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Contents

Vol. 6 No. 3 (2023)

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Research Articles	Pages
Comparison of The Efficacy of Intratesticular Lidocaine and Bupivacaine During Castration in Cats <i>Belemir Tan, Ertuğrul Elma, Zeynep Pekcan.....</i>	77-82
Intestinal Integrity Assessment with Diamine Oxidase Activity in Dogs with Atopic Dermatitis <i>Kerem URAL.....</i>	83-86
Prevalence of Trichuriasis in Shelter Dogs of Kırıkkale with Emphasis on Turkish Reports <i>Gozde Nur Akkus, Sinem Akdeniz, Kader Yıldız.....</i>	87-90
Preparation of Quail (<i>Coturnix coturnix</i>) Skeleton to Promote the Teaching Facilities of Avian Anatomy Laboratory <i>Swarup Kumar Kundu1, Zahid Hasan Rocky, Md. Amim Al Maruf, Ahanaf Tahmid Chowdhory, Abu Sayeed.....</i>	91-95
Comparison of Various Storage Conditions for The Stability of Escherichia coli O157:H7 Bacteriophage M8AEC16 <i>Nazlı Firdevs Aşlıoğlu, Naim Deniz Ayaz.....</i>	96-101

Comparison of The Efficacy of Intratesticular Lidocaine and Bupivacaine During Castration in Cats

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Abstract

In this study, it was aimed to evaluate the effectiveness of intratesticularly administered lidocaine and bupivacaine in castration in cats. 20 cats were divided into two groups regardless of breed and age. 80 microgram/kg medetomidine injection was administered for sedation. 4 mg/kg lidocaine or 1 mg/kg bupivacaine were injected intratesticularly in lidocaine and bupivacaine groups respectively. Castration was performed with routine methods. The reactions of the animals to the incision and the extraction of the funiculus spermaticus were recorded. After the operation, the animals were awakened by atipamezole. The pulse rate, respiratory rate and pain scores were evaluated for 6 hours at half-hour intervals. UNESP-Botucatu cat pain scale, Grimace cat pain scale and Glasgow cat pain scale were used to evaluate pain scores. A statistically significant difference was found between the groups in Grimace and Glasgow pain scores at the 150th min. and 180th min. postoperative evaluations ($p < 0.05$). A statistically significant difference was found between the groups in Botucatu pain score at the 60th, 90th, 150th, 180th, 300th and 330th min. ($p < 0.05$). No statistically significant difference was found in the pulse and respiration values ($p > 0.05$). As a result, it was determined that bupivacaine should be preferred to lidocaine for long-lasting surgical procedures and postoperative analgesia due to its long duration of action.

Keywords: Analgesia, bupivacaine, castration, cat, lidocaine.

INTRODUCTION

In order to prevent uncontrolled pregnancies and inconvenient reproductive behaviors such as escaping from home, irritability, shouting, and marking with urine, can be eliminated by neutering cats (Baran et al., 2016).

General anesthesia and regional (local) anesthesia are used to create anesthesia in companion animals (Topal, 2005). Local anesthetics are the only class of drugs that can completely block nociceptive impulses from reaching the cerebral cortex, and therefore represent the only way to completely prevent the patient from perceiving a nociceptive stimulus (Barletta and Reed, 2019).

Lidocaine is the most widely used local anesthetic. It has excellent therapeutic activity, fast action (5-10 min.), and is suitable for almost every clinical use (Vardanyan and Hrub, 2006; Barletta and Reed, 2019; Grubb and Lobprise, 2020a). The approximate duration of action of lidocaine without epinephrine is 30 to 60 minutes. Besides its use as a local and topical anesthetic agent, lidocaine treats all ventricular arrhythmias, mainly ventricular tachycardia, and ventricular premature complexes (Grubb and Lobprise, 2020a). Conditions affecting the central nervous system such as nervousness, agitation, depression, lethargy, and convulsions may occur due to lidocaine administration (Grubb and Lobprise, 2020a).

Bupivacaine is another local anesthetic drugs used in small animal medicine. It blocks the formation and transmission of nerve impulses by blocking the Na⁺ channels in the nerve membrane (Grubb and Lobprise

2020a). The onset of action of bupivacaine is slower than lidocaine (20 minutes), but it is long-acting (6-8 hours) and is more potent than other local anesthetics (Papich, 2020). Bupivacaine can be used for both local and epidural analgesia/anesthesia. Side effects after local infiltration are rare. Signs of toxicity in cats include bradycardia, arrhythmias, tremors, muscle twitches, and seizures. (Vardanyan and Hrub, 2006; Papich, 2020; Grubb and Lobprise, 2020a).

Pain negatively affects the health and well-being of animals, so pain management should always be part of the postoperative treatment (Mathews et al., 2014; Grubb et al., 2020a). When postoperative pain cannot be prevented, it causes complications such as lactic acidosis, increased protein catabolism, gastrointestinal ileus, decreased or complete cessation of food intake, delayed wound healing and prolonged hospitalization. Cardiovascular, pulmonary, gastrointestinal, urinary, and metabolic changes occur due to the operation and the postoperative discomfort (Duncan and Lascelles, 2007; Epstein et al., 2015).

As animals cannot express themselves verbally, pain assessment is difficult especially for cats and requires good observation (Johnson et al., 1993; Mathews, 2000; Pekcan, 2005). Pain assessment scales can be used for this purpose; some of these are UNESP-Botucatu cat pain scale, Glasgow composite pain scale and Grimace cat pain scale (Steagall and Monteiro, 2020).

This study is aimed to comparatively evaluate the

effectiveness of intratesticular administered lidocaine and bupivacaine for the prevention of postoperative pain before castration operation in cats.

MATERIALS AND METHODS

Animal Material and Creation of Groups

Twenty adult male cats admitted for elective orchietomy at the Kirikkale University Faculty of Veterinary Medicine Department of Surgery were studied. Each animal was randomly assigned to one of two groups of ten. They were considered to be healthy based on physical and haematological examination. Animal owners were informed about the operation to be performed. All procedures were carried out with the permission of the Kirikkale University Animal Experiments Local Ethics Committee, dated 13.12.2022, and numbered E.40905.

Preparation for Operation

All cats were fasted at least 6 hours and water was withheld for 2 h prior to anesthesia. The cats were allowed to rest for at least one hour in order to calm down, enable them to adapt to the environment, and the researcher observe temperament-behavior of the cats before the operation. 20 mg/kg ampicillin (Vilamoks-LA, Vilsan) were administered once to prevent infection presurgically. In order to provide premedication, 80 microgram/kg (0.08 ml/kg) intramuscular (IM) medetomidine (Tomidin 10 ml, ProVet) was administered to all cats in the study. After the cats sedated, 4 mg/kg lidocaine without adrenaline (Jetokaine simplex 20 mg/ml, Adeka) or 1 mg/kg bupivacaine was injected intratesticularly to the lidocaine or bupivacaine (Marcaine 0.5%, Aspen) groups, respectively (Grubb and Lobprise, 2020a). A 22 gauge needle was inserted into the center of the testicles with the tip of the needle pointed toward the spermatic cord, half the the calculated dose was injected into each testicle. In both groups, some of the local anesthetics were left subdermally while exiting the testis to provide anesthesia of the skin. While waiting for the time required for the local anesthetic to take effect 15 minutes for lidocaine group and 30 minutes for bupivacaine group, the cats were routinely prepared for the operation.

Surgical Procedure

The open prescrotal castration method was preferred as the surgical procedure. During castration the responses of the animals to the incision retraction of the incision and funiculus spermaticus were recorded. After the end of the operation atipamezole (Reversal 10 ml, ProVet) was administered to antagonize the sedative effect of the medetomidine and pain scale evaluations were started. Pain was evaluated and recorded postoperative 6 hours by the same person (BT). 0.2 mg/kg meloxicam was administered after 6 hours and cats were discharged with the owner postoperative instruction forms.

Pain Scale and Measurement of Vital Functions

The heart rate and respiratory rate of all cats were measured and recorded before the operation. During the operation, the reactions to the incision and the extraction of the funiculus spermaticus were recorded. In the study, pain assessments were made using the UNESP-Botucatu cat pain scale, Grimace cat pain scale and Glasgow cat pain scale in order to obtain concrete data in determining the degree of pain at 0th, 30th, 60th, 90th, 120th, 150th, 180th, 210th, 240th, 270th, 300th, and 330th minutes in the postoperative period. Facial expressions, heart and

respiratory rates were recorded to evaluate pain related physiological effects simultaneously (Figure 1, 2 and 3).



Figure 1. Ears forward, eyes open, mouth and mustache in a relaxed position and head above the shoulder line.



Figure 2. Ears slightly separated from each other, eyes half open, mouth position slightly tense, mustache position slightly curled or straightened, head in line with the shoulder line.



Figure 3. Ears outward, eyes closed, mouth stance taut, mustache stance straightened, head below shoulder line or slanted downwards.

Statistical analysis

In the study, the "Mann-Whitney U test" was used to compare groups at the same time, and the Friedmann test was used to compare different time periods in the same group. $p < 0.05$ was considered significant.

RESULTS

During incision increase in heart rate and movement of the hind legs recorded in two (L7 and L10) and three (B4, B8, and B10) cats in the lidocaine and bupivacaine group, respectively. One cat (B10) had a reaction to the

withdrawal of the funiculus spermaticus. Since the heart rates of these patients were recorded within the reference values, no other general anesthetic was added.

According to the results of the statistical analysis of the data; In the lidocaine group, changes in respiration and pulse over time were not significant in Grimace pain score, Glasgow pain score and Botucatu pain score ($p>0.05$). In the bupivacaine group, there were no significant changes in respiration and heart rate over time in the Grimace pain score, Glasgow pain score, and Botucatu pain score ($p>0.05$).

Table 1 and Table 2 show the time-dependent changes in respiration and pulse rate of cats treated with lidocaine and bupivacaine.

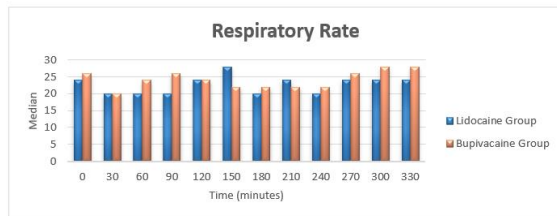


Table 1. Time-dependent variation of respiratory rate in cats treated with lidocaine and bupivacaine.

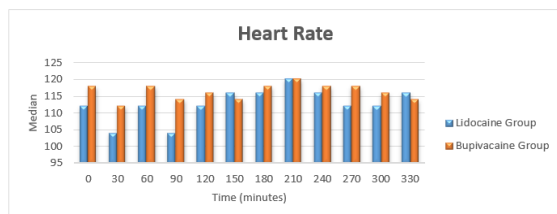


Table 2. Time-dependent variation of heart rate in cats treated with lidocaine and bupivacaine

In the intergroup comparisons, the Grimace pain score was recorded as 3 in the lidocaine group and 0.5 in the bupivacaine group at the 150th minute, and the median pain score was 2 in the lidocaine group at the 180th minute and 0 in the bupivacaine group. The pain score of the lidocaine group was higher than the bupivacaine group, and the difference was statistically significant ($p<0.05$) at 150th and 180th min. The median pain score of the lidocaine group was 3, while the median pain score of the bupivacaine group was 1 in the Glasgow pain score at the 150th and 180th minutes. It was determined that the pain score of the lidocaine group was higher than the bupivacaine group, and the difference was statistically significant ($p<0.05$) (Table 3 and 4).

