 ± 0.91 kg, whereas the females which had undergone caesarean section was 25.16 ± 1.44 kg. This resulted in a P-value of 0.0004 which shows strong evidence for the hypothesis of the study. This strongly indicates evidence for weight as an important factor of dystocia in females of English Bulldogs in this study. As a comparison, one female that undergone caesarean section with nearly the same height and length parameters as one with natural parturition is 1.6 kg heavier. The heaviest of the dogs in this study was 27.4 kg, whereas the heaviest in the group of natural parturition was 22.5 kg. These two dogs where in the same height and the length difference was 5 cm. In Johansen's thesis [9] there was not seen any correlation between the weight of the dams and the higher number of caesarean sections. In the article by Eneroth et al. [3] however, it was found that bodyweight, height and withers had some influence on the outcome of the parturition in Scottish terriers.

Obesity is defined as a disease in which excessive body fat has accumulated to such an extent, that the health of an animal may be adversely affected [6]. The cause of obesity is either excessive dietary intake or inadequate energy utilization, which results in positive energy balance [5]. Canine patients are typically seen as clinically obese when their body weight is at least 15 % above ideal weight of the breed. Since obesity is known to intensify and predispose to many different serious diseases and medical conditions, it is an important

disease to follow and control [7]. In reproduction, obesity is associated with multiple reproductive disorders. Infertility, ovulation dysfunction, foetal growth disorders and abortion is observed in some cases in clinical practice. Obese animals possess a higher risk of dystocia due to accumulation of fatty tissues around the pelvic cavity reducing the elasticity of the pelvic ligaments which is a must for parturition [14]. In other veterinary articles dystocia is marked as a possible consequence of obesity in dogs and cats [6, 7]. It is essential that only animals that are in optimal body condition should be bred. Overfeeding pregnant animals is commonly seen in veterinary practice, where many owners believe that high amounts of energy are required from the very beginning of pregnancy. This will result in fat deposition which will make the females more prone to have dystocia. Weight of the dams should therefore be monitored throughout the pregnancy and the weight should not exceed 25–30 % of the pre-breeding weight. [4].

Diagnostic imaging as computed tomography (CT) or radiography is essential for management of canine dystocia. Radiography is especially essential for determining foetal number, foetal misplacement and foetal-maternal incompatibility [15]. English Bulldogs are a brachycephalic breed and it is discussed that the breed's large head in relation to a narrow pelvis is the main reason for obstructive dystocia. Recent CT studies of bulldogs showed an overall narrower pelvis and a pelvic canal and a narrower pelvic outlet, which gives a higher risk of dystocia in the breed [2]. The study by D o b a k et al. [2] demonstrates that English bulldogs generally have a significantly smaller pelvis and pelvic diameter when compared to non-brachycephalic breeds of the same weight range. In the thesis of J o h a n s e n [9] it was also indicated that the pelvic canal was significantly shorter and lower in the dams suffering from dystocia compared to natural whelping dams in Scottish Terriers [3]. J o h a n s e n [9] and E n e r o t h et al. [3] did not observe any significant difference in *diameter transversa* in the two groups of dams in their studies, which is also the incidence in this study. That can indicate that the main problem with the pelvis of the Scottish Terriers is dorsoventral flattening of the pelvic inlet. In this study, there is not significant evidence to presume similar conformity, but it could be a possible description of the possible obstructive dystocia in the breed.

Contrary to expectations, this study did not find a significant difference between the diameter of the pelvis and the two groups of English Bulldog females. The P-value was 0.12, which showed no true evidence for the hypothesis of a narrower pelvic canal of the females giving birth through caesarean section. However, it could be significant for a possible future study with a higher number of animals for measuring pelvic diameter.

J o h a n s e n 's thesis [9] of radiographic pelvimetry of Scottish Terriers states that vertical diameters of the bitches that had given birth naturally were significantly longer than for bitches that had given birth solely by caesarean section. They also observed that the pelvic canal was significantly shorter and lower in the dams suffering from dystocia compared to dams that were naturally giving birth. However, there are differences in the breed of Scottish terriers and English Bulldogs, hence it is difficult to see the value of this resource. In an article written by C e l i m l i et al. [1], cats with dystocia had a significantly smaller pelvic dimension than cats which had a normal parturition. D o b a k et al. [2] also reveals that English bulldogs generally has a significantly smaller pelvis and pelvic diameter when compared to non-brachycephalic breeds of the same weight range. This is a very important factor for this study, because the results show low significance between the two groups of dams. Essentially, the theory of D o b a k et al. [2] points out the fact that the English Bulldogs' pelvic canal is smaller in general, hence the difference between the two groups can be minimal and there is a possibility of other factors making the difference in the problems of giving birth naturally, together with the main issue of a narrow pelvic canal.

There are some delimitations in this thesis. There are 11 dogs in the scientific study which limits the P-value of the scientific study. In this way, it would be useful to use this study as a preliminary study for a larger study where more subjects are used and more radiographic pelvimetry measurements are evaluated to get a larger picture of the dystocia in English Bulldogs.

CONCLUSIONS

The result of this study showed that the females which had undergone caesarean section and the females that had given birth *per via naturales* had only minor differences in height and length. The mean weight comparison for the two groups resulted in a P-value of 0.0004 which strongly indicates evidence for weight as an important factor of dystocia in females of English Bulldogs.

Contrary to expectations, this study did not find a significant difference between the diameter of the pelvis and the two groups of English Bulldog females. The P-value was 0.12, which is high and shows no true evidence for the hypothesis of a narrower pelvic canal of the females giving birth through caesarean section. However, it could be significant for a possible future study with a higher number of animals for measuring pelvic diameter. One can assume that if this had been a larger study with more research animals, the P-value would possibly been lower, and the number would been significant. This hypothesis cannot be concluded in this study.

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CONFLICT OF INTEREST

We declare that all listed authors are without a Conflict of Interest.

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PINEALOCYTES AND GLIA CELLS IN THE PINEAL GLAND OF THE AFRICAN STRAW-COLOURED FRUIT BAT (*EIDOLON HELVUM*)

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ABSTRACT

The mammalian pineal gland is a structure that in recent years has been extensively studied, due to its functions and the hormones it produces. Bats are the only known flying mammals, with the order having a large number of species. This study was carried out to investigate the gross and histological features of the pineal gland of the *Eidolon helvum*, the African fruit bat, using male and female subjects. Eight free flying *E. helvum* (4 males, 4 females) were captured using mist nets. The pineal gland was grossly observed to be very small in size, oval in shape, and covered by the pia mater. Histological examination revealed two populations of pinealocytes – Types I and II – oval structures possessing acidophilic cytoplasm and large, round nuclei. Pinealocytes were seen to appear singly or in clusters, having no particular arrangement. Sexual dimorphism was observed, with the females having less density in population of pinealocytes at the peripheral region of the gland. This was consistent in all subjects examined. Astrocytic appearance was typical with long and slender processes, and perivascular and capsular microglia were observed. The glia cells were observed to be abundant in the parenchyma and around the capsule. There was no sensitivity to NeuN antibody. Results obtained may find application in behavioural and comparative neuroscience.

Keywords: African fruit bat; astrocytes; *Chiroptera;* microglia; neuroanatomy; pineal gland; pinealocytes

INTRODUCTION

Bats, order *Chiroptera*, are the most abundant mammals and they form the biggest mammalian aggregation [15]. Bats are the only actively flying mammalian species with the capacity of echolocation. There are two sub-orders: *Megachiroptera* and *Microchiroptera* [9, 12, 13]. Pteropodids are mostly nocturnal mammals with an alternating circadian and photoperiodic system interphase. The *Eidolon helvum*, a member of the Pteropodidae family, is

not necessarily a nocturnal bat though it feeds at night. It is also called the straw-coloured fruit bat due to the silky yellowish colour of its exterior with black wings and a pale back hair [6, 11]. During the day, they rest and move among the colony. About 15 % of bats have been classified as being endangered or vulnerable by the International Union for Conservation of Nature Red list, due to a declining population [23].

The epithalamus is a subdivision of the diencephalon, located in the region of the posterior commissure that consist of the pineal gland and the habenular nuclei [1]. Embryologically, the pineal gland is an evagination of the diencephalic roof situated between the cerebral hemispheres and it is attached by a stalk to the roof of the third ventricle and bordered bilaterally by the rostral colliculi [1]. Virtually, all vertebrates possess a pineal organ. The pineal gland is associated with biorhythms, acting as an interface between the cyclic and the rhythmic vertebrate body. This results from the secretion of melatonin by the pineal gland. It is composed of cells called pinealocytes and cells of the nervous system called glial cells [14]. The pineal gland is attached to the brain at a region called the habenular nucleus [22]. The habenular comprises a small group of nuclei connecting the forebrain and midbrain within the epithalamus of the brain. It is divided into two asymmetric halves; the medial habenular and the lateral habenular**.** The medial and lateral nuclei are connected to each other, by the habenular commissure [20].

Justification for current research

In spite of the number of growing publications on the anatomy of the *Eidolon helvum* in recent years, careful electronic search at the time of this write-up did not reveal any stating the cell architecture of the pineal gland and the presence of any sexual dimorphism. This study aims to give an anatomical description, both gross and histological, of the pineal gland of the *Eidolon helvum*.

MATERIALS AND METHODS

Capture of experimental animals (bats)

Eight free flying bats (4 males and 4 females) were captured using mist nets set up along the flight path of the bats around known feeding areas between 6 pm and 10 pm. The nets with lengths ranging from 6 to 12 meters and a height of 3 to 6 meters, were tied to poles. The nets were checked every 10 minutes with infrared head lamps. Bats caught were carefully removed before being placed individually in a cloth holding-bag with a draw string closure for immediate transportation to the laboratory for species identification and sacrifice. Eight animals in total (4 males and 4 females) were used because *E. helvum* is classified as Near Threatened on the IUCN Red list [7] and they need to be conserved.

Identification of bats

Bat species were identified using external features. The fur of the African straw-coloured fruit bats has a yellow tinge and nearly always with an orange collar of fine textured hair (varying from very bright to rather indistinct), which usually extends round the back of the neck as well as the throat. They also possess a muzzle that tapers towards the nostrils. The sex of each bat was recorded.

Sacrifice

The bats were anaesthesized using a xylazine-ketamine combination of 10 $mg.kg^{-1}$ and 90 $mg.kg^{-1}$ respectively [2]. After confirming cessation of breathing and loss of response to tactile stimuli, the bats were perfused transcardially first with 0.9 % physiological saline, and next with 10 % buffered formalin (pH 7.4). The whole brain was harvested from the skull with the leptomeninges intact and immediately post-fixed in 10 % neutral buffered formalin for about 5 days. Prior to histological processing, gross structures of the brain were observed and captured. The brain was trimmed just immediately cranial to the pineal gland in the region of the *striae medullaris* and processed for histological staining. Thereafter, serial sections in coronal plane of 5 µm thickness were cut on a microtome, mounted on gelatin treated frosted end slides after floating on warm bath. The slides were oven dried for 1½ hrs and routinely stained with haematoylin and eosin (H&E). The same cutting procedure was repeated using a super frosted slide, oven dried and stained using cresyl violet [8].

Immunohistochemistry

The brains were prepared for immunohistochemistry following the protocol described by Usende et al. [21]. Briefly, the 5 µm thick brain sections were mounted on adhesive glass slides, and baked in an oven set at 60 ^{o}C for 1 hour 30 minutes to melt the wax. Deparaffinization of

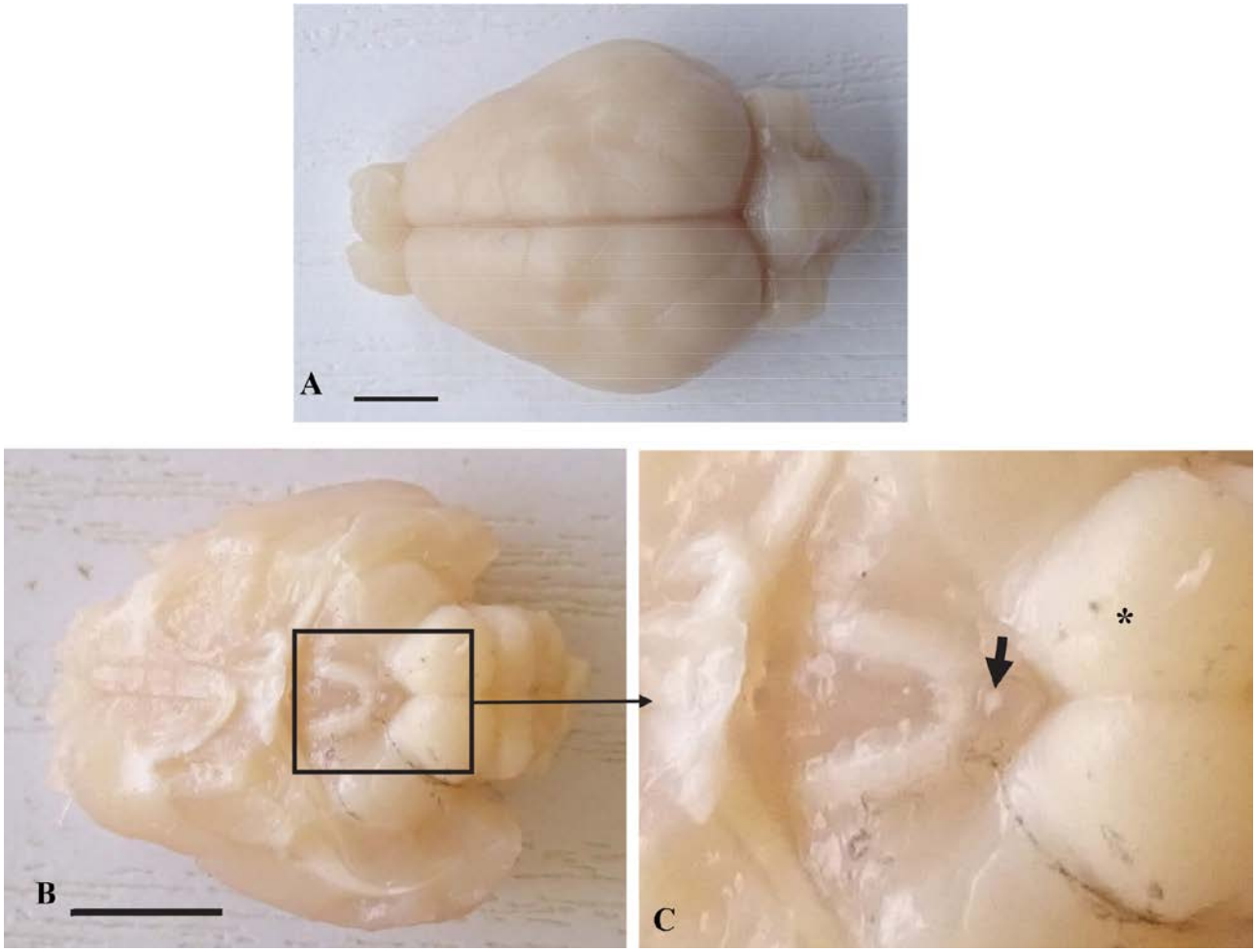

Fig. 1. Brain of the African fruit bat *(Eidolon helvum)*, dorsal view.
In **B** and **C**, part of the cerebrum had been removed to expose the pineal gland and colliculi. **C** is a magnification of boxed area in **B**. The pineal gland is depicted by the black arrow and the rostral colliculi by the asterisk. Scale bar 0.5 cm.

the sections was done in 2 changes of xylene, after which the sections were hydrated in decreasing concentration of alcohol to water. Sections were then rinsed in double distilled water before retrieval of antigen was achieved by incubating the sections in 10 mM citrate buffer at pH of 6.0 for 25 minutes to unmask the hidden antigenic site. In order to reduce non-specific antibody binding and to hinder endogenous peroxidase activities, sections were treated with 30 % H_2O_2/methanol. Sections were subsequently blocked by incubating in 2 % PBS milk while on a rocker for 60 minutes. Consecutive sections were immune-labeled with the following antibodies: rabbit anti-IBA 44 1 antibody (dilution 1:1000, Wako Pure Chemical Industries Ltd., Japan) for microglial cells, rabbit anti-glial fibrillary acidic protein (GFAP) (1: 1000; Dako, Denmark), to visualize astrocytes; rabbit anti-NeuN polyclonal antibody (dilution 1:500, EMD Millipore, USA) to visualize neurons. The antibodies were diluted in 1 % PBS milk and 0.1 % Triton X detergent (to facilitate quick penetration of antibody) and the samples underwent incubation over night at 40 °C. HRP-conjugated secondary antibodies were subsequently used following manufacturer's protocol (dilution 1:200, Abcam Inc, USA) to detect the bound antibody. The end product of entire reaction was improved with 3, 3'- diaminobenzidine a chromogen (1:25 dilution, Victor Laboratories, USA) for 5 minutes. Subsequently, sections were dehydrated in solutions of graded alcohol concentration, and alcohol removed by passing through two changes of xylene (5 minutes each), then mounted wet in permount (toluene based mountant, Atom Scientific, Manchester), cover slipped and allowed to dry. Microscopic examination was carried out using LEICA DM 500 microscope.

Pinealocytes observed were classified into dark and light based on appearance according to previous reports [4, 16–18].

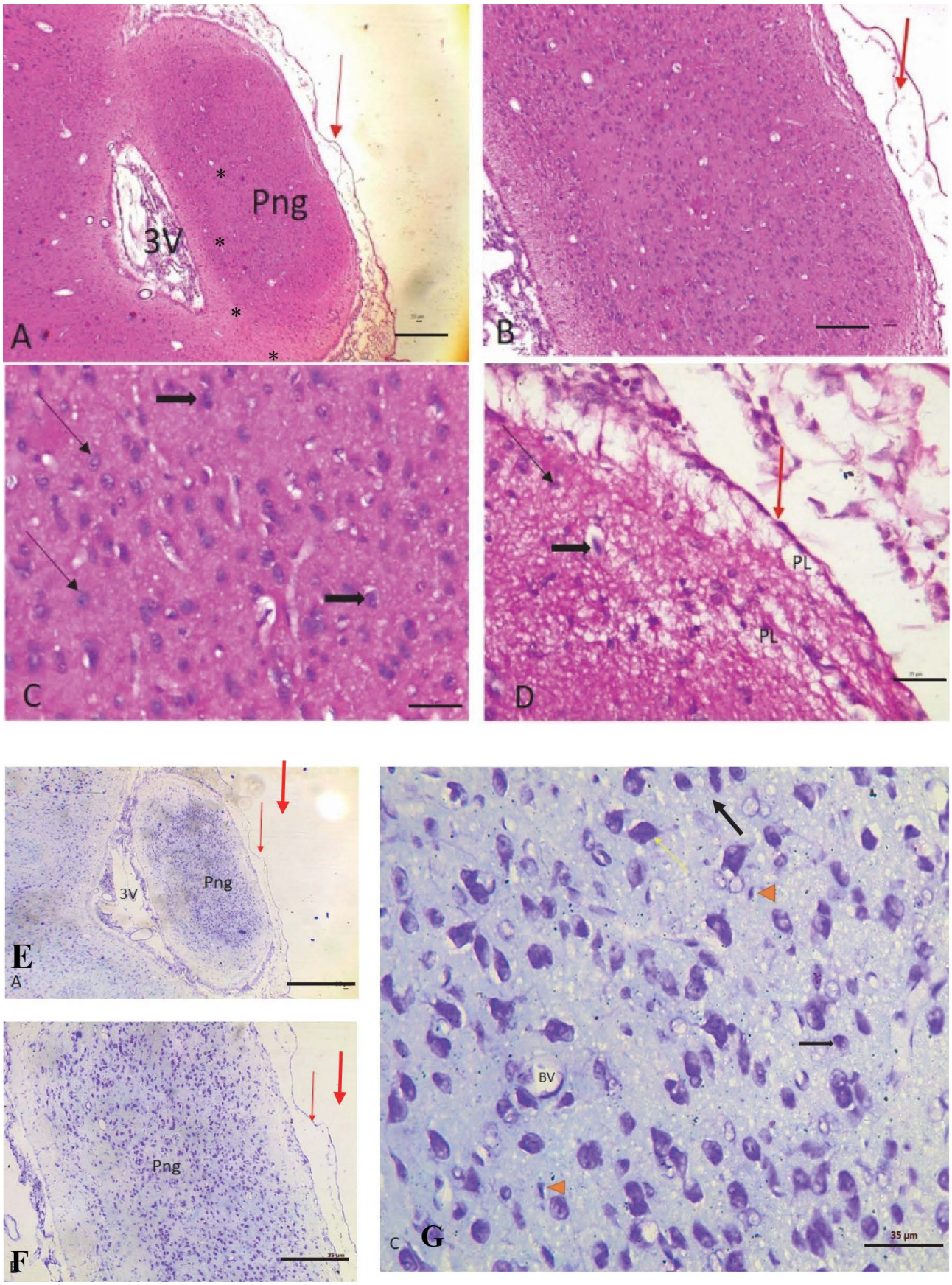

Fig. 2. Photomicrograph of the pineal gland of the female fruit bat, H & E (A – D), Cresyl violet (E – G).

A and **E** – Entire coronal section of pineal gland (Png) seen above the third ventricle (3V); **B** and **F** – Gland parenchyma showing pia capsule (red arrow); **C**, **D** and **G** – Pineal parenchyma showing the dark pinealocytes (long black arrows) and light pinealocytes (short black arrows). **F** – shows an astrocyte (arrow heads). The asterisks (*) in A is used to trace the condensation of cells, not seen in the male bat. Scale bar – 385 µm (**A** and **E**), 150 µm (**B** and **F**), 20 µm (**C** and **D**), 35 µm (**G**).

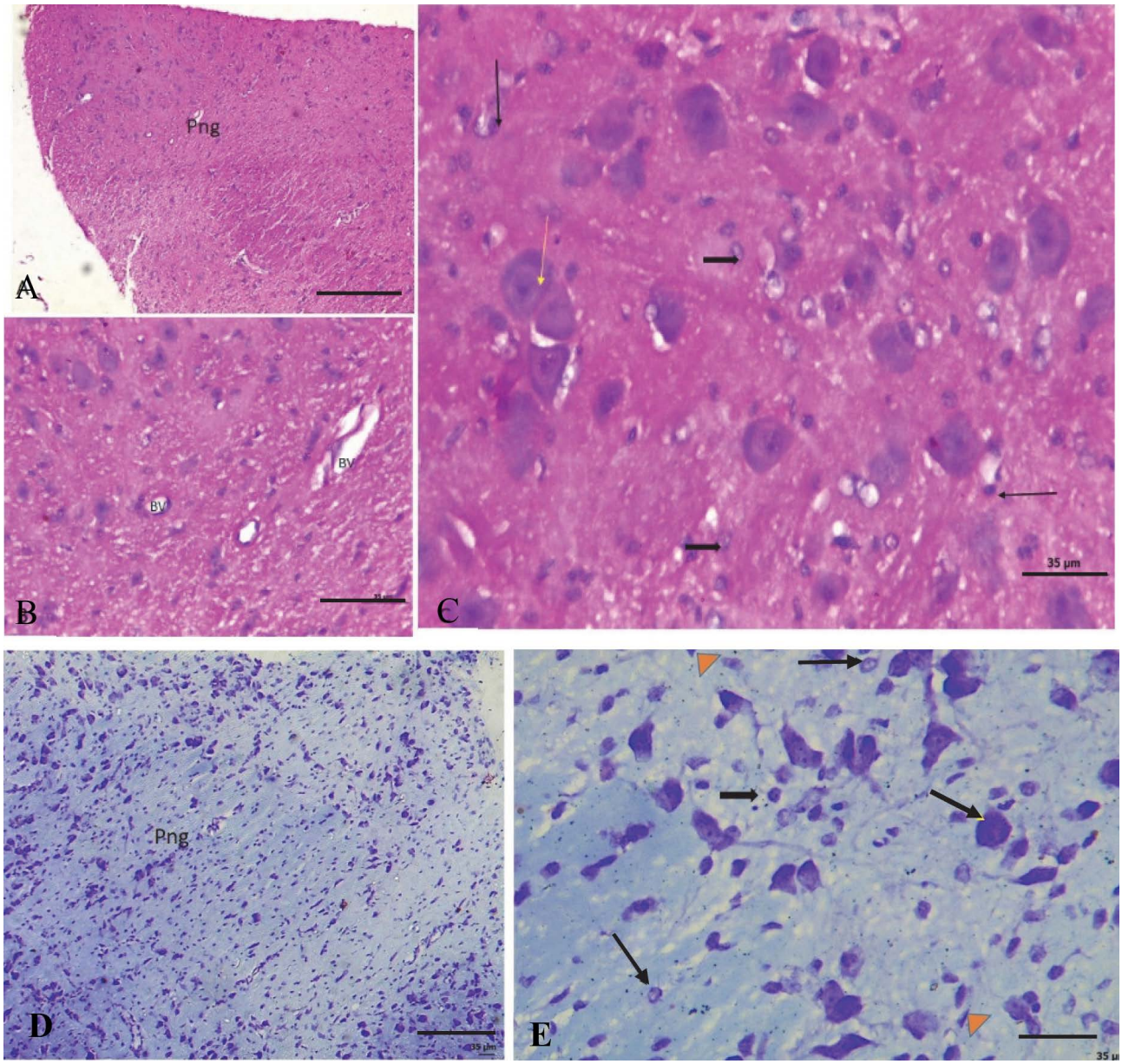

Fig. 3. Photomicrograph of the pineal gland of the male fruit bat, H & E (A – C), Cresyl violet (D and E).
A – Entire coronal section of pineal gland (*Png*) seen above the third ventricle (3V); **B** and **D** – Gland parenchyma, blood vessels (BV) are seen in **B**; **C** and **D** – Pineal parenchyma showing the dark pinealocytes (long black arrow), light pinealocytes (short black arrows) and astrocytes (arrow heads). Scale bar – 150 µm (**A** and **D**), 60 µm (**B**), 35 µm (**C** and **E**).

Ethical considerations

Ethical approval for this study was obtained from the Animal Care and Use Research Ethics Committee, of the University of Ibadan, Nigeria, with ethical code UI-ACUREC/App/2016/015. All bats were humanely handled.

RESULTS

The pineal gland of the fruit bat was observed to be very small, being a single rounded body about 2.1 mm in size and spherical in shape. It was situated immediately cranial to the rostral colliculi, along the midline; part of the cerebrum had to be removed to reveal the pineal. It had a creamy white colour after perfusion (Fig. 1). It should however be noted that the animals were perfused post-euthanasia, prior to dissection. This may probably affect the colour, but to little degree.

H and E and cresyl violet stains

Histological staining with H and E and cresyl violet revealed the pineal gland to be surrounded by a thick fibrous connective tissue capsule of pia matter (Figs. 2 and 4). The

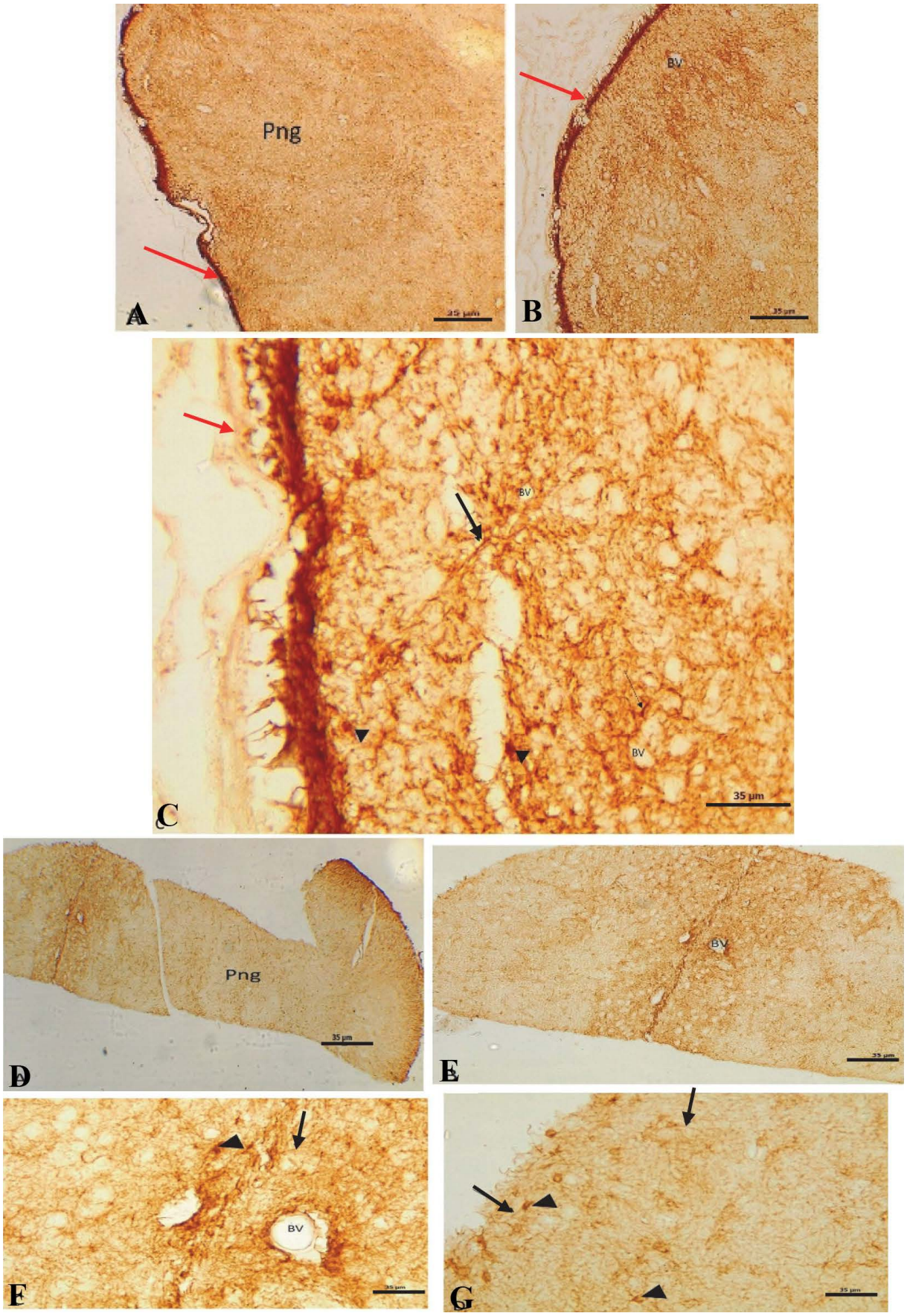

Fig. 4. Photomicrograph of the pineal gland of the female (A – C) and male (D – G) fruit bat showing immunoreactivity to GFAP.
A and **D** – entire coronal section of pineal gland (Png) and pia capsule (red arrows); **B** and **E** – Gland parenchyma showing blood vessel (BV); **C**, **F** and **G** – Pineal parenchyma showing astrocytic cells (arrow heads) with their long and slender processes (long black arrow). Scale bar – 105 µm (**A** and **B**), 35 µm (**C**, **F** and **G**), 70 µm (**D**), 140 µm (**E**).