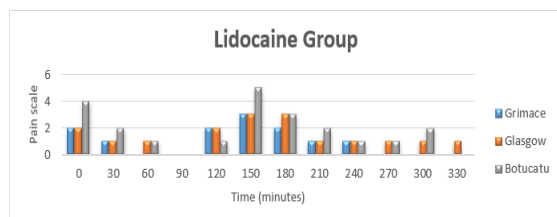


Table 3. Postoperative median pain scale values of cats treated with lidocaine

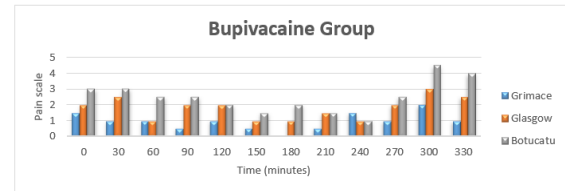


Table 4. Postoperative median pain scale values of cats administered bupivacaine.

The Botucatu pain scores at 60th, 90th, 300th, and 330th minutes, the medians were recorded as 1, 0, 2, 0 in the lidocaine group, and 2.5, 2.5, 4.5, and 4 in the bupivacaine group, respectively, and the pain score of the lidocaine group was lower than the bupivacaine group. The median scores of the lidocaine group were 5 and 3, respectively, and were 1.5 and 2 in the bupivacaine group, respectively. According to the pain scores at the 150th and 180th minutes, the bupivacaine group was found to be lower than that of the lidocaine group, and the difference was statistically significant ($p<0.05$) (Table 3 and 4).

Butorphanol was administered during postoperative period in three cats (L1, L5, and L10) in the lidocaine group and two cats (B6 and B10) in the bupivacaine group because of high pain scores and excluded from the study.

DISCUSSION AND CONCLUSION

In order to prevent uncontrolled reproduction and zoonotic diseases, as well as to prevent unwanted escape and urine marking in domestic cats, sterilization is frequently recommended. It is one of the most frequently performed operations in veterinary medicine. For the better surgical and postoperative period, researchers are still searching for better anesthetic combinations (Baran et al., 2016; Höglund et al., 2018).

Pain negatively affects recovery by causing stress in animals. Pain relief is important not only biologically and physiologically, but also ethically (Duncan and Lascelles, 2007; Gultekin, 2012). Pain can be prevented by several drugs like nonsteroidal antiinflammatory drugs, opioids, N-methyl-D-aspartat antagonists. Since the side effects of analgesics used in some animals are avoided, it has been suggested to use local anesthetics with fewer side effects instead (Barletta and Reed, 2019; Grubb and Lobprise 2020a). The aim of this study was to popularize the use of local anesthetics, which do not have many systemic side effects, for castration in cats.

Local anesthetic drugs like lidocaine and bupivacaine are one of the anesthetic drugs used for local blockage of motoric and sensoric function. It has been reported that high doses of lidocaine (toxic dose 8 mg/kg) and bupivacaine (toxic dose 5 mg/kg) cause complications such as ataxia, nystagmus, depression, seizures, bradycardia, hypotension and cardiovascular collapse (Barletta and Reed, 2019; Grubb and Lobprise, 2020a; Papich, 2020). In line with the drugs used in this study, no complications (respiratory arrest, cardiac arrest, death, etc.) or high-dose side effects occurred in any animal.

Evaluation of pain in animals can be difficult due to the inability to communicate verbally and the observers' lack of training and experience. In addition, animals may show different individual responses and behavior patterns to painful stimuli. While some animals show very severe pain symptoms for similar procedures, some may not (Mathews et al., 2000; Ansah et al., 2002; Pekcan, 2005; Gultekin, 2012). In this study, it was determined that 3 cats

in the lidocaine group and 2 cats in the bupivacaine group showed severe pain symptoms in the postoperative period, butorphanol was administered as a rescue analgesic and they were excluded from the study. This indicates that pain is an individual perception.

While assessing pain, it can be interpreted and evaluated differently by different observers. (Mathews, 2000; Dobromylskyj, 2001a). In an analgesia study conducted with 50 dogs, 3 different scoring systems were used by different observers and differences were observed in pain scores within the same hour (Holton et al., 1998; Pekcan, 2005). This creates difficulties in pain studies. In this study, scale interpretation was made by the same observer in 20 cats so interobserver differences were eliminated.

In most of the pain scales, the changes in behaviours while feeling pain are scored numerically and the total values are obtained from the pain scores (Mathews et al., 2014; Epstein et al., 2015;). There are no gold standard acute or chronic pain scoring systems for use in animals, but several scoring methods have become widely used around the world. All of these scales are largely based on analyzing behaviors (Holton, 1998; Steagall and Monteiro, 2019). In this study, the Botucatu cat pain scale, Grimace pain scale and Glasgow pain scales were used together to obtain accurate results. Each scale has both common and different components. Pain is more difficult to interpret in cats compared to dogs, so three scales were evaluated together. Arterial blood pressure assessment in the Botucatu pain scale could not be performed in some animals for technical reasons, although usage of the Botucatu scale was effective in determining and interpreting the presence of pain in cats, it was found not practical. Interpretation of the Glasgow cat pain scale was determined to be more practical in terms of its components and ease of application.

The average heart rate in cats is 110-140/minute (Fraser et al., 1991). Physiological parameters such as pulse, respiratory rate, and body temperature can be important indicators in terms of the presence and severity of pain. Even if a 20% change in physiological values is interpreted as the presence of pain, some other factors such as stress, fear, activity, anesthetic drug and other drugs can affect these physiological parameters (Pekcan, 2005). While other species show obvious symptoms such as vocalization in pain, this may not be the case in cats. Cats can show only postural changes against high-level pain, which creates difficulties during evaluation of pain (Glerum et al., 2001; Höglund et al., 2018; Er, 2019). In this study, there were also difficulties in the evaluation of pain from time to time. However, the pulse values remained within the reference limits during postoperative 6 hours in all cats.

Lidocaine, bupivacaine, and other local anesthetics are often administered to reduce the total dose of the general anesthetic drugs and also to provide some degree of postoperative analgesia. The approximate duration of action of lidocaine without epinephrine is 30 to 60 minutes. With epinephrine, this time can be extended to about 120 to 360 minutes. Bupivacaine has a duration of action of up to 8 hours when combined with epinephrine (Lim et al., 2021). The duration of conduction blockade, which is prolonged by at least 50% with the addition of epinephrine to lidocaine, it does not cause a clinical change in the blockade time when added to bupivacaine (Barletta and Reed, 2019; Grubb and Lobprise, 2020b; Bayram,

2020). Therefore, commercially produced bupivacaine preparations do not contain epinephrine.

The effects of lidocaine on the cardiovascular system are attributed to the blockade of sodium channels, which leads to a reduction in cardiac contractility at a rate proportional to their potential. Unfortunately, although the potency of bupivacaine is four times that of lidocaine, the potential for cardiac toxicity is also greater than that of lidocaine. Therefore, IV administration of lidocaine does not show any signs of toxicity, while IV administration of bupivacaine is contraindicated. Symptoms of toxicity in cats include bradycardia, arrhythmias, tremors, muscle twitches and seizures (Papich, 2020). In this study, in order to avoid any complications related to IV administration, the plunger of the injector was pulled back to prevent mislead IV injection and no cardiovascular complications were encountered.

When both active substances are compared, the onset of action of bupivacaine is four times longer than that of lidocaine, and the duration of action is also longer. This long duration of action is partially explained by the fact that the drug is very lipid soluble (Lim et al., 2021). When the onset times of lidocaine and bupivacaine were compared, it was determined that the operation was started earlier with a faster effect in cats using lidocaine, but when the increased rates and duration of the pain scales were examined, it was revealed that bupivacaine provided analgesia for a longer time.

Prolonging the presence of pain suppresses the immune system, causes the emergence of secondary diseases and delays the healing process by causing loss of appetite and cachexia. Insufficient food intake and loss of appetite cause hepatic lipidosis, especially in cats. (Mathews, 2000). With early postoperative food intake, catabolic protein metabolism is prevented and positive nitrogen balance is achieved. In this study, scale interpretations related to food intake were made in the postoperative period following the awakening of cats. Since the scale evaluations started from the 0th minute, the evaluations were interpreted as the cats' interest in food, and it was observed that the cats participating in the study were interested in food, 3 cats from the lidocaine group and 2 cats from the bupivacaine group were not interested in food.

The duration of anesthesia may be shorter when local anesthetics are applied to highly vascularized areas. It can also lead to direct intravascular injection, which carries the risk of incorrect infiltration technique and systemic toxicity (Lim et al., 2021). Although bupivacaine provides a longer duration of analgesia than lidocaine, some clinicians are more comfortable with lidocaine because the testicles are vascular and have a higher margin of safety if inadvertently injected IV (Grubb and Lobprise, 2020b). No complications were observed in our study. The local anesthetic given to the spermatic cord also moved upwards and relieved the pain caused by the surgical crushing of the cord and related vessels, and it was seen that the removed testes were anatomically larger than normal and haemorrhagic.

General anesthesia produces controlled and reversible loss of consciousness, sedation, analgesia and muscle paralysis however it has some morbidity and mortality side effects. In order to reduce these side effects, local anesthesia applications have recently gain popularity. Staying calm and still during operations is very important for the success rate of surgery. However animals may

become stressed and become active in unfamiliar environments such as the operating room for this reason local anesthesia without sedation is less preferred in veterinary medicine (Mathews, 2000; Grubb et al., 2020a;). In this study, medetomidine was preferred to provide either sedation and prevent the movement during the operation and no side effect was recorded related to the administration of medetomidine. Since our aim was to record postoperative pain after castration in cats, in order to eliminate the analgesic effect of medetomidine it was antagonized at the end of surgery.

Most of the anesthetic agents used in animals do not provide significant intra- and postoperative pain control, as they have little or no analgesic properties. (Lamont, 2008;). Medetomidine, a synthetic drug used both as a surgical anesthetic and analgesic, was preferred to provide preoperative sedation (Topal, 2005; Gultekin, 2012). As medetomidine is not enough for castration, local anesthetics were included in this study, and IV butorphanol was administered to cats whose pain score were higher than the acceptable score.

α_2 -adrenoceptor agonists, including medetomidine and dexmedetomidine, provide sedation, muscle relaxation and analgesia in cats (Robertson, 2018). The dose of medetomidine has been determined as 0.08 mg/kg (IV) and can be administered IM, IV, or SC. (Sinclair, 2003). With IV administration, medetomidine takes effect in 2 minutes, the sedative effect lasts for an average of 1-2 hours and can increase up to 6 hours depending on the dose given. The duration of the analgesic effect is approximately 1 hour and is shorter than the duration of sedation. The ability to reverse its effects with atipamezole is the major advantage of medetomidine, but this results in the reversal of analgesia in addition to sedation. (Topal, 2005; Çetinaslan and Apaydin, 2008; Robertson, 2018). In this study, the analgesic effect of medetomidine was eliminated by using atipamezole at the end of the operation to evaluate the analgesic efficacy of the applied local anaesthetics.