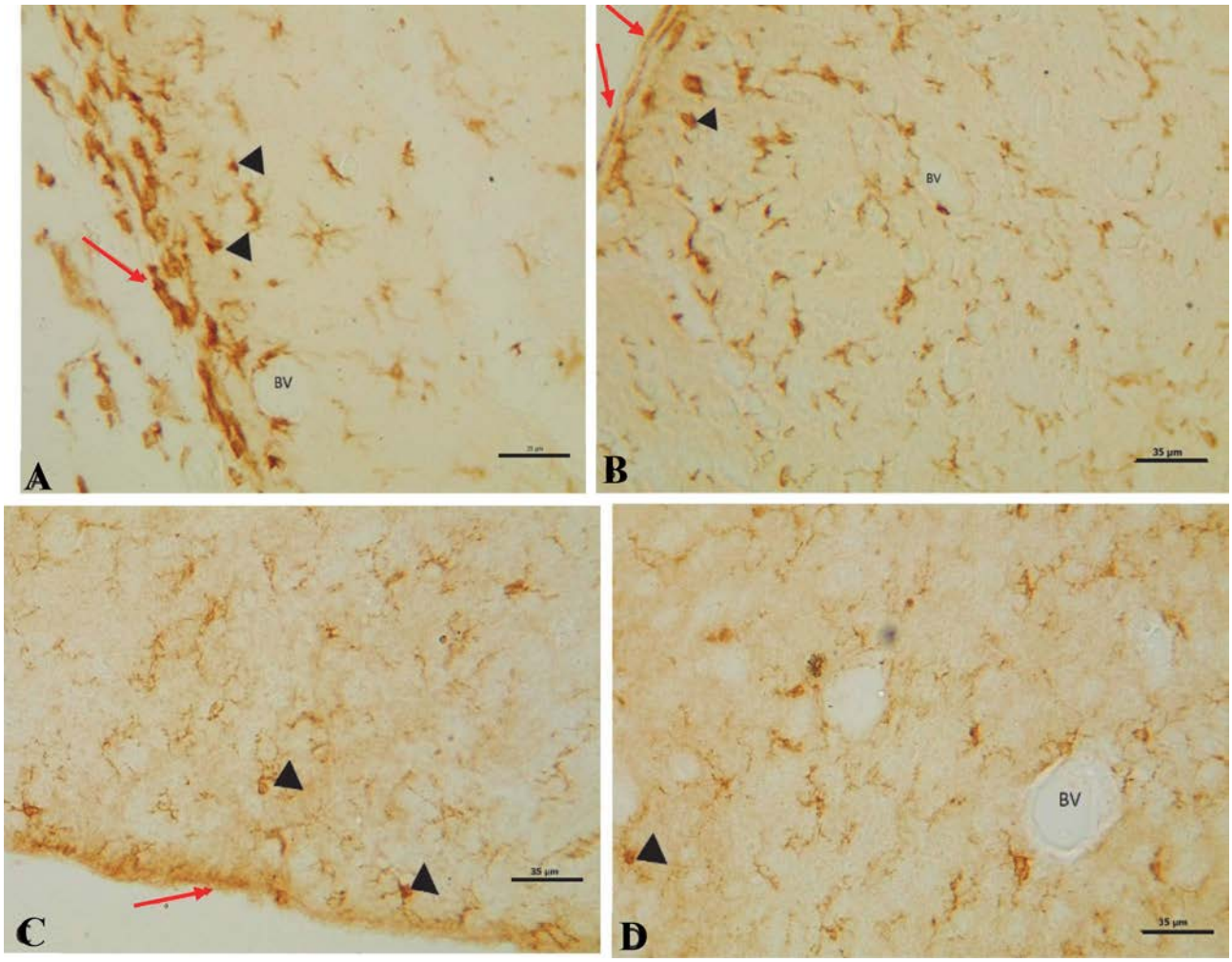

Fig. 5. Photomicrograph of the pineal gland of the female (A and B) and male (C and D) fruit bat showing immunoreactivity to IBA1.
A – Gland parenchyma showing expression of perivascular microglia around the blood vessel (BV); **B**, **C** and **D** – Pineal showing microglia expression in parenchyma (arrow heads) and pia capsule (red arrows). Scale bar – 35 µm for all micrographs.

pinealocytes appeared as round to oval shaped cells with a clear, abundant and acidophilic cytoplasm, and a large round nucleus (Figs. 2 and 3). Two categories of pinealocytes were observed: Type I being the light pinealocytes (possessing a lightly stained cellular content), and Type II being the dark pinealocytes (possessing a dark stained cellular content). The female bat pineal appeared less dense (pinealocyte-wise) at the periphery than within the parenchyma, a feature not observed in the male pineal (Figs. 2 and 3). In addition, „immediately inward" to the periphery, there was a line of condensation of cells (Fig. 2A) which was not seen in the males (Figure 3A). The arrangements of the pinealocytes did not follow a particular pattern. The blood supply of the gland occurred principally via the trabeculae of the connective tissue, where the majority of blood vessels were found (Fig. 3).

Immunohistochemical examination

Glial fibrillary acidic protein (GFAP): The pineal was densely populated with astrocytes, in both the male and the female, having the characteristic appearance, with the long and slender processes being distinct within the parenchyma, below the pia capsule and around the blood vessels (Fig. 4).

Iba1: Iba1 immunoreactivity in the pineal gland of the African fruit bat showed the expression of perivascular microglia round the blood vessel and capsular microglia in the pial capsule of the pineal gland (Fig. 5).

NeuN: The pinealocytes in the AGCR pineal gland had no immunoreactivity to NeuN antibody (Fig. 6).

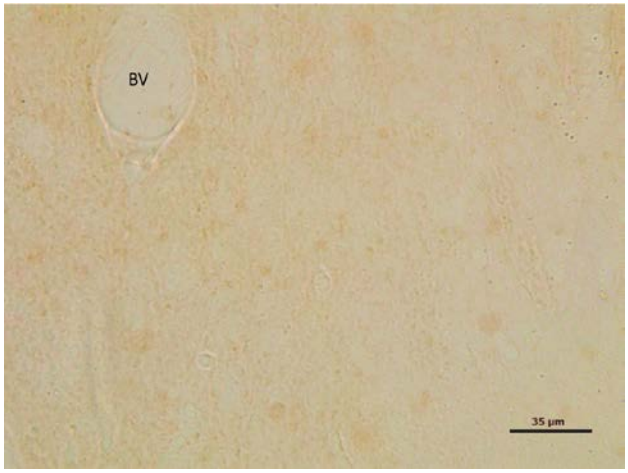

Fig. 6. Photomicrograph of the pineal gland of African fruit bat showing immunonegativity to NeuN antibody staining. BV indicates blood vessel.
Scale bar – 35 µm.

DISCUSSION

The *Chiroptera* (bats) are one of the most widely distributed and diverse group of mammals, with well over a thousand species. They are the only flying mammals, and also have a very wide range of feeding habits, roosting and social behaviours, and varied reproductive strategies [5]. They are seasonal breeders, roosting under varied conditions, from broad daylight, to total darkness, depending on the species [4]. These are some of the characteristics that make studying the pineal gland in the *Eidolon helvum* interesting.

Documented reports show varied sizes and gross appearance of the pineal in diverse bat species. This study showed the shape of the pineal gland in the *Eidolon helvum* to be spherical in shape, contrary to what was described in the *Rhinopoma microphyllum*, as oval, thin and antero-posteriorly flattened [3], whereas, in some species, the pineal was classified as being rudimentary (*Lonchophylla thomasi* and *Carollia perspicillata*), in some, could not be located (*Rhinonycteris aurantius* and *Triaenops persicus*), and so, were speculated to be either absent, or to consist of cell clusters which are diffusely arranged, making gross identification impossible [4]. Interestingly, some other Microchiroptera (*Peropteryx macrotis, Rhinolophus trifoliatus, Pipistrellus imbricatus, Tadarida mops*) examined showed relatively large pineal glands, superficially located and visible from the dorsal aspect of the brain, without the removal of any structure [4], contrary to this study where the cerebrum had to be partially removed to expose the pineal. However, grossly, the pineal gland observed in this study is generally consistent with the report of Bhatnagar et al. [4] that the gland is either round, conical or oval and is closely related to the 3rd ventricle. Therefore, the pineal gland of *E. helvum* can be classified as type A according to the Vollrath [24]. This was also consistent with a study of 191 bats with 47 Pteropodids where the pineal gland was either a Type A or Type AB [3].

Histologically, the parenchyma did not show a clear-cut 'dorsal-ventral' demarcation of cellular arrangement, as clearly defined in some other bats by Bhatnagar et al. [4], but nonetheless, there was a difference in cellular arrangement, with the periphery being less dense. In addition, our study revealed some sexual dimorphism with the cellular arrangement observed.

The pinealocytes are the major cellular component of the mammalian pineal gland, comprising about 90 % of all the cells, with glial cells also reported to occur in varying numbers [10]. Two types of pinealocytes are documented to be present in the pineal gland. The two types of pinealocytes observed were consistent with previous reports [17, 18]. The pinealocytes in bats reportedly present singly or in form of irregular cords or clusters [4]. However, observations from this study highlighted pinealocyte presenting more singly and with few clusters.

As of the time of writing this article, careful electronic search using Google Scholar and Pubmed (NIH) did not reveal any previous descriptive study on the bat brain using GFAP and/or IBA1 staining. Articles describing the glia cells used electron microscopy.

Astrocytic glia cells have a range of functions that indicate that they are involved in practically everything the brain does, varying from normal to pathological functions. Some of these functions include regulation of synaptic activity, synaptogenesis, neurogenesis, homeostasis, energy provision, ammonium detoxification, to mention just a few [19].

Previous reports assessing the glial cells in bats (pipistrelle bat) described glial cells as few and dispersed [18]. This description could not be said to be the same in this study, as the astrocytes and microglia were observed to be abundant in the capsular region and the parenchyma. The presence of these glia cells was observed in all the pineal glands harvested in this study. These bats were captured as free-ranging bats, travelling over quite long distances,

and exposed to the elements. It is understandable to have an appreciable presence of glia cells in the brain regions.

The absence of immunoreactivity to the anti-NeuN antibody, which specifically identifies the DNA-binding, neuron-specific protein NeuN, will need further investigation, as neurons are known to definitely be present in the pineal gland.

CONCLUSIONS

The pineal gland is associated with the circadian rhythm, which makes it an interesting study in nocturnal animals like the bat. In free-ranging migratory bats, elucidation of the histoarchitecture of the pineal may help to better understand the nocturnal habits of these species. In conclusion, this study highlights the presence of the two pinealocytes (light and dark) in the African straw-coloured bat, and the distribution of two types of glia cells – the astrocytes and the microglia. Further research will shed more light on the characteristics of the cells identified, highlight the presence of other cells in the pineal gland, and also give better understanding to the reason for the sexual dimorphism observed in the arrangement of the pinealocytes.

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Conflict of interest

The authors declare there are no conflicts of interest.

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COMPARISON OF THE EFFICIENCY OF DIAGNOSTIC TESTS USED TO PROVE GIARDIASIS IN TERMS OF THEIR PRACTICALITY AND USE IN THE VETERINARY CLINICAL PRACTICE

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ABSTRACT

Giardiasis is a protozoan disease that affects the health of animals, as well as other humans all over the world. Based on its host spectrum and genetic variability, *Giardia duodenalis* is classified into 8 assemblages (A–H). The present study was aimed at comparing the efficiency of the three most frequently used methods (the flotation method, the SNAP test and the ELISA assay) for the detection of giardiasis in carnivores in terms of the applicability thereof for the scientific purposes and the practicality of their application in the veterinary clinical practice. In the period from March 2020 to February 2022, a total of 173 faecal samples (141 samples collected from shelter dogs; 28 samples from pet dogs; and 6 samples from working dogs) were examined by applying the flotation method. The prevalence of *Giardia duodenalis* identified by the flotation method was 25 %. The SNAP test conducted with the fresh faecal samples revealed the high-level efficiency of 96 %, whereas the enzyme-linked immunosorbent assay (ELISA) achieved the efficiency of 65 %. By applying the nested PCR method, five samples were positively tested for assemblages C and D (*G. canis*) by the amplification of the *bg* and *tpi* loci. The dogs from shelters which were positive for *G. duodenalis* were also presented with a coinfection caused by other intestinal parasites, such as *Trichuris vulpis* (28.0 %) and parasites from the *Ancylostomatidae family* (8.0 %).

Key words: assemblages; dogs; ELISA assay; flotation methods; *Giardia duodenalis;* giardiasis; nested PCR; SNAP test

INTRODUCTION

Giardia duodenalis (also known as *G. intestinalis* or *G. lamblia*) is an intestinal parasite that occurs in developed, as well as developing countries. It is a cause of about 280 million cases of diarrhoea in humans per year [28]. Giardiasis is a zoonotic opportunistic disease with a cosmopolitan spread, afflicting primarily young and immunocompro-

mised individuals. Its developmental cycle is simple and direct, and it does not require any vector or intermediate host to infect a new host. The cycle is based on the alternation of the vegetative stage – a tropozoite, and an environmentally resistant infectious stage – a cyst. A cyst is the only stage of *Giardia duodenalis* in which it is able to survive for a long time outside a host, and it is responsible for launching a new infection cycle [20]. The disease is transmitted by the faecal-oral route and after the ingestion of food or water contaminated by the cysts. The disease is therefore classified as a waterborne and foodborne disease [11, 22, 30, 41, 42].

The basic method for confirming the giardiasis is a microscopic finding of *G. duodenalis* cysts in animal or human faeces. The prevalence of giardiasis may differ, depending on a selected examination method [39].

The standard technique that is used for the detection of *G. duodenalis* cysts is the flotation microscopic examination with the use of the flotation solution (Faust's solution – 33 % $ZnSO_4$). Since the cysts are excreted intermittently, sometimes with a pause lasting for several days, it is necessary to examine at least 3 separate faecal samples within the period of 5–7 days, or to examine the samples collected on three consecutive days [10, 37, 40]. The other diagnostic techniques that are frequently used include, for example, the rapid SNAP test, ELISA (enzyme-linked immunosorbent assay), IFA (indirect fluorescent antibody test) and DFA (direct fluorescent antibody test), whereas the PCR test is used to confirm the presence of assemblages [34, 38]. However, these techniques must be conducted in laboratories with the special equipment and the personnel with the required expertise, and they require higher financial costs.

The application of the commercial ELISA assay aimed at proving the presence of a coproantigen is a highly-sensitive technique (sensitivity: 99–100 %; specificity: 96–99 %), and its advantage is that it is not dependent on the presence of cysts in the examined samples [27, 33]. Veterinary clinics frequently use the SNAP tests, which are based on the principle similar to that of the ELISA assay; however, they provide very fast results – within 10–15 minutes [31, 33]. The presence of coproantigens is most frequently confirmed by applying the flotation method, while the ELISA assay and the SNAP test are the next two most frequently used techniques. Some authors state that compared to the microscopy, the IFA and DFA tests for the detection of *Giardia* cysts exhibit higher sensitivity and specificity (up to 100 %) [8, 32].

The genetic characterisation of *G. duodenalis* is conducted by applying the conventional nested PCR method with the exact identification of assemblages and sub-assemblages. There are several different protocols for the study of various gene loci (*gdh, tpi* and bg) with the use of specific primers [6, 18]. At present, the most frequently used protocol is the MLST (Multilocus Sequence Typing), which is based on multiple (up to five) genes. It is aimed at studying the genetic variability, the ability to divide the individual isolates into groups and detect the mixed infections. The real-time PCR method is also one of the methods that are used to detect the assemblages, primarily from human isolates. There are many research projects that are currently investigating into the confirmation of cysts in the environment and water [4, 31].

Based on the genetic differences, *G. duodenalis* is categorised into eight assemblages. Assemblages A (*G. duodenalis*) and B (*G. enterica*) are regarded as zoonotic and have been confirmed not only in humans but also in a wide spectrum of hosts [5, 9]. The remaining 6 assemblages are host-specific. Assemblages C and D (*G. canis*) are typical of dogs and other canids. In domestic mammals, such as cattle, sheep and goats, the assemblage E (*G. bovis*) was detected. The assemblage F was found in cats (*G. cati*), the assemblage G (*G. muris*) in rats, and the assemblage H (*G. simondi*) in marine mammals [14, 42].

The disease may be asymptomatic, acute or chronically causing catarrhal gastroenteritis. The symptomatology may vary, depending on the assemblage, the virulence of the infecting strain of *Giardia*, the current state of the host's immunity, the host's food, a coinfection with other enteropathogens, the composition and the function of the resident microbiota and the immunity modulation of *Giardia* (Variant-specific Surface Protein), while the environmental factors also play an important role [3, 4, 7, 8, 10, 14]. The interrupted excretion of cysts, a low count of cysts in faeces, and asymptomatic infections may complicate the diagnostics [23]. Farm animals, particularly the beef cattle, may suffer from diarrhoea, retarded growth, loss of weight (thus exhibit decreased productivity) and even death; all that results in significant economic losses [5, 18]. One of the main clinical symptoms of giardiasis in carnivores is diarrhoea, which is caused by the malabsorption combined with the hypersecretion [33]. The faeces are

characterised by a strong smell and the presence of mucus; they should never contain blood [13]. Giardiasis is more frequently detected in dogs from shelters that in pet dogs; this is primarily caused by the poor hygiene conditions in shelters, a higher concentration of individuals at one place, shared enclosures and the subsequent contamination of the environment with faeces containing the infectious stages of parasites in shelters [12, 16, 17].