The onset of action of bupivacaine is 20-30 minutes, the duration of action is in the range of 3-10 hours, the onset of action of lidocaine is 5-10 minutes and the duration of action is in the range of 1-1.5 hours. The addition of epinephrine to lidocaine can extend this time to approximately 120-360 minutes (Lim et al., 2021). In the Botucatu, Grimace and Glasgow pain scales, in which the efficacy of lidocaine was evaluated, an increase in pain scores was detected at the 150th and 180th minutes, which was thought to be due to the shorter duration of action of lidocaine compared to bupivacaine. Likewise, the Botucatu pain score is 60., 90., and 300. It is thought that the increases in pain scores detected at 330 minutes are due to the effect duration of bupivacaine.

As a result, it was determined that lidocaine and bupivacaine can be used as safe local anesthetics in terms of the heart and respiratory parameters and pain scales examined. Bupivacaine should be preferred to lidocaine for long-lasting surgical procedures and postoperative analgesia due to its long duration of action.

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Animal study and data collection: B.T., Analysis and interpretation: E.E., Statistical analysis and literature search: Z.P., Writing: B.T., Z.P.

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Ethical Approval

This study was conducted with the permission of the Kırıkkale University Local Ethics Committee for Animal Experiments with the decision No. E.40905. - 16 dated 13.12.2022.

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Intestinal Integrity Assessment with Diamine Oxidase Activity in Dogs with Atopic Dermatitis

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Abstract

Diamine oxidase (dAo) as a valuable biomarker, and a mirror of the integrity/mucosal function of the small intestine (sint). The levels of dAo in the serum and mucosa of the sint could be capable of determining as an assessment of sint barrier function. Given better understanding of gut-brain-skin axis in veterinary internal medicine field, relevant data is lacking through dogs with atopic dermatitis (atode) in which researches focus and rely on skin changes. However more data is crucial, which prompted the present author to perform this study and analyzed the relationship between integrity/mucosal function of sint to those of dogs with atode. Dogs enrolled and classified as suggested criterion for mild (10), moderate (35) and severe (60) skin lesions scoring based on CADESI-04. Commercially available Canine Diamine Oxidase ELISA Kit were purchased for dAo analytes. As detected by Quantitative Competitive ELISA, dAo levels (ng/mL) were detected as $5,28 \pm 1,19$, $2,594 \pm 0,76$ and $1,28 \pm 0,22$, with a statistical significance of Group III in contrast to Group I, suggesting that as severity of the atode elevates, circulating dAo levels were declined in correlation. It should not be unwise to draw preliminary suggestion that dAo levels could alter in relationship with disease activity in dogs with atode. This alterations, mainly deduction, should reflect disease activity, in which dogs in Group III (severe atode) represented lowest dAo values in contrast to other classified groups of disease.

Keywords: Atopic dermatitis, diamine oxidase, dog, intestinal integrity.

INTRODUCTION

For many long years, it was suggested that several frequent dermatological issues presented no association with diet. Contrarily researches coming from recent years, highlighted that diet could be capable of influencing outcome (Katta and Desai, 2014; Shmalberg, 2017). Furthermore diet, round the clock adjust gene expression (Hunter et al., 2008; Livingstone et al., 2014), in which genes encoding certain proteins is capable of switching on/off by lifestyle choices (to the present authors knowledge not only for human being but also for animals) i.e what people (animals) eat, and where/how they live (Anturaniemi et al., 2020; Geary et al., 2022). Diet could be capable of changing the game by gene expression through affecting gut microbiota (Singh et al. 2017, Anturaniemi et al., 2020; Geary et al., 2022). Given the resident microbiota is vital for maintenance of both functional and structural integrity of the gut and regulation of immunity (Furusawa et al., 2013; Purchiaroni et al., 2013), it should not be unwise to recognize 'gut-skin-brain axis' on behalf of investigating atode and its relationship with sint, which prompted the present author performing this study. Gut-skin-brain axis' needs to be explored in much details, specifically in dogs in relationship with dermatological issues and atode.

The term atode has took place used in veterinary field denoted inflammatory dermatitis with itching, in which foremost recognized frequently by an IgE antibody-related reaction (Halliwell, 2006). Following diagnosis of atode has been clarified, an elimination diet should be performed for possible detection of food allergens play a role in the

development of this disorder (Favrot et al., 2010). Proposed definitions through gut microbiota presented its effects on accompanying pathogenesis of atode were composed of i) immunity and inflammation (Bowe, 2011; Belkaid and Hand, 2014), ii) storage of blood lipids/fat (Musso et al., 2010 a,b) neuropeptide existence (Pincelli et al., 1990; Gueniche et al., 2010; Holzer and Farzi, 2014) and metabolic alterations Taking into account all aforementioned data, it should not be unwise to focus on the lack of literature for highlighting the association between intestinal integrity and atode among dogs, which was the purpose of this study.

MATERIALS AND METHODS

Diagnostic criterion and CADESI-04 scores

To those of dogs enrolled diagnosis was based on table 1 fully meeting all necessary criterion for a full diagnosis of atode. Available diagnostic tree evolved clinical signs (Griffin and DeBoer, 2001; Favrot et al., 2010), exclusion of other relevant dermatoses based on Favrot Criteria (Favrot et al., 2010, CADESI-04 scoring based on proposed benchmarks for mild (10), moderate (35) and severe AD (≥ 60) skin lesions (Olivry et al., 2014). In an attempt to exclude other relevant etiology; skin scraping, cutaneous cytology, dermatoscopy, epidermal corneometric analysis, serum biochemistry, endocrine panel results were all deemed available (in which not necessary data to show herein). The present research was approved by HADYK Aydın Adnan Menderes University Local Ethical Committee on Animal Experiments with number 64583101/2020/045 (9/7/2020).

Table 1. Diagnostic tree applied based on evidenced based veterinary medicine, at the present study.

Clinical signs	-Pruritus -Primary skin lesions (erythema), Secondary skin lesions (i.e. hyperpigmentation/lichenification) -Self-trauma (i.e. excoriations/self-induced alopecia) (Griffin and DeBoer, 2001).
-Excluding other pruritic dermatoses and active skin infection	Favrot et al. (2010)
CADESI-04 as a biomarker	-Proposed benchmarks for mild (10), moderate (35) and severe AD (≥ 60) skin lesions (Olivry et al 2014)

Intestinal integrity and mucosal function interpretation by use of Canine Diamine Oxidase ELISA Kit

This assay, as described by the available web site (<https://www.mybiosource.com/dao-canine-elisa-its/diamine-oxidase/739902>) was purchased by Turkish side Distributor (RDA Group, Istanbul) from the specified website of distributor. The latter assay presented sensitivity: 1.0 ng/mL and excellent specificity [Spike Recovery: 92-101% and Linearity 1:8 Range 994-109%]. There has been no significant cross-reactivity nor interference between dAo and its analogues (Mybiosource web site). The latter assay proven to have high sensitivity and specificity for detection of dAo. According to owner there was no prior cross-reactivity/interference between dAo and analogues. Samples included sera obtained from dogs with a diagnosis of atode. Assay Type was Quantitative Competitive, which was analyzed by ELISA Device available at RDA Group, Istanbul. Sensitivity was

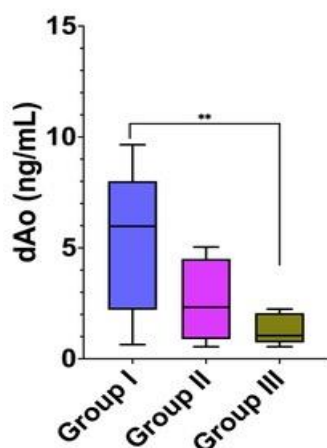
1.0 ng/mL, with a detection range of 0.312-20 ng/mL. Prior to analysis preparation and storage conditions evolved were 2-8 degree Celcius, immediately forwarded to RDA Group, Istanbul Kruskal Wallis one way ANOVA test was preferred as a rank based nonparametric methodology. P value was set as 0.01.

RESULTS

Regarding dAo values (mean $\pm$ standart deviation) statistical analytes showed p values set as $p = 0,299$ between Group II and III, $p = 0,008$ between Group I and III and $p = 0,158$ between Group I and II as was shown in fig. 1 and table 2. During study test kits were purchased previously were all gave available results. There was no error nor analytical fault during methodology.

Table 2. a, b: dAo activity among dogs enrolled at this study with atode. Different letters in same lime are statistically significant. Due to proposed benchmarks for atode based on CADESI-04 (Olivry et al 2014) Group I to III were denoted as mild, moderate and severe.

	Group I Mild (10)	Group II Moderate (35)	Group III Severe (≥ 60)
dAo	5,28 $\pm$ 1,19a	2,594 $\pm$ 0,76ab	1,28 $\pm$ 0,22b
P value		0,030	

**Figure 1.** Boxplot analytes showing dAo levels among dog with atode enrolled and classified as suggested criterion for Group I--mild (10), Group II--moderate (35) and Group III--severe (60) skin lesions scoring based on CADESI-04.

Selected clinical cases

All enrolled cases were diagnosed with atode as was also shown as a diagnostic tree at table I. Entire clinical cases were fully met the criteria shown at the latter table.

Different cases were presented at each group showed different levels of circulating dAo levels, selectedly shown at fig 2. None of the dogs were excluded from the study during trial, nor analysis were fault.

**Figure 2.** Serum dAo activities (ng/mL) were represented as 4.345, 3.210 and 0.765 to those of dogs with atode in groups I to III, respectively.

DISCUSSION AND CONCLUSION

The patho-physiological mechanisms underlying atode - related intestinal mucosal injury (inmi) have yet to be entirely elucidated in dogs. Prior researches denoted that inmi is a complex pathophysiological condition composed of multifactorial reasons [i.e. intestinal hypoperfusion, oxidative stress and inflammatory mediators] (Capurso et al., 2012; Rahman et al 2003). Hence, from a clinical point of view it is essential to analyze the association between injury of the intestinal mucosal barrier and atode ip herein.

Intestinal mucosal barrier could be damaged by i) ischemic reperfusion injury, ii) overwhelming production of inflammatory mediators, iii) microcirculatory alterations and iv) apoptosis (Zhang et al., 2007). Although this was not an etiological study, the present author did not evaluate predisposing factors or underlying reasons. Among those aforementioned factors microcirculation disorder causes intestinal barrier dysfunction through reactive oxygen species existence via xanthine oxidase/hypoxanthine buildup in intestinal environment (Tian et al., 2013). In humans with atopic elevated intestinal permeability (Ukabam et al., 1984; Pike et al., 1986; Caffarelli et al., 1993) without clear evidence was reported. Alterations among intestinal barrier functioning could represent mucosal damage as a probable consequence of local inflammatory response (Rosenfeldt et al., 2004). The latter valuable hypothesis prompted the present author to elucidate and establish the present study. Thus obtained results should be discussed cautiously, in which as the severity of the atopy increases, circulating dAo levels were decreased at the present study (fig. 1 and table 2).

It was well recognized that high-fat dietary conditions deduce biodiversity of intestinal microbiota along with elevated levels of lipopolysaccharides, consequently resulting with systemic inflammation via i) altering colonic epithelial integrity and barrier function, ii) diminishing mucus layer thickness, and iii) elevating pro-inflammatory cytokine levels (Morales et al., 2016; Deng et al., 2018). Interestingly 12 out of 17 dogs enrolled herein were receiving high fat and carbohydrate diet with an unknown origin (brand name) which could have hasten inmi to those of dogs.

The present author found that the decline in plasma dAo activity was associated with the severity of probable (without any histopathological evidence) mucosal injury. In the current study, in parallel line with the purpose, it was sought to determine the usefulness of plasma dAo activity level in estimating the severity of atopy in relationship with the small intestinal changes.

Given dAo adjust cell proliferation via degradation of polyamine, an essential substance for mitosis/meiosis (Kusche et al., 1975; Jänne et al., 1978; Baylin et al., 1978) it quickly detoxifies dietary histamine, preventing the allergy-like symptoms of histamine excess. dAo is peculiarly exist in enterocytes [through tip of small intestinal villi] (Baylin et al., 1978), there which it is libetared into the peripheral circulation into liver for inactivation (D'Agostino et al., 1986). dAo activity at the top level is presented within the small intestine, whereas lowest activity in the large intestine/stomach (Shakir et al., 1977; Bieganski, 1983) Plasma dAo activity decreased with inmi (Luk et al., 1980; Nakao et al., 2002), in which was the probable case obtained at this study. Circulating dAo levels (ng/mL) were detected as $5,28 \pm 1,19$, $2,594 \pm 0,76$ and $1,28 \pm 0,22$, with a statistical significance of Group III in contrast to Group I, denoting that as the severity of the atopy increases, circulating dAo levels were decreased at the present study (fig. 1 and table 2). Further studies are warranted in larger dog populations for better understanding the relationship.

Conflict of Interest

The present author has competing interest.

Authorship contributions

Concept: K.U., Design: K.U., Data Collection or Processing: K.U., Analysis or Interpretation: K.U., Literature Search: K.U., Writing: K.U

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Ethical Approval

This study was conducted with the permission of the Aydın Adnan Menderes University Local Ethics Committee for Animal Experiments with the decision No. 64583101/2020/045 - 16 dated 09.07.2020.

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Prevalence of Trichuriasis in Shelter Dogs of Kırıkkale with Emphasis on Turkish Reports

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Abstract

The whipworm, *Trichuris vulpis*, dwells in the caecum and infrequently the colon in the dog. The present study aimed to detect trichuriasis in shelter dogs in Kırıkkale province, Türkiye. The other reports originated from Türkiye on trichuriasis were addressed together with the results. The investigation was conducted from January through December of 2022. The faecal samples were obtained from 200 dogs (mixed breed, older than 1 year) from two different shelters in Kırıkkale. The faecal samples were analysed by centrifugal flotation both zinc sulfate and saturated salt solution. *Trichuris* spp. eggs were only found in 0.5% of the faeces (1/200), while other parasite eggs or oocysts were found in 21.5% of the faeces examined (43/200). The eggs of *Toxocara* spp. (6%, 12/200), *Toxascaris* sp. (2.5%, 5/200), *Taenia* spp. (2.5%, 5/200), hookworm eggs (0.5%, 1/200), *Isospora* spp. oocysts (11%) (22/200) and *Sarcocystis* spp. sporocysts (0.5%, 1/200) were detected. Only 3/200 (1.5%) of the dogs evaluated had mixed infections. Regarding the detection of all parasite eggs and oocysts, the zinc sulfate flotation technique outperformed the flotation with saturated salt solution. Canine trichuriasis has rarely been reported in Türkiye. The results of this study also support the previous reports.

Keywords: *Trichuris* spp., shelter dog, faecal examination, prevalence.

INTRODUCTION

Trichuris vulpis (Nematoda: Enoplida) lives in the caecum and rarely in the colon in dogs (Doganay, 2021). Male and female parasites are 4.5-7.5 cm long (Saari et al., 2019). The adult parasite has a whip-like morphology, and it is called “whipworm” (Traversa, 2011). The parasite develops without any intermediate host (monoxene) (Taylor et al., 2007). The first stage larva develops in the egg within a few months under suitable humidity and temperature conditions in nature (Anderson, 2000; Traversa, 2011; Doganay, 2021). The eggs including first-stage larvae (L1) are responsible for host infection (Taylor et al., 2007). Following oral ingestion of the eggs containing L1, larvae enter in the Lieberkuhn crypts and subsequently invade the mucosa of the large intestine in the host (Saari et al., 2019; Doganay, 2021). The prepatent period is 2-3 months (Traversa, 2011). Trichuriasis is generally asymptomatic in dogs (Doganay, 2021) and some clinical signs are observed during the prepatent period (Traversa, 2011). However, severe clinical signs can be observed in some dogs infected with trichuriasis (Taylor et al., 2007).

Canine trichuriasis has been reported in worldwide (Traversa, 2011). *Trichuris vulpis* is a neglected parasite in dogs and the prevalence rates are reported as 17.6-22.8% in Italy (Capelli et al., 2006; Liberato et al., 2018). Trichuriasis has also been detected in dogs in the neighbour countries of Türkiye (Papazahariadou et al., 2007; Mirzaei and Fooladi, 2012; Tavassoli et al., 2012;

Iliev et al., 2017; Diakou et al., 2019; Iliev et al., 2020; Shukur, 2021; Issa et al., 2022). *Trichuris* spp. is reported between 10.42-15.1% in dogs from Bulgaria (Iliev et al., 2017; Iliev et al., 2020), 3.5-9.6% from Greece (Papazahariadou et al., 2007; Diakou et al., 2019), 4.38-68% from Iraq (Shukur, 2021; Issa et al., 2022) in 4.3-36.2% from Iran (Mirzaei and Fooladi, 2012; Tavassoli et al., 2012). Diagnostic methods, climatic conditions, housing and feeding conditions and ages of sampled dogs are responsible for differences of prevalence (Traversa, 2011).

Dog trichuriasis has been rarely reported and the prevalence varies between 0.6-7.89% in Türkiye (Mimioglu et al., 1959; Doğanay, 1990; Zeybey et al., 1992; Unlu and Eren, 2007; Baklaya and Avcioğlu 2011; Cicek and Yilmaz, 2012; Gurler et al., 2015; Oge et al., 2017; Nas and Bicek, 2018; Afşar et al., 2022). In addition to necropsy and faecal examination results, *T. vulpis* eggs are reported as 2.27% in the hair samples of the dogs in Türkiye (Karaaslan, 2015). *Trichuris* spp eggs are recorded as 2.4% of the sand samples collected in some public parks located in Ankara, Türkiye (Oge and Oge, 2000) and as 21.42% in the sludge samples of the wastewater treatment plant in Elazığ, Türkiye (Obek et al., 2000). The present study, it was aimed to detect the prevalence of *Trichuris* spp. in dogs living in shelters in Kırıkkale. The results obtained were discussed together with the reports originating from Türkiye on this parasite.

MATERIALS AND METHODS

All animal handling was made according to the rules of the Animal Experiments Local Ethics Committee of Kirikkale University (E-60821397-010.99-129466). The study was carried out in a period between January and December 2022. The faecal samples were sampled from 200 dogs (mixed breed, over 1 year old) from two different shelters in Kirikkale province, Türkiye. The dogs live in separate kennels without contact with each other. Cleaning is made daily with pressurized tap water in the kennels. No attempt was made to collect faecal samples from dogs and they were sampled immediately after it has been dropped on the floor. Faecal consistency scores were recorded based on a modified faecal scoring system for dogs (<https://vmc.usask.ca/documents/fecal-scoring-system.pdf>). The samples were prepared using the centrifugal flotation technique both using the saturated salt solution and zinc sulfate (with a specific gravity of 1.3). After centrifugation (at 1500 rpm for 5 minutes), the upper part was put on a slide and covered by a cover slide and then they were examined under a light microscope (Leica DM100). Parasite eggs were diagnosed based on related references (Schmaschke, 2014).

RESULTS

In the present study, 145 of the faecal samples were taken from the dogs from Shelter 1 and 55 of them from Shelter 2. The parasitic eggs or oocysts were detected in 21.5% of faeces examined (43/200); however, *Trichuris* spp. eggs were observed in only 0.5% of the faeces (1/200). *Trichuris* spp. eggs were barrel- or lemon-shaped, yellowish-brown in colour, thick-walled, smooth on the surface, and have characteristic plugs at both ends symmetric and measured as 80x35 µm in diameter (Figure 1).



Figure 1. *Trichuris* spp. egg (x40).

Table 1. The results of faecal examination of the dogs in the shelters.

	Parasite species	Positive (n:200)	%
Helminths	<i>Trichuris</i> spp.	1	0.5
	<i>Toxocara</i> spp.	12	6
	<i>Toxascaris</i> sp.	5	2.5
	<i>Taenia</i> spp.	5	2.5
	Hookworm egg	1	0.5
Protozoon	<i>Isospora</i> spp. oocyst	22	11
	<i>Sarcocystis</i> spp. sporocyst	1	0.5

In the present study, the diagnosis rate of parasitic eggs or oocysts was slightly different based on the saturated solutions used the flotation technique. The parasite eggs or oocysts were detected as 7% of the dog faeces with the zinc sulfate flotation technique, whereas it was 1.5% during the analysis of the same samples with the saturated salt solution. While *Taenia* spp. eggs were observed only in the zinc sulfate flotation technique, they were not observed in the flotation with the saturated saline solution.

DISCUSSION AND CONCLUSION

The eggs of *T. vulpis* can survive in nature from rainy and cold winter season to hot summer months (Traversa, 2011). Dry conditions and direct sunlight can kill *Trichuris* spp. eggs (Bar and Bowman, 2012). *Trichuris* spp. eggs are inactivated at -20°C after 24 hours of incubation (Kines et al., 2021). In this study, the low trichuriasis rate in the sampled dogs (0.5%) may relate to the research area's climatic conditions. Kirikkale is located in the Central Anatolia which possesses a continental climate (cold winters and hot-dry summers). The infective stage larvae may not develop in the eggs on the soil because of the hot climatic conditions in this region. Canine trichuriasis has been reported as 0.7-7.89% in previous studies in the other parts of Central Anatolia, Türkiye (Gurler et al., 2015; Oge et al., 2017). Türkiye is surrounded by the sea on three sides and the coastal areas generally have a mild Mediterranean climate in contrary to the central part. There are some prevalence reports about dogs trichuriasis in the coastal regions of Türkiye (1.5- 6.9%) (Unlu and Eren, 2007; Gurler et al., 2015), however; there is no significant difference in the prevalence rate between the central and the coastal regions of Türkiye.

The prevalence of trichuriasis was low compared to toxocariasis in the dogs in this study (0.5% vs 6%). It is well known that *T. vulpis* eggs have a thick shell-like *Toxocara* spp., so the eggs of these parasites are known to resist natural conditions (Anderson, 2000; Taylor et al., 2007; Saari et al., 2019). However, the high prevalence of toxocariasis in the sampled dogs may be due to the different ways this parasite can infect the dog population such as intrauterine and transmammary transmission, eating of paratenic hosts or parasite eggs containing infective stage larvae. In contrast, *T. vulpis* is transmitted to the dog only in the oral way.