MATERIALS AND METHODS

In the period from October 2020 to February 2022, a total of 173 samples of dog faeces were examined. The samples were collected from the dogs kept in the shelter of the Union of Mutual Help of People and Dogs (UVP) near Košice. The other examined groups included the dogs from households in Košice and the surrounding regions, and the working dogs from Bratislava. There were two age groups: the dogs younger than 6 months and the dogs older than 6 months. All the samples were examined by applying the coprological flotation technique, based on the differences in the specific weights of the propagation stages and the flotation solution that is used. The post-flotation positive samples were subjected to the commercial diagnostic tests: SNAP, ELISA and PCR. The IDEXX SNAP *Giardia*® testing kit (IDEXX Laboratories Inc., Westbrook, Maine, USA) is used as a rapid enzyme immunoassay for the detection of *Giardia* antigen in the faeces of carnivores. A positive test result confirms the presence of the coproantigen and a high probability of the presence of cysts excreted with faeces into the environment. According to the manufacturer's instruction manual, it is possible to examine samples made of fresh faeces, faeces stored for a maximum of seven days at 2–8 °C, or frozen faeces. Therefore, the assay efficiency was tested using the fresh samples, as well as the samples that were frozen and then defrosted after 6, 9 and 12 months. A similar principle is used in the *Giardia lamblia* Antigen ELISA kit (EAGLE BIOSCIENCES, INC., Nashua, USA), which is intended for the qualitative detection of the *Giardia duodenalis* antigen in faeces. In our present study, the ELISA assay was conducted with the frozen samples only. The 26 positive samples with moderate to high intensity of infection were included in SNAP and ELISA detection. For the molecular identification of the assemblages, we used Nested – PCR method, amplifying the genes sequences *triose-phosphate isomerase* (*tpi*) and *β-giardin* (*bg*), following the protocols published by Sulaiman et al. 2003 and Feng, Xiao 2011 [13, 36].

RESULTS

Flotation method

Out of the total number of 173 samples, 141 faecal samples collected from the shelter dogs were examined during the study period. The positivity was confirmed in 32 samples, representing the 23 % prevalence. Out of 28 tested samples of faeces from the pet dogs, 9 were positive (32 %). The presence of *Giardia duodenalis* cysts in the working dogs was detected in 2 samples, out of the total number of 6 samples.

SNAP test

The SNAP test was used to analyse 43 samples identified as positive by applying the flotation method. Out of 26 samples of fresh faeces, the presence of the *Giardia duodenalis* coproantigen was confirmed in 25 samples. The efficiency of the SNAP test with the fresh faecal samples was 96 %.

Twenty six frozen samples were tested after 6, 9 and 12 months. After 6 months, the test revealed positivity in 17 samples, representing the 65 % efficiency. After another period of 3 months, the samples were tested again, while 18 of the samples were found positive; the efficiency of the SNAP test was 69 %. After 12 months, the samples were tested for the third time. The SNAP test efficiency was identical to that identified after 9 months. There were 18 positive samples (Table 1). Figure 1 shows that the results of the individual tests were identical.

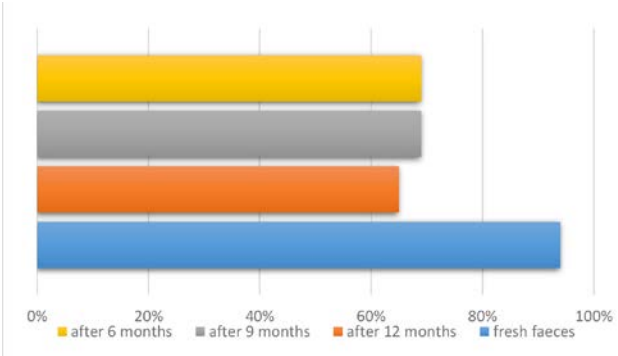

Fig. 1. Comparison of positivity in samples after 6, 9 and 12 months

Table 1. Examination of faeces by the SNAP test

Sample number	Fresh faeces	Frozen faeces after 6 m	Frozen faeces after 9 m	Frozen faeces after 12 m
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	-	-	+
6	+	-	-	+
7	+	-	-	+
8	+	+	+	+
9	+	-	-	-
10	+	-	-	+
11	+	+	+	+
12	+	-	+	+
13	+	+	+	-
14	+	+	+	+
15	+	+	+	+
16	+	+	+	+
17	+	+	+	+
18	+	-	-	-
19	+	+	+	+
20	-	+	+	+
21	+	+	+	+
22	+	-	-	-
23	+	+	+	-
24	+	+	+	-
25	+	-	-	-
26	+	+	-	-

m – months

ELISA assay

The ELISA assay was used to evaluate 26 selected samples that were identified as positive by applying the flotation method. The presence of coproantigen was detected in 17 samples (65 % efficiency).

Nested PCR method

The amplification of *bg* and *tpi* genes was carried out in 5 microscopically positive samples collected from the pet dogs. In the both cases, the amplification of 1 of the samples was unsuccessful. The genetic identification of the *G. duodenalis* species was confirmed by the direct sequencing of the PCR product. In 4 samples, the amplification of the *tpi* gene confirmed the presence of assemblage C, while the amplification of the *bg* gene identified, in addition to assemblage C, also assemblage D in one of the samples. Assemblages C and D are the specific assemblages that occur in dogs and other canids.

Coinfection with other endoparasites in the examined samples

Based on all 173 samples analysed by applying the flotation method, the dogs were found to be infected not only by *G. duodenalis* but also by other endoparasites. Their occurrence was only detected in the dogs kept in shelters. The frequency of the presence of parasites other than *G. duodenalis* was 26 %. The percentages of the occurrence of the individual endoparasites were as follows: *Trichuris vulpis* (38 %), followed by parasites from the *Ancylostomatidae* family (35 %), *Cystoisospora* spp. (15 %), and *Toxocara canis* (12 %). In 23 samples (13 %), various coinfections were detected. Out of them, 11 samples were found to be presented with a coinfection by *G. duodenalis* and other parasites. In these samples, the most frequently

identified coinfection was the coinfection by *G. duodenalis* and by *T. vulpis* (28 %).

A monoinfection by *G. duodenalis* was detected among the shelter dogs in 25 samples, representing 18 % of the total amount.

DISCUSSION

Endoparasitic intestinal infections affect the health not only of animals but also of humans, since many of them are transmitted zoonotically. In human populations, dogs are used for guarding, working, hunting and rescue purposes, but primarily as pets that are important companions in many households.

Despite the fact that they play an important role in human lives, they may be sources of parasites that infect humans. Giardiasis ranks among the most intensively spread parasitic diseases that occur in various regions all over the world. In the present study, the presence of cysts and the coproantigen was confirmed in the population of shelters dogs, pet dogs and working dogs. The prevalence of giardiasis varies and often depends on a number of factors, including an animal's age and health, its living conditions, a selected diagnostic method and a test group [39].

The application of the flotation method revealed the prevalence of giardiasis in 23 % (32/141) of the dogs kept in the shelter of the Union of Mutual Help of People and Dogs (UVP), while the prevalence in the pet dogs amounted to 32 % (9/28). The studies that included various groups of dogs, which lived in different living conditions, have confirmed that there were differences in the prevalence of giardiasis. A higher occurrence was identified in the dogs kept in shelters and in other groups. The factors which have negative effects on the spread of infections may include, in particular, an excessive concentration of dogs at one place and poor hygiene conditions. In the study conducted in 2014, authors A d a m o v á and Š t r k o l c o v á confirmed a 10.3 % higher prevalence in the very same shelter, while the frequency of the occurrence among the pet dogs amounted to 25.1 % (6/23) [1]. Three years later, M r a v c o v á et al. confirmed the 16.6 % (9/54) occurrence of this disease in the UVP [21]. In years 2010–2012 in Wroclaw, out of 128 examined faecal samples collected from the pet dogs, 27 were positive – the prevalence amounted to 21.1 % [24]. In Germany, the 9.5 % occurrence was identified in the pet dogs [8]. In a study conducted in Romania in the years 2008–2009, authors investigated into the occurrence of parasitic infections in pet dogs, shelter dogs, herding dogs and breeding dogs. The positivity rate for the shelter dogs amounted to 16.5 % (27/164); as for the dogs kept in breeding stations, it was 7.2 %; 4.8 % for the pet dogs; and 4.3 % for the herding dogs [19]. In a study conducted by U i t e r j w i j k and N i j s s e in 2018, significant differences were found between the different populations in the prevalence of *G. duodenalis*, while the highest prevalence of *G. duodenalis* was identified for the hunting dogs (64.9 %) and the lowest one for the pet dogs (7.9 %) [40].

In our present study, after the coproantigen was confirmed, the both tests (SNAP and ELISA) were used with the same samples in the same quantities. The IDEXX SNAP *Giardia*® test exhibited the highest efficiency – as much as 96 % (25/26) with the use of the fresh faecal samples. The tested samples also included the frozen faecal samples, stored at temperatures below -20 °C. In this present study, for the faecal samples that were defrosted after 6 months of the execution of the flotation method, the efficiency was proved to amount to 65 % (17/26). The efficiency of the SNAP test after 9 and after 12 months was 4 % higher than the efficiency after 6 months.

In a study conducted by U i t e r w i j k and N i j s s e in 2018, out of 4 applied diagnostic tests (IDEXX SNAP *Giardia*®, qPCR, CSF and DFA), the highest specificity (99.6 %) was identified for the IDEXX SNAP *Giardia*® [40]. In the present study, the Antigen ELISA assay for faecal *Giardia duodenalis* was used with the defrosted faecal samples, and the confirmed efficiency amounted to 65 % (17/26). The same percentage was achieved in the evaluation of the samples defrosted after 6 months by applying the SNAP test. In an investigation in Iraq, authors applied the ELISA assay and identified the 76.4 % sensitivity, compared to that of the microscopy, while the specificity amounted to 100 % [3]. In a 2018 study by S o m m e r, the prevalence of 30.6 % was identified by applying the ELISA assay for *Giardia* coproantigen [35].

In terms of the efficiency, the flotation method ranks among the most efficient techniques that are used in the diagnostics of the disease, considering the obtained results. The SNAP test is mostly used in private clinics, as it facilitates fast diagnostics of the disease. The use of the ELISA method is adequate primarily in laboratories that conduct

the epidemiological or clinical research. In the case the ELISA test is conducted with fresh faeces, the resulting percentage of positivity might be higher.

The results of our present study indicate that the SNAP test, conducted with fresh faeces, exhibited a high degree of efficiency, approaching to that of the flotation method. However, it is necessary to take into account that it is three times cheaper to apply the flotation method than to apply the SNAP test, and that it is more environment-friendly and has lower impact on the environment. One of the advantages of the SNAP test is that it is practical, fast and simple in terms of the required technical skills, compared to the flotation method and the ELISA assay.

All the samples tested for the presence of coproantigen by applying the SNAP test and the ELISA assay were flotation-positive, and the tests were conducted while strictly following the manufacturer's instructions; nevertheless, in 1 fresh sample, the SNAP test failed to detect the coproantigen. However, after 6, 9 and 12 months, the coproantigen was confirmed.

Despite the efficiency values of the SNAP test and the ELISA assay that were identified in the present study, the flotation method still remains to be the most efficient, economical, ecological and efficient method for the diagnostics of giardiasis.

The selected positive samples were used to identify the assemblages of *Giardia duodenalis* by applying the nested PCR. In ten microscopically positive samples, the PCR method was applied and the assemblages C and D (*G. canis*) were detected by the amplification of *bg* and *tpi* loci. In 2020, in a study conducted in Egypt, the PCR method was applied and the same assemblages were detected in dogs [29]. In the same year, the occurrence of assemblages C and D was also confirmed in a Polish study through the amplification of the β-giardin locus [23]. One year later in Columbia, the amplification of *bg*, *tpi* and *gdh* loci was applied and assemblages C and D were confirmed in 6 samples of dogs faeces [14]. A study by M r a v c o v á et al., conducted in 2017, confirmed the presence of assemblage C in 8 samples [21]. In the research conducted in Berlin in 2019, a total of 6 samples were detected, and in addition to the presence of assemblage C, they also confirmed the presence of assemblage A, assemblage D and a combination of assemblages A and B in the faecal samples [26]. In the research in Netherlands, conducted in 2001, authors came to the conclusion that assemblage A is associated with episodic diarrhoea, while persisting diarrhoea was associated with the genotype B [15].

In our present study, in addition to the presence of *Giardia duodenalis* in the population of shelter dogs, the presence of other endoparasite species was also confirmed. The highest occurrence was found for *Trichuris vulpis* (38 %) and parasites from the *Ancylostomidae* family (35 %). Similar results of the detected occurrence were published in Cuba in 2015, where the most frequently detected parasites were particularly *Ancylostoma caninum* (21.4 %) and *Trichuris vulpis* (16.3 %) [25]. In the present study, the presence of *Cystoisospora* spp. (15 %) and *Toxocara canis* (12 %) was also detected. In Romania, the dogs positive for *Giardia* spp. exhibited the coinfections with other intestinal parasites: *Toxocara canis* (26.9 %); *Cystoisospora ohiensis* (23.1 %); *Ancylostoma caninum* (17.3 %); *Uncinaria stenocephala* (13.5 %); *Neospora caninum* (9.6 %); *Sarcocystis* spp. (9.6 %); *Cystoisospora canis* (7.7 %); *Capillaria aerophilla* (5.8 %); *Strongyloides stercoralis* (3.8 %); *Dipylidium caninum* (1.9 %); and *Toxascaris leonina* (1.9 %) [19]. The results of the present research indicated the presence of coinfections by *Trichuris vulpis* (28 %) and by parasites from the *Ancylostomidae* family (8 %), while in 8 % of the samples, the coinfections by *Giardia* spp. and by the both above mentioned parasites were detected. The occurrence of parasites other than *G. duodenalis* exclusively in the shelter dogs may indicate a higher susceptibility to the infection due to the insufficient application of the deworming procedures with the use of efficient antiparasitic preparations, or due to an excessive environmental burden caused by the presence of cysts or eggs in the soil, and in some cases also due to their prolonged survival time.

CONCLUSIONS

The purpose of this study was to confirm the prevalence of Giardia duodenalis in dogs kept under different living conditions by using three methods (flotation method, SNAP and ELISA tests), and to compare the effectiveness of those methods in terms of their practical use, price, and use in veterinary clinical practice. The prevalence of giardiasis varies and often depends on a number of factors, including an animal's age and health, its living conditions, the selected diagnostic method and a test group. The One Health approach plays an important role in the diagnostics of giardiasis as the cause of diarrhoea in animals and

humans, and in the management of cysts spreading in the environment.

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TOPICAL APPLICATION OF HYPOTHERMIA IN A PORCINE SPINAL CORD INJURY MODEL

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ABSTRACT

Spinal cord injuries (SCIs) are catastrophic events in humans and animals. They often result in permanent loss of motor, sensory, and autonomic functions caudally from the site of the spinal cord (SC) lesion. The natural history of spontaneous recovery from SC trauma is disappointing and currently available therapeutic interventions fail to operate. Hence further research using bigger experimental animals or primates is necessary. The results of this study performed by the authors in 21 Göttingen-Minnesota-Liběchov female minipigs (3 sham controls, and 18 members of an experimental subgroup) showed that these animals are suitable for SCI research. All minipigs survived rather complex experiments carried out in general anaesthesia induced by 5 % thiopental solution administered i.v., maintained by endotracheal inhalation of 1.5 % sevoflurane with O_2 as well as a subsequent 9-week monitoring period. The experimental procedures comprised of L3 laminectomy, SCI inflicted by computer-controlled metallic piston crushing the SC with 8N, 15N, or 18N force. After the SCIs there were 9 minipigs left over during the next 5.5 hours in general anaesthesia, without application of hypothermia, then the surgical wounds were sutured, and the animals were allowed to awaken under supervision. Just 30 min following SCIs was in 6 minipigs started with the 5-hour application of 4 °C saline via perfusion chambers placed at the epicenter of the SCI, the chambers were removed, surgical wounds sutured, and animals were allowed to awaken. Just 30 minutes following the SCIs, there was in 3 minipigs started with a 5-hour administration of ≈24 °C saline at the epicentre of the SCIs, and then the perfusion chamber was removed, surgical wounds sutured, and the animals were allowed to awaken. The 5.5-hour local hypothermia and protracted general anaesthesia required monitoring of rectal temperature, and external warming of the minipig, if the temperature dropped below 36 °C. The currently available information on the therapeutic capacity of the method, and all technical aspects of its routine employment, needs validation in further experiments and preclinical trials.

Key words: experimental model; hypothermia; minipig; spinal cord trauma

INTRODUCTION

Spinal cord injuries (SCIs) imposed by mechanical force on the delicate structures of the spinal medulla (SM) are frightening pathological conditions associated with significant morbidity and mortality [20, 31]. Clinical symptomatology, characterized by paresis or paralysis, paraesthesia, pain, and cardiovascular, respiratory, bladder, bowel, and sexual dysfunction, is caused by a partial, or complete interruption of motor, sensory, and autonomic innervation of the body caudally from the site of the SM lesion. Due to the restricted regenerative capacity of the central nervous system of adult mammals, the consequences of SCI are usually permanent, and the situation of the affected individuals are further aggravated by frequently occurring life-threatening complications [2, 31]. That is why severe SCIs belong among the most challenging problems in both human and veterinary healthcare practice [2, 15, 31]. Since the regenerative therapeutic methods with promising results in rodent experimental models fail translation to bigger mammals and humans, the neuroprotective strategies become highly attractive [7, 10, 16]. The protective effects of hypothermia, are well-known and the method is still recommended as a therapeutic measure in critical situations [6, 11, 13, 24]. This inspired the authors to report on their experience with transdural cooling of the epicenter of spinal cord trauma in a porcine experimental model.

MATERIALS AND METHODS

A group of 21 female minipigs Göttingen-Minnesota-Liběchov crossbreed strains 5–8-month old weighing 25–35 kg were randomly divided into 7 subgroups: 1) Sham controls (n = 3); 2) Laminectomy of L3 vertebra, SCI by force 8N at a velocity of impactor movement 30 $mm.s^{-1}$, without hypothermia (n = 3); 3) L3 laminectomy, SCI 8N, a velocity of impactor movement 30 $mm.s^{-1}$, 5-hour hypothermia of the epicenter of injury by 4 °C saline (n = 3); 4) L3 laminectomy, SCI 15N, velocity of impactor movement 30 $mm.s^{-1}$, without therapy (n = 3); 5/ L3 laminectomy, SCI 15N, a velocity of impactor movement 30 $mm.s^{-1}$, 5-hour local hypothermia by 4 °C saline (n = 3); 6) L3 laminectomy, SCI 18N, a velocity of impactor movement 10 $mm.s^{-1}$ without therapy (n = 3); 7) L3 laminectomy, SCI 18N, a velocity of impactor movement 10 $mm.s^{-1}$, 5-hour local hypothermia of epicentre of injury by ≈24 °C (room temperature) saline (n = 3). The female minipigs were used with an aim to exclude the possibility of the development of penile prolapse, followed by the potential occurrence of decubital necrosis of the phallus – a life-threatening complication in paraplegic hogs [25].

For 3 days prior to the execution of the experimental procedures, minipigs were administered „Norostrep" (comprising of 100 mg penicillin, with 125 mg streptomycin in 1 ml of solution), intramuscularly (i. m.) one time a day. Approximately 30 min before the introduction of the general anesthesia for experimental procedure the minipig was premedicated by azaperonum (2 $mg.kg^{-1}$), and atropin (0.5 $mg.kg^{-1}$) administered i.m. After induction of general anesthesia with thiopental (10 $mg.kg^{-1}$) administered intravenously (i.v.), the minipig was intubated with an endotracheal cannula, placed on a volume-cycled ventilator of anesthetic apparatus and the anesthesia was maintained by inhalation of 1.5 % sevofluran with O_2. During the whole experimental procedure, i.e. L3 laminectomy, spinal cord contusion, and 5-hour therapeutic hypothermia application, the analgesia was supported by the administration of butorphanol (0.4 $mg.kg^{-1}$) via catheters inserted into the cephalic or auricular veins. Following the identification of the L3 vertebra with the help of plain X-ray in lateral projection (Fig. 1), the standard approach to the dural sac containing the spinal cord (SC) at the mid-lumbar level (L3 laminectomy) was performed using the same technique successfully employed in the thoracic part of the vertebral column previously [26]. In line with the recommendations of veterinary orthopedists, we carefully preserved facets to prevent the development of vertebral column instability [12]. The SCI was carried out by a 5 mm thick circular metallic rod driven by a stepping motor (ViDiTo, Košice, Slovakia) controlled by the computer software (FORCE, ViDiTi). To prevent a violent contraction of paravertebral muscles caused by a direct irritation of spinal medulla by the impactor resulting in further uncontrollable damage of delicate SM tissues, each minipig was intravenously administered muscle relaxant (Succinylcholin in a dose 100 mg) about 30 s before execution of the experimental trauma (Fig. 2). In minipigs from subgroups No. 2, 4, and 6, i.e., animals following SCI but without application of therapeutic hypothermia, the possible bleeding from small vessels was stopped by bipolar coagulation, blood clots

from epidural space removed by wet cottonoids and suction, laminectomy and both small skin incisions sutured. To ensure similar conditions for all minipigs, these animals were kept in endotracheal anaesthesia for another 5.5 hours.

In minipigs from subgroups No. 3 and 5, the 4 °C cold saline, and in minipigs from subgroup No 7 the saline at room temperature (≈24 °C) was applied to the spinal dural sac containing the spinal cord directly above the epicenter of SCI with the help of a perfusion chamber for five hours (≈300 min). The height of the chamber was 50 mm, and its weight was 4 g. The chamber which has a conical shape was manufactured from akrylonitrilbutadien (ABS plastic) by a 3D printer. The upper oval aperture (bound to the skin) has 40 x 30 mm, and the bottom oval aperture (bound to the spinal cord) has 20 x 14 mm. The two small apertures located opposite each other were destined for the insertion of infusion tubes providing for continuous circulation of cooling solutions. The perfusion chamber was freely placed on the *dura mater* without fixation to the spinal dural sac or paraspinal muscles. Each infusion tube was lead out through a separate small socket and skin incision located outside the laminectomy (Fig. 3). The inflow tube was connected to the infusion flask containing a 0.9 % solution of NaCl (normal saline). The flask was immersed in ice-cold water (4 °C). The circulation of the cooling solution in the perfusion chamber was carried out by a peristaltic pump (Heidolph Instruments GmbH, Germany), and the flow rate was maintained at 2 $ml.min^{-1}$. The outflow tube drained the excess saline from the perfusion chamber. The epidural temperature of saline in the vicinity of the SC injury site was monitored by the needle-tip thermometer (Omega Bio-Tek Inc., Norcross, Ga, USA). The rectal temperature of every minipig was monitored by the digital thermometer, at 15 min intervals. To minimize the fluctuations in body temperature of minipigs throughout the whole experimental procedure (it usually took about 6 hours). When the rectal temperature decreased below 36 °C, the animals were covered with a thermal blanket, and the warm air from a hair dryer was blown below the blanket. After execution of the experimental procedures, (SCI followed by 5.5-hour general anaesthesia and closure of laminectomy by the suture in anatomical layers, or SCI followed 30 min later by 5-hour local application of therapeutic hypothermia, then removal of perfusion chamber and suture of surgical wounds in anatomical layers) the

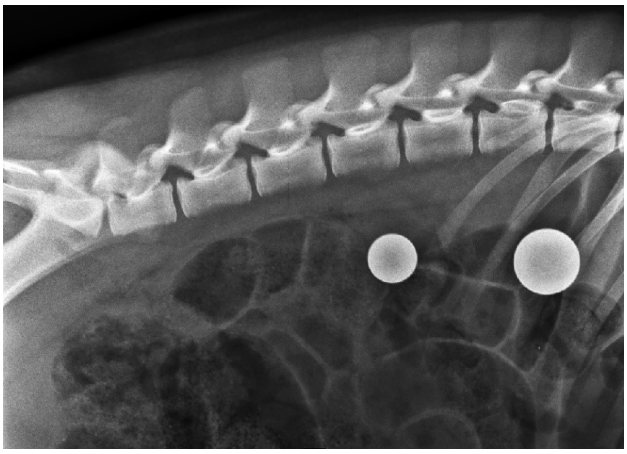

Fig. 1. Plain röntgenogram of one minipig in lateral projection helps to identify the L3 vertebra.
A personal observation.

minipigs were left to fully recover from anaesthesia. Later on they were placed into daily cleaned and disinfected pens for permanent housing. When fully conscious, the animals were offered drinking water *ad libitum*, and the next day, also a full diet. During three postoperative days, the pains were suppressed by intramuscular injections of 50 mg tramadol in 12-hour intervals, then penicillin with streptomycin („Norostrep“) in a dose of 0.5 $ml.30\ kg^{-1}$ per 24 hours for the next 10 days. To prevent the development of decubital necroses, the skin at the tails, pelvic extremities, and in the perianogenital region of animals was monitored daily. If skin abrasions appeared, they were treated with a 0.1 % solution of Rivanol and an aerosol of oxytetracycline applied in 12-hour intervals. The survival period of all minipigs utilised in our experiments was 9 weeks. For statistical analyses, we used Kruskall-Wallis test, $P < 0.05$ was regarded a statistically significant difference [10].

At the end of the survival period (9 weeks), all 21 minipigs were euthanased in deep thiopental anaesthesia (10 $mg.kg^{-1}$ of body weight i.v.) by transcardial perfusion with 5000 ml of heparinized saline and fixed with 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. The approximately 10 cm long parts of lumbar spinal cords with the epicenter of injury in the middle were carefully removed from the vertebral canal, dissected from the *dura mater*, and post-fixed in the same fixative at the temperature of 4 °C for another 24 hours. The next step was cutting the part of the spinal cord into seven 1 cm long segments comprising the epicenter, and three cranial, as well as, three caudal blocks. All 10 mm long pieces of SC tissue were cryopreserved in a 30 % neutral solution

of saccharose (30 % saccharose in 0.1 M PBS) at 4 °C for another two days, then the blocks were cut into 30 µm thick transverse sections by the cryostat (Leica Microsystems, Wetzlar, Germany) and stored in PBS for further histological and immunohistochemical processing.

Ethical considerations

The experimental protocols were prepared in compliance with the Animal Protection Act of the Slovak Republic No. 15/1995 and approved by the State Veterinary Administration in Bratislava (Decision No. 1749/10-221) as well as by the Ethical Commission of the Institute of Neurobiology, Slovak Academy of Sciences in Košice. All efforts were made to restrict the number as well as suffering of minipigs in particular stages of the experiments.

RESULTS

All minipigs, members of subgroups No. 2–7 (18 minipigs) survived rather complex experimental procedures (more than 6 hours lasting general anaesthesia, identification of the 3rd lumbar vertebra by the help of X-ray, fixation of the minipig in a special immobilization apparatus, expose the spinal dural sac containing the spinal medulla by L3 laminectomy, execution of SCI by a special contusion apparatus, implantation of perfusion chamber, 5-hour application of therapeutic hypothermia, removal of perfusion chamber and reconstruction of the surgical wound) without complications.

The experimental trauma caused complete paraplegia in all minipigs unrelated to the force of impact and velocity of impactor movement. The animals were not able to induce contraction of muscles innervated from spinal cord segments caudally from the site of injury, i.e. they did not move their tails or pelvic extremities, and they also lost voluntary control of defecation and urination (Fig. 4).

The temperature of saline in the perfusion chambers of minipigs from subgroups No. 3 and 5 decreased within several minutes to ≈17 °C, then mildly fluctuated influenced by warmer surrounding tissues. Further continual monitoring of the temperature of the cooling solutions showed that in minipigs from subgroup No. 3 it was possible to maintain it at 17.7 ± 1.04 °C and in minipigs from subgroup No. 5 at 17.65 ± 1.18 °C for the whole 5-hour application of therapeutic hypothermia.

The long-time monitoring (< 5.5 hours) of the temperature of the cooling solution in the perfusion chamber located above the epicenter of SCI (saline at room temperature, i. e. ≈24 °C) in minipigs from subgroup No. 7 did not exceed 24.06 ± 1.19 °C. The comparison of cooling solutions temperatures in the perfusion chambers of minipigs from subgroups No. 3 (17.7 ± 1.04 °C) and No. 5 (17.65 ± 1.18 °C) with cooling solution temperatures in minipigs from subgroup No. 7 showed a difference of 7.36 °C (subgroups 3:7), or 7.41 °C (subgroups 5:7), i. e. values statistically significant at $P < 0.05$.

The rectal temperature in all experimental animals mildly fluctuated. In minipigs from subgroup No. 3 was 36.18 ± 1.08 °C, in minipigs from subgroup No. 5 was 36.31 ± 0.92 °C and in minipigs from subgroup No. 7 it

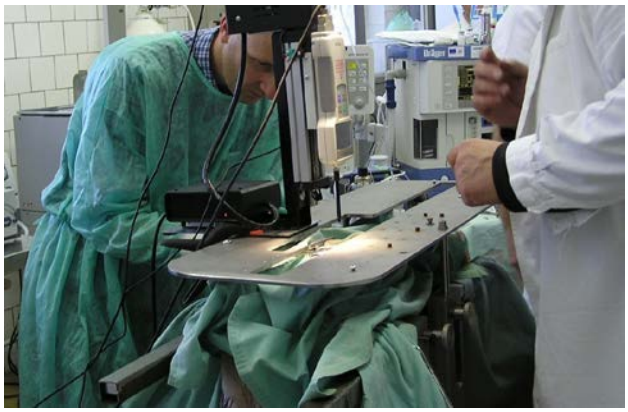

Fig. 2. Preparation of the computer-operated contusion apparatus and direction of the metallic impactor towards the spinal medulla exposed by L3 laminectomy in one of the minipigs.
A personal observation.

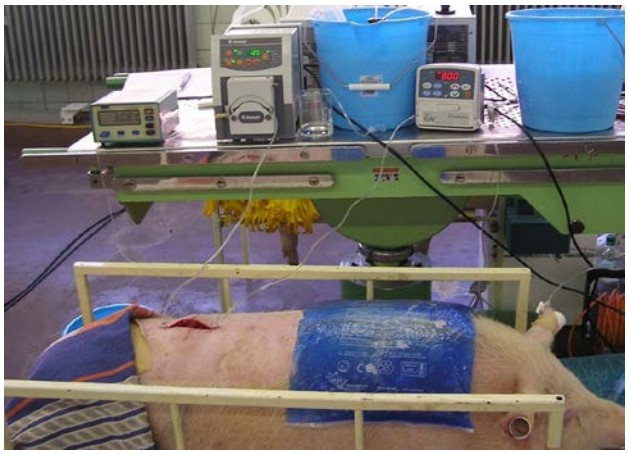

Fig. 3. Local application of 5-hour therapeutic hypothermia (4 °C saline) via the implanted perfusion chamber.
A personal observation.

Fig. 4. Demonstration of a motor deficit caused by SCI in experimental animals.
A personal observation.

was 36.06 ± 0.32 °C. It means the rectal temperature in all experimental animals during the 30 min preparatory phase and 5-hour period of 4 °C saline, or ≈24 °C saline application was relatively stable. The difference between average values of rectal temperature in minipigs from subgroups No. 3 and No. 5 was ≈0.13 °C (36.18 °C versus 36.31 °C). The difference between average values of rectal temperature in minipigs from subgroup No. 3 versus minipigs from subgroup No. 7 was ≈0.12 °C (36.18 °C versus 36.06 °C). The difference between average values of rectal temperature in minipigs from subgroup No. 5 versus minipigs from subgroup No. 7 was ≈0.25°C (36.31 °C versus 36.06 °C). Statistical analyses showed that despite the long-lasting anaesthesia (more than 6 hours) and 5-hour local spinal cord cooling by 4 °C or ≈24 °C saline, it was possible to maintain the core temperature of all minipigs at physiological value [8]. The expected negative effects of local spinal cord cooling and general anesthesia on the body temperature of a minipig weighing 28–35 kg [3] were eliminated by covering the minipig with a thermal blanket and intermittent blowing of warm air below it.