Dogs who live in shelters are exposed to various stressors, such as overcrowding or isolation, unfamiliar surroundings and noise, changing feeds, and restricted movement. Because of the stress factors and difficulty in preventing environmental contamination, animal shelters provide favourable conditions for the establishment and spread of parasitic infection (Raza et al., 2018). *Trichuris* spp. eggs are usually difficult to destroy in the environment (CFSPH, 2019). Prolonged desiccation and exposure to sunlight as well as heat treatment of solid surfaces, may ensure the elimination of eggs of the parasite (Raza et al., 2018; CFSPH, 2019). There are some data on the sensitivity of *Trichuris* spp. eggs to disinfectants (CFSPH, 2019). *Trichuris* spp. eggs are completely inactivated by some disinfectants such as 10% bleach and 10% iodine after five minutes of incubation, 95% ethanol after 48 hours, and 10% formalin after four weeks (Kines et al., 2021). In the present study, trichuriasis rate was detected as 0.5% in the sampled dogs. Any disinfectant has been not used in the sampled shelters. The low prevalence of the parasite can be explained by the faeces has been removed daily in the shelters. Separate kennels can reduce

possible parasitic contamination of the sampled dogs in the present study.

The clinical diagnosis of trichuriasis is made by faecal examination (Taylor et al., 2007). The flotation technique with saturated solutions generally is preferred (Traversa, 2011; Saari et al., 2019). The specific gravities of the saturated salt solution and zinc chloride solution are 1.20 and 1.30, respectively (Schnieder, 2006). Flotation carried out in saturated salt solution is generally used in laboratories because it is cheaper, however; the zinc sulfate flotation technique is known more reliable detection of *T. vulpis* infections (Zajac et al., 2002). It is probably due to the fact that comparing to other helminth eggs, *Trichuris* spp. eggs have a greater specific gravity (Zajac et al., 2002). Twice as many *T. vulpis* eggs have been found in the zinc sulfate centrifugal flotation technique (Gates and Nolan, 2009). Also, the zinc sulfate flotation technique is to be effective in the diagnosis of *Taenia* spp. eggs (Maurelli et al., 2018). In the present study, the eggs of *Taenia* spp. were found to only the zinc sulfate centrifugal flotation technique and this technique was more effective in terms of all parasite egg/oocyst detection (7% vs 1.5%).

Canine trichuriasis has rarely been reported in Türkiye from the past to the present. The results of this study also support this situation. The infective stage larvae may not develop in the eggs on the soil because of the hot climatic conditions in Türkiye.

Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Concept: G.N.A., S.A., Design: G.N.A., S.A., Data Collection or Processing: G.N.A., S.A., Analysis or Interpretation: G.N.A., S.A., K.Y., Literature Search: G.N.A., S.A., K.Y., Writing: G.N.A., S.A., K.Y.

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Preparation of Quail (*Coturnix coturnix*) Skeleton to Promote the Teaching Facilities of Avian Anatomy Laboratory

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Abstract

The stiff structure of the body, or skeleton, provides the body with its overall form, permits movement, generates blood cells, and protects delicate organs. This research was done to prepare the quail (*Coturnix coturnix*) skeleton for the avian anatomy museum and educational facilities. A total of five matured Quails (at the age of 6 months) were collected from a commercial quail farm, in Khulna, Bangladesh. The carotid artery and jugular vein were carefully dissected before skinning each quail with feathers, removing all viscera, combs, and wattles. To adequately disintegrate the muscles, ligaments, and tendons, different body sections of the bones were separated, wrapped in markin cloth, and cooked in a 3% solution of soda water for 1.5 hours. The body parts were then maintained in separate solutions for 5 days. After five days, the bones were thoroughly cleaned by soaking them in a 5% hydrogen peroxide solution for 30 minutes, and rinse them under running water. To maintain the order of the vertebrae in the various segments, a steel wire was inserted into the vertebral foramen. After proper sun drying for 25 days, a set of bony components from different parts (axial and appendicular) of the body was finally found and articulated to rise into a whole skeletal frame. Therefore, the present work denotes time-consuming and easy preparation of the avian skeleton as well as the skeletal framework will be helpful for veterinary students in the avian anatomy laboratory as well as to enhance the elegance of the anatomy museum.

Keywords: Avian morphology, framework, knowledge, quail, veterinary students.

INTRODUCTION

In both veterinary and human medical education, skeletons are the most important teaching tools for anatomy lessons (Kempa et al., 2016). Academicians and students need to comprehend what the bones, cartilage, and then a skeleton are in order to have a better comprehension of the human or animal body's structure (Atabo et al., 2019). Anatomical understanding helps a surgeon or clinical practitioner to identify a condition, develop their capacity to think about the pathophysiological mechanisms behind a certain bodily system, etc. (Turney, 2007). For veterinary anatomists, researchers, and practitioners to correctly draw up a skeletal framework of avian species, they must have a basic understanding of the avian skeleton (Baker et al., 2003). Skeletons are also utilized in forensic applications, such as the identification of carcasses or, in rare instances, bones that have been taken as evidence in other crimes, to identify fossils and for a variety of other purposes (Olson, 2003). The hollow-structured, strong bones that make up the avian skeletal system give the body a lightweight appearance (Jacob, 2023). The development of a robust skeletal system has also been intimately linked to egg production. Worldwide, the production of eggs in avian species is hampered by soft and fragile bones (Linden, 2014). However, the quail's skeleton is unique compared to other animals' and developed as powered flight progressed. A skeleton that provides a strong base and

rigidity for the attachment of flight muscles has resulted from a reduction in the total number of bones and the combination of numerous joints (Aspinall and Cappello, 2019). A skeleton can be prepared using a variety of techniques, including enzymatic maceration, burial maceration, cold water maceration, and more (Gofur and Khan, 2010). The processing of the skeletons of large and small animals requires different safety measures. Larger boiling times are required for large animal bones, particularly long bones, than for avian bones. Because excessive boiling causes a bone to become fragile and disintegrate (Hussain et al., 2007). The majority of anatomists and researchers prepare the skeleton using conventional procedures, placing special emphasis on the necessary timing. However, the authors of the present study emphasized more than just the expedient and economic aspect and they tried to prepare a quail skeleton that would last for a long time, helping educators and students in their basic anatomy education. As a result, this research will contribute to a greater knowledge of the avian skeleton and how it functions in relation to the skeletons of other birds. The information collected from this experiment might also be applied to teaching, improving student comprehension of comparative knowledge between large and avian anatomy by comparing various characteristics of the avian skeleton to that of other species.

MATERIALS AND METHODS

Sampling and equipment preparation

Five adult quails were taken from a commercial quail farm in Khulna, Bangladesh when they were around six months old. Prior to considering the utilization of the acquired quail for skeleton preparation, each quail's overall health, exterior appearance, eye mucous membrane, and all other body parts were meticulously inspected. Masks and rubber gloves were used as a safety precaution in accordance with the recommendations of Baker et al., (2003). The following supplies were utilized to create the desired skeleton: steel wire, 3% soda water solution, 5% hydrogen peroxide, a hardwood platform, adhesion agents (Fevicol), and varnish.

Animal Preparation and ethical guidelines

To drain all the blood from the body, captured quails were slaughtered by severing the carotid artery and jugular vein. A Littman stethoscope was then used to assess the sacrificial quail's heart rate in order to certify its demise. All the methods and procedures for killing the quails were approved by the Ethical Committee of Bangladesh Agricultural University (approval no. AWEEC/BAU/2023(34)).

Removal of skin and flesh

A scalpel, scissors, and forceps were used to trim the skin and muscles, which also helped to widen the abdominal cavity. Then, every organ of the digestive, respiratory, and reproductive systems was painstakingly removed, leaving only the visible, undamaged bones of the quail's structure. Finally, each segment of the body parts: the skull, thorax, forelimb, and hindlimb was cut away from the surrounding joint area and wrapped individually in clothing mainly because it minimizes the loss of tiny bones and helps to preserve the bones during boiling. In order to keep the spinal column aligned (from cervical to caudal), steel wires were specifically implanted.

Boiling and decomposing

The entire body segments coated with markin clothing were boiled for 1.5 hours in a metal container with soda water (sodium carbonate, Na_2CO_3) in a 3% solution. The appropriate digestion of each segment's fleshy portions is aided by the use of soda water as a macerating agent (Van Cleave, 2010; Simriti et al., 2019). The container was boiled and then left alone for five days. The bony segments' residual muscles correctly disintegrated throughout this period. The remainder of the muscles, ligaments, and other tissues were then completely removed with the use of a knife and forceps once the garments had been removed.

Cleaning and bleaching

After being collected, the skeletal pieces were immersed for 30 minutes in a 5% hydrogen peroxide (H_2O_2) bleaching solution to prevent further microbial deterioration, provide stability, and enhance the whitening look of the bones. In order to clean the bones, each bone fragment was lastly washed under running water.

Drying

For 25 days, the bones were correctly dried in the sun for

10 to 12 hours each day. After that, a cautious 50 ml varnish spray was applied to all the bony areas. Bones are given durability and protection from various microbial attacks by proper sun drying and varnishing.

Sequential arrangement of bones and construction of the skeletal frame

During the preparation of boiling and cleaning, different structures were disorganized. So, after completing sundry, different segments of the bony parts were articulated by using fevicol. Firstly, the axial part was taken into consideration for articulation. The nasal process of the premaxilla and hyoid bone were precisely organized in the correct position and the mandible was attached to the quadrate region by the fevicol. All the vertebrae of the column from cervical to caudal (although the lumbar and sacrum fuse together to form the synsacrum) were numbered and fixed to each other by adhesives. Firstly, a piece of heavy wire was pushed as far as possible inside the neural canal of the fused vertebra of the back. Then the first thoracic vertebrae were put in place, and then the cervical vertebrae were put on the wire and pushed into their normal positions. After the cervical vertebrae were in place and glued, it was then bent in an "S" shape curve to match the natural curve of the neck of the bird. The wire was cut and inserted into the skull. It was attached to the vertebral column with the support of wire and glue. Anteriorly (in the wing region), the scapula, coracoid, clavicle, humerus, radius-ulna, and carpometacarpus were articulated in sequence as well as the pairs of ribs were arranged in ascending order and attached to each thoracic vertebrae by fevicle. The Sternum was attached with the true rib pairs and coracoids on both sides by fevicle. Next, the hindlimb (leg) bones called the hip bones (ilium, ischium, and pubis), femur, tibia-fibula, and foot bones (metatarsals and digits) were articulated chronologically with fevicle glue. Finally, the skeletal structure was positioned on a wooden platform of the required dimensions according to the size of the animal for display in the avian anatomy museum and gross anatomical study in the academic lecture.

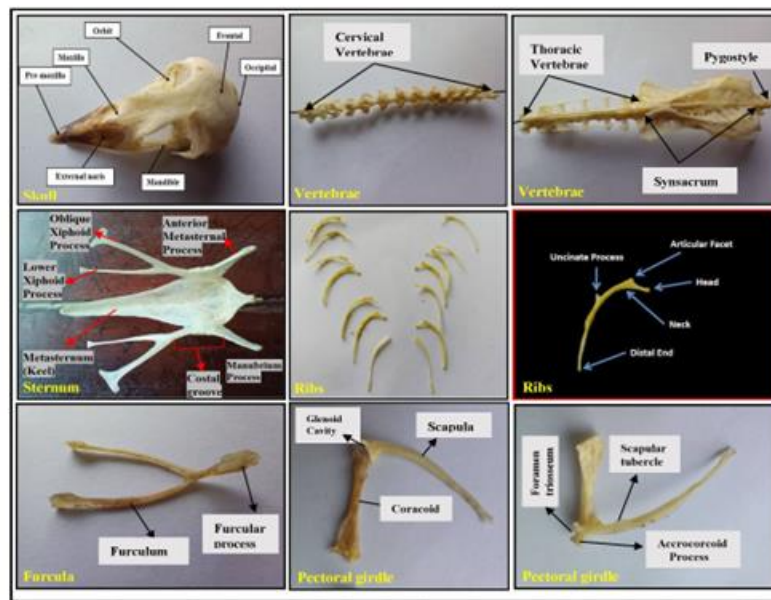
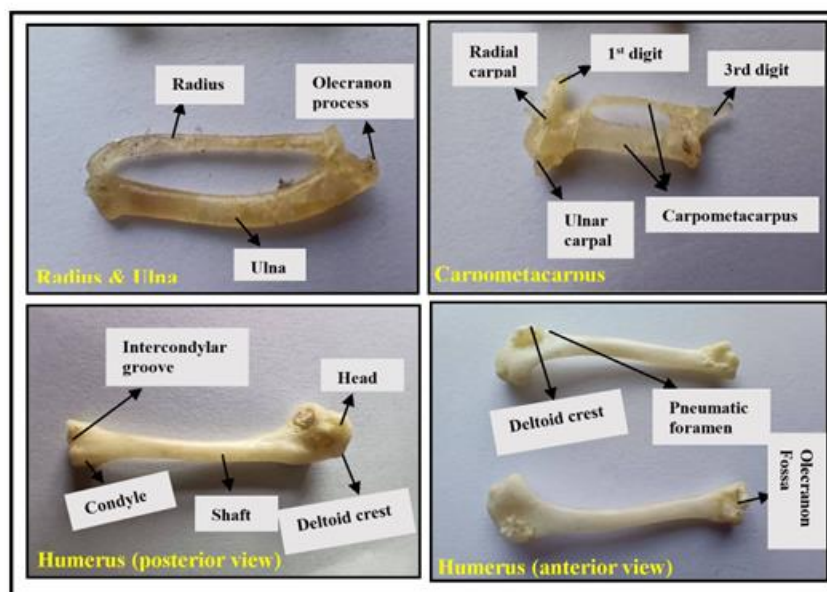
RESULTS

Compared to mammals, the avian skeletal system is light weighted. Mainly the bones are hollow structured which makes the weight light. Numerous bones fused together and make the bird's skeleton rigid. Table 1 shows the steps which were maintained with a specified time schedule.

In the present study of the quail skeleton, it has been designated into two parts: axial and appendicular parts. In the axial region: the skull; vertebrae: cervical; thoracic, lumbar, and sacral fused to form synsacrum and coccygeal vertebrae or caudal vertebrae (fused caudal vertebrae are called pygostyle); ribs, and breast bone or keel bone (Figure 1). On the other hand, in the appendicular skeleton: the forelimb or wing bone possesses scapula, coracoid, and clavicle (fusion of two clavicles form furcula or wishbone), humerus, radius-ulna, and carpometacarpus, phalanges (Figure 2). On the contrary, the hind limb or leg bone was formed by of pelvic girdle (ilium, ischium, and pubis), femur, tibia-fibula, and feet bone (tibiotarsus, tarsometatarsus, and phalanges) (Figure 3).

Table 1. Steps involved in time required for preparation of quail skeleton.

SI No.	Sequence maintained in the skeleton preparation	Required time
1.	Animal preparation	30 minutes
2.	Removal of skin and flesh	2 hours
3.	Boiling and decomposing	Boiling: 1.5 hours Decomposing: 5 days
4.	Cleaning and bleaching	Bleaching: 30 minutes Cleaning: 2 hours
5.	Drying	25 days
6.	Articulation and rising of the skeleton	1.5 hours

**Figure. 1:** Representative photographs depict the different bones (Skull, Vertebrae, Sternum, Ribs, Furculum, and Pectoral girdle) of the quail's skeleton. Identified structures (different colour arrows) of different bones illustrate the morphological characteristics of a quail's skeleton.**Figure. 2:** Representative photographs depict the different bones (Radius-ulna, Carpometacarpus, and Humerus) of the quail's skeleton. Identified structures (black arrow) of different bones illustrate the morphological characteristics of a quail's skeleton.

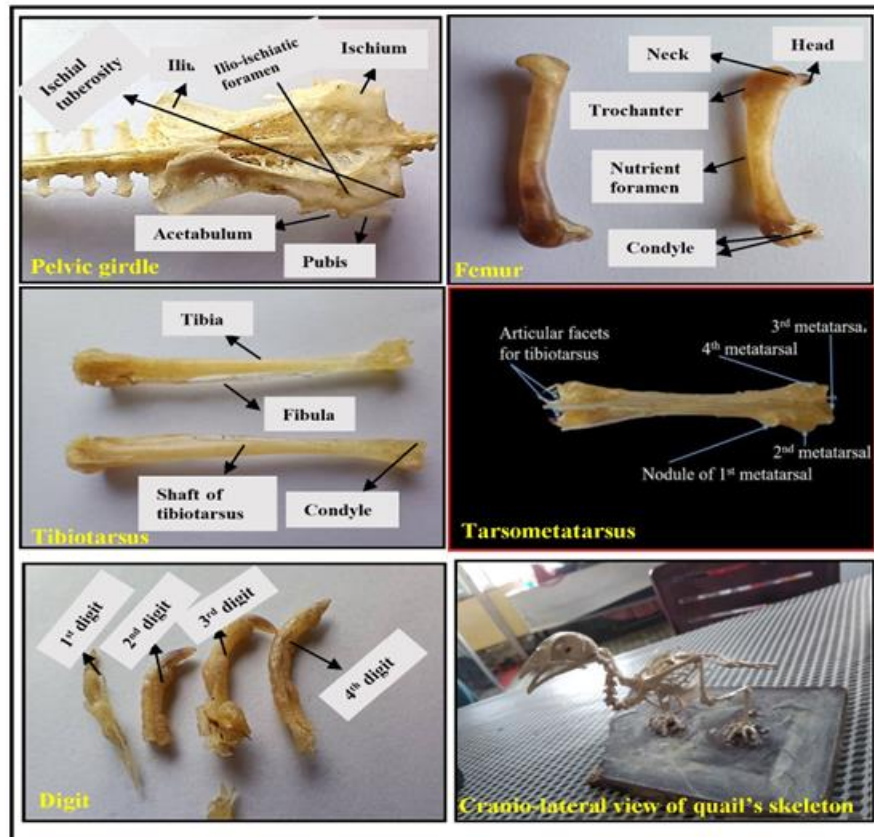


Figure. 3: Representative photographs depict the different bones (Pelvic girdle, Femur, Tibiotarsus, Tarsometatarsus, Digits and Cranio-lateral view of quail's skeleton) of the quail's skeleton. Identified structures (black arrow) of different bones illustrate the morphological characteristics of a quail's skeleton. Cranio-lateral view of a structured quail's skeletal frame is also presented.

DISCUSSION AND CONCLUSION

All vertebrates have skeletons because they need them for support and to protect internal organs and tissues. The skeletal structure of a bird is similar to that of other animals, but it is too light, which is what they need to fly while yet having the essential bodily support. Most aspects of the avian skeleton emphasize lightness and strength (Getty, 1975). Every stage of the quail skeleton preparation in the current investigation has been carried out with exceptional efficiency. Using the skeleton in the anatomy lab for a longer period of time was the primary goal of this preparation. The quail's fragile bones need extra care during the skinning and removal of the muscle before boiling. Gofur and Khan, (2010) likewise maintained this precaution. They also removed flesh by avoiding any damage to the bones. The boiling time was strictly maintained in the current bony management. As the excess boiling may cause any rupture or shrinking of the soft bones. The statement was fully agreed upon by Van Cleave, (2010). A 3% solution of soda water was used during boiling for the easy removal of the rest portion of the muscle as well as to properly decompose (Baker et al., 2003; Gofur and Khan, 2010) also in agreement with the procedure. They stated that using any other biological compounds may have corrosive actions on bones. After boiling, the decomposing period (Table 1) was taken into special consideration because, without proper decomposing of muscle, microorganisms may attack at any time in the bone which causes fragility or reduction of

longevity. Leon et al., (2004) also completed these steps with special care. Bones were then bleached into a 5% hydrogen peroxide (H_2O_2) solution for 30 minutes to enhance the whitening of the bone's surface according to the study of (Faruk and Das, 2023). Bones' impact strength, compressive strength, and shear strength also remained unaffected by the use of hydrogen peroxide (H_2O_2) solution which was revealed by De Paula, (2005). Curran et al., (2004) also suggested that, besides the hydrogen peroxide (H_2O_2) solution, different treatment methods may be applied to clean or disinfect the bones using ethanol, gamma radiation, acid washing, antibiotic solutions, etc. which have baleful effects on biochemistry and biomechanical integrity of the allograft tissues. The prepared bones were dried completely by sun rays. Then, the bones of different segments were articulated sequentially to form a skeletal structure and positioned on a wooden stage with the aid of a metal wire according to the direction of (Musa et al., 2015). According to the description of (Bairbre, 2008) as a fellow veterinary student or educator, or wildlife researcher, knowledge of avian anatomy helps them when providing avian care in a wildlife hospital. No one can develop a basic knowledge of comparative anatomy without learning avian anatomy. In conclusion, the morphology of different bird species and other big animals differs significantly. The importance of avian anatomy in the veterinary study is easily demonstrated by the importance of birds, thermoregulation mechanism, metabolism, behavioral pattern, flight

mechanism, the fusion of the bones of the different regions (furculum, pygostyle, synsacrum, etc.), cardiac output, large volume of oxygen capturing capacity, and unique digestive procedure data. The veterinarian can better care for avian species by having an understanding of their anatomical structures, especially when it comes to medicine administration. Various organs and bone's morphological makeup also aids in identifying species. The quail's skeleton will therefore make a significant contribution to academics and students' understanding of anatomy, as well as improve the aesthetics of the avian anatomy museum.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Authorship Contributions

Concept: S.K.K., Design: S.K.K., Data Collection or Processing: S.K.K., Z.H.R., M.A.A.M., A.T.C., Analysis or Interpretation: S.K.K., A.S., Literature Search: S.K.K., A.S., Writing: S.K.K. Critical Review: S.K.K., Z.H.R., M.A.A.M., A.T.C., A.S.

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Comparison of Various Storage Conditions for The Stability of *Escherichia coli* O157:H7 Bacteriophage M8AEC16

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Abstract

In this study, bacteriophage M8AEC16, which is lytic to *Escherichia coli* O157:H7, isolated and characterized within the scope of our previous studies, was used to determine the stability during 6 months of storage at +4°C, -20°C and -85°C using glycerol, skimmed milk powder and carrageenan as cryoprotectants. For this purpose, bacteriophage M8AEC16, was enriched with *E. coli* O157:H7 ATCC 43895 and then phage suspension groups were prepared by adding three different cryoprotectants (test groups) in addition to those without cryoprotectant (control). Bacteriophage suspensions were stored at 3 different storage temperatures (+4°C, -20°C and -85°C) for six month period. During storage, phage titers were determined on double-layer LB agar by making serial dilutions of phage suspensions on the 0th day and at the end of each month. As a result of the study, it was observed that stability was maintained at all temperatures for all groups during the first two months. However, in the third and fourth months, a decrease in the counts of bacteriophages were observed at -85°C in the glycerol added group. In addition to the control group, in the fifth month, carrageenan provided the highest preservation at all temperatures. In the sixth month, +4°C glycerol provided the best preservation, while carrageenan provided the highest preservation at other temperatures compared to the other test groups. In conclusion, the most successful result was obtained at +4°C for the preservation of *Escherichia coli* O157:H7 phage M8AEC16 considering the cost, usefulness and effectiveness.