DISCUSSION

Spinal cord traumatic lesions result in serious neurological disorders that substantially alter the body functions of affected people or animals. The central nervous system in mammals does not regenerate [2, 15, 20]. The pathophysiology of SCI is possible to divide into two phases. The primary phase (primary injury) is initiated by the mechanical impact afflicted on the spinal medulla. The external force disrupts axons, blood vessels, and cell membranes. The rapidity of such an unexpected action prevents the application of any therapeutic measure during the primary phase. By contrast, the secondary phase (secondary injury) is a complex cascade of molecular events, such as disturbances in ionic homeostasis, development of spinal cord edema, ischemia, hemorrhage, free radical production, and action, inflammatory response, apoptosis, and cellular death. It takes a much longer time, so different therapeutic measures may be employed [21]. The notable fact is that all secondary phase SCI processes are stimulated by increased and mitigated by decreased body temperature, i.e. they are temperature dependent [9, 11]. Although the exact molecular pathways of the secondary injury are still not completely understood, therapeutic strategies inhibiting or delaying these pathological processes may contribute to the amelioration of the deficit after SCIs [2]. Therapeutic hypothermia (TH) is defined as a controlled reduction of a core body temperature (CBT) 1 centigrade below the physiological rate [3, 6, 8, 9]. There are two techniques for the application of TH – local (regional) and systemic (general) TH [9, 16]. The CBT reduction below 35 °C is considered mild hypothermia, the reduction of CBT to 34–32 °C is modest hypothermia, the reduction of CBT to 32–30 °C is moderate, and the reduction of CBT below 30 °C is considered profound hypothermia [29]. The positive effects of TH are mediated by deceleration of enzymatic activity, reduction of oxygen consumption, preservation of normal transmembrane ion gradients, improvement of adenosine triphosphate (ATP) accumulation, support of tight junctions between endothelial cells in spinal cord capillaries forming blood spinal cord barrier, suppression of edema formation, reduction of oxidative stress, and free radical generation, diminishing the release of proinflammatory cytokines, mitigation of inflammatory cell infiltration, the calpain-mediated proteolysis and mitochondrial dysfunction, leading to cell death. An especially important fact is that TH can discontinue the apoptotic pathway at the early stages of the secondary phase of SCI and improve outcomes [2, 16]. On the other hand, the lowering of CBT increases the blood viscosity (2 % per 1 °C), a decrease of CBT causes shivering and increases oxygen consumption, induces insulin resistance, suppresses leukocyte and phagocyte chemotaxis, and impairs immune functions [13]. A convenient alternative for bypassing the negative effects of systemic hypothermia is local cooling of

the epicentre of SCI. This method we decided to test in our experiments [10].

It is evident that animal models are necessary to study complex problems associated with spinal cord traumatic lesions. An ideal experimental model should imitate as closely as possible the situation arising in humans following SCI, be easily reproducible, provide the opportunity to impose exactly defined type and measure of spinal cord damage, help to identify pathological processes, facilitate to assess therapeutic options following SCIs, and their safety [1, 5, 10, 18, 19].

The traumatic SCIs in both, people and animals usually originate in traffic accidents, falls from a height, human-human, human-animal, animal-animal violent interactions, and degenerative intervertebral disc disease [4, 31]. The damage of the spinal medulla in these types of injuries is caused by displaced fragments of broken or luxated vertebrae, extruded intervertebral discs, osteophytes, or ligaments, which act as impactors that contuse the fragile neural tissue. Due to the high incidence of this type of SCI, we decided to use the contusion type of spinal cord trauma in our experiments. The contusion apparatus (ViDiTo, Košice, Slovakia) controlled by the computer software (FORCE, ViDiTi) was employed to reliably replicate the mechanism and force of impacts [10, 19].

The rat and mice experimental models are most frequently used in SCI research since these animals are small, easy to manipulate, relatively cheap, and have demonstrated similar functional, electrophysiological, and morphological outcomes to bigger mammals and humans [1, 5, 7, 18, 28]. However, many therapeutic interventions, which have shown positive results in rodents, failed in other experimental animals, and primates [1, 5]. The problems with the translation of positive achievements from rats and mice may be explained by the smaller size of rodents, their different metabolism, as well as the arterial blood supply to the spinal cords, and the more specific organization of the central nervous system in dogs, cats, pigs, apes, and humans in comparison with rodents [1, 7, 14, 17, 22, 28]. Undoubtedly, the highest similarity to human afferent and efferent spinal cord tract organization, thoracic extremities sensorimotor functions, as well [1]. However, the apes are too expensive, they need special attention, working with them requires experienced personnel, and they are difficult to handle [18]. Accordingly, the majority of neuroscientists prefer to use less challenging experimental models. It is well known that pigs share many morphological and physiological features with people, and their potential for modeling human brain and spinal cord pathological conditions has been widely recognized [1, 5, 18, 19, 30]. Positive experience with the Göttingen-Minnesota-Liběchov minipig crossbreeding strains inspired us to proceed with our experiments and try to assess the therapeutic potential of local hypothermia following spinal cord contusion injury in a slightly modified porcine SCI model [10, 19, 26, 27]. Modifications consisted of a shift of experimental SCI from thoracic to lumbar region, administration of muscle relaxant immediately before spinal cord trauma, contusion of spinal medulla with bigger impact force (to original 8N and 15N we added 18N), and increase in the temperature of cooling solution (besides the originally 4 °C saline we began to use also saline at room temperature, i.e. ≈24 °C). The main reason to shift the location of experimental SCI from the caudal thoracic to the lumbar region was an effort to facilitate localization of the L3 vertebra and to it matching the spinal cord segment by plain X-ray. In this manner it was possible to provide for more exact reproducibility of the experimental conditions in animals having the variable number of vertebrae [22, 23, 30].

CONCLUSIONS

This presented study showed that Göttingen-Minnesota-Liběchov minipigs are suitable experimental animals for SCI research. The surgical approach to the porcine spinal medulla through the L3 laminectomy is a standard procedure in the hands of an experienced surgeon, but the skeletotopic localization of the chosen vertebra for laminectomy by plain X-ray is a condition *sine qua non*. The execution of SCIs by a computer-operated contusion apparatus gaurantees reliability and reproducibility of the type and extent of spinal cord lesions. The special perfusion chamber, inserted into the laminectomy, facilitates the precise application of cooling solutions on the epicenter of SCI. The 5.5-hour local hypothermia and protracted general anesthesia require monitoring of rectal temperature and external warming of the minipig if the temperature subsides below 36 °C. The currently available information on the therapeutic capacity of the method and all technical aspects of its routine clinical employment needs further validation in additional preclinical trials.

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DYSPLASIA OF *TROCHLEA FEMORIS* IN DOGS

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ABSTRACT

This article provides a comprehensive analysis of *dysplasia trochlea* in dogs, summarizing the most recent findings in the field and highlighting important new findings, as well as, areas that require further research. The anatomy of the knee and the *trochlea femoris*, as well as its function in maintaining knee joint stability, are covered in the first section. The accurate diagnosis of orthopaedic disorders, and injuries are necessary for efficient treatment, and this is made possible by understanding the anatomy of the knee. The factors that can affect the onset, development, and course of the disease are included in the section that follows, which discusses the etiology, and pathophysiology. Veterinary surgeons must measure trochlear femur dysplasia in dogs in order to determine the severity of the condition and the best course of treatment. It is typically assessed by looking at X-ray images and assigning a score, however, this can differ from one individual to another. New measurement techniques that give a better level of objectivity and precision, like CT scans or MRIs, are now being tested.

Key words: dog; dysplasia; orthopaedics; *trochlea femoris*

INTRODUCTION

The knee joint is a complex synovial joint with condyloid femorotibial, femoropatellar, and proximal tibiofibular articulations [7, 14, 46]. The changing any of the joint's anatomical components might cause dysfunction and damage to other structures [7]. Osseous, fibrocartilaginous, and ligamentous tissues make up the dog's knee joint, which often causes lameness [32]. The quadriceps, patella, trochlear groove, patellar ligament, and tibial tuberosity make up the knee extensor mechanism. Any variation in this arrangement of structures might produce anatomical changes in the distal femur and proximal tibia during growth, resulting in patellar instability [11].

The femoropatellar joint, formed by the asymmetrically shaped articular surface of the patella, and the femoral trochlear groove, is an incongruent joint that is often impacted by painful, and disabling degenerative processes like

osteoarthritis. Incongruent joints, which trade stability for a range of motion, often develop pathogenic processes [9].

Dogs with trochlear dysplasia have shallow femur grooves, causing patella dislocation. This syndrome may be induced by persistent instability and lateral tracking, birth, or a combination of causes [2]. Patellar luxation severity affects symptoms. These include hindlimb lameness, structural anomalies such as femur or tibia bending, discomfort, and reluctance to move [47]. Medial patellar luxation affected 73 % and lateral luxation 27 % of large breed dogs [3]. The condition known as medial patellar luxation (MPL) exerts strain on the cranial cruciate ligament (CCL), resulting in its degeneration and subsequent rupture. The development of secondary osteoarthritis (OA) can be attributed to the presence of MPL [1].

MATERIALS AND METHODS

A thorough literature review was performed to fully investigate trochlear dysplasia in dogs. Electronic databases such as PubMed, Web of Science, and Google Scholar were exhaustively searched for the most recent publications. The search terms encompassed „trochlear dysplasia in dogs", „patellar groove dysplasia", „patellar luxation", „patellar instability", „patellofemoral instability", and different combinations of these terms. After the search, we selected studies that were directly relevant to trochlear dysplasia in dogs. This research looked into trochlear dysplasia's causes, progression, diagnostic techniques, clinical presentation, available treatments, and results. Titles and abstracts were screened, and then papers with potential relevance were evaluated in full text. The chosen studies that fulfilled the objectives of this evaluation were used to gather data.

Anatomical features of the *trochlea femoris* and its role in patellar stability

Femoris muscle and patellar tendon complexes include the largest sesamoid bone, the patella. It acts as a biomechanical lever, increasing the quadriceps force during knee extension. Additionally, it centralizes the quadriceps muscle's divergent forces and transfers the tension around the femur to the patellar tendon. The complicated patellofemoral joint, where the patella meets the femoral trochlea, can become unstable [44, 45].

The intercondylar notch of the patellofemoral joint extends into the trochlea. The functionality of the extensor mechanism and the stability of the patellofemoral joint depend on the shape of the trochlear sulcus. Trochlear dysplasia, also known as a shallow sulcus, can result in diminished angle and depth of the groove, a small medial facet, and anterior knee pain [4, 24].

The bone contour and cartilage depth determine the concave shape of the trochlea [49, 53, 55]. The depth and angle of the trochlear fossa on the femur are important factors that provide valuable insights because they affect the stability of the patella in the trochlear fossa. The deepest point of the trochlear groove is midway between the proximal face of the lateral ridge and 50 % of the sagittal length of the femoral trochlea in a healthy dog's knee joint. The patella is thickest in the distal half and less than half the depth of the trochlear groove. The patella's midsagittal connection to the trochlear groove is particularly evident [38]. Pettazzoni [42] measured the patellar bone's maximal craniocaudal thickness and trochlear groove depth using three-dimensional multiplanar reconstructive computed tomography. The mean maximum trochlear groove depth was 46 % of the patellar thickness [42]. Trochlear dysplasia has a significant impact on patellofemoral kinematics, contact pressure, contact area, and stability and appears in a variety of geometric configurations [54].

Etiology and pathogenesis

A survey of the literature in the field of human medicine revealed that most cases of dysplasia arise due to abnormal forces acting on the patellofemoral joint [21]. According to a study conducted on rabbits, inappropriate patellar positioning can cause excessive flattening of the distal femoral groove, which can increase the risk of patellar instability [20]. A study by L i et al. investigated the effect of patellofemoral instability on trochlear groove development in growing rabbits. Their research found that the pattern of patellar dislocation in young rabbits can cause dysplasia in the femoral trochlea. If patellar dislocation is present, it can affect the normal function of the quadriceps mechanism, leading to secondary bone changes [31].

The absence of physiological pressure of the patella on the articular cartilage of the trochlear groove during growth in dogs can prevent the development of a sufficiently deep and wide groove, which is known as trochlear hypoplasia [11]. This was confirmed in a study conducted on growing

rats. It was confirmed that surgical removal of the patella and dislocation of the patella in growing rats can lead to a dysplastic trochlear groove. Along with changes in the bony structure of the trochlear groove, early remodelling of both the articular cartilage and the underlying subchondral trabeculae occurs postoperatively. Therefore, inadequate loading of the patellar bone after birth may affect the morphological development of the *trochlea femoris* [58].

Recent research has shown the association between certain anatomical traits and patellar luxation in dog breeds. G a r n o e v a found in 2022 that tiny female dogs of particular breeds are more prone to medial patellar luxation due to anatomical factors. A lower patellar thickness and shorter medial femoral condyle allow the kneecap to leave the trochlear groove [16]. Pugs, French Bulldogs, and English Bulldogs without clinical patella luxation were examined for trochlear sulcus depth by M a t c h w i c k et al. [33]. A validated computed tomography ratio of maximum trochlear sulcus depth and maximum craniocaudal patella thickness was used. The study discovered that French and English Bulldogs had shallower trochlear sulcuses than Pugs, suggesting that breed-specific traits may affect them [33].

L o n g o et al. found that dogs with medial patellar luxation exhibited a different femoral trochlear groove angle. Small breeds had a shallower femoral trochlear groove angle than medium/large breeds [30].

Femoral rotational osteotomy with anteversion increased trochlear dysplasia in a new approach. Image analysis confirmed that trochlear shape greatly influences femoral anteversion. Femoral anteversion flattens and dysplasticizes the trochlea. Trochlear dysplasia flattens the femoral groove, yet this investigation discovered a boss in the anterior proximal trochlea. This boss will prevent the patella from smoothly engaging into the groove when the knee is flexed [20, 21, 22, 29, 57].

Trochlear dysplasia research has advanced. T a n g et al. revealed that patellar hypermobility and dislocation produce trochlear dysplasia, which worsens with age. The study indicated that inadequate or excessive loading can cause trochlear dysplasia [51]. Recently, an abnormality of the trochlear bone with preterm birth and obstetric presentation in rats has been detected. The obstetric presentation can cause trochlear abnormalities in neonatal knees. Trochlear dysplasia risk increases as the foetus stays *in utero* [28].

Clinical signs and diagnostics

Up to 85 % of patients with symptomatic patellar instability had trochlear dysplasia, which B r a t t s t r ö m [5] and D e j o u r [10] identified as a major contributor to repeated patella subluxation.

The forces required for subluxation or dislocation can be influenced by the depth and angle of the slopes on the articular facets on either side [20].

According to P o w e r s [43], patients with patellofemoral discomfort often have a smaller groove, and people who feel pain beyond 27 degrees of flexion show a considerable loss in groove depth. Significant research has been conducted on the distal femoral sulcus angle – a prominent landmark in the trochlear groove. A recent MRI study found that people with patellar instability have a less curved distal trochlear groove compared to people without pain [13, 20]. Furthermore, according to H o d e l et al., high-degree patellofemoral dysplasia in humans commonly coexists with frontal and axial leg misalignment [19]. The evaluation of abnormal trochlear development in small dogs can involve three stages of classification, with indications of mild and moderate trochlear dysplasia including a shallow trochlear groove and hypoplasia of the medial femoral condyle [15].

Higher sulcus angle values, as well as noticeably lower levels of trochlear depth, medial trochlear inclination, and the ratio of patellar thickness to trochlear depth, were indicators of changes in the depth and form of the trochlea [15].

The potential effects of patellar dislocation in growing rabbits are clarified by a recent study by D a i et al. [8]. It showed that this condition can result in severe subchondral bone loss and trochlear dysplasia, underscoring the significance of appropriate care and therapy. Furthermore, it was discovered that the cartilage degenerative alterations brought on by patellar dislocation will get worse with time. The expression of MMP-13, an enzyme that breaks down cartilage, increased whereas TRPV4 and collagen II, which are crucial for maintaining healthy cartilage, were expressed less. These results are in line with earlier studies implying that improper patellofemoral joint biomechanics and added strain can cause cartilage degradation [20]. In order to avoid long-term problems including trochlear dysplasia and cartilage degeneration, it emphasizes the significance of early diagnosis and adequate care of patellar dislocations.

Imaging techniques that can be used to diagnose trochlear dysplasia of the femur in dogs

A complex system of osseous, articular, fibrocartilaginous, and ligamentous components make up the stifle joint. However, accurate diagnostic imaging is challenging to achieve due to its constrained joint space, complex composition, and tiny component structures. Furthermore, the overlap between several tissue types can make it difficult to make a diagnosis using just one imaging modality [32]. With the help of lateral radiography views [35], skyline radiographic views [34], and CT images [52], trochlear depth assessment has been established. The use of sedation or anaesthetic may even be necessary with these techniques due to the need for proper placement. Planning surgical operations for people with reoccurring patellar instability and trochlear dysplasia can be made easier with a greater understanding of imaging techniques and the production of 3D models of patellofemoral joints affected by dysplasia [26].

Radiological evaluations are regarded as a crucial component of the treatment of patellar instability. However, assessments of patellar instability are affected by the knee posture, which cannot always be consistent between examinations [59]. The distal, middle, and proximal trochlear grooves cannot be distinguished in the skyline view, which can only provide a 2D evaluation of the 3D structure. Therefore, it might not provide a reliable and consistent measurement of the trochlear groove's depth and angle [20]. In contrast, G a r n o e v a claimed that tangential X-ray images of the canine stifle joints can sufficiently evaluate the groove's depth and shape [15].

In dogs, adding ultrasonography to clinical and radiographic exams can help with stifle joint disease diagnosis. Sonography can give a view of the soft tissue components of the joint whereas radiography can evaluate the bony components of the joint and their interactions [25]. When performing joint imaging, sound waves move more slowly through joint fluid and more quickly through bone. Ultrasonography is therefore particularly useful for examining soft tissue features in the stifle joint. This method is efficient for finding arthropathies, neoplasia, meniscal anomalies, cartilage, muscles, tendons, and ligaments. In a recent study, ultrasonography was found to be a reliable technique for determining the trochlear groove's depth and evaluating the femoral groove [36].

A standardized approach for precisely measuring the femoral trochlear groove using CT has been developed [30, 38, 42] The femoral trochlea can be evaluated in 3D using CT scanning, which has the benefit of allowing precise point identification when measuring [38]. The ability to manipulate 3D reconstructed pictures provided by CT scanning also makes it easier to identify specific points of interest [38, 39].

In general, radiography, CT scans, and MRI scans can all be used to evaluate the sulcus angle accurately, but only CT and MRI scans can be used to precisely measure the trochlear depth [48]. The Insall-Salvati (IS) ratio was the sole measurement used for CR, CT, and MRI that showed strong consistency and agreement between various observers as well as within the same observer [56].

Approaches for assessing femoral trochlear dysplasia

The trochlear dysplasia of the femur can be evaluated using a variety of techniques, such as:

1. Dejour system of classification: According to the Dejour categorization system, dysplasia can be divided into four types: Type A, Type B, Type C, and Type D (Table 1) [23]. It has been determined that Dejour's evaluation of trochlear depth is an important diagnostic tool for trochlear dysplasia and for estimating the possibility of future patella dislocation because it was the sole measurement judged pertinent for this use [41]. This measurement helps predict future risks of patellofemoral discomfort. The lateral inclination of the trochlea has emerged as a nearly perfect form of dependable measurement approach and may serve as a new benchmark for quantifying trochlear dysplasia in contrast to Dejour's method, which is unreliable [40].

2. The Insall-Salvati index: This index is calculated by dividing the maximum length of the patella – measured from the lowermost point to the uppermost point of the patella – by the length of the patellar tendon, which is measured from the lowermost point of the patella to the tibial tuberosity [56]. The Insall-Salvati ratio has drawbacks despite being a helpful tool. One of these restrictions is the ratio's insensitivity to the patella's morphology. The Insall-Salvati ratio can have misleading values because of the abnormal patella [17].

3. Caton-Deschamps index: This ratio is calculated by dividing the distance between the lowermost portion of the patellar articular surface and the anterosuperior point of the tibial plateau by the length of the patellar articular surface [56].

4. Tuberositas tibae and trochlear groove (TT-TG) distance: According to H i n g e l b a u m et al., the TT-TG

Table 1. Imaging characteristics of the various forms of trochlear dysplasia according to the Dejour classification [23].

Dejour type	Lateral radiograph	Axial image
Type A	Crossing sign	Shallow trochlea or symmetric concave trochlea
Type B	Crossing sign and supratrochlear spur	Flat or convex trochlea
Type C	Crossing sign and double contour	Asymmetry of the trochlea faces; lateral facet convex with hypoplastic medial facet
Type D	Crossing sign, supratrochlear spur, and double contour	Asymmetry of the trochlea faces; vertical slope demonstrating a "cliff" pattern

distance is increased between the tuberositas tibae and the trochlear groove [18]. The increasing TT-TG distance, or the distance between the tibial tubercle and the trochlear groove, is one of the main factors that raises the risk of patellofemoral instability. The externalization of the tibial tubercle and the medialization of the trochlear sulcus in trochlear dysplasia instances serve as objective indicators of the extensor mechanism's dysfunction [12, 37].

5. Sulcus angle: The measurement of the femoral trochlear groove's angle can be used to gauge the groove's depth before surgery and determine whether a deepening of the groove is required [30]. The surface area measurements for the articular cartilage and the subchondral bone contour have been shown to differ in the field of human medicine [36; 50].

6. Trochlear depth: The depth of the trochlear groove is a key factor in ensuring that the patella is stable over the front of the knee [20].

The sulcus angle, trochlear depth, and Dejour's classification are the metrics that have received the most attention [48]. Analysing these characteristics before surgery can enhance surgical planning and decision-making [15].

Methods of measuring trochlear dysplasia of the femur

Several measurements have been developed and presented to describe trochlear dysplasia, but experts cannot agree on which measurements should be used to diagnose the condition or choose the best course of action [41].

The sulcus angle, anterior and posterior condylar angles, and the heights of the medial and lateral femoral condyles are some of the factors that define the morphology of the trochlear anatomy. Measuring several morphometric parameters is required for the radiographic assessment of trochlear morphology in dogs. According to a study by G a r n o e v a [15], five of these indicators are significant for the identification of canine trochlear dysplasia. There were measured six morphometric factors to look into how trochlear dysplasia develops in dogs. The trochlear sulcus angle (SA), lateral and medial trochlear inclination angles (LTI; MTI), trochlear groove depth (TD), patella thickness (PaT), and the ratio of trochlear groove depth to patella thickness (PaT/TD) are among the measurements that are comparable to those employed in human medicine [15].

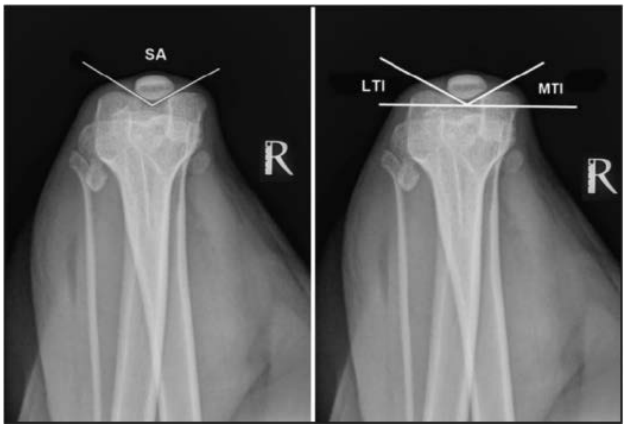

Fig. 1. Measurement of trochlear sulcus angle (SA) on the left and measurement of lateral and medial trochlear inclination (LTI; MTI) on the right [15].

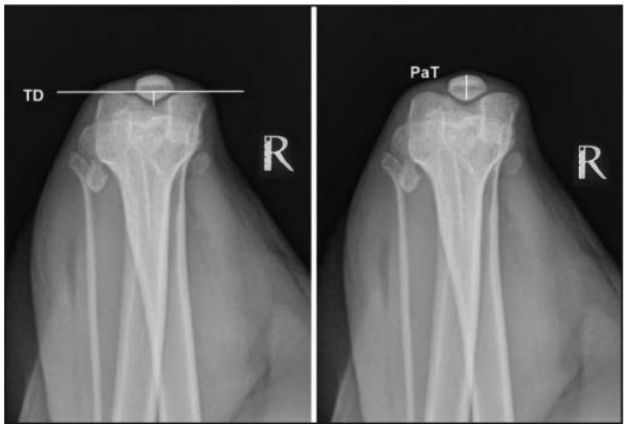

Fig. 2. Measurement of trochlear depth (TD) on the left and measurement of patellar thickness on the right [15].

In a study by M o c h i z u k i and H o n n a m i [36], six characteristics were assessed on typical transverse scans of the femur (Figure 3). In the transverse pictures, a baseline was created perpendicular to the caudal condyles as a point of reference. The angles between the facet inclination and the baseline were used to measure the medial trochlear slope (MTS) and lateral trochlear slope (LTS).

The distance between the most cranial points of the medial and lateral trochlear ridges and the most caudal point of the femoral trochlea was used to calculate the medial trochlear ridge height (MRH) and lateral trochlear ridge height (LRH). The distance between the intercondylar fossa and the most caudal point of the femoral trochlea was used to calculate the height of the middle portion of the femoral trochlea (HMF). The distance between the intercondylar fossa and the baseline measured the height of the caudal section of the femoral trochlea (HCF) [36].

For each section of the measured groove, C a r n e i r o et al. [6] discovered a substantial difference in the outcomes. This shows that the trochlear groove measurements should be tailored to the dog's breed and that it's crucial to standardize the values and narrow the range of variance.

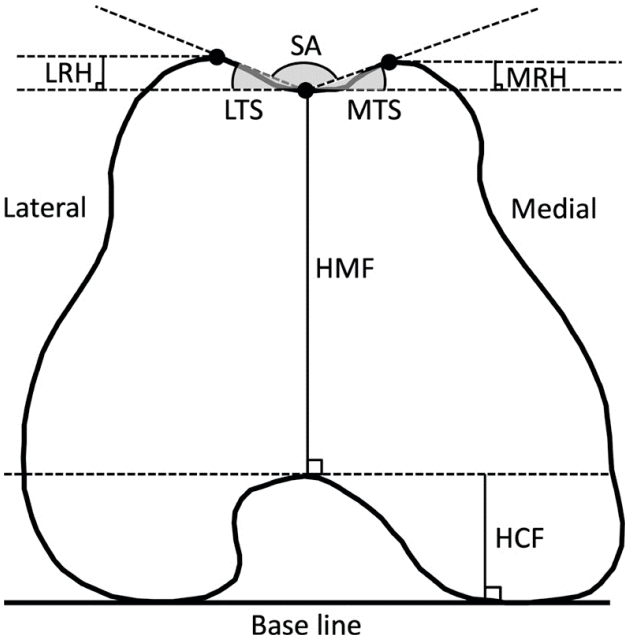

Fig. 3. Transverse image of the femur with six parameters [36].

CONCLUSIONS

In this article, we analysed the results of several research that focused on trochlear dysplasia in dogs. Our goal was to gain a better understanding of the problem as well as the best ways to treat it in canine patients. Following a comprehensive review of the relevant published material, we have uncovered numerous important concepts that are associated with trochlear dysplasia. It appears that a combination of genetics and environmental variables is to blame, but we need further research to fully understand how this process works. Dogs that have trochlear dysplasia are more likely to suffer from patellar luxation since the condition is caused by abnormalities in the groove of the trochlea. In summing up, this analysis drives home the point that early diagnosis and treatment of trochlear dysplasia in dogs is extremely important. Veterinarians, breeders, and dog owners must work together to reduce the occurrence of this orthopaedic disorder in canine populations and the impact it has on these populations. This study provides new information that adds to what is already known about trochlear dysplasia in dogs and stresses the need for additional research as well as careful monitoring to enhance the health of dogs that are affected by the condition.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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BIOCHEMICAL MARKERS OF LIVER DAMAGE IN CATTLE NATURALLY INFECTED WITH FASCIOLA HEPATICA

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ABSTRACT

Fascioliasis is a worldwide spread parasitic disease with a high impact on the economy and public health in many countries. Therefore, it is important to apply prevention, effective treatment, and education campaigns, to prevent the disease. This work aimed to determine the biochemical markers of liver damage caused by *Fasciola hepatica* during 21 days (D0 – D21) post albendazole (ABZ) therapy in the blood sera. This study included 24 cows naturally infected with *Fasciola hepatica,* randomly divided into a control group (n = 12) and a group treated *per os* with ABZ (7.5 mg per kg body weight) (n = 12). The activity of hepatic enzymes – aspartate aminotransferase (AST), alanine aminotransferase (ALT) and cholestatic enzymes – gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP), were measured in both groups. The activity of AST has significantly increased in the course of the experiment (D0 – D21) in both groups (P < 0.001). ALT activity during D0 – D21 was significantly decreased in both groups (P < 0.001). Activity of GGT was not significantly changed in the control group in the course of the experiment (P > 0.05). Contrarily, the activity of GGT in the treated group was significantly decreased (P < 0.01) during D0 – D21. The activity of GGT in the blood sera of the treated group was significantly lower in comparison with the control group in D7 and D21 (P < 0.01). This decreased GGT activity in the blood sera of the treated group may be caused by the effect of albendazole resulting in fasciola elimination and bile flow improvement. The results of ALP determination show no significant changes in ALP activity between groups. This can lead to the conclusion that the most useful marker of cholestasis caused by *F. hepatica* is the GGT activity and may help to evaluate the ABZ therapy effect in cattle.

Key words: alanine aminotransferase; albendazole; alkaline phosphatase; aspartate aminotransferase; fascioliasis; γ-glutamyl transferase

INTRODUCTION

Fascioliasis is a common zoonotic parasitic disease that affects ruminants worldwide and is known to cause significant economic losses in the livestock industry. The most important species of liver flukes are *F. hepatica* and *F. gigantica*. *Fasciola hepatica* is the most widespread species, may be found in more than 70 countries around the world [10]. The only fasciola free area is Antarctica [1]. Lymnae snails as intermediate hosts are required for complete life cycle of *F. hepatica* [2, 29]. Therefore *F. hepatica* is limited to areas where high soil moisture, high rainfall and poorly drained soils leading to standing waters are present [6, 10, 11]. There is also need for temperature range of 10–25 °C for development of both *F. hepatica* and the snail *L. trunculata* [11]. The disease has a high negative impact on economic conditions in the livestock industry annually [2, 4, 5, 10, 28]. There is a decreased milk production in dairy cattle, decreased fertility, nutrients conversion and requirement for anthelmintic drugs administration [2, 28]. Distribution of fascioliasis around the world is a dynamic process and may change with years, therefore monitoring of its prevalence is important to determine the risk [2]. Increasing risk of fascioliasis is caused by multiple factors – climate changes, anthelmintic resistance and absence of clinical signs in cattle [16, 22]. Humans living in the endemic areas of fascioliasis are at high risk of the infection. Fascioliasis is defined as plant/food-borne disease [18]. Additionally, individuals living in endemic regions for this disease are at considerable risk of contracting it. Studies showed that roughly 50 million people are currently affected, with over 180 million more at risk of infection annually [4, 13, 18]. The incidence of human natural fascioliasis is mostly correlated with regions of occurrence of animal fascioliasis [2, 23]. As such, it is crucial to take preventative measures in both dairy and meat livestock production to minimize the spread of this disease.

In cattle the infection can be more severe to develop evident clinical signs. The liver and biliary system are mainly affected in infected cattle. The liver parenchyma may show pathological changes such as fibrosis, haemorrhage, or necrosis, and bile ducts may become obstructed [21]. Therefore, it is important to monitor a herd for the presence of the infection. There are many diagnostic methods available to identify the presence of fascioliasis. Several diagnostic methods are available to identify the presence of fascioliasis, such as detecting fasciola eggs or antigens in the faeces, antibodies in blood serum, or using bulk milk tests for antibodies in milk. Biochemical analysis may be very helpful and enhance laboratory diagnostics of fascioliasis [31]. In addition to diagnostic methods it is important to use effective treatment of the infection. The treatment of fascioliasis based on the use of anthelmintics. The efficacy of the drug against different stages of liver flukes should be considered [25]. However, there has been an increasing risk of anthelmintic resistance in fascioliasis treatment in the last few decades. In the studies by Novobilský and Höglund [17], Overend and Bowen [20], and Sanabria et al. [24] the authors discussed the presence of resistance of *F. hepatica* resistance to various anthelmintics (triclabendazole, closantel, albendazole).