Keywords: Bacteriophage, carrageenan, cryoprotectant, glycerol, preservation, skimmed milk powder.

INTRODUCTION

Bacterial pathogens are of serious importance for public health. According to the World Health Organization (WHO), it has been reported that approximately 600 million people become ill and 420 thousand people die every year as a result of consuming contaminated foods (WHO, 2023). These pathogens can contaminate foods at all stages of production such as slaughtering, milking, fermentation, packaging, and storage. Good Manufacturing Practices (GMP), Hazard Analysis Critical Control Points (HACCP) and other risk assessment practices may be insufficient in the control of pathogenic bacteria (Seçkin and Baladura, 2010).

The other issue with food-borne pathogens is antimicrobial resistance. Appropriate and extensive use of antimicrobials causes resistance in bacteria. In this case, existing antimicrobials may not be effective in treating these bacteria. Therefore, alternative control methods are needed to both protect public health and meet the demand of consumers who demand "minimally processed and chemical-free products". In this context, among these alternative methods, bacteriophages can be used for the biocontrol of bacterial pathogens effectively (Cufaoglu and Ayaz, 2019).

Bacteriophages (phages), which are obligate intracellular parasites, are defined as bacteria-killing viruses. It has been shown that they do not harm mammalian cells, but only infect specific bacterial cells.

They can be found in all kinds of environments where bacteria can survive, such as human and animal skin, intestinal flora, fermented foods, sewage water, air and soil. Bacteriophages, like other viruses, multiply only in case of infection. They do not have their metabolic systems and ribosomes. Therefore, they cannot produce genetic information or synthesize proteins (Gökçe, 2010; Jończyk et al., 2011; Gümüştas 2015).

There are two types of life cycles: lytic and lysogenic. The proliferation strategy followed by phages after the penetration stage determines their life cycle (Salmond and Fineran, 2015). The time it takes for bacteriophages to cause infection after encountering bacteria and the formation of new phages; is examined in four stages: adsorption, penetration, latent period and lysis period (Arda, 2011).

Phage therapy studies started with D'Herelle on patients with dysentery and plague. In the following years, phage cocktails such as "Pyophage" (contains phages targeting about 20 different pathogenic gastrointestinal bacteria) and "Intestiphage" (contains phages targeting *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Proteus*, and *E. coli*) were used both as treatment and prophylactic against gas gangrene, and positive results were recorded (Abedon et al., 2011). In addition to their use for therapeutic purposes in humans and animals, bacteriophages have spread to a wide field of study by being used in the field of molecular biology, identification

of bacteria, biotechnology studies, agricultural applications, biosanitation, bioprotection and biocontrol (Kasimoglu Dogru et al., 2017).

The use of bacteriophages in the field of food safety is very important. Bacteriophages are used in the food industry for equipment and contact surface disinfection (biosanitation), decontamination (biocontrol) and shelf-life extension (bioprotection) of carcasses, processed foods, fresh vegetables, and fruits (Gencay et al. 2016; Kasimoglu Dogru et al. 2017; Kekillioglu et al., 2019). Bacteriophages can be applied by adding them into foods, applying them to the surface, immersing the food in phage-containing water, or attaching them to food packaging materials. The most preferred method is application to the surface as a spray, as it is easy to apply on foods such as cheese, sausage, and meat fillet (Pérez Pulido et al., 2016; Doğru et al., 2017).

Like all microorganisms, bacteriophages must first be preserved under appropriate conditions to be used in studies and applications. In order to choose the right preservation method, many criteria such as phage stability, population, genetic change, preservation of purity, frequency of use and cost should be evaluated. Many studies have been conducted and some methods have been recorded for long-term preservation of bacteriophages. These methods; freezing (Adams, 1950), drying (Prouty, 1953), lyophilization (freeze drying) (Harris, 1954) and encapsulation (Saygılı and Karagözlü, 2017; Malik, 2021). It has been reported that many factors such as storage temperature, storage time, preservatives (cryoprotectant) and phage density before processing affect stability when choosing appropriate storage conditions. It has also been stated that in addition to the existing methods, alternative methods such as hydrocolloids such as carrageenan and gelation techniques can be used in future studies (Malik, 2021).

In this study, the stability of *Escherichia coli* O157:H7 phage M8AEC16, which was isolated and characterized within the scope of previous studies, was observed during the storage at +4°C, -20°C and -85°C using cryoprotectants such as glycerine, skimmed milk powder and carrageenan.

MATERIALS AND METHODS

Activation and enrichment of phage culture:

This study aimed to determine the storage stability of lytic *E. coli* O157:H7 bacteriophage M8AEC16 which was classified in the family *Myoviridae* under the order *Caudovirales* with the A1 morphotype (Gencay et al., 2016). Bacteriophage M8AEC16 stored at -85°C was activated and enriched in the fresh culture of *E. coli* O157:H7 ATCC 43895 (ECO157) in Tryptic Soy Broth (TSB; Oxoid) for 24 hours at 37°C. The enriched phage suspension was centrifuged (10,000 x g) and then subjected to a 0.22 µm diameter millipore filter (Guo et al., 2019). The presence of the plaques was revealed with the double-layer agar method. For this purpose, 3-4 ml of Luria Bertani (LB; Sigma-Aldrich) Soft Agar (LB Broth + 0.75% agar) containing 200 µl of ECO157 (10⁸ cfu/ml) was poured on LB agar as a second layer. Then filtered phage suspensions were inoculated on LB soft agar (Xie et al., 2015). Following the spot plating at room temperature, petri dishes were incubated at 37°C for 24 h. The next day, the plaques on the agars were observed (Xie et al., 2015).

Determination of titers of phage stock

Phage titers were detected by spot plating on double-layer LB agars. For this purpose, the log phase ECO157 as a host bacteria and M8AEC16 phage filtrate from which the phage was isolated were incubated in TSB at 37°C overnight. At the end of the incubation, serial dilutions of the supernatant obtained were prepared by centrifuging at 10,000 x g for 5 minutes and passing it through a 0.22 µm diameter millipore filter. Then, 200 µl of log phase bacteria were added to 3 ml of LB soft agar and poured onto the previously prepared LB agar as a second layer. After the second layer was congealed at room temperature, 10 µl of each phage dilution was spotted. After incubation at 37°C overnight clear plaques were calculated as plaque-forming unit (pfu/ml) (Kishi et al., 2018).

Preservation of phage groups and determination of titers during storage

In addition to the control phage suspension which didn't contain cryoprotectant and test phage groups, contained 20% of one of the cryoprotectant substances (glycerol, skimmed milk powder and carrageenan as a gelling agent) were stored under three different temperatures (+4°C, -20°C and -85°C).

During storage, titer determination was performed on double-layer LB agar by making serial dilutions of phage suspensions on the 0th day and 1st, 2nd, 3rd, 4th, 5th, 6th months. The results obtained were calculated as pfu/ml and compared with the initial titers. Then the reduction in the number of phages and the stability of the phages under storage conditions were investigated. These phage counts were carried out in three repetitions.

Statistical Analysis

In order to determine the most effective preservation method, control (without cryoprotectant) and test phage groups (with cryoprotectant: carrageenan, skimmed milk powder or glycerol) bacteriophages were stored at +4°C, -20°C and -85°C for 6 months. In order to determine the effect of cryoprotectants, statistical analysis were carried out with the Greenhouse-Geisser test and the Sphericity Assumed test to determine whether the change of concentration at constant temperature according to the elapsed time is significant and whether the change of the concentration according to the elapsed time is significant when keeping the cryoprotectant constant and comparing the results at different storage temperatures (Alpar, 2010).

RESULTS

In the study, the stability of M8AEC16 bacteriophage, which is lytic to *E. coli* O157:H7, in the presence of glycerol, skimmed milk powder, or carrageenan cryoprotectants during 6 months of storage at +4°C, -20°C and -85°C was examined, and the analysis results are shown in Table 1.

When the results of the study were examined, no significant decrease (0.78 log pfu/ml) (p>0.05) was observed in the number of control bacteriophages stored at +4°C without cryoprotectant for 6 months. In parallel, no decrease in phage stocks was observed in the counts between the 0th day and the 3rd month in the cryoprotectant groups, and it was noted that the phages maintained their stability at storage temperatures (+4°C, -20°C, and -85°C).

In the 4th month of storage at +4°C, a decrease of 0.75 to 1.31 log pfu/ml was observed in all cryoprotectant-added phage stocks, and the least decrease in the number of bacteriophages occurred in the glycerinated phage stock, and the highest decrease was in the carrageenan added stock. In addition, while a 1 log pfu/ml decrease was recorded in skimmed milk powder and glycerol phage stocks at -20°C, no significant decrease was observed in

carrageenan phage titer ($p>0.05$). On the other hand, at -85°C, a decrease of approximately 1 log pfu/ml was observed in all cryoprotectant-added phage stocks. When the counts of the storage for the 4th month were examined, in addition to the control phage stock, the highest stability values were observed in the carrageenan-added phage stock stored at -20°C.

Table 1. Counts of bacteriophages detected under storage conditions (log pfu/ml).

Storage period	Number of bacteriophages (log pfu/ml)									
	+4°C		+4°C		-20°C			-85°C		
	Control	C	SMP	G	C	SMP	G	C	SMP	G
0 th day	17,78	16,78	16,95	16,70	16,78	16,95	16,70	16,78	16,95	16,70
1 st month	17,70	16,70	16,95	16,60	16,70	16,93	16,47	16,70	16,68	16,00
2 st month	17,70	16,70	16,95	16,30	16,70	16,75	16,47	16,70	16,07	16,00
3 st month	17,60	16,70	16,90	16,30	16,70	16,70	16,47	16,60	16,00	15,94
4 st month	17,60	15,47	15,84	15,95	16,70	15,95	15,70	15,70	15,84	15,70
5 st month	17,78	14,00	14,84	15,95	15,70	15,30	15,70	15,60	15,30	15,47
6 st month	17,00	14,00	14,60	15,78	15,00	15,30	15,82	15,60	15,30	15,47

C: Carrageenan, **SMP:** Skimmed Milk Powder, **G:** Glycerol

In the 5th month of the study, a decrease was observed in all cryoprotectant-added phage stocks at +4°C. However, the most serious decrease compared to the previous month was in the phage stocks with added carrageenan (1.47 log pfu/ml) and skimmed milk powder (1 log pfu/ml), while no decrease was observed in the glycerol stock. In the study, a decrease was observed in all phage stocks with cryoprotectant added at -20°C and -85°C, but the only phage stock that did not show a decrease compared to the previous month was the one containing glycerol at -20°C.

Finally, in the 6th month counts, no significant decrease in phage stocks (0 - 0.70 log pfu/ml) was observed compared to the previous month, and the highest decrease occurred in the phage stock with carrageenan at -20°C at the level of 0.70 log pfu/ml. On the other hand, considering the six-month storage period, the decrease in the number of bacteriophages in the control group was recorded in the range of 0.78 to 2.78 log pfu/ml. Accordingly, the least decrease in the number of bacteriophages following the control group was detected in phage stocks containing glycerol at -20°C and +4°C, with 0.88 and 0.92 log pfu/ml, respectively. On the other hand, the highest decrease in bacteriophage titer was 2.35 and 2.78 in stocks containing skimmed milk powder and carrageenan stored at +4°C, respectively.