The aim of this study was to evaluate the biochemical markers of liver damage hepatic and cholestatic enzymes – caused by fascioliasis during 21 days post the albendazole (ABZ) therapy in the control and the treated group.

MATERIALS AND METHODS

Animals

Our experimental study included 24 Charollais cattle aged 3–7 years, weighing from 400 to 900 kilograms, non-lactating. The cattle were located in the endemic zone of fascioliasis in the North-East part of Slovakia with semi-extensive farming system. All 24 animals were positive for *Fasciola hepatica* eggs. The last anthelmintic drug was administered 12 months before this study. All 24 animals were divided randomly to two equal groups. In the untreated group (control group), no anthelmintic drug was administered for fasciola infection treatment study. Animals in the treated group (ABZ) were administered albendazole orally at a dose of 7.5 mg per kg body weight (Albendavet 10 %®, DIVASA-FARMAVIC S.A., Spain).

Sampling

In both groups blood samples were obtained at the beginning of the experiment (D0) and animals in the treated group were administered albendazole afterwards. Later, on the 7th (D7) and 21st (D21) day of the experimental study, additional blood samples were collected from individuals in both groups (Figure 1). Serum was obtained from these blood samples for biochemical analysis.

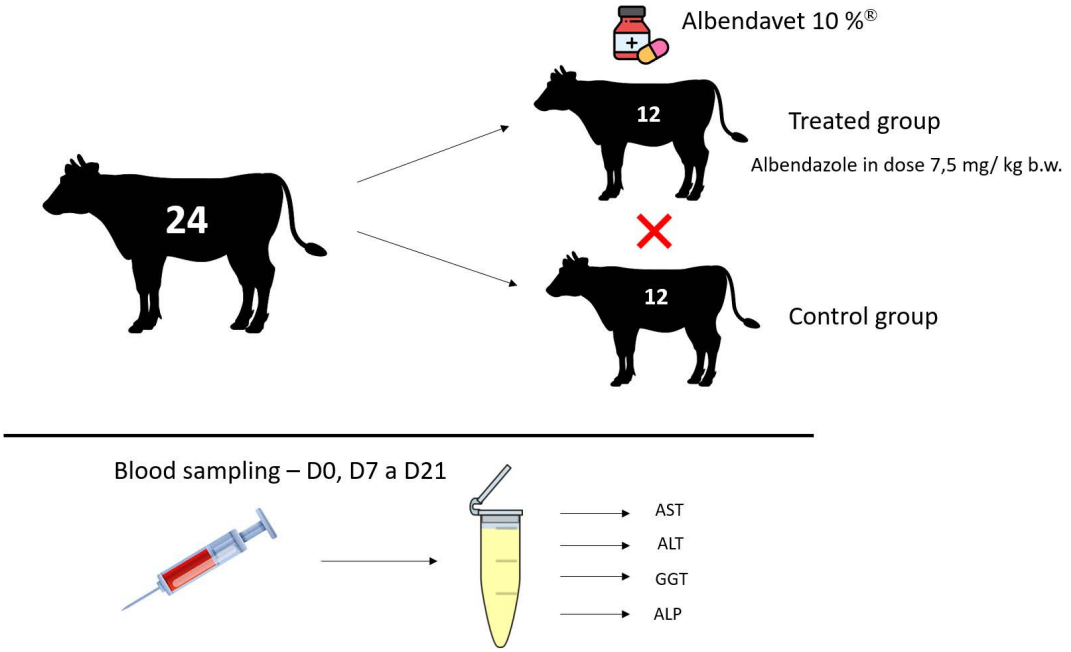

Fig. 1. Scheme of the experiment

Biochemical analysis

To monitor the development of liver injury and the effects of anthelmintic treatment, levels of four biochemical parameters were determined in the blood serum. The following hepatic and cholestatic enzymes were detected: aspartate aminotransferase (AST); alanine aminotransferase (ALT); gamma-glutamyl transferase (GGT); and alkaline phosphatase (ALP). Commercial kits from Erba Lachema (Brno, Czech Republic) and a spectrophotometer ONDA V-10 PLUS from Labbox (Spain) were used to determine all parameters.

Calibration curves were used to determine the activities of hepatic enzymes (AST and ALT), while kinetic spectrophotometric methods were used to determine the activities of cholestatic enzymes (GGT and ALP). The enzymatic activity was calculated using the following formula:

$$GGT/ALP(\mu kat/l) = \frac{\Delta_{sam}}{\Delta_{cal}} \cdot C_{cal}$$

Δ_{sam} = absorbance change of sample
Δ_{cal} = absorbance change of calibrator
C_{cal} = calibrator concentration

Statistics

Statistical analysis of the obtained data was performed using Student t-test in the Microsoft Excel 2021 program (Table 1).

Ethical consideration

The study was approved by the Ethics Committee of the Institute of Parasitology of the Slovak Academy of Sciences in Košice, Certificate of approval No. 03/2021.

RESULTS

The activity of AST was significantly increased in the course of the experiment (D0 – D21) in both control and treated groups ($P < 0.001$). ALT activity during D0 – D21 was significantly decreased in both groups ($P < 0.001$). The activity of GGT was not significantly changed in the control group in the course of the experiment ($P > 0.05$). Contrarily, the activity of GGT in the treated group was significantly decreased ($P < 0.01$) during D0 – D21. The activity of GGT in the blood sera of the treated group was significantly lower in comparison with the control group in D7 and D21 ($P < 0.01$). All measured serum enzymatic activity levels and statistical analysis are shown in Figure 2.

Table 1. Statistical analysis

	Mean 1	Mean 2	t-value	P	Valid N1	Valid N2	SD 1	SD 2
Control (1) - treated (2) group								
ALT (D0)	0.2002	0.1020	5.0441	0.000172	10	10	0.054	0.030
ALT (D7)	0.1697	0.1374	1.4314	0.170600	10	9	0.050	0.049
ALT (D21)	0.1033	0.0534	4.3267	0.000407	10	10	0.026	0.026
AST (D0)	0.4744	0.5168	-1.8755	0.077650	10	10	0.055	0.045
AST (D7)	0.7057	0.7952	-3.2708	0.007396	10	9	0.035	0.075
AST (D21)	0.7747	0.8619	-6.3661	0.000006	10	10	0.034	0.027
GGT (D0)	0.4560	0.3980	1.1263	0.279100	10	10	0.143	0.078
GGT (D7)	0.3814	0.2825	3.5475	0.002496	10	9	0.066	0.055
GGT (D21)	0.3842	0.2833	3.1476	0.008501	10	10	0.094	0.038
ALP (D0)	0.8436	0.8331	0.0380	0.970100	10	10	0.581	0.657
ALP (D7)	0.8621	0.8026	0.2364	0.816000	10	9	0.592	0.504
ALP (D21)	1.1205	1.1995	-0.2051	0.839900	10	10	0.787	0.932
Control group								
AST D0 - D21	0.4744	0.7747	-14.6932	3.00E-10	10	10	0.0552	0.0336
ALT D0 - D21	0.2002	0.1033	5.1400	0.000189	10	10	0.0536	0.0260
GGT D0 - D21	0.4560	0.3842	1.3281	0.203300	10	10	0.1430	0.0939
ALP D0 - D21	0.8436	1.1205	-0.8948	0.383700	10	10	0.5807	0.7873
Treated group								
AST D0 - D21	0.5168	0.8619	-20.5512	2.85E-12	10	10	0.0455	0.7873
ALT D0 - D21	0.1020	0.0534	3.8842	0.001135	10	10	0.0302	0.0256
GGT D0 - D21	0.3980	0.2833	4.1726	0.001084	10	10	0.0781	0.0381
ALP D0 - D21	0.8331	1.1995	-1.0167	0.324300	10	10	0.6568	0.9315

DISCUSSION

Internal parasites as *Fasciola hepatica* are likely to influence general health status in infected animals. Fascioliasis affects mainly the liver and biliary system function. The determination of biochemical parameters can help identify pathological changes in the hepatobiliary system of affected animals [2]. In the case of severe infection, loss of hepatic a biliary system function may occur. Those pathological changes can be detected by analyses of some biochemical markers when elevation of their activities are expected in blood sera. Increased activity of enzymes indicates damage to those tissues in which the enzymes are found. Cell death or tissue damage and therefore subsequently increased permeability of cell membranes and the transfer of enzymes into the intercellular space and then blood circulation occur. As enzymatic markers of hepatic and biliary function are AST, ALT, GGT and ALP determined commonly [7]. Since AST and ALT are not enzymes specific for the liver parenchyma, they can be examined together with GGT and ALP to monitor liver damage [27]. In the early stage of *F. hepatica* infection, changes occur mainly in the activity of AST and ALT as enzymes related to hepatic function when juvenile forms of flukes migrate in the liver parenchyma [19]. According to O t t e r [19], activity of AST is high in the liver parenchyma and is an indicator of acute and chronic liver injury. Increased serum activity of AST occurs 3–4 weeks post infection. However, the increase in AST serum activity may be also associated with pathological changes in kidneys, pancreas, skeletal muscles or erythrocytes. Therefore, the increase in AST serum activity is not specific to liver injury. Serum activity of ALT is normally measured to monitor hepatic function in animals. However according to H o f f m a n and S o l t e r [9] serum ALT activity is decreased in cattle, horses, sheep

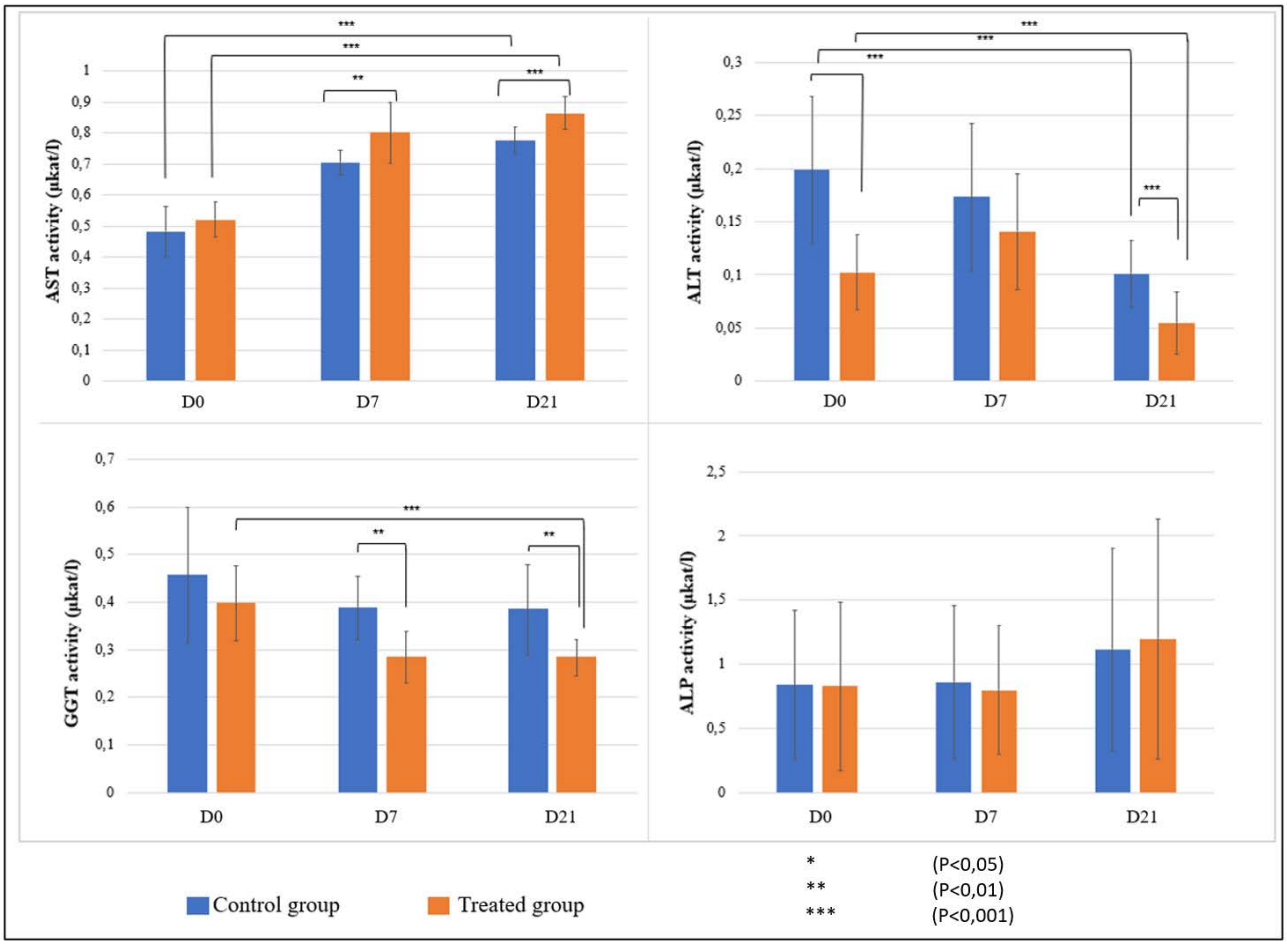

Fig. 2. Serum enzymatic activity levels during 21 days (D0 – D21) post albendazole therapy and statistical analysis
AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGT – gamma glutamyl transferase; ALP – alkaline phosphatase

and pigs in comparison with other animal species. Therefore, it is not routinely monitored in these animal species. In our study we noticed significantly decreased activity of ALT and increased activity of AST in the course of the experiment in both groups. Our results of AST activity resemble those of Simesen and Nansen [26]. On the contrary, Kowalczyk et al. [12] and Matanović et al. [14] reported increased serum AST activity in infected group comparing with the control group. Hodžić et al. [8] did not note any difference in AST activity between infected and control groups. These findings of researchers support the claim made by Hoffman and Solter [9] that the AST activity has a limited diagnostic sensitivity for fascioliasis (46 % sensitivity).

High activity of GGT is present in the biliary system and kidneys. In kidney injury, GGT is eliminated mainly by urine [3, 30]. Therefore, increased blood GGT activity is related primarily to liver and pancreas pathologies. In our study, we noticed decreased serum GGT activity in the treated group compared to the control group in D7 and D21. In the course of our study, the activity of GGT was significantly decreased only in the group treated with albendazole. The decreased level of serum GGT activity may be associated with the flukicidal effect of albendazole administration in this group of cattle. Albendazole affects the adult forms of the liver fluke present in the biliary ducts. After albendazole administration, elimination of flukes from biliary ducts occurs, the bile flow is improved and serum GGT activity is decreased. Similar findings were observed in the study by Bulgin et al. [3], Hodžić et al. [8], Kowalczyk et al. [12] and Matanović et al. [14]. These authors reported increased GGT serum activity in infected group in comparison with the control group. However, Kowalczyk et al. [12] noted that GGT serum activity may be affected also by other factors like age of cattle or raising cattle on pasture. ALP is present in several body tissues such as the liver, bones, kidneys, intestinal mucosa and placenta. Infestation with

a small number of flukes may not be sufficient to cause an increase in ALP serum activity. Additionally, bile stasis usually causes increase in ALP activity but cattle and sheep are an exception [3]. According to H o f f m a n and S o l t e r [9], in comparison with ALP activity the serum GGT activity is a more sensitive marker of extrahepatic cholestasis, cholangiohepatitis and liver cirrhosis. In our study, we did not note any statistically significant changes in ALP activity during the experiment. B u l g i n et al. [3] pointed out that compared to the GGT activity more severe infection is needed to produce change in ALP serum activity.

Increased serum activity of hepatic enzymes, ALT and AST, is associated mainly with the acute stage of liver injury. However, other organ tissues responsible for increased serum activity should be considered. On the contrary, increased GGT and ALP activity in the blood serum is associated with the chronic stage of the infection when adult forms of *F. hepatica* are located in the biliary system.

CONCLUSIONS

The monitoring of hepatic and biliary system injury in cattle with *F. hepatica* infestation revealed that determining the GGT activity is the most reliable parameter. GGT activity can serve as a highly effective diagnostic tool for identifying pathological changes in these systems. Regular blood biochemistry monitoring can aid in the diagnosis and monitoring of fascioliasis in cattle. It is recommended to monitor also other blood biochemical parameters, such as globulins, total proteins, urea, and glutamate dehydrogenase (GLDH). For the most accurate diagnosis, a combination of coprological examination and blood biochemical analysis should be used.

Conflict of interest

The authors declare that they have no conflict of interest.

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