When the temperature was kept constant and the effect of cryoprotectants was examined, the measurements differed over time when the change in concentration according to the elapsed time was determined whether it was significant or not. ($p<0.001$). However, the interaction between temperature and time was not significant. In other words, the change over time did not differ at different temperatures. ($p=0.460$; >0.05). It was statistically determined that the difference between temperature groups was not significant. ($p>0.05$). When we kept the cryoprotectant constant and compared the results at

different storage temperatures and examined whether the change in concentration over time was significant, the measurements differed over time ($p<0.001$). Additionally, the interaction between group and time was significant ($p=0.039$; <0.05). In other words, it was statistically determined that the difference between cryoprotectant substances was significant.

The change in bacteriophage stock counts depending on temperature and cryoprotectant variables during 6 months of storage are shown in Figures 1 to 6.

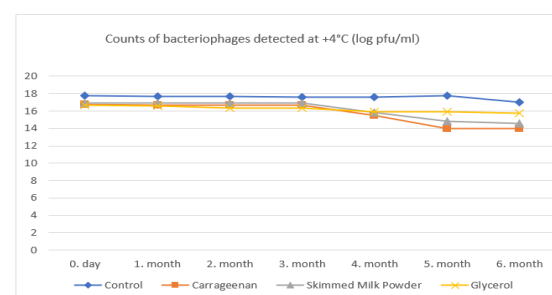


Figure 1. Counts of bacteriophages detected at +4°C.

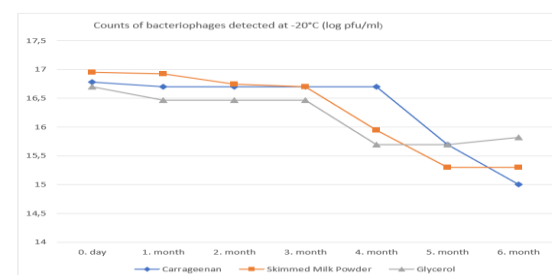


Figure 2. Counts of bacteriophages detected at -20°C.

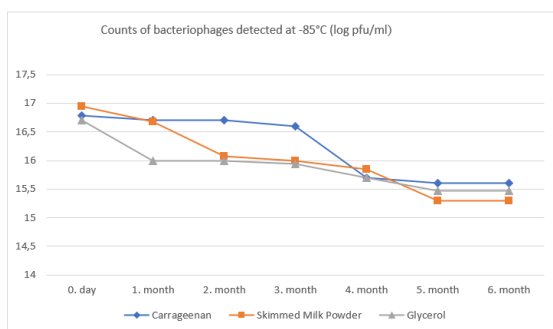


Figure 3. Counts of bacteriophages detected at -85°C.

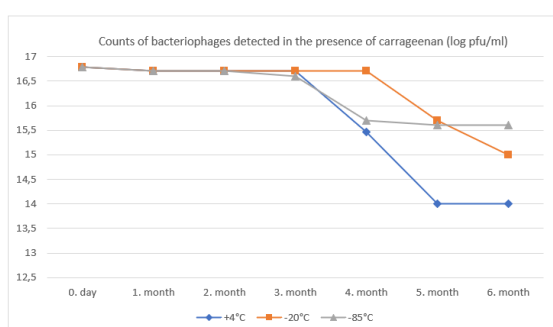


Figure 4. Counts of bacteriophages detected in the presence of carrageenan.

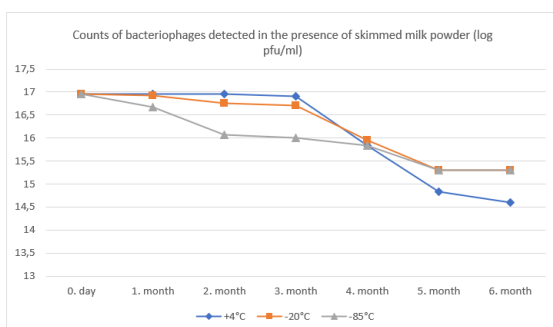


Figure 5. Counts of bacteriophages detected in the presence of skimmed milk powder.

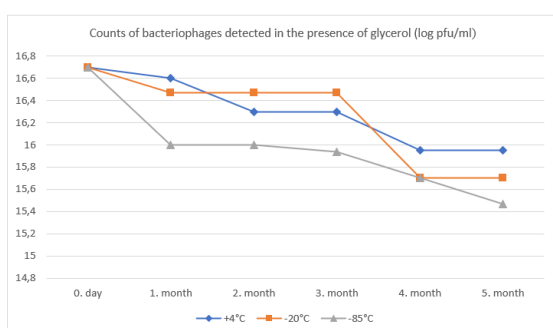


Figure 6. Counts of bacteriophages detected in the presence of glycerol.

DISCUSSION AND CONCLUSION

In a study investigating the long-term preservation of bacteriophages with standard cooling without a decrease in phage activity; the stability of 5 different bacteriophages

(JHP, RLP, RSP, SaPL, IPL) were examined one year after being frozen at different temperatures (-20°C, -80°C, -196°C). In the study, 50% glycerol was used for preservation at -20°C and -80°C. When bacteriophages stored at 4°C were checked after 1 year, it was observed that there was no decrease in their titers (Clark et al., 1962; Clark and Geary., 1973; Verbeke, 2015; Alvi et al., 2018). According to the study, An 84-100% decrease (from 8 log units to 17 log units) was recorded in the titers of phages stored at 37°C. Similarly, a 69-100% decrease (from 8 log units to 15 log units) was observed in the titers of phages stored at 25°C. While a decrease from 5 log units to 12 log units (between 25-70%) was recorded at -20°C, a decrease between 45-100% was observed at -80°C (Alvi et al., 2018). In the study, a loss of stability was noted in freezing conditions at -20°C and -80°C, while at 25°C and 37°C, very little recovery was achieved after 12 months. As a result, the most reasonable temperature for storing tailed bacteriophages was reported as +4°C which is compatible with the findings of our study.

In another study conducted on the long-term preservation of bacteriophages, the effect of the structure of the phage on stability was investigated (Ackerman et al., 2004). Lysates were maintained at +4°C and -80°C (in liquid nitrogen) with glycerol. Lysogenic phages were stored at -80°C with 15% glycerol. According to studies, it has been stated that lipid-free, tailed cubic structured, or filamentous phage lysates can be stored for 5-10 years, but the titers will decrease by 1 log per year (Vieu and Croissant 1966; Steele, 1976), while it has been stated that more meticulous studies are required for lipid-containing phages. It has been stated that chloroform should not be preferred for storage because it inactivates one third of tailed and lipid-containing filamentous phages (Ackerman et al., 2004). Unlike our study findings, it has been stated that lysates should not be stored alone at +4°C and should be supported by lyophilization and deep freezing. In the study, the survival of the phages was not guaranteed even in deep freezing at -80°C or in liquid nitrogen (Ackermann et al., 2004).

In a study on four different *Staphylococcus* bacteriophages, the effect of storage at different temperatures using the lyophilization method and different stabilizers on stability was compared. In the study, philPLA35 and philPLA88, which belong to the *Siphoviridae* family, and philPLA-RODİ and philPLA-CIC bacteriophages, which belong to the *Myoviridae* family, were used. Phages were preserved in the presence of trehalose, sucrose, glycerol, and skim milk. In the study, phages were stored at 20-25°C for 6 months, at 4°C, -20°C, and -80°C for 24 months, and at -196°C for 12 months. Stability was lost in phages after 6 months at 20-25°C. In *Siphoviridae*, stability was preserved at -20°C for all stabilizers (there was a decrease in infected philPLA88). This finding is different from our study findings since a decrease was detected in all cryoprotectant added phage (*Myoviridae*) groups at -20°C after 6 months of storage. It is thought that this difference may be due to the classification of the tested phages in different families. Similarly in the study, *Myoviridae* phages, in which sucrose and trehalose were used as stabilizers, could not maintain their stability at -20°C and a decrease in titer was observed. No serious loss of stability was observed for all phages in the presence of all stabilizers at -80°C. For *Siphoviridae* and *Myoviridae*, decreases of less than 1 log unit were observed after 24 months. Similar results were recorded after 12 months in samples stored at -196°C in

liquid nitrogen (Gonzales-Menendes et al., 2018). In this study, it was clearly observed that the results of *Siphoviridae* phages were different from our study, but similar findings were obtained with *Myoviridae* phages.

In another study, the effects of preservatives and storage temperature on the stability of phages preserved using the lyophilization method were compared. Three different phages used in this study (ECP311 against *Escherichia*, KPP235 against *Klebsiella* and ELP140 against *Enterobacter*) were tested on the 0th, 1st, 5th, 10th, 15th, and 20th day at +4°C and 37°C in the presence of glucose, sucrose, mannitol, gelatine, sorbitol, and polyethylene glycol as cryoprotectant substances. As a result of the study, it was observed that sucrose, gelatin, and their combinations were successful in preserving phage viability after lyophilization. In the study, while viability was preserved for up to 20 months at 4°C, a decrease in activity was noted after 10 months at 37°C (Manohar and Ramesh, 2019).

In a different study which was conducted comparing the effects of encapsulation and microencapsulation on stability, and it was observed that microencapsulation provided better stability for phages. Alginate encapsulation and microencapsulation methods were used in the study. In the study, 4 different bacteriophages stored at +4°C and +20°C were checked in the 1st, 2nd, and 3rd months. Only phPLA-RODI maintained its stability when encapsulation was performed at +4°C. All bacteriophages lost their stability at +20°C. In microencapsulation application, while all phages preserved their hyphens at +4°C, stability losses occurred at -20°C (Gonzales-Menendes et al., 2018). These results showed that cold storage (+4°C) can be used effectively for the preservation of these types of phages instead of frozen storage (-20°C or -85°C), which requires higher energy costs and special devices. Similar to these studies; it was observed that stability was maintained at all tested temperatures (+4°C, -20°C, and -85°C) and with all tested cryoprotectants (glycerol, skimmed milk powder, and carrageenan) during the first two months. However, in the third and fourth months, a decrease in glycerol was observed at -85°C. In the fifth month, carrageenan provided the highest preservation at all temperatures. In the sixth month, +4°C glycerol provided the best preservation, while carrageenan provided the highest preservation at other temperatures.

As a result, when the effects of storage temperatures on the stability of the lytic M8AEC16 phage against *Escherichia coli* O157:H7 were evaluated, the most successful result was obtained in storage without cryoprotectant at +4°C. In the cryoprotectant group the best preservation was determined at +4°C and -20°C in the presence of glycerol. In addition to this study, when the results of the published articles were examined, it was observed that there is no correlation with the findings of the studies that used similar conditions for the storage of bacteriophages. It is thought that these differences are mostly due to the type of bacteriophage. For this reason, it was concluded that it is important to determine the appropriate storage conditions for each phage included in the culture collection and stocked for use as a biocontrol agent.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Authorship Contributions

Concept: N.F.A, N.D.A, Design: N.F.A, N.D.A, Data Collection or Processing: N.F.A, N.D.A, Analysis or Interpretation: N.F.A, N.D.A, Literature Search: N.F.A, Writing: N.F.A, N.D.A.

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